The double-stranded RNA-induced apoptosis pathway is involved in the cytopathogenicity of cytopathogenic Bovine viral diarrhea virus

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Bovine viral diarrhea virus (BVDV), which is classified in the genus Pestivirus, family Flaviviridae, can be divided into two biotypes according to its ability to induce a cytopathic effect in tissue culture cells. The mechanisms through which cytopathogenic (cp) BVDV induces cell death and non-cytopathogenic (ncp) BVDV causes persistent infection without producing cell death remain unclear. Here, it was found that the overexpression of four apoptosis-related cellular mRNAs in cells infected with cpBVDV could also be caused by synthetic dsRNA. In fact, it was found that the amount of dsRNA produced by cpBVDV considerably exceeded the amount yielded by ncpBVDV. To evaluate the possible involvement of dsRNA in the induction of apoptosis, this study examined whether RNAi-mediated depletion of two dsRNA-reactive cellular factors, dsRNA-dependent protein kinase and 2',5'-oligoadenylate synthetase 1, resulted in the prevention of cpBVDV-induced apoptosis. Although the induction of apoptosis was reduced after the suppression of either factor alone, the simultaneous silencing of both factors resulted in an almost complete inhibition of apoptosis without affecting viral titre. These results showed that dsRNA is the main trigger of apoptosis in cpBVDV-infected cells and that the cytopathogenicity of BVDV depends on the yield potential of dsRNA. In contrast, ncpBVDV yielded minimal levels of dsRNA, thereby establishing a persistent infection without inducing apoptosis. This report supports the significance of viral dsRNA as a trigger of innate immune responses.

INTRODUCTION

A persistent viral infection often causes profound disease in host animals. However, viruses must avoid or overcome the defensive mechanisms of the host to establish persistent infection. The innate immune responses of host cells, such as apoptosis and interferon (IFN) synthesis, provide a crucial first line of defence by eliminating virus-infected cells before the onset of a virus-specific immune response (reviewed by Chawla-Sarkar et al., 2003). As previous studies (Gregory et al., 1991; Levine et al., 1993; Yeung et al., 1999) have suggested that the prevention of apoptosis could convert a lytic infection into a persistent infection, evading apoptosis seems to be crucial for establishing persistent infection and viruses have evolved various survival strategies by suppressing apoptosis and IFN responses (Cebulla et al., 1999; Roulston et al., 1999).

Bovine viral diarrhea virus (BVDV), which is classified in the genus Pestivirus of the family Flaviviridae, is a virus that can cause lifelong persistent infection. BVDV can be divided into two biotypes, which are distinguished by their effect on cultured cells. Non-cytopathogenic (ncp) strains do not cause cell death, while cytopathogenic (cp) strains induce cytopathic effects (CPE) via apoptosis (Zhang et al., 1996; Hoff & Donis, 1997). In the field, only ncpBVDV can establish persistent infections via transplacental infection of a bovine fetus in conjunction with the acquisition of immuno-tolerance against the infecting ncpBVDV. Immunotolerant calves develop mucosal disease following superinfection with cpBVDV. As only ncpBVDV can establish persistent infection in vivo, the pathology associated with infection by cpBVDV seems to correlate with the cell-killing property of cp strains. Accordingly, clarifying the cpBVDV-derived factor(s) that triggers apoptosis might lead to a better understanding of the mechanisms involved in persistent infection.

cpBVDV-induced apoptosis has been reported to be triggered through the intrinsic pathway (Grummer et al., 2002), extrinsic pathway (Yamane et al., 2005) and endoplasmic reticulum stress-mediated activation of caspase-12 induced by virus replication (Jordan et al., 2002). Several reports have stated that increased viral RNA replication occurs in cells infected with cpBVDV (Mendez et al., 1998; Vassilev & Donis, 2000; Becher et al., 2001; Lackner et al., 2004). However, the cpBVDV-derived factor(s) that induces cell death via apoptosis has not been determined.

