Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites

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The polyprotein of infectious bursal disease virus (IBDV), an avian birnavirus, is processed by the viral protease, VP4. Previous data obtained on the VP4 of infectious pancreatic necrosis virus (IPNV), a fish birnavirus, and comparative sequence analysis between IBDV and IPNV suggest that VP4 is an unusual eukaryotic serine protease that shares properties with prokaryotic leader peptidases and other bacterial peptidases. IBDV VP4 is predicted to utilize a serine–lysine catalytic dyad. Replacement of the members of the predicted catalytic dyad (Ser-652 and Lys-692) confirmed their indispensability. The two cleavage sites at the pVP2–VP4 and VP4–VP3 junctions were identified by N-terminal sequencing and probed by site-directed mutagenesis. Several additional candidate cleavage sites were identified in the C-terminal domain of pVP2 and tested by cumulative site-directed mutagenesis and expression of the mutant polyproteins. The results suggest that VP4 cleaves multiple (Thr/Ala)–X–Ala motifs. A trans activity of the VP4 protease of IBDV, and also IPNV VP4 protease, was demonstrated by co-expression of VP4 and a polypeptide substrate in Escherichia coli. For both proteases, cleavage specificity was identical in the cis- and trans-activity assays. An attempt was made to determine whether VP4 proteases of IBDV and IPNV were able to cleave heterologous substrates. In each case, no cleavage was observed with heterologous combinations. These results on the IBDV VP4 confirm and extend our previous characterization of the IPNV VP4, delineating the birnavirus protease as a new type of viral serine protease.

Introduction

Infectious bursal disease, also named Gumboro disease, is a contagious virus disease of young chickens that is of considerable economical importance in the poultry industry. Infectious bursal disease virus (IBDV) specifically infects B cells present in the bursa of Fabricius. IBDV, like infectious pancreatic necrosis virus (IPNV) and Drosophila X virus (DXV), is a member of the Birnaviridae (Dobos et al., 1995). Birnaviruses contain two double-stranded RNA segments, A and B, within a non-enveloped, single-shelled icosahedral virion, about 65 nm in diameter (Dobos et al., 1979; Böttcher et al., 1997). Segment B (approximately 2–8 kbp) encodes VP1, a 100 kDa protein with RNA polymerase activity (Morgan et al., 1988). Segment A (approx. 3–3 kbp) encodes a polyprotein precursor, which is co-translationally processed into three proteins, pVP2 (also designated VPX), VP3 and VP4 (Hudson et al., 1986). The pVP2 to VP2 conversion involves the cleavage of pVP2 near its C terminus (Azad et al., 1987). VP2 and VP3 are the major structural proteins of virions, whereas VP4 is a virus-encoded protease essential for processing (Azad et al., 1987; Jagadish et al., 1988; Kibenge et al., 1997), which belongs to the U43 family according to the classification of the MEROPS website (http://www.bi.bbsrc.ac.uk/world/Labs/peptidase/unkfam.htm). A second open reading frame, partially overlapping the 5′ polyprotein gene, encodes the non-structural protein VP5 (Mundt et al., 1995).

We have recently reported the characterization of the IPNV VP4 protease (Petit et al., 2000), showing that this protease may use a catalytic serine–lysine dyad, a catalytic site previously identified in several prokaryotic peptidases and hydrolases (for a review, see Barrett & Rawlings, 1995). The IPNV VP4 cleavage sites were identified by N-terminal

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sequencing. They were probed by mutagenesis and found to be defined by the (Ser/Thr)→X-Ala | (Ser/Ala) motif. For IBDV, the canonical His→Asp→Ser catalytic triad found in classical chymotrypsin-like proteases has been proposed as the VP4 active site (Brown & Skinner, 1996). Immunoelectron microscopy data from IBDV-infected cells showed that the VP4 protein does not appear to be a constituent of mature virions, but is mainly associated with tubules 24 nm in diameter (Granowitz et al., 1997). Recently, site-directed mutagenesis experiments allowed the identification of two tripeptides, Leu-511→Ala-513 and Met-754→Ala-756, that are essential for processing at the pVP2→VP4 and VP4→VP3 junctions. A secondary pVP2→VP4 processing site was also detected upstream of the Leu→Ala site (Sanchez & Rodriguez, 1999).

With the identification of catalytic residues and of the cleavage sites of IBDV VP4, the present analysis extends our previous characterization of the IPNV protease and leads us to propose that the birnavirus VP4 defines a novel group of serine proteolytic enzymes.

