Expression of the NS3 protease of cytopathogenic bovine viral diarrhea virus results in the induction of apoptosis but does not block activation of the beta interferon promoter

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Bovine viral diarrhea virus (BVDV; genus Pestivirus) can exist as two biotypes, cytopathogenic (CP) and non-cytopathogenic (NCP). The CP form differs from NCP by the continual expression of free non-structural protein 3 (NS3). CP BVDV infection of cultured cells induces apoptosis, whereas NCP BVDV infection has been reported to block the induction of beta interferon (IFN-β).

To investigate the viral mechanisms underlying these effects, NS3 or NS2–3 proteins of NCP and CP BVDV biotypes, together with the cognate NS3 co-factor NS4A, were expressed in cells, and their effect on apoptosis and induction of IFN-β was investigated. Expression of NS3/4A resulted in increased activity of caspase-9 and caspase-3, indicating induction of the intrinsic apoptosis pathway. Mutational analysis revealed that a protease-inactive NS3/4A was unable to induce apoptosis, suggesting that NS3 protease activity is required for initiation of apoptosis during CP BVDV infection. The ability of NS2–3 to modulate activation of the IFN-β promoter was also investigated. These studies confirmed that, unlike the related hepatitis C virus and GB virus-B, BVDV proteases are unable to inhibit TLR3- and RIG-I-dependent activation of the IFN-β promoter. These data suggest that BVDV NS3/4A is responsible for regulating the levels of cellular apoptosis and provide new insights regarding the viral elements associated with CP biotype pathogenesis.

INTRODUCTION

Within the family Flaviviridae, the genus Pestivirus contains economically important pathogens of domesticated animals: classical swine fever virus (CSFV), border disease virus, bovine viral diarrhea virus (BVDV) types 1 and 2 and atypical BVDV (Liu et al., 2009). All viruses within this genus can exist as both non-cytopathogenic (NCP) and cytopathogenic (CP) biotypes. The two biotypes are differentiated from one another according to post-infection effects observed in cultured cells, where CP biotype infection induces cytopathic effects. BVDV infection of non-pregnant cattle results in a wide spectrum of clinical manifestations including asymptomatic presentation, erosion of the digestive tract, haematological damage and haemorrhagic syndrome. Furthermore, transplacental infection can result in abortion, significant fetal abnormalities and the birth of persistently infected calves (Duffell & Harkness, 1985; Roeder et al., 1986; Brownlie, 1991).

Persistently infected calves represent a critical element of the BVDV life cycle, both in terms of epidemiology, as these animals are the most efficient transmitters of the virus (Niskanen & Lindberg, 2003; Fulton et al., 2005), and with regard to the molecular biology of BVDV. Mutation of the NCP virus genome (by a variety of genetic mechanisms such as insertion of cellular sequences, deletion, duplication or point mutation of viral sequences) in a persistently infected animal can result in the generation of a CP biotype virus (Meyers & Thiel, 1996; Kummerer et al., 2000).

The pestivirus genome comprises a single open reading frame encoding a polyprotein of approximately 4000 aa, flanked by 5’ and 3’ untranslated regions (Collett et al., 1988; Meyers et al., 1989; Moormann et al., 1990). Co- and post-translational processing of the viral polyprotein produces 11 or 12 viral proteins depending on biotype. In general, NCP viruses produce 11 proteins, one of which is a fusion of NS2 and NS3 (NS2–3), although recently it has been demonstrated that limited cleavage of NS2–3 to

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release NS3 occurs early in infection and is essential for mediating genome replication and translation (Lackner et al., 2004, 2005, 2006). In contrast, CP viruses produce 12 proteins as a result of genetic changes that permit the constitutive expression of NS3 (Meyers & Thiel, 1996).