It has been reported that dsRNA is formed mainly as a replicative intermediate during the replication of RNA
viruses and is an active component of virus infection that stimulates host antiviral responses, including apoptosis (Clemens, 1997; Williams, 1997; Gil & Esteban, 2000) and the production of cytokines such as IFNs and nitric oxide (Heitmeier et al., 1998; Blair et al., 2002; Auch et al., 2004). Two cellular factors, dsRNA-dependent protein kinase (PKR) and 2′,5′-oligoadenylate synthetase (OAS), can be induced by type I IFNs and are activated by binding to dsRNA (reviewed by Chawla-Sarkar et al., 2003). Activated PKR can phosphorylate the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α), leading to the arrest of translation and cell death (Kaufman, 1999). Activated OAS synthesizes 2′,5′-oligoadenylates and, in turn, activates RNase L, which causes apoptosis through the degradation of ssRNA including viral and cellular RNAs (Castelli et al., 1998). Thus, the PKR and OAS systems induce apoptosis by inhibiting the synthesis of viral and host proteins through independent mechanisms.

In this study, we showed that cpBVDV, but not ncpBVDV, produces a large amount of dsRNA in host cells, as reported previously (Mendez et al., 1998; Vassilev & Donis, 2000; Lackner et al., 2004). Subsequently, to analyse the involvement of the interactions between cpBVDV-produced dsRNA and dsRNA-binding cellular proteins in the execution of apoptosis, we examined the potential of PKR- and OAS-1-targeted RNA interference (RNAi) to inhibit cpBVDV-induced apoptosis.

METHODS

Reagents. Synthetic polynucleotides [polyinosinic–polycytidylic acid, poly(IC); polyinosinic acid, poly(I)] and 2-aminopurine were purchased from Sigma.

Cells and viruses. Primary bovine fetal muscle (BFM) cells were maintained as described previously (Yamane et al., 2005). BVDV strains KS86-1cp and KS86-1ncp (genotype 1) were used (Nagai et al., 2001, 2003) and inoculated as described in the figure legends.

Apoptosis assays. Caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays were performed as described previously (Yamane et al., 2005).

RT-PCR of apoptosis-related cellular mRNAs. RT-PCR of apoptosis-related cellular mRNAs was performed as described previously (Yamane et al., 2005). The primers used to amplify bovine OAS-1 mRNA (369 bp product) were 5′-TCTCAGCTTTGTGCTGAGGT-3′ (sense) and 5′-TGAGCTGCTGAATTCTGG-3′ (antisense). PCR amplification of bovine OAS-1 was performed for 40 cycles.

Immunoblot analysis. At a selected time post-inoculation (p.i.), cells were treated as described previously (Jordan et al., 2002). Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham). Membranes were blocked overnight at 4°C in blocking buffer (0.1% Tween 20, 5% non-fat milk powder in PBS). After blocking, membranes were incubated with primary polyclonal antibody against PKR (D-20; Santa Cruz Biotechnologies) or against phosphorylated eIF2α (Biomol International) or against OAS-1 (GenWay Biotech) (each diluted 1:3000 with PBS containing 1% BSA and 0.1% Tween 20) for 2 h. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody for the detection of PKR and phosphorylated eIF2α, and horseradish peroxidase-conjugated rabbit anti-chicken IgG was used for OAS-1 detection (each diluted 1:5000 with PBS containing 2% non-fat milk powder and 0.1% Tween 20). Immunoreactive protein bands were detected using an enhanced chemiluminescence (ECL) detection kit (Amersham). Immunoblotting of β-actin, which served as a loading and transfer control, was conducted essentially as described above but with the use of a mouse anti-β-actin monoclonal antibody (1:3000 dilution; Chemicon International) as the primary antibody and a peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution) as the secondary antibody.

Extraction of RNA and RNA transfection. RNA samples were extracted using TRIzol reagent (Invitrogen) from 6 × 10^6 mock-infected BFM cells or BFM cells infected with KS86-1ncp or KS86-1cp at an m.o.i. of 1 at 48 h p.i. Each RNA sample was treated with 1 U RNase I (Promega), which specifically digests ssRNA, or RNase III (New England Biolabs), which digests only dsRNA, for 30 min at 37°C. The enzymes were inactivated by heating at 70°C for 20 min. The resultant RNA samples were then transfected using Lipofectamine 2000 reagent (Invitrogen) into 6 × 10^6 confluent BFM cells according to the manufacturer’s protocol. Twenty-four hours after transfection, photographes were taken and apoptosis assays were performed as described above.

