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

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We have identified a region related to the protease domain of bacterial and organelle ATP-dependent Lon proteases in virus protein 4 (VP4) of infectious bursal disease virus strain P2 (IBDVP2), a two-segmented double-stranded RNA virus. Unlike canonical Lons, IBDVP2 VP4 possesses a proteinase activity though it lacks an ATPase domain. Ser652 and Lys692 of IBDVP2 VP4 are conserved across the Lon/VP4 family and are essential for catalysis. Lys692 has the properties of a general base, increasing the nucleophilicity of Ser652; a similar catalytic dyad may function in the other Lons. VP4 can cleave *in trans* and is responsible for the interdomain proteolytic autoprocessing of the pVP2–VP4–VP3 polypeptide encoded by RNA segment A. VP2, which is later derived from pVP2, and VP3 are major capsid proteins of birnaviruses. Results of the characterization of a range of the IBDVP2 VP4 mutants in cell cultures implicate VP4 in *trans*-activation of the synthesis of VP1, putative RNA-dependent RNA polymerase encoded by RNA segment B, and in cleavage rate-dependent control of process(es) crucial for the generation of the infectious virus progeny.

Keywords: autoproteolysis/birnavirus/capsid biogenesis/La (Lon) protein/serine–lysine protease

Introduction

Escherichia coli Lon (La) protease maintains cell homeostasis through ATP-dependent breakdown of abnormal proteins and a number of short-lived regulator proteins to low molecular weight products (Goldberg, 1992; Gottesman, 1996). *Escherichia coli* survival under extreme stress conditions as well as development and vegetative growth in other bacteria involve Lon (Gottesman, 1996). This protein is also conserved in mitochondria of different species, where its proteolytic activity is used for respiration (Suzuki *et al.*, 1994). A multidomain organization of Lon proteases includes a conserved combination of the ATPase and protease domains (Figure 1A, bottom protein) (Chin *et al.*, 1988; Amerik *et al.*, 1991). Polypeptide substrates allosterically switch Lon from the latent ADP-bound to activated ATP-bound form by inducing release of ADP and binding of Mg-ATP molecules (Waxman and

Goldberg, 1986; Goldberg, 1992). In the presence of non-hydrolyzable analogs of ATP, Lon retains peptidase activity (Goldberg and Waxman, 1985) and, accordingly, an isolated Lon protease domain (Lon^{pro}) proved to be a peptidase rather than a proteinase (Rasulova *et al.*, 1998; van Dijk *et al.*, 1998).

The Lon^{pro} contains a conserved Ser residue indispensable for the proteolytic activity (Amerik *et al.*, 1991; Fischer and Glockshuber, 1993), although Lon was not found to be related to any other serine proteases (Chung and Goldberg, 1998). The latter enzymes employ Ser as the principal nucleophile of the catalytic dyad or triad residues, respectively Ser–Lys or different combinations of three residues, with Ser–His–Asp being the most common (Dodson and Wlodawer, 1998). Few dyad-containing protease families have been described (Black, 1993; Little, 1993; Keiler and Sauer, 1995; Dalbey *et al.*, 1997), and a unique β -sheet fold is conserved in two of them (Peat *et al.*, 1996; Paetzel *et al.*, 1998). In contrast, catalytic triads exist in many forms and are grafted upon different folds identified in serine proteases of cellular and viral origin (Babe and Craik, 1997; Dodson and Wlodawer, 1998).

Viral proteinases have evolved to regulate replication and expression of viral and cellular genomes through irreversible conversion of selected proteins from one form to another by limited cleavage(s) (Kräusslich and Wimmer, 1988; Dougherty and Semler, 1993; Gorbalenya and Snijder, 1996). An especially astonishing diversity has been described for RNA viral proteinases. The Birnaviridae viral protein 4 (VP4), also known as non-structural (NS) protein in some viruses, is among the few RNA viral proteinases that have not yet been classified and, furthermore, resisted detailed characterization (Dobos, 1995; Leong and Mason, 1998). The Birnaviridae family includes viruses of three genera infecting birds, fish and insects, respectively: Avibirnaviruses (prototyped by infectious bursal disease virus, IBDV); Aquabirnaviruses (infectious pancreatic necrosis virus, IPNV); and Entomobirnaviruses (*Drosophila* X virus, DXV). The birnavirus genome consists of two double-stranded RNAs: segment A (~3.1–3.4 kb) and segment B (~2.7–3.0 kb) (Dobos, 1995; Chung *et al.*, 1996). Segment A encompasses two overlapping open reading frames (ORFs), with the large one encoding the NH₂-pVP2–VP4–VP3-COOH polypeptide (PP) (Figure 1A, top protein), and the small ORF, whose position is genus specific, encoding a protein called VP5 in IBDV. The integrity of VP4 was shown to be essential for the proteolytic processing of PP at the two interdomain junctions in reticulocyte lysates and in *E. coli* (Duncan *et al.*, 1987; Jagadish *et al.*, 1988; Manning *et al.*, 1990). Therefore, VP4 is believed to be a proteinase. pVP2 (VPX) is produced from PP through cleavage of a scissile bond, probably between Ser and Ala in DXV

(Chung *et al.*, 1996), and, later, is processed further to VP2 by an unknown mechanism. VP2 and VP3 form, respectively, the outer and inner layers of the icosahedral virions, which also include several molecules of VP1, putative RNA-dependent RNA polymerase (RdRp) encoded by segment B (Dobos, 1995; Bottcher *et al.*, 1997; Lombardo *et al.*, 1999). VP4 was also detected in virions (Kibenge *et al.*, 1988; Magyar and Dobos, 1994), although it may be a contamination from unique type II tubules (Granzow *et al.*, 1997). VP5 was not reported in

In this report, we predict and provide experimental evidence to show that IBDV VP4 and its homologs in the other birnaviruses form a unique branch of the Lon family whose Lon^{pro} is not associated with an ATPase domain. This non-canonical RNA viral Lon proteinase with a

Table 1. Oligonucleotides used for the construction of full-length cDNA clones of IBDVP2 RNA segment A mutants

Nucleotide sequence of primers ^a	Designation	Orientation	Coordinates ^b	Plasmid
GGAAAC gc TGGAAATCTAGCCATAG	S652A	Sense	2078–2102	pP2AS652A
GGAAAC ct TGGAAATCTAGCCATAG	S652C	sense	2078–2102	pP2AS652C
GGAAAC c GTGGAAATCTAGCC	S652R	sense	2078–2098	pP2AS652R
GGAAAC Ac TGGAAATCTAGCCATAG	S652T	sense	2078–2102	pP2AS652T
CCAATCCATGTGGCTATG g CGGGAG	T674A	sense	2132–2156	pP2AT674A
CATGTGGCTATG gt GGAGCCCTCAATGCTTGTGG	T674C	sense	2138–2172	pP2AT674C
CCAATCCATGTGGCTATG t CGGGAG	T674S	sense	2132–2156	pP2AT674S
CAACTTAAGGCCAAGTCGGTGTGCAGTGGCGAGCTTGGT	S690A	antisense	2186–2239	pP2AS690A
Ggc TCTAAAGCTTAC				
AGCAC gc GCTCGCCACTGCACACC	K692A	sense	2198–2222	pP2AK692A
AGCAC c ATCTCGCCACTGCACACC	K692H	sense	2198–2222	pP2AK692H
AGCAC c AGCTCGCCACTGCACACC	K692Q	sense	2198–2222	pP2AK692Q
AGCAC cg GCTCGCCACTGCACACC	K692R	sense	2198–2222	pP2AK692R
CAACTTAAGGCCAAGTC Gc TGTGCAGTGGC	H697Q	antisense	2210–2239	pP2AH697Q
ATGGAGGTGGCCGACCTCAACTCTC	1409	sense	1409–1433	–

^aMutated codons are in bold and replaced wild-type nucleotides are in lower case. The *A*/III restriction sites in two oligonucleotides are underlined.

