Bovine Viral Diarrhea Virus NS3 Serine Proteinase: Polyprotein Cleavage Sites, Cofactor Requirements, and Molecular Model of an Enzyme Essential for Pestivirus Replication

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Members of the Flaviviridae encode a serine proteinase termed NS3 that is responsible for processing at several sites in the viral polyproteins. In this report, we show that the NS3 proteinase of the pestivirus bovine viral diarrhea virus (BVDV) (NADL strain) is required for processing at nonstructural (NS) protein sites 3/4A, 4A/4B, 4B/5A, and 5A/5B but not for cleavage at the junction between NS2 and NS3. Cleavage sites of the proteinase were determined by amino-terminal sequence analysis of the NS4A, NS4B, NS5A, and NS5B proteins. A conserved leucine residue is found at the P1 position of all four cleavage sites, followed by either serine (3/4A, 4B/5A, and 5A/5B sites) or alanine (4A/4B site) at the P1' position. Consistent with this cleavage site preference, a structural model of the pestivirus NS3 proteinase predicts a highly hydrophobic P1 specificity pocket. trans-Processing experiments implicate the 64-residue NS4A protein as an NS3 proteinase cofactor required for cleavage at the 4B/5A and 5A/5B sites. Finally, using a full-length functional BVDV cDNA clone, we demonstrate that a catalytically active NS3 serine proteinase is essential for pestivirus replication.

Pestiviruses such as bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and border disease virus (BDV) comprise a group of economically important animal pathogens affecting cattle, pigs, and sheep (for reviews, see references 61 and 92). These enveloped positive-sense RNA viruses are classified as a separate genus in the family Flaviviridae. Two additional genera of Flaviviridae are the classical flaviviruses, such as yellow fever virus (YFV) and dengue virus, and the hepaciviruses (hepatitis C virus [HCV]) (73).

Pestivirus genome RNAs are typically ~12.5 kb in size and consist of 5' and 3' noncoding regions that flank a long open reading frame (ORF) encoding a single viral polyprotein (see reference 58 for a review). Like those of HCV and the picornaviruses, genome-length pestivirus mRNAs are believed to be uncapped (22), and capping is not necessary for infectivity of non-CP BVDV, cleavage at the 2/3 site leads to the production of both NS2-3 and NS3. For BVDV, the situation is more complex. BVDV isolates can be either cytopathic (CP) or non-CP in cell culture. Remarkably, production of NS3 generally correlates with cytopathogenicity. In the case of non-CP BVDV, cleavage at the 2/3 site does not occur and only uncleaved NS3-2 is observed. In CP BVDV biotypes, both NS3 and NS2-3 products are found in virus-infected cells. Several molecular events can lead to production of NS3, including duplication and rearrangement of pestivirus sequences, insertion of cellular sequences, and large in-frame deletions resulting in subgenomic defective interfering RNAs
(see reference 58 for a comprehensive review of this subject). In the case of the American prototype CP BVDV strain, NADL, a 270-base portion of a bovine mRNA is inserted in the NS2 region (called cnh), resulting in an in-frame insertion of 90 amino acid residues. By mechanisms that are not understood, this insertion leads to partial processing at the 2/3 site, production of both NS2-3 and NS3, and cytopathogenicity in cell culture (54).

Downstream cleavages in the pestivirus NS region (at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites) appear to be mediated by a serine protease activity present in the N-terminal one-third of the NS3 coding region (97). This chymotrypsin-like serine protease domain consists of approximately 180 residues and is present in all members of the Flaviviridae (73). In the flaviviruses, the upstream NS2B protein forms a complex with NS3 and is absolutely required as a cofactor for processing (73). The flavivirus enzyme has a preference for basic amino acids (Arg or Lys [13]) at the P1 cleavage site position (following the nomenclature proposed by Schechter and Berger [82]) and is required for virus replication (17). For the HCV NS3 protease, whose crystal structure has recently been described (41, 52), the downstream 54-residue NS4A polypeptide is an integral component of the serine protease (41) and dramatically stimulates cleavage at some sites (73). The HCV enzyme prefers cyansteine at the P1 position, although threonine is found at the 3/4A processing site (73). For the pestivirus NS3 serine protease, neither the cleavage site specificity nor the cofactor requirements have been clearly defined. Transient expression of truncated BVDV polyproteins has shown that an amino substitution for the putative serine nucleophile (Ser-1842) abolishes cleavage at all four downstream sites (97). Evidence for a pestivirus serine protease cofactor stems from the observation that polypeptide sequences downstream of NS3 are required for cleavage at the 5A/5B site (97).

