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Chapter 779

Birnavirus VP4 Processing Endopeptidase

Databanks

MEROPS name: infectious pancreatic necrosis birnavirus Vp4 peptidase
MEROPS classification: clan SJ, family S50, peptidase S50.001
Tertiary structure: Available
Species distribution: family Birnaviridae
Reference sequence from: infectious pancreatic necrosis virus (UniProt: Q703G9)

MEROPS name: avian infectious bursal disease birnavirus Vp4 peptidase
MEROPS classification: clan SJ, family S50, peptidase S50.002
Species distribution: known only from avian infectious bursal disease virus
Reference sequence from: avian infectious bursal disease virus (UniProt: P22351)

MEROPS name: Drosophila X virus Vp4 peptidase
MEROPS classification: clan SJ, family S50, peptidase S50.003
Species distribution: known only from Drosophila x virus
Reference sequence from: Drosophila X virus
PDB accession numbers: 2GEF for BSNV VP4, 2PNM and 2PNL for IPNV VP4, 3P06 for TV-1 VP4 proteases

Name and History

Birnaviruses form a distinct family of double-stranded RNA (dsRNA) viruses infecting diverse animal species from vertebrates (fish and avian species) and invertebrates, including insects, mollusks and rotifers [1]. The family comprises six divergent genetic clusters, four of which are recognized as the genera: *Avibirnavirus* (prototyped by infectious bursal disease virus, IBDV); *Aquabirnavirus* (infectious pancreatic necrosis virus, IPNV); *Blosnavirus* (Blotched Snakehead

virus, BSNV) and *Entomobirnavirus* (Drosophila X virus, DXV). The two other clusters, with no taxonomic rank assigned so far, include the Drosophila B virus (DBV) and the Tellina virus 1 (TV-1), respectively. The virions are nonenveloped, icosahedral particles with a capsid made by the viral protein 2 (VP2, [2]); the number 2 following 'VP' corresponds to the relative migration position of this protein – from top to bottom – among other virion proteins in SDS-PAGE. The birnavirus genome is made by two dsRNA segments, A and B, each consisting of about 3–3.5 thousand base pairs. Genomic segment A encodes a polyprotein precursor (PP) with the NH_2-pVP2-VP4-VP3-COOH protein organization; a small protein, called VP5 in IBDV, is encoded in a separate overlapping open reading frame. In BSNV and TV-1, the PP includes an additional polypeptide – called X and flanked by the pVP2 and VP4 – to form the pVP2-X-VP4-VP3 protein organization. Segment B encodes the viral RNA-dependent RNA polymerase (VP1) a fraction of which is covalently bound to the 5′-ends of the two strands of the both genomic RNA segments [3].

The ***birnavirus virus protein 4 processing endopeptidase*** (***VP4***) is autoproteolytically released from the PP along with the VP2 precursor pVP2 and VP3 *in vivo* in infected cells [4–7] and *in vitro* in cell-free reticulocyte lysates [8,9]. VP4 remained an enigmatic protease for more than a decade (for a detailed historical account see Leong & Mason [10]). Two groups have independently presented conclusive evidence for VP4 to be a Ser-Lys protease that is distantly related to bacterial Lon proteases (see Chapters 781 and 782) [8,9,11]. VP4 was coined 'non-canonical Lon proteinase' to distinguish it from the bacterial, ATP-dependent homologs [8]. The substrate specificity was first identified for the DXV VP4 protease cleaving between Ser500 and Ala501 and, likely, Ser723 and Ala724 residues (hereafter the PP nomenclature specific for a particular birnavirus) [12].