Methods

■ Construction of the IBDV segment A (IBDA) expression vector pUC-IBDA. The complete IBDV segment of Gumboral CT IBDV vaccine strain (Mérial) was amplified by RT→PCR by standard techniques and procedures (Sambrook et al., 1989) with the following oligonucleotides, 5′ AGAGAATTCTAATACGACTCACTATAGGAGTAGTC 3′ and 5′ AGAGAATTCTGTTGCGTTCGCGGCGGAA 3′ and 5′ AGAGAATTCTGTGACGGGCCGCGAGAAGCATTGG 3′, and cloned into the EcoRI site of pUC19 to generate the plasmid pUC-IBDA. Thus, the 5′ terminus of the IBDV segment (GGTACGAGGTAGTC... ) was downstream of the T7 promoter. The entire segment was sequenced and compared with published segment A sequences. The polyprotein sequence was found to be 99% identical to the IBDA sequence reported as accession No. Lejal and others (1999).

■ Site-directed mutagenesis of pUC19/IBDA. Mutations were introduced by using Phusion DNA polymerase with the QuickChange site-directed mutagenesis kit (Stratagene) as described by the manufacturer. For some mutations, a novel restriction site (EcoRI) was introduced (Table I), and sequence analysis was carried out to confirm the amino acid changes.

■ Construction of plasmids expressing truncated IBDV segments. To obtain a reading frame carrying the pVP2−VP4 and VP4→VP3 cleavage sites (construct VP4−3α0), a PCR was carried out with the Phusion DNA polymerase, pUC19/IBDA as template and oligonucleotides 5′ GGGAATTCAGGAACTGCTCGAGCCGCGTC 3′ and 5′ CGGAAATTCAGGAACTGCTCGAGCCGCGTC 3′ and 5′ CGGAAATTCAGGAACTGCTCGAGCCGCGTC 3′ and 5′ CGGAAATTCAGGAACTGCTCGAGCCGCGTC 3′ as template and 3′ termini and self-ligated into the EcoRI site of pUC19 to generate the plasmid pUC-IBDA. Thus, the 5′ terminus of the IBDV segment (GGTACGAGGTAGTC... ) was downstream of the T7 promoter. The entire segment was sequenced and compared with published segment A sequences. The polyprotein sequence was found to be 99.4% identical to the IBDA sequence reported as accession No. Lejal and others (1999).

■ Construction of plasmids expressing truncated IPNV segments A (IPNA). The complete IPNA segment of the IPNV SP strain was cloned by RT→PCR into the pBluescript II SK− phagemid (Stratagene) (unpublished results). To obtain a reading frame carrying the IPNV protease activity, the resulting plasmid was digested with NcoI (nt 1596) and SalI (present in the polylinker) and the insert was cloned into the pUC19 and Xhol sites of a modified pET-22b vector (Novagen), in which the pelB leader sequence was deleted and the Ncol restriction site was located on the initiation codon. For a plasmid expressing the junction of pVP2 and VP4, the pET-28b vector was cut with EcoRI, treated with Klenow fragment to fill recessed 3′ termini and self-ligated to generate pET28-Asel. Thus, a stop codon was located after the EcoRI site. The IPNA gene was cut with BgIII and the pVP2−VP4 junction domain was cloned in the BamHI site of pET28-Asel.

■ In vitro expression, protein labelling and immunoprecipitation. In vitro T7-driven expression was carried out by using the TNT Quick coupled transcription/translation system (Promega) as described by the manufacturer, except that reactions were performed in a final volume of 11 μl. The DNA template (1 μg) was incubated for 1.5 h at 30 °C, aliquots of 2–3 μl were submitted to 10% SDS–PAGE, essentially according to Laemmli (1970), and gels were dried for autoradiography.

