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Characterisation and Mutational Analysis of an ORF 1a-Encoding Proteinase Domain Responsible for Proteolytic Processing of the Infectious Bronchitis Virus 1a/1b Polyprotein

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Coronavirus gene expression involves proteolytic processing of the mRNA 1-encoded polyproteins by viral and cellular proteinases. Recently, we have demonstrated that an ORF 1b-encoded 100-kDa protein is proteolytically cleaved from the 1a/1b fusion polyprotein by a viral-specific proteinase of the picornavirus 3C proteinase group (3C-like proteinase). In this report, the 3C-like proteinase has been further analysed by internal deletion of a 2.3-kb fragment between the 3C-like proteinase-encoding region and ORF 1b and by substitution mutations of its catalytic centre as well as the two predicted cleavage sites flanking the 100-kDa protein. The results show that internal deletion of ORF 1a sequences from nucleotide 9911 to 12227 does not influence the catalytic activity of the proteinase in processing of the 1a/1b polyprotein to the 100-kDa protein species. Site-directed mutagenesis studies have confirmed that the predicted nucleophilic cysteine residue (Cys^{2922}) and a histidine residue encoded by ORF 1a from nucleotide 8985 to 8987 (His^{2820}) are essential for the catalytic activity of the proteinase, and that the QS(G) dipeptide bonds are its target cleavage sites. Substitution mutations of the third component of the putative catalytic triad, the glutamic acid 2843 (Glu^{2843}) residue, however, do not affect the processing to the 100-kDa protein. In addition, cotransfection experiment shows that the 3C-like proteinase is capable of *trans*-cleavage of the 1a/1b polyprotein. These studies have confirmed the involvement of the 3C-like proteinase domain in processing of the 1a/1b polyprotein, the predicted catalytic centre of the proteinase, and its cleavage sites.

INTRODUCTION

Avian infectious bronchitis virus (IBV) is the prototype species of the Coronaviridae, a family of enveloped viruses with large positive-stranded RNA genomes. Its genomic RNA is 27.6 kb in length and contains 10 distinct open reading frames (ORFs) (Boursnell *et al.*, 1987). Six mRNA species, including a genome-length mRNA (mRNA 1) and five subgenomic mRNA species (mRNAs 2–6), are produced in virus-infected cells and are involved in expression of viral structural and nonstructural proteins. The four virion structural proteins spike, membrane, nucleocapsid, and small membrane are encoded by mRNA 2, mRNA 4, mRNA 6, and the third ORF of mRNA 3, respectively (Stern and Sefton, 1984; Liu and Inglis, 1991). Recently, four small nonstructural proteins encoded by mRNA 3 and mRNA 5 have been identified in virus-infected cells (Smith *et al.*, 1990; Liu *et al.*, 1991; Liu and Inglis, 1992a).

Nucleotide sequencing of the genomic RNA of IBV has shown that the 5′-terminal unique region of mRNA 1 contains two large ORFs (1a and 1b), with ORF 1a having the potential to encode a polypeptide of 441 kDa and 1b a polypeptide of 300 kDa (Boursnell *et al.*, 1987) (Fig. 1a). The downstream ORF 1b is likely to be produced as a fusion protein of 741 kDa with 1a by a ribosomal frameshift (Brierley *et al.*, 1987, 1989). The 1a/1b fusion polyprotein is expected to be cleaved by viral or cellular proteinases to produce functional products associated with viral RNA replication (Fig. 1a). Significant progress has recently been achieved in identification and characterisation of the products encoded by IBV mRNA 1. These include the identification of an 87-kDa protein encoded by the 5′-most section of ORF 1a and a 100-kDa polypeptide encoded by ORF 1b in both IBV-infected and plasmid DNA-transfected cells using region-specific antisera (Liu *et al.*, 1994, 1995). Both viral papain-like proteinase encoded by IBV sequence from nucleotide 4680 to 5550 and cellular proteinases have been demonstrated to be involved in proteolytic processing of the 1a polyprotein to the 87-kDa protein, and a picornavirus 3C-like proteinase (3C-like proteinase) located in IBV ORF 1a between nucleotides 8937 and 9357 has been shown to be capable of proteolytic processing of the 1a/1b fusion polyprotein to the 100-kDa protein species (Liu *et al.*, 1994, 1995).

We report here experiments designed to characterise the 3C-like proteinase by internal deletion and substitution mutations of its predicted catalytic centre and the cleavage sites. Data presented show that deletion of ORF 1a sequences from nucleotide 9911 to 12227 has no effect on proteolytic cleavage of the 1a/1b polyprotein to the 100-kDa protein species, though the production of the 100-kDa protein is significantly increased. Site-directed mutagenesis studies have confirmed that the predicted nucleophilic cysteine (Cys^{2922}) residue and the histidine

