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Identification of a 24-kDa Polypeptide Processed from the Coronavirus Infectious Bronchitis Virus 1a Polyprotein by the 3C-like Proteinase and Determination of Its Cleavage Sites

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We report here the identification of a 24-kDa polypeptide in IBV-infected Vero cells by immunoprecipitation with a region-specific antiserum raised in rabbits against the IBV sequence encoded between nucleotides 10928 and 11493. Coexpression, deletion, and mutagenesis studies have demonstrated that this protein is encoded by ORF 1a from nucleotide 10915 to 11544 and is released from the 1a polyprotein by the 3C-like proteinase-mediated proteolysis. A previously predicted Q-S ($Q^{3462}S^{3463}$) dipeptide bond encoded by the IBV sequence from nucleotide 10912 to 10917 is identified as the N-terminal cleavage site, and a Q-N ($Q^{3672}N^{3673}$) dipeptide bond encoded by the IBV sequence between nucleotides 11542 and 11547 is identified as the C-terminal cleavage site of the 24-kDa polypeptide.

INTRODUCTION

The avian infectious bronchitis virus (IBV) is the prototype species of the *Coronaviridae,* a family of enveloped viruses with large positive-stranded RNA genomes. Six mRNA species (mRNAs 1–6) are involved in the expression of IBV genes. Among them, the 27.6-kb genome-length mRNA, mRNA 1, is composed of two large overlapping ORFs (1a and 1b) at the 5′-unique region and has the capacity to encode a fusion polyprotein of 741 kDa by a ribosomal frameshifting mechanism (Boursnell *et al.,* 1987; Brierley *et al.,* 1987, 1989). Proteolytic processing of the 441-kDa 1a polyprotein and the 741-kDa 1a/1b fusion polyprotein to smaller functional products is expected to be mediated by viral proteinases. Three proteinase domains, namely, two overlapping papain-like and a picornavirus 3C-like proteinase domains, have been predicted to be encoded by the ORF 1a sequences from nucleotide 4243 to 5553 and from 8937 to 9357, respectively (Gorbalenya *et al.,* 1989; Lee *et al.,* 1991).

Recently, considerable progress has been made in the identification and characterization of the ORF1a-encoding proteinase activities as well as their cleavage products. The two overlapping papain-like proteinase domains and cellular proteinase(s) were demonstrated to be involved in processing of the 1a polyprotein to an 87-kDa protein (Liu *et al.,* 1995). The 3C-like proteinase is responsible for processing of the 1a and 1a/1b polyproteins to a 10- and a 100-kDa protein (Liu *et al.,* 1994, 1997; Liu and Brown, 1995). Proteolytic processing of the 1a and 1a/1b polyproteins by the equivalent proteinase activities of human coronavirus and mouse hepatitis virus has also been documented. In the case of human coronavirus, four mature products have been identified so far. These include a 34-kDa protein representing the 3C-like proteinase, a 105-kDa protein corresponding to the 100-kDa protein of IBV, a 71-kDa protein with an ATPase activity and a 41-kDa protein processed from the 1b polyprotein (Ziebuhr *et al.,* 1995; Grotzinger *et al.,* 1996; Heusipp *et al.,* 1997a,b). Similarly, three mature products, including two N-terminal cleavage products of 28 and 65 kDa and a 27-kDa protein identified as the 3C-like proteinase, have been reported for mouse hepatitis virus (Denison and Perlman, 1987; Denison *et al.,* 1995; Lu *et al.,* 1995).

In this communication, we show that the IBV 3C-like proteinase is involved in processing of the 1a polyprotein to a 24-kDa mature viral product. By using a region-specific antiserum (anti-24) raised in rabbits against the IBV sequence encoded between nucleotides 10928 and 11493, the 24-kDa polypeptide was immunoprecipitated from IBV-infected but not from mock-infected Vero cells. Coexpression, deletion, and mutagenesis studies demonstrated that this polypeptide was encoded by ORF 1a between nucleotides 10915 and 11544 and was cleaved from the 1a polyprotein by the 3C-like proteinase. Substitution mutation studies suggested that the predicted $Q^{3462}S^{3463}$ dipeptide bond was responsible for the release of the N terminus, and the $Q^{3672}N^{3673}$ dipeptide bond was responsible for the release of the C terminus of the 24-kDa protein.

