PROTEOLYTIC PROCESSING OF THE POLYPROTEIN ENCODED BY ORF1b OF THE CORONAVIRUS INFECTIOUS BRONCHITIS VIRUS (IBV)

D. X. Liu,^{1,2} S. Shen,¹ H. Y. Xu,¹ and T. D. K. Brown²

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

²Division of Virology
Department of Pathology
University of Cambridge
Tennis Court Road
Cambridge, CB2 1QP, United Kingdom

1. ABSTRACT

We present here evidence demonstrating that four previously predicted Q-S(G) cleavage sites, encoded by the IBV sequences from nucleotide 15129 to 15134, 16929 to 16934, 18492 to 18497, and 19506 to 19511, respectively, can be recognised and *trans*-cleaved by the 3C-like proteinase. Five mature products with sizes of approximately 100 kDa, 65 kDa, 63 kDa, 42 kDa and 35 kDa are released from the ORF1b polyprotein by the 3C-like proteinase-mediated cleavage at these positions. Meanwhile, expression of plasmids containing only the ORF1b region showed no autocleavage of the polyprotein encoded, suggesting that the 3C-like proteinase may be the sole proteinase involved in processing of the 1b polyprotein. These data may therefore represent a complete processing map of the polyprotein encoded by ORF1b of mRNA1.

2. INTRODUCTION

Three proteinase domains, including two overlapping papain-like proteinase domains encoded by the IBV sequences from nucleotide 4242 to 5553 and one trypsin-like

proteinase domain of the picornavirus 3C proteinase group (3C-like proteinase) encoded between nucleotides 8937 and 9357, have been predicted to be encoded by ORF 1a (Gorbalenya *et al.*, 1989; Lee *et al.*, 1991). The papain-like proteinase domains were demonstrated to be involved in proteolytic processing of the 1a polyprotein to an 87 kDa mature viral product (Liu *et al.*, 1995). However, it is likely that only a limited number of cleavages may be mediated by this proteinase. More important role in proteolytic processing of the mRNA1-encoding polyprotein may be played by the 3C-like proteinase domain, as more than ten Q-S(G) dipeptide bonds located in the 1a/1b polyproteins are predicted to be the cleavage sites for this proteinase (Gorbalenya *et al.*, 1989). Indeed, we have reported the identification of a 100 kDa polymerase domain-containing protein, released from the 1a/1b polyprotein by the 3C-like proteinase-mediated cleavage at two predicted Q-S dipeptide bonds (Liu *et al.*, 1994; Liu and Brown, 1995). More recently, a 10 kDa mature viral product encoded by ORF 1a from nucleotide 11545 to 11878 was shown to be cleaved from the 1a polyprotein by the same proteinase (Liu *et al.*, 1997).

In this communication, we report experiments designed to map the 3C-like proteinase-dependent cleavage sites in the polyprotein encoded by the ORF 1b region of mRNA1. Three ORF 1b-specific antisera, V58, V60 and V17 were used in the study. Co-transfection, deletion and site-directed mutagenesis studies demonstrated that four previously predicted Q-S(G) cleavage sites, encoded by the IBV sequences from nucleotide 15129 to 15134, 16929 to 16934, 18492 to 18497 and 19506 to 19511, respectively, were recognised and cleaved by the 3C-like proteinase to release five mature products with sizes of approximately 100 kDa, 65 kDa, 63 kDa, 42 kDa and 35 kDa. Information gained from this study could be used to guide further identification and characterisation of the ORF 1b-specific viral products.

3. MATERIALS AND METHODS

3.1. Transient Expression of IBV Sequences in Vero Cells Using a Vaccinia-T7 Expression System

Open reading frames placed under control of the T7 promoter were expressed transiently in eukaryotic cells using the vaccinia virus-T7 expression system (Fuerst *et al.*, 1986).