Activity

The proteolytic activities of IBDV, IPNV, BSNV and TV-1 VP4s were demonstrated in *E. coli* and reticulocyte lysates [8,9,11,13,14] and, additionally for the IBDV VP4, in cRNA transfected cells *in vivo* [8]. The authentic IBDV and IPNV PP proteolytic processing has also been reported in transient expression systems in eukaryotic cells [15–18]. When expressed in these systems, PP or its fragments, containing VP4 with upstream or downstream sequences, were processed to VP4 and (truncated versions of) pVP2, (X), and VP3. The further conversion of pVP2 into VP2 was evident only when virus-like particles were formed, suggesting that the pVP2-to-VP2 proteolytic conversion is linked to virus morphogenesis [19–21]. The IPNV and IBDV VP4s may cleave in *cis* and *trans*, although the latter, less efficient activity has been demonstrated only in studies in *E. coli* and reticulocyte lysates [8,9,11]. The PP cleavage at the VP4↓VP3 versus the pVP2↓VP4 site was found to be more sensitive to point mutations in the IBDV and IPNV VP4 (and not observed in *trans* for PP formed in reticulocyte lysates). The VP4↓VP3[6,15] and pVP2↓VP4 [5,22] interactions do not appear to be crucial for the cleavage at the distal site. The proteolytic processing of the IPNV PP was claimed to be resistant to inhibitors of all protease classes, although the details of this analysis was only published in a thesis [10]. Unlike the canonical Lon, VP4 does not depend on ATP to cleave its protein substrate [8].

Cleavage Specificity

VP4 proteases of different birnaviruses were shown to cleave the VP4↓VP3 and the pVP2↓VP4 (and the pVP2↓X and X↓VP4) junctions cotranslationally during the PP synthesis. Consequently, PP may never be formed *in vivo*. Subsequent serial cleavages at the C-terminus of pVP2 yield the mature VP2 protein and, depending on the virus, three (IPNV, TV-1 and DXV) or four (IBDV and BSNV) peptides that remain associated with the virion. Stepwise conversion of pVP2 to VP2 takes place only upon particle assembly [19–21,23]. It involves a large quaternary maturation complex formed by pVP2 that is processed by VP4 in association to VP3 interacting with VP1 [23]. It seems that a conformation of VP3, which is produced under the VP4 control, may affect the cleavage [19]. The VP4 involvement in the final cleavage leading to the mature VP2 – between VP2 and the largest peptide in IBDV (pep46) – was postulated based upon similarities of this site with the proven VP4 cleavage sites sequences [24]. Alternatively, this cleavage may be VP4-independent and requires an Asp431 in VP2 [25].

The structure of the discussed cleavage sites were identified by different methods including the N-terminal sequencing of the C-terminal cleavage products, site-directed mutagenesis of these sites, mass spectrometry identification of the virus-associated peptides or their combination. Some cleavage sites in the least characterized birnaviruses were assigned by comparative sequence analysis with experimentally established sites [9,11–14,18,24,26]. A birnavirus-wide comparison of the established and putative VP4-mediated cleavage sites in the different birnaviruses (Figure 779.1) showed the relatively strong conservation of the P3 and P1 positions. With the notable exception of Pro in the P3 position of BSNV, they are commonly occupied by the small amino acid residues Ala and Ser. For the P1′ and P2′ positions, while no consensus amino acid can be identified for TV-1 and DBV, Ala is most frequent in the P1′ position and Ser, Asp and Gly are in the P2′ position. Thus, the cleavages likely occur at the [A/T]XA↓[A/F][SDG] sites in IBDV, [T/S]X[A/S]↓[A/S/W]G sites in IPNV, [P/A]XA↓[A/F/S][S/D/G] in BSNV, [A/G]XS↓[A/F][S/D] in DXV, AXA↓