■ N-terminal sequence determination of VP3 and VP4 polypeptides expressed in E. coli. Two hundred and fifty ml of an overnight liquid culture of E. coli BL21 (DE3) carrying the construct VP4−3α0 was diluted in an equal volume of LB medium containing kanamycin (50 μg/ml) and expression was induced with 1 mM IPTG for 4 h at room temperature. The induced cells were collected by centrifugation at 5000 × g for 5 min and resuspended in 50 ml of 50 mM Tris–HCl (pH 8), 2 mM EDTA. After an additional centrifugation (5000 × g, 5 min), cells were resuspended in 15 ml of 50 mM Tris–HCl (pH 8), 60 mM NaCl with a cocktail of protease inhibitors without EDTA (Boehringer Mannheim). Lysozyme was added to a concentration of 300 μg/ml and the mixture was placed in an ice bath for 30 min. Fifty μl benzonase (Boehringer) and 42 μl of 1 M MgCl₂ was added and the mixture was incubated for 10 min. The lysates were centrifuged at 13000 × r.p.m. for 1 h at 4 °C. The supernatants were diluted in 20 mM Tris–HCl (pH 8), 5 mM imidazole, 0.5 M NaCl. The pellet was resuspended in the same buffer supplemented with 6 M urea and solubilized overnight at 4 °C with mild agitation. This material was clarified by centrifugation (13000 × g, 30 min). The VP3 and VP4 polypeptides were solubilized in the buffer containing urea and they were processed for Ni²⁺ affinity chromatography under denaturing conditions as described by Novagen. The VP3 polypeptides were eluted by the 200 mM imidazole buffer. About 1 nmol VP3 was processed for
Table 1. Amino acid substitutions introduced into the IBDV VP2–4–3 polyprotein

<table>
<thead>
<tr>
<th>Construct</th>
<th>Amino acid substitution(s)</th>
<th>Wild-type sequence*</th>
<th>Mutated sequence†</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBDV A487E–A488F</td>
<td>Ala-487 → Glu, Ala-488 → Phe</td>
<td>CAGGCTGCTTCA</td>
<td>CAGGAATTCCTCA</td>
</tr>
<tr>
<td>IBDV A494E–A495F</td>
<td>Ala-494 → Glu, Ala-495 → Phe</td>
<td>CGAGCGCGGTCA</td>
<td>CGAGAATTCTCA</td>
</tr>
<tr>
<td>IBDV A501E–A502F</td>
<td>Ala-501 → Glu, Ala-502 → Phe</td>
<td>AGAGCTGCCTCA</td>
<td>AGAGAATTCTCA</td>
</tr>
<tr>
<td>IBDV A512E–A513F</td>
<td>Ala-512 → Glu, Ala-513 → Phe</td>
<td>CTCGCCGCGCAC</td>
<td>CTCGAATTCGAC</td>
</tr>
<tr>
<td>IBDV A487E–A488F, A494E–A495F</td>
<td>Ala-487 → Glu, Ala-488 → Phe</td>
<td>CAGGCTGCTTCA</td>
<td>CAGGAATTCCTCA</td>
</tr>
<tr>
<td>IBDV A501E–A502F, A512E–A513F</td>
<td>Ala-501 → Glu, Ala-502 → Phe</td>
<td>AGAGCTGCCTCA</td>
<td>AGAGAATTCTCA</td>
</tr>
<tr>
<td>IBDV A494E–A495F, A501E–A502F, A512E–A513F</td>
<td>Ala-494 → Glu, Ala-495 → Phe</td>
<td>CAGGCTGCTTCA</td>
<td>CAGGAATTCCTCA</td>
</tr>
<tr>
<td>IBDV S652A</td>
<td>Ser-652 → Ala</td>
<td>AACAGTTGA</td>
<td>AACGCTGGA</td>
</tr>
<tr>
<td>IBDV S652T</td>
<td>Ser-652 → Thr</td>
<td>AACAGTTGA</td>
<td>AACACTGGA</td>
</tr>
<tr>
<td>IBDV S652C</td>
<td>Ser-652 → Cys</td>
<td>AACAGTTGA</td>
<td>AACTGTTGA</td>
</tr>
<tr>
<td>IBDV K692A</td>
<td>Lys-692 → Ala</td>
<td>ACCAGCTC</td>
<td>ACCGCCGCTC</td>
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<tr>
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<td>Lys-692 → Arg</td>
<td>ACCAGCTC</td>
<td>ACCAGGCCTC</td>
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<tr>
<td>IBDV K692H</td>
<td>Lys-692 → His</td>
<td>ACCAGCTC</td>
<td>ACCCCACCTC</td>
</tr>
<tr>
<td>IBDV A755E–A756F</td>
<td>Ala-755 → Glu, Ala-756 → Phe</td>
<td>ATGCGCTGCATCA</td>
<td>ATGGAATTCCTCA</td>
</tr>
</tbody>
</table>

