The Amino-Terminal Domain of Bovine Viral Diarrhea Virus N\textsuperscript{pro} Protein Is Necessary for Alpha/Beta Interferon Antagonism

Laura H. V. G. Gil,\textsuperscript{1}† Israrul H. Ansari,\textsuperscript{1}§ Ventzislav Vassilev,\textsuperscript{4}‡ Delin Liang,\textsuperscript{1}‡ Vicky C. H. Lai,\textsuperscript{2} Wei Dong Zhong,\textsuperscript{2} Zhi Hong,\textsuperscript{2} Edward J. Dubovi,\textsuperscript{3}§ and Ruben O. Donis\textsuperscript{1}‡

Departments of Veterinary and Biomedical Sciences, University of Nebraska—Lincoln, Lincoln, Nebraska 68583-0905; Population and Diagnostic Medicine, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 18453; and Valeant Pharmaceuticals, Valeant Plaza, 3300 Hyland Avenue, Costa Mesa, California 92626

Received 3 November 2004/Accepted 24 October 2005

The alpha/beta interferon (IFN-\alpha/\beta) system is the first line of defense against viral infection and a critical link between the innate and adaptive immune responses. IFN-\alpha/\beta secretion is the hallmark of cellular responses to acute RNA virus infections. As part of their survival strategy, many viruses have evolved mechanisms to counteract the host IFN-\alpha/\beta response. Bovine viral diarrhea virus (BVDV) (genus Pestivirus) was reported to trigger interferon production in infected cultured cells under certain circumstances or to suppress it under others. Our studies with various cultured fibroblasts and epithelial bovine cells indicated that cytopathic (cp) BVDV induces IFN-\alpha/\beta very inefficiently. Using a set of engineered cp BVDVs expressing mutant N\textsuperscript{pro} and appropriate controls, we found that the IFN-\alpha/\beta response to infection was dependent on N\textsuperscript{pro} expression and independent of viral replication efficiency. In order to investigate whether the protease activity of N\textsuperscript{pro} is required for IFN-\alpha/\beta antagonism, we engineered N\textsuperscript{pro} mutants lacking protease activity by replacement of amino acid E\textsubscript{22}, H\textsubscript{99}, or C\textsubscript{99}. We found that E\textsubscript{22} and H\textsubscript{99} substitutions abolished the ability of N\textsuperscript{pro} to suppress IFN, whereas C\textsubscript{99} had no effect, suggesting that the structural integrity of the N terminus of N\textsuperscript{pro} was more important than its catalytic activity for IFN-\alpha/\beta suppression. A catalytically active mutant with a change at a conserved N\textsuperscript{pro} region near the N terminus (LSP) in both BVDV biotypes did not antagonize IFN-\alpha/\beta production, confirming its involvement in this process. Taken together, these results not only provide direct evidence for the role of N\textsuperscript{pro} in blocking IFN-\alpha/\beta induction, but also implicate the amino-terminal domain of the protein in this function.

The genus Pestivirus comprises an important group of animal pathogens that includes agents such as Bovine viral diarrhea virus (BVDV) and Classical swine fever virus (CSFV). Based on their molecular phylogenies, as well as physical, chemical, and biological properties, the genus Pestivirus is classified in the family Flaviviridae (74). The pestivirus genome consists of a single RNA molecule approximately 12 kb in length and with positive polarity (14, 43). The enveloped virions deliver the genomic RNA to target cells by fusion with endocytic membranes (21). Pestiviruses replicate exclusively in the cytoplasm, generally without inducing death of the infected cultured cells, i.e., most pestiviruses are noncytopathic (ncp) (27).

Much of the economic impact of pestivirus infections results from their tropism for the fetus. Fetal infection with ncp BVDV during the first trimester of gestation results in the birth of calves that are persistently infected and immunotolerant to the viral antigens. These newborn calves develop into apparently healthy animals, which remain viremic for life. The ncp BVDV virus pool within persistently infected animals often gives rise to cytopathic (cp) mutants, which cause a fatal syndrome known as mucosal disease (8, 11, 46, 47, 49, 54). Cp BVDV variants induce apoptosis in cultured cells (29, 30, 32, 76, 81). It is thought that immunologic tolerance allows unchecked replication of these cp BVDV variants, triggering the pathogenic cascade associated with mucosal disease (9). The biochemical hallmark of cp variants is the production of high levels of NS3 as a free protein after the early phase of infection (17, 39, 57). In contrast, ncp BVDV variants produce largely NS2-3 and small amounts of NS3 during the early phase of infection (39). The pathogenesis of the fatal mucosal disease syndrome is poorly characterized; however, excessive stimulation of innate immune responses may play a significant role. Alpha/beta interferons (IFN-\alpha/\beta) are powerful cytokines which regulate cells from most organ systems in the body with regard to their permissiveness to viral infection and many other specialized functions, e.g., differentiation of dendritic cells (25, 50, 55, 70).

