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Further Characterization of the Coronavirus Infectious Bronchitis Virus 3C-like Proteinase and Determination of a New Cleavage Site

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Coronavirus infectious bronchitis virus (IBV) encodes a trypsin-like proteinase (3C-like proteinase) by ORF 1a, which has been demonstrated to play a pivotal role in proteolytic processing of gene 1-encoded polyproteins. In our previous studies, the proteinase was identified as a 33-kDa protein in IBV-infected cells, and its catalytic center was shown to consist of H\(^{2820}\) and C\(^{2922}\) residues. It is released from the 1a and 1a/1b polyproteins by autoprocessing at two Q–S dipeptide bonds (Q\(^{2779}\)–S\(^{2780}\) and Q\(^{3086}\)–S\(^{3087}\)). In this report, further characterization of the two cleavage sites demonstrates that the N-terminal Q\(^{2779}\)–S\(^{2780}\) site is tolerant to mutations at the P1 position. Deletion of the C-terminal region of the proteinase shows that a significant amount of the enzymatic activity is maintained upon deletion of up to 67 amino acids, suggesting that the extreme C-terminal region may be dispensable for the proteolytic activity of the proteinase. Analysis of the autoprocessing kinetics in vitro reveals that proteolysis at the Q\(^{2779}\)–S\(^{2780}\) site is the first cleavage event mediated by this proteinase. This is followed by cleavage at the Q\(^{3086}\)–S\(^{3087}\) site. The occurrence of both cleavage events in intact cells is potentially rapid and efficient, as no intermediate cleavage products covering the proteinase were detected in either IBV-infected or transfected cells. Immunofluorescence microscopy and subcellular fractionation studies further show differential subcellular localization of the proteinase in IBV-infected cells and in cells expressing the 3C-like proteinase alone, indicating that additional roles in viral replication might be played by this protein. Finally, a Q–A (Q\(^{3379}\)–A\(^{3380}\)) dipeptide bond encoded by nucleotides 10,663 to 10,668 was demonstrated to be a cleavage site of the proteinase.

INTRODUCTION

The avian coronavirus infectious bronchitis virus (IBV) is now categorized under a new order, Nidovirales (Cavanagh, 1997; de Vries et al., 1997). Its 27.6-kb genome-length mRNA, mRNA1, is composed of two large overlapping ORFs (1a and 1b) at the 5′-unique region and encodes a fusion polyprotein of 741 kDa by a unique frameshifting mechanism (Boursnell et al., 1987; Briefly et al., 1987, 1989). Proteolytic processing of the 441-kDa 1a and 741-kDa 1a/1b fusion polyproteins to smaller mature products is mediated by viral proteinases. Three proteinase domains, namely, two overlapping papain-like proteinase domains and a picornavirus 3C-like proteinase domain (3C-like proteinase) (Fig. 1a), have been predicted to be encoded by ORF 1a (Boursnell et al., 1987; Gorbalenya et al., 1989; Lee et al., 1991).

In recent years, progress has been made in the identification and characterization of the proteinase activities as well as their cleavage products. The first papain-like proteinase domain is involved in proteolytic cleavage of the N-terminal region of the 1a and 1a/1ab polyproteins to release an 87-kDa protein and a 195-kDa protein and the N-terminus of a 41-kDa product in IBV-infected cells (Liu et al., 1995; Lim and Liu, 1998; Lim et al., 2000). Similar enzymatic activities were also reported for the first papain-like proteinase domain of human and murine coronaviruses. In mouse hepatitis virus (MHV), two N-terminal cleavage products (p28 and p65) have been reported (Hughes et al., 1995; Bonilla et al., 1997; Baker et al., 1989, 1993; Dong and Baker, 1994; Gao et al., 1996; Schiller et al., 1998). The same domain of human coronavirus (HCV) processes the two polyproteins to release p9 (Herold et al., 1998).

Important roles in processing of the 1a and 1a/1b polyproteins are also played by the 3C-like proteinase domain. Characterization of the proteinase activities has shown that it cleaves the bulk of the polyproteins at conserved Q–S (G and N) dipeptide bonds, resulting in the release of more than 10 mature products (Liu et al., 1994, 1995, 1997, 1998; Ng and Liu, 1998; Tibbles et al., 1999; Ziebuhr and Siddell, 1999; Grotzinger et al., 1996; Heusipp et al., 1997a, b; Lu et al., 1995, 1998; Schiller et al., 1998). The proteinase was identified as a 27-kDa protein for MHV (Lu et al., 1998) and a 34-kDa protein for HCV (Ziebuhr et al., 1995). The catalytic center of the proteinase consists of a His residue and a Cys residue (Liu and Brown, 1995; Lu et al., 1995; Seybert et al., 1997; Ziebuhr et al., 1995, 1997).