In this study, we have examined several aspects of pestivirus NS3 serine protease function, using cDNA cloning of the CP BVDV NADL strain. Expression of full-length NADL polypeptides with active or inactive NS3 proteases was used to clarify the cleavages mediated by this viral protease. NS proteases produced by serine protease-dependent cleavage were isolated and their N-terminal sequences were determined, allowing us to define the cleavage site specificity of the pestiviral enzyme and compare this with those of other members of the Flaviviridae. Besides determining precise boundaries for the pestivirus NS proteins for future functional studies, definition of these cleavage sites allowed us to investigate the role of downstream polypeptides, in particular NS4A, in NS3 serine protease-mediated processing. Finally, using a functional NADL cDNA clone capable of producing infectious RNA transcripts (54), we analyzed the effect of the Ser-1842-to-alanine mutation, which inactivates the NS3 serine protease, on BVDV replication.

MATERIALS AND METHODS

Cell cultures, virus stocks, and plaque assays. The BHK-21 and BSC-40 cell lines were maintained in Eagle's minimal essential medium (MEM) supplemented with 2 mM l-glutamine, nonessential amino acids, penicillin, streptomycin, and 10% fetal bovine serum (FBS). The 59 cell line, obtained from the American Type Culture Collection, was maintained in complete TNE-FH medium (Invitrogen) supplemented with amphotericin B and 10% heat-inactivated FBS. VTF7-3, a vaccinia virus recombinant expressing the T7 DNA-dependent RNA polymerase (30), was amplified in BSC-40 monolayers and, after purification, titers were determined by plaque assay on BSC-40 monolayers (38). The baculovirus recombinant bacD2-133, which expresses residues 1591 to 3988 of the NADL polypeptide (68), was amplified and its titer was determined by using Sf9 cells in a 150-mm-diameter dish (approximately 2 £ 106 cells) infected with baculovirus recombinant bacD2-133 (68) at a multiplicity of infection of 5 PFU per cell. After adsorption for 60 min at room temperature, the inoculum was removed and replaced with fresh medium. At 48 h postinfection, the infected monolayers were lysed and the supernatant fluid was harvested and used for virus titer determination by plaque assay on BSC-40 monolayers (38). The bacu-

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FIG. 1. The BVDV polyprotein, region-specific antisera, and expression constructs. The diagram at the top represents the BVDV strain NADL polyprotein, with the locations of the cleavage sites, the structural proteins (shaded boxes), and the NS proteins (open boxes) indicated. Enzymes responsible for proteolytic processing include host signal peptidase ( ), the Npro autoprotease, the NS3 serine proteinase ( ), and as yet unidentified activities (7). Region-specific antisera used in this study are indicated by the subregions (short gray bars) used for immunization. These regions include the following amino acids: 873 to 1015 (D31; E2 specific), 1335 to 1351 (α1335; NS2 specific), 1807 to 2093 (G40; NS3 specific), 2133 to 2480 (B10; react with NS3, helicase region), 2396 to 2414 (α2396; NS4A specific), 2890 to 3192 (α262; NS5A specific), and 3409 to 3692 (B3B; NS5B specific). K1 antiseraum (57) (kindly provided by R. Stark and H.-J. Thiels) was produced against the amino acid 2555 to 2766 subregion of the CSFV polyprotein (homologous to residues 2645 to 2856 of the NADL polyprotein) and reacts with BVDV NS4B (57). Processing sites for the NS3 serine proteinase, as determined in this study by the N-terminal sequences of NS4A (N-terminal residue 2636), NS4B (residue 2427), NS5A (residue 2774), and NS5B (residue 3270), are indicated (vertical shaded lines). Although the precise location of the NADL 2/3 cleavage site has not been determined, Gly-1808 corresponds to the NS3 N terminus determined for a number of other CP pestiviruses (58). Expression constructs, detailed in Materials and Methods, are shown below and drawn to scale to indicate the region of the NADL polyprotein expressed. T7-driven mammalian expression constructs were either pACNR (full-length) (54) or pT3 (all others) derivatives. The baculovirus recombinant (bacD2-133) (68) used to produce NADL NS5A and NS5B is also shown. Regions of the BVDV polyprotein expressed by these constructs are given at the left, as are short names used in the text and subsequent figures. Asterisks denote N- or C-terminally truncated proteins. Mutant derivatives in which the NS3 protease serine nucleophile (Ser-1842) was inactivated by replacement with alanine are indicated (S1842A).

washed twice with cold PBS, harvested by being scraped into PBS, and collected by centrifugation at 10,000 × g for 10 min. The cell pellet was resuspended in ice-cold hypotonic buffer (25 mM Tris-Cl [pH 8.0], 1 mM MgCl2, 10 mM KCl, 20 μg of PMSF per ml, 5 μg of leupeptin per ml, 50 μg of antipain per ml, 1 μg of pepstatin per ml, 0.02% 2-mercaptoethanol) and lysed by 20 strokes with a tight-fitting Dounce homogenizer. The disrupted cells were pelleted by centrifugation at 8,000 × g for 10 min, and the pellet was resuspended in hypotonic buffer containing 1% Triton X-100. After shaking at 4°C for 30 min, the detergent-insoluble fraction was pelleted at 8,000 × g for 10 min. The resulting pellet containing the majority of the BVDV NS proteins was solubilized in SDS sample buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon polyvinylidene difluoride membranes and localized by Coomassie blue staining. Partial amino acid sequence analyses of radiolabeled and unlabelled protein samples were performed as described previously (14, 53).