^bThe positions in IBDVP2 RNA A (Mundt and Muller, 1995) complementary to the primers.

narrow specificity employs the conserved Ser–Lys catalytic dyad to perform cleavages controlling IBDV reproduction at several levels. The chemistry of proteolysis used by VP4 is likely to be conserved across the whole Lon family.

Results

VP4 of birnaviruses contains the conserved Ser–Lys dyad in a domain related to Lon^{pro}

Using bioinformatics tools, we identified a domain related to Lon^{pro} in VP4 of birnaviruses (Figure 1). This Lon, as well as some other proteins theoretically identified in genomes of Archaea (van Dijk *et al.*, 1998) and *Caenorhabditis elegans*, does not include Lon^{ATPase} (Figure 1) and, therefore, will be called non-canonical Lon. The domain conserved across the Lon/VP4 proteases and called the Lon^{pro} core is composed of ~80 amino acids including six invariant residues, identified in IBDVP2 as Gly560, Gly675, Pro706, Ser652, Thr674 and Lys692 (Figure 1B, line 130564_PP_IBDVP2, positions with a violet or red background). The latter three residues could be catalytic, and indeed, counterparts of Ser652 have already been shown to be essential for Lons of bacteria (Amerik *et al.*, 1991; Fischer and Glockshuber, 1993; Roudiak *et al.*, 1998) and mitochondria (Rep *et al.*, 1996). The conserved Ser and Lys residues of Lons can be aligned arbitrarily with the catalytic Ser–Lys dyad of proteases prototyped by two *E.coli* proteins: LexA (LexA-like family) (Little, 1993; Peat *et al.*, 1996) and leader protease (LPP-like) (Dalbey *et al.*, 1997) (Figure 1B). No statistically significant sequence similarity between Lon-related and the Ser–Lys proteases was detected. However, counterparts for five out of seven β -strands of UmuD' (β 1, β 2, β 3, β 5 and β 6), a LexA-like protease with a resolved three-dimensional structure (Peat *et al.*, 1996), could also be predicted in the Lon/VP4 proteases (Figure 1B). The discrepancy between the two sets of secondary structures may result from the <75% reliability of the secondary structure prediction methods (Rost and Sander, 1996). Thus, the UmuD' core fold may be conserved in Lon/VP4 proteases. We predicted from these analyses that birnavirus VP4s employ the Ser–Lys catalytic

Fig. 2. IBDVP2 proteins directed by RNA A in chicken embryonic cells (CEC) and in reticulocyte lysates. The positions of the viral proteins and the sizes of the marker proteins are indicated. A 25 μ l aliquot of [³⁵S]methionine-labeled cell lysates from mock-infected and infected CEC (Mundt *et al.*, 1995) as well as 12.5 μ l of the [³⁵S]methionine-labeled transcription and translation (TNT) programmed by a plasmid containing a DNA copy of wild-type IBDVP2 RNA A (pP2Awt) was precipitated with α -VP2, α -VP3, α -VP4 or α -IBDVP2 and processed for SDS–PAGE and autoradiography (see Materials and methods).

dyad as part of a Lon^{pro}-related domain mediating PP processing in an ATP-independent manner.

Conserved Ser652 and Lys692 are essential for processing of the IBDVP2 PP *in vitro*

To test the predictions of the model (Figure 1), we chose to characterize the IBDVP2 VP4. We have observed that the PP directed by IBDVP2 genome segment A transcripts was processed completely in reticulocyte lysates to three products equivalent to pVP2, VP3 and VP4, identified in infected cells using differential radioimmunoprecipitation analysis (RIPA) (Figure 2). This result was consistent with observations of researchers studying other birnaviruses such as IPNV (Manning *et al.*, 1990) and IBDV strain OH (Kibenge *et al.*, 1997). We will refer to the assay in Figure 2 as a monomolecular reaction since its design allows, but does not imply, self-processing of PP by *cis* cleavages. This *in vitro* system was employed to study

Fig. 3. Proteolytic activity of VP4 mutants as analyzed by monomolecular reaction in reticulocyte lysates. (A) Effect of mutations of VP4 on the PP processing in reticulocyte lysates. A 12.5 μ l aliquot of the [35 S]methionine-labeled TNT programmed by pP2AwT and its mutated derivatives was precipitated with α -IBDVP2 and analyzed as described in Materials and methods. (B) Differential RIPA of the PP processing of S652C in reticulocyte lysates. The analysis was conducted as described in Figure 2 except for using α -VP4-precipitated labeled proteins from 24.0 μ l of the lysate. (C) Kinetics of cleavages mediated by S652C VP4. The TNT directed by S652C was conducted and terminated as described in (A) and was either processed immediately for protein analysis (0 h) or incubated for the indicated time and processed thereafter.

how the PP processing is affected by substitutions of five residues, including the presumed catalytic Ser652 and Lys692 dyad (Table I; Figure 1B, residues marked by #). The PP processing was abolished when Lys692 was replaced by Ala, His, Gln or Arg, or when Ser652 was replaced by Ala, Thr and Arg (Figure 3A). In contrast, three mutants, T674S, S690A and H697Q, were indistinguishable from the parent, while only a partial inhibition of the PP processing, mostly at the pVP2–VP4 junction, was observed in T674C and T674A mutants (Figure 3A). The significant activity of the T674A mutant implied that the hydroxyl group of Thr674 is probably not involved in the catalysis. Unlike what was observed for the other S652 mutants, the PP processing was only slowed in S652C. The unprocessed PP and two intermediate precursors, pVP2–VP4 and VP4–VP3, detected after the termination of the transcription and translation (TNT) of S652C (Figure 3B), were converted steadily and completely into pVP2, VP3 and VP4 in a time-dependent manner (Figure 3C). These results are consistent with the model (Figure 1) and show that Cys can replace the presumed catalytic Ser652 efficiently.

The VP4 can process IBDVP2 PP to pVP2 and VP4–VP3 in trans in vitro

The mutations at Ser652 and Lys692 might block the PP processing due to misfolding of the cleavage sites rather than by inactivating the active site of VP4. To rule out this interpretation, a selective inactivation of a *trans*-acting VP4 proteinase tested against the 35 S-labeled S652R PP (*trans*-cleavage assay; see Materials and methods) was demonstrated *in vitro*. The substrate proved to be processed by the protease in a time-dependent manner for at least 12 h, and this processing was abolished by an S652A mutation of the protease (Figure 4). The PP was processed *in trans* at one site only, presumably the pVP2–VP4 junction, with the generation of stable pVP2 and VP4–VP3 precursors (Figure 4). In contrast to the monomolecular reaction (Figure 3A), the PP was cleaved very inefficiently in the *trans*-cleavage reaction; this could be a property of

Fig. 4. Kinetic analysis of the PP processing in reticulocyte lysates *in trans*. A 9 μ l aliquot of the [35 S]methionine-labeled substrate (S652R) was incubated with 9 μ l of non-labeled active (wt) or inactivated (S652A) *in vitro* generated VP4 proteinase for the indicated time and the products of the reactions were analyzed by SDS–PAGE as described in Materials and methods. The positions of IBDVP2 products and molecular weight markers are indicated. Two exposures, one short and one long, of the gel are presented for the high and low molecular weight zones, respectively, indicated by two double-headed arrows.

the PP processing or a result of the set-up of the *trans*-cleavage assay (e.g. low protease/substrate ratio).