The 3C-like proteinase domain is flanked by hydrophobic regions in the coronaviral genomes. In vitro studies in
MHV have shown that its enzymatic activity can be greatly enhanced by the addition of canine pancreatic microsomal membranes to the translation reaction mixture (Pinon et al., 1997; Schiller et al., 1998). The proteolytic activity of the proteinase could be inhibited by a variety of cysteine and serine inhibitors and was eliminated by the cysteine proteinase inhibitor E64d (Lu et al., 1996; Pinon et al., 1997; Seybert et al., 1997). More recently, immunofluorescence studies using region-specific antisera against several 1a and 1a/1b cleavage products including the 3C-like proteinase have suggested that the proteinase may be associated with the viral RNA replication–transcription machinery (Schiller et al., 1998; Ziebuhr and Siddell, 1999; Shi et al., 1999; Denison et al., 1999; van der Meer et al., 1999).

In a previous study, we identified the IBV 3C-like proteinase as a 33-kDa protein in IBV-infected Vero cells (Lim et al., 2000). The proteinase was shown to be self-cleaved from the polyproteins at the two previously predicted Q–S dipeptide bonds (Q2779–S2780 and Q3086–S3087) in an in vitro processing assay (Tibbles et al., 1996). The in vitro proteolytic activity was shown to be strictly dependent on the addition of canine microsomal membranes (Tibbles et al., 1996). Here, we show, by deletion and mutational analyses, that the N-terminal cleavage site of the 3C-like proteinase is tolerant to mutations and the extreme C-terminal region may be dispensable for the enzymatic activity. The proteinase could be self-cleaved to the 33-kDa mature form in vitro in rabbit reticulocyte lysate without the addition of canine microsomal membranes. Immunofluorescence staining and cellular fractionation studies demonstrated that the proteinase is associated mainly with the membrane fraction in virus-infected cells. However, a free distribution pattern was observed when the proteinase was overexpressed in intact cells. Finally, instead of the previously predicted Q3086–G3086 dipeptide bond, the Q3087–A3088 dipeptide bond was identified as a cleavage site of the proteinase.

**RESULTS**

Tolerance of the N-terminal cleavage site (Q2779–S2780) of the 3C-like proteinase to mutations

The previously predicted Q2779–S2780 dipeptide bond was shown to be the N-terminal cleavage site of the 3C-like proteinase in an in vitro processing assay (Tibbles et al., 1996). Substitution mutagenesis was carried out to investigate the effects of mutations on the self-cleavage efficiency of the proteinase at this position. The Q2779–S2780 dipeptide bond was first mutated to E2779–S2780, giving rise to pIBV9Q1Q2779–E (Fig. 1a). Expression of pIBV9ΔIQ2779–E led to the detection of three protein species (Fig. 1b, lane 2). In addition to the 33-kDa protein, two products of approximately 43 and 48 kDa, respectively, were also detected (Fig. 1b, lane 2). The 43-kDa protein may represent a fusion product containing the 33-kDa protein and the product encoded by nucleotides 8693 to 8865, and the 48-kDa protein may represent the full-length product encoded by this construct (Fig. 1a). As a significant amount of the 33-kDa protein was detected from the expression of this construct (Fig. 1b, lane 2), this result indicates that an E–S dipeptide could be used efficiently by the 3C-like proteinase at this position. Substitution of the Q2779 residue with a K was then carried out, giving rise to pIBV9Δ1Q2779–K (Fig. 1a). Expression of this construct resulted in more efficient detection of the 43- and 48-kDa products (Fig. 1b, lane 3). However, a trace amount of the 33-kDa protein was still detected (Fig. 1b, lane 3). These results suggest that cleavage at the Q2779–S2780 dipeptide bond may be tolerant to mutations and the Q to E substitution is better tolerated than the Q–K substitution.

**Mutation and deletion analyses of the C-terminal region of the 3C-like proteinase**

Mutation of the C-terminal cleavage site (Q3086–S3087 dipeptide bond) of the 3C-like proteinase to E3086–S3087 was subsequently made, giving rise to pIBV9Q3086–E (Fig. 2a). Expression of pIBV9Q3086–E led to the detection of a novel product with an apparent molecular mass of approximately 35 kDa (Fig. 2b, lane 2). No 33-kDa protein was observed (Fig. 2b, lane 2). This was unexpected, as no further cleavage site was predicted to be located 30–60 bp downstream of the Q3086–S3087 dipeptide bond. To investigate the possibility that the C-terminus of the 35-kDa protein may be derived from a novel cleavage site located 10 to 20 amino acid residues downstream of the Q3086 residue, two deletion plasmids, pIBV9Q3086–Δ1 and pIBV9Q3086–Δ2, were constructed. As can be seen in Fig. 2a, the IBV sequence present in pIBV9Q3086–Δ1 and pIBV9Q3086–Δ2 was expressed from the expression of the pIBV9Q3086–Δ1 plasmid, as indicated by the appearance of two novel protein species (Fig. 2a, lane 3).
was terminated at nucleotide position 9911. Expression of this construct, once again, led to the detection of the 35-kDa protein (Fig. 2b, lane 3). Meanwhile, a novel product migrating more slowly than the 35-kDa protein was also detected (Fig. 2b, lane 3). Its apparent molecular mass of 39 kDa suggests that it is a fusion product containing the 35-kDa protein and the product encoded by nucleotides up to 9911. pIBV9Q3086–E was constructed by introducing a UGA termination codon immediately downstream of the Q3086 residue (Fig. 2a). Interestingly, expression of this plasmid led to the detection of a single protein species comigrating with the 35-kDa protein processed from pIBV9Q3086–E (Fig. 2b, lane 4), suggesting that the E3086 residue is actually the C-terminus of the 35-kDa protein expressed from pIBV9Q3086–E and pIBV9Q3086–E1. These results demonstrate that mutation of the Q3086 residue to an E only marginally reduces the cleavage efficiency occurring at the Q3086–S3087 dipeptide bond, but alters the mobility of the 33-kDa protein on SDS–PAGE.