Sequence alignments and molecular modeling. Sequence alignments were generated by using the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, Wis.) and were used to map the side chains lining the S1 site. The BVDV model was constructed by using elastase (protein database [PDB] entry 1HNE) as a framework, while the YFV model was based on trypsin (PDB entry 1TP). Side chains surrounding the S1 sites were generated by using Quanta96 (Molecular Simulations Inc., San Diego, Calif.). Graphic representations of the pockets were generated by using the program GRASP (66).

For transfection, 1 μg or 100 ng of transcript RNA was added to 200 μl of RNase-free PBS containing 5 μg of Lipofectin (Gibco-BRL). Samples were incubated for 10 min at 4°C. During this time, MDBK monolayers (50 to 70% confluent) grown in six-well plates (35-mm diameter) were washed twice with RNase-free water. For RNA-free PBS containing 5 μg of Lipofectin (Gibco-BRL). Samples were incubated for 10 min at 4°C. During this time, MDBK monolayers (50 to 70% confluent) grown in six-well plates (35-mm diameter) were washed twice with RNase-free PBS. After the final wash was removed, the transfection mixtures were added to the cell monolayers, which were then incubated for 10 min at room temperature with occasional rocking. A 2-ml volume of DMEM containing 10% HS was added to each well, the contents were mixed, and the supernatant was removed. The cells were washed once more with the same medium and overlaid with 1.5% low-melting-point agarose in MEM containing 5% HS. Following incubation for 3 to 4 days at 37°C, the agarose plugs were removed, and BVDV plaques were visualized by staining with crystal violet (74).

Infectivity analyses of transcribed full-length NADL RNA. Template DNAs were prepared by linearization of pACNR/NADL and pACNR/NADL S1842A DNAs with Sse8387I. Full-length uncapped RNAs were transcribed by using the T7 RNA polymerase MEGAscript system (Ambion). [3H]UTP was included during transcription to monitor the yield and integrity of the RNA products (75). After transcription, template DNA was degraded by digestion with DNase I (Ambion), and RNA products were extracted with phenol and then chloroform, concentrated by ethanol precipitation, and resuspended at 2 μg/ml in RNase-free water.

RESULTS

Mutation of Ser-1842 blocks processing at downstream sites. Previous experiments examining expression of various truncated BVDV polypeptides in mammalian and insect cell systems demonstrated that an active NS3 serine protease was required for processing at downstream sites (97). Discordant results were obtained regarding processing at the 2/3 junction: apparent NS3-dependent cleavage was observed in mammalian cells (97) but not in the insect cell system (68). We reexamined this issue, using the vaccinia virus-T7 system to drive expression of the entire BVDV polyprotein in mammalian cells. Expression constructs consisted of the T7 promoter followed by the full-length BVDV (strain NADL) cDNA, which had been assembled from sequenced clones described elsewhere (54). At position 1842, the parental construct encodes the serine residue believed to be the nucleophile of the pestivirus NS3 serine protease catalytic triad (97). A mutant derivative, S1842A, contained an alanine substitution at this position. Polyprotein processing phenotypes were examined by metabolic labeling followed by immunoprecipitation with rabbit polyclonal antisera specific for E2, NS2, NS3, or NS5A. Extracts from BVDV-infected MDBK cells were radiolabeled and processed in parallel to provide markers for authentic viral cleavage products.

As shown in Fig. 2, identical BVDV-specific proteins of the expected sizes for E2, NS2, NS3, and NS5A (18, 20) were observed in BVDV-infected cells and for the transfected parental construct. For the S1842A mutant, proteins comigrating
with E2 and NS2 were observed; however, NS3 and NS5A were not detected. Rather, large uncleaved polyproteins immunoprecipitating with NS3- and NS5A-specific antisera were observed. Other immunoprecipitations demonstrated that NS4B and NS5B were produced in BVDV-infected cells and for the parental construct but not for the S1842A mutant (data not shown). These observations are consistent with previous results (97) and demonstrate that the NS3 serine protease is required for processing at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites but not for processing in the E2-NS2 region or at the 2/3 site (see Discussion).

**Definition of NS3 serine protease-dependent cleavage sites.** Although the polyprotein cleavage sites for the flavivirus and HCV serine proteinases have been determined (73), the sites cleaved by the pestivirus NS3 serine protease have not been previously identified. To determine the sequences at the four NS3 protease cleavage sites, the N-terminal sequences of NS4A, NS4B, NS5A, and NS5B were determined. Sufficient quantities of BVDV-specific proteins for N-terminal sequence analysis were not readily obtained from virus-infected MDBK cells. Since the vaccinia virus-T7 and baculovirus expression systems appear to faithfully reproduce authentic BVDV polyprotein processing at serine protease-dependent sites (68, 97) (Fig. 2), these systems were used as sources of BVDV-specific cleavage products.