Much work has been undertaken to determine the molecular mechanisms of the cytopathic effect induced following infection with CP BVDV. These studies have clearly shown that CP infection induces apoptosis (Zhang et al., 1996; Lambot et al., 1998), and this is associated with the accumulation of viral RNA (Vassilev & Donis, 2000) and the cleavage of poly(ADP-ribose) polymerase (PARP) (Hoff & Donis, 1997). CP infection appears to induce the intrinsic apoptotic cascade, exemplified by activation of mitochondrion-dependent caspase-9, cytochrome c release from mitochondria (Grummer et al., 2002) and caspase-12 cleavage, also implicating the endoplasmic reticulum (ER) stress-mediated apoptotic pathway (Jordan et al., 2002). However, there is also evidence for the extrinsic induction of apoptosis as upregulation of tumour necrosis factor-α production has been observed (Yamane et al., 2005). Furthermore, the dsRNA-activated proteins 2′,5′-oligoadenylate synthetase and protein kinase R may play a critical role in the induction of apoptosis following CP BVDV infection (Yamane et al., 2006). Transient expression of the NS3 proteins from BVDV and CSFV (without the presence of NS4A, the NS3 co-factor; Xu et al., 1997) has been shown to induce apoptosis (St Louis et al., 2005; Xu et al., 2007). However, the mechanism by which CP BVDV and NS3/4A induce these effects has yet to be elucidated.

In contrast, NCP BVDV infection has been demonstrated to protect against dsRNA-induced apoptosis, probably through the action of Erns to degrade the RNA (Iqbal et al., 2004) and inhibit the production of alpha and beta interferon (IFN-α and -β) (Schweizer & Peterhans, 2001; Baigent et al., 2002; Bendfeldt et al., 2003). Interestingly, IFN regulatory transcription factor 3 (IRF3) DNA-binding activation was blocked in both CP- and NCP-infected cells, yet CP BVDV infection still resulted in the production of type I IFN, implicating another member of the IRF family in the response to CP infection (Baigent et al., 2002, 2004). Once again, however, the mechanisms underlying these effects have yet to be elucidated in detail.

To characterize the viral elements underlying the cytopathology of CP BVDV infection, we analysed the induction of apoptosis and activation of the IFN-β promoter in cells expressing either the NS3/4A proteins of CP BVDV, or the NS2–4A region of the polyprotein of either NCP or CP BVDV. In pestiviruses, the N protein polypeptide and Erns have been shown to limit IFN production (Ruggli et al., 2003, 2005; Iqbal et al., 2004; La Rocca et al., 2005; Gil et al., 2006; Hilton et al., 2006; Bauhofer et al., 2007; Seago et al., 2007), but no effect has been reported for the NS2–3 or the NS3 protein. The lack of effect of NS2–3 and NS3 contrasts strikingly with many other members of the family Flaviviridae (discussed below) and warrants further investigation. Here, we demonstrated using exogenous expression of NS2–4A, NS3/4A and related constructs that CP NS3/4A induces apoptosis in cultured cells and that protease activity (but not helicase activity) is essential for this effect. Our investigations also showed that neither NCP nor CP NS2–4A was able to block activation of the IFN-β promoter, in agreement with published data (Hilton et al., 2006). These data suggest that the cytopathology of CP BVDV may result from the ability of mature NS3/4A to induce apoptosis, rather than a loss in the ability of CP BVDV to prevent IFN induction.

