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CHARACTERISATION OF A PAPAIN-LIKE PROTEINASE DOMAIN ENCODED BY ORF1a OF THE CORONAVIRUS IBV AND DETERMINATION OF THE C-TERMINAL CLEAVAGE SITE OF AN 87 kDa PROTEIN

K. P. Lim and D. X. Liu

Institute of Molecular Agrobiology
59A The Fleming
1 Science Park Drive
Singapore 118240

1. ABSTRACT

Our previous studies have shown that two overlapping papain-like proteinase domains (PLPDs) encoded by the IBV sequence from nucleotides 4155 to 5550 is responsible for cleavage of the ORF 1a polyprotein to an 87 kDa protein. In this study, we demonstrate that only the more 5' one of the two domains, PLPD-1 encoded between nucleotides 4155 and 5031, is required for processing to the 87 kDa protein. Site-directed mutagenesis studies have shown that the Cys^{1274} and His^{1435} residues are essential for the PLPD-1 activity, suggesting that they may be the components of the catalytic centre of this proteinase. Coexpression and immunoprecipitation studies have further revealed that PLPD can interact with the 87 kDa protein. Meanwhile, data obtained from the construction and expression of a series of deletion mutants have indicated that the 87 kDa protein is encoded by the 5'- most 2600 bp part of ORF1a. Further deletion and mutagenesis studies are underway to determine precisely the C-terminal cleavage site of the 87 kDa protein.

2. INTRODUCTION

Proteolytic processing of the 741 kDa polyprotein encoded by mRNA 1 is one of the strategies employed by the Coronavirus IBV in replication of its genome. Two overlapping viral papain-like proteinase domains (PLPDs) belonging to the class of cysteine pro-

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teinases and a trypsin-like proteinase domain belonging to the picornavirus 3C proteinase supergroup are predicted to be encoded by the IBV sequence from nucleotide 4155 to 5550 and from 8864 to 9787, respectively (Gorbalenya *et al.*, 1989). The two overlapping PLPDs have been demonstrated to be responsible for cleavage of the 1a polyprotein to a novel 87 kDa protein, as the release of this 87 kDa product was observed only when the IBV sequence from nucleotide 4859 to 5753 was included in the expression plasmids (Liu *et al.*, 1995).

In this communication we report that only the first PLPD encoded between nucleotides 4242 and 5031 is required for *cis*-cleavage of the 87 kDa protein at its C-terminus. Substitution of either of the two putative core residues, Cys^{1274} and His^{1435}, totally abolished the proteinase activity, thereby allowing us to conclude that they may be components of the catalytic centre of PLPD-1. The C-terminal cleavage site of the 87 kDa protein was meanwhile determined by construction and expression of a series of deletion constructs. It was shown that the C-terminal scissile bond of the 87 kDa protein was most likely located in the vicinity of Gly^{673} residue. In addition, evidence presented indicated that the 87 kDa protein may interact with products encoded by the IBV sequence from nucleotide 2548 to 5753.

3. METHODS AND MATERIALS

3.1. In Vivo Expression of IBV Sequences

IBV sequences placed under the control of a T7 promoter were transiently expressed in Vero cells using the vaccinia recombinant virus-T7 expression system as described before (Liu *et al.*, 1994).

3.2. Polymerase Chain Reaction

Amplification reactions of the respective template DNAs with appropriate primers were performed with Native Pfu DNA polymerase (Stratagene) under standard buffer conditions with 2 mM $MgCl_2$.

3.3. Site-Directed Mutagenesis Studies

Site-directed mutagenesis was performed as previously described (Liu *et al.*, 1997).

3.4. Radioimmunoprecipitation

Plasmid DNA-transfected Vero cells were lysed with RIPA buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS) and precleared by centrifugation at 12,000 rpm for 5 minutes at 4°C. Immunoprecipitation was performed as described previously (Liu *et al.*, 1994).

3.5. Cell-Free Transcription and Translation

Plasmid DNA was transcribed and translated *in vitro* with the TnT T7-coupled reticulocyte lysate system (TnT system) according to the instructions of the manufacturer (Promega). Reaction products were separated by SDS-PAGE and detected by autoradiography.