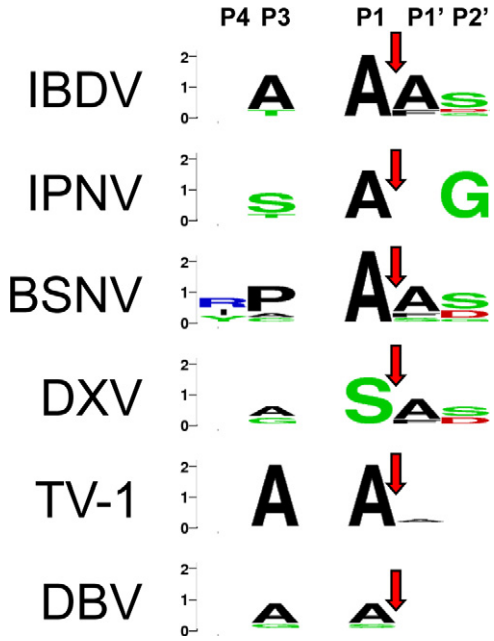

FIGURE 779.1 Sequence logos for the birnavirus polyprotein cleavage sites. The five-six cleavage sites sequences identified for each of the six birnaviruses (infectious bursal disease virus (IBDV), infectious pancreatic necrosis virus (IPNV), Blotched Snakehead virus (BSNV), Drosophila X virus (DXV), Tellina virus 1 (TV-1), Drosophila B virus (BDV)) were aligned and submitted to logo representation [35]). The characters representing the amino acids are stacked on top of each other for each position in the aligned sequences. The height of each letter is made proportional to its frequency, and the letters are shown in the descending order of frequency. The height of the entire stack reflects the information content of the sequences at that position (bits). Note the lack of a dominating amino acid residue at the position P2.

in TV-1, and [A/G]X[A/S]↓ in DBV. Despite the sequence similarities between the VP4 cleavage sites of different birnaviruses, VP4s of IPNV and IBDV were unable to cleave non-cognate substrates indicating the involvement of other species-specific determinants [9]. These and other determinants of substrate specificity were elucidated in the crystal structures of the IPNV and TV-1 VP4s in complex with peptide substrates [27,28] (see below).

Structural Chemistry: Fold, Catalytic Site and Substrate Recognition

Bioinformatics, molecular genetics and biochemical analyses first identified VP4 as a Ser-Lys catalytic dyad protease containing from 226 to 247 amino acid residues and including an ~90 amino acid domain conserved across the Lon/VP4 proteases (core) at its C-terminus [8,9,11]. The Ser652 and Lys692 of IBDV and their counterparts in IPNV, BSNV and TV-1 proved to be indispensable for the catalysis. From all mutations tested in the IBDV Ser652 position, the PP processing tolerated only a Ser652Cys replacement [8]. The proteolytic activity of the IBDV Ser652Cys mutant was selectively inactivated by exposure to 3.5 mM *N*-ethylmaleimide. Likewise, the proteolytic activity of the IBDV Lys692Arg but not four other Lys692 mutants was activated in the monomolecular reaction at pH 11.0. Collectively, these results strongly imply that Lys692 plays the role of a general base and activates the hydroxyl of Ser652 for catalysis [8]. The chemistry of proteolysis used by VP4 is likely to be conserved across the whole Lon protease family.

In contrast to the conservation of the core domain in VP4 and Lon, no statistically significant similarity was detected between the primary structures of VP4 and other proven Ser-Lys proteases. However, the relative position and distance between the catalytic Ser and Lys residues, and results of secondary structure predictions are compatible with the VP4 core adopting a variant of the β-sheet fold conserved in UmuD′ and leader bacterial protease, prototypes of two other, distantly related, Ser-Lys families (see Chapters 772–778 and this chapter) [8].

These and other aspects were fully addressed in the crystal structures of the *E.coli* expressed VP4s of BSNV, IPNV and TV-1 solved by the group of M. Paetzel [27–29] (Figure 779.2). The VP4s from these three birnaviruses were found to adopt similar protein folds made of two separate domains. Domain I is predominately formed by β-strands mainly arranged in an antiparallel fashion. This β-sheet domain houses the substrate groove and specificity pockets. Domain II adopts an α/β fold, with the nucleophilic serine residing at the amino-terminal end of the first α-helix and the general-base lysine being part of the second helix. The VP4 fold distantly resembles those of other Ser-Lys proteases.