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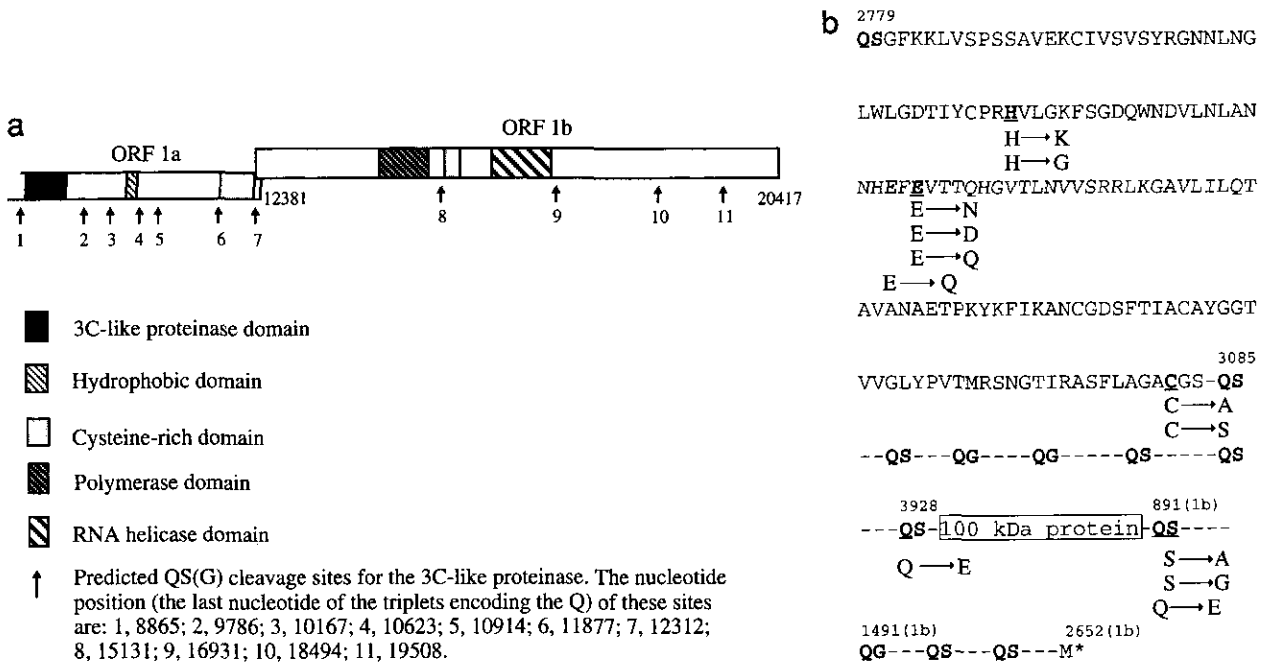

FIG. 1. (a)Diagram of the structure of ORFs 1a and 1b, showing the locations of the putative functional domains and the predicted cleavage sites of the 3C-like proteinase. (b) Deduced amino acid sequence of the predicted 3C-like proteinase domain encoded by ORF 1a between nucleotide 8937 and 9357. The putative catalytic triad, His^{2820}, Glu^{2843}, and Cys^{2922}, is shown with bold print and underlined, and the Glu^{2841} is outlined. Also shown are all the substitution mutations introduced at the putative catalytic centre of the proteinase and two of its cleavage sites. The first amino acid position of ORF 1b-encoded polyprotein is counted from the methionine residue encoded by nucleotides 12458 to 12460.

2820 (His^{2820}) residue are essential for the catalytic activity of the proteinase and that the flanking QS dipeptide bonds are its target cleavage sites. Substitution mutations of the third component of the putative catalytic triad, the glutamic acid 2843 (Glu^{2843}) residue, however, do not influence the catalytic activity of the proteinase required for releasing the 100-kDa protein. This report therefore represents the first effort to characterise in detail the 3C-like proteinase encoded by a coronavirus.

MATERIALS AND METHODS

Site-directed mutagenesis

Site-directed mutagenesis was carried out, as previously described (Liu and Inglis, 1992b), using single-stranded DNA templates prepared from plasmid pIBV14Δ1 and appropriate oligonucleotide primers (see Table 1 for the list of the oligonucleotide primers used).

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 as described previously (Liu *et al.*, 1993). Briefly, semi-confluent monolayers of Vero cells were infected with 10 PFU/cell of a recombinant vaccinia virus (vTF7-3), which expresses the bacteriophage T7 RNA polymerase, and then transfected with appropriate plasmid DNA using lipofection (Gibco–BRL) according to the manufacturer's instructions. After incubation of the cells at 37° for 4 hr, 25 μCi/ml [^{35}S]methionine was added directly to the medium. The radiolabelled cells were harvested at 18 hr postinfection.

Radioimmunoprecipitation

Plasmid DNA-transfected Vero cells were lysed with RIPA buffer (50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 1% sodium deoxycholate, 0.1% SDS) and precleared by centrifugation at 12,000 rpm for 5 min at 4° in a microfuge. Immunoprecipitation was carried out as described previously (Liu *et al.*, 1994).