RESULTS

Expression of BVDV non-structural proteins

To evaluate the effects of BVDV NS2 and NS3 expression on apoptosis and IFN induction, the coding sequences from NCP7 and CP7 biotypes were amplified from the full-length clones pA/BVDV and pA/BVDV-Ins (Meyers et al., 1996) and cloned into the mammalian expression vectors pSG5 and pSG6. To ensure correct functioning of the viral proteases, the NS4A co-factor was included in all of the expression constructs. To establish that correct processing of the NS2–3 fusion protein was occurring, MDBK cells were infected with BVDV viruses or electroporated with the appropriate expression plasmids and the cells were lysed over a 48 h time period. A 125 kDa product was detected in both NCP7 and CP7 virus-infected lysates, corresponding to uncleaved NS2–3 (Fig. 1a). An 80 kDa product was also observed in the CP7-infected lysates at all time points. This corresponded to the mature NS3 protein. Our data showed that the mature NS3 protein was the more abundant product compared with NS2–3 in CP7-infected cells. In agreement with Lackner et al. (2004), we observed significant levels of cleaved NS3 protein at the early time points in NCP7-infected cells; however, this protein species was absent at subsequent time points and this correlated with increased levels of the NS2–3 fusion protein. Maximal expression of the BVDV proteins was observed between 24 and 48 h. Immuno-reactive proteins of the same apparent molecular masses were present in MDBC cells electroporated with either NS3 alone (Fig. 1b) or NS2–4A expression constructs (Fig. 1c). Expression of the mature NS3 protein was observed in NCP7 NS2–4A-expressing cells at earlier time points and maximal expression of the proteins of interest occurred between 24 and 48 h. The subcellular localization of NS3 was verified by immunofluorescence (Fig. 1d) at 12 h post-electroporation. In all cases, NS3 exhibited a cytoplasmic distribution and co-localized extensively with calreticulin, a classical marker of the ER. This distribution was also observed in MDBK cells 12 h after infection with either NCP7 or CP7 BVDV (Fig. 1e). As shown previously (Zhang et al., 2003), both NS2–3 and NS3 were excluded from the nucleus. By analogy to hepatitis C virus (HCV) NS3, it is likely that the presence of NS4A results in
Fig. 1. Expression of BVDV non-structural proteins. (a) MDBK cells were infected with NCP7 or CP7 BVDV, harvested at the indicated time points and Western blotted with a mouse anti-NS3 mAb (upper panels) or antibody against glyceraldehyde-3-phosphate dehydrogenase (GADPH; Abcam) (lower panel) as an internal control. (b) MDBK cells were electroporated with pSG5.NS3/4A and harvested at the indicated time points, prior to Western blotting for NS3 or GAPDH. (c) MDBK cells were electroporated with pSG6.NCP7 NS2–4A or pSG6.CP7 NS2–4A, harvested at the indicated time points and Western blotted for NS3 or GAPDH. (d) MDBK cells expressing the indicated constructs were stained with anti-NS3 mAb. Calreticulin was used to stain the ER. A high-power view of the inset is shown on the right. (e) MDBK cells were infected with NCP7 or CP7 BVDV and stained as in (d) at 12 h p.i.
cytoplasmic retention of NS2–3 and NS3 (Wölk et al., 2000).

**Infection with CP BVDV induces apoptosis**

MDBK cells were infected with NCP7 or CP7 BVDV and the induction of apoptosis was monitored by measuring annexin V binding to cellular membranes at 48 h post-infection (p.i.). As a positive control, cells were treated with thapsigargin, a powerful inducer of apoptosis that functions by releasing calcium from intra-cytoplasmic stores. As shown in Fig. 2(a), both thapsigargin treatment and infection with CP7 BVDV elevated annexin V binding, suggesting the induction of apoptosis, whereas infection with NCP7 BVDV had little effect. We quantified the number of apoptotic cells by counting cells that exhibited nuclear fragmentation following BVDV infection. Fig. 2(b) shows that, in the presence of NCP7 BVDV infection, levels of fragmentation similar to that of uninfected cells (<10%) were observed. After infection with CP7 BVDV, the levels of nuclear fragmentation approached 50% (Fig. 2b). Taken together, these data confirmed that infection with CP7 BVDV is able to induce an apoptotic response. A similar pattern of virus-induced apoptosis was observed with the strains Pe515 (data not shown) and NADL (Bendfeldt et al., 2003), suggesting that apoptosis is a common outcome of infection with CP BVDV.

**BVDV NS3/4A induces apoptosis**

We investigated the induction of apoptosis in MDBK cells expressing either NCP7- or CP7-derived NS2–4A proteins. Consistent with the virus data, we observed elevated levels of annexin V binding and nuclear fragmentation in cells expressing CP NS2–4A but not in those expressing NCP NS2–4A (Fig. 2c, d), confirming that the ability of CP BVDV infection to induce apoptosis is conferred, at least in part, by the NS2–4A proteins.

As NCP NS2–4A did not induce apoptosis, this implied that the pro-apoptotic factor was either the cleaved NS2 or NS3 protein. To investigate this, we measured levels of apoptosis in cells expressing NS3/4A alone (Fig. 2c, d). NS3/4A expressing cells showed a 30% increase in apoptotic levels, suggesting that free NS3, rather than NS2, was responsible for the induction of apoptosis in CP BVDV-infected cells. This is consistent with the observation that the strain Pe515 CP virus, which is thought not
to produce NS2 (Meyers et al., 1992), is able to induce apoptosis.

