Short Communication

Peptides resulting from the pVP2 C-terminal processing are present in infectious pancreatic necrosis virus particles

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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.

Infectious pancreatic necrosis virus (IPNV) causes an acute, contagious disease of young salmonid fish that is of significant importance in aquaculture (Wolf *et al.*, 1960). IPNV, like infectious bursal disease virus (IBDV), drosophila X virus (DXV) and blotched snakehead virus (BSNV), is a member of the *Birnaviridae* (John & Richards, 1999; Leong *et al.*, 2000). Birnavirus particles are single-shelled, non-enveloped viruses with $T=13$ icosahedral capsids and are about 70 nm in diameter (Ozel & Gelderblom, 1985; Böttcher *et al.*, 1997). VP2 and VP3 are assumed to form the outer and inner layers of the virions, respectively. The virus particles contain several VP1 molecules and the bisegmented double-stranded RNA genome (Dobos *et al.*, 1979; Dobos, 1995). In IBDV and BSNV, four small peptides have been found in the virus particles (Da Costa *et al.*, 2002, 2003). VP3 was shown to interact with both VP1 and genomic RNA (Lombardo *et al.*, 1999; Tacken *et al.*, 2000, 2002; Maraver *et al.*, 2003).

The genomic segment B encodes VP1, the putative viral RNA-dependent RNA polymerase (Gorbalenya *et al.*, 2002). Translation of genomic segment A yields a polyprotein, pVP2–VP4–VP3 (Kibenge *et al.*, 1988; Muller *et al.*, 2003) and a small protein (VP5) dispensable for replication (Mundt *et al.*, 1997; Yao *et al.*, 1998). The polyprotein processing is controlled by VP4, the viral protease (Birghan *et al.*, 2000; Lejal *et al.*, 2000; Petit *et al.*, 2000).

The complete proteolytic cascades involved in processing the IBDV and BSNV polyproteins and generating VP2, the structural peptides and VP3 have been characterized. The polyproteins are cotranslationally cleaved to generate pVP2 (the precursor of VP2), VP4 and VP3. Cleavage sites have been identified at the pVP2–VP4 and VP4–VP3 junctions (Sanchez & Rodriguez, 1999; Lejal *et al.*, 2000; Da Costa *et al.*, 2003). For both viruses, processing of pVP2 generates VP2 and four small peptides derived from the C terminus of pVP2 (Da Costa *et al.*, 2002, 2003). These peptides are associated with the virus particles. For IPNV, only the VP4 target sites generating pVP2, VP4 and VP3 have been defined, being between aa 508–509 and 734–735 of the polyprotein (Petit *et al.*, 2000). In this study we characterized the final processing of the IPNV polyprotein. By using mass spectrometry and N-terminal sequencing, we identified peptides derived from the C-terminal domain of pVP2 that were present in the virus particles.

Fig. 1(a) shows a multi-alignment of the C-terminal domains of the birnavirus pVP2s. The sequence comparison is anchored to the multiple cleavage sites identified in the C terminus of pVP2 of BSNV and IBDV and to the cleavage site mapped at the pVP2–VP4 junction of IPNV and DXV (Chung *et al.*, 1996; Sanchez & Rodriguez, 1999; Petit *et al.*, 2000; Da Costa *et al.*, 2002, 2003). We predicted from this alignment that pVP2 processing of IPNV might result in the generation of mature VP2 (aa 1–442 of the polyprotein) and three peptides (aa 443–486, 487–495 and 496–508 of the polyprotein). The $[M+H]^+$ theoretical monoisotopic masses of the three peptides were expected to be 4796·59, 949·43 and 1451·73 Da, respectively. As most IBDV and