* The codon(s) for the wild-type residue(s) is shown in bold.
† Nucleotide substitutions are shown in bold. Introduced EcoRI sites are underlined.
automated N-terminal Edman sequencing with a Perkin Elmer Applied Biosystems Procise 494A sequencer with the manufacturer’s reagents and methods. For the VP4 polypeptide, an aliquot of the material eluted from the column by the binding buffer (5 mM imidazole, 0.5 M NaCl, 6 M urea, 20 mM Tris–HCl, pH 7.9) was loaded on a 10% polyacrylamide gel for blotting on a ProBlott membrane (Applied Biosystems). The band was visualized by staining with 0.1% Coomassie blue R250 in methanol:acetic acid:water (40:1:59) (1 min at room temperature) and destaining in 50% methanol. The polypeptide of interest was submitted for N-terminal sequencing.

Protease trans-activity assay. Cultures of recombinant E. coli BL21 harbouring the two plasmids were precultured overnight in 2 ml LB medium containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml) at 37 °C with shaking. The cultured cells were diluted in an equal volume of the same medium with 1 mM IPTG and shaken for a further 4 h. The cultured cells were then harvested and suspended in water and a portion of the supernatant was submitted to SDS–PAGE for Coomassie blue staining or for blotting on a ProBlott membrane. The processed products were visualized by Coomassie blue staining for N-terminal sequencing.

Results

Identification of the catalytic residues of the IBDV VP4 protease

We showed recently that Ser-633 and Lys-674 of the IPNV polyprotein are catalytic residues of the VP4 protease (Petit et al., 2000). An alignment of two regions of the VP4 proteases of IPNV, IBDV and DXV shows that Ser-633 and Lys-674 are conserved in the VP4 sequences of IBDV and DXV (Fig. 1). The identified members of the IBDV VP4 putative catalytic dyad, Ser-652 and Lys-692 of the polyprotein, were replaced by various other residues by site-directed mutagenesis to confirm their critical importance (see also Table 1). Alanine was selected first because it is small and has a chemically inert side chain. Thus, indirect effects on catalysis were minimized. Other substitutions were carried out with other related residues. The full-length segment A polyproteins carrying these mutations were expressed by using an in vitro T7-driven expression system, and their processing was analysed by PAGE of radiolabelled products (Fig. 1b). Processing of the wild-type polyprotein yielded the expected cleavage products, with a main VP2 form (pVP2, 46 kDa), VP3 (33 kDa) and VP4 (30 kDa), and with no uncleaved precursor (Fig. 1b). The replacement of Ser-652 (by alanine or threonine) and Lys-692 (by alanine, arginine or histidine) completely inactivated polyprotein processing. The mutant with the Ser-652 to cysteine substitution was able to cleave the pVP2–VP4 and VP4–VP3 sites, but not with wild-type efficiency. These results were in good agreement with those observed previously for the predicted IPNV VP4 active site (Petit et al., 2000).

Identification and mutagenesis of the cleavage site at the VP4–VP3 junction

As the IBDV VP4 protease is active in E. coli (Azad et al., 1987; Jagadish et al., 1988), we chose this expression system to determine the cleavage site at the VP4–VP3 junction by direct sequence analysis of the N terminus of VP3. For this purpose, we engineered the VP4–3Δ0 construct encoding a 5′-truncated form of the polyprotein with part of pVP2, VP4 and VP3 fused to two 6× His tags located at the N and C termini (Fig. 2a). The VP4–3Δ0 polypeptide was expressed in E. coli and VP3–His protein was found in the inclusion bodies. VP3–His...
Identification and mutagenesis of the cleavage site at the VP3–VP4 junction

A similar approach was used to map the N terminus of VP4. The VP4–3Δ0 polypeptide was expressed in E. coli and the inclusion body material that was not retained on the Ni²⁺ column was blotted on a membrane for N-terminal sequencing of the VP4 protein. Sequence analysis of the first seven amino acids was carried out (Fig. 2b). This sequence was identical to the polyprotein sequence starting at Ala-756, suggesting that cleavage occurred at the Ala-755–Ala-756 dipeptide. Next, we tested the effect of the double A755E–A756F substitution on the processing of the polyprotein expressed in vitro (Fig. 3). This substitution was expected to interfere strongly with the processing of the polyprotein between VP4 and VP3. Indeed, this double mutation resulted in the generation of pVP2 and the uncleaved VP4–3 polypeptide.