Both BVDV and HCV NS3 comprise two domains with distinct enzymic activities – an N-terminal serine protease domain and a C-terminal helicase domain. To test whether either of these enzymic activities was required for the induction of apoptosis, we constructed three mutants. Two of these targeted the active-site serine of the protease, S1752 (polyprotein numbering). An S1752T mutant was previously reported to prevent trans cleavage (within the polyprotein) but retained the ability to function in cis, by cleaving NS3 from NS4A, whereas S1752A eliminated both cis and trans cleavage by NS3 (Tautz et al., 2000). Both of these mutants abolished the ability of NS3/4A to induce apoptosis (Fig. 2c, d). In contrast, a mutation within the active site of the helicase (H1922A; Gu et al., 2000) had no effect on the ability of NS3/4A to induce apoptosis (Fig. 2c, d). We concluded that the ability of CP BVDV to induce apoptosis is not only dependent on the presence of cleaved NS3, but also requires the proteolytic activity of NS3.

**BVDV NS3/4A induces an intrinsic apoptotic pathway**

To verify further that CP BVDV induces apoptotic cell death, we analysed caspase-3 activity in BVDV-infected MDBK cells at 48 h p.i. As shown in Fig. 3(a), both thapsigargin treatment and infection with CP7 BVDV significantly increased caspase-3 activity, as judged by cleavage of a luminescent substrate, whereas infection with NCP7 BVDV had little effect. To correlate caspase-3 activation directly with expression of NS3, the population of cells expressing NS3 was analysed by flow cytometry for cleaved caspase-3 at 48 h p.i. Infection with CP7 BVDV resulted in increased caspase-3 cleavage in NS3-positive cells, whereas the NS3-positive population of cells from an NCP7 infection showed little cleaved caspase-3 (Fig. 3b). The caspase-3 activity assays were repeated in cells expressing NS2–4A from NCP7 and CP7 BVDV and confirmed that the level of caspase-3 activity was dependent on the proteolytic activity of NS3/4A (Fig. 3c).

Importantly, these differences in caspase-3 activity were not due to differential expression of the BVDV proteins, as all NS3 proteins were expressed to similar levels as judged by Western blotting. To verify the role of caspase-3 for CP BVDV-mediated apoptosis, we analysed cleavage of the caspase-3 substrate PARP in cells expressing BVDV NS3. Flow cytometry demonstrated increased levels of the cleaved form of PARP after 48 h of NS3 expression (Fig. 3d). Importantly, levels of PARP cleavage were reduced in NS3/4A-expressing cells incubated with the specific caspase-3 inhibitor Z-DEVD-FMK. These results strongly suggest that CP BVDV-induced cell death is caused by caspase-3-dependent apoptosis.

Caspase-3 activity is regulated by upstream initiator caspases (Porter & Janicke, 1999). Perturbation of mitochondrial integrity by the intrinsic apoptosis pathway results in the release of cytochrome c into the cytosol and activation of caspase-9 (Luu et al., 2002). As shown in Fig. 3(e), caspase-9 activity was increased 10-fold in cells expressing either CP7 NS2–4A or NS3/4A and this was dependent on the protease activity of NS3/4A. In addition to the intrinsic pathway, the extrinsic pathway has also been linked with CP BVDV-induced apoptosis (Yamane et al., 2005). This pathway is classically associated with death receptor-mediated apoptosis and is mediated by activation of caspase-8 (Kiechle & Zhang, 2002). We examined the level of caspase-8 activity in cells expressing NS2–4A from NCP and CP BVDV. Levels of caspase-8 increased slightly in cells expressing CP NS2–4A (threelfold; Fig. 3f) and were again dependent on the protease activity of NS3/4A. However, these increases were significantly less than those observed for caspase-9 and caspase-3.