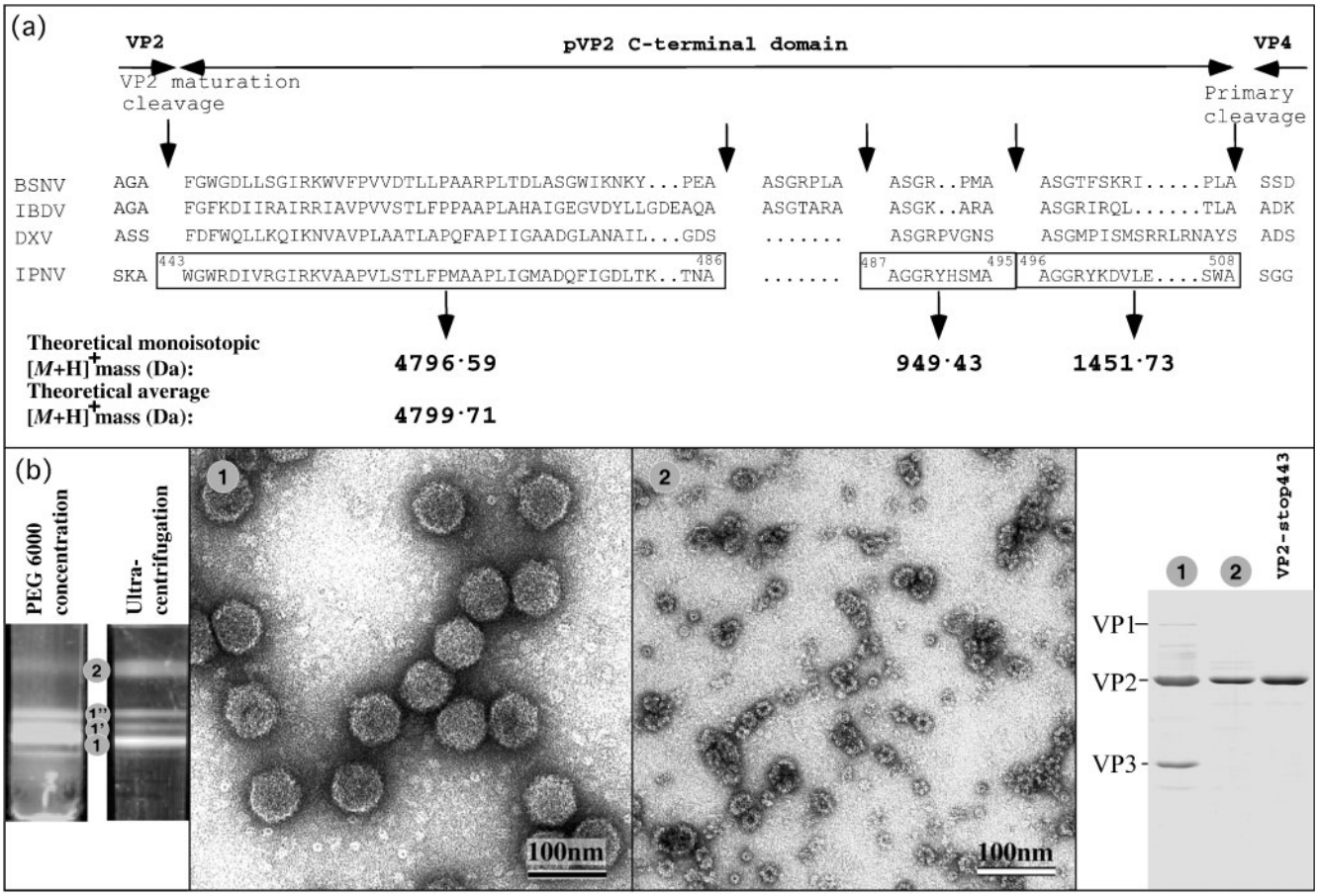
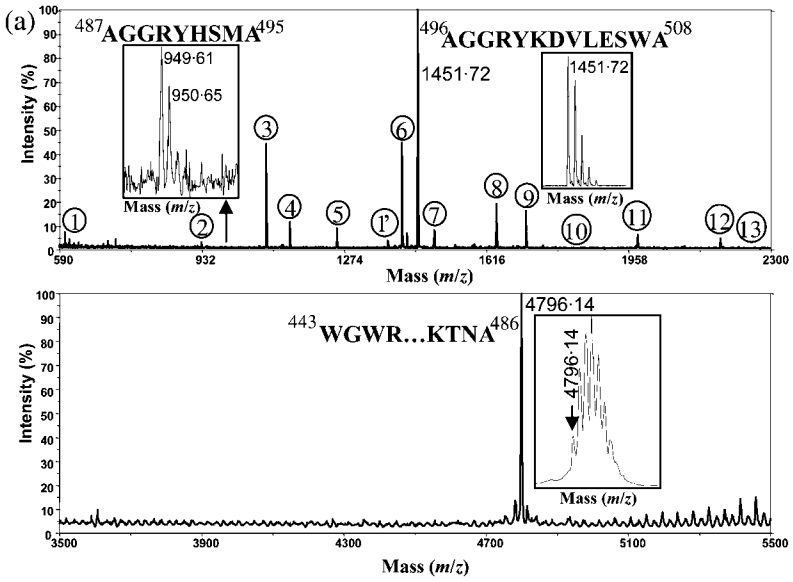