3.6. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of viral polypeptides was performed using 10% polyacrylamide gels (Laemmli, 1970). The [^{35}S]-labelled polypeptides were detected by autoradiography of the dried gels.

3.7. Construction of Plasmids

Plasmid pIBV1a2 (formerly referred to as pKT1a2) which covers the IBV sequence from nucleotide 362 to 5753 (Liu *et al.*, 1995), was used to construct pIBV1a2Δ1, pIBV1a2Δ4, pIBV1a2Δ5 and pIBV1a2Δ8. Plasmid pIBV1a2Δ1 which covers the IBV sequence from nucleotide 1311 to 5753, was constructed by cloning a BamHI/MluI digested-PCR fragment into BglII/MluI digested pIBV1a2. BglII digests pIBV1a2 at a position immediately upstream of the viral sequence and MluI digests the IBV sequence at nucleotide position 3997. An artificial AUG initiation codon in an optimal context (AC*AUG*G) was introduced immediately upstream of the viral sequence. The sequences of the upstream primer (LDX-3) and downstream primer (LDX-4) used for the construction of this plasmid are indicated in Table 1. Plasmid pIBV1a2Δ4 was constructed by cloning a 2.7 kb BamHI/MluI digested PCR fragment containing the IBV sequence from nucleotides 362 to 3047 into BglII/MluI digested pIBV1a2, resulting in the deletion of the region between nucleotides 3047 and 3997. The upstream and downstream primers used are LKP-1 and LKP-2 respectively (Table 1). Plasmid pIBV1a2Δ5 contains the IBV sequence from nucleotide 362 to 5027. It was constructed by ligation of a 1.03 kb DNA fragment, obtained by digestion of pIBV1a2 with MluI and SpeI, into MluI/XbaI digested pIBV1a1 (Liu *et al.*, 1995). SpeI cuts the IBV sequence at nucleotide position 5027.A second series of deletion mutants was made by introducing a UAA termination codon into different positions of the viral sequences. The first plasmid, pIBV1a2Δ8 which contains a stop codon at the nucleotide position 2856, was constructed by cloning a BamHI/MluI digested PCR fragment into BglII/MluI digested pIBV1a2. The upstream primer was LKP-1 and the downstream primer was LKP1a2Δ8. Plasmids pIBV1a2Δ10, pIBV1a2Δ11, and pIBV1a2Δ12 contain an UAA codon at nucleotide positions 2601, 2547 and 2517 respectively. The primers used to introduce these mutations are LKP1a2Δ10, LKP1a2Δ11 and

Table 1. Primers used for PCR amplification and site-directed mutagenesis

Primer name	Nucleotide sequences	ORF1a position
Deletion mutagensis primers		
LDX-3	5′ -ACG(CGGATCCAC)C**ATG**GGTTCTAAG- 3′	1304-1323
LDX-4	5′ -TCTGTTTGCAAGTTACATCG- 3′	4060-4041
LKP-1	5′ -(TCTCAGGGA)TCCCCCCACATACC - 3′	370-383
LKP-2	5′ -ACGCTA(ACGCGT)TCATCAAGAGGCAG- 3′	3047-3022
LKP1a2Δ8	5′ -CTCTCATAGACGCG*TTA*GATCAAATGGC- 3′	2870-2843
LKP1a2Δ10	5′ -ATAGG(CCCGGG*TTA*)AGGTGGTATCT - 3′	2612-2584
LKP1a2Δ11	5′ -CAGT(CCCGGG*TTA*)TGCTTTGCAAACCAC - 3′	2557-2530
LKP1a2Δ12	5′ -CATTATT(CCCGGG*TTA*)TTGAGACATTGGTG - 3′	2530-2501
Catalytic residue mutagenesis primers		
LKP1a2Δ4$^{C1274-S}$	5′ -CGATGGAAAT(AGC)TGGATTAGTTCAGC- 3′	4338-4364
LKP1a2Δ4$^{H1435-K}$	5′ -CTAATAGTGGC(AAG)TGTTATACACAAGC- 3′	4826-4853