To investigate the possibility that the C-terminal region of the 3C-like proteinase may contain sequences nonessential for the catalytic activity of the proteinase, five deletion constructs, pIBV9Δ19, pIBV9Δ20, pIBV9Δ21, pIBV9Δ22, and pIBV9Δ23, were made and expressed in intact cells. These constructs encode proteins with deletions of 3, 7, 33, 67, and 135 amino acid residues, respectively, from the C-terminus of the 3C-like proteinase. Expression of pIBV9Δ19 and pIBV9Δ20 showed that almost full enzymatic activity was maintained upon the deletions. As can be seen, a 35-kDa product representing the C-terminally truncated form of the 3C-like proteinase was detected in both cases (Fig. 2b, lanes 6 and 7). No full-length product was observed from the expression of pIBV9Δ19 (Fig. 2b, lane 6), but a trace amount of the full-length product was detected from the expression of pIBV9Δ20 (Fig. 2b, lane 7). Progressively less enzymatic activity was observed when pIBV9Δ21 and pIBV9Δ22 were expressed. In each case, two products representing the full-length proteins and the C-terminally truncated 3C-like proteinase were detected (Fig. 2b, lanes 8 and 9). Expression of pIBV9Δ23 showed the detection of the full-length product only (Fig. 2b, lane 10), indicating that the catalytic activity was abolished by this deletion. These results suggest that the extreme C-terminal region is not essential for the catalytic activity of the 3C-like proteinase.

Characterization of the in vitro enzymatic activity of the 3C-like proteinase

Studies on the 3C-like proteinase of human and murine coronaviruses have demonstrated that microsomal membranes are not strictly required for the in vitro cleavage activity but addition of the membranes can greatly enhance the autoprocessing activity of the proteinase.

FIG. 2. (a) Diagram showing the IBV sequences present in pIBV9Q3086–E, pIBV9Q3086–E1, pIBV9Q3086–E2, pIBV9Δ19, pIBV9Δ20, pIBV9Δ21, pIBV9Δ22, and pIBV9Δ23. The viral products generated from cleavage by the 3C-like proteinase and the full-length products encoded by some plasmids are illustrated. The molecular masses of these products were estimated according to their migration on SDS–PAGE. The Q3086 residue is encoded by nucleotides 9784–9786. (b) Mutational and deletion analyses of the Q3086–S3087 dipeptide bond and the C-terminal region of the 3C-like proteinase. Plasmids pIBV9, pIBV9Q3086–E, pIBV9Q3086–E1, pIBV9Q3086–E2, pIBV9Δ19, pIBV9Δ20, pIBV9Δ21, pIBV9Δ22, and pIBV9Δ23, respectively, were transiently expressed in Cos-7 cells using the vaccinia-T7 system. The cells were labeled with [35S]methionine–cysteine, lysates were prepared, and the labeled polypeptides were immunoprecipitated with anti-3C. Gel electrophoresis of the polypeptides was performed on an SDS–12.5% polyacrylamide gel and polypeptides were detected by fluorography.
The expression of pIBV1a9 was observed after chase for 20 min (Fig. 4b). It was first seen after chase for 40 min (Fig. 4b, lane 14).

Differential subcellular localization of the 3C-like proteinase in virus-infected and transfected cells

Determination of the subcellular localization of ORF 1a-encoded viral products from the Nidovirales family, namely, MHV-A59, MHV-JHM, HCV-229E, and equine arteritis virus (EAV), was the subject of several recent reports (Shi et al., 1999; Schiller et al., 1998; Ziebuhr and Siddell, 1999; van der Meer et al., 1998, 1999). It was interesting to note that immunofluorescence staining of viral infected cells using anti-3C antiserum showed distinct punctuate perinuclear staining patterns (Schiller et al., 1998; Shi et al., 1999; Denison et al., 1999; van der Meer et al., 1999), suggesting that the proteinase may be associated with intracellular membranes. To study the subcellular localization patterns of the IBV 3C-like proteinase, the proteinase was tagged to an 11-amino-acid T7 tag (MASMTGQQMQGL derived from the major capsid protein of bacteriophage T7, and a highly specific anti-T7 monoclonal antibody (Novagen) was used to stain cells overexpressing the fusion protein. This procedure is used to avoid the high background of the anti-3C polyclonal antiserum when it was used in indirect immunofluorescence assays. As can be seen in Figs. 5A and 5B, staining of Vero cells expressing the T7 tagged 3C-like proteinase with anti-T7 antibody showed a diffuse cytoplasmic staining profile. Indirect immunofluorescence staining of IBV-infected Vero cells with anti-3C antiserum showed a distinct perinuclear punctate pattern at 13 h postinfection (Fig. 5D), although high background was also observed for the mock-infected cells (Fig. 5C). This distribution pattern is similar to those reported for other coronaviruses and arteriviruses (Schiller et al., 1998; Shi et al., 1999; van der Meer et al., 1998). These results suggest that the IBV 3C-like proteinase may exhibit differential subcellular distribution patterns in virus-infected cells and in transfected cells. When it was expressed individually, a diffuse distribution profile was observed. However, the proteinase was shown to be associated with intracellular membranous structures in virus-infected cells. Subcellular fractionation of pIBV3C-transfected and IBV-infected cells was then carried out to investigate this possibility further. Two fractions, namely, the membrane and cytosol fractions, were collected and subjected to immunoprecipitation with anti-3C antiserum. Figure 6a shows that approximately equal amounts of the 33-kDa proteinase were detected from both fractions (Fig. 6a, lanes 1 and 3). Quantification by densitometry confirmed that the membrane and cytosol fractions contain 50.19