Fig. 1. (a) Sequence alignment of pVP2 C-terminal domains of birnavirus polyproteins. The single-letter code is used to indicate amino acids. The alignment is anchored to the multiple cleavage sites identified for BSNV and IBDV in this domain (Da Costa *et al*., 2002, 2003), and to the pVP2–VP4 cleavage site identified for IPNV (Petit *et al*., 2000) and proposed for DXV (Chung *et al*., 1996). IPNV predicted peptides are boxed and their theoretical monoisotopic $[M+H]^+$ masses indicated. (b) Purification of IPNV virus particles. Left: two density gradients are shown; that on the left was carried out after a preliminary PEG 6000 concentration of virus, that on the right was obtained after virus ultracentrifugation. Middle: material collected from bands 1 and 2 present in the gradients was negatively stained with 1 % uranyl acetate. Right: polypeptide identification by SDS-PAGE analysis of bands 1 and 2, and of the material produced by a baculovirus expressing a mutated IPNV polyprotein having a stop codon at position 443 (VP2-stop443). The VP2-stop443 was purified as described previously (Chevalier *et al*., 2002). The gel was stained with Coomassie blue and the protein assignment carried out by tryptic digestion of the material present in the band, followed by mass spectrometry analysis.

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



(b)

WGWRDIVRGIRKVAAPVLSTLFP...KTNA		AGGRYKDLESWA
WGWRDIVRGIRKVAAPVLS	(13)	AGGRYKDLESW (1′)
WGWRDIVRGIRKVAAPVL	(12)	or GGRYKDLESWA (1′)
WGWRDIVRGIRKVAAPV	(11)	
WGWRDIVRGIRKVAA	(10)	
WGWRDIVRGIRKVA	(9)	
WGWRDIVRGIRKV	(8)	
WGWRDIVRGIRK	(7)	
WGWRDIVRGIR	(6)	
WGWRDIVRGI	(5)	
WGWRDIVRG	(4)	
WGWRDIVR	(3)	
WGWRDIV	(2)	
WGWR	(1)	

(c)

	Mass (Th.)	Mass (Exp.)		Mass (Th.)	Mass (Exp.)
①	604·29	604·26	⑧	1640·94	1640·93
②	931·47	931·47	⑨	1711·98	1711·97
③	1087·57	1087·57	⑩	1783·03	1783·00
④	1144·59	1144·60	⑪	1979·14	1979·09
⑤	1257·58	1257·67	⑫	2092·23	2092·13
⑥	1413·78	1413·77	⑬	2179·25	2179·19
⑦	1541·87	1541·85	①	1380·69	1380·70

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 AGGRYHSMA 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 1′) 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] (1′). (c) Comparison of experimental (Exp.) and theoretical (Th.) masses (Da) of the 14 peptides.

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 1451·73 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 4796·59 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 peptides 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] (Fig. 2b, c). All possessed the same N terminus as peptide [443–486]. No complementary peptide harbouring the corresponding C-terminal domains of peptide [443–486] was identified. These observations suggest that a fraction of peptide [443–486] was cleaved by a carboxypeptidase with low specificity. A cellular carboxypeptidase, or possibly VP4, might be associated with these cleavage events. Finally, a peptide, with an $[M+H]^+$ mass of 1380·70, deriving from the amino- or carboxy-cleavage of peptide [496–508], was also detected.

To confirm the existence of these peptides in the virus particles, we carried out N-terminal sequencing on the

(a)

[443–486] [487–495] [496–508]

.SKA WGWRDIVRGIRKVAA…GDLTKTNA AGGRYHSMA AGGRYKDVLESWA SGG.

(b)

Amino acid position	Amino acid (picomoles)	[443–486]	[487–495]	[496–508]
1	A(169),W(36)	W	A	A
2	G(65)	G	G	G
3	W(19),G(-20)	W	G	G
4	R(29)	R	R	R
5	Y(37),D(31)	D	Y	Y
6	I(65),K(24),H(6)	I	H	K
7	V(43), D(11),S(5)	V	S	D
8	R(45),V(37),M(10)	R	M	V
9	L(16),G(13),A(12)	G	A	L
10	I(44),E(26)	I		E
11	S(13),R(12)	R		S
12	K(20),W(3)	K		W
13	V(32),A(26)	V		A
14	A(39)	A		
15	A(29)	A		

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.

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.