The production of IFN-\alpha/\beta in virus-infected cells is transcriptionally regulated (45). IFN-\alpha/\beta gene transcription is activated by multiple signals, the best characterized being viral infection, in which signaling is thought to actually be mediated by double-stranded RNA (dsRNA) (26, 38, 79). Viral infec-
tions, particularly those caused by RNA viruses, involve the production of cRNA molecules in the form of extended dsRNA structures. dsRNA, as well as endoplasmic reticulum stress, oxidative stress, and additional uncharacterized viral signals, activates a cascade of events that converge to activate several transcription factors, such as NF-κB and ATF-2/c-jun, as well as interferon regulatory factor 3 (IRF-3) and IRF-7 (24, 43, 38, 68, 69, 79). The resulting enhanceosome is responsible for the surge of IFN-α/β gene transcription, leading to translation and secretion. IFN-α/β molecules bind interferon receptors (IFN-α/βR) present in virtually all nucleated cells of the body to activate an antiviral program in the target cells. To counter this antiviral response of host cells, many viruses have evolved effectors that actively interfere with the IFN-α/β system or with the downstream interferon-stimulated gene products that mediate the antiviral state (7, 23, 24, 28, 48, 51, 73, 78, 82). Viruses that target the cellular IFN-α/β system have two general modes of action: they either suppress the production of IFN-α/β or interfere with the IFN-α/βR signaling pathway (7, 24, 67).

Previous studies have shown that infection of cultured cells with cp BVDV triggers IFN-α/β production in macrophages and in some nonmammalian tissue cultures, whereas no IFN-α/β is produced by ncp-BVDV production in macrophages and in some nonmammalian tissue cultures, whereas no IFN-α/β is produced by ncp-BVDV. Both cp and ncp BVDVs have been shown to block the function of IRF-3, which is required for IFN-α/β transcription (5, 6). Thus, cp-BVDV infection is reported to either induce or suppress IFN-α/β in infected cells. The Npro and Ems proteins have been reported to participate in IFN-α/β suppression during CSFV infection (34, 41, 64). The Npro protein is dispensable for the growth of BVDV and CSFV in cell culture, but deletion mutants are growth impaired in cell culture (40, 71). The growth impairment of CSFV Npro deletion mutants has been recently linked to interferon antagonism (64). Recently, it was demonstrated that the Npro protein of CSFV can impair IRF-3 activity (33, 41). The genetic analysis of Npro mutants of pestiviruses is complicated by the fact that changes in the RNA sequence that encode this protein can affect polyprotein translation and cleavage of the capsid protein, as well as host cell IFN-α/β responses. The RNA sequences encoding Npro that are adjacent to the internal ribosome entry site (IRES) element mediate translation of the viral polyprotein (53, 60). In this report, we show that cp-BVDV infections do not induce detectable levels of IFN-α/β and that the phenotype depends on Npro expression. We also found that antagonism to IFN-α/β production by viral infection requires the N-terminal domain of Npro, whereas its endoprotease function seems dispensable for this purpose.

**MATERIALS AND METHODS**

**Cells and viruses.** Bovine testicle cells express epithelial markers and were derived as described previously (18). NCL1 bovine uterine cells are also epitheloid (E. J. Dubovi, unpublished data), whereas CRIB cells resistant to BVDV infection (20) were derived from MDBK cells. All cells were grown in minimum essential medium (MEM) supplemented with 10% equine serum (ES; HyClone) and 1% penicillin-streptomycin antibiotics (Life Technologies). The NCL1-ISR-E-Luc-Hygro cell line (L. Gil and R. Donis, unpublished data) is a stable bovine cell line selected for integration of the luciferase reporter under transcriptional control of an IFN-stimulated response element (ISRE); it was cultured in the same medium supplemented with 300 μg/ml hygromycin (A. G. Scientific, Inc.).

pNADLp15 (p NADL), N-dINs (ncp NADL), N-H/B, and 5B-1HR are modified viruses derived from a plasmid containing the full-length cDNA of the BVDV strain NADL genome, as described elsewhere (3, 40, 76). The mutant 5B-1HR was derived by reverse genetics from the NADL strain of BVDV by insertion of a 19-amino-acid epitope tag at position 704 of NS5B (insertion of 57 residues) and insertion of a 19-amino-acid epitope tag at position 704 of NS5B (insertion of 57 residues). Gag polyprotein of CSFV can impair IRF-3 activity (33, 41). The genetic analysis of Npro mutants of pestiviruses is complicated by the fact that changes in the RNA sequence that encode this protein can affect polyprotein translation and cleavage of the capsid protein, as well as host cell IFN-α/β responses. The RNA sequences encoding Npro that are adjacent to the internal ribosome entry site (IRES) element mediate translation of the viral polyprotein (53, 60). In this report, we show that cp-BVDV infections do not induce detectable levels of IFN-α/β and that the phenotype depends on Npro expression. We also found that antagonism to IFN-α/β production by viral infection requires the N-terminal domain of Npro, whereas its endoprotease function seems dispensable for this purpose.