The Catalytic Site

In BSNV, the N^{ζ} of the general base Lys729 is coordinated by the nucleophile Ser692 O^{γ} that together define the catalytic dyad of the protease [29]. The N^{ζ} of Lys729 is also coordinated via hydrogen bonds to the $O^{\gamma}1$ of Thr712 and the O of Pro590. The two catalytic and two catalytic-coordinating residues are universally conserved in all known birnavirus proteases [27]. The accessible surface area of Lys729 N^{ζ} is 5.5 Å as compared to an average of 51 Å for the N^{ζ} of other lysine residues in BSNV VP4. When a cognate cleavage site (a substrate) was modeled into the substrate-binding pocket of VP4, the N^{ζ} of Lys729 was found to be completely buried. This suggests that the ε-amino group of Lys729 can be deprotonated by change in local structural environment that decreases its pK_a and enables it to act as a general base during the catalysis.

An important contributor to the catalytic machinery in serine proteases is the oxyanion hole that neutralizes the developing negative charge on the scissile carbonyl oxygen atom of the substrate during the formation of the tetrahedral intermediates. Typically, two main chain amide hydrogen atoms that serve as hydrogen bond donors to

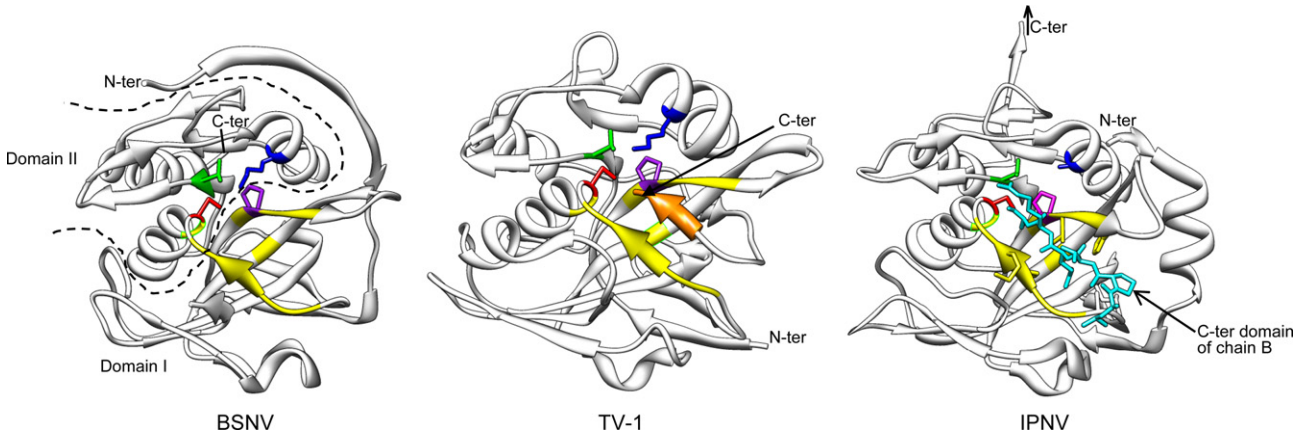

FIGURE 779.2 The structural architecture of BSNV, IPNV and TV-1 VP4 proteases. Shown are proteases in a ribbon representation depicting β-strands and α-helices. The four absolutely conserved residues forming the catalytic site (Ser in red, Lys in blue, Thr in green and Pro in magenta) are depicted with their bonds. Amino acid residues comprising the extended crevice which is involved in the substrate binding are indicated in yellow. (Left) In the BSNV VP4, the two structural domains are separated by a dashed line. The protease structure was solved with the substrate-binding site unoccupied. (Middle) The TV-1 VP4 structure revealed an intramolecular (*cis*) acyl-enzyme complex. (Right) Acyl-enzyme complex obtained with a truncated form of the IPNV VP4 and formed between the nucleophilic serine with the C-terminal residue of a neighbouring VP4 (chain B, colored in cyan). Note that the catalytic lysine was substituted by an alanine.

