

Expression and characterization of a recombinant murine coronavirus 3C-like proteinase

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The replication of coronaviruses involves proteolytic processing of the gene 1 translation products, pp1a and pp1ab. One of the key enzymes in this process is predicted to be a virus-encoded 3C-like proteinase. In this report, we describe a bacterial system that has allowed us to express and characterize a recombinant murine coronavirus (MHV-JHM) 3C-like proteinase. The partially purified protein has been shown to exhibit proteolytic activity in *trans* and mutation analysis has been used to demonstrate the indispensability of Cys-3495 for enzymatic activity. Finally, the effect of class-specific proteinase inhibitors on the *trans* cleavage activity of the MHV 3C-like proteinase has been used to demonstrate the functional and structural homology of this enzyme to the picornavirus 3C proteinases.

The coronavirus gene 1 encompasses approximately 20–22 kb and is composed of two open reading frames (ORFs), ORF 1a and ORF 1b (Bournsnel *et al.*, 1987; Lee *et al.*, 1991; Herold *et al.*, 1993; Eleouet *et al.*, 1995). ORF 1a encodes a polyprotein, pp1a, with a molecular mass of approximately 450–500 kDa. ORF 1b is expressed together with ORF 1a as a large polyprotein, pp1ab, with a molecular mass of approximately 750–800 kDa. The translation of pp1ab is mediated by a mechanism involving (–1) ribosomal frame-shifting (Brierley *et al.*, 1987, 1989; Bredenbeek *et al.*, 1990; Herold & Siddell, 1993). Sequence comparison of four coronavirus genomes has revealed a number of putative functional domains encoded in gene 1. These include an RNA-dependent RNA polymerase, a helicase domain and several proteinase domains. The putative cleavage sites of one proteinase domain, the 3C-like proteinase, have also been predicted for several coronaviruses (Gorbalenya *et al.*, 1989; Lee *et al.*, 1991; Herold *et al.*, 1993; Eleouet *et al.*, 1995).

Recent data suggest that the coronavirus 3C-like proteinase domain, encoded in ORF 1a, plays a central role in the

processing of gene 1 translation products (Liu *et al.*, 1994; Ziebuhr *et al.*, 1995; Liu & Brown, 1995; Tibbles *et al.*, 1996; Grötzing *et al.*, 1996). For example, in the case of the murine coronavirus MHV-A59, it has been shown that a protein with autoproteolytic activity can be expressed by the *in vitro* translation of a synthetic RNA encoding the predicted 3C-like proteinase domain, together with its flanking regions (Lu *et al.*, 1995). In the infectious bronchitis virus (IBV) system, *in vitro* translation studies have also shown that the efficient release of the 3C-like proteinase from larger precursor molecules requires the presence of membrane preparations (Tibbles *et al.*, 1996). The IBV 3C-like proteinase appears to be rapidly degraded in reticulocyte lysate systems (Tibbles *et al.*, 1995).

Using a different approach, we have demonstrated that in human coronavirus (HCV) 229E-infected cells the major form of the 3C-like proteinase is a 34 kDa polypeptide encompassing amino acids 2966–3267 of the HCV 229E ORF 1a gene product (Ziebuhr *et al.*, 1995). We have also shown that bacterially expressed HCV 229E 3C-like proteinase exhibits enzymatic activity in *cis* and in *trans* (Ziebuhr *et al.*, 1995; Grötzing *et al.*, 1996). In the experiments reported here, we have extended this approach to the study of the MHV 3C-like proteinase.