**RNA transcription and electroporation.** RNA transcripts were prepared using a cRNA transcript kit in vitro transcription kit as recommended by the manufacturer (Ambion). The concentration of purified RNA was determined by staining with RiboGreen RNA quantification reagent (Molecular Probes), as described previously (77).

The in vitro-transcribed RNAs were transfected into bovine uterine cells by electroporation as described previously (3). Briefly, bovine uterine cells were trypsinized, washed twice with MEM, and resuspended at 2 × 10⁵ cells/ml in optimax (120 mM KCl, 0.35 mM CaCl₂, 10 mM K₂HPO₄/K₂HPO₄, pH 7.6, 25 mM HEPES, pH 7.6, 2.0 mM EGTA, 5.0 mM MgCl₂ plus 2.0 mM of ATP and 5.0 mM of glutathione (72). Unless otherwise indicated, 3 μg of transcribed RNA was mixed with 0.1 ml of cells in suspension and immediately pulsed with a BTX-600 ElectroSquarePorator (320 V; 48 Ω). The electroporated cells were diluted in 10 ml of MEM-ES and plated on tissue culture dishes.

**Virus preparation.** All experiments aimed at measuring IFN-α/β responses to virus infection were performed using pelleted virus preparations to infect cells. Viruses were inoculated onto NCL1 cells with seed virus at an input multiplicity of infection (MOI) of 1. The cell culture fluid was harvested at 24 h postinfection (p.i.) and clarified by low-speed centrifugation (2,000 × g) for 30 min. The clarified culture fluid was centrifuged at 100,000 × g for 2 h. The supernatant was discarded, and the virus-containing pellet was washed, resuspended in a small volume of MEM with 5% horse serum, and frozen in aliquots. Stocks used in these studies had titers of 7.5 to 7.7 median tissue culture infective doses (CTD).
RNA infectivity determination by infectious-center assay. An infectious-center assay was used to quantify the specific infectivity of in vitro-transcribed RNA. Tenfold dilutions of electroporated bovine uterine cells were added to preseeded monolayers of MDBK ce lls grown to 60% confluence. Following 4 h of incubation, the cells were washed and overlaid with 0.1% agarose in MEM-5% equine serum. The plates were incubated for 3 days at 37°C, and infectious centers were visualized and counted by staining the plaques as described above.

Northern blots. Bovine testicle cells (106 cells; 60-mm dish) were infected with purified BVDV at 10 PFU/cell. At 1 h postinfection, the cell monolayers were washed with 5 ml of PBS, followed by the addition of 5 ml of MEM containing 5% ES. The total RNA from bovine uterine cells was prepared by using an RNasy total-RNA kit (QIAGEN). Ten micrograms of total RNA was separated on a formaldehyde-containing agarose gel, transferred onto a nylon membrane (Hybond N+; Amersham Biosciences), and probed by hybridization to a DNA fragment spanning nucleotides 6040 to 6432 of BVDV-NADL labeled with [α-32P]dATP by a random priming method (Prime-a-Gene Labeling System, Promega). The membranes were twice washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)+0.1% sodium dodecyl sulfate (SDS) at room temperature and once in 0.1× SSC+0.1% SDS at 60°C for 30 min and exposed to X-ray film (O-Mat; Kodak). The blots were rehybridized with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probe to ascertain the lane loading and transfer efficiency. Scanning densitometry was achieved by PhosphorImager analysis with ImageQuant software (Molecular Dynamics). The signal intensities from BVDV-specific bands were normalized by comparison to the signal from the cellular housekeeping gene GAPDH mRNA.

Western blots. Proteins were separated by SDS-polyacrylamide gel electrophoresis (10% or 12% acrylamide) and transferred onto a nitrocellulose membrane (Hybond; Amersham Life). The membrane was blocked with 5% (wt/vol) dried skim milk in PBS containing 0.005% Tween 20. For the detection of the Npro protein, membranes were probed with polyclonal antibody specific against the Npro protein of BVDV, followed by incubation with a secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody. The NS3 or NS2-3 protein of BVDV was detected by using the monoclonal antibody 20.10.6.6, which is specific for the pestivirus nonstructural protein NS3, and the bound antibodies were detected by horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham). The membranes were rinsed five times in PBS containing 0.005% Tween 20 after primary and secondary antibodies. The membrane-bound immune complexes were revealed by the enhanced-chromiluminescence detection system (Amersham), and images were captured on X-ray film.