SDS–polyacrylamide gel electrophoresis

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

Construction of plasmids

Plasmid pIBV11, which covers IBV sequences from nucleotide 8693 to 16980 (Liu *et al.*, 1994), was used to construct plasmids pIBV14 and pIBV14Δ1. Plasmid pIBV14 was made by deletion of IBV sequences from nucleotide 15537 to 16788 by digestion of pIBV11 with

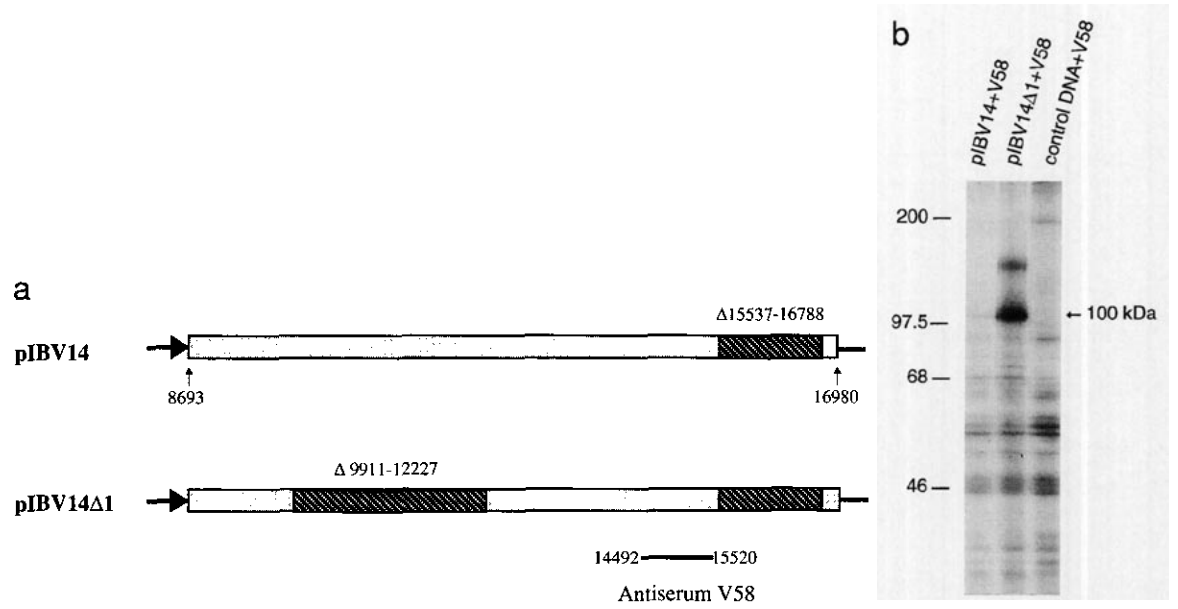

FIG. 2. (a)Diagram showing IBV sequence present in plasmids pIBV14 and pIBV14Δ1 and the sequence used to raise antiserum V58. (b) Analysis of transiently expressed ORF 1a and 1b products from plasmids pIBV14 and pIBV14Δ1, using the vaccinia/T7 recombinant virus expression system. Cells were labelled with [^{35}S]methionine, lysates were prepared, and polypeptides were immunoprecipitated with antiserum V58. Polypeptides were separated on a 10% SDS–polyacrylamide gel and detected by fluorography. Control DNA, plasmid DNA without IBV sequence.

restriction endonuclease *Pst*I and religation with T4 DNA ligase. Plasmid pIBV14 was then digested by restriction enzymes *Pvu*II and *Sna*BI, which cut IBV sequences at nucleotide positions 9911 and 13046. It was then religated with a *Sna*BI fragment (containing IBV sequences from nucleotide 12227 to 13046) of pIBV14, giving plasmid pIBV14Δ1. The RNA sequences present in plasmids pIBV14 and pIBV14Δ1 are shown in Fig. 2a.

Several mutants with alterations at the putative catalytic centre of the 3C-like proteinase domain and two of its cleavage sites were made by site-directed mutagenesis using single-stranded DNA templates prepared from plasmid pIBV14Δ1. All the mutants were detected by nucleotide sequencing, and the mutations were confirmed by transformation into *Escherichia coli* strain TG1 and resequencing. Figure 1b shows the substitutions introduced.

RESULTS

Effect of internal deletion of IBV sequences from nucleotide 9911 to 12227 on processing to the 100-kDa polypeptide

We have recently reported the identification of a 100-kDa polypeptide in IBV-infected Vero cells using a region-specific antiserum V58, which recognises IBV sequences encoded between nucleotides 14492 and 15520. Expression and deletion analyses indicated that this protein is encoded by IBV sequence information from nucleotides 8693 to 15537 and is cleaved from the 1a/1b polyprotein by the putative 3C-like proteinase domain located in ORF 1a between nucleotides 8937 and 9357 (Liu *et al.*, 1994). To support further the requirement for the 3C-like domain in processing of the polyprotein to the 100-kDa protein, plasmid pIBV14Δ1 was made by internal deletion of IBV sequences between nucleotides 9911 and 12227 from pIBV14 (Fig. 2a) and was expressed transiently in Vero cells using the vaccinia/T7 system (Fuerst *et al.*, 1986). As shown in Fig. 2b, expression of pIBV14Δ1 led to efficient production of the 100-kDa protein, reinforcing our previous observation that the 3C-like proteinase domain is responsible for proteolytic processing of 1a/1b polyprotein to the 100-kDa protein and suggesting that the deleted region is not essential for processing of the polyprotein region encoded by this plasmid. Furthermore, this deletion results in much more efficient production of the 100-kDa protein species (Fig. 2b), and reduction in size of the inserted IBV fragment from 6827 to 4511 bp greatly facilitates mutagenesis studies of the catalytic centre of the proteinase and its cleavage sites.