LKP1a2Δ12. The PCR fragments obtained for these constructs were digested with BamHI and SmaI, and then cloned into BglII/SmaI-digested pKT0 (Liu *et al.*, 1994). Two mutants with alterations at the putative catalytic residues Cys^{1274} (pIBV1a2Δ4$^{C1274-S}$) and His^{1435} (pIBV1a2Δ4$^{H1435-K}$) were created by PCR amplification using pIBV1a2Δ4 as templates. The respective mutation primers used were LKP1a2Δ4$^{C1274-S}$ and LKP1a2Δ$^{H1435-K}$ (Table 1). All constructs made by site-directed mutagenesis were selected and confirmed by automated sequencing.

Regions of the IBV sequences presented in these constructs are illustrated in Fig. 1.

4. RESULTS

4.1. Further Analysis of the Involvement of the Two Papain-Like Proteinase Domains in Processing of the 1a Polyprotein to the 87 kDa Protein

It was previously shown that when a construct (pIBV1a1) containing the IBV sequence from nucleotide 362 to 4858 was expressed, only the full-length product of 220 kDa was observed (Liu *et al.*, 1995). However, expression of the IBV sequence up to nucleotide 5753 (pIBV1a2), resulted in the detection of three protein species: a 250 kDa full-length product and two cleavage products of 160 kDa and 87 kDa (Liu *et al.*, 1995), implying that processing of the full length polypeptide had occurred. These results suggest strongly that the region

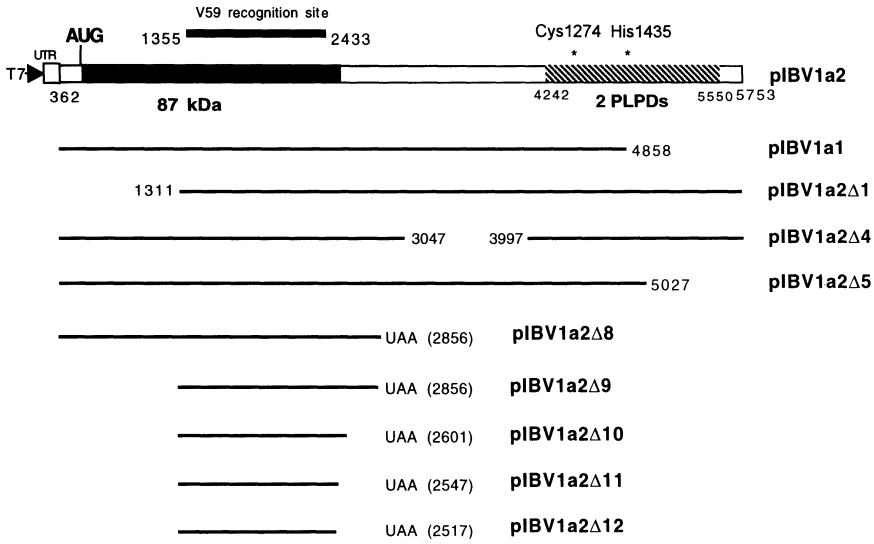

Figure 1. Diagram of the ORF1a region presented in plasmid pIBV1a2, showing the locations of the 87 kDa protein, the two PLPDs and the region recognised by antiserum V59. The positions of the putative C-terminal cleavage site (Gly^{673}↓Gly^{674} dipeptide) of the 87 kDa protein and the catalytic residues of the PLPD-1 (Cys^{1274} and His^{1435}) are also indicated. Also shown are the regions of ORF1a present in plasmids pIBV1a1 and the various deletion mutants. The nucleotide positions of the UAA termination codon introduced are indicated in the brackets.

from nucleotide 4859 to 5753 is essential for the processing of the 1a polyprotein to the 87 kDa protein. As computer prediction indicates that two putative overlapping PLPDs may be encoded from nucleotide 4155 to 5550 (Lee *et al.*, 1991), it is highly likely that this cleavage is mediated by the two proteinase domains. However, it was not clear if both domains were required for the cleavage. To clarify this issue, plasmid pIBV1a2Δ5 (Fig. 1) which contains the IBV sequence from nucleotide 362 to 5027 was constructed and expressed in Vero cells using the vaccinia virus-T7 expression system (Fuerst, 1986). The deletion showed little, if any, effect on the processing to the 87 kDa protein. As can be seen from Figure 2, expression of pIBV1a2Δ5 resulted in the detection of the 87 kDa protein as well as the full length 225 kDa protein and 136 kDa cleavage product. This data indicates that only the first PLPD is responsible for the release of the 87 kDa protein from its precursor.