RESULTS AND DISCUSSION

Identification of a 24-kDa polypeptide in IBV-infected Vero cells

We have previously shown that a novel ORF 1a-specific 10-kDa polypeptide encoded by ORF 1a from nucle-

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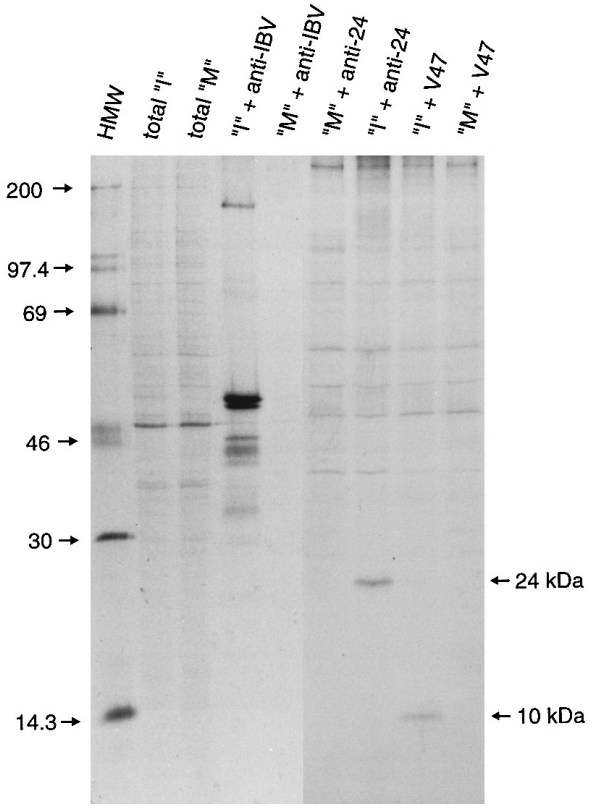

FIG. 1. Detection of a 24-kDa protein in IBV-infected Vero cells. IBV-infected (I) and mock-infected (M) Vero cell monolayers in 35-mm dishes were labeled with [^{35}S]methionine for 4 h at 6 h postinfection. Cell lysates were prepared by adding 1 ml of RIPA buffer to each dish. The lysates were either analyzed (15 μl) directly or immunoprecipitated (200 μl) with anti-IBV, anti-24, and antiserum V47. Polypeptides were separated on an SDS–15% polyacrylamide gel and detected by fluorography. Lanes labeled HMW, total "I", total "M", "I"+anti-IBV, and "M"+anti-IBV were exposed for 1 day, and the other lanes were exposed for 14 days. HMW, high-molecular-weight protein markers (numbers indicate kilodaltons).

otide 11545 to 11877 was processed from the 1a polyprotein by the 3C-like proteinase (Liu *et al.,* 1997). In an effort to identify more viral polypeptides encoded by ORF 1a, a region-specific antiserum, anti-24, was raised in rabbits against a bacterially expressed protein containing the IBV sequence encoded between nucleotides 10928 and 11493. This antiserum was then used to test if specific viral products can be identified from IBV-infected Vero cells. For this purpose, confluent monolayers of Vero cells were infected with IBV at a multiplicity of infection of approximately 2 PFU per cell. The cells were labeled with [^{35}S]methionine at 6 or 14 h postinfection and were harvested 4 h after labeling. Cell lysates were prepared and immunoprecipitated with anti-IBV, anti-24, and antiserum V47. Antiserum V47 was raised against the IBV sequence encoded between nucleotides 11488 and 12600 and was used successfully in the identification of a 10-kDa protein in IBV-infected cells (Liu *et al.,* 1997). Figure 1 shows the results of this experiment using lysate prepared from Vero cells harvested at 10 h postinfection. As can be seen, a protein with an apparent molecular weight of approximately 24 kDa was specifically precipitated with anti-24 antiserum from IBV-infected, but not from mock-infected Vero cell lysates (Fig. 1). Immunoprecipitation of the same cell lysate with antiserum V47, once again, led to the detection of the 10-kDa protein (Fig. 1) (also see Liu *et al.,* 1997). No obvious difference in abundance of the 24-kDa protein was observed from lysates prepared from cells harvested at 10 and 18 h postinfection (data not shown).

Release of the 24-kDa protein from the 1a polyprotein by the 3C-like proteinase

The detection of the 24-kDa protein in IBV-infected Vero cells with a region-specific antiserum indicates that it may be encoded by the corresponding region of ORF 1a. This possibility was investigated by coexpression of plasmid pBP5 (Fig. 2a) in Vero cells with pIBV3C, a plasmid containing the IBV sequence coding for the 3C-like proteinase (Liu *et al.,* 1997). The cells were labeled with [^{35}S]methionine for 4 h at 14 h posttransfection, and the expression of IBV-specific products was analyzed by immunoprecipitation with anti-24 and antiserum V47. As shown in Fig. 2b, transfection of pBP5, once again, showed the expression of the 60-kDa 1a termination and the 72-kDa 1a/1b fusion products (see also Liu *et al.,* 1997); both products can be efficiently precipitated by anti-24 and antiserum V47. Coexpression of pBP5 with pIBV3C resulted in the detection of the 10-kDa protein and a product with an apparent molecular weight of approximately 24 kDa; the 10- and 24-kDa products were specifically immunoprecipitated by anti-24 and antiserum V47, respectively (Fig. 2b). In addition, three more products with apparent molecular masses of 55, 42, and 38 kDa were immunoprecipitated by both antisera (Fig. 2b). They may represent intermediate processing products (see Fig. 2a); the identity of these products was explored in the following sections.