By using the vaccinia virus-T7 system to drive expression of pTM3/BVDV1598-3035 (2/+5A−), NS4A and NS4B were metabolically labeled with either [3H]leucine, [3H]valine, [35S]methionine, or [3H]isoleucine, immunoprecipitated with region-specific antisera, separated by electrophoresis on SDS-polyacrylamide gels, and electroblotted onto Immobilon P membranes. The appropriate bands were localized and sequenced for 14 or 15 cycles of Edman degradation. Representative results are shown in Fig. 3. For each protein, the deduced sequence was sufficient to unambiguously define its N terminus within the BVDV polyprotein. For NS4A, leucine residues were recovered at positions 7, 8, and 11 and valine was recovered at position 9. These data establish the N terminus of NS4A as residue 2363. For NS4B, valine was recovered at position 5, isoleucine residues were recovered at positions 8 and 12, and a methionine residue was recovered at position 9. These data establish the N terminus of NS4B as residue 2427.

For NS5A and NS5B, sufficient quantities of unlabeled proteins could be isolated by SDS-PAGE of a detergent-washed membrane fraction derived from recombinant baculovirus bacD2-133-infected cells (Fig. 1). N-terminal sequences obtained from these samples establish the N termini of NS5A and NS5B as residues 2774 and 3270 of the NADL polyprotein, respectively (Fig. 3).

Assuming that C-terminal trimming or additional cleavages do not occur, BVDV NS4A is 64 amino acid residues in length (predicted molecular mass, 7,179 Da), NS4B is 347 residues (predicted molecular mass, 38,312 Da), NS5A is 496 residues (predicted molecular mass, 55,716 Da), and NS5B is 719 residues (predicted molecular mass, 81,918 Da). These predicted sizes are in rough agreement with the apparent molecular weights of the BVDV strain NADL NS proteins estimated by SDS-PAGE (18, 20, 95).

Inspection of the sequences surrounding the NADL cleavage sites and comparison with the homologous regions deduced from the nucleotide sequences of other pestiviruses reveal an invariant leucine residue at the P1 position of NS3 serine protease-dependent cleavage sites (74). The apparent preference for a leucine residue at the P1 position differs from that of the classical flaviviruses and HCV (see Discussion). At the P1′ position, a serine residue is found at the 3/4A, 4A/4B, and 5A/5B sites, whereas an alanine is present at the 4A/4B site. Other than these conserved residues at the P1 and P1′ positions, the aligned sequences surrounding the cleavage sites did not reveal any additional common features that might be important for determining cleavage site specificity of the NS3 serine protease. Clearly, other determinants must be important for site-specific cleavage, since 17 additional Leu-Ser dipeptide sequences are present in the...
NADL NS polyprotein (data not shown), yet cleavages at these sites are not apparent.

BVDV NS4A functions as a serine proteinase cofactor. Previous studies demonstrated that the BVDV NS3 serine proteinase expressed alone was not sufficient for cleavage at the 5A/5B site (97). Specifically, a proteinase construct spanning polyprotein residues 1591 to 2191 was active for trans cleavage at the 4A/4B, 4B/5A, and 5A/5B sites of a substrate beginning in the middle of the proteinase domain and extending to the C terminus of the BVDV polyprotein (residues 1761 to 3988). This proteinase was not, however, capable of cleaving at the 5A/5B site of a substrate which began just downstream of the NS5A N terminus (from residues 2796 to 3988). By using a proteinase construct extending into the NS4B region (residues 1591 to 2645), the same 5A-5B substrate could be cleaved efficiently in trans. These results suggested that sequences between residues 2191 and 2645 were required for serine proteinase-dependent processing at the 5A/5B site. This region of the polyprotein encompasses the C-terminal 25% of the NS3 RNA helicase domain, the complete NS4A protein, and the first 219 residues (63%) of NS4B.