4.2. Mutational Analysis of the Putative Catalytic Dyad of PLPD-1

Data presented above demonstrated that only the first of the two overlapping papain-like proteinase domains is required for processing of the 1a polyprotein to the 87 kDa

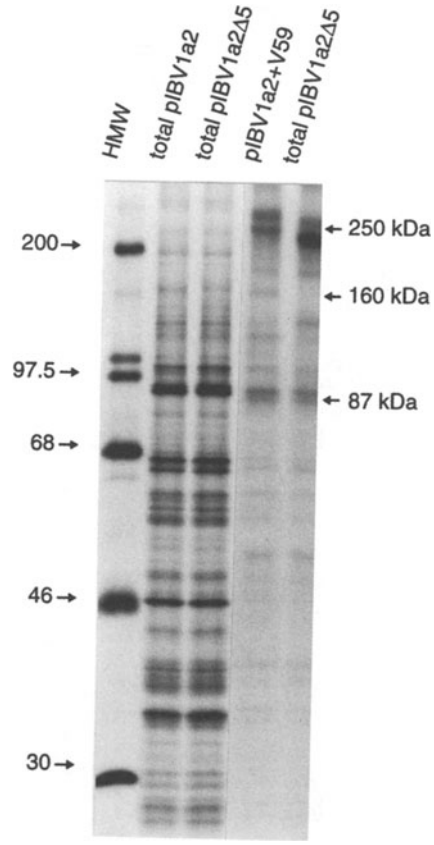

Figure 2. In vivo expression of pIBV1a2 and pIBV1a2Δ5 in Vero cells using the vaccinia recombinant virus-T7 expression system. The transfected cells were labelled with [^{35}S] methionine and the cell lysates were subjected to immunoprecipitation with antiserum V59 as indicated above each lane. SDS-PAGE was performed on an SDS-10% polyacrylamide gel. HMW-high molecular weight markers (numbers indicate kilodaltons).

product. We then sought to test if the two predicted residues, Cys^{1274} and His^{1435}, is associated with the catalytic activity of the proteinase (Lee *et al.*, 1991). To facilitate this study, plasmid pIBV1a2Δ4 (Fig. 1), containing an internal deletion of 0.96 kb from nucleotide 3047 to 3997, was constructed and used to make the mutant constructs. By monitoring the production of the 87 kDa protein *in vivo*, we were able to test the effect of the mutation on the proteinase activity. Using pIBV1a2Δ4 as the template, the putative nucleophilic residue Cys^{1274} was mutated to a Ser residue (pIBV1a2Δ4$^{C1274\text{-}S}$) and the predicted His^{1435} residue was mutated to a Lys (pIBV1a2Δ4$^{H1435\text{-}K}$). Fig. 3 shows that expression of pIBV1a2Δ4 yielded both the 190 kDa full length polypeptide and the 87 kDa protein. However, expression of pIBV1a2Δ4$^{C1274\text{-}S}$ and pIBV1a2Δ4$^{H1435\text{-}K}$ resulted in the detection of only the 190 kDa full-length product by antiserum V59 (Fig. 3). No 87 kDa protein was detected from the two mutant constructs (Fig. 3). This result demonstrated that substitutions of Cys^{1274} with a Ser and His^{1435} with a Lys disrupt the proteinase activity required for the re-