the developing oxyanion form oxyanion holes. In the VP4 structure of BSNV without a bound substrate, the main chain NH of the Ser692 and Gln691 residues point towards the binding site; these residues most likely contribute atoms to the oxyanion hole. In contrast, the NH group of the highly conserved Gly690, whose equivalent in many serine proteases forms the oxyanion hole, is pointing away from the binding site. The TV-1 VP4 acyl-enzyme structure [27] revealed the structural conservation of the oxyanion hole that is formed by the main chain amide nitrogen of the catalytic Ser738 and Asn737 (TV-1 amino acid numbering). Ser738 is within hydrogen bonding distance to the scissile carbonyl oxygen. Because the main chain amide nitrogen of Asn737 is too distant to contribute to the oxyanion stabilization, it is possible that the TV-1 VP4 oxyanion hole is only formed during the transition states when the tetrahedral oxyanion is present.

The acyl-enzyme intermediate complex structure determination of the TV-1 VP4 [27] allowed the identification of a potential 'deacylating' ('nucleophilic') water. It is coordinated by hydrogen bonds to Ser738 O^{γ}, Lys778 N^{ζ}, Pro649 O, and Thr760 $O^{\gamma}1$.

The Substrate-Binding Site

Surface analysis of the BSNV VP4 showed that the largest pocket on the surface of VP4 corresponds to an extended bent crevice that incorporates the catalytic residues at one end [29]. The dimensions of the crevice are consistent with the binding of an extended polypeptide and the topology of the surface suggests that the crevice is the S1/S3 binding pocket. Their sizes are consistent with the size of the side chains at the P1 and P3 positions of the cleavage site. The position of the binding site relative to the nucleophilic serine hydroxyl group shows that Ser692 O^{γ} attacks from the *si*-face of the scissile bond.

The TV-1 VP4 crystals revealed a continuous electron density from the catalytic Ser738 O^{γ} to the carbonyl carbon of Ala830, the C-terminus of VP4 and P1 residue for the VP4↓VP3 cleavage site [27]. Therefore, the structure revealed an intramolecular acyl-enzyme complex with the three last residues of TV-1 VP4 stabilized by hydrogen bonding interactions with the VP4 residues constituting the cleavage-site recognition groove and specificity pockets. Consistent with results obtained for the BSNV VP4, no binding pockets were identified for the P2, P4 and P5 residues whose side chains are pointing away from the substrate-binding site.

The crystal structure of the IPNV VP4 revealed the molecular details of an acyl-enzyme complex formed between the enzyme and an internal VP4 cleavage site of another VP4 molecule in *trans* [28]. This complex was described using a truncated enzyme in which the general base lysine was substituted by an alanine. In the complex, the nucleophilic Ser633 O^{γ} forms an ester bond with the main chain carbonyl of the C-terminus of a neighboring VP4 molecule in which the internal site is located. The substrate specificity is determined by interactions involving the S1, S3, S5 and S6 substrate-binding pockets and respective residues of the substrate.

Birnaviruses differ in respect to the conservation of VP4 cleavage sites in the PP. Alanine is conserved at positions P1 and P3 of all cleavage sites in TV-1 [14]. In contrast, in IPNV alanine is conserved only at the P1 position while a serine residue is predominantly found at the P3 position of the cleavage sites. Consistently with these patterns, analysis of the molecular surfaces for the S1 and S3 specificity pockets of the TV-1 VP4 revealed that the S1

and S3 are shallow and hydrophobic, complementary of the alanine methyl group side chains at the P1 and P3 positions. Likewise, the IPNV S1 was also shallow and hydrophobic, while the S3 pocket is deeper and hydrophilic, allowing it to adapt with a greater variety of residues at the P3 position. Water molecules at the bottom of the pocket may play a role in the fit for a larger variety of side chains (see Figure 779.1) [27]. A comparison of the substrate-binding grooves with bound substrates revealed a similar hydrogen bonding pattern spanning residues P1 to P5 with an average hydrogen bonding distance of 3.0 Å for both TV-1 and IPNV VP4s [27,28]. Both substrate-binding grooves utilize a water molecule within the interaction distance, but on the opposite site of the substrate.