This observation is strikingly reminiscent of HCV polyprotein processing, in which NS4A functions as an integral component of the NS3 serine proteinase, leading to a dramatic stimulation in cleavage at certain sites (see Discussion). Having defined the boundaries of BVDV NS4A, we tested the ability of this 64-residue protein to function as a proteinase cofactor. The vaccinia virus-T7 system was used for expression of various BVDV polypeptides (Fig. 1), and the products were analyzed by metabolic labeling, immunoprecipitation, and SDS-PAGE (Fig. 5). The proteinase substrate (4B*-5B) initiated 37 residues downstream from the NS4B N terminus and terminated at the end of the BVDV ORF. The proteinase construct (2*-3) contained 82 C-terminal residues of NS2 (assuming that NS3 begins at residue 1680 [58]) and the entire NS3 coding region except for two C-terminal amino acids. Transfection of the substrate construct alone yielded a major band of 170 kDa that reacted with antisera specific for NS4B, NS5A, and NS5B. A series of smaller, less prominent products was also observed, presumably as a result of premature termination and/or degradation. This pattern was unchanged when the substrate was coexpressed with the 2*-3 proteinase or an inactive derivative (2*-3 S1842A; Fig. 5, a3hel panel). Only when both the 2*-3 proteinase and NS4A were coexpressed with the substrate were cleavage products observed. The NS4B-specific antiserum recognized a product which migrated slightly faster than the full-length NS4B marker produced by the 2*-5B polyprotein (Fig. 5, top panel). The NS5A-specific antiserum recognized a doublet migrating at 58 kDa, and the NS5B-specific antiserum recognized a 75-kDa product that was not immunoprecipitated by anti-NS4B and is therefore likely to be 5A-5B. Taken together, these data suggest that NS4A functions as an NS3 serine proteinase cofactor that is required for efficient cleavage at both the 4B/5A and the 5A/5B sites. Interestingly, the level of NS4A
accumulation when the protein was expressed independently was much lower than when the protein was expressed in the context of the 2*-5A* polyprotein (Fig. 5, bottom panel, lane 3). Slightly increased accumulation of NS4A was observed when it was coexpressed with substrate and active or inactive NS3 proteinase (Fig. 5, bottom panel, lanes 9 and 10). Despite this low level of NS4A, a majority of the substrate was processed in the presence of active proteinase and NS4A.

A catalytically active serine protease is essential for BVDV replication. Cleavages mediated by the NS3 serine proteinases of viruses from all three genera of Flaviviridae are believed to be important for virus replication. For flavivirus YFV, this was formally demonstrated by examining the effects of mutations that inactivate the serine proteinase or block cleavage at specific sites in the context of an infectious molecular clone (16, 17, 65). Recently, we constructed a full-length cDNA clone of the NADL strain of BVDV from which infectious RNA could be transcribed (54). The transcribed pestivirus RNA was of high specific infectivity, and recovered virus was similar to the parental virus in terms of plaque phenotype, growth properties, and the pattern of virus-specific proteins. We used this system to test the infectivity of full-length RNA transcripts containing the S1842A mutation. The yields of full-length transcripts produced in the transcription reaction from the wild-type (pACNR/NADL) and the S1842A mutant templates were similar (data not shown). Transcribed RNAs were transfected into MDBK monolayers, which were then either overlaid with agarose to assay directly for plaque formation or incubated in culture medium for 2 days before being assayed for release of infectious virus. Both Lipofectin (~10^3 to 10^4 PFU/µg of RNA) and electroporation (10^4 to >10^5 PFU/µg of RNA) transfection methods were used in different experiments. Figure 6 shows the results of a representative Lipofectin-mediated RNA transfection/plaque assay. More than 10^3 plaques were observed for the wild-type parent; none were observed for the S1842A mutant. In a second experiment, electroporation with the wild-type transcripts resulted in the appearance of complete CP effect within 2 days and yielded 2.9 × 10^4 PFU/µg of RNA and virus titers in the culture medium of >2 × 10^5 PFU/ml. In contrast, monolayers transfected with the S1842A

![FIG. 4. Alignment of sequences flanking the serine proteinase-dependent cleavage sites. As determined from the N-terminal sequence data shown in Fig. 3, the 10 amino acid residues on either side of the 3/4A, 4A/4B, 4B/5A, and 5A/5B cleavage sites are aligned for BVDV strain NADL and selected pestivirus sequences. As shown at the right, NS4A, NS4B, NS5A, and NS5B begin at NADL polyprotein residues 2363, 2427, 2774, and 3270, respectively. The corresponding polyprotein residues for the other aligned pestivirus sequences are also given. The P1 and P1' positions are shown (boldface). The pestivirus sequences are from the following sources: NADL (19); BVDV SD-1 (23); BVDV Osloss (21); BVDV type 2 (77); CSFV Alfort-Tübingen (55); CSFV Brescia (63); and BDV BD31 (accession no. U70263).](http://jvi.asm.org/)

![FIG. 5. NS4A functions as an NS3 serine protease cofactor. BHK-21 cell monolayers were infected with vTF7-3 alone (lane 5) or infected with vTF7-3 and transfected with the indicated plasmids (plus sign over the respective lanes) (see Fig. 1). Between 3 and 7 h postinfection, the cells were metabolically labeled as described in Materials and Methods. Cell lysates were immunoprecipitated with the following BVDV-specific rabbit antisera: NS4B-specific K1 (α4B), NS3A-specific 62D (α5A), NS3B-specific B3B (α5B), NS3-specific B10 (α3hel), and NS4A-specific α2396 (α4A). Immunoprecipitated proteins were solubilized and separated by electrophoresis on 8% (α5A, α5B, and α3hel) or 14% (α4B and α4A) polyacrylamide–SDS gels. Only the relevant portions of the gels are shown; BVDV-specific proteins are indicated at the right.](http://jvi.asm.org/)
mutant RNA failed to exhibit any CP effect and yielded no plaque-forming virus in the medium, even after a prolonged culture period (7 days). To rule out the possibility that the S1842A mutation led to a replication-competent but non-CP virus, transfected cells were analyzed for production of BVDV antigens by immunostaining or by metabolic labeling and immunoprecipitation. BVDV-specific antigens were readily detected for both CP BVDV and non-CP BVDV control RNAs. In contrast, no BVDV-specific proteins were detected for the S1842A mutant by either method (data not shown). These results indicate that the NS3 serine proteinase activity is essential for efficient pestivirus replication.

