17

EXPRESSION, PURIFICATION, AND ACTIVITY OF RECOMBINANT MHV-A59 3CLpro

A. C. Sims,[1,3] X. T. Lu,[2,3] and M. R. Denison[1,2,3]

[1]Department of Microbiology and Immunology
[2]Department of Pediatrics
[3]Elizabeth B. Lamb Center for Pediatric Research
Vanderbilt University Medical Center
Nashville, Tennessee

1. ABSTRACT

The 3C-like proteinase (3CLpro) of MHV-A59 is predicted to mediate the majority of proteolytic processing events within the gene 1 polyprotein. We have overexpressed 3CLpro in *E. coli* as a fusion protein with maltose binding protein (MBP). The MBP-3CLpro fusion protein was purified from contaminating *E. coli* proteins by amylose column chromatography, and r3CLpro was cleaved from the fusion protein by factor Xa. Recombinant 3CLpro (r3CLpro) was able to cleave a polypeptide substrate containing mutated inactive 3CLpro and portions of the flanking domains. R3CLpro cleaved substrate completely within 5 minutes and the activity of r3CLpro was sensitive to inhibition by serine and cysteine proteinase inhibitors; however, it was not inhibited by EDTA, suggesting that metal ions were not critical for 3CLpro activity.

2. INTRODUCTION

Expression of viral proteins mediating RNA transcription and replication is presumed to occur primarily if not exclusively from gene 1 of MHV. Translation of the 22 kb gene 1 has been demonstrated *in vitro* and *in cyto*, resulting in expression of a number of proteins (Denison and Perlman, 1986; Denison *et al.*, 1992). Gene 1 is composed of two overlapping open reading frames, ORF 1a and ORF 1b. Translation of ORF 1a is calcuated to result in expression of a polyprotein of 495 kDa (pp1a) and translation of ORFs 1a and 1b together via a ribosomal frameshift would result in expression of an 803 kDa polyprotein (pp1ab). The ORF 1a portion of the polyprotein contains two experimentally

Coronaviruses and Arteriviruses, edited by Enjuanes *et al.*
Plenum Press, New York, 1998

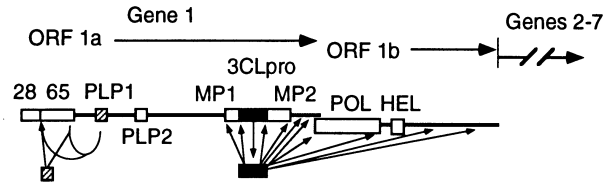

Figure 1. Location of proteinase domains and cleavage sites in the gene 1 polyprotein. This schematic shows the location of known (PLP-1, 3CLpro) and predicted (PLP-2) proteinases in the gene 1 polyprotein. Both PLP-1 and 3CLpro have been shown to be capable of acting *in trans*. Predicted cleavage sites for 3CLpro (Lee *et al.*, 1991) are indicated by arrows.

confirmed proteinase activities; a papain-like proteinase (PLP-1) and a 3C-like proteinase (3CLpro). PLP-1 is known to cleave the amino-terminal gene 1 proteins p28 and p65 (Dong and Baker, 1994; Hughes *et al.*, 1995). 3CLpro has been shown to cleave itself from the gene 1 polyprotein (Lu *et al.*, 1995). In addition 3CLpro is predicted to mediate all of the gene 1 polyprotein cleavages in pp1ab carboxy terminal to 3CLpro; these include processing of the putative polymerase and helicase proteins encoded in ORF 1b (Lee *et al.*, 1991) (Figure 1). Thus the activity of MHV 3CLpro is presumed to be critical to virus replication.

In this study we have cloned the precise domain of 3CLpro and have overexpressed the proteinase in *E. coli* as a fusion protein with maltose binding protein. Following purification and cleavage of r3CLpro, we have demonstrated its activity against cleavage sites in the ORF 1a polyprotein and have characterized the determinants of 3CLpro activity against *in vitro* translated substrate. Our results demonstrate that MHV 3CLpro can be expressed in *E. coli*, and that the proteinase has unique features of activity compared with other 3C or 3C-like proteinases.

3. METHODS

3.1. Cloning and Expression of Recombinant 3CLpro (r3CLpro) in *E. coli*

The region of MHV gene 1 encoding the functional 3CLpro was amplified by PCR from the existing clone pGpro (Lu *et al.*, 1995). The left primer began at nt 10209 and the right at nt 11112; neither primer had additional nucleotides. The amplified fragment was ligated into Xmn 1 digested pMAL-c2 (New England Biolabs) and the ligated plasmid was transformed into nm522 *E. coli* cells. The recombinant plasmid (pMAL MPB-r3Clpro) was transformed into DH5a cells for protein expression. Following induction, *E. coli* were lysed per manufacturer's instructions and the clarified lysate passed over an amylose column. The bound MBP-r3CLpro fusion protein was eluted from the column and cleaved with factor Xa (obtained from Paul Bock) at 0.1% for 12 hours at 4°C. Cleavage of MBP and r3CLpro was confirmed by visualization of proteins on SDS-PAGE and Coomassie Blue staining (Figure 2). The elutate containing separated MBP and r3CLpro was used for all experiments without additional purification.