RNAi procedure. We designed Stealth RNAi (Invitrogen) against bovine PKR and OAS-1. The Stealth RNAi against bovine PKR was 5′-GCGUCUAAUAACGUCUCCAGAAA′-3′ (sense) and 5′-UUGGAGAGUACUAAAGGCCG-3′ (antisense) and against bovine OAS-1 was 5′-GGATTCCCGACCCCAAGTCAAAC-3′ (sense) and 5′-UUUGUUGAUUGUGGUGGAGC-3′ (antisense). Stealth RNAi controls for PKR and OAS-1 contained the same base composition but in a random order. At 4 h before virus infection, BFM cells were seeded in 48-well plates in 200 μl antibiotic-free DMEM supplemented with 10% FCS and incubated at 37°C in a humidified 5% CO2 atmosphere. BFM cells were infected with cpBVDV at an m.o.i. of 2 for 1 h at 37°C. After the inoculum was removed by washing the cells in FCS-free DMEM, transfection with 25 pmol Stealth RNAi against PKR or OAS-1 or with each control was performed using Lipofectamine 2000 reagent according to the manufacturer’s protocol. The medium was replaced 4 h post-transfection with DMEM supplemented with 5% FCS. At 24 h p.i., cells were assayed for the inhibition of PKR and OAS-1 mRNA expression by quantitative real-time PCR as described below.

Quantitative real-time PCR. The reverse transcriptase reaction to initiate cDNA synthesis was performed with SuperScript III RNaseH− reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. To determine whether the RNA was single- or double-stranded, RNA samples were treated with RNase I or RNase III as described above. The viral negative-strand RNA was primed with 0.8 μM sense primer 324 [5′-ATGCC(T/A)TAGTAGGACCTACA-3′; 5′ non-coding region (NCR)] and the viral positive-strand RNA was primed with antisense primer 326 [5′-TCAACTCATTGGCCGATGTC-3′; 5′ NCR] as described previously (Vilcek et al., 1994). A quantitative real-time PCR assay was performed using a Smart Cycler II (Cepheid). To measure the amount of viral RNA, a Cycleave RT-PCR pestivirus detection kit (TaKaRa) was used according to the manufacturer’s protocol. Quantification of OAS-1 and PKR mRNAs was performed with SYBR Premix Ex Taq (TaKaRa) using 1 μl reverse-transcribed cDNAs. The primers used to amplify bovine PKR mRNA were 5′-CTGGTGCTTACCACTGTTTTC-3′ (sense) and 5′-CTCAATGGGTGGCTTCTCT-3′ (antisense) and the primers of bovine OAS-1 mRNA were the same as described above for RT-PCR of apoptosis-related cellular mRNAs. All samples were analysed in triplicate. In each run, tenfold serial dilutions of each synthetic transcript were tested in duplicate to establish a standard curve to calculate the amount of each RNA present in the sample. The
measured amounts of RNA were normalized to the amount of glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in each sample, as described previously (Yamane et al., 2005).

**RESULTS**

**Overexpression of apoptosis-related cellular mRNAs and proteins induced by cpBVDV can also be caused by transfection of synthetic dsRNA**

Previously, we carried out a comprehensive investigation of apoptosis-related cellular mRNAs, which showed that overexpression of Mx1, iNOS and TNF-α mRNAs was caused by cpBVDV infection in primary BFM and primary bovine testicle cells (Yamane et al., 2005). In this study, we additionally found overexpression of OAS-1 mRNA (Fig. 1a), and participation of the OAS and RNase L system, which degrades ssRNA including viral RNA and cellular mRNA, was indicated by degradation of GAPDH mRNA in cpBVDV-infected cells (data not shown). In addition, overexpression of PKR mRNA following cpBVDV infection was detected by quantitative real-time PCR (data not shown) and protein overexpression of PKR and phosphorylated eIF2α in cpBVDV-infected cells (Fig. 1b) was also confirmed, as reported recently (Gil et al., 2006).