To verify if the 24-kDa protein identified from coexpression of pBP5 and pIBV3C is the same product detected in IBV-infected cells, Vero cell lysates prepared from IBV-infected and plasmid-transfected cells were immunoprecipitated with anti-24 antiserum and the viral products were analyzed by SDS–PAGE. The results obtained are shown in Fig. 2c. As can be seen, comigration of the 24-kDa protein species was clearly observed, suggesting that they are the same product.

The 24-kDa protein is a mature cleavage product of the 1a polyprotein

As shown in Figs. 2b and 2c, coexpression of pBP5 with pIBV3C led to the detection of five cleavage products of 10, 24, 38, 42, and 55 kDa by immunoprecipitation with anti-24 and antiserum V47. Since both the 10- and 24-kDa proteins were detected in IBV-infected cells and the 10-kDa protein was a mature viral product, it was of

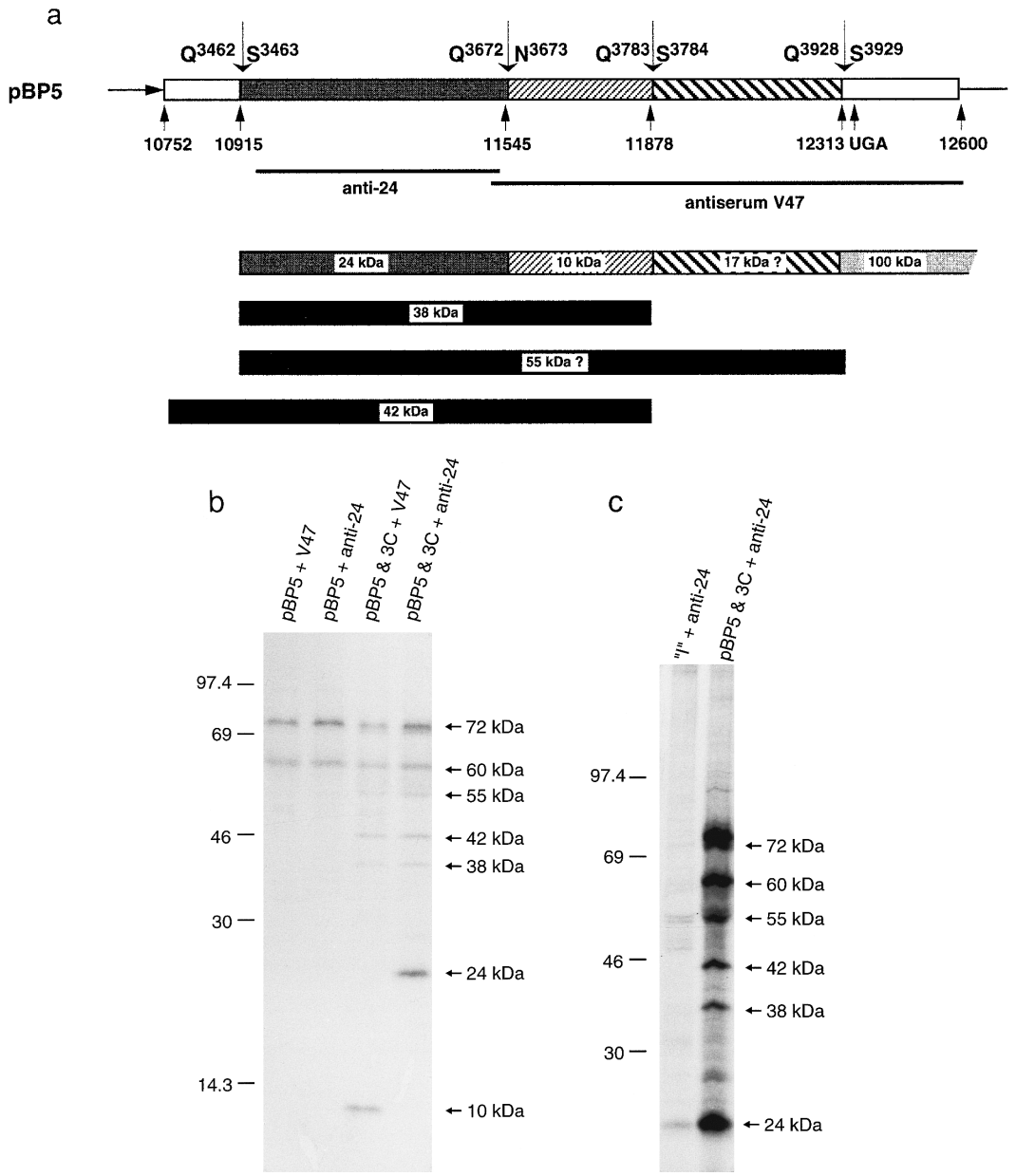

FIG. 2. (a) Diagram showing the IBV sequence present in plasmid pBP5, the locations of four cleavage sites and the sequences used to raise anti-24 and antiserum V47. Also shown are the cleavage products encoded by this region of the IBV genome. The first nucleotide position of the IBV sequences encoding the four cleavage products and the UGA termination codon for ORF 1a encoded by nucleotides 12382 to 12384 are also indicated. (b) Analysis of transiently expressed mRNA 1 products from cotransfection of plasmids pBP5 and pIBV3C. Plasmid DNAs were expressed in Vero cells using the vaccinia virus-T7 expression system described in Fuerst *et al.