In vitro autoprocessing kinetics of the 3C-like proteinase

The in vitro processing system was then used to study the autoprocessing kinetics of the IBV 3C-like proteinase. Plasmids pIBV9Q3086–EΔ1 and its parental construct pIBV1a9Δ1 were expressed in reticulocyte lysate and pulse-chased for up to 2 h. The full-length 48-kDa product was observed from the expression of both constructs after chase for 10 min, and its expression gradually decreased toward the end of the time course (Fig. 4b). The appearance of a 39-kDa product, representing the fusion protein containing the 3C-like proteinase and the product encoded by nucleotides 9787 to 9910, was observed after chase for 20 min (Fig. 4b). It peaks after chase for 60 min and then starts to decline (Fig. 4b). The 33-kDa protein was first seen from the expression of pIBV1a9Δ1 after chase for 30 min and

FIG. 3. Time course analysis of the autoprocessing of the 3C-like proteinase expressed from pIBV1a7Δ1 in rabbit reticulocyte lysates. A 10-fold excess of unlabeled methionine was added to the in vitro translation reaction after incubation at 30°C for 10 min. Aliquots were subsequently taken from the reaction after incubation for 0, 1, 2, 3, 4, 5, 6, 7, and 8 h. The [35S]methionine-labeled products were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography.

(Lu et al., 1996; Pinon et al., 1997; Schiller et al., 1998; Heusipp et al., 1997a). However, the microsomal membranes were shown to be dispensable for the in vitro activity of the IBV 3C-like proteinase (Tibbles et al., 1996). To clarify this discrepancy, plasmid pIBV1a7Δ1, which covers nucleotides 7999 to 9786 and contains the upstream hydrophobic region (M1), was expressed in vitro in a rabbit reticulocyte lysate system and pulse-chased for up to 8 h. Aliquots were removed at the indicated time and the reactions were stopped by the addition of RNase A. The 33-kDa protein was detected after incubation for 1 h and its expression steadily increased toward the end of the time course (Fig. 3). The expression of the full-length 54-kDa protein meanwhile decreased rapidly over time (Fig. 3). The addition of microsomal membranes did not significantly increase the autoprocessing rate of the 33-kDa protein (data not shown). These results demonstrate that autoprocessing of the IBV 3C-like proteinase could occur in vitro in reticulocyte lysate in the absence of the membranes.
and 49.66%, respectively, of the 3C-like proteinase. In a control experiment, the IBV membrane protein was exclusively detected in the membrane fraction (Fig. 6a, lane 6). Similarly, IBV-infected Vero cells were fractionated into the membrane and cytosol fractions. Immunoprecipitation and quantification assays showed that 76% of the 33-kDa proteinase was detected in the membrane fraction (Fig. 6b, lane 3), while only 24% of the protein was detected in the cytosol (Fig. 6b, lane 5). Once again, the IBV membrane protein was detected only in the membrane fraction (Fig. 6b, lane 4).

Identification of the Q<sup>3379</sup>–A<sup>3380</sup> dipeptide bond as a cleavage site of the 3C-like proteinase