3.2. Polymerase Chain Reaction (PCR)

Appropriate primers and template DNAs were used in amplification reactions with Pfu DNA polymerase (Stratagene) under standard buffer conditions with 2 mM MgCl₂. PCR reaction conditions were 30 cycles of 95°C for 45 seconds, X°C for 45 seconds and 72°C for X minutes. The annealing temperature and the extension time were adjusted according to the melting temperature of the primers used and the length of the PCR fragments synthesised.

3.3. Site-Directed Mutagenesis

Site-directed mutagenesis was carried out, as previously described (Liu *et al.*, 1994), using single stranded DNA templates prepared from pIBV1b5 and appropriate oligonucleotide primers.

Substitution mutation of the Q^{1491(1b)}-G^{1492(1b)} dipeptide bond was introduced, as previously described (Liu *et al.*, 1997), by two rounds of PCR with two pairs of primers.

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 pre-cleared by centrifugation at 12,000 rpm for 5 minutes at 4°C in a microfuge. Immunoprecipitation was carried out as described previously (Liu *et al.*, 1994).

3.5. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of virus polypeptides was carried out with 10% polyacrylamide gels (Laemmli, 1970). Labelled polypeptides were detected by autoradiography or fluorography of dried gels.

3.6. Construction of Plasmids

Plasmid pIBV1b3, which covers the IBV sequence from nucleotide 15135 to 16931, was constructed by cloning an NcoI/BamHI digested-PCR fragment into NcoI/BamHI digested pKT0 (Liu *et al.*, 1994). An artificial AUG initiation codon in an optimal context (ACCAUGG) located immediately upstream of the viral sequence and a UAG termination codon located immediately downstream of the viral sequence were meanwhile introduced by PCR. The sequence of the upstream PCR primer is 5'-CGACTTCCATG GCTTGTGGCGTT-3', and the sequence of the downstream primer is 5'-CCAAAG-GATCCTATTGCAGACTTG-3'. Plasmid pIBV1b4 was constructed by cloning an SnaBI/DraI fragment containing the IBV sequence from nucleotide 18930 to 20874 into EcoRV/SmaI digested pKT0, and selected by nucleotide sequencing. Plasmid pIBV1b5 was made by cloning an NcoI/BamHI digested PCR fragment, which covers the IBV sequence from nucleotide 16932 to 20490, into NcoI-BamHI digested pKT0. An artificial AUG initiation codon in an optimal context (ACCAUGG) located immediately upstream of the viral sequence was introduced by PCR. The sequence of the upstream PCR primer is 5'-ACAAGTCCATGGGTACAGGTT-3', and the sequence of the downstream primer is 5'-GCACCCCGGGATCCTGCCAAC-3'.

Plasmids pIBV3C and pIBV5, which cover the IBV sequence from nucleotide 8871 to 9786 and from 10752 to 16980, respectively, were described previously (Liu *et al.*, 1994; Liu *et al.*, 1997).

Plasmid pIBV20 was made by cloning a 3,665 bp PCR fragment, which contains the IBV sequence from nucleotide 16841 to 20506, into EspI- and BamHI-digested pIBV1b3 (EspI cuts the IBV sequence at nucleotide position 16840, the BamHI site was located immediately downstream of the IBV sequence and was introduced by PCR with primer 5'-GCACCCCGGGATCCTGCCAAC-3'.

Plasmid pIBV14E1Q^{891(1b)}-E, which covers the IBV sequences from nucleotide 8693 to 16980 with deletions of the regions from nucleotide 9911 to 12227 and from 15537 to 16788, respectively, and contains a Q^{891(1b)} to E mutation, was described previously (Liu and Brown, 1995). Plasmid pIBV21, which covers the IBV sequences from nucleotide 8693 to 17630 with the two deletions in pIBV14E1Q^{891(1b)}-E and contains substitution mutations at both the Q^{891(1b)}-S^{892(1b)} and Q^{1491(1b)}-S^{1492(1b)} sites, was constructed as follows. A 1,242 bp PCR fragment, which covers the IBV sequence from nucleotide 16788