* (1986). Fifty to eighty percent confluent monolayers of Vero cells in 35-mm dishes were infected with a recombinant vaccinia virus (vTF7-3) and were transfected with plasmid DNA using DOSPER according to the instructions of the manufacturer (Boehringer Mannheim). Cells were labeled with [^{35}S]methionine for 4 h at 14 h posttransfection and total cell lysates were prepared by adding 500 μl of RIPA buffer to each dish. Two hundred microliters of the lysates was immunoprecipitated with antiserum V47 and anti-24. Polypeptides were separated on an SDS–16% polyacrylamide gel and detected by fluorography. Numbers indicate molecular masses in kilodaltons. (c) Comigration of the 24-kDa protein detected from IBV-infected (I) and plasmids (pBP5 and pIBV3C)-cotransfected Vero cells in SDS–PAGE. Cell lysates were prepared as indicated in the legends for Fig. 1 and b and the same amount of lysate was immunoprecipitated with anti-24. Viral polypeptides were separated on an SDS–16% polyacrylamide gel and detected by fluorography. Numbers indicate molecular masses in kilodaltons.

interest to know if the 24-kDa protein was also a mature cleavage product. For this purpose, Vero cells were co-transfected with pBP5 and pIBV3C, but were labeled with [^{35}S]methionine for 13 h at 6 h posttransfection and were chased with 10-fold excess of cold methionine up to 9 h. As shown in Figs. 3a and 3b, after prolonged labeling, only the 24- and 10-kDa proteins were detected by immunoprecipitation with anti-24 and antiserum V47, respectively. This is consistent with our previous observation that only the 10-kDa protein was detectable by