Two oligonucleotides, 5' TCT GGT ATA GTG AAG ATG GT 3' and 5' TTA CTG TAG CTT AAC ACC AGC TAG 3', were used to amplify the predicted 3C-like proteinase coding sequence of MHV-JHM (Lee *et al.*, 1991). The amplification procedure, which involved RT-PCR from poly(A)⁺ RNA of MHV-JHM-infected Sac(–) cells (Herold *et al.*, 1996), also positioned a termination codon at the end of the 3C-like proteinase coding sequence. The 912 bp PCR product was treated with T4 DNA polymerase, phosphorylated and ligated to *Xmn*I-digested pMal-c2 DNA (New England Biolabs). The proteinase coding region of the resultant plasmid, pMal-MHVpro, was sequenced and found to correspond to the published sequence (Lee *et al.*, 1991; EMBL/GenBank accession no. M55148). pMal-MHVpro was used to transform competent *E. coli* TBI cells. The plasmid encodes a 75 kDa fusion protein consisting of the maltose-binding protein of *E. coli* and the predicted MHV-JHM 3C-like proteinase domain. The fusion protein was expressed, partially purified and the MHV 3C-like proteinase was released from the fusion protein by factor Xa cleavage (Ziebuhr *et al.*, 1995; Herold *et al.*, 1996).

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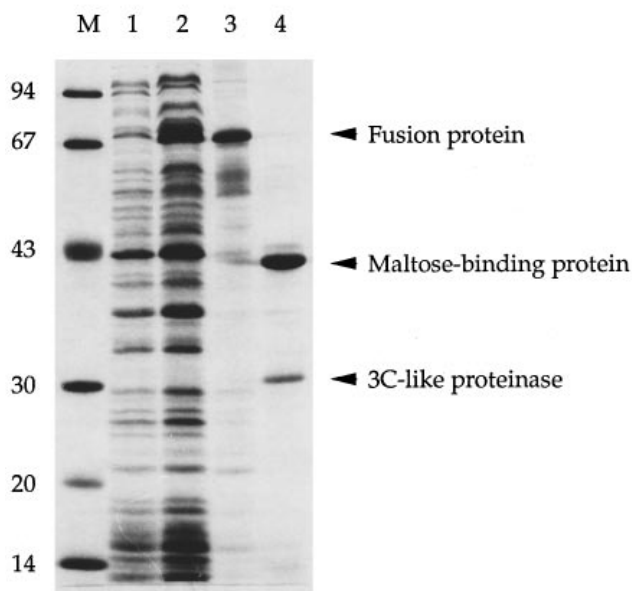
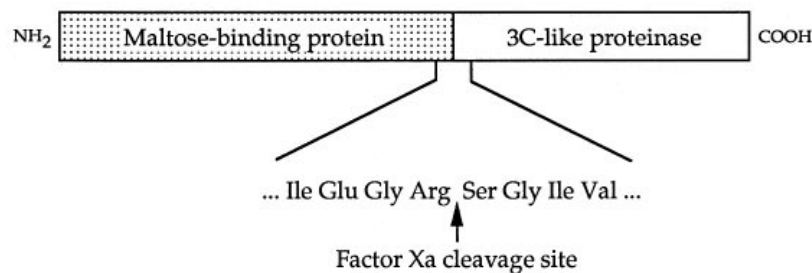


Fig. 1. Expression and partial purification of the MHV-JHM 3C-like proteinase. Cell lysates from non-induced or IPTG-induced *E. coli* TB1 (pMal-MHVpro) bacteria (lanes 1 and 2, respectively) were separated on an SDS-15% polyacrylamide gel and stained with Coomassie brilliant blue. The partially purified MBP-3C-like proteinase fusion protein (lane 3) and the factor Xa-cleaved fusion protein (lane 4) were also analysed and are indicated. Protein molecular mass markers (kDa) are shown in lane M.

As is shown in Fig. 1, bacterial expression and partial purification of the fusion protein, followed by factor Xa cleavage, results in a highly enriched proteinase preparation.