Two procedures were used to purify IPNV particles (Fig. 1b, left panel). Before density centrifugation in CsCl, the virions were concentrated by either ultracentrifugation or low-speed centrifugation after overnight incubation in 10 % polyethylene glycol 6000 (PEG). When the virus was ultracentrifuged, band 2 was denser than when the material had been concentrated with PEG. In contrast, the virus bands contained more material after PEG treatment. Electron microscopy revealed isometric particles in band 2 (Fig. 1b, middle). SDS-PAGE (Fig. 1b, right) and mass spectrometry analysis (see below) showed that these particles contained only VP2. These results suggest that the IPNV virus particles were partially altered by ultracentrifugation, and the dissociated VP2 self-assembled into structures that did not contain VP3.

The existence of peptides derived from pVP2 suggests that aa [443–508] represent the domain that is processed during pVP2 maturation. To determine whether the pVP2-processed domain involved residues upstream of residue 442, we engineered a baculovirus driving expression of the IPNV polyprotein with a stop codon at position 443, and compared the electrophoretic mobility of the mature VP2 (present on virus particles) with this truncated form. To this end, plasmid SK-ΔIPNA (Petit *et al.*, 2000) was used as a template to amplify the VP2 reading frame (the first 442 codons of the polyprotein) by PCR. The PCR product was cloned into pFastBac1 (Gibco-BRL) using suitable restriction sites. The resulting plasmid was used to generate the recombinant baculovirus BacVP2IPNV by standard procedures. Production and purification by density centrifugation of the baculovirus-expressed IPNV VP2 were carried out as described previously (Chevalier *et al.*, 2002). As shown in Fig. 1(b), the virion-associated protein VP2, the VP2 released from the virus particles and the VP2-stop443 co-migrated. As determined by mass spectrometry analysis, the experimental masses of viral VP2 and VP2-stop443 were 48 578·3 and 48 531·6 Da, respectively. These masses fit well with the theoretical $[M+H]^+$ mean mass of a VP2 extending from residues 1 to 442 with an initial acetylated methionine (48 569·9 Da), suggesting that the pVP2 characteristic domain does not extend upstream of residue 442.

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 endoprotease 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

Birghan, C., Mundt, E. & Gorbalenya, A. E. (2000). A non-canonical Lon proteinase lacking the ATPase domain employs the Ser–Lys catalytic dyad to exercise broad control over the life cycle of a double-stranded RNA virus. *EMBO J* **19**, 114–123.

Böttcher, B., Kiselev, N. A., Stel'Mashchuk, V. Y., Perevozchikova, N. A., Borisov, A. V. & Crowther, R. A. (1997). Three-dimensional structure of infectious bursal disease virus determined by electron cryomicroscopy. *J Virol* **71**, 325–330.

Caston, J. R., Martinez-Torrecuadrada, J. L., Maraver, A., Lombardo, E., Rodriguez, J. F., Casal, J. I. & Carrascosa, J. L. (2001). C terminus of infectious bursal disease virus major capsid protein VP2 is involved in definition of the T number for capsid assembly. *J Virol* **75**, 10815–10828.

Chevalier, C., Lepault, J., Erk, I., Da Costa, B. & Delmas, B. (2002). The maturation process of pVP2 requires assembly of infectious bursal disease virus capsids. *J Virol* **76**, 2384–2392.

Chung, H. K., Kordyban, S., Cameron, L. & Dobos, P. (1996). Sequence analysis of the bicistronic drosophila X virus genome segment A and its encoded polypeptides. *Virology* **225**, 359–368.

Da Costa, B., Chevalier, C., Henry, C., Huet, J.-C., Petit, S., Lepault, J., Boot, H. & Delmas, B. (2002). The capsid of infectious bursal disease virus contains several small peptides arising from the maturation process of pVP2. *J Virol* **76**, 2393–2402.

Da Costa, B., Soignier, S., Chevalier, C., Henry, C., Thory, C., Huet, J.-C. & Delmas, B. (2003). Blotched snakehead virus is a new aquatic birnavirus that is slightly more related to avibirnavirus than to aquabirnavirus. *J Virol* **77**, 719–725.

Dobos, P. (1995). The molecular biology of infectious pancreatic necrosis virus (IPNV). *Annu Rev Fish Dis* **5**, 25–54.

Dobos, P., Hill, B. J., Hallett, R., Kells, D. T., Becht, H. & Teninges, D. (1979). Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. *J Virol* **32**, 593–605.

Dorson, M., Castric, J. & Torchy, C. (1978). Infectious pancreatic necrosis virus of salmonids: biological and antigenic features of a pathogenic strain and a nonpathogenic variant selected in RTG-2 cells. *J Fish Dis* **1**, 309–320.