Although the factor(s) leading to overexpression remains unclear, we found that poly(IC), a synthetic dsRNA

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**Fig. 1.** Transfection of poly(IC) induces overexpression of four apoptosis-related cellular mRNAs (Mx1, iNOS, TNF-α and OAS-1), similar to cpBVDV infection, and induces cell death via apoptosis in BFM cells. (a) Overexpression of the four mRNAs induced by cpBVDV infection or transfection with poly(IC). At 4 h after transfection of poly(I) (lane I) or poly(IC) (lane IC) or 24 h after infection with cpBVDV (KS86-1cp; lane cp) or ncpBVDV (KS86-1ncp; lane ncp) at an m.o.i. of 1, total RNA was isolated and RT-PCR was performed as described in Methods. (b) Expression of PKR and phosphorylated eIF2α (P-eIF2α) induced by poly(IC) or cpBVDV. After BFM cells were treated with poly(IC) or infected with cp- or ncpBVDV as described in (a), each protein was detected by immunoblotting as described in Methods. (c) Caspase-3 is activated only in the presence of poly(IC) transfected using Lipofectamine 2000 (LF). BFM cells were mock-treated or treated with poly(IC) or infected with cp- or ncpBVDV as described in (a), each protein was detected by immunoblotting as described in Methods. (c) Caspase-3 is activated only in the presence of poly(IC) transfected using Lipofectamine 2000 (LF). BFM cells were mock-treated or treated with 10 µg ml⁻¹ of poly(I) or poly(IC) with (filled bars) or without (shaded bars) LF. The activity of caspase-3 was measured at 24 h after transfection as described in Methods. Each activity was standardized to the value of mock-treated cells without LF, which was set at 100%. (d) The activity of poly(IC)-induced caspase-3 is slightly reduced by prior infection with ncpBVDV. BFM cells were mock-infected (open bars) or infected with KS86-1ncp at an m.o.i. of 1 at 18 h (shaded bars) before transfection of poly(IC) at the indicated concentrations. The activity of caspase-3 was measured at 24 h after poly(IC) transfection. Each activity was standardized to the value of mock-infected cells without poly(IC), which was set at 100%. **, P < 0.01 (determined using Student’s t-test).
analogue, could induce these four apoptosis-related cellular mRNAs (Mx1, iNOS, TNF-α and OAS-1) and proteins (PKR and phosphorylated eIF2α), similar to cpBVDV infection (Fig. 1a, b). In contrast, following transfection of poly(I), a ssRNA analogue, overexpression of only two mRNAs, Mx1 and iNOS, was induced, even at a concentration of 10 μg ml⁻¹ (Fig. 1a). When we tested various concentrations of poly(IC), overexpression of the four apoptosis-related mRNAs was observed at doses greater than approximately 50 ng ml⁻¹ (data not shown). Furthermore, cpBVDV-like cell death and activation of caspase-3, the apoptosis executor enzyme, occurred when poly(IC) was transfected into cells using Lipofectamine 2000 (Fig. 1c), but not when poly(IC) was simply added in the supernatant, even at a high concentration (200 μg ml⁻¹; data not shown).

**ncpBVDV reduces apoptosis induced by transfected poly(IC) but cannot completely inhibit apoptosis in BFM cells**

It has been shown previously that ncpBVDV inhibits signal transduction induced by poly(IC) added to the culture supernatant (Schweizer & Peterhans, 2001; Iqbal et al., 2004) and that induced by transfected poly(IC) (Schweizer & Peterhans, 2001; Gil et al., 2006). In contrast to primary bovine turbinate cells used in the previous study of Schweizer & Peterhans (2001), BFM cells induced apoptosis only when poly(IC) was transfected (Fig. 1c). All cells transfected with poly(IC) at concentrations greater than 50 ng ml⁻¹ underwent apoptosis, regardless of whether they had been infected previously or not with ncpBVDV (Fig. 1d). A slight reduction in caspase-3 activity was observed in the ncpBVDV-infected cells at a poly(IC) concentration of 5 μg ml⁻¹, which seemed to be an effect of the Ems protein secreted from BVDV-infected cells (Iqbal et al., 2004). Furthermore, overexpression of the four apoptosis-related cellular mRNAs stimulated by poly(IC), as shown in Fig. 1(a), was not inhibited by prior infection with ncpBVDV, even at the low poly(IC) concentration of 50 ng ml⁻¹ (data not shown). These results indicated that ncpBVDV does not inhibit signal transduction stimulated by intracellular poly(IC) in BFM cells.