to 17630 and contains a Q^{1491(1b)} to M^{1491(1b)} mutation, was generated by two rounds of PCR. The sequence of the oligonucleotide primer used to introduce the mutation is 5'-ACAAGTCCCATGGGTACAGGT-3', and the sequence of the complementary primer is 5'-AACAAACCTGTACCCATGGGAC-3'. This PCR fragment was then digested with PstI (which cuts the IBV sequence at nucleotide 15537), gel-purified and ligated into PstI-SmaI digested pIBV14Δ1Q^{891(1b)}-E, giving plasmid pIBV21. The construct was selected by restriction digestion with NcoI and confirmed by nucleotide sequencing.

Two mutant constructs, pIBV1b5Q^{2350(1b)}-E and pIBV1b5Q^{2012(1b)}-E, with alterations at the putative Q^{2350(1b)}-S^{2351(1b)} and Q^{2012(1b)}-S^{2013(1b)} cleavage sites, respectively, were made by site-directed mutagenesis with the single stranded DNA templates prepared from plasmid pIBV1b5. The sequences of the oligonucleotide primers used to introduce the mutations are: 5'-TCCACAGCTTGAATCAGCATG-3' and 5'-TTCAGCTCTCGAGTCTATC GAC-3', respectively.

4. RESULTS

4.1. Characterisation of Two Antisera Raised against Bacterial-Viral Fusion Proteins Containing the C-Terminal Regions of the ORF 1b-Encoding Polypeptide

We have recently reported the identification of a 100 kDa protein encoded by the IBV sequence from nucleotide 12313 to 15131 in IBV-infected Vero cells with an ORF 1b-specific antiserum V58 (Liu *et al.*, 1994; Liu and Brown, 1995). No viral products, however, were detected by using two other ORF 1b-specific antisera, V60 and V17 (Liu *et al.*, 1994; Liu, unpublished observations). Antisera V60 and V17 were raised in rabbits against β-galactosidase-IBV fusion proteins containing the IBV sequences encoded from nucleotide 16066 to 16783 and from 19154 to 20649, respectively (Figure 1a). The specificity and affinity of these two antisera for their target proteins were tested by immunoprecipitation against *in vitro* synthesised ORF 1b products.

As the results shown in Figure 1b, expression of plasmid pIBV1b3, which contains the IBV sequence from nucleotide 15132 to 16932, led to the synthesis of a polypeptide of approximately 65 kDa, consistent with the calculated molecular weight of 69 kDa of the full-length product encoded by this construct. Immunoprecipitation study showed that the 65 kDa protein can be efficiently and specifically precipitated by antiserum V60 (Figure 1b).

To characterise antiserum V17, plasmid pIBV1b5, which contains IBV sequence from nucleotide 16930 to 20874, was linearised at different positions by restriction enzymes (Figure 1a), and transcribed and translated in the TnT system. As shown in Figure 1c, transcription and translation of SacI-digested pIBV1b5 led to the synthesis of a polypeptide of approximately 140 kDa, representing the full-length product encoded by this construct. Transcription and translation of BstBI-, MboII- and StuI-digested pIBV1b5 resulted in the synthesis of proteins of approximately 110 kDa, 98 kDa and 85 kDa, respectively (Figure 1c). Immunoprecipitation studies showed that the 140 kDa full-length product as well as the 110 kDa and 98 kDa proteins could be efficiently precipitated by antiserum V17 (Figure 1c). However, the 85 kDa protein could not be precipitated by the same antiserum. These results confirm the specific recognition of the target products by the antiserum.