Mutation of the putative catalytic triad of the 3C-like proteinase domain

Computer-aided analysis has predicted that the 3C-like proteinase domain is located in the 1a polyprotein between amino acids 2779 and 3085, corresponding to ORF 1a sequences from nucleotide 8937 to 9357 (Gorbalenya *et al.*, 1989, see also Fig. 1). Three important residues, His^{2820}, Glu^{2843}, and Cys^{2922}, are predicted to form a catalytic triad associated with the catalytic activity of the proteinase (Fig. 1). To test this prediction, site-directed mutagenesis was carried out using appropriate oligonucleotide primers (Table 1) and single-stranded DNA templates were prepared from pIBV14Δ1.

TABLE 1

Oligonucleotide Primers Used in Site-Directed Mutagensis Analysis of the Catalytic Centre of the 3C-like Proteinase Domain and Its Cleavage Sites

Mutants	Nucleotide sequences
WT	5′-^{9281}TGCGGGAGCC**TGTGGTTCAGTTG**^{9304}-3′
C^{2922}-A	TGCGGGAGCC**GCT**GGTTCAGTTG
C^{2922}-S	GGGAGCC**TCT**GGTTCAGTTG
WT	^{9045}CATGAGTTT**GAA**GTTACAACTC^{9067}
E^{2843}-N	CATGAGTTT**AAT**GTTACAACTC
E^{2843}-Q	CATGAGTTT**CAA**GTTACAACTC
E^{2843}-D	CATGAGTTT**GAT**GTTACAACTC
WT	^{9036}GCTAATAATCAT**GAGTTTGAAGT**^{9059}
E^{2841}-Q	GCTAATAATCAT**CAG**TTTGAAGT
WT	^{8975}CGTCCTCGT**CAT**GTATTGGGT^{8997}
H^{2820}-K	CTGTCCTCGT**AAG**GTATTGGGT
H^{2820}-G	CTGTCCTCGT**GG**TGTATTGGGT
WT	^{15122}GACTTTACAAT**CT**TGTGGCG^{15142}
$S^{892(1b)}$-G	GACTTTACAA**GG**TTGTGGCG
$S^{892(1b)}$-A	GACTTTACAA**GC**TTGTGGCG
WT	^{15118}CTACGACTTTA**CAA**TCTTGTGG^{15140}
$Q^{89(1b)}$-E	CTACGACTTTA**GAA**TCTTGTGG
WT	^{12300}ATCTTCTGTT**CAA**TGAGTTGC^{12321}
Q^{3928}-E	ATCTTCTGTT**GAA**TGAGTTGC

We first analysed the catalytic role of the presumed nucleophilic residue Cys^{2922} in proteolytic cleavage of the polyprotein by substitution mutations of the residue, expression of the mutants in Vero cells, and monitoring the production of the 100-kDa protein in transfected cell lysates. Two mutants were made by substitution of this residue either with an alanine (plBV14Δ1$^{C2922\text{-}A}$) or with a serine (plBV14Δ1$^{C2922\text{-}S}$), and they were expressed in Vero cells using the vaccinia/T7 system. As can be seen from Fig. 3, a polypeptide of approximately 180-kDa was immunoprecipitated from plBV14Δ1$^{C2922\text{-}A}$-transfected cell lysate with antiserum V58. This protein comigrated on SDS–PAGE with the plBV14Δ1$^{C2922\text{-}A}$-derived polypeptide synthesised in reticulocyte lysates (data not shown), suggesting that it represents the full-length product encoded by this construct. No 100-kDa polypeptide was detected from the same lysate with antiserum V58. In addition, a polypeptide of approximately 48 kDa was also immunoprecipitated from plBV14Δ1$^{C2922\text{-}A}$-transfected cell lysate with antiserum V58 (Fig. 3). As antiserum V58 has been shown to cross-react with the products encoded by the 3′-terminus of ORF 1a (Liu *et al.*, 1994), this protein species may therefore represent the full-length 1a stop product encoded by this construct. These results demonstrate that substitution of the Cys^{2922} residue with an alanine abolishes the catalytic activity of the proteinase required for release of the 100-kDa protein. *Expression of* plBV14Δ1$^{C2922\text{-}S}$ in Vero cells, however, led to partial processing of the polyprotein encoded by this construct. The 100-kDa protein was immunoprecipitated from plBV14Δ1$^{C2922\text{-}S}$-transfected Vero cell lysate with antiserum V58 (Fig. 3). Furthermore, a polypeptide with an apparent molecular weight of approximately 155 kDa was also precipitated with antiserum V58 (Fig. 3), suggesting that certain catalytic functions of the proteinase are maintained after mutation of the Cys^{2922} residue to a serine.