To demonstrate the enzymatic activity of the recombinant MHV 3C-like proteinase *in trans*, we have used a synthetic 15-mer peptide, NH₂-Leu-Cys-Thr-Thr-Ser-Phe-Leu-Gln-Ser-Gly-Ile-Val-Lys-Met-Val-COOH (N3C), representing amino acids 3343–3357 of the MHV-JHM ORF 1a gene product. This peptide contains a previously characterized 3C-like proteinase cleavage site (Gln-3350/Ser-3351; Lu *et al.*, 1995). The identity and purity of the synthetic peptide were confirmed by mass spectrometry and HPLC (JERINI-Bio Tools). The cleavage reactions were performed at 24 °C in a total volume of 20 µl buffer (5 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.25 mM EDTA, 0.25 mM DTT) containing 1 µM recombinant 3C-like proteinase and 1 mM peptide. Following a 120 min incubation,

the reaction products were analysed by reversed-phase HPLC (600E system controller, Waters) in a continuous gradient of 92% eluent A [0.1% aqueous trifluoroacetic acid (TFA)]/8% eluent B (0.1% TFA in 90% acetonitrile/10% water) up to 100% eluent B (25 min, flow rate 1 ml/min, detection at 215 nm) on a Delta Pak C₁₈ column (3.9 × 150 mm, 300 Å, Waters). Fig. 2(c) shows that recombinant MHV 3C-like proteinase cleaved the substrate peptide completely under these conditions. The substrate peptide, which was eluted after 15.5 min, was converted into two products that eluted from the column after approximately 11 and 13 min.

To exclude the possibility that co-purification of a bacterial proteinase was responsible for the observed proteolysis, we introduced mutations into the coding sequence of the MHV 3C-like proteinase. The predicted catalytic residue, Cys-3495, was substituted by Gly, Ala, Val, Arg, Asp and Ser using a