4.2. Transient Expression of ORF 1b in Eukaryotic Cells

Plasmids covering the ORF1b region were then expressed in Vero cells by using the vaccinia virus-T7 expression system, and analysed by immunoprecipitation with three ORF-1b-specific antisera. As can be seen, expression of plasmid pIBV5, which contains the IBV sequence from nucleotide 10752 to 16980, resulted in the detection of two major protein species with apparent molecular weights of approximately 60 kDa and 200 kDa, respectively, representing the ORF 1a termination product and the full-length product encoded by the construct (also see Liu *et al.*, 1994). The 200 kDa full-length protein can be immunoprecipitated by both antisera V58 and V60 (Figure 2). No 100 kDa and any other cleavage products were detected (Figure 2). Similarly, expression of pIBV20, which covers the IBV sequence from nucleotide 15135 to 20506, led to the detection of a product with an apparent molecular weight of approximately 190 kDa, representing the full-length polypeptide encoded by this construct. This 190 kDa protein can be immunoprecipitated by both antisera V60 and V17 (Figure 2). No other products were detected by either antiserum (Figure 2), confirming that there is no autoprocessing of the polypeptide encoded by this region of ORF 1b.

4.3. Determination of the C-Terminal Cleavage Site of the Putative Metal-Binding and RNA-Helicase Protein

Data presented above suggest that there is no autocleavage of the polypeptide encoded by ORF1b. In a previous report, we confirmed that a previously predicted QS (Q^{891(1b)}S^{892(1b)}) dipeptide bond is the C-terminal cleavage site of the 100 kDa protein (Liu and Brown, 1995). Three other QS(G) dipeptide bonds in the 1b polypeptide were also predicted to be the cleavage sites of the 3C proteinase (Gorbalenya *et al.*, 1989). The first of these sites is the Q^{1491(1b)}G^{1492(1b)} dipeptide bond. Substitution of the Q^{1491(1b)} residue with an M was therefore carried out to test if this is a scissile bond of the 3C-like proteinase. As shown in Figure 3, expression of pIBV14 I Q^{891(1b)}-E led to the detection of a protein of 125 kDa (see also Liu and Brown, 1995). Expression of pIBV21, however, resulted in the detection of a protein migrating in SDS-PAGE much more slowly than the 125 kDa protein species (Figure 3). The apparent molecular weight of approximately 155 kDa of this product suggested that it is a fusion protein comprising the 125 kDa protein and the product encoded by the IBV sequence from nucleotide 16932 to 17630. This result indicates that mutation of the Q^{1491(1b)} residue to an M blocked the cleavage occurred at this position.

4.4. Analyses of the Putative Q²³⁵⁰-S²³⁵¹ and Q²⁰¹²-S²⁰¹³ Cleavage Sites

During the course of studying the ORF1b expression, we have consistently observed a very low level of expression of constructs covering the 3C-like proteinase domain and the complete ORF1b sequence. In order to analyse the two putative cleavage sites (Q²³⁵⁰-S²³⁵¹ and Q²⁰¹²-S²⁰¹³) located in the C-terminal one third region of the 1b polypeptide, co-transfection approaches were used. As shown in Figure 4a, expression of plasmid pIBV1b5, which covers the IBV sequences from nucleotide 16932 to 20490, led to the synthesis of a 140 kDa polypeptide, representing the full-length product encoded by this construct. Once again, no processing of this polypeptide to smaller products were observed (Figure 4a). However, co-transfection of pIBV1b5 with pIBV3C, a plasmid containing the whole 3C-like proteinase domain, resulted in the detection of five protein species. In addition to the 140 kDa full-length product, four other protein species with apparent molecular

weights of approximately 105 kDa, 77 kDa, 42 kDa and 35 kDa, respectively, were also detected (Figure 4a). To reveal the identities of these products, plasmid pIBV1b4, which contains the IBV sequence from nucleotide 18930 to 20874, was expressed in Vero cells. As shown in Figure 4a, transfection of pIBV1b4 alone led to the synthesis of a polypeptide of approximately 60 kDa, consistent with the calculated full-length product of 60 kDa