**Quantification of viral ssRNA and dsRNA using real-time PCR**

To examine whether there were differences in the yields of viral RNA between cpBVDV and ncpBVDV, we quantified the amounts of positive-sense ssRNA and dsRNA of each virus using real-time PCR. Viruses were inoculated at an m.o.i. of 5 to analyse the viral RNA replication kinetics of synchronous infections. As shown in Fig. 2, levels of cpBVDV viral RNA started to increase from 8 h p.i.; however, levels of ncpBVDV had decreased by 8 h p.i. At 24 h p.i., the copy number of positive-sense ssRNA in cpBVDV-infected cells was more than approximately 100 times that found in ncpBVDV-infected cells, whilst the amount of dsRNA in cpBVDV-infected cells was 100–200 times that in ncpBVDV-infected cells (Fig. 2a, c). In addition, in the case of multicycle infections at an m.o.i. of 0.1, viral ssRNAs of the ncp and cp strains were found to increase in the same manner by 48 h p.i. (data not shown), suggesting that each strain replicated with the same efficiency, regardless of m.o.i. Similar results were obtained using two other cp and ncp virus pairs, including BVDV genotype 2 (IS4ncp and IS5cp; KZ91ncp and KZ91cp; Nagai et al., 2001) (data not shown). In contrast, the copy number of negative-sense ssRNA (an indicator of replication) derived from cpBVDV was 100–200 times greater than that from ncpBVDV, corresponding to the difference in the yield of viral dsRNA (Fig. 2b, c).

**Viral dsRNA extracted from BFM cells induces apoptosis**

To examine whether viral dsRNA by itself was sufficient to induce CPE via apoptosis, extracted RNA was transfected into BFM cells. Samples of RNA extracted from mock- and BVDV-infected cells were treated with or without RNase I or RNase III and each sample was transfected into the same number of normal BFM cells. Only cells transfected with RNA extracted from cpBVDV-infected cells, either without RNase treatments or with RNase I treatment, induced CPE-like cell death (Fig. 3a). Cell death was also shown to be triggered via apoptosis as indicated by an ELISA-based TUNEL assay (Fig. 3b). These results showed that cpBVDV yielded a large amount of dsRNA, enough to induce apoptosis, whilst ncpBVDV replicated at a minimum level, yielding an undetectable amount of dsRNA.

**Apoptotic sensitivity to poly(IC) is decreased after RNAi-mediated knockdown of PKR and/or OAS-1**

We assessed the effects of RNAi-mediated silencing of PKR and OAS-1 mRNA in cpBVDV-infected BFM cells as described in Methods. Following RNAi treatment, the quantity of PKR mRNA extracted from cells transfected with PKR small interfering (si)RNA was reduced by approximately 85% (Fig. 4a; iPKR) and OAS-1 mRNA was reduced by approximately 90% in the OAS-1 siRNA-transfected cells (Fig. 4a; iOAS). After administration of the siRNA mixture against PKR and OAS-1, the PKR and OAS-1 mRNAs were simultaneously depleted (Fig. 4a; iMix). The RNAi-mediated reduction of PKR or OAS-1 protein and that of both proteins through the double knockdown in cpBVDV-infected cells was confirmed by Western blotting (Fig. 4b).

We next examined the inhibition of poly(IC)-induced caspase-3 activity to examine cellular sensitivity against dsRNA after RNAi-mediated depletion of PKR and OAS-1. Poly(IC)-induced caspase-3 activity was reduced by approximately 30% after knockdown of PKR and by approximately 15% after knockdown of OAS-1 compared with the activity in the siRNA-transfected controls (Fig. 4c). These results showed that the participation of OAS-1 in the induction of poly(IC)-induced apoptosis was less than that of PKR;
however, the OAS-1 pathway was also involved in executing apoptosis. Following the double knockdown of PKR and OAS-1, caspase-3 activity induced by poly(IC) was decreased to the same level as that of mock-treated cells (Fig. 4c; iMix). These results suggested that the apoptotic sensitivity of BFM cells to transfected dsRNA was functionally depleted through the administration of PKR and OAS-1 RNAi.