Mutagenesis of potential additional cleavage sites in the C-terminal domain of pVP2

On the basis of the identification of the cleavage sites at the pVP2–VP4 and VP4–VP3 junctions (an Ala–Ala dipeptide was identified as the cleavable peptidyl bond for both cleavage sites), an analysis of the junction between the VP2 and VP4 domains allowed us to postulate that pVP2 contains three potential additional cleavage sites for VP4 at its C terminus, with three Ala–Ala dipeptides at positions 487–488, 494–495, and 500–501.

was purified by Ni²⁺ affinity chromatography and direct sequence analysis of its first seven amino acids was carried out (Fig. 2b). The resulting amino acid sequence was identical to the polyprotein sequence starting at Ala-756, suggesting that cleavage occurred at the Ala-755–Ala-756 dipeptide. Next, we tested the effect of the double A755E–A756F substitution on the processing of the polyprotein expressed in vitro (Fig. 3). This substitution was expected to interfere strongly with the processing of the polyprotein between VP4 and VP3. Indeed, this double mutation resulted in the generation of pVP2 and the uncleaved VP4–3 polypeptide.

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Fig. 4. Mutagenesis of the cleavage site at the VP2–VP4 junction. (a) Schematic representation of the IBDA polyprotein. The amino acids of the VP2–VP4 junction domain are indicated. The four Ala–Ala doublets of this domain are underlined. The arrow indicates the N-terminal part of the VP4 protein with Ala-513 as its N-terminal residue. (b) A number of single and cumulative double substitutions of the Ala–Ala doublets were introduced at positions 512–513, 501–502, 494–495 and 487–488. Expression of the wild-type polyprotein (wt) and the mutants was carried out as described in Fig. 2. The locations of viral polypeptides are indicated.

and 501–502 (Fig. 4a). To analyse the involvement of these Ala–Ala dipeptides as additional cleavage sites, we tested the effect of Ala–Ala to Gln–Leu substitutions at these sites. Fig. 4(b) shows that the four simultaneous double substitutions at positions 487–488, 494–495, 501–502 and 512–513 completely inhibited the generation of pVP2 and shorter pVP2 forms, but generated an uncleaved pVP2–VP4 polypeptide and the VP3 protein, suggesting that all potential cleavage sites at the pVP2–VP4 junction domain are blocked in this construct. In contrast, when only two (positions 501–502 and 512–513) or three (positions 494–495, 501–502 and 512–513) double substitutions were engineered, a pVP2–VP4 polypeptide, but also shorter pVP2 forms, were generated. Mutants with only one double substitution at positions 487–488, 494–495 or 501–502 showed wild-type processing. It must also be noted that, in mutants carrying the double substitution 512–513, the VP4 derivatives were not clearly identified. Immunoprecipitation studies are needed in order to clarify this observation further. These results suggest that three alternative cleavage sites are present in the C-terminal part of pVP2 and that the processing of pVP2 to the VP2 mature form may be associated with these sites.

**Trans-activity of the IBDV and IPNV VP4 proteases**

To measure the ability of the VP4 protease to cleave a substrate *in trans*, we developed a co-expression assay that detects cleavage activity by co-transformation of *E. coli* with the expression plasmids pET/VP4–His as the source of proteolytic activity and pET/VP2–4j IBDV as the substrate (Fig. 5). pET/VP2–4j encodes a portion of the IBDV segment A genome leading to expression of amino acids 490–687, which include the C-terminal domain of pVP2 and the first 175 amino acids of VP4. Thus, the cleavage site between pVP2 and
VP4 (P1–P1′ position: residues 512–513) and two potential alternative cleavage sites (P1–P1′ positions residues 494–495 and 501–502) are present in this construct. As shown in Fig. 5(b), we found that the VP2–4j polypeptide disappeared only when the VP4 protease was co-expressed. As a result of the cleavage, a polypeptide of 23 kDa was generated. Processing of the VP2–4j polypeptide was not due to experimental artefacts, as demonstrated by co-expression of VP2–4j with an inactive protease (by mutation of Ser-652 to Thr; VP4–His S652T) (Fig. 5). To map the cleavage site on VP2–4j, the bacterial material was blotted on a membrane for N-terminal sequencing. The first eight amino acids of the 23 kDa band were identified as NH$_2$-ADKGYEVV, corresponding to the N terminus of VP4. These results indicated that IBDV VP4 can act efficiently in trans and that the substrate specificity was identical in the cis- (self-cleavage activity) and trans-activity assays.