The Q<sup>3365</sup>–G<sup>3366</sup> dipeptide bond encoded by nucleotides 9784–9786 was previously predicted to be a cleavage site of the 3C-like proteinase (Gorbalenya et al., 1989). In a separate report, the Q<sup>3379</sup>–A<sup>3380</sup> dipeptide bond, which is located 14 amino acids downstream of the Q<sup>3365</sup>–G<sup>3366</sup> dipeptide bond and is conserved in all four sequenced coronaviruses (Lu et al., 1998), was also predicted to be a cleavage site of the 3C-like proteinase (Lee et al., 1991). The equivalent Q–A dipeptide bond of human coronavirus was actually identified as a genuine cleavage site (Ziebuhr and Siddell, 1999). In an in vitro study, Tibbles et al. (1999) recently reported that the Q<sup>3365</sup>–G<sup>3366</sup> dipeptide bond may not be used by the IBV 3C-like proteinase. However, the cleavability of the Q<sup>3379</sup>–A<sup>3380</sup> dipeptide bond was not mentioned in their report (Tibbles et al., 1999). To further study whether either site is used by the IBV 3C-like proteinase, deletion and mutational analyses were carried out. As antiserum against this specific region was not available, the two downstream cleavage sites were disabled by site-directed mutagenesis, and anti-24 and antiserum V47 were used to immunoprecipitate the potential cleavage products (Ng and Liu, 1998) (Fig. 7a). Expression of pIBV1a16 resulted in the detection of the 33-kDa protein and a
The 46-kDa protein may represent the C-terminal cleavage product with its N-terminus derived from cleavage at either the Q<sup>3365</sup>–G<sup>3366</sup> or Q<sup>3379</sup>–A<sup>3380</sup> dipeptide bond. Mutation of the Q<sup>3365</sup> residue to an E did not change the expression pattern of the construct (Fig. 7b, lanes 2 and 4), indicating that the Q<sup>3365</sup>–G<sup>3366</sup> dipeptide bond may be not used. To confirm this possibility, the Q<sup>3365</sup>–G<sup>3366</sup> dipeptide bond was deleted from pIBV1a16, giving rise to pIBV1a16<sub>D1</sub> (Fig. 7a). As can be seen in Fig. 7b, expression of pIBV1a16<sub>D1</sub> resulted in the detection of both the 33- and the 46-kDa proteins and a product of approximately 52 kDa (lanes 5, 6, and 7). The 52-kDa protein may represent an intermediate cleavage product containing the 46-kDa protein and product encoded by the upstream region in this construct (Fig. 7a). As the 46-kDa cleavage product was still detected from this deletion construct, this result rules out the possibility that the Q<sup>3365</sup>–G<sup>3366</sup> dipeptide bond could be used by the 3C-like proteinase.

Similar deletion and mutational studies were then carried out to study whether the Q<sup>3379</sup>–A<sup>3380</sup> dipeptide bond is used by the proteinase to release the 46-kDa protein. Mutation of the Q<sup>3379</sup> to an E led to the detection of only the 33- and 52-kDa proteins (lanes 5, 6, and 7). The 52-kDa protein may represent an intermediate cleavage product containing the 46-kDa protein and product encoded by the upstream region in this construct (Fig. 7a). As the 46-kDa cleavage product was still detected from this deletion construct, this result rules out the possibility that the Q<sup>3379</sup>–A<sup>3380</sup> dipeptide bond could be used by the 3C-like proteinase.

**DISCUSSION**

The IBV 3C-like proteinase plays a major role in processing of the gene 1-encoded polyproteins to smaller functional products associated with viral RNA replication. Previous studies using an in vitro processing assay showed that this proteinase may be released from the 1a polyprotein by self-cleavage at two Q–S dipeptide bonds.
with anti-24 and anti-3C, respectively. Polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography.

molecular masses of these products were estimated according to their migration on SDS–PAGE. The Q 2779–S2780, Q 3086–S3087, Q 3365–G3366, Q 3379–A3380, and the substitution mutations that were introduced. The viral products generated from cleavage by the 3C-like proteinase are illustrated, and the computational studies present here clearly indicate that cleavage and enzymatic activities of the proteinase. The ki

-enzymatic activities of the proteinase reported here resemble the equivalent proteinase of human and murine coronaviruses. For example, the 3C-like proteinases of both human and murine coronaviruses are active when expressed in vitro (Heusipp et al., 1997a; Lu et al., 1995; Schiller et al., 1998), and autoprocessing of the MHV 3C-like proteinase was slightly increased by the addition of microsomal membranes when only the upstream hydrophobic region was presented (Pinon et al., 1997).

The mutagenesis studies presented here support the conclusion that the Q 2779–S2780 and Q 3086–S3087 dipeptide bonds are the N- and C-terminal cleavage sites, respectively, of the 3C-like proteinase. Interestingly, substantial cleavage was observed after mutation of either site to E–S, suggesting that the P1 position at these two sites are more tolerant to mutagenesis. However, much less tolerance to alterations at the P1 position was observed for the rest of the 11 cleavage sites identified so far (Liu et al., 1997, 1998; Liu and Brown, 1995; Ng and Liu, 1998). The reasons for this discrepancy are uncertain, but it may reflect the differing cleavage efficiency between the cis- and trans-enzymatic activities of the proteinase. The kinetic studies present here clearly indicate that cleavage first takes place at the Q 2779–S2780 dipeptide bond, which is followed by cleavage at the Q 3086–S30870 dipeptide bond, resulting in the release of the 3C-like proteinase. Cleavage at these two sites is therefore mediated by the cis activity of the proteinase. This process is very rapid and efficient, as no precursor containing the proteinase was detected even when the region was overexpressed in intact cells. Processing at other sites is thus likely mediated by the "free" 3C-like proteinase in trans. To argue against this speculation, Pinon et al. (1999) have recently reported that mutations of either the P1 or the P2 residue of the N-terminal Qsite abolished in vitro autoprocessing of the MHV 3C-like proteinase.