DISCUSSION

Results presented here and elsewhere (see references 58 and 73 for reviews) reveal both striking similarities and distinct differences in NS3 serine proteinase-mediated processing in the Flaviviridae. For all three genera, inactivation of this enzyme blocks polyprotein cleavage at multiple sites. The pestivirus and HCV serine proteinases cleave at four downstream sites in the NS region (3/4A, 4A/4B, 4B/5A, and 5A/5B). The flavivirus enzyme, in addition to cleaving at three downstream sites (3/4A, 4A/2K, and 4B/5), also mediates additional upstream cleavages in the structural and NS2 regions, including autocatalytic cleavage at the 2B/3 site. Other aspects of flavivirus NS region processing are also distinct from those of pestiviruses and HCV, including a signal peptidase cleavage that generates the N terminus of NS4B (47) and a lack of further processing of the 100-kDa NS5 protein.

N-terminal sequence analysis of the BVDV NS3 serine proteinase-dependent cleavage products shows that the specificity of the pestiviral enzyme differs from that of the flaviviruses and HCV. At all four sites, leucine was found at the P1 position, and either serine, alanine, or asparagine was found at the P1' position. Other than the asparagine at the P1' position of the BVDV 5A/5B site, these P1-P1' residues are conserved among pestivirus isolates (Fig. 4). Inspection of residues immediately flanking these sites has not revealed any other common features which might contribute to substrate specificity of the enzyme. For viruses from all three genera of the Flaviviridae, amino acids with short side chains seem to be preferred at the P1' position. In contrast, while the BVDV NS3 proteinase prefers leucine at the P1 site, the HCV proteinase favors cysteine or threonine (6, 33, 42, 43, 45) and the flavivirus enzyme prefers basic residues (reviewed in reference 13) (Table 1).

Structural comparisons of the P1 binding sites of a number of chymotrypsin-fold serine proteinases indicate that substrate specificity is determined mainly by a small number of residues (67). Mutations of these residues can change the specificity of an enzyme, as demonstrated for trypsin (36), α-lytic protease (46), and other members of this enzyme family, including the HCV NS3 proteinase (27). These residues are located near the catalytic serine residue in both the tertiary structure and primary polypeptide sequence (Table 1). The high degree of sequence conservation surrounding this serine allows sequence alignments in this region to be constructed with a high level of confidence. This in turn allows construction of homology models which can be used to predict or rationalize cleavage specificity (35).

Models for the P1 specificity pockets of the NS3 serine proteinases of the Flaviviridae compared to those of chymotrypsin, elastase, and trypsin are shown in Fig. 7. Residues known or predicted to contribute to P1 specificity include positions 189, 190, 213, 216, and 228 (chymotrypsin numbering system [35]; Table 1). In particular, the residue at position 216 defines the depth of the P1 specificity pocket, whereas the residues at positions 190 and 213 help to determine its width. The specificity site in the BVDV proteinase model resembles those of elastase and chymotrypsin; glycine at position 216 results in a deeper binding pocket, but asparagine at position 190 limits its width (Fig. 7). This is consistent with the leucine substrate specificity that we observe for BVDV proteinase and is also observed for Streptomyces griseus protease B, which has a glycine at position 216 and valine at 190 (39). Chymotrypsin can accommodate larger hydrophobic residues due to the presence of Gly-216 and Ser-190. For the HCV proteinase, the crystal structure (41, 52) confirmed the prediction that its P1 specificity pocket would resemble that of elastase (69). In both enzymes, residues other than glycine are located at position 216 (Table 1). The combination of this and relatively large hydrophobic residues at positions 190 (leucine) and 213 (phenylalanine) effectively seals off a shallow hydrophobic P1 specificity pocket, consistent with the observed preference for cysteine. The flavivirus NS3 proteinase has specificity for basic residues, as does trypsin (32). In trypsin, this specificity has been shown to be due to Asp-189 (10). Sequence alignments suggest that the flavivirus NS3 proteinase also has an aspartate at this location (8, 31). Changing this aspartate to lysine in trypsin, which is the residue found at this position in the BVDV alignment, does not enhance the specificity of trypsin.