Ser652Cys proteinase acting in trans is inactivated selectively by N-ethylmaleimide

The ability of VP4 to cleave *in trans* was used to address the reactivity of the predicted catalytic Ser652. We decided to characterize a partially active mutant S652C (Figure 3) whose presumed catalytic nucleophile Cys652 could be probed with *N*-ethylmaleimide (NEM), an SH-alkylating compound (Tschantz *et al.*, 1993). The wild-type and mutated (S652C) proteinases were found to cleave the pVP2–VP4 junction *in trans* with a comparable efficiency (Figure 5). This activity of the S652C but not of the wild-type VP4 was completely eliminated when these proteases were exposed to NEM (Figure 5). Hence, the Cys652 does deliver an SH group crucial for the authentic PP processing, and, by implication, the deprotonated OH group of Ser652 in the wild-type VP4 is the nucleophile, which attacks substrates.

Fig. 5. The effect of NEM treatment of S652C VP4 on its *trans*-cleavage activity. *In vitro* generated unlabeled wild-type, S652C and S652R VP4 were exposed (+) or not (-) to NEM and then incubated with [³⁵S]methionine-labeled substrate (S652R) as described in Materials and methods. Viral proteins were immunoprecipitated using α -IBDVP2 serum (Mundt *et al.*, 1995). The positions of products of VP4-mediated cleavage are indicated on the gel by dots. For other details, see the legend to Figure 4.

Lys692Arg proteinase, but not the other Lys692 mutants, is activated at pH 11.0

To verify the catalytic role of Lys692, we have studied the PP proteolytic processing of the Lys692 mutants further in reticulocyte lysates upon a pH shift to 11.0. At neutral pH, the prototype Ser-Lys protease, LexA protein, normally is activated by RecA protein through a mechanism presumably reducing the pK_a of the catalytic Lys. Alternative, 'non-cofactor' activation of the autodigestion of wild-type LexA or its catalytic Lys $\rightarrow$ Arg mutant could be achieved at alkaline pH values close to the pK_a of Lys and Arg, when both residues are partly deprotonated (Lin and Little, 1989; Little, 1993). We reasoned, therefore, that if VP4 does belong to the Ser-Lys proteases, its wild-type version must be active at neutral pH because of a unique spatial microenvironment around the catalytic Lys692, reducing the pK_a of this residue (Paetzel and Dalbey, 1997). Because the K692R mutant is proteolytically defective at neutral pH (Figure 3A), this microenvironment must be not nearly as efficient with respect to mutated Lys692 $\rightarrow$ Arg. It could be speculated, however, that the alkaline pH might instead be sufficient to activate the K692R mutant. Accordingly, when different samples of the TNT-generated PPs mutated at the Lys692 position were incubated at pH 8.0 and 11.0 for 24 h, a selective and pronounced activation of pVP2-VP4 processing was observed in the K692R mutant at pH 11.0 (Figure 6). An apparently authentic, but residual and pH-independent cleavage of PP was also detected in the K692H mutant (Figure 6); such cleavage was not evident after a 1 h incubation (Figure 3A). This weak activity of the K692H mutant fits the original model as well (see Discussion).

VP4 can cleave PP in trans in the absence of ATP in vitro

Lon proteases cleave polypeptide substrates in reactions strongly dependent on Mg-ATP (Goldberg, 1992). We determined whether the same requirements hold for IBDVP2 VP4. In the experiments described above, ATP and Mg^{2+} necessary to drive the TNT reactions might also have been used by the IBDVP2 proteinase to cleave PP. The 'endogenous' ATP and Mg^{2+} , along with other

Fig. 6. pH dependence of the proteolytic activity of Lys692 VP4 mutants. The TNT-generated [³⁵S]methionine-labeled proteins of five K692 codon mutants were incubated at a pH of 8.0 or 11.0 for 24 h as described in Materials and methods. As a positive control, proteins identified in the *trans*-cleavage assay of wild-type VP4 using S652R substrate are shown. For other details, see the legends to Figures 4 and 5.

Fig. 7. Effect of ATP on the *trans*-cleavage activity of VP4. The TNT-generated [³⁵S]methionine-labeled PP (S652R; substrate), and unlabeled wild-type and inactivated (S652A) VP4 were either subjected to ultrafiltration (+) or not (-). The substrate and VP4 (wild-type or S652A) were combined and incubated for 16 h. ATP and/or $MgCl_2$ were added (+) or not (-) to the mixtures where indicated. For other details, see Materials and methods and the legends to Figures 4 and 5.

low molecular weight compounds, were removed by three cycles of ultrafiltration. The final concentration of ATP ranged from 6 to 10 nM in different samples, i.e. approximately three orders below the concentration optimal to promote the Lon proteolytic activity (Waxman and Goldberg, 1985). The ultrafiltered samples of the wild-type protease and S652R substrate (see Materials and methods) were used in the *trans*-cleavage assay. In the absence of ATP, VP4 retains proteolytic activity (Figure 7). This activity was not affected by added ATP but was slightly stimulated by Mg^{2+} or Mg-ATP (Figure 7). The latter effect is likely to be caused by Mg^{2+} alone, although we are aware that our analysis was complicated by the fast hydrolysis of the added ATP in samples containing Mg^{2+} (not shown), probably due to stimulation of a cellular ATPase. This complication could be resolved by testing purified VP4 proteinase against a purified substrate not currently available.

VP4 is a trans-activator of expression of VP1, putative RNA-dependent RNA polymerase

To address the biological role of the VP4-driven PP processing, the production of IBDVP2-specific proteins in cells transfected with wild-type or mutated RNA A in conjunction with wild-type RNA B was analyzed (Table I; see Materials and methods). Owing to a low efficiency of transfection, infection starts in a minority of cells (Mundt and Vakharia, 1996). As a result, only VP3 and VP4 were identified reliably by RIPA in some virus variants; the other virus proteins were masked by mock-labeled pro-

Fig. 8. Biological properties of VP4 mutants. (A) Confluent Quail muscle-7 cells were transfected by cRNA transcribed from wild-type pP2B (RNA B) mixed with cRNA transcribed from wild-type or mutated pP2A (RNA A; Table I) and transfected cells were processed for the analysis of virus-specific proteins by RIPA with subsequent 12% SDS-PAGE and autoradiography. Shown is a fragment of a gel exposed for 30 days. Underneath the gel are results of α -IBDVP2-based (top line) or α -VP1-based (second line) IFAs of CEC transfected by wild-type RNA B mixed with wild-type or mutated RNA A and analyzed at 24, 48 and 72 h post-transfection. The third line (infectivity) summarizes results of α -IBDVP2-based IFAs of cells infected by viruses, which were derived from passaging of combined medium and cytoplasmic extracts obtained from transfected cells. + and -, IFA-positive and -negative cells, respectively. For experimental details see Materials and methods. (B) An extract of the IFA results summarized in the top and second lines underneath the gel in (A) is given for three time points and two transfected mixtures.

teins, which dominated heavily elsewhere in the gel (gel in Figure 8A; data not shown). VP3 and (very) faint VP4 were detected in cells transfected by either wild type or any of three mutants, T674S, S690A and H697Q (Figure 8A, gel). Faint VP3 bands were also detected in three mutants, S652C, T674A and T674C, while neither VP3 nor VP4 was detected in the other seven mutants with a lesion in the catalytic Ser-Lys dyad (Figure 8A, gel). Remarkably, this clustering of viruses in three groups is perfectly correlated with, respectively, full, partially defective and abrogated PP processing directed by the same variants of VP4 in reticulocyte lysates (compare Figures 3A and 8A, gel). We believe, therefore, that a monomolecular reaction in reticulocyte lysates adequately models *in vivo* proteolytic properties not only of wild-type VP4 (Figure 2) but also of VP4 mutants.