Another interesting observation is that mutation of the Q 2779 residue to an E alters the mobility of the 33-kDa proteinase on SDS–PAGE, yet almost full enzymatic activity of the proteinase is maintained. Mutation of the equivalent Q 3086 residue of HCV-229E 3C-like proteinase to an A exerts no effect on the proteinase activity, although information on the mobility of this mutant on SDS–PAGE was not available (Ziebuhr et al., 1997). Deletion of up to 7 amino acids from the C-terminus of the IBV 3C-like proteinase renders no significant effect on the enzymatic activity of the proteinase. Progressive loss of the activity was observed when further deletions were made. Interestingly, significant enzymatic activity was still maintained when the C-terminal 67 amino acid residues were deleted. Furthermore, the three mutant forms (the Q 3086–E mutant and the two mutants with deletions of 3 and 7 amino acids) migrate on SDS–PAGE significantly more slowly than the wild-type proteinase. These results suggest that the extreme C-terminal region may be able to fold into a unique structure independent of the catalytic domain, which is located at the N-terminal half of the protein. Cotranslational folding of a functionally active proteinase domain may occur even before the whole proteinase is synthesized. This argument would be supported by several recent studies on eukaryotic protein folding. It was demonstrated that rapid and efficient folding of modular model proteins depends on sequential folding of their domains during synthesis (Netzer and Hartl, 1997; Nicola et al., 1999; Louis et al., 1999). Cotranslational folding of two proteinase domains with enzymatic activities was observed when the novel polypeptide chains were still bound to the ribosome (Nicola et al., 1999; Louis et al., 1999). This is in contrast to the protein folding in prokaryotic system, in which the folding of a functional domain occurs posttranslationally (Netzer and Hartl, 1997). This different folding mechanism may explain the data reported by Ziebuhr et al. (1997), showing that the HCV-229E 3C-like proteinase expressed in bacteria was inactivated by deletion of the C-terminal 33 amino acid residues.

Immunofluorescence staining of coronavirus-infected cells using anti-3C antibodies reveals distinct perinuclear, punctate staining patterns in several recent reports (van der Meer et al., 1998, 1999; Schiller et al., 1998; Shi et al.)

FIG. 7. (a) Diagram of plBV1a16, plBV1a16Q3086–E, plBV1a16Δ1, plBV1a16Δ1Q2779–E, and plBV1a16Δ2, showing the locations of the cleavage sites and the substitution mutations that were introduced. The viral products generated from cleavage by the 3C-like proteinase are illustrated, and the molecular masses of these products were estimated according to their migration on SDS–PAGE. The Q 2779–S2780, Q 3086–S3087, Q 3365–G3366, Q 3379–A3380, Q 3402, and Q 2372 residues are encoded by nucleotides 8863–8888, 9784–9789, 10,621–10,626, 10,663–10,656, 10,912–10,914 and 11,542–11,544, respectively. (b) Mutation and deletion analyses of the Q 2779–A 2372 dipeptide bond. Plasmids plBV1a16Δ1, plBV1a16Δ1Q2779–E, and plBV1a16Δ2 were expressed in Cos-7 cells using the vaccinia-T7 system. Cells were labeled with [35S]methionine–cysteine and total lysates were immunoprecipitated with anti-24 and anti-3C, respectively. Polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography.
et al., 1999). As rapid self-cleavage of the 3C-like proteinase from the polyprotein precursors was reported for these viruses, these staining patterns should be contributed mainly by the staining of the mature 3C-like proteinase. In this report, we present evidence showing that the IBV 3C-like protein displays a free distribution pattern when it was expressed in intact cells alone. However, in IBV-infected cells, the proteinase is associated mainly with the intracellular membranes. Two possibilities may be responsible for these different distribution patterns. First, the 3C-like proteinase may be sequestered to cellular membranous compartments by interacting with other viral or cellular proteins. Recently, we showed that anti-3C antiserum could immunoprecipitate a protein band of about 170 kDa in IBV-infected cells (Lim et al., 2000). The same protein species could also be detected by several other IBV region-specific antisera (Lim et al., 2000). As the 170-kDa protein may be an IBV-induced host protein, it is tempting to speculate that the IBV 3C-like proteinase together with other viral products may interact with this protein to form a multiprotein complex involved in viral replication. The second possibility is that the IBV 3C-like proteinase may be able to bind to viral RNA. In poliovirus, the viral 3C proteinase was shown to be an RNA binding protein (Andino et al., 1993). The 3C proteinase and polymerase precursor (3CD) together with a cellular protein can form a ribonucleoprotein complex with the first 90 nucleotides of the poliovirus genome (Andino et al., 1993). This complex may play an important role in the replication of polioviral RNA. We are currently unclear whether the IBV 3C-like proteinase may be involved in the formation of coronavirus replication complexes.

MATERIALS AND METHODS

Virus and cells

The egg-adapted Beaudette strain of IBV (ATCC VR-22) was obtained from the American Type Culture Collection (ATCC) and was adapted to Vero cells as described previously (Ng and Liu, 1998). The virus was passaged three times in 11-day-old chicken embryos and then adapted to Vero cells (ATCC CCL-81) by a series of passages at 24- to 48-h intervals. The cytopathic effects, including syncytium formation and rounding-up of cells, were initially observed after three passages in Vero cells. Virus stocks were prepared after the 62nd passage by infecting monolayers of Vero cells at a multiplicity of infection of approximately 0.1 PFU/cell. The virus was harvested at 24 h postinfection and the titer of the virus preparation was determined by plaque assay on Vero cells.