### Table 1. Sequence alignment of residues surrounding the P1 specificity pocket of chymotrypsin-like serine proteinases

<table>
<thead>
<tr>
<th>Enzyme and source</th>
<th>P1 specificitya</th>
<th>P1 specificity pocketb (residue)</th>
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<tbody>
<tr>
<td></td>
<td>189</td>
<td>190</td>
</tr>
<tr>
<td>NS3, pestiviruses</td>
<td>L</td>
<td>K</td>
</tr>
<tr>
<td>Protease B, S. griseus</td>
<td>L</td>
<td>C</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>F, Y</td>
<td>W</td>
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<tr>
<td>NS3, HCV</td>
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<td>Elastase</td>
<td>A, V</td>
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<td>NS3, flavivirus</td>
<td>K, R</td>
<td>D</td>
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<tr>
<td>Tryptsin</td>
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a For the listed serine proteinases, residues preferred at the P1 position are indicated. The single-letter amino acid code is used (see Fig. 3 legend).

b Residue numbers refer to the standard chymotrypsin numbering system as described by Greer (35).
Towards acidic residues, possibly because the lysine side chain is reoriented away from the P1 specificity site (32).

Previous work suggested that sequences downstream of the BVDV NS3 serine proteinase domain were required for processing at the 5A/5B site (97). Trans-processing experiments reported here show that BVDV NS4A is essential for cleavage at both the 4B/5A and the 5A/5B sites. Similar observations hold for HCV (73), in which the 54-residue NS4A protein forms a complex with the NS3 serine proteinase domain (7, 25, 51, 81) and enhances processing at downstream sites (5, 26, 49, 89). HCV NS4A is required for cleavage at the 4B/5A site, but unlike in BVDV, processing at the HCV 5A/5B site occurs with reasonable efficiency in the absence of the cofactor (see reference 86 and citations therein). Structural analysis of an HCV proteinase-NS4A peptide complex (41) has shown that a central hydrophobic region of NS4A, which is essential for cofactor activity (7, 11, 50, 84, 93), forms an integral component of the N-terminal domain of the proteinase (41). For the flaviviruses, the upstream NS2B protein forms a stable complex with the NS3 serine proteinase domain after autocatalytic cleavage at the 2/3 site (3, 15). NS2B is required for cleavage at all NS3 proteinase-dependent sites (1, 3, 12, 29). A central region in flavivirus NS2B, which is highly conserved and rich in charged residues, has been implicated in cofactor function (15, 28). In future experiments with the pestivirus NS4A cofactor, it will be interesting to examine requirements for cleavage at the 3/4A and 4A/4B sites, to define residues in NS4A important for cofactor function, and to elucidate its mechanism of action, whether similar to or distinct from those of the other genera.

Production of NS3 is a hallmark of CP BVDV strains and occurs by varied mechanisms (58). In several cases, the proteinases responsible for the N-terminal (2/3) cleavage of the NS3 polypeptide have been defined. In CP BVDV strains Osloss, CP14, and CP1, insertion of cellular ubiquitin sequences adjacent to NS3 creates a processing site for the cellular enzyme ubiquitin carboxyterminal hydrolase. In other CP BVDV strains (PeS15, CP6, CP4), duplications and rearrangements of BVDV RNA sequences have led to the juxtaposition of Npro immediately upstream of an additional copy of the NS3 coding sequence. Similarly, in the defective CP BVDV isolate CP9, Npro and NS3 are found adjacent to one another as a result of a large in-frame deletion. For these various gene fusions, autocatalytic cleavage by Npro is believed to produce the proper NS3 N terminus, corresponding to Gly-1590 (using the numbering for the non-CP strain SD-1 polyprotein sequence [23]). For two CP strains, NADL and CP7, short in-frame insertions of either cellular (NADL) or duplicated viral (CP7) sequences in the NS2 region upstream of the putative NS3 cleavage site lead to NS3 production (54, 56, 91). Recent work on CP7 has clearly shown that cleavage at the 2/3 site is independent of the NS3 serine proteinase activity (91). Previous studies of NADL gave discordant results regarding the involvement of the NS3 serine proteinase in this cleavage (68, 97). Data reported here, where NS2 was produced normally after inactivation of the NS3 serine proteinase, suggest the involvement of a distinct proteolytic activity. This discrepancy may be related to aberrant processing in the NS2 region when truncated (versus full-length) polyproteins are used for expression (98). Although additional work is needed to definitively establish the NS3 N terminus, preliminary microsequence analysis of NADL NS3 has yielded cysteine residues at positions 5 and 13, which is consistent with Gly-1680 as the N-terminal residue (98).
polyprotein position is identical to that predicted for the CP
strains described above (e.g., Gly-1590 in the SD-1 sequence).