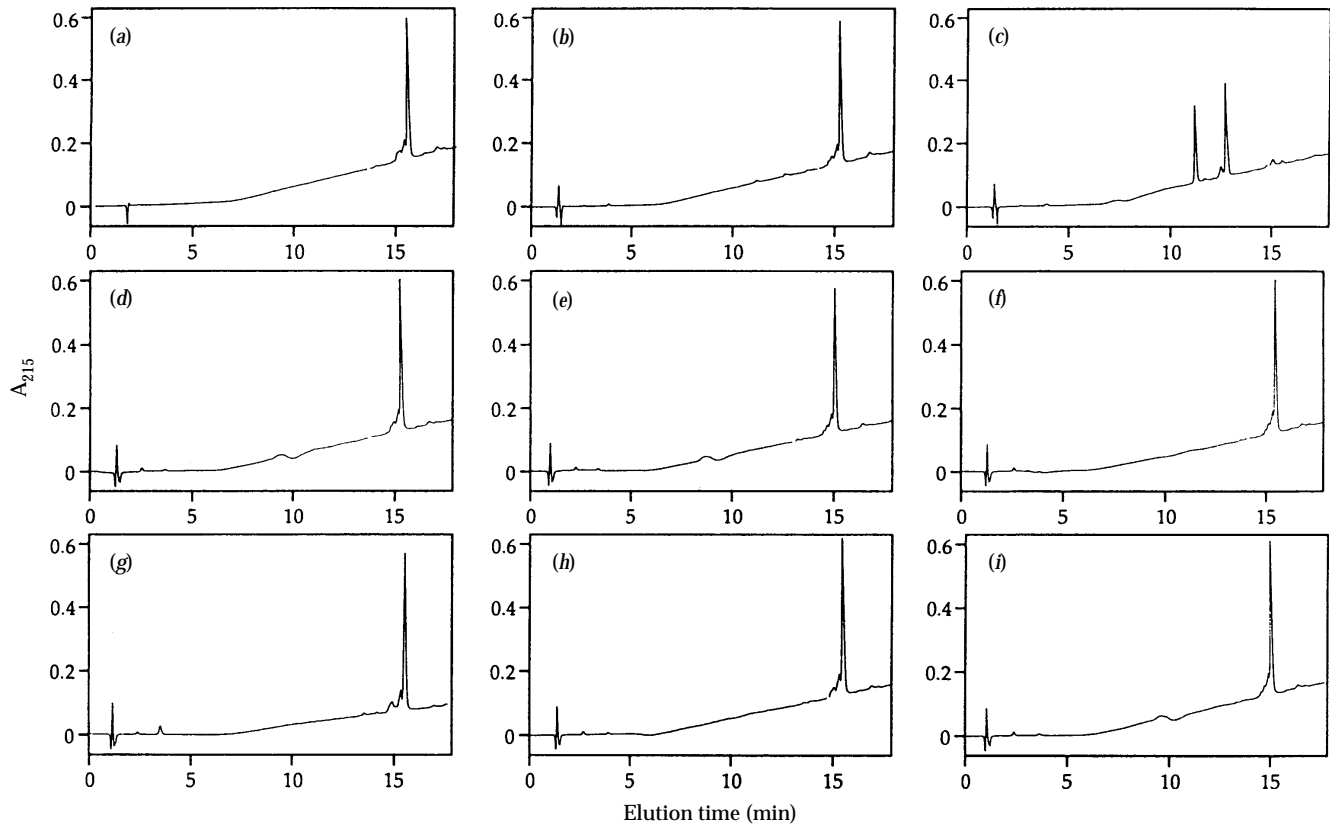


Fig. 2. Mutagenesis of the predicted active site residue, Cys-3495. Reversed-phase HPLC analysis of peptide N3C and its cleavage products after incubation with wild-type or mutant MHV-JHM 3C-like proteinase. Peptide N3C (1 mM) was incubated at 24 °C in a total volume of 20 μ l with (a) water for 2 h, (b) 1 μ M 3C-like proteinase (wild-type) for 20 s, (c) 1 μ M 3C-like proteinase (wild-type) for 2 h, (d) 1 μ M mutant 3C-like proteinase (C3495G) for 2 h, (e) 1 μ M mutant 3C-like proteinase (C3495A) for 2 h, (f) 1 μ M mutant 3C-like proteinase (C3495V) for 2 h, (g) 1 μ M mutant 3C-like proteinase (C3495S) for 2 h, (h) 1 μ M mutant 3C-like proteinase (C3495R) for 2 h or (i) 1 μ M mutant 3C-like proteinase (C3495D) for 2 h.

recombination-PCR method (Yao *et al.*, 1992; Herold *et al.*, 1996). No enzymatic activity was detected with any of the mutated proteins (Fig. 2). These data strongly indicate that the predicted catalytic residue, Cys-3495 (Gorbalenya *et al.*, 1989; Lee *et al.*, 1991; Lu *et al.*, 1995), is indispensable for the proteolytic activity of the MHV 3C-like proteinase. It might, therefore, indeed represent the nucleophilic residue required for catalysis. These data are consistent with the results reported by Lu *et al.* (1995), which show that an exchange of the corresponding Cys of the MHV-A59 3C-like proteinase for an Arg residue abolishes cleavage *in cis* and *in trans*.

Interestingly, we could not observe any proteolytic activity for the Cys \rightarrow Ser (C3495S) mutant of the MHV-JHM 3C-like proteinase (Fig. 2g). Whereas 1 μ M of wild-type proteinase cleaved more than 90% of 1 mM peptide within 10 min, no cleavage products were generated using the C3495S-3C-like proteinase, even after prolonged incubation for up to 4 h (data not shown). This result contrasts with the findings of Tibbles *et al.* (1996), who reported a residual activity of the corresponding IBV 3C-like mutant proteinase in an *in vitro* translation system, albeit only after a long incubation time.

Taken together, these data suggest that the substitution of the putative catalytic Cys-3495 residue by results in a dramatic reduction, if not a complete loss of proteolytic activity. It remains to be demonstrated whether or not any residual activity would be sufficient to provide the biological function necessary for virus replication.

In an additional set of experiments, we investigated the enzymatic properties of the recombinant MHV 3C-like proteinase. The approximate turnover number for the proteinase was estimated from the rate of substrate turnover at an estimated enzyme concentration. Cleavage of 1 mM peptide N3C was 90% complete within 10 min in the presence of approximately 1 μ M proteinase. This corresponds to a turnover number of approximately 1.5 s^{-1} , assuming that the enzyme was saturated under these conditions and the observed rate represents V_{max} . This value should be considered, therefore, a lower limit for the turnover number but it is comparable to the turnover numbers of recombinant picornavirus 3C proteinases (Malcolm *et al.*, 1992). The enzyme is relatively insensitive to varying ionic strength. NaCl concentrations between 50 and 500 mM did not influence the