Vero cells and Cos-7 cells were grown at 37°C in 5% CO₂ and maintained in Dulbecco’s modified minimal essential medium (Gibco BRL, Life Technologies) supplemented with 10% newborn calf serum.

Radiolabeling of IBV-infected and mock-infected Vero cells

Confluent monolayers of Vero cells grown on 60-mm dishes were infected with IBV at a multiplicity of infection of approximately 2 PFU/cell. The cells were labeled for 4 h with 25 μCi/ml [35S]methionine–cysteine at 6 h postinfection before harvesting.

Transient expression of plasmid DNAs using the vaccinia virus-T7 expression system

Semiconfluent monolayers of Cos-7 cells grown on 60-mm dishes were infected with 10 PFU/cell of vTF7-3 (Fuerst et al., 1986) and transfected with 2.5 μg of plasmid DNAs using the vaccinia virus-T7 expression system (Boehringer Mannheim). After incubation of the cells at 37°C for 7 h, the cells were incubated in methionine- and cysteine-free medium (ICN) for 30 min and labeled with 25 μCi/ml [35S]methionine–cysteine (35S Express Protein Labelling Mix, NEN Life Science) for 13 h. The cells were scraped off the dishes in phosphate-buffered saline (PBS) and recovered by centrifugation at 12,000 rpm for 1 min.

Cell fractionation of [35S]methionine–cysteine-labeled cells

The radiolabeled cells were resuspended in hypotonic buffer (1 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 15 mM NaCl) containing 2 μg/ml leupeptin and 0.4 mM phenylmethylsulfonyl fluoride (PMSF) and broken by 20 strokes with a Dounce cell homogenizer as described (van der Meer et al., 1998). Cell debris and nuclei were removed by centrifugation at 3500 rpm for 10 min at 4°C. The membranes were pelleted by ultracentrifugation in a Beckman TLA 120.1 rotor (65,000 rpm for 30 min at 4°C). Membrane pellets were resuspended in immunoprecipitation buffer [20 mM Tris–HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.5% SDS, 0.1% sodium deoxycholate, 0.5% Nonidet-P40 (NP-40)] and the cytosol fraction (supernatant) was adjusted to the same volume and ion/detergent conditions with 2X immunoprecipitation buffer. Samples were precleared by centrifugation and incubated with anti-3C or anti-M for 30 min at room temperature, followed by 30 min incubation with protein A–Sepharose CL-4B (Sigma). Immunoprecipitated beads were washed three times with the immunoprecipitation buffer and analyzed by SDS–PAGE.

SDS-polyacrylamide gel electrophoresis

SDS–PAGE was carried out with a 12.5% polyacrylamide concentration, and the labeled polypeptides were detected by autoradiography or fluorography of dried gels.
**Cell-free transcription and translation**

Plasmid DNAs were expressed in rabbit reticulocyte lysate using a transcription coupled translation system (TnT, Promega) in the presence of [35S]methionine (Amersham-Pharmacia Biotech) according to the instructions of the manufacturer. Briefly, 1 μg of plasmid DNA was incubated in a 50-μl reaction mix with or without 5 μl of canine pancreatic microsomal membranes (Promega). Aliquots were either analyzed directly by SDS–PAGE or immunoprecipitated with polyclonal antiserum as described previously (Liu et al., 1997).

**Polymerase chain reaction (PCR)**

Appropriate primers and template DNAs were used in amplification reactions with cloned Plu DNA polymerase (Stratagene) under standard buffer conditions with 2 mM MgCl₂.

**Indirect immunofluorescence microscopy**

Cells were grown on coverslips and infected with IBV or transfected with appropriate plasmid DNAs. After being washed with PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ (PBSCM), the cells were fixed with 4% paraformaldehyde (in PBSCM) for 30 min at room temperature and permeabilized with 0.1% saponin (in PBSCM), followed by incubation with specific antiserum (anti-3C) at room temperature for 1 h before being mounted.

**Site-directed mutagenesis**

Site-directed mutagenesis was carried out by two rounds of PCR with two pairs of primers as described for pIBV9 (Liu et al., 1997). The mutations introduced were verified for pIBV9 and its derivatives by DNA sequencing.

**Construction of plasmids**

Plasmids pIBV9 (formerly known as pKT205), pIBV3C, pBP5Δ5, and pIBV1a7Δ1 were described previously (Liu et al., 1994, 1997; Lim et al., 2000). The IBV sequences present in these constructs are nucleotides 8693 to 10,925, 8665 to 9786, 11,306 to 11,877, 6870 to 9786, and 7999 to 9786, respectively.

Plasmid pIBV9Δ1Q2779–E, which contains the Q2779–E mutation and a deletion of nucleotides 9911 to 10,925 from pIBV9, was constructed as follows. A PCR fragment covering nucleotides 8693 to 10,637 was generated by two rounds of PCR with pIBV9 as the template. The PCR fragment was digested with BglII and PvuII and cloned into BglII- and Smal-digested pKTO (Liu et al., 1994) (see Fig. 2a).