Figure 1. (A). Diagram showing IBV sequences present in plasmids pIBV1b3 and pIBV1b5, and the sequences used to raise antisera V17 and V60. Also shown are the restriction sites used to linearise pIBV1b5. (B). Testing of the reactivities of antiserum V60 to the *in vitro* synthesised target polypeptides. The polypeptides were transcribed and translated from pBP5 and pIBV1b3 in reticulocyte lysate with the TnT coupled translation system (Promega). Plasmid DNA was added to reticulocyte lysate at approximately 200 µg/ml. [³⁵S] methionine-labelled translation products were separated on an SDS-12.5% polyacrylamide gel directly or after immunoprecipitation with either antiserum V47, which recognises the IBV sequence encoded between nucleotides 11488 and 12600 (Liu *et al.*, 1994), or antiserum V60, and detected by fluorography. Numbers indicate molecular mass in kilodaltons. Plasmid pBP5 covers the IBV sequence from nucleotide 10752 to 12600 (Liu *et al.*, 1994). (C). Testing of the reactivities of antiserum V17 to the *in vitro* synthesised target polypeptides. The polypeptides were transcribed and translated from SacI-, BstBI-, MboII- and StuI-digested pIBV1b5 in reticulocyte lysate with the TnT translation system, and were separated on an SDS-12.5% polyacrylamide gel directly or after immunoprecipitation with antiserum V17.

Figure 1C.

encoded by this construct. As expected, two additional products with apparent molecular weights of 25 kDa and 35 kDa, respectively, were produced from co-expression of pIBV1b4 with pIBV3C (Figure 4a). The 35 kDa protein co-migrates with the 35 kDa protein produced from the co-expression of pIBV1b5 and pIBV3C (Figure 4a), indicating that they are the same cleavage products from the C-terminal region of the 1b polyprotein.

To confirm further if cleavage did occur at the Q^{2350} - S^{2351} cleavage site, substitution of the Q^{2350} residue with an E was produced by site-directed mutagenesis using the DNA template prepared from pIBV1b5, giving a mutant construct pIBV1b5 $Q^{2350(1b)}$ -E. Expression of pIBV1b5 $Q^{2350(1b)}$ -E in Vero cells resulted in the synthesis, once again, of the full-length 140 kDa polyprotein (Figure 4a). Co-expression of pIBV1b5 $Q^{2350(1b)}$ -E and pIBV3C led to the formation of a polypeptide co-migrating with the 77 kDa protein detected from co-transfection of pIBV1b5 and pIBV3C (Figure 4a). No other cleavage products were detected (Figure 4a), suggesting that mutation of the Q^{2350} residue to E did block the cleavage occurred at this position. These results confirm that the Q^{2350} - S^{2351} dipeptide bond is responsible for releasing the N-terminus of the 35 kDa and the C-terminus of the 42 kDa proteins. The 77 kDa protein detected from co-expression of pIBV1b5 and pIBV3C is therefore representing an intermediate cleavage product encoded by the IBV sequence from nucleotide 18496 to 20414.

Figure 2. Analysis of transiently expressed mRNA1 products from transfection with plasmids pIBV5 and pIBV20, using the vaccinia virus-T7 expression system. Cells were labelled with [35 S] methionine, lysates were prepared, and polypeptides were either analysed directly, or immunoprecipitated with either antiserum V17, V58 or V60. Polypeptides were separated on an SDS-12.5% polyacrylamide gel, and detected by fluorography. HMW-high molecular weight markers (numbers indicate kilodaltons).

Substitution of the Q²⁰¹² residue of the predicted Q²⁰¹²-S²⁰¹³ cleavage site with an E was subsequently made by site-directed mutagenesis, giving the mutant construct pIBV1b5Q²⁰¹²-E. Co-expression of this plasmid with pIBV3C led to the synthesis of the full-length 140 kDa protein (Figure 4b). In addition, two polypeptides co-migrating, respectively, with the 105 kDa and the 35 kDa proteins detected from the co-expression of pIBV1b5 and pIBV3C, were also detected (Figure 4b). No 42 kDa and 77 kDa proteins, however, were observed (Figure 4b). These results indicate that mutation of the Q²⁰¹² residue to E abolished the cleavage occurred at this position.