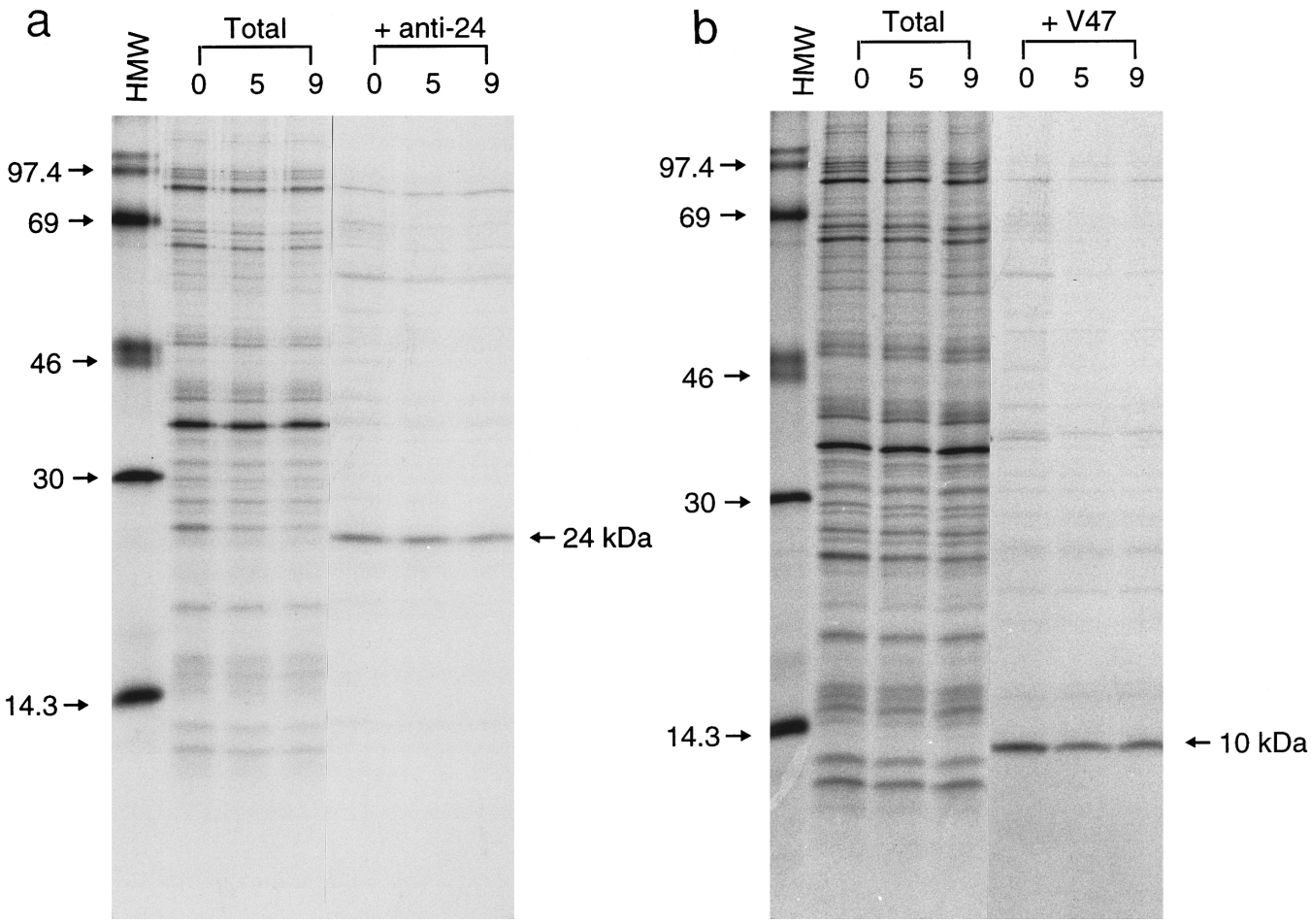

FIG. 3. Pulse–chase analysis of the expression, processing, and accumulation of the 24 (a) and 10 (b) kDa proteins from cotransfection of pBP5 and pIBV3C. Fifty to eighty percent confluent monolayers of Vero cells in 35-mm dishes were infected with a recombinant vaccinia virus (vTF7-3) and were transfected with plasmid DNA using DOSPER. Cells were labeled with [^{35}S]methionine for 13 h at 6 h posttransfection and were chased with 10-fold excess of cold methionine for 0, 5, and 9 h as indicated above each lane. Cell lysates were prepared by adding 500 μl of RIPA buffer to each dish and were either analyzed (15 μl) directly or immunoprecipitated (200 μl) with anti-24 (a) and antiserum V47 (b). Polypeptides were separated on SDS–16% polyacrylamide gels and detected by fluorography. HMW, high-molecular-weight protein markers (numbers indicate kilodaltons).

antiserum V47 from coexpression of pBP5 and pIBV3C (Liu *et al.,* 1997). The amount of both products remained constant after chase for 9 h (Figs. 3a and 3b). These results suggest that, like the 10-kDa polypeptide, the 24-kDa protein is also a mature product. The 38-, 42-, and 55-kDa proteins shown in Figs. 2b and 2c are therefore intermediate cleavage products.

Mutational analysis of the N- and C-terminal cleavage sites of the 24-kDa protein

Mutagenesis studies were then carried out to define the N- and C-terminal cleavage sites of the 24-kDa protein. As anti-24 antiserum was raised against the IBV sequence immediately upstream of the 10-kDa protein, it is likely that the N-terminal cleavage site (the $Q^{3672}N^{3673}$ dipeptide bond) of the 10-kDa protein may be the C-terminal cleavage site of the 24-kDa protein. Based on the calculated molecular mass of the 24-kDa protein, a previously predicted QS ($Q^{3462}S^{3463}$) dipeptide bond encoded by ORF 1a from nucleotide 10912 to 10917 may therefore be the N-terminal cleavage site of the 24-kDa protein (Gorbalenya *et al.,* 1989). To test these possibilities, plasmids containing substitution mutation of the Q residue of the two putative scissile bonds to an E were constructed and expressed in Vero cells using the vaccinia/T7 expression system (Fig. 4a).

The putative N-terminal QS ($Q^{3462}S^{3463}$ dipeptide bond) cleavage site was first analyzed by this approach. Plasmid pBP5Q^{3462}-E, which covers the IBV sequence from nucleotide 10752 to 11877 and contains the Q^{3462} to E mutation, was constructed and expressed. As can be seen, expression of pBP5Q^{3462}-E alone resulted in the detection of a protein of approximately 42 kDa (Fig. 4b), representing the full-length product of this construct. Interestingly, this product comigrated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with the 42-kDa intermediate processing product detected from coexpression of pBP5 and pIBV3C, indicating