We next analysed the role of the Glu^{2843} residue in formation of the catalytic centre of the proteinase. Three mutants were made by mutation of Glu^{2843} to Asp (plBV14Δ1$^{E2843\text{-}D}$), Asn (plBV14Δ1$^{E2843\text{-}N}$), and Gln (plBV14Δ1$^{E2843\text{-}Q}$). As expected, substitution of Glu^{2843} with Asp did not affect the proteinase activity required for release of the 100-kDa protein. As shown in Fig. 4, the 100-kDa protein was detected following transfection of plBV14Δ1$^{E2843\text{-}D}$ into Vero cells; neither full-length product nor intermediately processed species were immunoprecipitated. It is surprising, however, that expression of both plBV14Δ1$^{E2843\text{-}N}$ and plBV14Δ1$^{E2843\text{-}Q}$ in Vero cells also resulted in detection of only the 100-kDa protein species (Fig. 4). These results suggest that the Glu^{2843} residue may not be essential for the catalytic activity of the proteinase required for release of the 100-kDa protein.

It is therefore possible that other glutamic acid residues may be required for the catalytic activity of the proteinase. Examination of the amino acid composition of the 3C-like proteinase reveals that a glutamic acid

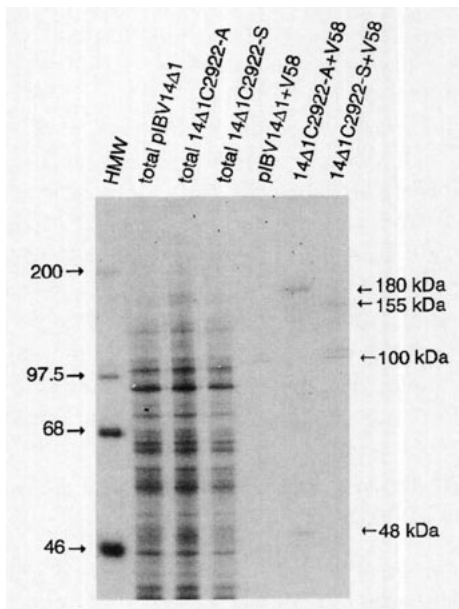

FIG. 3. Mutational analysis of the putative nucleophilic residue (Cys^{2922}) of the 3C-like proteinase. The mutants were transiently expressed in Vero cells, using the vaccinia/T7 recombinant virus expression system, as described in the legend for Fig. 2. Polypeptides were separated on a 10% SDS–polyacrylamide gel and detected by fluorography.

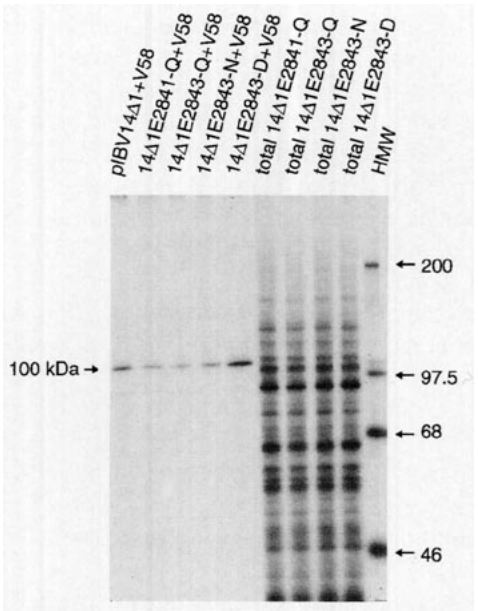

FIG. 4. Mutational analysis of Glu^{2843} and Glu^{2841} residues at the catalytic centre of the 3C-like proteinase. The mutants were transiently expressed in Vero cells, using the vaccinia/T7 recombinant virus expression system. Polypeptides were separated on a 10% SDS–polyacrylamide gel and detected by fluorography.

(Glu^{2841}) residue is located two amino acids upstream of the Glu^{2843} (see Fig. 1b) and might be required for formation of the catalytic centre of the proteinase. To test this possibility, Glu^{2841} was changed to Gln (pIBV14Δ1$^{E2841\text{-}Q}$) by site-directed mutagenesis. As shown in Fig. 4, expression of pIBV14Δ1$^{E2841\text{-}Q}$ in Vero cells again showed complete processing of the polyprotein to the 100-kDa protein. Only the 100-kDa species was detected from the transfected cell lysates by immunoprecipitation with antiserum V58 (Fig. 4).

Finally, the third component of the predicted catalytic triad, the histidine (His^{2820}) residue at amino acid position 2820 of the 1a polyprotein, was mutated to lysine (pIBV14Δ1$^{H2820\text{-}K}$) and glycine (pIBV14Δ1$^{H2820\text{-}G}$) (see Fig. 1b). As shown in Fig. 5, expression of pIBV14Δ1^{H2820K} and pIBV14Δ1$^{H2820\text{-}G}$ in Vero cells resulted in formation of the full-length 180-kDa protein species and the 48-kDa 1a stop product. Both products were immunoprecipitated from the transfected cell lysate with antiserum V58, but no 100-kDa protein species was detected (Fig. 5).