PvuII cuts the PCR fragment at a position 9911 and BglII digests the PCR fragment at a position 19 nucleotides upstream of the viral sequence. The primers used to introduce the Q2779–E mutation are LN-10 (5'-GGTAGTAGTTAGAGGCTCGGGTTTAAG-3') and LN-11 (5'-CAGTTTCCTTAACCCAGACTCTCATCT-3'), and the primers used for the second round of PCR are T7 (5'-TAAAGAGCTCACTAGG-3') and LN-8 (5'-GTCACCACAAATTCTCTAAAGTAT-3'). Plasmid pIBV9ΔQ2779–K was generated as described for pIBV9ΔQ2779–E by mutation of the Q2779 residue to a K with primers LN-32 (5'-GGTAGTAGTTAAGAAGCTGTTTAAG-3') and LN-33 (5'-CAGTTCTCTAAACAGACTCTCATCT-3').

Plasmid pIBV9Q3086–E contains the Q3086–E mutation and a deletion of nucleotides 10,637 to 10,925 from pIBV9. The mutation and deletion were introduced by two rounds of PCR with pIBV9 as the template. The PCR fragment was digested with BglII and cloned into BglII- and EcoRV-digested pKTO, giving rise to pIBV9Q3086–E. The mutation primers are LN-12 (5'-GGGTAGATTAGAGGCTCTTTTGTA-3') and LN65 (5'-GTCAGTGAGCTCTATCCAGTGTGTAATGC-3') and the two primers used for the second round of PCR are T7 and LN-8. Plasmid pIBV9Q3086–EΔ1, which covers nucleotides 8693 to 9910 and contains the Q3086–E mutation, was constructed by religation of PvuII- and Smal-digested pIBV9Q3086–E, resulting in the deletion of nucleotides 9911 to 10,637. Plasmid pIBV9Q3086–EΔ2 was constructed by introducing a termination codon (UAG) immediately downstream of the viral sequences. The constructs were made by ligation of PCR fragments covering the appropriate regions into pKTO. The constructs were then digested with BglII and Smal-digested pIBV9, resulting in the deletion of nucleotides 9911 to 10,925.

Plasmids pIBV9Δ19, pIBV9Δ20, pIBV9Δ21, pIBV9Δ22, and pIBV9Δ23 cover nucleotides 8693 to 9777, 8693 to 9765, 8693 to 9687, 8693 to 9585, and 8693 to 9381, respectively, and contain a UAG termination codon immediately downstream of the viral sequences. The constructs were made by ligation of PCR fragments covering the appropriate regions into pKTO. The constructs were then digested with BglII and Smal-digested pIBV9, resulting in the deletion of nucleotides 8693 to 11,877 and contains both Q3626–E and Q373C–E mutations. The two mutations were introduced into a PCR fragment covering nucleotides 8693 to 11,877 as follows. The Q373C–E mutation was incorporated into the PCR fragment covering nucleotides 8693 to 11,877 and contains both Q3626–E and Q373C–E mutations. The two mutations were introduced into a PCR fragment covering nucleotides 8693 to 11,877 as follows. The Q373C–E mutation was incorporated into the PCR fragment covering nucleotides 8693 to 11,877 as follows.
by using pBP5Δ5O3672–E (Liu et al., 1997) as the template, and the Q3462–E mutation was introduced by primers X-38 (5′-CAACTGTATAGAATCGGTAC-3′) and LN-2 (5′-CTTGAGTAAATCATGTAATTTAGCCTCAACTGTAG-3′). This PCR fragment was cloned into pKT0, giving rise to pIBV1a16. Plasmid pIBV1a16Q3462–E, which contains the Q3462–E mutation (see Fig. 8a), was constructed by two rounds of PCR with pIBV1a16 as the template. The primers used to introduce the Q3462–E mutation were LN-7 (5′-TCGA-CAAATATCGCTTATAGGAATTGT-3′) and LN-8.

Plasmid pIBV1a16Δ1 covers nucleotides 8693 to 11,877 with a deletion of nucleotides 9911 to 10,634. It was made by digestion of pIBV1a16 with BstEII (which cuts at nucleotide 10,634), end-repair with Klenow, religation with Ndel (cuts at nucleotide 9911), and religation with T4 DNA ligase, resulting in the deletion of nucleotides 9911 to 10,634; pIBV1a16Δ2 was constructed by deletion of nucleotides 9911 to 10,669 from pIBV1a16.

Plasmid pIBV1a16Δ1O3379–E, which covers nucleotides 8693 to 11,877 with the Δ3379 to E mutation, was made by two rounds of PCR with pIBV1a16Δ1 as the template. The primers used to introduce the Δ3379 to E mutation are LN-22 (5′-TCACTTGACAAGTTGAGCTTAATTAGT-3′) and LN-23 (5′-TACATCTACAAATGCTCGTCTAATTAGT-3′), and the cloning primers are T7 and LDX-7 (5′-GCTC-CAGGATCTACTGTAAGAACAC-3′).

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