Reporter plasmids and electroporation. pIFN-beta/CAT expresses chomaphenicol acetyltransferase (CAT) under the control of the IFN-β enhancer element (19), pISRE-Luc expresses firefly luciferase under the control of an IFN-β promoter cassette derived from the hepatitis C virus (HCV) NS3 protease domain. The two Npro deletion mutants were deletion mutants, one (N-dNpro) in which the Npro protease domain was completely deleted and the other (N-H/B) in which Npro was replaced with a heterologous autocatalytic cleavage cassette derived from the hepatitis C virus (HCV) NS3 protease domain. The luciferase assay was performed by using the dual-luciferase (firefly and Renilla) assay system (Promega) according to the manufacturer's protocol. Expression levels were normalized for transfection efficiency by dividing the firefly luciferase or CAT values by those of a co-transfected Renilla luciferase plasmid. The luciferase activity was measured as described above. This assay is sensitive to bovine IFN-α/β and detected IFN-α/β levels as low as 5 IU/ml (Gil and Donis, unpublished).

RESULTS

IFN-α/β secretion by cells infected with Npro mutants of BVDV. The presence of a nonstructural protein at the 5’ end of the polyprotein is a defining feature of pestiviruses. This polypeptide, termed Npro, is an unconventional protease capable of autocatalytic cleavage at its own C terminus (65, 80). To characterize the role of the Npro protein, a series of Npro BVDV mutants were used (Fig. 1). Two of the Npro mutants were deletion mutants, one (N-dNpro) in which the Npro protein was completely deleted and the other (N-H/B) in which Npro was replaced with a heterologous autocatalytic cleavage cassette derived from the hepatitis C virus (HCV) NS3 protease domain. The Npro deletion mutants were previously found to be phenotypically similar (40). The growth curve of a mutant virus lacking Npro showed that the virus progeny levels were ~10-fold lower than those of wild-type (wt) virus at each of the time points (Fig. 2B) (40). This observation was correlated with an ~3-fold reduction of specific infectivity of the RNA compared to that of the wt virus (Fig. 2A). The infectivity of the RNA, growth kinetics, plaque size, and RNA accumulation revealed that the N-H/B chimeric virus is similar to the N-dNpro mutant in its replication efficiency (Fig. 2A to D). The third Npro mutant, termed N-Pro18GFP, contains the jellyfish green fluorescent protein (GFP) gene cloned within the N-terminal region of Npro, between codons 18 and 19 (Fig. 1). Although the autocatalytic protease activity of this Npro chimeric remained intact (data not shown), the virus formed small plaques like the other two mutants (Fig. 2C). The analysis of cellular responses to infection by Npro deletion and chimeric mutants is complicated by the fact that Npro mutant viruses have reduced replication efficiency and, consequently, delayed induction of apoptosis compared to the wt virus. Thus, to test the hypothesis that increased cytokine production and secretion in cells infected with Npro mutants is not attributable to a decreased impairment of host cell metabolism compared to that of cells infected by wt virus, we also developed and char-
characterized an isogenic BVDV mutant with an altered NS5B polymerase gene, termed 5B-1HR. Comparative analysis of this mutant with the Npro deletion viruses revealed that it displays a degree of replication deficiency similar to that observed with Npro mutants (Fig. 2C and D).

We used our panel of mutant chimeric and mutant cp BVDVs and several bovine cell lines to assess the effects of viral infection on IFN-α/β production. To this end, we first used the VSV bioassay to measure IFN-α/β levels in the culture media of infected cells. No detectable IFN was present in the culture medium, suggesting that induction of IFN-α/β proteinase activity of Npro are lethal due to improper formation of infectious virions (40). To overcome this problem, we engineered a virus whose genome consisted of the N-H/B chimeric BVDV, the open box represents the HCV-derived HCV NS3 protease domain (residues 3 to 181), and the patterned boxes represent NS4A cofactors and the NS4B/5A cleavage site. The amino acid sequences (single-letter code) of Npro (partial) and the NS4A-NS5B boundary (underlined) are indicated. The organization of Npro shows that only the initiator methionine was retained; the rest of Npro was deleted. The genome organization of N-Npro18GFP shows the enhanced GFP (EGFP) protein (open box) cloned between amino acids 18 and 19 of the Npro protein. Mutant 5B-1HR (bottom) is different from the wt only at the C terminus of NS5B; this region is shown expanded to highlight the linker insertion (underlined).