This analysis was extended by characterizing the expression of RNAs A and B by immunofluorescence assay (IFA). In preliminary experiments, we detected the expression of

RNA A transfected alone using α -IBDVP2 antiserum, although we failed to observe the expression of RNA B transfected alone using α -VP1 antiserum (not shown). This indicated that RNA B may need RNA A in order to be expressed. When wild-type RNA B was transfected along with different variants of RNA A, the α -IBDVP2-positive signal was observed for every pair tested (Figure 8A, top line underneath the gel). Although α -IBDVP2 can recognize VP1 in addition to PP-derived products, the observed signal was attributed mostly to the latter, RNA A-encoded proteins since, depending on the virus, α -VP1 recognized far fewer or no cells at all (Figure 8A, second line underneath the gel; data not shown; see also below). Thus, we concluded that the synthesis of PP-related proteins but not VP1 was directed by every mutant tested. The expression of RNA B was observed only in VP4 mutants with no visible defect in PP processing, i.e. T674S, S690A and H697Q, as well as in the S652C mutant with complete, albeit a slowed, PP processing (Figure 8A, second line underneath the gel). This implies that VP4, most probably through its proteolytic activity, *trans*-activates the expression of RNA B.

Ser652Cys IBDVP2 mutant with a slowed PP processing does not yield a viable virus progeny

We have also analyzed the ability of VP4 mutants to yield infectious progeny. The α -IBDVP2-based IFA was employed to monitor the production of the IBDVP2 antigens in chicken embryonic cells (CEC) infected by virus. The virus was collected after two passages of culture medium, which was combined with cytoplasmic extracts prepared from the cells 24, 48 or 168 h post-transfection (see Materials and methods). The only mutants with no defect in PP processing were those that were IFA positive (Figure 8A, bottom line underneath the gel), and this result was confirmed when transfected cell extracts were titrated directly by using a plaque assay (not shown). Hence, the inability to support the expression of RNA B and the lack of infectivity are correlated for all virus variants except S652C, thus demonstrating that our results are consistent. To prove further that S652C has a distinct phenotype, the expression of IBDVP2 proteins in wild-type- and S652C-transfected CEC was analyzed by IFA, using α -IBDVP2 and α -VP1 at 24, 48 and 72 h post-transfection. During this time, the number of cells positive for both antisera has grown steadily, approximately from one to two orders for wild-type RNA, but has been left unchanged for the S652C variant, with only a few cells staying α -VP1 positive (Figure 8B; data not shown). This result indicates that no infectious particles were formed by the S652C mutant for the period that was sufficient to spread the wild-type infection from isolated, initially transfected cells to plate-wide. Collectively, these results imply that, besides being involved in the PP proteolytic processing and *trans*-activation of VP1 expression, VP4 controls downstream process(es) in the IBDVP2 replicative cycle that consume the PP proteolytic products and VP1.

Discussion

The Birnaviridae is a poorly studied virus family. It is characterized by a unique blend of properties typical for

either Reoviridae or Picornaviridae. These two RNA virus families, with life cycles that lack a DNA stage, are often considered prototypes, respectively, of double-stranded and positive single-stranded RNA viruses. Birnavirus virions, like cores of Reoviridae (Grimes *et al.*, 1998), have $T = 13$ symmetry (Bottcher *et al.*, 1997), include a segmented double-stranded genome and have few RdRp molecules (Dobos, 1995). This core composition in the Reoviridae is a result of two concurrent processes, RNA replication and virion assembly (Patton, 1994); a similar coordination might occur in the Birnaviridae. Indeed, IBDV virion-like particles incorporate RdRps during assembly, but not after its completion (Lombardo *et al.*, 1999). Furthermore, IBDVP2 segment B encoding RdRp failed to express and replicate independently from segment A encoding capsid proteins (E.Mundt, unpublished observation). In contrast, in Picornaviridae, RNA replication is not capsid dependent (Richards and Ehrenfeld, 1990). Birnaviruses, however, resemble picornaviruses in other respects, as they can initiate a complete infectious cycle with synthetic mRNAs (Mundt and Vakharia, 1996), may use protein priming for RNA replication (Magyar *et al.*, 1998) and generate capsid proteins from a common precursor by proteolytic processing (Kibenge *et al.*, 1997). This processing is performed by viral protease(s) that differ functionally and structurally in the two viral families.

The birnavirus virions contain few, if any, molecules of unprocessed PP (Magyar and Dobos, 1994) and, thus, the precursor cleavages may trigger virion biogenesis, as was observed in picornaviruses (Palmenberg, 1990). An obvious purpose of the PP processing is to separate the major capsid proteins VP2 (pVP2) and VP3 from VP4, which, unlike VP2 and VP3, may have few, if any, encapsidated molecules. It is no wonder, therefore, that abolishing the PP processing through active site inactivation of VP4 is correlated with a loss of infectivity of IBDVP2. However, it was much more surprising when the same mutations were found to be correlated with the no-expression phenotype of RNA B. Apparently, VP4 itself, or through proteins produced under its control, *trans*-activates expression of RNA B encoding RdRp (Figure 8A) by an unknown mechanism. In poliovirus, one of the picornaviruses, translation of virion RNA was demonstrated to be up-regulated by an unknown mechanism involving viral 2A protease (Hambidge and Sarnow, 1992). In surrogate systems, however, induced transient expression of birnavirus RNA B was observed in the absence of RNA A (Magyar and Dobos, 1994; Lombardo *et al.*, 1999). As shown for the S652C mutant phenotype, even when PP is processed and RNA B is expressed, infectious progeny may not be formed (Figures 3 and 8A). Again, the molecular mechanism underlying the S652C phenotype is completely obscure, but slowed PP processing of this mutant indicates that virion biogenesis may be coupled with the PP cleavages in a cleavage rate-restricted manner, a mechanism that fits with the above-proposed coordination of virion maturation and RNA replication. Because of these characteristics, the VP4 proteinase is emerging as a key regulator of the birnavirus life cycle.

The unique biological properties of VP4 are matched by its unprecedented (for a viral protease) relationship with cellular Lon (Figure 1) (Babe and Craik, 1997; Chung

and Goldberg, 1998). The canonical Lons are multidomain proteins important for homeostasis in bacteria (Goldberg, 1992) and mitochondria (Suzuki *et al.*, 1994) and the assembly of protein complexes in mitochondria (Rep *et al.*, 1996). When expressed separately, two Lon domains, Lon^{ATPase} and Lon^{pro}, complemented the respective deficient versions of the yeast mitochondrial Lon *in trans* and were enzymatically active *in vitro* (van Dijl *et al.*, 1998). The VP4 precursor PP also has a complex domain organization, while the mature VP4 is a natural variant of Lon, having a simple domain organization resembling, in this respect, the *C.elegans* F43E2 ORF product, an uncharacterized, non-canonical cellular Lon (Figure 1A). In the PP, VP4 may interact with its neighbors, although the VP4-VP3 (Jagadish *et al.*, 1988; Kibenge *et al.*, 1997) and pVP2-VP4 (Duncan *et al.*, 1987; Magyar and Dobos, 1994) interactions do not appear to be crucial for the cleavage at the distal site. Whether those cleavages require interactions between the proteins that they separate remains to be seen.