Mutation of the two putative QS cleavage sites flanked the 100-kDa protein

C-terminal deletion data presented previously indicated that a predicted QS cleavage site encoded by nucleotides 15129 to 15135 may be responsible for releasing the C-terminus of the 100-kDa protein (Liu *et al.*, 1994). To test this possibility, substitution mutations of both the glutamine 891(1b) ($Gln^{891(1b)}$) and serine 892(1b) ($Ser^{892(1b)}$) residues were produced by site-directed mutagenesis. Three mutants were made, two by substitution of the $Ser^{892(1b)}$ with either Ala (pIBV14Δ1$^{S892(1b)\text{-}A}$) or Gly (pIBV14Δ1$^{S892(1b)\text{-}G}$), and one by changing the $Gln^{891(1b)}$ to Glu (pIBV14Δ1$^{Q891(1b)\text{-}E}$) (see Fig. 1b). As shown in Fig. 6a, expression of pIBV14Δ1$^{S892(1b)\text{-}A}$ and pIBV14Δ1$^{S892(1b)\text{-}G}$ resulted in efficient detection of the 100-kDa protein species, suggesting that both mutations did not affect the cleavage of the proteinase at this target site. Mutation of the $Gln^{891(1b)}$ residue to Glu, however, completely blocked the processing to the 100-kDa protein. A polypeptide of approximately 125 kDa was immunoprecipitated from pIBV14Δ1$^{Q891(1b)\text{-}E}$-transfected Vero cells (Fig. 6a). The apparent molecular weight of 125 kDa of this protein on SDS–PAGE is consistent with the calculated molecular weight of a processed product covering the 100-kDa protein-encoding region, but with the C-terminal cleavage occurring at the next QG ($Q^{1491(1b)}G^{1492(1b)}$) dipeptide bond (see Fig. 1), 181 amino acid residues downstream of the $Q^{891(1b)}S^{892(1b)}$ site in this construct. These results confirm that the $Q^{891(1b)}S^{892(1b)}$ dipeptide bond is cleaved by the 3C-like proteinase to release the C-terminus of the 100-kDa protein.

We next analysed the N-terminal cleavage site of the 100-kDa protein. A QS dipeptide bond ($Q^{3928}S^{3929}$), encoded by ORF 1a from nucleotide 12310 to 12315, is predicted to be the cleavage site responsible for release of the N-terminus of the 100-kDa protein (see Fig. 1). One substitution mutation was made by changing the Gln^{3928} to a Gln (pIBV14Δ1$^{Q3928\text{-}E}$). As shown in Fig. 6b, substitu-

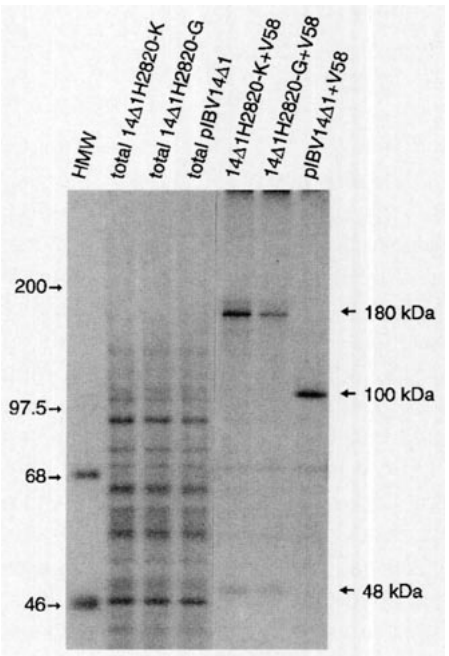

FIG. 5. Mutational analysis of the His^{2820} residue at the catalytic centre of the 3C-like proteinase. The mutants were transiently expressed in Vero cells using the vaccinia/T7 recombinant virus expression system. Polypeptides were separated on a 10% SDS–polyacrylamide gel and detected by fluorography.

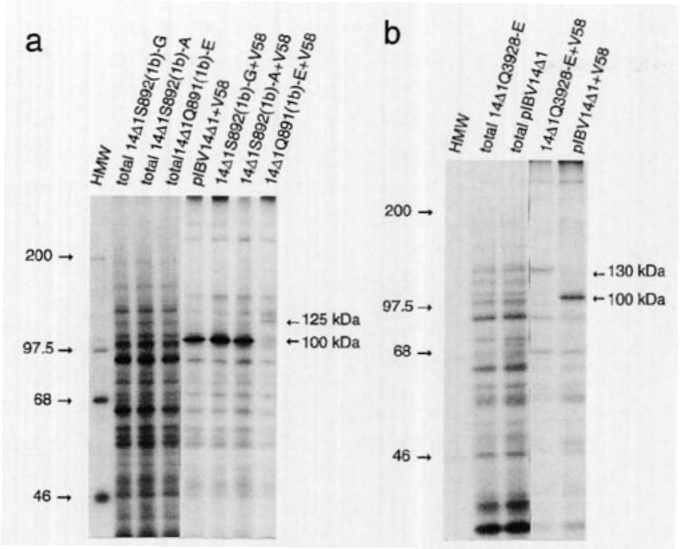

FIG. 6. (a)Mutational analysis of the predicted QS cleavage site responsible for release of the C-terminus of the 100-kDa protein. The mutants were transiently expressed in Vero cells using the vaccinia/T7 recombinant virus expression system. Polypeptides were separated on a 10% SDS–polyacrylamide gel and detected by fluorography. (b) Mutational analysis of the predicted QS cleavage site responsible for release of the N-terminus of the 100-kDa protein.