Preparation

VP4 can be produced as a recombinant protein in prokaryotic and eukaryotic expression systems. Regardless of the virus origin, all VP4s characterized so far were found soluble when expressed in *E. coli* and amenable for structural and biochemical studies (see Chung & Paetzel [27] and references therein). However, some differences between VP4s from diverse birnaviruses were also revealed. For instance, recombinant expression of the IPNV VP4 results in the generation of several truncated by-products likely produced through the utilization of internal cleavage sites [11]. Consequently, expression of a proteolytically inactive VP4 mutant (with a replacement of the catalytic Ser or Lys residue) yielded a stable full-length protein. In contrast, wild-type VP4s from BSNV, TV-1 and IBDV have long half-life times and are stable at high concentrations. An additional distinguishing feature of the IBDV VP4 is that it forms tubules made by homo-multimers in infected cells and heterologous expressing systems. These tubules (named type II tubules to differentiate from those formed by pVP2 in infected cells) are 25 nm in diameter. They may change in appearance during isolation [30] and can be recovered by differential centrifugations from *E. coli* or cellular lysates. Purifications of other recombinant VP4s were mainly carried out using recombinant His-tagged proteins by nickel-affinity and size-exclusion chromatographies.

Biological Aspects

VP4 regulates the birnavirus replication cycle at several levels. VP4 was detected in IBDV and IPNV virions [22,31], although it may be a contamination from unique type II tubules in IBDV [30]. The indispensability of the VP4 proteolytic activity for the virus viability was proved using both IBDV and IPNV ([8]; E. Mundt, unpublished data). Further insight into the biological roles of VP4 has been gained using IBDV. The VP4-mediated proteolytic processing of PP is critical for the formation of virion particles, of which two major components, pVP2/VP2 and VP3, are proteolytically derived. Virions of IBDV also contain four peptides derived upon the conversion of pVP2 into VP2. Two IBDV mutants, in which a locus encoding either the first or last VP2 peptide was deleted, were found to be noninfectious [24]. When the PP processing was blocked or heavily impaired by a mutation of the active site of VP4, the expression of VP1 encoded by RNA segment B was not evident in transfected cells [8]. VP4 might trans activate the expression of RNA B (VP1) through the production of VP3 that is known to interact with VP1 [23,32,33] and may bind virus RNAs [19,34]. Complete but slowed PP processing in cells transfected by the active site Ser652Cys mutant was apparently not sufficient for the generation of infectious progeny, indicating that virion biogenesis may be coupled with the PP cleavages in a cleavage-rate restricted manner [8].

Distinguishing Features

The birnavirus VP4s form a unique branch of the Lon protease family, which protease domain has a narrow substrate specificity and neither needs ATP for its activity nor contains an ATPase domain. Proteases with a similar domain organization are encoded by eukaryotes [8].

The property of the IBDV VP4 to form tubules with a diameter of 25 nm *in vivo* is not shared with the VP4 of other birnaviruses. Mouse monoclonal antibodies directed against VP4 of IBDV strain P2 were described [30].

Related Peptidases

For related peptidases see Chapters 781 and 782 on Lon proteases, and a separate chapter (780) on TV-1 VP4 peptidase.

Further Reading

The papers of Birghan *et al.* [8], Lejal *et al.* [9], Petit *et al.* [11], da Costa *et al.* [24], Chung & Paetzel [27], Lee *et al.* [28], and Feldman *et al.* [29] are recommended.

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