**BVDV NS2–4A does not block the induction of IFN-β**

It is well established that infection with NCP BVDV does not induce type I IFNs (Schweizer & Peterhans, 2001; Gil et al., 2006). Studies have traced this immune evasion, in part, to the N-terminal BVDV protease Npro (Hilton et al., 2006). Furthermore, recent studies suggest that CP variants of BVDV may also share this property and can effectively suppress IFN-β induction (Baigent et al., 2004; Chen et al., 2007). Given that NCP- and CP-derived NS2–4A differed in their ability to induce apoptosis, we investigated whether the two biotypes had a differential effect on the induction of an IFN response. Therefore, we set out to determine whether the NS2–4A proteins of BVDV play a role in the immune-evasion properties of NCP BVDV. To test this, HEK-293 cells expressing Toll-like receptor 3 (HEK–TLR3) were transfected with a reporter construct containing the IFN-β promoter together with constructs expressing BVDV NS2–4A or NS3/4A. As a control for these experiments, cells were also transfected with a construct expressing HCV NS3/4A (Aoubala et al., 2001). To stimulate the reporter, cells were treated with a synthetic dsRNA, poly(I:C). As expected, poly(1:C) treatment induced a significant increase in luciferase levels, consistent with induction of the IFN-β promoter (Fig. 4a), and co-transfection with the HCV NS3/4A wild type reduced this increase. Importantly, a previously described HCV NS3/4A construct containing a mutation in the DEAD-box motif of helicase (NS3/4A D316A/E1317A) failed to eliminate induction of the IFN-β promoter (data not shown). We have shown previously that this mutation disrupts the interaction between NS3 and NS4A and reduces NS3 protease activity by 20-fold (Aoubala et al., 2001; data not shown). Co-transfection with the BVDV NS3/4A construct had no effect on stimulation of the IFN-β promoter by poly(1:C) treatment, which is in agreement with recently published data (Hilton et al., 2006; Chen et al., 2007b). Furthermore, we verified that any inhibition of dsRNA-mediated IFN-β induction was not being masked by BVDV NS3/4A-mediated apoptosis, as none of the active-site mutants...
described above was able to block stimulation of the IFN-β promoter (data not shown). Given that BVDV NS3/4A was unable to block stimulation of the IFN-β promoter, we then tested whether NCP NS2–4A or CP NS2–4A was able to modulate the response to dsRNA. As shown in Fig. 4(a), neither NCP nor CP NS2–4A had an effect on the IFN-β promoter in response to poly(I:C). Western blotting was used to confirm that all viral proteins were expressed at similar levels.

Retinoic acid inducible gene I (RIG-I) signalling also contributes to defence against members of the family
**Flaviviridae** (Loo et al., 2008). To assess whether NS2–4A could affect RIG-I signalling, we expressed the IFN-β reporter plasmid in MDBK cells and challenged them with Sendai virus (SeV), an activator of RIG-I. Infection with SeV led to a small but significant increase in IFN-β promoter-driven reporter levels (Fig. 4b). Significantly, NS2–4A proteins from NCP and CP BVDV were unable to inhibit SeV-mediated induction of IFN-β (Fig. 4b). In the same experiment, HCV NS3/4A was able to block activation of the reporter. The SeV data were confirmed by studies using the essential mitochondrial adaptor protein IFN-β promoter stimulator 1 (IPS-1). Overexpression of this protein led to an increase in luciferase levels (Fig. 4c). Importantly, levels of the reporter were not affected by proteins from the NCP or CP strains of BVDV. By comparison, HCV NS3/4A effectively blocked activation of the reporter, consistent with the cleavage of IPS-1 by HCV NS3/4A to inhibit RNA helicase-mediated induction of IFN-β (Meylan et al., 2005).