tion of the Gln^{3928} residue with a Glu (pIBV14$\Delta 1^{Q3928\text{-}E}$) abolished the production of the 100-kDa protein. Instead of the 100-kDa protein species, a major polypeptide of approximately 130 kDa was immunoprecipitated from pIBV14$\Delta 1^{Q3928\text{-}E}$-transfected Vero cell lysate with antiserum V58 (Fig. 6b). This result suggests that mutation of the Gln^{3928} residue to a Glu blocked the proteolytic cleavage of the polyprotein from occurring at this site and led to formation of a novel protein species with extra N-terminal sequences. However, the migration of this protein on SDS–PAGE is somehow slower than what is expected, since mutation of this cleavage site should result in addition of only 70 amino acid residues to the N-terminus of the 100-kDa protein, and hence produce a protein of approximately 110 kDa. The reason for this discrepancy is currently unclear, but presumably it reflects the complexity of the factors governing polypeptide migration on SDS-PAGE.

The *trans*-cleavage of the 1a/1b polyprotein by the 3C-like proteinase

As shown in Fig. 2b, internal deletion of IBV sequences from nucleotide 9911 to 12227 did not block the processing of the 1a/1b polyprotein to the 100-kDa protein from, confirming the *cis*-activity of the 3C-like proteinase. It was then of interest to see if *trans*-cleavage of the 1a/1b polyprotein could also be mediated by this proteinase. For this purpose, we coexpressed in Vero cells plasmid pKT205, which covers IBV sequence from nucleotide 8693 to 10925 (Liu *et al.*, 1994) and therefore provides *trans*-activity of the 3C-like proteinase, and one of the mutants, pIBV14$\Delta 1^{C2922\text{-}A}$, which lacks the proteinase activity. As expected, expression of pKT205 alone did not produce any products detectable by immunoprecipitation with antiserum V58 (Fig. 7). Expression of pIBV14$\Delta 1^{C2922\text{-}A}$, once again, resulted in synthesis of the full-length 180-kDa protein and the 48-kDa 1a stop product (Fig. 7, also see Fig. 3). No 100-kDa protein was detected (Fig. 7). Cotransfection of pKT205 and pIBV14$\Delta 1^{C2922\text{-}A}$, however, led to processing of the 180-kDa full-length product to the 100-kDa protein (Fig. 7). In addition, the 48-kDa 1a stop product was also seen to be processed to undetectable products (Fig. 7). These results suggest that *trans*-cleavage of the polyprotein did occur in this cotransfection experiment and further indicate that all the cleavage sites contained in this construct may be sensitive to the *trans*-activity of the proteinase.

DISCUSSION

We have recently reported the identification of a 100-kDa protein in IBV-infected Vero cells using a region-specific antiserum V58 (Liu *et al.*, 1994). Our previous data suggested that this novel protein is encoded by the 5′-portion of ORF 1b up to nucleotide 15520 and may be cleaved from the 1a/1b fusion polyprotein by the putative picornavirus 3C-like proteinase domain located in ORF 1a region from nucleotide 8937 to 9357. Several lines of evidence presented here confirm that the 3C-like proteinase domain is responsible for proteolytic cleavage of the 1a/1b polyprotein at the predicted QS dipeptide bonds to release the 100-kDa protein. First, internal deletion of ORF 1a sequences from nucleotide 9911 to 12227 does

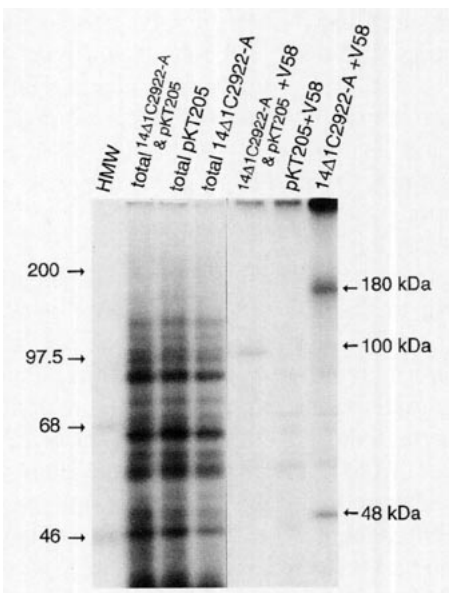

FIG. 7. The *trans*-cleavage of the 1a/1b polyprotein mediated by the 3C-like proteinase. Plasmids pKT205 and pIBV14$\Delta 1^{C2922\text{-}A}$ were transiently coexpressed in Vero cells, using the vaccinia/T7 recombinant virus expression system. Polypeptides were separated on a 10% SDS–polyacrylamide gel and detected by fluorography.