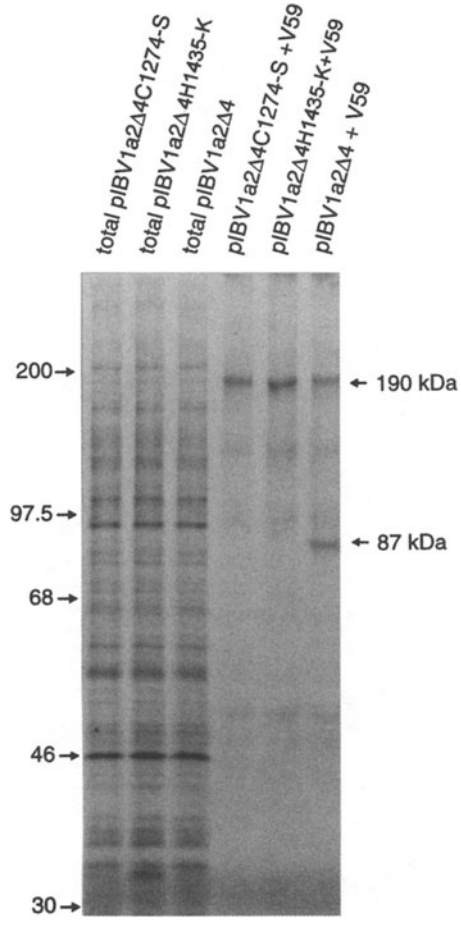

Figure 3. Mutational analysis of the putative catalytic residues Cys^{1274} and His^{1435} of PLPD-1. Plasmids pIBV1a2Δ4, pIBV1a2Δ4$^{C1274\text{-}S}$ and pIBV1a2Δ4$^{H1435\text{-}K}$ were transiently expressed in Vero cells using the vaccinia recombinant virus-T7 expression system. The plasmid transfected cells were labelled with [^{35}S] methionine and the lysates were subjected to immunoprecipitation with V59. The polypeptides were separated on an SDS-10% polyacrylamide gel. HMW-high molecular weight markers (numbers indicate kilodaltons).

lease of the 87 kDa protein. It is therefore suggested that the Cys^{1274} and His^{1435} may be core residues of the catalytic centre of PLPD-1.

4.3. Deletion Analysis of the C-Terminal Cleavage Site of the 87 kDa Protein

The target cleavage site of PLPD-1 was next analysed by deletion analysis. The first deletion mutant, pIBV1a2Δ8, was constructed by introducing a UAA termination codon into pIBV1a2 at the nucleotide position 2856. Expression of this plasmid led to the synthesis of a product with an apparent molecular mass of 93 kDa, which appeared much larger than the expected size of 87 kDa in SDS-PAGE (Fig. 4a). This implies that the actual cleavage site responsible for releasing the C-terminus of the 87 kDa protein is located at least 250 bp upstream of this position.

To rule out the possibility that the size difference observed was caused by N-terminal cleavage of the pIBV1a2-encoded polyprotein, pIBV1a2Δ1 was constructed by deletion of the IBV sequence from nucleotide 362 to 1310. Expression of this construct resulted in the detection of three protein species of 220 kDa, 160 kDa and 58 kDa, representing the full-length and the cleavage products, respectively (Fig. 4b). The migration of the 58 kDa protein in SDS-PAGE suggested that it may be the 5'-truncated version of the 87 kDa protein. Based on pIBV1a2Δ1, plasmids pIBV1a2Δ9, pIBV1a2Δ10, pIBV1a2Δ11 and pIBV1a2Δ12 (Fig. 1) were then constructed by inserting the UAA stop codon into pIBV1a2Δ1 at nucleotide positions 2856, 2601, 2547 and 2517 respectively, and were expressed in Vero cells. As shown in Fig. 4b, transfection of pIBV1a2Δ9 and pIBV1a2Δ10 resulted in the synthesis of the full-length stop products migrating in SDS-PAGE more slowly than the 58 kDa protein. Their approximate sizes were 65 kDa and 61kDa, respectively. Plasmids pIBV1a2Δ11 and pIBV1a2Δ12 encoded proteins with apparent molecular masses of 58 kDa and 52 kDa. The product encoded by pIBV1a2Δ11 had an electrophoretic mobility in SDS-PAGE almost identical to that of the 58 kDa protein encoded by pIBV1a2Δ1 (Fig. 4b). As the UAA termination codon in pIBV1a2Δ11 was inserted immediately downstream of the deduced amino acid position 673, it is likely that the C-terminal cleavage site of the 87 kDa protein is in the vicinity of this residue.