**DISCUSSION**

Induction of apoptosis plays a significant role in the pathology of CP BVDV infection, both in infected cells and in relation to the clinical manifestations of mucosal disease. Although expression of free NS3 (i.e. not fused to NS2) is considered a marker of cytopathogenicity, it is not clear whether NS3 directly induces apoptosis. Previously, it was shown (St Louis et al., 2005) that BVDV NS3, expressed in the absence of NS4A from an adenoavirus vector, and CSFV NS3 expressed as a green fluorescent protein fusion (Xu et al., 2007) were able to induce apoptosis. However, the physiological significance of these results is questionable for two reasons. Firstly, in the case of HCV, NS3 expressed in the absence of NS4A exhibits an aberrant subcellular localization (predominantly nuclear) and is rapidly degraded (half-life of 3 h), in comparison with NS3 expressed in the context of the NS3/4A complex (Wölk et al., 2000). Secondly, an N-terminal deletion of 50 residues had no effect on the ability of NS3 to induce apoptosis (St Louis et al., 2005), and, as previously demonstrated, such a deletion would be predicted to abolish protease activity (Tautz et al., 2000). To clarify this situation, we used transient expression of wild-type and protease/helicase mutant derivatives of BVDV NS3, both alone and in the context of the NS2–3 fusion protein, which in all cases included NS4A. Our data showed that expression of either NS3/4A or CP NS2–4A mediated significant increases in apoptosis, which were not observed in NCP NS2–4A-expressing cells, indicating that free NS3/4A is able to induce apoptosis. Furthermore, our data demonstrated that CP7 BVDV induces significant caspase-3 activation compared with mock-infected cells (Fig. 3). Importantly, protease mutants of NS3/4A (Fig. 3c) or CP NS2–4A (data not shown) failed to induce caspase-3 activity, suggesting a role for the protease activity in the induction of apoptosis. In an effort to determine the mechanism for CP BVDV-induced apoptosis, we measured the activation of caspase-9, a protease activated by the ER and mitochondrial stress (Masud et al., 2007). Our data showed that caspase-9 was activated in cells infected with CP7 BVDV or expressing NS3/4A. In this regard, CP BVDV infection can induce ER stress-mediated apoptosis and activate caspase-12 (Jordan et al., 2002). It is possible that proteolytic activation of an ER stress-sensing factor such as ATF-6, or indeed the direct cleavage of caspase-12 by NS3/4A, may be at least partially responsible for the induction of apoptosis seen following CP BVDV infection. Interestingly, we also noted a small increase in the levels of caspase-8 activity in cells infected with CP7 BVDV (Fig. 3f). Caspase-8 activity is associated with extrinsic receptor-mediated cell death. Whether the activation of caspase-8 is directly caused by BVDV, by an interaction or upregulation of Fas expression, or by cross-talk with caspase-9 remains to be determined.

During these studies, we were able to confirm that cells infected with NCP BVDV produced cleaved NS3 protein at early time points (Lackner et al., 2004). However, we did not observe significant levels of apoptosis in these cells. Currently, we lack a mechanism to explain this observation. It is possible that, during the early stages of virus
infection when mature NS3 is being produced by NCP BVDV, a host protein is able to protect against apoptosis. This implies that the protector protein is rapidly depleted (by 12 h p.i.) and that continued expression of NS3, seen in CP BVDV-infected cells, induces apoptosis.

A number of flaviviruses have been shown to induce apoptosis following infection, including Langat virus (Prikhod’ko et al., 2002), dengue virus (Despré’s et al., 1996, 1998), West Nile virus (Parquet et al., 2001; Yang et al., 2002), Japanese encephalitis virus (Tsao et al., 2008) and HCV (Deng et al., 2008). Furthermore, studies have indicated the involvement of flaviviral NS3 protease domains in the induction of apoptosis (Prikhod’ko et al., 2002; Shafee & AbuBakar, 2003; Ramanathan et al., 2006; Tsao et al., 2008). In conjunction with the data presented here, this suggests that induction of apoptosis by NS3 is a common theme in the flaviviruses and merits further investigation.