**Double knockdown of OAS-1 and PKR with RNAi results in complete inhibition of cpBVDV-induced cell death**

The effect of PKR and OAS-1 knockdown on the induction of apoptosis in cpBVDV-infected cells was analysed. In a time-course experiment, cpBVDV-induced caspase-3 activity was reduced by approximately 30 % at 48 h p.i. following the administration of PKR RNAi (Fig. 5a), corresponding to the change observed with poly(IC)-induced caspase-3 activity. As observed with the suppression of caspase-3, RNAi-mediated silencing of PKR in cells infected with cpBVDV resulted in a delay in the appearance of CPE and a reduction in visible cell death (Fig. 5b). In addition, cpBVDV-induced caspase-3 activity was reduced by approximately 15 % following the administration of OAS-1 RNAi (Fig. 5c), which corresponded to the reduction following transfection of poly(IC). However, the reduction in apoptosis in OAS-1 siRNA-transfected cells was not clearly observed when compared with that in control siRNA-transfected cells (data not shown). When we tested the double knockdown of PKR and OAS-1, caspase-3 activity induced by cpBVDV infection was dramatically decreased, in the same manner as that induced by poly(IC), and cpBVDV-induced cell death was completely inhibited as demonstrated by microscopy at 48 h p.i. (Fig. 5d), although the viral titres remained unaffected (Fig. 5e). Although we cannot exclude the possibility that cpBVDV may induce apoptosis via additional triggers of PKR or OAS-1, these results strongly suggest that each of these factors independently activates the pro-apoptotic signalling pathways against dsRNA and that both pathways synergistically participate in the execution of apoptosis. In addition, cpBVDV-induced apoptosis occurred in the same fashion as that stimulated by synthetic dsRNA, suggesting that viral dsRNA is a main trigger of cpBVDV-induced apoptosis.

![Fig. 2. Amounts of viral positive-sense ssRNA (a), negative-sense ssRNA (b) and dsRNA (c) yielded by cpBVDV significantly exceed those produced by ncpBVDV. The amount of viral positive- or negative-sense ssRNA or dsRNA was determined by quantitative real-time PCR as described in Methods. BFM cells were infected with cpBVDV (KS86-1cp) or ncpBVDV (KS86-1ncp) at an m.o.i. of 5. Each genome copy number was normalized to the amount of cellular GAPDH mRNA and standardized to the positive-sense ssRNA copy number of ncpBVDV at 0 h p.i., which was set at 1.](http://vir.sgmjournals.org)
The molecular feature that distinguishes the cp from the ncp biotype of BVDV is the efficient generation of discrete NS3 protein, which is thought to correlate with an upregulated synthesis of viral RNA, throughout infection with cpBVDV (Meyers & Thiel, 1996; Lackner et al., 2004). We focused on the effect of viral RNA accumulation in the induction of apoptosis due to the efficient RNA replication of cpBVDV as shown in Fig. 2 (also reported by Mendez et al., 1998; Vassilev & Donis, 2000; Lackner et al., 2004). In this study, dsRNA, which was derived from an increased level of viral RNA replication, was shown to be highly involved in the execution of apoptosis by interactions with the dsRNA-reactive cellular factors, PKR and OAS-1.

We found that the upregulation of four apoptosis-related cellular mRNAs observed in cpBVDV-infected cells could also be induced by transfection of synthetic dsRNA. Among the set of four factors, iNOS has been shown to serve as an anti-apoptotic factor and TNF-α has been revealed to participate in enhancing cpBVDV-induced apoptosis as an