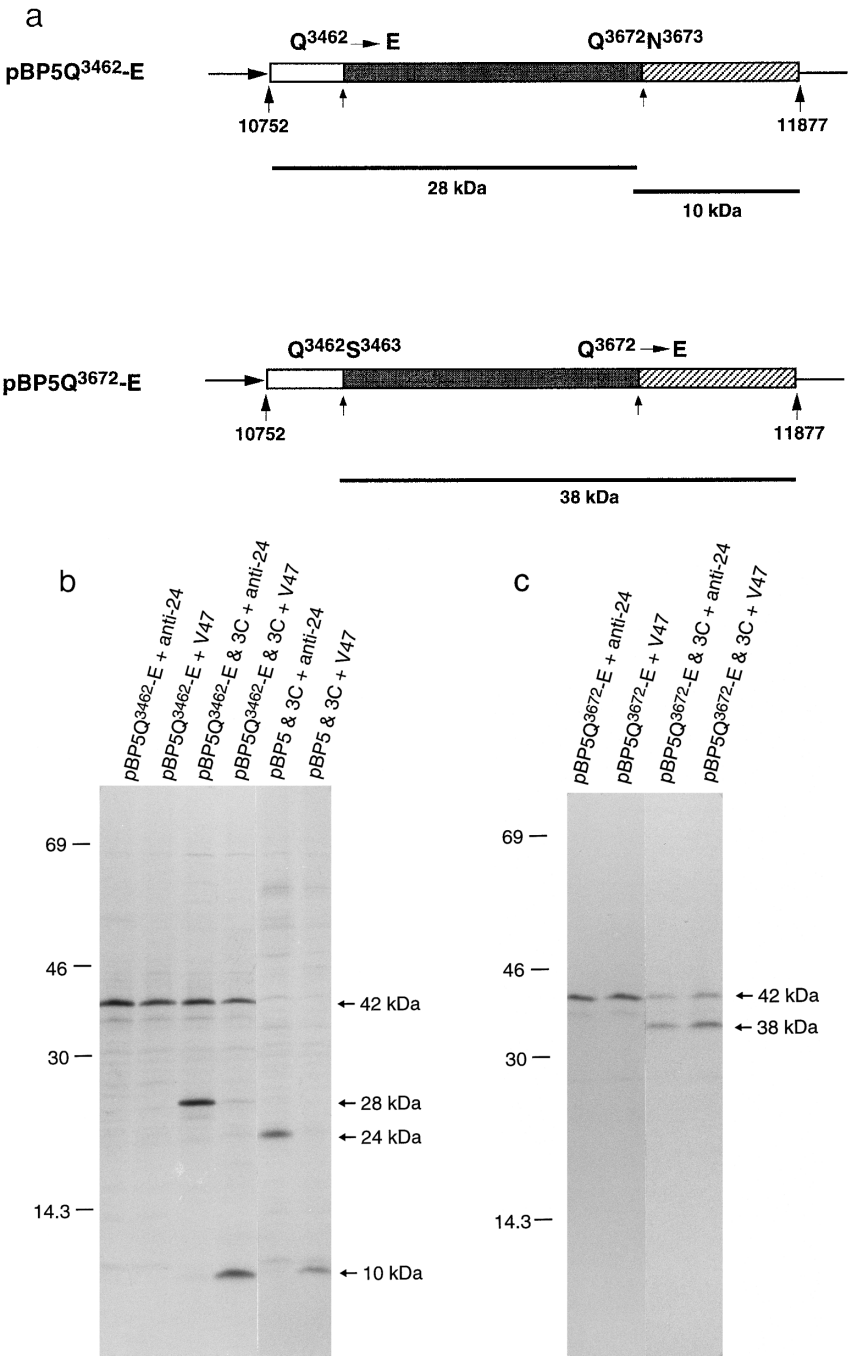

FIG. 4. (a) Diagram of the structures of plasmids pBP5Q^{3462}-E and pBP5Q^{3672}-E, showing the locations of the cleavage sites and the substitution mutations introduced. The predicted protein species recognized by antiserum V47 and anti-24 are also shown. (b) Mutational analysis of the N-terminal predicted cleavage site (Q^{3462}-S^{3463}) of the 24-kDa protein. Plasmid pBP5Q^{3462}-E was coexpressed with pIBV3C in Vero cells using the vaccinia virus-T7 expression system. Cells were labeled with [^{35}S]methionine and total cell lysates were either analyzed directly or immunoprecipitated with antiserum V47 and anti-24. Polypeptides were separated on an SDS–16% polyacrylamide gel and detected by fluorography. Numbers indicate molecular masses in kilodaltons. (c) Mutational analysis of the C-terminal predicted cleavage site (Q^{3672}-N^{3673}) of the 24-kDa protein. Numbers indicate molecular masses in kilodaltons.

that they may encode by the same region of ORF1a (Fig. 4b). Coexpression of pBP5Q^{3462}-E with pIBV3C, however, led to the detection of the 10-kDa protein and a 28-kDa novel product (Fig. 4b). The efficient immunoprecipitation of the 28-kDa product with anti-24 antiserum indicates that it may be a fusion protein containing the 24-kDa protein and the upstream sequence (see Fig. 2a). These results demonstrate that mutation of the Q^{3462}S^{3463} to ES blocks cleavage occurring at this position.

Similarly, substitution mutation of the Q^{3672}N^{3673} dipeptide bond was carried out to test if it is indeed the C-terminal cleavage site of the 24-kDa protein (Fig. 4a). As can be seen, expression of plasmid pBP5Q^{3672}-E led to the synthesis of the full-length 42-kDa protein (Fig. 4c).