Although infection with NCP BVDV blocked IFN induction in cell culture, previous studies have provided
contradictory results when describing the ability of CP BVDV to trigger IFN (Chen et al., 2007a). Recent work has focused on the ability of the N^pro protein to subvert IFN activation by increasing the proteasomal degradation of the IRF3 transcription factor (Hilton et al., 2006; Seago et al., 2007). However, N^pro is functional in both CP and NCP strains of BVDV, and yet persistence is established only in fetuses infected with the NCP virus (Charleston et al., 2001; Peterhans et al., 2003). The fact that both NCP and CP BVDV share similar mechanisms for eliminating the expression of IRF3-mediated host defence suggests that this by itself is not sufficient for persistence. Therefore, we explored the possibility that the NS2–4A fusion protein from NCP BVDV may act as an antagonist of IFN-β activation. Interestingly, neither the NCP nor CP biotype NS2–4A protein was able to inhibit an IFN-β promoter-driven reporter activated by either of the major pathogen receptors TLR3 or RIG-I. Furthermore, unlike the related viruses HCV and GB virus-B, the N3/4A protease of BVDV was unable to inhibit the activation of IFN-β. The significance of the differential mechanisms for immune evasion by hepaciviruses and pestiviruses has yet to be determined. Our data are therefore consistent with a distinct biochemical action of BVDV NS3/4A. We propose that the switch from NCP to CP biotype does not necessarily involve a loss in the ability of N^pro to block dsRNA signalling, but rather is a gain in the ability to induce apoptosis. This would result in the marked cytopathogenicity associated with CP BVDV infection. Further experimentation in the context of virus-infected cells and transient expression is now required to elucidate the mechanisms by which NS3/4A modulates apoptotic signalling cascades.

METHODS

Virus stocks. Stocks of CP7 and NCP7 biotypes of BVDV (Corapi et al., 1988) were kindly provided by Munir Iqbal (Institute for Animal Health, Compton Laboratory, UK). Madin–Darby bovine kidney (MDBK) cell monolayers were infected with virus at a m.o.i. of 5. Infection efficiencies ranged from 70 to 80% as judged by routine immunofluorescence staining for the E2 glycoprotein.

Plasmid constructs. NS3/4A was amplified by PCR using the plasmid pa/BVDV as template (Meyers et al., 1996) and cloned into the eukaryotic expression vector pSG5 (Green et al., 1988) to generate pSG5 NS3/4A. To generate constructs containing the coding sequences for NS2 to NS4A, a modified pSG5 vector (designated pSG6) was first generated by ligation of oligonucleotides with SbfI and XmaI restriction sites into BamHII-digested pSG5. A KpnI/BglII fragment of pa/BVDV, lacking the 5′ and 3′ termini of the NS2–4A coding region, was cloned into the Litmus28i vector. The 3′ terminus of NS3 and the complete NS4A sequence were amplified by PCR, digested with BglII and SmaI and ligated onto the 3′ end of the Litmus28i vector containing the incomplete NS2/3 coding sequences. The 5′ end of the NS2–4A coding sequence was generated by PCR from either CP (pa/BVDV) or NCP (pa/BVDV-Ins, a derivative of pa/BVDV reconstructed to remove the 27 bp insertion present in NS2 of CP7) templates, either containing or lacking the 27 bp insertion responsible for BVDV CP7 cytopathogenicity (Meyers et al., 1996). The complete NCP and CP NS2–4A coding sequences were then ligated into pSG6 using SbfI and XmaI. Mutated derivatives were generated by overlap PCR (Higuchi et al., 1988). All DNA inserts were sequenced to completion to confirm identity. Primer sequences are available on request. The constructs expressing HCV NS3/4A have been described previously (Aoubala et al., 2001). The sequence encoding IPS-1 was amplified from murine liver total RNA by RT-PCR using Superscript III (Invitrogen) (primer sequences are available on request). The resulting product was cloned into pCR2.1 and sequenced to completion. The insert was then digested with BamHII and NotI and cloned into pCMV5.

Cell culture. MDBK cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% heat-inactivated horse serum, 100 U penicillin ml^-1 and 100 μg streptomycin ml^-1. HEK293 cells stably expressing TLR3 were purchased from InvivoGen and cultured in DMEM supplemented with 10% heat-inactivated calf serum, 100 U penicillin ml^-1, 100 μg streptomycin ml^-1 and 10 μg blasticidin ml^-1.