4.4. Interaction of the 87 kDa Protein with Products Encoded by the Papain-Like Proteinase Domains

When pIBV1a2 and its derivatives were expressed *in vivo* , detection of a 160 kDa protein was consistently observed (Fig. 2 and see Liu *et al.*, 1995). It was previously postulated that the 160 kDa protein was an intermediate cleavage product containing the 87 kDa protein, as it could be immunoprecipitated by antiserum V59. However, this possibility was ruled out by the expression of the N- and C- terminal deletion constructs pIBV1a2Δ1 and pIBV1a2Δ5. Expression of pIBV1a2Δ1 showed the detection of the 160 kDa protein (Fig. 4b). Expression of pIBV1a2Δ5, however, resulted in the synthesis of a 136 kDa product; no 160 kDa protein was detected (see Fig. 2). It is therefore likely that the 160 kDa product was a C-terminal cleavage product released from the 250 kDa polyprotein encoded by pIBV1a2. Detection of the 160 kDa protein by the N-terminal specific antiserum V59 may be due to the interaction of this protein with the 87 kDa protein species.

To investigate this possibility, C-terminal specific antiserum V16 which recognised the IBV sequence between nucleotides 4864 and 5576, was used in immunoprecipitation

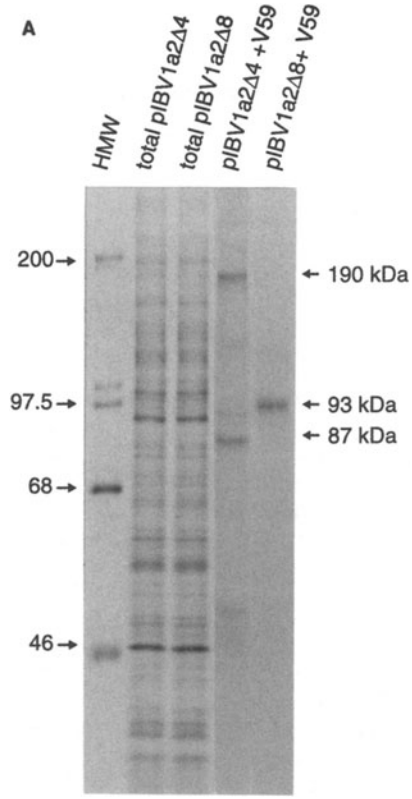

Figure 4. Determination of the C-terminal cleavage site of the 87 kDa protein by deletional studies. A) *In vivo* expression of pIBV1a2Δ8 and pIBV1a2Δ4 in Vero cells using the vaccinia recombinant virus-T7 expression system. The cells were labelled with [^{35}S] methionine and the plasmid-transfected cell lysates were subjected to immunoprecipitation with antiserum V59 as indicated above each lane. The proteins synthesised were separated on an SDS-10% polyacrylamide gel. HMW-high molecular weight markers (numbers indicate kilodaltons). B) Immunoprecipitation of the proteins encoded by pIBV1a2Δ1, pIBV1a2Δ9, pIBV1a2Δ10, pIBV1a2Δ11, and pIBV1a2Δ12 were done with V59.

studies. As shown in Figure 5a, immunoprecipitation of pIBV1a2Δ1-transfected cell lysate with V16 led to the detection of the 220 kDa, 160 kDa and 58 kDa protein species. The same three protein species could be immunoprecipitated by V59 (Fig. 5a).

To test the specificities of antisera V59 and V16, plasmids pIBV1a2Δ8 (covers the IBV sequences between nucleotides 362 and 2856) and pIBV2P (contains the IBV sequences between nucleotides 3832 and 5757) was expressed in vitro. As can be seen from Figure 5b, pIBV1a2Δ8 encoded a polypeptide with an apparent molecular weight of 93 kDa. This protein could only be immunoprecipitated by antiserum V59 (Fig 5b). Similarly, pIBV2P encoded a 74 kDa protein, which could only be recognised by antiserum V16 (Fig. 5b).

