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) proteinase 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 (Lonpro) proved to be a peptidase rather than a proteinase (Rasulova et al., 1998; van Dijl et al., 1998).

The Lonpro contains a conserved Ser residue indispensable for the peptolytic activity (Amerik et al., 1991; Fischer and Gockshuber, 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 graft 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) (Krausslich and Wimmer, 1988; Dougherty and Semler, 1993; Gorbalyenya 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 (~3.3–3.4 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 lyastes 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...
virions (Kibenge et al. 1997; Lombardo et al. 1997) and, later, is processed further to VP5, which is not reported in IBDV virions and is not essential for IBDV reproduction in tissue culture (Mundt et al. 1997) and in chickens (Yao et al. 1998). The replication of Birnaviridae does not include a DNA stage and might be primed by VP1 (Dobos, 1995).

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 Lonpro is not associated with an ATPase domain. This non-canonical RNA viral Lon proteinase with a
narrow specificity employs the conserved Ser–Lys catalytic dyad to perform cleavages controlling IBV replication 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

Using bioinformatics tools, we identified a domain related to Lon in VP4 of birnaviruses (Figure 1). This Lon, as well as some other proteins theoretically identified in genomes of Archaea (van Dijl 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 the sizes of the marker proteins are indicated. A 25 μl aliquot of [35S]methionine-labeled cell lysates from mock-infected and infected CEC (Mundt et al., 1995) as well as 12.5 μl of the [35S]methionine-labeled transcription and translation (TNT) system was employed to study IBVDP2 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 μl aliquot of [35S]methionine-labeled cell lysates from mock-infected and infected CEC (Mundt et al., 1995) as well as 12.5 μl of the [35S]methionine-labeled transcription and translation (TNT) programmed by a plasmid containing a DNA copy of wild-type IBVDP2 RNA A (pP2Awt) was precipitated with α-VP2, α-VP3, α-VP4 or α-IBDP2 and processed for SDS–PAGE and autoradiography (see Materials and methods).

![Fig. 2. IBVDP2 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 μl aliquot of [35S]methionine-labeled cell lysates from mock-infected and infected CEC (Mundt et al., 1995) as well as 12.5 μl of the [35S]methionine-labeled transcription and translation (TNT) programmed by a plasmid containing a DNA copy of wild-type IBVDP2 RNA A (pP2Awt) was precipitated with α-VP2, α-VP3, α-VP4 or α-IBDP2 and processed for SDS–PAGE and autoradiography (see Materials and methods).](image)

Conserved Ser652 and Lys692 are essential for processing of the IBVDP2 PP in vitro

To test the predictions of the model (Figure 1), we chose to characterize the IBVDP2 VP4. We have observed that the PP directed by IBVDP2 genome segment A transcripts was processed completely in reticulocyte lysates to three products equivalent to pVP2, pVP3 and pVP4, 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
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 35S-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 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 probe 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.
**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 pKa of the catalytic Lys. Alternative, ‘non-cofactor’ activation of the autodigestion of wild-type LexA or its catalytic Lys→Arg mutant could be achieved at alkaline pH values close to the pKa 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 pKa 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→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 PP mutants 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 Mg2+ necessary to drive the TNT reactions might also have been used by the IBDVP2 protease to cleave PP. The ‘endogenous’ ATP and Mg2+, along with other 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 Mg2+ or Mg-ATP (Figure 7). The latter effect is likely to be caused by Mg2+ alone, although we are aware that our analysis was complicated by the fast hydrolysis of the added ATP in samples containing Mg2+ (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-
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 (Hambridge 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 protease 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$^{\text{NTPase}}$ and Lon$^{\text{pro}}$, 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–VP2 (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$^{\text{NTPase}}$ is part of the mechanism activating Lon$^{\text{pro}}$ upon polypeptide binding to an allosteric site (Goldberg, 1992; Smith et al., 1999). In an isolated form, the Lon$^{\text{pro}}$ cleaved low molecular weight substrates only (Rasulova et al., 1998; van Dijl et al., 1998). It is likely that the cooperation between Lon$^{\text{NTPase}}$ and Lon$^{\text{pro}}$ has evolved to ensure a high efficiency of digestion of selected polypeptide substrates and to prevent Lon$^{\text{pro}}$ 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$^{\text{pro}}$.

The conserved core of Lon$^{\text{pro}}$ 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 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$^{\text{pro}}$ core whose size varies (Figure 1A). This N-terminal region may fold separately or be part of the same domain including Lon$^{\text{pro}}$ 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
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 (Autschl et al., 1997), and with hidden Markov models trained on multiple sequence alignments using the HMMER 1.8.4 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**

CIE derived from embryonated specific-pathogen-free eggs (VALO, Lohman, 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 (IBDV-P2; Schobries, 1987) and for IFA’s. Infection of CEC or QM-7 with IBVDV, virus plaque titration and preparation of cell-infected extracts followed Mundt et al. (1995).

**Antisera**

Antibodies used include: (i) α-P2, mouse monoclonal antibodies (mAbs) (a generous gift of Dr A.van Loon, Intervet, Boxmeer, The Netherlands); (ii) α-P3 and α-P1, rabbit polyclonal antisera obtained by repeated injections of partly purified VP3 or VP1, respectively, expressed in E.coli (E.Mundt, unpublished); (iii) α-P4, the mAb described in Granzow et al. (1997); and (iv) α-IBDP2, a rabbit polyclonal serum obtained against IBDP2 virions (Mundt et al., 1995).

**Site-directed mutagenesis of segment A of IBDV-VP2**

To produce IBDV-VP2 mutants, a full-length cDNA clone of segment A of IBDV-VP2 was first constructed by RT-PCR using RNA template isolated from the purified IBDV-VP2 virions (Muller, 1986; Mundt and Muller, 1995), and following Mundt and Kahara (1996). The constructed plasmid containing the cDNA A under the control of the T7 promoter (pP2Awt) was linearized with BsrGI, blunted with Klenow and cleaved by EcoRI. The cDNA A was isolated by agarose gel electrophoresis and ligated into the EcoRI-EcoRV-cleaved BlII-SK plasmid (Stratagene, Heidelberg, Germany). This plasmid and the oligonucleotides described in Table 1 were used to produce all mutants except S690A and H697Q according to Kunkel et al. (1987). Plasmids carrying mutations were cleaved with SacII–DraII to produce cDNA fragments, which were ligated into the SacII–DraII-cleaved pP2Aw. 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 SacII–AflII. The SacII–AflII 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 PCR 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 [35S]methionine for 60 min unless specified otherwise. Protein synthesis was stopped by the addition of RNase A to 10 μg/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 μl of α-IBDP2 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 [35S]methionine-labeled S652R normally was used as substrate for unlabeled wild-type or mutated VP4 programmed by, respectively, pP2Aw 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 [35S]methionine-labeled K692 mutant samples of 30 μl were divided into two halves. One half was supplemented with 1.6 μl 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 (Prismus 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 PP containing wild-type or S652C VP4 (200 μg/ml) 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 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 subsequent procedures 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.

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 (pPB2B; Mundt and Vakharia, 1996) linearized with either BsgI or PstI. 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 infection of CEC, and used for infection of CEC, and used for infection of CEC, and used for infection of CEC.

Acknowledgements

We are grateful to Norbert Tautz for helping to initiate this study, Axel Karger for helpful hints regarding the experimental design, Stan Burt and Jacob Maizel for commenting on the manuscript, and Maritta Grau for correcting the text. This study has been funded in part by Deutsche Forschungsgemeinschaft grant MU 1244/1-2 and with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. NO.1-C0-56000. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US Government.

References