Transfection and electroporation. Plasmid DNA was introduced into MDBK cells by polyethyleneimine transfection (Polysciences) according to the manufacturer’s instructions or by electroporation. Briefly, cells were trypsinized, washed twice and resuspended at a density of 2 × 10^6 cells ml^-1 in PBS. A volume of 400 μl (8 × 10^6 cells) was mixed with 1 μg plasmid DNA and placed in a 4 mm electroporation cuvette. Cells were pulsed in a Bio-Rad Gene Pulser II at 270 V, 950 F, and resuspended in an appropriate volume of medium. Electroporation efficiencies ranged from 40 to 60%, as judged by immunofluorescent staining of cells with an anti-BVDV NS3 monoclonal antibody (mAb 8.12.7; Corapi et al., 1990).

Immunodetection of NS3. Anti-BVDV NS3 antibody (mAb 8.12.7) was used in the detection of NS3 by both Western blotting and immunofluorescence staining. For the latter, the cells on glass coverslips were processed by fixing with methanol at −20 °C, followed by permeabilization in methanol/acetone at −20 °C. Cells were washed with PBS and blocked in PBS/1% BSA for 30 min. Cells were then incubated with anti-NS3 antibody for 1 h in PBS/1% BSA and washed with PBS prior to incubation with Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen Molecular Probes) in PBS/1% BSA for 1 h at room temperature. Cells were then fixed in 1% paraformaldehyde and resuspended in an appropriate volume of PBS. Immunofluorescent staining was performed using Citifluor (Agar Scientific). The cells were washed and mounted on coverslips with mounting medium. The cells were then viewed using a Zeiss 510-META laser-scanning confocal microscope under an oil immersion ×63 objective lens (NA = 1.40). Alexa Fluor 488 (494 nm excitation, 519 nm emission) was excited using an argon laser fitted with 488 nm filters and Alexa Fluor 594 (550 nm excitation, 570 nm emission) was excited using a helium/neon laser fitted with 543 nm filters. The images shown are representative and are displayed as single optical sections of 50 μm thickness. The thresholds of each channel were set at 10% of the maximum intensity and maintained throughout image capture.

Flow cytometry assays. Cells were detached from monolayers. For intracellular staining, cells were fixed in paraformaldehyde and permeabilized with Triton X-100. Cells were stained with primary antibody (anti-BVDV NS3, anti-cleaved caspase-3 (Cell Signalling Technologies) or anti-cleaved PARP (Cell Signalling Technologies)) or fluoresein isothiocyanate-labelled annexin V (Roche) at room temperature for 15–30 min according to the manufacturer’s protocol. For annexin V assays, cells were mixed with binding buffer containing 5 μg propidium iodide ml^-1 and analysed immediately using a FACScalibur (BD Biosciences). For other assays, cells were incubated with labelled secondary antibody for 30 min at room temperature.
before analysis using a FACSCalibur. The resulting data were analysed using Flowjo software (Treestar).

**Nuclear fragmentation assays.** Cell monolayers were fixed in 3% paraformaldehyde (5 min) and permeabilized in ice-cold methanol/acetone (5 min) prior to staining with 4’,6-diamidino-2-phenylindole (DAPI). Randomly chosen fields were observed by fluorescence microscopy, and cells with fragmented nuclei were considered to be apoptotic. Data were expressed graphically as the percentage of apoptotic cells.

**Luminescent caspase assays.** Levels of activated cysteine protease caspase-3, caspase-8 and caspase-9 were determined using a Caspase-Glo kit (Promega) according to the manufacturer’s instructions. Briefly, substrate was added to cultured cells in a 12-well dish. The plate was incubated at room temperature for 30–60 min. Samples were analysed on an EG&G Berthold luminometer. The resulting values were then converted to a fold increase from the mock-infected/transfected sample, to demonstrate the increase in caspase activity.

**Dual-luciferase reporter assays.** Reporter assays were carried out as described previously (Macdonald et al., 2003). Briefly, cells expressing BVDV/HCV proteins were mock treated, incubated with poly(I:C) or challenged with SeV for 16 h and then lysed and assayed for luciferase activities using Dual-Luciferase Stop & Glo reagents (Promega) and a luminometer (EG&G Berthold). All assays were performed in triplicate, and each experiment was repeated a minimum of three times. Promoter activity was calculated by dividing the relative luciferase activity of stimulated cells by that of mock-treated cells.

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