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Pestivirus NS2-3/NS3 Serine Peptidase

**DATABANKS**

*MEROPS name:* pestivirus NS3 polyprotein peptidase  
*MEROPS classification:* clan PA, subclan PA(S), family S31, peptidase S31.001  
*Species distribution:* known only from bovine viral diarrhea virus 1  
*Reference sequence from:* bovine viral diarrhea virus 1 (UniProt: Q01499)

**Name and History**

The genera *Pestivirus*, *Hepacivirus* and *Flavivirus* are grouped together in the family *Flaviviridae*. Pestiviruses are bovine viral diarrhea virus (BVDV-1, BVDV-2), classical swine fever virus (CSFV) and border disease virus of sheep (BDV). The single-stranded RNA genome of pestiviruses contains one long open reading frame (ORF) with about 4000 codons. The putative polyprotein is cleaved co- and post-translationally by host cell- and virus-encoded proteases. The existence of a chymotrypsin- or trypsin-like serine protease in the N-terminal domain of pestiviral nonstructural protein NS3 (p80) was predicted by comparative amino acid pattern analyses [1,2]. Subsequently, transient expression studies in the T7 vaccinia virus system [3–5] and expression via baculovirus recombinants [6] provided experimental evidence for this assumption. The studies revealed that NS2-3 (p125) as well as NS3 are proteolytically active serine proteases. The serine protease domain residing in NS2-3/NS3 is essential for processing of the polyprotein downstream of NS2-3 and, with respect to genomic localization, function and amino acid motifs is homologous to the NS3 serine proteases of hepaciviruses (Chapter 688) and flaviviruses (Chapter 687).

Within the family *Flaviviridae*, the existence of unprocessed NS2-3 as a mature viral protein is unique for pestiviruses. It was demonstrated for BVDV and CSFV that uncleaved NS2-3 is essential for the production of infectious virions [7,8]. Moreover a cellular chaperone of the J-domain family can induce NS2-3 cleavage in *cis* or in *trans* [9]. It turned out that this chaperone acts as cofactor of a viral autoprotease residing in NS2 [10,11]. The limiting amounts of this cellular cofactor available in the infected cell are responsible for the observed temporal modulation of NS2-3 cleavage by the NS2 protease [12]. Accordingly, in noncytopathogenic (noncp) pestivirus-infected cell cultures efficient NS2-3 cleavage and thereby NS3 release is only observed in the first hours after infection. Even though uncleaved NS2-3 is present in pestivirus-infected cells, certain pestivirus isolates have evolved mechanisms for a highly efficient release of NS3 from the polyprotein (see Further Reading below).

Interestingly, high-level expression of NS3 is correlated with cytopathogenicity of the respective viruses.

**Activity and Specificity**

Processing of the pestiviral polyprotein downstream of NS2-3 is mediated by the NS2-3/NS3 serine protease. The cleavage generating the C terminus of NS2-3/NS3 is believed to occur only in *cis*. The cleavages at the NS4A↓4B, NS4B↓5A and NS5A↓5B sites can be mediated in *trans* [3,4,17].

The NS2-3/NS3 serine protease is not involved in processing at the NS2-3 cleavage site [15,18,19].

The cleavage sites recognized by the NS2-3/NS3 serine protease have been identified [4,5]. The amino acids at the P1/P1’ positions are conserved between pestivirus species. Leu at P1 was found to be the only position...
conserved at all four cleavage sites. At P1' Ala is found in almost all pestiviral isolates at the NS4A-4B site; isolate Bungowannah [20] is an exception coding for Ser at this position. Ser is also found at the P1' position for all other cleavage sites. Remarkably, BDV represents an exception since the P1' position of the functional NS5A-5B cleavage site is covered by an Asn residue [21]. Inhibitory activity against the NS3 protease was demonstrated with a boron-modified peptidyl mimic of the NS4A/4B cleavage site in vitro with enzyme expressed in and purified from E. coli [22].