FIG. 1. Genome structures of wild-type cp BVDV strain NADL and engineered derivatives. The genome organization of BVDV strain NADL and its encoded proteins is shown (center), with the cis cleavage of Npro denoted by a curved arrow. In the genome structure of the HCV NS3 protease-dependent BVDV (N-H/B) chimeric BVDV, the open box represents the HCV-derived HCV NS3 protease domain (residues 3 to 181), and the patterned boxes represent NS4A cofactors and the NS4A/5B cleavage site. The amino acid sequences (single-letter code) of Npro (partial) and the NS4A-NS5B boundary (underlined) are indicated. The organization of Npro shows that only the initiator methionine was retained; the rest of Npro was deleted. The genome organization of N-Npro18GFP shows the enhanced GFP (EGFP) protein (open box) cloned between amino acids 18 and 19 of the Npro protein. Mutant 5B-1HR (bottom) is different from the wt only at the C terminus of NS5B; this region is shown expanded to highlight the linker insertion (underlined).

The high levels of IFN-α/β released into the media of cells infected with Npro deletion mutants could result from transcriptional or posttranscriptional regulation events. To investigate these possibilities, cells were transiently transfected with human IFN-β–CAT reporter or ISRE-Luc reporter plasmids and subsequently infected with wt or Npro mutant viruses. Cells infected with the Npro mutants showed 40- to 70-fold- and 80- to 145-fold-increased levels of activation of the IFN-β and ISRE promoters at 20 h.p.i. compared to the wt virus or the 5B-1HR mutant (Fig. 3A and B). Cells infected with N-dINS (ncp NADL) and wt NADL showed background levels of IFN-α/β promoter activation identical to those of mock-infected cells, in agreement with our earlier data (Table 1). These results implicate IFN-α/β gene transcription regulation as the major determinant of secretion cytokine levels and reveal the possible autocrine or paracrine action of the secreted IFN-α/β in the activation of the ISRE enhancer element.

Role of the catalytic domain of Npro in IFN-α/β antagonism. Recent studies showed that the HCV NS3/4A serine protease blocks the phosphorylation of IRF-3, a key cellular antiviral signaling molecule (22). To investigate the importance of the protease activity of Npro in IFN-α/β antagonism, we utilized a reverse genetics approach targeting residues E_22, H_49, and C_69, individually known to be essential for the catalytic activity of Npro in pestiviruses (65). However, mutations that abolish the protease activity of Npro are lethal due to improper maturation of the capsid protein, which in turn prevents the formation of infectious virions (40). To overcome this problem, we engineered a virus whose genome consisted of the N-H/B genome (Fig. 1) with the Npro protein cloned upstream of HCV NS3 protease (Fig. 4A). The restored virus, termed N-
Npro-H/B produced plaques slightly larger than those of the parental virus N-H/B (2.45 ± 0.44 mm in diameter versus 1.7 ± 0.29 mm), which we hypothesized stemmed from the restoration of Npro function (Fig. 4B). To determine whether the improved growth phenotype of N-Npro-H/B involved IFN-α/β inhibition, we analyzed the production of IFN-α/β. No detectable IFN-α/β activity was present in the medium of bovine testicle cells infected with wt NADL, 5B-1HR, and the different Npro mutant viruses was performed using bovine testicle cells; the plates were stained with crystal violet after incubation at 37°C for 4 days. Mean plaque diameter values ± standard errors of the mean are shown. (D) RNA accumulation in bovine uterine cells infected with 5B-1HR, wt NADL, N-dINS, N-dNpro, and N-H/B; Northern analysis of total cellular RNA extracted from bovine cells harvested 16 h after infection with an MOI of 10. RNA ac., normalized viral RNA accumulation.

**TABLE 1. IFN production in bovine cells**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Testicle</th>
<th>Uterine</th>
<th>MDBK</th>
<th>CRIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt NADL</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>N-dINS</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>5B-1HR</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>N-dNpro</td>
<td>600</td>
<td>2,400</td>
<td>150</td>
<td>&lt;5</td>
</tr>
<tr>
<td>N-H/B</td>
<td>600</td>
<td>2,400</td>
<td>150</td>
<td>&lt;5</td>
</tr>
<tr>
<td>N-Npro18GFP</td>
<td>600</td>
<td>2,400</td>
<td>150</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Poly (I · C)b</td>
<td>4,800</td>
<td>4,800</td>
<td>4,800</td>
<td>4,800</td>
</tr>
</tbody>
</table>

*a 1U/ml in culture medium from BVDV-infected CRIB cells collected at 24 h p.i.

*b 100 μg/ml of poly(I · C) for 24 hours.
lacked the 20-kDa protein and expressed only a fusion protein of approximately 40 kDa, comprising the mutated Npro and the HCV protease. Interestingly, the cleavage of Npro by N-Npro-H/B is incomplete, and substantial amounts of the Npro-HCV protease fusion protein are detected. Regardless of the cause of the reduced catalytic function of Npro as an endoprotease, the plaques formed by the parental virus N-Npro-H/B and the N-Npro-H/B-C69A mutant were comparable in size (1.3 ± 0.3 mm and 1.4 ± 0.3 mm in diameter, respectively), suggesting similar replication efficiencies. However, the plaques produced by the N- Npro-H/B-E22L and N- Npro-H/B-H49V mutants were smaller (0.6 ± 0.19 mm and 0.76 ± 0.29 mm, respectively) (Fig. 5C).

