The capsid of birnaviruses contains two proteins, VP2 and VP3, which derive from the processing of a large polyprotein, NH$_2$–pVP2–VP4–VP3–COOH. The proteolytic cascade involved in processing the polyprotein, and in the final maturation of pVP2 (the precursor of VP2), has recently been shown to generate VP2 and four structural peptides in infectious bursal disease virus and blotched snakehead virus. The presence of peptides in infectious pancreatic necrosis virus particles was investigated using mass spectrometry and N-terminal sequencing of virus particles. Three peptides deriving from the C terminus of pVP2 (residues 443–486, 487–495 and 496–508 of the polyprotein) and 14 additional peptides produced by further processing of peptides [443–486] and [496–508] were identified. These results indicate that the presence of several virus-encoded peptides in the virions is a hallmark of birnaviruses.
BSNV pVP2-derived peptides have been identified on virus particles, we postulated that their IPNV homologues should be identifiable in purified IPNV virions.

IPNV used in this study (strain 31-75, serotype Sp; Dorson et al., 1978) was propagated in the rainbow trout gonad-2 cell line. The genome of this strain has been cloned and sequenced (database accession numbers AJ622822 and AJ622823). To identify the predicted peptides by mass spectrometry, the virus particles were purified by density centrifugation as shown in Fig. 1(b) (left panel). Two gradients are shown, resulting from two different purification procedures (see below). Four main bands were observed in the CsCl gradients. Electron microscopy revealed that the three lower bands (1, 1' and 1'') contained typical birnavirus particles, and the upper band (2) contained particles with a diameter of about 25 nm (Fig. 1b, middle panel). SDS-PAGE showed that the virus particles contain the expected VP1 (90 kDa), VP2 (54 kDa) and VP3 (31 kDa) structural proteins, and a very small amount of pVP2. The difference in density between the virus particles present in bands 1, 1' and 1'' could be explained by differences in the amount of nucleic acid present in particles. An analogous band profile has been described for another birnavirus, IBDV (Muller & Becht, 1982; Da Costa et al., 2002).

Mass spectrometry analysis was carried out on the virus particles present in band 1 using a Voyager-DE STR time-of-flight mass spectrometer (Applied Biosystems). One
main peptide with a monoisotopic \([M+H]^+\) mass of 1451.72 was identified (Fig. 2a, top panel). This \([M+H]^+\) mass fitted well with the theoretical mass of the predicted 13 aa long peptide extending from Ala-496 to Ala-508, which is 1451.72 Da. As cleavage at the pVP2–VP4 junction occurs between aa 508 and 509, this peptide is indeed the C terminus of pVP2. An additional peptide with an \([M+H]^+\) mass of 4796.14 was detected (Fig. 2a, lower panel). No other peptide with the same range of mass (3500–5500 Da) was detected. This \([M+H]^+\) mass fitted well with the mass of a peptide extending from Trp-443 to Ala-486, which is 4796.14 Da and represents the N-terminal part of this pVP2 domain. The presence of these two peptides from residues [443–486] and [496–508] in the virus particles prompted us to locate the putative 9 aa peptide derived from residues 487–495. To identify this peptide, which had a theoretical \([M+H]^+\) monoisotopic mass of 949.43 Da, we analysed the magnified signal in this mass range. As shown in Fig. 2(a) (top panel), a peptide with an \([M+H]^+\) monoisotopic mass of 949.61 was detectable. Fourteen additional signals were also identified by mass spectrometry in the mass range 590–2300 Da (Fig. 2a, top panel). Thirteen of these appeared to be cleaved products derived from peptide [443–486] (1–13) and of peptide [496–508] (14). (c) Comparison of experimental (Exp.) and theoretical (Th.) masses (Da) of the 14 peptides.