5. DISCUSSION

A number of positive-stranded RNA viruses employs proteolytic processing as one of the strategies for their gene expression. Post-translational processing of the 741 kDa polyprotein encoded by the 27.6 kb genome-length mRNA 1 of the Coronavirus IBV into

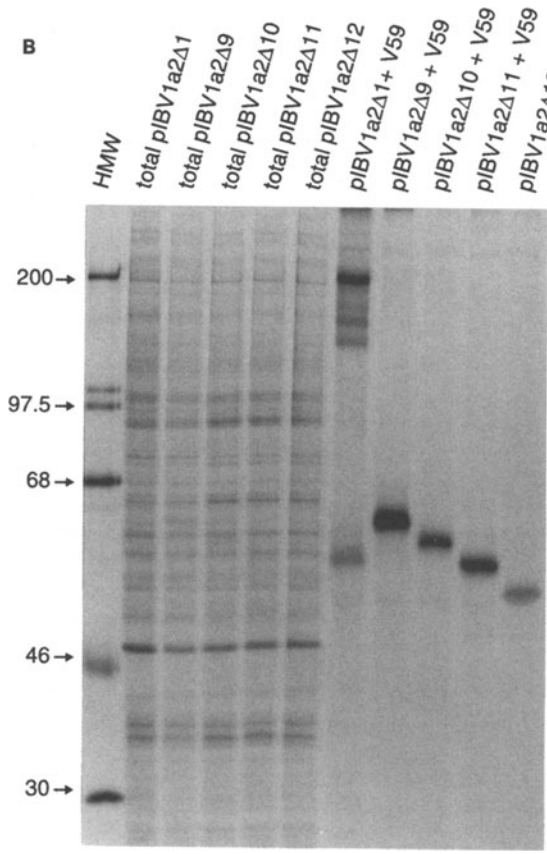

Figure 4B.

smaller functional proteins is probably mediated by both viral and cellular proteinases. One of such cleavage product is an 87 kDa protein identified previously from IBV-infected Vero cells by using a region-specific antiserum V59 (Liu *et al.*, 1995). Data obtained from expression of IBV sequence up to nucleotide 5753 have shown that inclusion of the predicted sequence encoding the two putative overlapping papain-like proteinase domains was required for releasing the 87 kDa protein from the full length precursor. Through the expression of a construct lacking the second of the overlapping papain-like proteinase domains, we demonstrated that this domain was not essential for the *in vivo* processing of the 1a polyprotein to the 87 kDa product. Therefore, only PLPD-1 encoded between nucleotides 4242 and 5031 was required for the *cis* -cleavage of the 87 kDa protein from the 1a polyprotein precursor. Site-directed mutagenesis studies confirmed the previously predicted Cys^{1274} and His^{1435} residues as members of the catalytic centre of PLPD-1. The lack of proteolytic activity when either of the catalytic residues was substi-

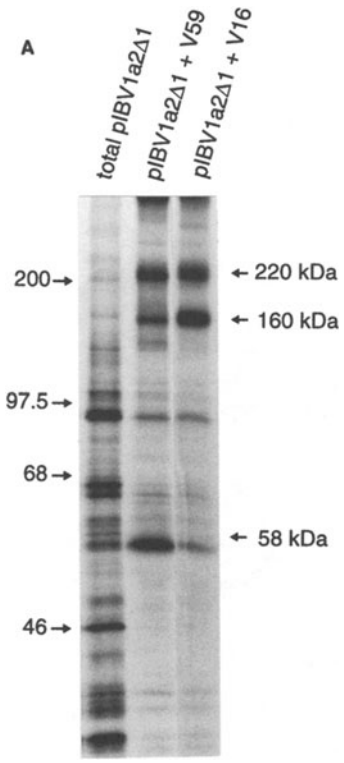

Figure 5. A) Analysis of polypeptides synthesised from *in vivo* expression of pIBV1a2Δ1. The cells were labelled with [^{35}S] methionine and the lysate was subjected to immunoprecipitation with antisera V59 and V16 as indicated above each lane. Electrophoresis of the polypeptides were performed on an SDS-10% polyacrylamide gel. HMW-high molecular weight markers (numbers indicate kilodaltons). B) Immunoprecipitation of *in vitro* translation products of RNAs cotranscribed from pIBV1a2Δ8 and pIBV2P in reticulocyte lysate using the transcription-coupled-translation system (Promega). Approximately 200μg/ml of plasmid DNA was added to reticulocyte lysate and the proteins were labelled with [^{35}S] methionine. The polypeptides were separated on an SDS-10% PAGE either directly or after immunoprecipitation with antisera V59 and V16 and detected by autoradiography. Numbers indicate molecular mass in kilodaltons.