Coexpression of pBP5Q^{3672}-E with pIBV3C resulted in the detection of a novel product with an apparent molecular weight of approximately 38 kDa; neither the 24- nor the 10-kDa product was generated from the coexpression of these two constructs (Fig. 4c). The 38-kDa protein can be efficiently precipitated with both anti-24 and antiserum V47, suggesting that it may represent a fusion product containing the 24- and 10-kDa proteins (see Figs. 2a and 4a). We have noted that the apparent molecular weight of 38 kDa of the fusion protein is slightly larger than its calculated molecular weight of 35 kDa. This discrepancy may be due to some minor conformational changes of the fusion protein. Nevertheless, these results demonstrate that a substitution mutation of the $Q^{3672}N^{3673}$ dipeptide bond to EN abolishes cleavage occurring at this position and suggest that the $Q^{3672}N^{3673}$ dipeptide bond is the C-terminal cleavage site of the 24-kDa protein. The 24-kDa protein is therefore encoded within the IBV sequence from nucleotide 10915 to 11544.

The relative amount of the 24- and 10-kDa proteins presented in antoradiographs shown in Figs. 1, 2a, and 3 was then analyzed by scanning densitometry (data not shown). Taking account of the relative immunoprecipitation efficiency of the two antisera against their common targets and the methionine content of the two products (the 24-kDa protein contains 4 and the 10-kDa protein contains 3), it was noted that a very similar amount of the 24- and 10-kDa proteins was detected in IBV-infected as well as plasmid DNA-transfected cells. As the two products were processed from the same precursors and no intermediate cleavage product was detected with either antiserum, it is reasonable to assume that, at least at early stages of the IBV infection cycle, these two proteins are produced and accumulated at very similar rates.

In addition to the 38- and 42-kDa intermediate cleavage products, coexpression of pBP5 with pIBV3C also led to the detection of an intermediate cleavage product of 55 kDa (Figs. 2b and 2c). From its apparent molecular weight of 55 kDa, this product may be a fusion protein covering both the 24- and the 10-kDa proteins as well as a putative downstream cleavage product of 17 kDa (see Fig. 2a). As this putative product was flanked by two confirmed cleavage sites of the 3C-like proteins, i.e., the C terminal cleavage site ($Q^{3783}S^{3784}$) of the 10-kDa protein and the N-terminal cleavage site ($Q^{3928}S^{3929}$) of the 100-kDa protein (see Fig. 2a), and no other potential cleavage site was found to be located between these two sites, it is unlikely that further cleavage may occur in this region. To support this, we have reported that coexpression of pIBV3C with pBP5Δ4Q^{3783}-E, which covers the IBV sequence from nucleotide 11568 to 12312 and contains a mutation of the $Q^{3783}S^{3784}$ to ES, resulted in the detection of only the full-length product and trace amount of cleavage product derived from cleavage at the $Q^{3783}S^{3784}$ site (Liu *et al.,* 1997). No other cleavage product was detected (Liu *et al.,* 1997), suggesting that this putative 17-kDa protein may represent a mature cleavage product of the 1a polyprotein. Attempts have been made to raise antiserum against this region, but no success has been achieved so far. We are currently unclear if this is due to the low antigenicity of the product encoded by this particular region.

The identification of a 24-kDa product reported here adds a new member to the growing list of nonstructural products processed from the 741-kDa 1a/1b fusion polyprotein encoded by IBV mRNA1. These include an 87-kDa protein encoded by the first 2.6-kb region of ORF1a and a 10- and a 100-kDa protein encoded by the IBV sequences from nucleotide 11545 to 11877 and 12313 to 15131, respectively (Liu *et al.,* 1994, 1995, 1997; Liu and Brown, 1995). The 100-kDa protein may be the viral polymerase as a putative RNA-dependent RNA polymerase domain was identified in the region coding for this protein (Gorbalenya *et al.,* 1989). So far, no functional roles have been assigned to the 87-, 24-, and 10-kDa protein species. As systems for molecular genetic analysis of the IBV replication machinery are not yet available, it is still difficult to determine the functions of these products in the virus life cycle. However, analysis of their amino acid sequences by computer-aided programs showed that the 24- and 10-kDa proteins may contain hydrophobic/transmembrane domains (data not shown), indicating that they may be hydrophobic proteins. Small hydrophobic viral proteins are usually associated with host cell membranes. This membrane-association property may play an important role in viral RNA replication, as it could act as anchorage of replication complexes. For example, a 6-kDa viral protein from tobacco etch virus was recently shown to be an integral membrane protein associated with large vesicular compartments derived from the endoplasmic reticulum of the host cells (Schaad *et al.,* 1997). This interaction is proposed to be essential for targeting of the viral RNA replication complexes to membranous sites of replication. Small hydrophobic viral proteins, such as the enterovirus 2B protein, have also been shown to be able to increase membrane permeability of the host cells, causing membrane lesions and facilitating release of the virus progeny (van Kuppeveld *et al.,* 1997). It is currently unclear if similar functions may be played by the 24- and 10-kDa proteins during the IBV replication cycle.