not influence the 3C-like proteinase-mediated proteolytic processing of the polyprotein to the 100-kDa protein. Taken together with the N-terminal deletion data previously reported (Liu *et al.*, 1994), this result suggests strongly that the 3C-like proteinase is involved in processing of the 1a/1b polyprotein. Second, substitution mutations of the presumed nucleophilic cysteine residue (Cys^{2922}) to alanine and a histidine (His^{2820}) residue to either a lysine or a glycine completely abolished the proteolytic activity of the 3C-like proteinase; while partial loss of the catalytic activity was observed when the Cys^{2922} residue was substituted by a serine. Finally, alteration of the Gln^{3928} and $Gln^{891(1b)}$ residues of the predicted QS cleavage sites encoded by nucleotides 12310 to 12315 and 15129 to 15135, respectively (Gorbalenya *et al.*, 1989), blocked the proteolytic processing of the polyprotein to the 100-kDa protein species.

The observation that substitution of the Cys^{2922} residue with alanine completely abolished the catalytic activity of the 3C-like proteinase suggests that this residue may play an essential role in formation of the catalytic centre of the proteinase. Recently, increasing numbers of Cys-active-centre viral proteinases have been identified in animal and plant viruses, including picornaviruses, comoviruses, and potyviruses (Gorbaleya *et al.*, 1986; Bazan and Fletterick, 1988). This type of viral proteinase was originally classified as a cysteine proteinase, but they are now considered to belong to the trypsin superfamily of serine proteinases. Two recent reports on the X-ray crystal structures of the 3C proteinases from two viruses of the picornavirus family reveal similarities in folding of the proteinase polypeptides, in RNA-binding sites, and in cleavage mechanisms between the viral proteinases and cellular serine proteinases of the trypsin or chymotrypsin class (Matthews *et al.*, 1994; Allaire *et al.*, 1994). In fact, poliovirus 3C proteinase has been reported to be able to use serine as its nucleophilic residue (Lawson and Semler, 1991; Kean *et al.*, 1993). Similarly, catalytic activity of the IBV 3C-like proteinase was partially maintained after substitution of the Cys^{2922} residue with a serine (Fig. 3).

Mutation of the Cys^{2922} to serine leads to partial processing to the 100-kDa protein species. Both the 100-kDa protein and a 155-kDa intermediate cleavage product were detected by immunoprecipitation with the C-terminal-specific antiserum V58 (Liu *et al.*, 1994). Furthermore, only a trace amount of the full-length polyprotein of 180 kDa was immunoprecipitated with antiserum V58 from the same lysates. These results indicate that an efficient cleavage of the full-length 180-kDa polyprotein had occurred, resulting in removal of the 3C-like proteinase domain and sequences upstream of the 100-kDa protein-encoding region. It is therefore likely that individual cleavage sites have differential sensitivity to the amino acids substituted at the catalytic centre of the IBV proteinase. Similar observations are also documented with the picornavirus 3C-like proteinase (Lawson and Semler, 1991; Kean *et al.*, 1993). Jackson and colleagues have recently reported differential effects of substitution mutations of Glu^{71} and Cys^{147} residues of the putative catalytic triad of the poliovirus 3C proteinase on cleavage at different sites of the poliovirus polyprotein (Kean *et al.*, 1993). Substitutions of Glu^{2841} and Glu^{2843} residues with amino acids of similar or distinct properties do not affect the catalytic activity of the proteinase required for release of the IBV 100-kDa protein species from its precursor (Fig. 4). The reason for this is currently not clear. It may, however, simply reflect the particular tolerance of the QS cleavage sites flanking the 100-kDa protein to the amino acids substituted. Development of an *in vitro* processing system and an attempt to identify more mature cleavage products are underway to address this issue further.

Our previous deletion results indicated that the C-terminus of the 100-kDa protein is specified by ORF 1b sequences close to nucleotide 15120, raising the possibility that proteolysis at a predicted QS cleavage site encoded by nucleotides 15129 to 15135 may lead to releasing the C-terminus of this protein (Gorbalenya *et al.*, 1989). This is supported by the observation that substitution of the $Gln^{891(1b)}$ residue with a Glu completely blocked the cleavage at this position. However, alterations of the $Ser^{892(1b)}$ residue to either Ala or Gly had no effect on the proteolytic processing of the polyprotein to the 100-kDa protein, suggesting that both Gln–Ala and Gln–Gly dipeptide bonds can be recognised and cleaved by the IBV 3C-like proteinase. For picornavirus 3C proteinases, it has been reported that most cleavages occurred at the Gln–Gly peptide bond; less commonly, cleavages were also observed between Gln–Ser, Gln–Ala, Glu–Ser, or Glu–Gly pairs (Palmenberg, 1990). Systematic substitution mutations of both Gln and Ser (Gly) residues are required to provide a complete picture of the cleavage specificities of the IBV 3C-like proteinase.

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