The birnavirus PP does not contain an ATPase domain (Figure 1A), and its processing can proceed in the absence of ATP *in vitro* (Figure 7). In the canonical Lon proteases, the Lon^{ATPase} is part of the mechanism activating Lon^{pro} upon polypeptide binding to an allosteric site (Goldberg, 1992; Smith *et al.*, 1999). In an isolated form, the Lon^{pro} cleaved low molecular weight substrates only (Rasulova *et al.*, 1998; van Dijl *et al.*, 1998). It is likely that the cooperation between Lon^{ATPase} and Lon^{pro} has evolved to ensure a high efficiency of digestion of selected polypeptide substrates and to prevent Lon^{pro} from cleaving proteins in an uncontrollable fashion (Goldberg, 1992). As a result, as yet undefined determinants, which must be essential for the intrinsic endoproteolytic activity of VP4, may have been modified or tightly down-regulated in cellular Lon^{pro}.

The conserved core of Lon^{pro} is located in the C-terminal part of VP4 shown to be essential for the pVP2-VP4 processing of IPNV in *E.coli* (Manning and Leong, 1990) and of IPNV and IBDV in reticulocyte lysates (Duncan *et al.*, 1987; Manning *et al.*, 1990; Kibenge *et al.*, 1997). The ~100 N-terminal amino acids unique for VP4 are also essential for the proteolytic activity (C.Birghan, E.Mundt and A.E.Gorbalenya, unpublished data). Cellular Lon proteases also have a comparably positioned region upstream of the Lon^{pro} core whose size varies (Figure 1A). This N-terminal region may fold separately or be part of the same domain including Lon^{pro} core. The properties of purified IBDV VP4 have not yet been described.

The canonical Lon proteases have broad cleavage specificity and cleave *in trans*. However, in the process of self-maturation, a yeast mitochondrial Lon protease cleaves off a propeptide at a selected Ala-Lys bond co-translationally (Wagner *et al.*, 1997; van Dijl *et al.*, 1998). The latter reaction resembles the *modus operandi* of the birnavirus VP4 processed from PP autocatalytically. In the DXV PP, VP4 is likely to cut the Ser500-Ala501 bond (Chung *et al.*, 1996), which is aligned with a region of IBDV to which the pVP2-VP4 cleavage site was roughly mapped (Figure 1A; C.Birghan and E.Mundt, unpublished data). Large pieces of the IBDVP2 PP containing the pVP2-VP4 cleavage site were not processed by VP4 in reticulocyte lysates *in trans* (C.Birghan, E.Mundt and

A.E.Gorbalenya, unpublished observations), indicating that the primary structure determinants are not sufficient for proper processing. The cleavage at the VP4–VP3 versus the pVP2–VP4 site was found to be more sensitive to point mutations in VP4 (Figures 3 and 6) and not observed *in trans* (Figure 4).

The Ser652 and Lys692 conserved in VP4 were shown to carry polarized side chains indispensable for the PP processing (Figures 3A, 5 and 6) and, thus, formed a catalytic dyad (Lin and Little, 1989; Tschantz *et al.*, 1993; Keiler and Sauer, 1995). VP4 of the other birnaviruses, as well as cellular canonical and non-canonical Lon proteins, are likely to use a similar chemistry of proteolysis. For the IPNV VP4 (C.Birghan, E.Mundt and A.E.Gorbalenya, unpublished observations) and Lons from *E.coli* (Amerik *et al.*, 1991; Fischer and Glockshuber, 1993), *Mycobacterium smegmatis* (Roudiak *et al.*, 1998) and yeast mitochondria (Rep *et al.*, 1996; Wagner *et al.*, 1997), counterparts of the catalytic Ser652 have already been shown to be essential. The context of Ser652 also resembles that of the catalytic Ser of chymotrypsin-like proteases (Amerik *et al.*, 1991; A.E.Gorbalenya, unpublished data). Although this observation was used previously to suggest that it may be part of the catalytic Ser–His–Asp triad (Brown and Skinner, 1996), the latter is not supported by the results of our analysis. In the Ser–Lys proteases, an ϵ -amino group of Lys plays the role of a general base and activates the hydroxyl of Ser for catalysis (Paetzel and Dalbey, 1997). This activation may proceed differently from that in the serine triad proteases using Asp–His for Ser polarization; that would explain the unique inhibitor profiles of VP4 (Dobos, 1977; Leong and Mason, 1998) and Lon (Waxman and Goldberg, 1985).

A pronounced activation of the K692R VP4 protease by alkaline pH implies that in the wild-type VP4, active at physiological pH, the catalytic Lys692 must have, due to a microenvironment, a pK_a lower than the 10.5 usual for Lys (Paetzel and Dalbey, 1997). The pK_a of catalytic Lys of viral and cellular Lons may differ, and *E.coli* Lon was the most active at pH 9.0–9.5 (Waxman and Goldberg, 1985). Surprisingly, K692H, another mutant of VP4, showed residual activity at pH 8.0 and 11.0 (Figure 6). The Ser–His catalytic dyad previously was demonstrated in a catalytic antibody (Zhou *et al.*, 1994) and an esterase from *Streptomyces scabies* (Wei *et al.*, 1995). Furthermore, endoplasmic reticulum signal peptidases, a subset of the LPP-like Ser–Lys proteases, have His in place of the catalytic Lys (Figure 1) (van Dijk *et al.*, 1992; VanValkenburgh *et al.*, 1999). However, against this background, the catalytic Lys→His or His→Lys mutants of LPPs proved to be inactive (Tschantz *et al.*, 1993; van Dijk *et al.*, 1995; VanValkenburgh *et al.*, 1999). Thus, the K692H VP4 activity can be attributed to special properties of this enzyme and/or to a high sensitivity of the monomolecular assay. In any event, a certain flexibility of the VP4 Ser–Lys dyad is evident and it may reflect a natural potential of this site to evolve. It would be interesting to see whether this property is related to the RNA origin of VP4.

Materials and methods

Computer sequence analyses

Amino acid sequences were derived from the Genpeptides database and protein structures from the Brookhaven database (PDB). Sequence

alignments were produced using the ClustalX program (Thompson *et al.*, 1997) and the Macaw workbench (Schuler *et al.*, 1991). The non-redundant sequence database was searched with single sequences (Altschul *et al.*, 1997), and with hidden Markov models trained on multiple sequence alignments using the HMMER1.8.4 package (Eddy, 1996). These alignments were also sent as input for the PhD program (Rost and Sander, 1996) to predict secondary structure.

Viruses and cells

CEC derived from embryonated specific-pathogen-free eggs (VALO, Lohmann, Cuxhaven, Germany) and Quail muscle-7 (QM-7) cells were grown in Dulbecco's minimal essential medium, supplemented with 10% fetal calf serum. They were used for transfection experiments, propagation of IBDV strain P2 (IBDVP2; Schobries, 1987) and for IFAs. Infection of CEC or QM-7 with IBDVP2, virus plaque titration and preparation of cell-infected extracts followed Mundt *et al.* (1995).