To extend our observations on another birnavirus protease, we constructed two expression plasmids, pET/VP4–3 IPNV as the source of IPNV VP4 proteolytic activity and pET/VP2–4j IPNV for substrate (Fig. 6). pET/VP2–4j IPNV encodes the junction domain between VP2 and VP4, leading to expression of amino acids 480–624 (Fig. 6a). As shown in Fig. 6(b), co-expression of the two polypeptides resulted in the partial cleavage of VP2–4j IPNV to generate a 15 kDa band. To determine the cleavage site in this assay, the 15 kDa protein was processed for N-terminal sequencing. The first seven residues were identified as NH$_2$-SGGPDGK, residues corresponding to the N terminus of IPNV VP4 (Petit et al., 2000). Similarly to the results reported above for IBDV, this result shows that the substrate specificity for IPNV VP4 was identical in the trans- and cis-activity assays.

The VP4 proteases of birnaviruses are species specific

To determine whether the proteases of IBDV and IPNV could cleave heterologous polypeptide substrates, the VP4 of IBDV was co-expressed in E. coli with VP2–4j IPNV and the
Fig. 6. Trans-cleavage assay of the IPNV VP4 protease in E. coli. (a) Schematic representation of the recombinant substrate and the VP4 protease produced by the expression plasmids. The arrow indicates the N-terminal part of the IPNV VP4 protein with Ser-509 as its N-terminal residue. (b) The substrate (VP2–4j IPNV) and the protease (M-VP4–3 IPNV, a 5’-truncated form of the polyprotein) were expressed in single transformants (first and third lanes) or co-expressed (second lane) with the substrate and the protease expression vectors and by IPTG induction. Proteins were submitted to PAGE analysis and revealed by Coomassie blue staining. N-terminal sequencing of the VP2–4j cleavage product reveals cleavage of the peptidyl bond between Ala-508 and Ser-509. The locations of polypeptides and molecular mass markers (in kDa) are indicated.

Discussion

In a previous study, we have shown that the VP4 protease of IPNV, a fish birnavirus, has an unusual active site never previously identified in a viral protease (Petit et al., 2000). We have proposed that this protease contains in its active site Lys-674, which acts as a general base in the activation of the nucleophilic Ser-633. These two residues are conserved in the VP4 sequences of all strains of IBDV and DXV.

In this study, we first used site-directed mutagenesis to ascertain the importance of the two homologous residues of IBDV VP4 for its protease activity. Ser-652 and Lys-692 were critical for this function. It is very likely that Ser-652 represents the nucleophilic residue because its substitution by threonine or alanine completely abolished the self-cleavage activity of the polyprotein, whereas its substitution by cysteine, which can act as the nucleophile in several viral proteases (Babe & Craik, 1997), yielded a mutant protein with good activity. Finally, all of the substitutions of Lys-692 resulted in complete inactivation of the protease activity, suggesting strongly that this residue acts as a general base at the catalytic site. Thus, these residues appear to be the functional counterparts of Ser-633 and Lys-674 of IPNV VP4. These results confirm our previous proposition (Petit et al., 2000) that the birnavirus VP4s may use a serine–lysine catalytic dyad. Such a catalytic serine–lysine dyad has been characterized from prokaryotic serine proteases and hydrolases such as α-Ala–α-Ala peptidase A and the β-lactamases (with the catalytic motif Ser–X–X–Lys).

VP4 of IPNV with VP2–4j IBDV (Fig. 7). In both cases, no cleavage of the VP2–4j polypeptides occurred, in contrast to our observations with authentic substrates, indicating that the birnavirus proteases recognize different substrate-cleavage motifs.
and from peptidases such as the bacterial signal (or leader) peptidases and from the LexA repressor family (with these catalytic residues distant on the primary sequence) (Strynadka et al., 1992; Paetzel et al., 1998; Peat et al., 1996; Barrett & Rawlings, 1995). This catalytic dyad has only been described in eukaryotes from the two subunits of the mitochondrial inner membrane protease of the yeast Saccharomyces cerevisiae (Nunnari et al., 1993).