Mutagenesis studies presented in this communication suggested that the $Q^{3462}S^{3463}$ and $Q^{3672}N^{3673}$ dipeptide bonds were the N- and C-terminal cleavage sites of the 24-kDa protein, respectively. Examination of the amino acid sequences shows that both cleavage sites have a Leu residue at the P2 position. From the results shown in Figs. 3b and 3c, it was also noted that a QA ($Q^{3575}A^{3576}$) dipeptide bond encoded by the IBV sequence from nucleotide 11251 to 11256 could not be recognized and cleaved by the 3C-like proteinase. The amino acid residue at the P2 position of this dipeptide bond is an Asp. These observations are consistent with our working hypothesis that the consensus amino acid sequence of the

IBV 3C-like proteinase-dependent cleavage sites at P2-P1-P1′ positions may be L(V)-Q-S(GNA). Identification and mutational analysis of more cleavage sites are underway to generalize this conclusion.

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 by Alonso-Caplen *et al.* (1984). Briefly, 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 34th 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 were grown at 37°C in 5% CO_2 and maintained in Glasgow's modified minimal essential medium supplemented with 10% newborn calf serum.

Labeling of IBV-infected cells with [^{35}S]methionine

Confluent monolayers of Vero cells were infected with IBV at a multiplicity of infection of approximately 2 PFU/cell. Prior to being labeled, the cells were incubated in methionine-free medium for 30 min. After 4 h of labeling with [^{35}S]methionine, the cells were scraped off the dishes in phosphate-buffered saline, recovered by centrifugation, and stored at −80°C.

Transient expression of IBV sequences in Vero cells by using a vaccinia virus-T7 expression system

ORFs placed under the control of a T7 promoter were expressed transiently in eukaryotic cells as described in Fuerst *et al.,* (1986). Semiconfluent monolayers of Vero cells were infected with 10 PFU per cell of a recombinant vaccinia virus (vTF7-3) which expresses the T7 phage RNA polymerase and then transfected with appropriate plasmid DNA by using DOSPER liposomal transfection reagent according to the instructions of the manufacturer (Boehringer Mannheim). After incubation of the cells at 37°C overnight (12 h), the cells were incubated in methionine-free medium for 30 min prior to being labeled. After 4 h of labeling with [^{35}S]methionine, the cells were lysed with radioimmunoprecipitation assay buffer (RIPA) [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS] and removed from the dishes. The lysates were then precleared by centrifugation at 15,000 rpm for 5 min at 4°C and stored at −80°C.

Radioimmunoprecipitation

Radioimmunoprecipitation with polyclonal rabbit antisera was carried out as described previously (Liu *et al.,* 1991).

SDS–polyacrylamide gel electrophoresis

SDS–PAGE of viral polypeptides was carried out with 15 and 16% polyacrylamide concentrations (Laemmli, 1970). Labeled polypeptides were detected by autoradiography or fluorography of dried gels.

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 $MgCl_2$.

Site-directed mutagenesis

Site-directed mutagenesis was carried out by two rounds of PCR with two pairs of primers as described (Liu *et al.,* 1997). The mutations introduced were verified by automated nucleotide sequencing.

Construction of plasmids

Plasmids pIBV3C and pBP5, which cover the IBV sequences from nucleotide 8869 [this position was mislabeled in Liu *et al.,* (1997)] to 9786 and from 10752 to 12600, respectively, were described before (Liu *et al.,* 1997).

Plasmids pBP5Q^{3462}-E and pBP5Q^{3672}-E, covering the IBV sequence from nucleotide 10752 to 11877 and containing, respectively, the Gln^{3462} to Glu and Gln^{3672} to Glu mutations, were created by two rounds of PCR with pBP5 as templates (Liu *et al.,* 1997). The sequence of the primer used to introduce the Gln^{3462} to Glu mutation is 5′-CAACTGTATTAGAATCGGTTAC-3′, and the sequence of the primer used to introduce the Gln^{3672} to Glu mutation is 5′-GATGTTGTTTTGGAGAATAAT-3′. The sequence of the upstream primer used for the second round of PCR is 5′-TAATACGACTCACTATAGGG-3′, which is corresponding to the T7 promoter sequence; the sequence of the downstream primer is 5′-GCTCCAGGATCCTACTGTTAAGACAAC-3′, which contains a region complementary to the IBV sequence from nucleotide 11866 to 11877 and a UAG termination codon immediately downstream of the viral sequence. The two PCR fragments were then digested with *Bg1*II (*Bg1*II cuts the PCR fragment between the viral and the T7 promoter sequences) and cloned into *Bg1*II and *Eco*RV-digested pKTO (Liu *et al.,* 1994), giving rise to pBP5Q^{3462}-E and pBP5Q^{3672}-E, respectively. The mutations introduced were confirmed by automated nucleotide sequencing.

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