Table 1. Effect of proteinase inhibitors on the activity of MHV-JHM 3C-like proteinase

Inhibitor class	Inhibitor	Concentration	Inhibition*
Serine proteases	PMSF	2.0 mM	+
	Aprotinin	0.3 mM	—
	Benzamidine	2.5 mM	—
Cysteine proteases	E-64	0.4 mM	—
	ZnCl ₂	2.0 mM	+
Cysteine/serine proteases	Leupeptin	2.5 mM	—
Metallo-proteases	EDTA	1.0 mM	—

* The designation '+' indicates more than 90% inhibition, the designation '—' less than 10% inhibition under the conditions described in the text.

proteolytic activity, and even at 1 M NaCl more than 50% activity was retained. The pH optimum of the MHV 3C-like proteinase activity was found to be between pH 7 and 8. The activity dropped significantly at values above pH 9; below values of pH 5 no activity was detectable (data not shown).

On the basis of computer-assisted analyses, Gorbalenya *et al.* (1989) have predicted a structural relatedness of the coronavirus 3C-like proteinase domain with the picornavirus 3C proteinases. To test this prediction, we investigated the inhibitor susceptibility of the recombinant MHV 3C-like proteinase. Using the *trans* cleavage assay system described above, we tested a range of class-specific inhibitors. Table 1 demonstrates that inhibitors of both serine and cysteine proteinases abolish or markedly reduce the proteolytic activity of the MHV 3C-like proteinase. Thus, the inhibitor profiles of the MHV 3C-like proteinase and the poliovirus 3C proteinase (Baum *et al.*, 1991) are similar. Secondly, the inhibitor profile of the MHV 3C-like proteinase corresponds, in general, to the inhibitor susceptibility of the IBV 3C-like proteinase (Tibbles *et al.*, 1996). One exception, however, is the inhibitor E-64. This drug has been demonstrated to inhibit the IBV 3C-like proteinase, but even at exceedingly high concentrations of E-64 we could not observe inhibition of the MHV proteinase activity. This discrepancy needs to be further investigated by calculating inhibition constants using purified enzyme preparations and (different) peptide substrates.

In conclusion, we have been able to confirm the proteolytic activity in *trans* of bacterially expressed, recombinant MHV 3C-like proteinase. This experimental approach, which has also proven useful for the study of the HCV 229E 3C-like proteinase (Ziebuhr *et al.*, 1995; Gröttinger *et al.*, 1996), appears to provide a general strategy for the investigation of coronavirus 3C-like proteinases. The approach should be useful to elucidate the structurally and catalytically important residues of the

coronavirus 3C-like proteinases, to define the substrate specificity of the enzymatic activity associated with this protein, and to investigate the 3C-like proteinase-mediated processing of coronavirus polyproteins.