Gorbalenya, A. E., Pringle, F. M., Zeddam, J. L., Luke, B. T., Cameron, C. E., Kalmakoff, J., Hanzlik, T. N., Gordon, K. H. & Ward, V. K. (2002). The palm subdomain-based active site is internally permuted in viral RNA-dependent RNA polymerases of an ancient lineage. *J Mol Biol* **324**, 47–62.

John, K. R. & Richards, R. H. (1999). Characteristics of a new birnavirus associated with a warm-water fish cell line. *J Gen Virol* **80**, 2061–2065.

Kibenge, F. S. B., Dhillon, A. S. & Russell, R. G. (1988). Biochemistry and immunology of infectious bursal disease virus. *J Gen Virol* **69**, 1757–1775.

Lejal, N., Da Costa, B., Huet, J.-C. & Delmas, B. (2000). Role of Ser-652 and Lys-692 in the protease activity of infectious bursal disease virus VP4 and identification of its substrate cleavage sites. *J Gen Virol* **81**, 983–992.

Leong, J. C., Brown, D., Dobos, P., Kibenge, F. S. B., Ludert, J. E., Müller, H., Mundt, E. & Nicholson, B. (2000). Family *Birnaviridae*. In *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*, pp. 481–490. Edited by M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle & R. B. Wickner. San Diego: Academic Press.

Lombardo, E., Maraver, A., Caston, J. R., Rivera, J., Fernandez-Arias, A., Serrano, A., Carrascosa, J. L. & Rodriguez, J. F. (1999). VP1, the putative RNA-dependent RNA polymerase of infectious bursal disease virus, forms complexes with the capsid protein VP3, leading to efficient encapsidation into virus-like particles. *J Virol* **73**, 6973–6983.

Maraver, A., Clemente, R., Rodriguez, J. F. & Lombardo, E. (2003). Identification and molecular characterization of the RNA polymerase-binding motif of infectious bursal disease virus inner capsid protein VP3. *J Virol* **77**, 2459–2468.

Martinez-Torrecuadrada, J. L., Caston, J. R., Castro, M., Carrascosa, J. L., Rodriguez, J. F. & Casal, J. I. (2000). Different architectures in the assembly of infectious bursal disease virus capsid proteins expressed in insect cells. *Virology* **278**, 322–331.

Muller, H. & Becht, H. (1982). Biosynthesis of virus-specific proteins in cells infected with infectious bursal disease virus and their significance as structural elements for infectious virus and incomplete particles. *J Virol* **44**, 384–392.

Muller, H., Islam, M. R. & Raue, R. (2003). Research on infectious bursal disease – the past, the present and the future. *Vet Microbiol* **97**, 153–165.

Mundt, E., Kollner, B. & Kretzschmar, D. (1997). VP5 of infectious bursal disease virus is not essential for viral replication in cell culture. *J Virol* **71**, 5647–5651.

Ozel, M. & Gelderblom, H. (1985). Capsid symmetry of viruses of the proposed Birnavirus group. *Arch Virol* **84**, 149–161.

Petit, S., Lejal, N., Huet, J.-C. & Delmas, B. (2000). Active residues and viral substrate cleavage sites of the protease of the birnavirus infectious pancreatic necrosis virus. *J Virol* **74**, 2057–2066.

Sanchez, A. B. & Rodriguez, J. F. (1999). Proteolytic processing in infectious bursal disease virus: identification of the polyprotein cleavage sites by site-directed mutagenesis. *Virology* **262**, 190–199.

Tacken, M. G. J., Rottier, P. J. M., Gielkens, A. L. J. & Peeters, B. P. H. (2000). Interactions *in vivo* between the proteins of infectious bursal

disease virus: capsid protein VP3 interacts with the RNA-dependent RNA polymerase, VP1. *J Gen Virol* **81**, 209–218.

Tacken, M. G., Peeters, B. P., Thomas, A. A., Rottier, P. J. & Boot, H. J. (2002). Infectious bursal disease virus capsid protein VP3 interacts both with VP1, the RNA-dependent RNA polymerase, and with viral double-stranded RNA. *J Virol* **76**, 11301–11311.

Wolf, K., Snieszko, S. F., Dunbar, C. E. & Pyle, E. (1960). Virus nature of infectious pancreatic necrosis in trout. *Proc Soc Exp Biol Med* **104**, 105–108.

Yao, K., Goodwin, M. A. & Vakharia, V. N. (1998). Generation of a mutant infectious bursal disease virus that does not cause bursal lesions. *J Virol* **72**, 2647–2654.