Fig. 3. Extracted viral dsRNA from cpBVDV-infected cells induces CPE via apoptosis. (a) Transfection of extracted dsRNA from cpBVDV-infected cells resulted in the appearance of cell death. RNA samples extracted from mock-infected BFM cells (Ct) or BFM cells infected with KS86-1ncp (ncp) or KS86-1cp (cp) were mock-treated (left panels) or treated with RNase I (centre panels) or RNase III (right panels) and transfected into confluent BFM cells as described in Methods. Cell death was observed 24 h later. (b) The apoptotic index was determined using an ELISA-based TUNEL assay. RNA samples were extracted from mock-infected BFM cells as a control (Ct) and from cells infected with ncpBVDV (KS86-1ncp; lane ncp) and used for transfection. Similarly, RNA samples from cpBVDV-infected cells were mock-treated (cp) or treated with RNase I (cp I) or RNase III (cp III), transfected into BFM cells and incubated for 24 h. The assay was performed as described in Methods. **, *P* < 0.01 (determined using Student’s t-test).
Cytopathogenic BVDV causes dsRNA-induced apoptosis

Overexpression of the four apoptosis-related cellular mRNAs was not observed in ncpBVDV-infected cells, indicating that ncpBVDV yields only low levels of ssRNA, as overexpression of Mx1 and iNOS mRNAs was shown to be caused by synthetic ssRNA (Fig. 1a). Quantification of the levels of viral RNA, as shown in Fig. 2, supported the suggestion that minimal RNA replication occurred in ncpBVDV-infected cells. In the case of HCV, the limitation of viral RNA replication via the degradation of N55B, which functions as an RNA-dependent RNA polymerase, is thought to be important in escaping host cell defences (Gao et al., 2003). Thus, the maintenance of low-level RNA replication, as observed in ncpBVDV infection, would be a crucial strategy for establishing persistent infection, given that the difference in the RNA replication levels between ncpBVDV and cpBVDV was the common feature among all three pairs of BVDV strains tested. Conversely, as ncpBVDV and cpBVDV both share the ability to inhibit an IFN-related antiviral response (Baigent et al., 2004) or a signal transduction against dsRNA (Iqbal et al., 2004), it can be speculated that the excessive amount of dsRNA yielded by cpBVDV surpasses the capacity of BVDV to inhibit the antiviral responses.

In this study, the participation of the PKR pathway and the subsequent phosphorylation of eIF2α, which leads to the restriction of mRNA translation, were shown to be involved in cpBVDV infection (Fig. 1b). As the inhibition of eIF2α kinase by 2-aminopurine increased the viral titre of cpBVDV up to tenfold and as a slight increase in the viral titre was observed by the knockdown of PKR (Fig. 5e), protein extrinsic factor (Yamane et al., 2005). In this study, we revealed that dsRNA could trigger the intrinsic apoptotic factors PKR and OAS-1 and initiate apoptosis through interactions with both factors; these are thought to be key events in the cytopathogenicity of BVDV. RNAi-mediated knockdown of either PKR or OAS-1 in cpBVDV-infected cells resulted in a reduction in caspase-3 activity and the delay of cell death. With the double knockdown of both PKR and OAS-1, the apoptotic activity of cpBVDV-infected cells was inhibited to almost the same level as that observed in mock-infected cells. These results indicated that a pro-apoptotic synergistic effect of OAS-1 and PKR was induced by accumulated viral dsRNA in cpBVDV-infected cells and that both factors independently play critical roles in initiating apoptosis. Viral titres of cpBVDV were unchanged when faced with the double knockdown of the two factors compared with titres in the mock-transfected and siRNA-transfected control (Fig. 5e); thus, the inhibition of apoptosis appeared to be attributable to the insensitivity of host cells to the PKR- and OAS-mediated antiviral pathways probably stimulated by cpBVDV-derived dsRNA, which convert a cytopathic infection of cpBVDV to a non-cytopathic one. Furthermore, the functions of PKR and OAS-1 have been suggested to be associated with clearance of hepatitis C virus (HCV) infection (Knapp et al., 2003), supporting the idea that both factors might play crucial roles in preventing a persistent infection.