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References

- Baum, E. Z., Bebernitz, G. A., Palant, O., Mueller, T. & Plotch, S. J. (1991). Purification, properties, and mutagenesis of poliovirus 3C protease. *Virology* **185**, 140–150.
- Bournsnel, M. E. G., Brown, T. D. K., Foulds, I. J., Green, P. F., Tomley, F. M. & Binns, M. M. (1987). Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. *Journal of General Virology* **68**, 57–77.
- Bredenbeek, P. J., Pachuk, C. J., Noten, A. F. H., Charité, J., Luytjes, W., Weiss, S. R. & Spaan, W. J. M. (1990). The primary structure and expression of the second open reading frame of the polymerase gene of the coronavirus MHV-A59; a highly conserved polymerase is expressed by an efficient ribosomal frameshifting mechanism. *Nucleic Acids Research* **18**, 1825–1832.
- Brierley, I., Bournsnel, M. E. G., Binns, M. M., Billimoria, B., Blok, V. C., Brown, T. D. K. & Inglis, S. C. (1987). An efficient ribosomal frameshifting signal in the polymerase-encoding region of the coronavirus IBV. *EMBO Journal* **6**, 3779–3785.
- Brierley, I., Digard, P. & Inglis, S. C. (1989). Characterization of an efficient coronavirus ribosomal frameshift signal: requirement for an RNA pseudoknot. *Cell* **57**, 537–547.
- Eleouet, J.-F., Rasschaert, D., Lambert, P., Levy, L., Vende, P. & Laude, H. (1995). Complete sequence (20 kilobases) of the polyprotein-encoding gene 1 of transmissible gastroenteritis virus. *Virology* **206**, 817–822.
- Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P. & Blinov, V. M. (1989). Coronavirus genome: prediction of putative functional domains in the nonstructural polyprotein by comparative amino acid sequence analysis. *Nucleic Acids Research* **17**, 4847–4861.
- Gröttinger, C., Heusipp, G., Ziebuhr, J., Harms, U., Süß, J. & Siddell, S. G. (1996). Characterization of a 105-kDa polypeptide encoded in gene 1 of the human coronavirus 229E. *Virology* **222**, 227–235.
- Herold, J. & Siddell, S. G. (1993). An elaborated pseudoknot is required for high frequency frameshifting during translation of HCV 229E polymerase mRNA. *Nucleic Acids Research* **21**, 5838–5842.
- Herold, J., Raabe, T., Schelle-Prinz, B. & Siddell, S. G. (1993). Nucleotide sequence of the human coronavirus 229E RNA polymerase locus. *Virology* **195**, 680–691.
- Herold, J., Siddell, S. G. & Ziebuhr, J. (1996). Characterization of coronavirus RNA polymerase gene products. *Methods in Enzymology* **275** (in press).
- Lee, H.-J., Shieh, C.-K., Gorbalenya, A. E., Koonin, E. V., La Monica, N., Tuler, J., Bagdzhadzhyan, A. & Lai, M. M. C. (1991). The complete sequence (22 kilobases) of murine coronavirus gene 1 encoding the putative proteases and RNA polymerase. *Virology* **180**, 567–582.
- Liu, D. X. & Brown, T. D. K. (1995). Characterization and mutational analysis of an ORF 1a-encoding proteinase domain responsible for proteolytic processing of the infectious bronchitis virus 1a/1b polyprotein. *Virology* **209**, 420–427.
- Liu, D. X., Brierley, I., Tibbles, K. W. & Brown, T. D. K. (1994). A 100-kilodalton polypeptide encoded by open reading frame (ORF) 1b of the

coronavirus infectious bronchitis virus is processed by ORF 1a products. *Journal of Virology* **68**, 5772–5780.

Lu, Y., Lu, X. & Denison, M. R. (1995). Identification and characterization of a serine-like proteinase of the murine coronavirus MHV-A59. *Journal of Virology* **69**, 3554–3559.

Malcolm, B. A., Chin, S. M., Jewell, D. A., Stratton-Thomas, J. R., Thudium, K. B., Ralston, R. & Rosenberg, S. (1992). Expression and characterization of recombinant hepatitis A virus 3C proteinase. *Biochemistry* **31**, 3358–3363.

Tibbles, K. W., Brierley, I., Cavanagh, D. & Brown, T. D. K. (1995). A region of the infectious bronchitis virus 1a polyprotein encoding the 3C-like protease domain is subject to rapid turnover when expressed in rabbit reticulocyte lysate. *Journal of General Virology* **76**, 3059–3070.

Tibbles, K. W., Brierley, I., Cavanagh, D. & Brown, T. D. K. (1996). Characterization in vitro of an autocatalytic processing activity associated with the predicted 3C-like proteinase domain of the coronavirus avian infectious bronchitis virus. *Journal of Virology* **70**, 1923–1930.

Yao, Z., Jones, D. H. & Grose, C. (1992). Site-directed mutagenesis of herpesvirus glycoprotein phosphorylation sites by recombination polymerase chain reaction. *PCR Methods and Applications* **1**, 205–207.

Ziebuhr, J., Herold, J. & Siddell, S. G. (1995). Characterization of a human coronavirus (strain 229E) 3C-like proteinase activity. *Journal of Virology* **69**, 4331–4338.

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