Most of NS2-3 remains uncleaved in cells infected with noncp pestiviruses due to the cofactor dependency of the NS2 autoprotease [11,12]. So far it is believed that the NS2-3 and the NS3 serine proteases basically exert identical cleavage reactions. However, the processing kinetics of free NS3 seems to be significantly faster than the one of the NS2-3 protease. This assumption is derived from comparative studies on polyprotein processing in cells infected by either cp or noncp CSFV [23].

For HCV the NS3 serine protease dependent cleavage sites show a consensus motif for P6 (Asp- or Glu), P1 (Thr or Cys) and P1' (Ser or Ala) (see also Chapter 688). The corresponding protease of flaviviruses cleaves after two amino acids with basic side chains (P2 and P1) which are usually followed by amino acids with small side chains (see also Chapter 687).

**Structural Chemistry**

Processing studies and protein sequencing data revealed that the N terminus of NS2-3 is generated by cellular signal peptidase [24]. The N-terminal part of NS2 is highly hydrophobic leading to anchoring of NS2/NS2-3 to intracellular membranes. It is assumed that the C termini of NS2-3 and NS3 are identical. Protein sequencing approaches identified Gly1590 (numbering according to noncp BVDV strain SD-1 [25]) as the N terminus of NS3 of two cp BVDV strains [14,15]. The genome structures of cp pestiviruses imply that the N-terminal region of NS3 appears likely [13]. Accordingly, NS2-3 and NS3 of BVDV exhibit lengths of 1137 and 684 amino acids, respectively. While NS3 starting with Gly1590 is catalytically active at all cleavage sites, N-terminal truncation of NS3 by only 6 amino acids led to a strongly reduced cleavage selectivity. While NS3 starting with Gly1590 is catalytically active at all cleavage sites, N-terminal truncation of NS3 by only 6 amino acids led to a strongly reduced cleavage selectivity at the NS4A-4B site [17]. After a deletion of the N-terminal 20 amino acids only NS3 and a minor amount of NS5B were released from the polyprotein. An N-terminal truncation by 45 amino acids finally interfered also with the cis-cleavage at site NS3-4A. The minimal NS3 serine protease domain shown to be active in trans on an NS5A-5B substrate encompasses so far 209 amino acids [17]. However, it should be pointed out that for pestiviral RNA replication the authentic N terminus of NS3 is essential [26].

For flaviviruses and HCV the minimal length of a catalytically active NS3 serine protease is about 180 amino acids [27,28]. Thus the length of the serine protease is very similar for all members of the Flaviviridae. A model developed by Gorbalenya and coworkers ([29]; see also Bazan & Fletterick [11]) proposed for the pestiviral protease a catalytic triad consisting of His1658, Asp1686 and Ser1752. Mutagenesis studies revealed that His1658 and Ser1752 are indispensable for all cleavages mediated by the NS2-3/NS3 serine protease. In contrast, Asp1686 was not essential for cis cleavage at the NS2-3/NS3-4A site but for all other cleavages. An exchange of Ser1752 by Cys led to a protease with significant (cis) activity at the NS2-3/NS3-4A site. Interestingly, also the replacement of Ser1752 by Thr yielded detectable cleavage at this site even though at strongly reduced levels [17]. This unusual finding is reminiscent of an earlier report [30] for the capsid protease of Sindbis virus (Chapter 504), an alphavirus of the family Togaviridae underlining the similarity between these proteases as proposed earlier.

For its activity the NS2-3/NS3 serine protease requires NS4A as a cofactor [3,4,17]. NS4A has a length of 64 amino acids [4,5]; the cofactor function has been mapped to a 38-amino-acid peptide in the middle of NS4A [5]. NS4A is essential at least for the cleavages at the NS4B-5A and the NS5A-5B sites [4]. It is still unclear whether the NS4A cofactor is also essential for the cleavages at the NS3-4A and NS4A-4B sites. N-terminally truncated NS4A-4B no longer encompassing an active NS4A cofactor domain was no substrate for the NS2-3/NS3 serine protease; however, in this assay cleavage could not be restored by co-expression of authentic NS4A (Tautz, N., unpublished data). Accordingly, the cofactor requirements in this processing step are still to be determined.