**Table 1**

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**Fig. 2.** Characterization of the peptides present in IPNV particles. (a) Mass spectrometry analysis of IPNV particles. Two mass/charge windows ranging from 590 to 2300 and 3500 to 5500 are presented. Magnified signals showing the isotopic pattern of selected peptides are inserted in each window. Top, the main signal identified at a mass/charge monoisotopic (m/z) ratio of 1451.72, representing the signature of peptide AGGRYKDVLESWA. Magnification of the signal allowed identification of peptide AGGRYHSMAs with an experimental \([M+H]^+\) mass of 949-61. Below, the main signal was identified at a mean m/z ratio of 4796.14, the signature of the peptide extending from Trp-443 to Ala-486. Fourteen additional signals (1–13 and 14) were identified on the mass/charge windows, ranging from 499 to 2300. (b) Assignment of the 14 peaks as cleaved products of peptide [443–486] (1–13) and of peptide [496–508] (14). (c) Comparison of experimental (Exp.) and theoretical (Th.) masses (Da) of the 14 peptides.
purified virus, as described for IBDV (Da Costa et al., 2002). Because the main capsid proteins, VP2 and VP3, have been reported to possess a blocked amino terminus (Dobos, 1995), only the N termini of the peptides should be detected. The results are presented in Fig. 3. The N-terminal amino acids of peptide [443–486] were identified through 15 Edman degradation cycles. In particular, residue Trp at positions 1 and 3, and residues Asp and Ile at positions 5 and 6, respectively, provided a signature for this peptide. In addition, an Ala at position 1 and a Gly at positions 2 and 3 were revealed, compatible with the predicted N-terminal sequences of both peptides [496–508] and [487–495]. The presence of these short peptides was validated by the identification of residues (His, Lys), (Ser, Asp), (Met, Val) and (Ala, Leu) at positions 6 to 9, respectively. Thus N-terminal sequencing results are consistent with the mass spectrometry analysis. In summary, three peptides corresponding to amino acid residues [443–486], [487–495] and [496–508] were detected in virus particles. A large number of peptides deriving from carboxy-terminal processing of peptide [443–486] were also detected.

Fig. 3. N-terminal sequencing of the purified virus. (a) Sequence of the IPNV pVP2 C-terminal domain (aa 443–508 of the polyprotein). Arrows indicate cleavage sites. (b) Nature of amino acids (single-letter code) and amount (in differential picomoles, the amount in picomoles of an amino acid at one position after background subtraction of its amount detected at the previous position) revealed at each Edman degradation cycle (1–15). The sequences of the three peptides are aligned on the residues identified.

In this study we showed that processing of pVP2 of IPNV is similar to the maturation of the IBDV and BSNV pVP2s. Three peptides (not four, as for IBDV and BSNV) define the C-terminal domain of pVP2. For IPNV, peptide [443–486] is processed further to generate a large number of additional peptides. For BSNV, processing of the peptide [443–486] homologue was also observed (Da Costa et al., 2003). Although processing of the IPNV peptide [443–486] appeared to involve a carboxypeptidase, an endopeptidase appeared to control the cleavages of its BSNV homologue. Similar additional peptides were not identified in IBDV virions (Da Costa et al., 2002).

Three target cleavage sites were identified in the pVP2 maturation process. We previously proposed two of these (486–487, 495–496) as targets for the IPNV VP4 protease.
(Petit et al., 2000). These two sites, and the primary cleavage site at the pVP2–VP4 junction, were defined by the motif [S/T]XA ↓ A. This consensus sequence shared some similarity with the sequence SKA ↓ W surrounding the maturation cleavage site at position 442–443, suggesting that VP4 could be involved in the cleavage generating the mature VP2.

When the virus particles were altered by ultracentrifugation, the released VP2 was able to self-assemble into particles with a diameter of about 25 nm. These observations suggest that the VP2 of IPNV has assembly properties similar to the VP2/pVP2 of IBDV (Martinez-Torrecuadrada et al., 2000; Caston et al., 2001; Chevalier et al., 2002).

The role of the birnavirus structural peptides remains to be elucidated. We favour the hypothesis that they may be involved in virus entry into the target cells, but it cannot be ruled out that they play a role in capsid assembly or genome encapsidation.

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References