Fig. 4. Functional depletion of PKR and OAS-1 by the administration of RNAi. (a) Reduction of cpBVDV-induced PKR and OAS-1 mRNA by each RNAi. BFM cells were infected with KS86-1cp at an m.o.i. of 2 for 1 h. Cells were washed with DMEM and each siRNA was transfected as described in Methods. At 24 h after transfection of siRNA against PKR (iPKR) or control (iCt-PKR), against OAS-1 (iOAS) or control (iCt-OAS) or against both PKR and OAS (iMix) or control (iCt-Mix) in cpBVDV-infected cells, total RNA was extracted and quantitative real-time PCR was performed as described in Methods. (b) RNAi-mediated reduction of PKR and OAS-1 protein induced by cpBVDV in BFM cells. At 24 h after transfection of each siRNA as described above in cpBVDV-infected cells, lysates were collected and Western blotting was performed to detect PKR, OAS-1 and β-actin. (c) Knockdown of PKR or and OAS-1 reduces the apoptotic sensitivity of BFM cells to poly(IC). Transfection of each siRNA as described above was performed at 36 h before transfection of 500 ng poly(IC) ml⁻¹. After 12 h, the level of caspase-3 activity was measured and standardized to the value of poly(IC)-transfected normal BFM cells, which was set at 100%. *, P<0.05; **, P<0.01 (determined using Student’s t-test).
Fig. 5. Apoptosis of cpBVDV-infected cells is inhibited after RNAi-mediated double knockdown of PKR and OAS-1. (a) Caspase-3 activity induced by cpBVDV is reduced after knockdown of PKR. BFM cells were mock-infected (Mock) or infected with KS86-1ncp (ncp) or KS86-1cp (cp) at an m.o.i. of 2, 1 h before transfection of siRNAs against PKR (iPKR) or control (iCt-PKR). Caspase-3 activity was assayed at the indicated time points. Each activity was standardized to the value of mock-infected cells at 24 h p.i., which was set at 100 %. (b) cpBVDV-induced CPE is visibly reduced by PKR siRNA transfection. Results are shown at 48 h after infection. (c) cpBVDV-induced caspase-3 is inhibited by the double knockdown of PKR and OAS-1. BFM cells were mock-infected (Mock) or infected with KS86-1cp (cp) or KS86-1ncp (ncp) at an m.o.i. of 2, 1 h before transfection of siRNA against OAS-1 (iOAS) or control (iCt-OAS), or against both PKR and OAS (iMix) or control (iCt-Mix). At 48 h p.i., caspase-3 activity was assayed and standardized to the value of the mock-transfected control, which was set at 100 %. (d) cpBVDV-induced CPE is visibly inhibited by the RNAi-mediated double knockdown of both PKR and OAS-1. Results are shown at 48 h after infection. (e) Viral titres of cpBVDV. At 48 h p.i., cpBVDV viral titres generated from mock-transfected cells, cells transfected with siRNAs and cells treated with 100 μM 2-aminopurine (2-AP), an inhibitor of eIF2α kinases, were calculated as TCID₅₀ ml⁻¹ using Madin–Darby bovine kidney cells. *, P<0.05; **, P<0.01 (determined using Student's t-test).
synthesis of cpBVDV might be limited following the activation of PKR and subsequent eIF2α phosphorylation. However, despite the induction of antiviral factors such as Mx1 or OAS-1, which restrict viral RNA replication (Castelli et al., 1998; Haller et al., 1998), the mechanism through which cpBVDV replicates more efficiently than ncpBVDV remains to be elucidated (Lackner et al., 2004).

The difference between the biotypes, which can be distinguished in vitro, needs to be clarified, as transmission of BVDV to a fetus and persistent infection are apparently correlated with the biotype of BVDV. In this study, all of the ncpBVDV strains tested were shown to evade apoptosis and to produce a sufficiently small amount of dsRNA to escape recognition by cellular dsRNA-reactive factors. In contrast, the BVDV biotype with cytopathogenic properties commonly correlated with the productivity of dsRNA and the extracted dsRNA from cpBVDV-infected cells induced apoptosis in the absence of viral proteins, as shown in Fig. 3(a), suggesting that dsRNA is a key factor of cytopathogenicity.

Although the observation in vitro might be different from that in vivo, as apoptosis and the IFN response are manifested from the earliest stages of pregnancy (Śpíchal et al., 1994), transplacental and persistent infection could be affected by the dsRNA-induced innate immune responses. Although dsRNA-induced apoptosis is known to correlate with negative-strand RNA viruses (Takizawa et al., 1996) and DNA viruses (Kibler et al., 1997), these results show that, in the case of positive-strand RNA viruses also, dsRNA can be a main trigger of apoptosis.

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REFERENCES


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