We next analyzed IFN-α/β induction in the culture media of cells infected with each protease mutant (Fig. 5D). Interestingly, cells infected with the E22 and H49 mutants induced high levels of IFN. In contrast, the C69 mutant induced background levels of IFN-α/β, suggesting that the catalytic endoprotease activity of Npro is dispensable for the IFN-α/β antagonist function. In this context, the lack of IFN-α/β antagonism of mutants E22 and H49 could be explained by disruption of a hypothetical IFN-α/β regulatory domain located near the N terminus of Npro.

A conserved domain at the N terminus of Npro is essential for IFN antagonism. Alignment of multiple pestivirus Npro sequences revealed a 5-amino-acid sequence motif (LLYKT; codons 8 to 12) near the amino terminus of the protein as the largest conserved block in the region. In order to study the contribution of this conserved domain to IFN antagonism, we constructed a mutant viral genome termed N-L8P, in which the

FIG. 3. Induction of IFN-β promoter and IFN signaling in bovine cells. (A) Activation of the IFN-β promoter by infection with Npro mutants. Bovine cells were electroporated with IFN-CAT (A) and ISRE-Luc (B) reporter plasmids, and after 24 h of incubation, they were mock infected or infected with different BVDVs as indicated. The data were normalized by Renilla luciferase expression, and the induction was calculated relative to the mock-infected cells. The error bars indicate standard deviations.

FIG. 4. Viable BVDV mutants expressing Npro and HCV NS3 protease. (A) Genome organizations of N-H/B and N-Npro-H/B viruses. Npro protein (black-filled box) was cloned upstream of the HCV-NS3 protease of the N-H/B virus. The HCV NS5A-NS5B boundary amino acids are underlined. (B) Plaque phenotypes of wt NADL, N-H/B, and N-Npro-H/B. Bovine testicle cells were infected with the different viruses for 4 days at 37°C. Plaque formation was revealed by crystal violet staining. Mean plaque diameters ± standard error values are shown. (C) Interferon induction by the Npro restored virus (N-Npro-H/B). Harvested culture media were assayed for interferon using the ISRE reporter cell line as described in Materials and Methods. The induction was calculated relative to values from mock-infected cells. The error bars indicate standard deviations.
FIG. 5. Structure and characterization of BVDV mutants with catalytically inactive N\textsuperscript{pro}. (A) Schematic representation of N\textsuperscript{pro} protease mutants. The N-terminal region of the N-N\textsuperscript{pro}-H/B genome is shown at the top. The amino acid sequences (single-letter code) of N\textsuperscript{pro} (partial), the conserved domain (open box), and the 3 amino acids known to be critical for catalysis (boldface) are indicated. (B) Western blot analysis of N\textsuperscript{pro} of wt NADL, N-N\textsuperscript{pro}-H/B, and catalytic mutants. Cell lysates were analyzed in a Western blot probed with anti-N\textsuperscript{pro} polyclonal antibody. The positions of N\textsuperscript{pro}-HB fusion protein, N\textsuperscript{pro} (arrowheads), and a nonspecific band (*) are indicated. (C) Plaque phenotypes of wt NADL, N-N\textsuperscript{pro}-H/B, and N-N\textsuperscript{pro}-H/B protease mutants. Bovine testicle cells were infected with the different viruses for 4 days at 37°C and stained with crystal violet. Mean plaque diameter values ± standard errors of the mean are shown. (D) IFN-α/β induction by catalytically inactive N\textsuperscript{pro} mutants. Cells were infected with different N\textsuperscript{pro} protease mutants at an MOI of 1 for 24 h. Harvested culture media were assayed for IFN using the ISRE reporter cell line as indicated above. The induction was calculated relative to values from mock-infected cells. Incr, increase. The error bars indicate standard deviations.
highly conserved leucine at position 8 was changed to proline. The N-L8P virus formed considerably smaller plaques than the wt virus (1.9 ± 0.4 mm versus 2.9 ± 0.95 mm in diameter), resembling the plaques formed by the viruses with Npro deleted (Fig. 6A). This was correlated with an ∼68% reduction in progeny yield at various times after infection (Fig. 6D). The stabilities of Npro and its endoproteinase were examined by Western blot analysis of the steady-state levels of the protein in infected cells. The Western blots of bovine testicle cells infected with the N-L8P mutant revealed that Npro expression levels and endoproteinase activity were similar to those of the parental viruses (Fig. 6B). Interestingly, culture medium from cells infected with N-L8P at an MOI of 10 for 24 h were harvested and assayed for IFN in the ISRE reporter cell line as indicated above. The induction was calculated relative to the values from mock-infected cells. The error bars indicate standard deviations. (D) Growth curve of wt NADL and N-L8P viruses. Bovine uterine cells seeded in 35-mm dishes (5 × 10⁵ cells/dish) were infected at a multiplicity of infection of 0.3. Culture media were harvested at appropriate times and cleared by centrifugation, and the infectivity was determined by endpoint dilution as described in Materials and Methods.