Antisera

Antibodies used include: (i) α -VP2, mouse monoclonal antibodies (mAbs) (a generous gift of Dr A.van Loon, Intervet, Boxmeer, The Netherlands); (ii) α -VP3 and α -VP1, rabbit polyclonal antisera obtained by repeated injections of partly purified VP3 or VP1, respectively, expressed in *E.coli* (E.Mundt, unpublished); (iii) α -VP4, the mAb described in Granzow *et al.* (1997); and (iv) α -IBDVP2, a rabbit polyclonal serum obtained against IBDVP2 virions (Mundt *et al.*, 1995).

Site-directed mutagenesis of segment A of IBDVP2

To produce IBDVP2 mutants, a full-length cDNA clone of segment A of IBDVP2 (cDNA A) was first constructed by RT-PCR using RNA template isolated from the purified IBDVP2 virions (Muller, 1986; Mundt and Muller, 1995), and following Mundt and Vakharia (1996). The constructed plasmid containing the cDNA A under the control of the T7 promotor (pP2Awt) was linearized with *Bsr*GI, blunt ended with Klenow and cleaved by *Eco*RI. The cDNA A was isolated by agarose gel electrophoresis and ligated into the *Eco*RI–*Eco*RV-cleaved pBluescript II SK-plasmid (Stratagene, Heidelberg, Germany). This plasmid and the oligonucleotides described in Table I were used to produce all mutants except S690A and H697Q according to Kunkel *et al.* (1987). Plasmids carrying mutations were cleaved with *Sac*II–*Dra*III to produce cDNA fragments, which were ligated into the *Sac*II–*Dra*III-cleaved pP2Awt. Two S690A and H697Q were generated using PCR and two primers: oligonucleotide 1409 and another containing a mutagenized codon for S690 or H697 (Table I). The PCR fragments produced were amplified, purified with phenol/chloroform extraction and ethanol precipitation, and digested with *Sac*II–*Afl*III. The *Sac*II–*Afl*III fragments were purified by agarose gel electrophoresis, and ligated into the cleaved pP2A to obtain derivatives pP2AS690A and pP2AH697Q, respectively. All mutations were verified by DNA sequencing.

In vitro translation of segment A RNA

Plasmid DNA for *in vitro* transcription was obtained with a plasmid miniprep kit (Quantum Prep R; Bio-Rad, München, Germany) and used in TNT coupled with a reticulocyte lysate system (Promega, Heidelberg, Germany). Translation was conducted in either the presence or absence of [35 S]methionine for 60 min unless specified otherwise. Protein synthesis was stopped by the addition of RNase A to 10 μ g/ml and cycloheximide to 0.3 mg/ml with subsequent incubation at 30°C for 10 min (Lin and Rice, 1995). The labeled products, either immediately or after additional manipulations (see Results), were precipitated by 2 μ l of α -IBDVP2 or other sera under RIPA with subsequent 12% SDS–PAGE of the precipitates (Sambrook *et al.*, 1989). Radioactively labeled proteins were visualized by autoradiography.

Trans-cleavage assay for the VP4 proteinase

The TNT-generated [35 S]methionine-labeled S652R normally was used as substrate for unlabeled wild-type or mutated VP4 programmed by, respectively, pP2Awt and its derivatives (Table I) in the TNT. After termination of the TNT reactions (Lin and Rice, 1995; see above), equal volumes of substrate and enzyme were combined and incubated for a designated time in the thermal cycler. The PP processing products were analyzed as described above.

Analysis of the pH sensitivity of VP4 proteinase mutated at the K692 codon

TNT-generated [35 S]methionine-labeled K692 mutant samples of 30 μ l were divided into two halves. One half was supplemented with 1.6 μ l of 1 M 3-(cyclohexylamino) propane sulfonic acid (CAPS; ICN, Aurora,

USA) buffer (pH 11.0), and the other with 1.6 µl of 50 mM Tris-HCl pH 8.0 (buffer A). All samples were incubated at 30°C for 24 h in a thermal cycler with a heated lid (Primus cycler; MWG, Ebersberg, Germany), and the PP processing was analyzed as described above.

Probing the S652C VP4 with NEM

The ability of NEM to inactivate the *trans*-cleavage activity of the wild-type or S652C VP4 was analyzed by adopting the protocol of Tschantz *et al.* (1993) with modifications. The IBDVP2 PPs containing wild-type or S652C VP4 (enzymes) were generated using TNT not supplemented with [³⁵S]methionine (see above). Each sample was divided into two 30 µl aliquots, one incubated with 3.3 µl of buffer A and the other with 3.3 µl of 35 mM NEM (Sigma, Steinheim, Germany) in buffer A at room temperature for 30 min. The probes were then supplemented with 3.3 µl of 50 mM dithiothreitol (DTT) and incubated at 30°C for 30 min. The processed enzymes were combined with an equal volume of the [³⁵S]methionine-labeled S652R PP (substrate) and incubated at 30°C for 3 h with subsequent analysis of proteins as described above.

Analysis of ATP dependence of the VP4 proteinase activity

The ATP dependence of VP4 was analyzed in the *trans*-cleavage assay, which included an additional step. The volumes of [³⁵S]methionine-labeled S652R substrate and non-labeled wild-type and S652A enzymes were adjusted to 400 µl with buffer A and the samples were concentrated to ~10 µl using Centrux UF-0.5, 10 kDa MWCO (Schleicher & Schuell, Dassel, Germany) and centrifugation at 10 500 g for 30 min. After three cycles of this procedure, the volumes of the samples were adjusted to 25 µl with buffer A, and equal volumes of an enzyme and the substrate were combined. The samples were supplemented with Na-ATP or MgCl₂, or both, to final concentrations of 1 and 5 mM, respectively, and incubated at 30°C for 16 h followed by standard analysis of proteins.

Detection of the IBDVP2-encoded proteins and virus in transfected cells by RIPA and IFA

Full-length transcripts of the IBDVP2 genome were obtained from plasmids containing cDNA A (Table I) and wild-type cDNA B (p2Bwt; Mundt and Vakharia, 1996) linearized with either *Bsr*GI or *Pst*II. The subsequent procedure was as in Mundt and Vakharia (1996), except for: (i) the transcription mixtures were not purified by phenol/chloroform extraction; and (ii) ~300 000 QM-7 or CEC in 3 ml of culture medium were used for transfection or infection. Two types of analysis of IBDVP2-specific proteins produced in transfected cells were conducted. At 4 h post-transfection, QM-7 cells were rinsed with phosphate-buffered saline (PBS), overlaid with methionine- and cysteine-free MEM (Gibco-BRL) and incubated for 1 h. After a new round of rinsing with PBS, cells were overlaid with 200 mCi of Tran³⁵S-LabelTM (1.000 Ci/mmol; ICN, Costa Mesa, CA) and incubated for 60 min. Thereafter, cells were overlaid with medium containing serum and maintained until 24 h post-transfection. At this point, cells were processed for RIPA using α-IBDVP2 as described (Muller and Becht, 1982). For an alternative analysis, transfected cells were grown on sterile coverslips and were processed for IFA using α-IBDVP2 or α-VP1 at 12, 24, 48 or 72 h post-transfection. To analyze virus production, transfected cells were maintained on a plate until 24, 48 or 168 h post-transfection. They were then frozen, thawed and combined with the culture medium, and centrifuged at 700 g. The supernatants obtained were clarified further by filtration through 0.45 µm filters and used for infection of CEC, which were observed for 3–7 days. These infected cells were processed for a new round of virus passaging on CEC as described above for transfected cells. For the next, final infection, cells were grown on sterile coverslips and were subjected to IFA using α-IBDVP2 at 24 or 48 h post-infection.