If this result is confirmed, then the 90-amino-acid cellular
insert in NS2 of the NADL virus somehow promotes cleavage
at a site 53 residues downstream. Whether the NADL and CP7
inserts target the BVDV polyprotein for site-specific cleavage
at the 2/3 site by a cellular enzyme or activate a cryptic activity
residing in the NS2-3 region remains to be determined. It is
interesting that NS3 is still produced, albeit inefficiently, by
cSFV strains that do not contain insertions or rearrangements
such as those described for CP BVDV (58). It is also noteworthy
that in the case of HCV, the NS2-3 region encodes a Zn2+-stimulated proteolytic activity, distinct from the NS3
serine protease, which is responsible for efficient autoca-
talytic cleavage at the 2/3 site (34, 37, 71).

On the basis of the cleavage site determinations reported
here, features of the pestivirus NS proteins (except NS2, for
which the N terminus remains to be determined) are summa-
rized in Table 2. The NS3 protease/RNA helicase is the most
highly conserved NS protein and predicted to be hydrophilic
with a net positive charge. The NS3 proteinase is 64 residues
long, quite acidic, and also highly conserved among pestivi-
rases. Similar to the 54-residue HCV NS4A, the N-terminal
third of the pestivirus NS4A protein is hydrophilic and the
C-terminal portion is highly charged and acidic. The N-termi-
nal hydrophilic region of HCV NS4A is believed to be re-
sponsible for membrane association and stabilization of the
NS3-4A proteinase complex (89). The central region of BVDV
NS4A contains a number of conserved charged residues and in
this respect differs from HCV NS4A, which contains mainly
uncharged hydrophobic residues that also stabilize interactions
with NS3 required for cofactor function (see discussion in reference 41). Little is known about the function of NS4B
for members of the Flaviviridae. Pestivirus NS4B is basic with
a number of hydrophobic regions, perhaps indicative of mem-
brane association. Although the predicted molecular mass of
NS4B is 38 to 39 kDa, the protein migrates at ~30 kDa as
determined by SDS-PAGE. Whether this reflects additional
processing at the C terminus, other posttranslational modifi-
cations, or simply aberrant migration is unknown. NS3A is a
hydrophilic protein, a presumed replicase component, and
shows considerable divergence between BVDV and CSFV iso-
lates. Recent work has shown that HCV N5A and flavivirus
NS5 proteins are serine phosphoproteins (40, 72, 90). The
highly basic NS5B protein is predicted to encode the pestivirus
RNA-dependent RNA polymerase. This activity was recently
demonstrated for partially purified preparations of HCV NS5B
(9) and dengue virus NS5 (88) but has not yet been reported for
pestivirus NS5B.

Processing of the polypeptides of positive-strand animal
RNA viruses regulates important steps in virion assembly and
RNA replication function. This is also believed to be the case for
NS regions cleavages in the polyproteins of the Flaviviridae.
Using a functional BVDV CDNA clone, we have now shown
that the NS3 serine protease is essential for pestivirus repli-
cation. Previous studies with YFV demonstrated that muta-
tions in NS3 serine protease catalytic residues (17) or in the
NS2B cofactor (15) or substitutions affecting cleavage at spe-
cific sites (2, 16, 65) impaired or blocked flavivirus replication.
Given the numerous similarities among the NS3 proteinases
and polyprotein-processing schemes of members of the Flavi-
viridae, it seems likely that the HCV proteinase is also neces-
sary for replication of hepaciviruses, further validating its
choice as a target for antiviral drug development. Future stud-
ies will focus on determining the steps in viral RNA replication
which are regulated by NS3-mediated processing events.

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ADDITIONAL MATERIALS AND METHODS

Tantz et al. (J. Virol. 71:5415–5422, 1997) have indepen-
dently determined the serine proteinase-dependent processing

TABLE 2. Properties of pestivirus NS proteins generated by NS3 serine proteinase-mediated cleavages

<table>
<thead>
<tr>
<th>Protein</th>
<th>No. of amino acid residues</th>
<th>Predicted polyopeptide molecular mass (kDa)</th>
<th>pI</th>
<th>Hydropathy</th>
<th>Amino acid identity vs BVDV-1 (%)</th>
<th>Function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS3</td>
<td>683</td>
<td>75–76</td>
<td>7.3–8.1</td>
<td>Hydrophilic</td>
<td>97–100 91–92 89–90 91–92</td>
<td>Ser protease, RNA helicase</td>
</tr>
<tr>
<td>NS4A</td>
<td>64</td>
<td>7–7</td>
<td>4.6–5.2</td>
<td>Hydrophobic/hydrophilic</td>
<td>93–100 78–83 86–89 88–89</td>
<td>Ser protease cofactor</td>
</tr>
<tr>
<td>NS4B</td>
<td>347</td>
<td>38–39</td>
<td>7.5–8.8</td>
<td>Hydrophobic</td>
<td>92–97 84–86 76–78 76–77</td>
<td>Replicase component?</td>
</tr>
</tbody>
</table>

* * *

Possible or predicted functions are indicated by a question mark. See Discussion for further details.
sites for BVDV strain CP7. Their results (CP7) and ours (NADL) are in complete agreement.

REFERENCES