FIG. 6. IFN-α/β induction by L8P Npro mutant BVDV. (A) Plaque morphology of N-L8P mutant compared to wt NADL. Bovine testicle cells were infected with the different viruses for 4 days at 37°C. Plaque formation was revealed by crystal violet staining. Mean plaque diameters ± standard errors of the mean are shown. (B) Western blot analysis of Npro protein expression and processing. Lysates from cells infected for 20 h with wt-NADL probed with anti-Npro polyclonal antibody. The migration of the Npro protein is indicated. (C) IFN production by cells infected with N-L8P mutant BVDVs. Cell culture media from cells infected with N-L8P at an MOI of 10 for 24 h were harvested and assayed for IFN in the ISRE reporter cell line as indicated above. The induction was calculated relative to the values from mock-infected cells. The error bars indicate standard deviations. (D) Growth curve of wt NADL and N-L8P viruses. Bovine uterine cells seeded in 35-mm dishes (5 × 10⁵ cells/dish) were infected at a multiplicity of infection of 0.3. Culture media were harvested at appropriate times and cleared by centrifugation, and the infectivity was determined by endpoint dilution as described in Materials and Methods.

Next, we tested whether N-dINS-L8P virus could block IFN-α/β induction by dsRNA treatment. For this purpose, we used the PINBA previously described (44, 61). The results of this experiment demonstrated that, unlike N-dINS, cells persistently infected with N-dINS-L8P virus are not able to inhibit IFN-α/β induction by dsRNA and consequently were nonsusceptible to VSV infection (Table 2). Taken together, these results strongly suggest that the N-terminal domain of the BVDV Npro protein plays a critical role in IFN-α/β suppression.
DISCUSSION

We exploited a reverse genetics approach to analyze the role of Npro in virus-host interactions. We first studied two BVDV mutants with Npro deleted and one Npro mutant derived from the same infectious clone but structurally distinct at the junction between the IRES element and the polyprotein open reading frame. We also analyzed a mutant with intact Npro that is phenotypically similar to the mutants with Npro deleted with regard to replication efficiency and RNA accumulation. Contrary to the prevailing notion that wt NADL infection induced IFN-α/β, we found that, at least in our systems, infection with wt NADL was indistinguishable from mock infection or ncp BVDV infection. Assay sensitivity cannot explain these differences, because we used a highly sensitive reporter cell line to detect IFN-α/β in concentrations as low as 5 IU/ml (data not shown). Because these observations were the same in different cell lines, we cannot attribute the failure of wt NADL to induce IFN-α/β to host factor variability. Analysis of IFN-α/β in the supernatants of bovine cells infected with different cp BVDV strains detected only low levels of IFN-α/β, which suggests that cp BVDV is a poor IFN-α/β inducer (data not shown).

In contrast to the wt NADL, mutants with Npro deleted elicited very high levels of IFN-α/β, regardless of the type of bovine cell line used. The unrestrained IFN-α/β production triggered by infection with an Npro deletion mutant appeared to contribute significantly to the reduced growth of these mutants. The reduced efficiency of IRES function in the Npro deletion mutants might further contribute to the replicative impairment.

The importance of Npro for IFN-α/β suppression was made clear by the restoration of Npro to the N-H/B deletion mutant virus. N-Npro-H/B showed no IFN-α/β induction and produced

<table>
<thead>
<tr>
<th>TABLE 2. PINBA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell type</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Bovine testicle cells</td>
</tr>
<tr>
<td>Bovine testicle cells</td>
</tr>
<tr>
<td>Bovine testicle cells</td>
</tr>
<tr>
<td>N-dINS</td>
</tr>
<tr>
<td>Bovine testicle cells</td>
</tr>
<tr>
<td>N-dINS-L8P</td>
</tr>
<tr>
<td>Bovine testicle cells</td>
</tr>
</tbody>
</table>

<sup>a</sup> +, present.

<sup>b</sup> CPE, cytopathic effect.