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References

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3389–3402.
- Amerik, A., Antonov, V.K., Gorbalenya, A.E., Kotova, S.A., Rotanova, T.V. and Shimbarevich, E.V. (1991) Site-directed mutagenesis of La protease. A catalytically active serine residue. *FEBS Lett.*, **287**, 211–214.
- Babe, L.M. and Craik, C.S. (1997) Viral proteases: evolution of diverse structural motifs to optimize function. *Cell*, **91**, 427–430.
- Black, M.T. (1993) Evidence that the catalytic activity of prokaryote leader peptidase depends upon the operation of a serine–lysine catalytic dyad. *J. Bacteriol.*, **175**, 4957–4961.
- Bottcher, B., Kiselev, N.A., Stel'Mashchuk, V.Y., Perevozchikova, N.A., Borisov, A.V. and Crowther, R.A. (1997) Three-dimensional structure of infectious bursal disease virus determined by electron cryomicroscopy. *J. Virol.*, **71**, 325–330.
- Brown, M.D. and Skinner, M.A. (1996) Coding sequences of both genome segments of a European 'very virulent' infectious bursal disease virus. *Virus Res.*, **40**, 1–15.
- Chin, D.T., Goff, S.A., Webster, T., Smith, T. and Goldberg, A.L. (1988) Sequence of the *lon* gene in *Escherichia coli*. A heat-shock gene which encodes the ATP-dependent protease La. *J. Biol. Chem.*, **263**, 11718–11728.
- Chung, C.H. and Goldberg, A.L. (1998) Endopeptidase La. In Barrett, A.J., Rawlings, N.D. and Woessner, J.F., Jr (eds), *Handbook of Proteolytic Enzymes*. Academic Press, San Diego, CA, pp. 527–531.
- Chung, H.K., Kordyban, S., Cameron, L. and Dobos, P. (1996) Sequence analysis of the bicistronic *Drosophila* X virus genome segment A and its encoded polypeptides. *Virology*, **225**, 359–368.
- Dalbey, R.E., Lively, M.O., Bron, S. and van Dijk, J.M. (1997) The chemistry and enzymology of the type I signal peptidases. *Protein Sci.*, **6**, 1129–1138.
- Dobos, P. (1977) Virus-specific protein synthesis in cells infected by infectious pancreatic necrosis virus. *J. Virol.*, **21**, 242–258.
- Dobos, P. (1995) The molecular biology of infectious pancreatic necrosis virus (IPNV). *Annu. Rev. Fish Dis.*, **5**, 25–54.
- Dodson, G. and Wlodawer, A. (1998) Catalytic triads and their relatives. *Trends Biochem. Sci.*, **23**, 347–352.
- Dougherty, W.G. and Semler, B.L. (1993) Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes. *Microbiol. Rev.*, **57**, 781–822.
- Duncan, R., Nagy, E., Krell, P.J. and Dobos, P. (1987) Synthesis of the infectious pancreatic necrosis virus polyprotein, detection of a virus-encoded protease and fine structure mapping of genome segment A coding regions. *J. Virol.*, **61**, 3655–3664.
- Eddy, S.R. (1996) Hidden Markov models. *Curr. Opin. Struct. Biol.*, **6**, 361–365.
- Fischer, H. and Glockshuber, R. (1993) ATP hydrolysis is not stoichiometrically linked with proteolysis in the ATP-dependent protease La from *Escherichia coli*. *J. Biol. Chem.*, **268**, 22502–22507.
- Goldberg, A.L. (1992) The mechanism and functions of ATP-dependent proteases in bacterial and animal cells. *Eur. J. Biochem.*, **203**, 9–23.
- Goldberg, A.L. and Waxman, L. (1985) The role of ATP hydrolysis in the breakdown of proteins and peptides by protease La from *Escherichia coli*. *J. Biol. Chem.*, **260**, 12029–12034.
- Gorbalenya, A.E. and Snijder, E.J. (1996) Viral cysteine proteinases. *Persp. Drug Discov. Design*, **6**, 64–86.
- Gottesman, S. (1996) Proteases and their targets in *Escherichia coli*. *Annu. Rev. Genet.*, **30**, 465–506.
- Granzow, H., Birghan, C., Mettenleiter, T.C., Beyer, J., Kollner, B. and Mundt, E. (1997) A second form of infectious bursal disease virus-associated tubule contains VP4. *J. Virol.*, **71**, 8879–8885.
- Grimes, J.M., Burroughs, J.N., Gouet, P., Diprose, J.M., Malby, R., Zientara, S., Mertens, P.P. and Stuart, D.I. (1998) The atomic structure of the bluetongue virus core. *Nature*, **395**, 470–478.
- Hambidge, S.J. and Sarnow, P. (1992) Translational enhancement of the poliovirus 5' noncoding region mediated by virus-encoded polypeptide 2A. *Proc. Natl Acad. Sci. USA*, **89**, 10272–10276.
- Henikoff, S. and Henikoff, J.G. (1992) Amino acid substitution matrices from protein blocks. *Proc. Natl Acad. Sci. USA*, **89**, 10915–10919.
- Jagadish, M.N., Staton, V.J., Hudson, P.J. and Azad, A.A. (1988) Birnavirus precursor polyprotein is processed in *Escherichia coli* by its own virus-encoded polypeptide. *J. Virol.*, **62**, 1084–1087.