The IBDV VP4 cleavage sites have been determined by direct N-terminal sequence analysis of the cleavage products generated in E. coli, and they were probed by mutagenesis. The N-terminal residues of VP4 and VP3 were identified as Ala-513 and Ala-756, respectively. This was in accordance with the identification of the tripeptides Leu-511–Ala–Ala-513 and Met-754–Ala–Ala-756 as critical for cleavage of the pVP2–VP4 and VP4–VP3 junctions, respectively (Sanchez & Rodriguez, 1999). The two cleavage sites thus identified possess structural similarities. They are characterized by the (Thr/Ala)–X–Ala motif, with alanines in the P1–P1′ positions and a threonine or an alanine in the P3 position. Even though they were not directly determined by N-terminal sequence determination, three other (putative) cleavage sites in the C-terminal part of pVP2 (P1–P1′ positions 487–488, 494–495, 501–502) were characterized indirectly. Firstly, these cleavage sites were identified by sequence comparison: the P1–P1′ residues were conserved as an alanine doublet, the P3 residue as an alanine and the P2′–P3′ residues (Ser–Gly) were identical for these three cleavage sites. Secondly, only cumulative mutagenesis of the P1–P1′ residues of all four Ala–Ala doublets (487–488, 494–495, 501–502 and 512–513) abolished processing completely at the mutated junction, thus revealing the existence of three alternative cleavage sites at these positions.

The pVP2 to VP2 conversion observed in infected cells involves one (or multiple) cleavage(s) of pVP2 near its C terminus, an event proposed to be associated with VP4 protease activity (Azad et al., 1987; Kibenge et al., 1997). The identification of three alternative cleavage sites for VP4 in the C-terminal domain of pVP2 argues strongly for the implication of VP4 in the pVP2 to VP2 conversion. It is noteworthy that, for IBDV, we identified (by N-terminal sequencing and mutagenesis) four cleavage sites between the VP2 and VP4 domains, whereas only three cleavage sites were defined for IPNV (Petit et al., 2000). This probably indicates that during the assembly of the virus particle, an event probably associated with the conversion of pVP2 to the VP2 mature form, different structural constraints exist for the maturation of the IBDV and IPNV particles.

The IBDV cleavage motif thus defined, (Thr/Ala)–X–Ala, was not fully conserved for the cleavage sites of IPNV and DXV. The IPNV VP4 cleavage sites were defined by the consensus motif (Ser/Thr)–X–Ala, whereas the DXV cleavage site between the VP2 and VP4 domains could be defined as an Ala–X–Ser motif (Chung et al., 1996). Our results indicate that, even though these cleavage site motifs have strong similarities, the VP4 proteases of birnaviruses are species specific, since they do not cleave heterologous substrates. In addition, it is interesting to note that, for bacterial signal peptidases (which possess the serine–lysine catalytic dyad), alanine residues are the most common residues at the P1 and P3 positions, giving the so-called 1, 3 or Ala–X–Ala rule. The similarities observed between their catalytic sites and substrate cleavage sites suggest that these birnavirus proteases and prokaryotic signal peptidases are structurally related.

A trans-cleavage assay of IBDV and IPNV VP4 was developed, which detects cleavage activity in E. coli. By using a co-expressed substrate, the cleavage activity was detected by SDS–PAGE and Coomassie blue staining. We determined by N-terminal sequencing that cleavage specificity was conserved for IBDV, we identified (by N-terminal sequencing and mutagenesis) four cleavage sites between the VP2 and VP4 domains, whereas only three cleavage sites were defined for IPNV (Petit et al., 2000). This probably indicates that during the assembly of the virus particle, an event probably associated with the conversion of pVP2 to the VP2 mature form, different structural constraints exist for the maturation of the IBDV and IPNV particles.

The IBDV cleavage motif thus defined, (Thr/Ala)–X–Ala, was not fully conserved for the cleavage sites of IPNV and DXV. The IPNV VP4 cleavage sites were defined by the consensus motif (Ser/Thr)–X–Ala, whereas the DXV cleavage site between the VP2 and VP4 domains could be defined as an Ala–X–Ser motif (Chung et al., 1996). Our results indicate that, even though these cleavage site motifs have strong similarities, the VP4 proteases of birnaviruses are species specific, since they do not cleave heterologous substrates. In addition, it is interesting to note that, for bacterial signal peptidases (which possess the serine–lysine catalytic dyad), alanine residues are the most common residues at the P1 and P3 positions, giving the so-called 1, 3 or Ala–X–Ala rule. The similarities observed between their catalytic sites and substrate cleavage sites suggest that these birnavirus proteases and prokaryotic signal peptidases are structurally related.
be possible to develop a more convenient cleavage assay that would allow the screening of birnavirus protease inhibitors.

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References


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