FIG. 7. N-dINS-L8P mutant characterization. (A) N-dINS and N-dINS-L8P mutant focus formation. A focus-forming assay was performed as described in Materials and Methods. Mean plaque diameters ± standard errors of the mean are shown. (B) Western blot analysis of NS3 and NS2-3 expression. Cell lysates from cells infected with wt NADL, N-dINS, N-L8P, and N-dINS-L8P at an MOI of 18 for 18 h were analyzed in a Western blot probed with anti-NS3 monoclonal 20.10.6. The positions of NS2-3 protein, NS3 (arrowheads), and nonspecific bands (*) are indicated. (C) IFN production analysis in acute infection by BVDV Npro mutants. Bovine testicle cells were infected with the different BVDV strains at an MOI of 1. The supernatants were removed from infected cells after 24 h, clarified by centrifugation, added to the ISRE-Luc-Hygro reporter cell line, and incubated for 8 h. The luciferase assay was realized as described in Materials and Methods. The induction was calculated based on the mock-infected cells. The error bars indicate standard deviations.
plaques larger than those of the parental virus (N-H/B), which induced IFN-α/β production. Interestingly, transient expression of Npro by plasmid transfection did not result in functional complementation; the growth of the Npro deletion mutants could not be rescued, presumably because of inefficient Npro expression in transfected bovine cells. Previous reports implicating a viral proteolytic activity in the suppression of host IFN-α/β response by HCV NS3 prompted us to examine whether the Npro endoprotease has an analogous function (22). Mutagenesis of Npro effectively disabled its catalytic activity, confirming the findings of Rumennapf et al. (65). One of the catalytically inactive Npro mutants (C69) showed efficient suppression of IFN-α/β induction, strongly suggesting that the catalytic activity of Npro is dispensable for this function, although the unlikely possibility that an undetected residual protease activity is responsible for IFN-α/β suppression cannot be ruled out. However, the two other mutants (E22 and H49) with a catalytically disabled Npro lost IFN-α/β-antagonistic activity. Although this could point to the importance of the catalytic activity of Npro for IFN-α/β suppression, it is also possible that the structural changes that disable catalysis also disrupt an overlapping domain involved in IFN-α/β antagonism. The second hypothesis is consistent with the identification of Lα as a critical amino acid in a conserved region of the N-terminal domain. Lack of IFN-α/β suppression by mutants L8P, E22L, and H49V suggests that the N-terminal 49 amino acids of Npro are essential for the suppression of IFN-α/β production in infected cells. In addition, the cloning of N-dINS-L8P did not induce high levels of IFN-α/β production as expected. One reason for this unexpected outcome of N-L8P versus N-dINS-L8P might be related to different amounts of dsRNA being formed during replication. Previous data showed that the amount of RNA present in cells infected with cp BVDV vastly exceeds that in cells infected with ncp biotypes (76). Therefore, it is tempting to speculate that a large amount of this viral RNA is implicated in the IFN-α/β-inducing activity of the N-L8P virus.

The identification of Npro as a potential suppressor of IFN-α/β production by BVDV-infected cells provides a candidate target for intervention, since it is becoming increasingly clear that this function is part of the essential machinery of animal viruses. HCV was recently shown to have developed a mechanism to suppress IFN-α/β transcription activation by way of its NS3 protease, by targeting a host factor upstream of IRF-3 (22). Many other RNA and DNA viruses have proteins with demonstrated antagonism for the IFN-α/β system. In the case of BVDV, suppression of IFN-α/β production has traditionally been described as a fundamental property of ncp biotype strains, whereas its role in cp BVDV infections was incompletely defined (6, 12, 66). The similar levels of NS2-3 expression in cells infected with N-dINS and N-INS-L8P suggest that NS2-3 expression may not be essential to prevent IFN-α/β induction.

These findings also have implications for pestivirus vaccine design. The manipulation of the N-terminal region of Npro may allow the engineering of avirulent viruses which could be used as live vaccines. Because suppression of innate immune responses is likely to prove critical for the establishment of persistent fetal infections, it is tempting to speculate that these viruses may be safer than existing vaccines for the developing fetus.

The identification of Npro as a suppressor of IFN-α/β is also consistent with a significant amount of experimental and circumstantial data that associate BVDV infections with immunosuppression. In vitro studies have shown that lymphocytes, macrophages, and neutrophils from normal donor animals infected with BVDV were functionally impaired (37, 52, 62). BVDV infections are known to increase susceptibility to other pathogenic bacteria (58, 59) and viruses, e.g., BVDV and respiratory syncytial virus coinfections result in enhanced respiratory syncytial virus lung pathology (10). The immunosuppression caused by BVDV infection also interferes with immunological assays for Mycobacterium bovis, which compromises the diagnosis of tuberculosis (13). Further studies are needed to determine if these interactions involve the suppression of IFN-α/β by Npro.

ACKNOWLEDGMENTS

This work was supported by USDA/NRI grant 2002-35204-11619 to R.O.D.

We are grateful to all the current and past members of the Donis laboratory for reagents, protocols, and helpful discussions. We also thank F. Osorio for generous gifts of virus strains. We are indebted to L. Babiuk for a gift of IFN-α, to L. Zhang for a gift of pIFN-β-CAT, and to X. Cao for antibodies.

REFERENCES