5. DISCUSSION

We have recently reported that an ORF 1a-encoding proteinase of the picornavirus 3C proteinase group is responsible for cleavage of the polyproteins encoded by mRNA1 to a 10 kDa and a 100 kDa mature viral products (Liu *et al.*, 1994; Liu *et al.*, 1997). Deletion and site-directed mutagenesis studies demonstrated that the 100 kDa protein was encoded by IBV sequence from nucleotide 12313 to 15131 (Liu *et al.*, 1994; Liu and Brown, 1995). Two Q-S dipeptide bonds, encoded by the ORF 1a sequence from nucleotide 12310 to 12315, and by ORF 1b from nucleotide 15129 to 15134, respectively, were identified to be the cleavage sites responsible for releasing the 100 kDa protein from the 1a/1b fusion polyprotein (Liu and Brown, 1995). In this report, we show that the 3C-like proteinase is also able to mediate cleavage at three other sites located in the ORF 1b polyprotein, resulting in the releasing of four more cleavage products approximately 65 kDa, 63 kDa, 42 kDa and 35 kDa, respectively.

Among the five protein species identified in this study, only the 100 kDa protein has actually been detected from IBV-infected cells (Liu *et al.*, 1994). Attempts were made to detect the rest four protein species in virus-infected cells, but were unsuccessful so far. It is currently uncertain if these proteins represent genuine viral products. However, as important functional domains, such as the metal-binding and RNA helicase domains, were predicted to be located in these proteins, some of them may be essential for the replication of viral RNA. Failure to detect these products may simply reflect the rapid degradation of these products in virus-infected cells during the virus infection cycle. Alternatively, complex mechanisms may be involved in regulation of the expression and accumulation of these products. Further investigations are underway to explore these possibilities.

Our previous mutagenesis data demonstrated that the Q residue of a Q-S cleavage site is very sensitive to substitution mutations. Substitution of the Q residue with an E totally block the cleavage occurred at the corresponding site (Liu and Brown, 1995). Substitution of the Q residue with an M also blocks the cleavage occurred at a Q-G dipeptide bond. The S residue, however, is more tolerant to similar substitution mutations. Substitution of the S residue with either an A or S does not inhibit the cleavage (Liu and Brown, 1995). It seems that a wide range of Q-X (X represents any amino acid residue) dipeptide bonds might be used by the 3C-like proteinase domain to release mature viral products. To support this hypothesis, we have recently identified a Q-N dipeptide bond responsible for the release of a 10 kDa mature viral product from the 1a polyprotein (Liu *et al.*, 1997).

Figure 3. Mutational analysis of the C-terminal cleavage site of the putative metal-binding and RNA helicase protein. Plasmids were transiently expressed in Vero cells, using the vaccinia virus-T7 expression system. The transfected cells were labelled with [35 S] methionine, lysates were prepared, and polypeptides were either analysed directly, or immunoprecipitated with antiserum V58. Polypeptides were separated on an SDS-12.5% polyacrylamide gel, and detected by fluorography. HMW-high molecular weight markers (numbers indicate kilodaltons).

Figure 4. Analysis of transiently expressed products from pIBV1b4- and pIBV1b5-transfected Vero cells (a), and mutational analyses of the predicted $Q^{2350(1b)}S^{2351(1b)}$ (a) and $Q^{2012(1b)}S^{2013(1b)}$ cleavage sites (b). The transfected cells were labelled with [^{35}S] methionine, lysates were prepared, and polypeptides were either analysed directly, or immunoprecipitated with antiserum V17. Polypeptides were separated on an SDS-12.5% polyacrylamide gel, and detected by fluorography. HMW-high molecular weight markers (numbers indicate kilodaltons).

Systematic mutagenesis of the cleavage dipeptides and their context is required to address this issue further.

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