tuted even though the second proteinase domain was still intact further reinforces the conclusion that only the first PLPD is required for the processing to the 87 kDa protein.

Unlike MHV, processing of the ORF1a polyprotein into smaller products by the PLPDs was not observed during the *in vitro* translation of constructs containing this region of ORF1a (Liu *et al.*, 1995). Microsequencing of the C-terminal cleavage site of the 87 kDa protein could not be performed as insufficient templates could be generated by using the *in vivo* expression system. In order to define the C-terminal boundary of the 87 kDa protein, we constructed and expressed a series of deletion constructs by introducing a UAA triplet at different positions of the IBV sequences. By comparing the migration of the termination products in SDS-PAGE with the cleavage product, this approach allows us to define Gly^{673}-Gly^{674} dipeptide as a potential cleavage site for releasing the C-terminus of the 87 kDa protein. Mutagenesis studies are underway to confirm this speculation.

We have previously reported that the 160 kDa species is probably the intermediate cleavage product covering the 87 kDa protein, as it could also be immunoprecipitated by region-specific antiserum V59. By construction and expression of N- and C-terminal deletion constructs, we showed that the 160 kDa protein species was actually not encoded from the 5'-end of ORF1a, as the full-length 160 kDa protein was observed even when the 5'-end of ORF1a up to nucleotide 1311 was deleted. Therefore, the 160 kDa protein species probably represents the C-terminal cleavage product resulted from cleavage of the

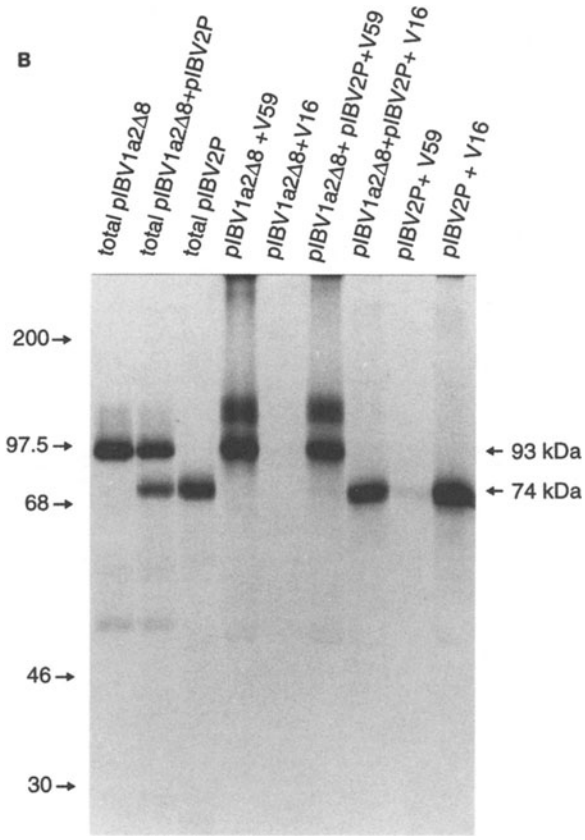

Figure 5B.

250 kDa polypeptide encoded by pIBV1a2, at the Gly^{673}-Gly^{674} dipeptide bond. This result meanwhile rules out the possibility that a second PLPD cleavage site may be located in this portion of the 1a polyprotein.

Evidence presented confirmed that the detection of the 160 kDa protein by N-terminal specific antiserum is due to the interaction of the 160 kDa protein with the 87 kDa protein. We are currently uncertain if this interaction is essential for the functions of the products encoded by this region of the viral RNA.

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