- Keiler, K.C. and Sauer, R.T. (1995) Identification of active site residues of the Tsp protease. *J. Biol. Chem.*, **270**, 28864–28868.
- Kibenge, F.S., Dhillon, A.S. and Russell, R.G. (1988) Biochemistry and immunology of infectious bursal disease virus. *J. Gen. Virol.*, **69**, 1757–1775.
- Kibenge, F.S., Qian, B., Cleghorn, J.R. and Martin, C.K. (1997) Infectious bursal disease virus polyprotein processing does not involve cellular proteases. *Arch. Virol.*, **142**, 2401–2419.
- Kräusslich, H.G. and Wimmer, E. (1988) Viral proteinases. *Annu. Rev. Biochem.*, **57**, 701–754.
- Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.
- Lawrence, C.E., Altschul, S.F., Boguski, M.S., Liu, J.S., Neuwald, A.F. and Wootton, J.C. (1993) Detecting subtle sequence signals: a Gibbs sampling strategy for multiple alignment. *Science*, **262**, 208–214.
- Leong, J.-A.C. and Mason, C. (1998) Infectious pancreatic necrosis virus endopeptidase. In Barrett, A.J., Rawlings, N.D. and Woessner, J.F., Jr (eds), *Handbook of Proteolytic Enzymes*. Academic Press, San Diego, CA, pp. 1593–1596.
- Lin, C. and Rice, C.M. (1995) The hepatitis C virus NS3 serine proteinase and NS4A cofactor: establishment of a cell-free *trans*-processing assay. *Proc. Natl Acad. Sci. USA*, **92**, 7622–7626.
- Lin, L.L. and Little, J.W. (1989) Autodigestion and RecA-dependent cleavage of Ind⁻ mutant LexA proteins. *J. Mol. Biol.*, **210**, 439–452.
- Little, J.W. (1993) LexA cleavage and other self-processing reactions. *J. Bacteriol.*, **175**, 4943–4950.
- Lombardo, E., Maraver, A., Caston, J.R., Rivera, J., Fernandez-Arias, A., Serrano, A., Carrascosa, J.L. and 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.
- Magyar, G. and Dobos, P. (1994) Evidence for the detection of the infectious pancreatic necrosis virus polyprotein and the 17-kDa polypeptide in infected cells and of the NS protease in purified virus. *Virology*, **204**, 580–589.
- Magyar, G., Chung, H.K. and Dobos, P. (1998) Conversion of VP1 to VPg in cells infected by infectious pancreatic necrosis virus. *Virology*, **245**, 142–150.
- Manning, D.S. and Leong, J.C. (1990) Expression in *Escherichia coli* of the large genomic segment of infectious pancreatic necrosis virus. *Virology*, **179**, 16–25.
- Manning, D.S., Mason, C.L. and Leong, J.C. (1990) Cell-free translational analysis of the processing of infectious pancreatic necrosis virus polyprotein. *Virology*, **179**, 9–15.
- Muller, H. (1986) Replication of infectious bursal disease virus in lymphoid cells. *Arch. Virol.*, **87**, 191–203.
- Muller, H. and 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.
- Mundt, E. and Muller, H. (1995) Complete nucleotide sequences of 5'- and 3'-noncoding regions of both genome segments of different strains of infectious bursal disease virus. *Virology*, **209**, 10–18.
- Mundt, E. and Vakharia, V.N. (1996) Synthetic transcripts of double-stranded Birnavirus genome are infectious. *Proc. Natl Acad. Sci. USA*, **93**, 11131–11136.
- Mundt, E., Beyer, J. and Muller, H. (1995) Identification of a novel viral protein in infectious bursal disease virus-infected cells. *J. Gen. Virol.*, **76**, 437–443.
- Mundt, E., Kollner, B. and Kretzschmar, D. (1997) VP5 of infectious bursal disease virus is not essential for viral replication in cell culture. *J. Virol.*, **71**, 5647–5651.
- Paetzel, M. and Dalbey, R.E. (1997) Catalytic hydroxyl/amine dyads within serine proteases. *Trends Biochem. Sci.*, **22**, 28–31.
- Paetzel, M., Dalbey, R.E. and Strynadka, N.C. (1998) Crystal structure of a bacterial signal peptidase in complex with a β -lactam inhibitor [published erratum appears in *Nature*, **396**, 707]. *Nature*, **396**, 186–190.
- Palmenberg, A.C. (1990) Proteolytic processing of picornaviral polyprotein. *Annu. Rev. Microbiol.*, **44**, 603–623.
- Patton, J.T. (1994) Rotavirus replication. *Curr. Top. Microbiol. Immunol.*, **185**, 107–127.
- Peat, T.S., Frank, E.G., McDonald, J.P., Levine, A.S., Woodgate, R. and Hendrickson, W.A. (1996) Structure of the UmuD' protein and its regulation in response to DNA damage. *Nature*, **380**, 727–730.
- Rasulova, F.S., Dergousova, N.I., Starkova, N.N., Melnikov, E.E., Rumsh, L.D., Ginodman, L.M. and Rotanova, T.V. (1998) The isolated proteolytic domain of *Escherichia coli* ATP-dependent protease Lon exhibits the peptidase activity. *FEBS Lett.*, **432**, 179–181.
- Rep, M., van Dijk, J.M., Suda, K., Schatz, G., Grivell, L.A. and Suzuki, C.K. (1996) Promotion of mitochondrial membrane complex assembly by a proteolytically inactive yeast Lon [published erratum appears in *Science*, **275**, 741]. *Science*, **274**, 103–106.
- Richards, O.C. and Ehrenfeld, E. (1990) Poliovirus RNA replication. *Curr. Top. Microbiol. Immunol.*, **161**, 89–119.
- Rost, B. and Sander, C. (1996) Bridging the protein sequence–structure gap by structure predictions. *Annu. Rev. Biophys. Biomol. Struct.*, **25**, 113–136.
- Roudiak, S.G., Seth, A., Knipfer, N. and Shrader, T.E. (1998) The Lon protease from *Mycobacterium smegmatis*: molecular cloning, sequence analysis, functional expression and enzymatic characterization. *Biochemistry*, **37**, 377–386.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schobries, H.D. (1987) Virus infections of domestic poultry in industrial poultry production. *Arch. Exp. Veterinarmed.*, **41**, 846–850.
- Schuler, G.D., Altschul, S.F. and Lipman, D.J. (1991) A workbench for multiple alignment construction and analysis. *Proteins*, **9**, 180–190.
- Smith, C.K., Baker, T.A. and Sauer, R.T. (1999) Lon and Clp family proteases and chaperones share homologous substrate-recognition domains. *Proc. Natl Acad. Sci. USA*, **96**, 6678–6682.
- Suzuki, C.K., Suda, K., Wang, N. and Schatz, G. (1994) Requirement for the yeast gene LON in intramitochondrial proteolysis and maintenance of respiration [published erratum appears in *Science*, **264**, 891]. *Science*, **264**, 273–276.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, **25**, 4876–4882.
- Tschantz, W.R., Sung, M., Delgado-Partin, V.M. and Dalbey, R.E. (1993) A serine and a lysine residue implicated in the catalytic mechanism of the *Escherichia coli* leader peptidase. *J. Biol. Chem.*, **268**, 27349–27354.
- van Dijk, J.M., de Jong, A., Vehmaanpera, J., Venema, G. and Bron, S. (1992) Signal peptidase I of *Bacillus subtilis*: patterns of conserved amino acids in prokaryotic and eukaryotic type I signal peptidases. *EMBO J.*, **11**, 2819–2828.
- van Dijk, J.M., de Jong, A., Venema, G. and Bron, S. (1995) Identification of the potential active site of the signal peptidase SipS of *Bacillus subtilis*. Structural and functional similarities with LexA-like proteases. *J. Biol. Chem.*, **270**, 3611–3618.
- van Dijk, J.M., Kutejova, E., Suda, K., Perecko, D., Schatz, G. and Suzuki, C.K. (1998) The ATPase and protease domains of yeast mitochondrial Lon: roles in proteolysis and respiration-dependent growth. *Proc. Natl Acad. Sci. USA*, **95**, 10584–10589.
- Van Valkenburgh, C., Chen, X., Mullins, C., Fang, H. and Green, N. (1999) The catalytic mechanism of endoplasmic reticulum signal peptidase appears to be distinct from most eubacterial signal peptidases. *J. Biol. Chem.*, **274**, 11519–11525.
- Wagner, I., van Dyck, L., Savel'ev, A.S., Neupert, W. and Langer, T. (1997) Autocatalytic processing of the ATP-dependent PIM1 protease: crucial function of a pro-region for sorting to mitochondria. *EMBO J.*, **16**, 7317–7325.
- Waxman, L. and Goldberg, A.L. (1985) Protease La, the lon gene product, cleaves specific fluorogenic peptides in an ATP-dependent reaction. *J. Biol. Chem.*, **260**, 12022–12028.
- Waxman, L. and Goldberg, A.L. (1986) Selectivity of intracellular proteolysis: protein substrates activate the ATP-dependent protease (La). *Science*, **232**, 500–503.
- Wei, Y., Schottel, J.L., Derewenda, U., Swenson, L., Patkar, S. and Derewenda, Z.S. (1995) A novel variant of the catalytic triad in the *Streptomyces scabies* esterase. *Nature Struct. Biol.*, **2**, 218–223.
- Yao, K., Goodwin, M.A. and Vakharia, V.N. (1998) Generation of a mutant infectious bursal disease virus that does not cause bursal lesions. *J. Virol.*, **72**, 2647–2654.
- Zhou, G.W., Guo, J., Huang, W., Fletterick, R.J. and Scanlan, T.S. (1994) Crystal structure of a catalytic antibody with a serine protease active site. *Science*, **265**, 1059–1064.

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