NS4A undergoes a stable interaction with NS3. Since N-terminal truncations of NS3 by only six amino acids abrogate NS4A binding, an interaction of NS4A with the N-terminal region of NS3 appears likely [17]. Interestingly, in the HCV system NS4A also represents a cofactor of the NS3 protease and binds to the N-terminal region of NS3 (for review see De Francesco & Steinkühler [27]). Comparative structural models of the NS3 proteases of different members of the family Flaviviridae are published [4].

**Preparation**

Enzymatically active proteases have been expressed using the T7 Vaccinia virus system [3,17], the baculovirus system [6], E. coli [5,22] and in vitro translation [31]. Fusion of GST to the N terminus of NS3 did not interfere with the activity of the protease [17]. NS3 serine protease expressed in and purified from E. coli has been used for inhibitor development [22]. To study the helicase activity...
of NS3 the active enzyme has been partially purified by affinity chromatography from eukaryotic cells [31].

**Biological Aspects**

Experiments with an infectious cDNA clone of BVDV revealed that an active NS2-3/NS3 serine protease is essential for RNA replication [4]. Moreover, RNA replication also depends on the authentic N terminus of NS3 [26].

Polypeptide cleavage kinetics studied by pulse chase experiments with cp BVDV strain NADL infected cells showed that cleavages at the NS3-4A and NS4B-5A sites are very rapid; cleavages at the NS4A-B and NS5A-B sites occur in a delayed fashion and the respective precursor molecules were detected [32]. This indicated that processing takes place in a temporally regulated manner. Recently, a significant difference in the processing kinetics of the NS4-5 region was observed between cp and noncp CSFV [23]. The results of this study imply that the serine protease of NS3 shows faster cleavage kinetics than the one of NS2-3.

Since high level expression of NS3 is linked to cytopathogenicity of pestiviruses the differences observed in the CSFV system between the proteolytic activities of NS2-3 and NS3 may be of biological significance for pestiviruses in general. In this context, it will be interesting to see additional data on the enzymatic activities of the purified NS2-3 and NS3 serine proteases.

Along those lines it was nicely demonstrated that expression of NS3 together with NS4A led to induction of apoptosis while expression of NS2-3/4A did not [33]; this observation offers one explanation for the link between the cp biotype and deregulated NS3 expression. The latter is connected to the cytopathogenic biotype of BVDV whose appearance in animals persistently infected with noncp BVDV leads to a lethal disease [13,16]. While NS2-3 is co-translationally associated with ER-derived membranes, NS3 appears to localize differently after being cleaved off [34]. Accordingly, discrepancies in the effect of either the NS2-3 or the NS3 serine protease on cell viability may be related to their subcellular localization and the resulting shift in the panel of accessibl cellular substrates.

**Distinguishing Features**

The expression of uncleaved NS2-3 by pestiviruses is a unique feature within the family Flaviviridae. All members of the other two genera of this virus family show efficient processing of NS2-3. Moreover cleavage of NS2-3 is essential for the viability of these viruses [35,36].

With respect to cofactor requirements the pestiviral protease resembles the hepaciviral enzyme (see above). Furthermore, for pesti- and hepaviruses the NS2 region is not essential for proteolytic function of the serine protease. This is in contrast to the flavivirus system where NS2B is an essential cofactor of the NS3 serine protease [28]. These findings demonstrate a closer relationship between the serine proteases of pestiviruses and hepaviruses in comparison to the respective enzyme of the flaviviruses. The significant residual activity of the NS2-3/NS3 serine protease after replacement of the active site serine by threonine stands for an interesting parallel to the capsid protease of Sindbis virus, an alphavirus (family Togaviridae), and suggests a common ancestor of these enzymes.

Monoclonal antibody 8.12.7, generated against BVDV NS3 in the laboratory of E.J. Dubovi (Cornell University, Ithaca, N.Y.) displays a very broad reactivity against NS3/NS2-3 molecules of the different pestivirus species and is thus a highly valuable tool [37].

**Further Reading**

For mechanisms for a highly efficient release of NS3 from the polyprotein, see Meyers & Thiel [13], Meyers et al. [14], Kümmere et al. [15], and Becher & Tautz [16]. Reviews have been provided by Lindenbach et al. [28].

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