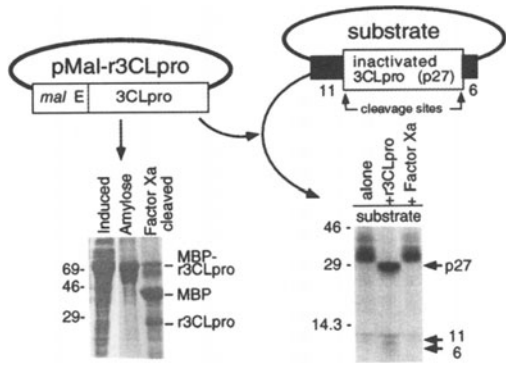

Figure 2. Expression of r3CLpro in E. coli and *trans* cleavage of substrate by r3CLpro. The pMAL MBP-r3CLpro construct is shown in the upper left. The gel below the construct shows results of expression in *E. Coli*. Induced lane shows total *E. coli* proteins following induction. The MBP-r3CLpro was purified on amylose resin (amylose) and then cleaved with factor Xa (factor Xa). The location of MBP-r3CLpro, MBP alone, and r3CLpro alone are shown. The substrate construct shows the organization and apparent molecular masses (see Methods). Gel shows electrophoresis of substrate (alone), substrate incubated with r3CLpro (+r3CLpro), and substrate incubated with factor Xa (+Factor Xa). Locations of cleavage products are shown to the right of the gel.

3.2. Cloning and Expression of Substrate for 3CLpro

The plasmid pGproH41Q was used to program *in vitro* translation in a combined transcription/translation reticulocyte lysate system, as previously described (Lu *et al.*, 1995). pGproH41Q expresses a polypeptide containing mutated inactive 3CLpro and 11 kDa and 6 kDa fragments of the flanking amino and carboxy terminal domains respectively (Figure 2). Although the protein expressed from pGproH41Q (hereafter referred to as "substrate") is unable to autoproteolytically cleave itself, the cleavage sites flanking the mutated proteinase are intact and can be cleaved in trans. Translation was performed in the presence of [^{35}S]methionine for 90 minutes.

3.3. *Trans* Cleavage Assays

Elutate containing r3CLpro, MBP and factor Xa was added directly to reticulocyte lysate reactions containing [^{35}S]met labeled substrate. Usually 2 ul of substrate was incubated with r3CLpro in a total volume of 10 ul. Incubations were performed at 30°C for the times indicated in each experiment. *Trans* cleavage experiments were terminated by addition of 2X Laemlli buffer or by snap freezing in dry ice/ethanol. Reaction products were separated on SDS- gradient polyacrylamide gels and analyzed by fluorography.

4. RESULTS

4.1. Cloning, Expression, and Activity of r3CLpro

The pMAL-r3CLpro plasmid directed expression of large amounts of soluble MBP-r3CLpro fusion protein in DH5a cells following induction with IPTG for 4 hours at 32°C

(Figure 2). Typically 20–50 mg/l of fusion protein was obtained under these conditions. Cleavage of MBP-r3CLpro by factor Xa was never complete, despite prolonged incubation at a variety of temperatures, even with the addition of fresh aliquots of factor Xa.

The substrate for 3CLpro was translated in a combined *in vitro* transcription/translation reticulocyte lysate system (TnT) and radiolabeled with [^{35}S]met. Radiolabled substrate was incubated in the absence of proteinase (alone), in the presence of r3CLpro (+ r3CLpro) or in the presence of factor Xa alone for 1 hour at 30°C (Figure 2). Under these conditions r3CLpro completely processed the substrate to p27 and fragments of 11 and 6 kDa. These data demonstrated that 3CLpro was able to cleave *in trans* at its amino and carboxy termini. and that this process was rapid and complete.

4.2. Determinants of r3CLpro Activity *in Vitro*

We next defined the extent of r3CLpro activity against the *in vitro* translated substrate (Figure 3). Radiolabled substrate was incubated with excess r3CLpro at 30°C and samples were taken a various times from 0.5 to 240 minutes.

Since no intermediate cleavage products were identified, even at very early times, it appeared that r3CLpro was able to cleave at both cleavage sites concurrently. Substrate was 50% cleaved at the earliest time point examined, 0.5 min. indicating that proteinase/substrate interaction was quite rapid. Complete cleavage of substrate into the "mature" components of 11, 27 and 6 kDa occurred by 4.5 min. The rapidity and extent of cleavage was much greater than we have previously observed with 3CLpro expressed *in vitro* or purified from virus-infected cells.

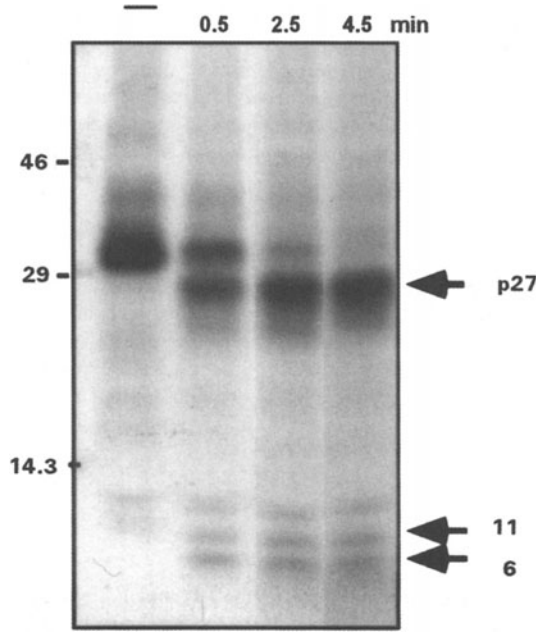

Figure 3. r3CLpro substrate cleavage. Substrate was incubated alone (-) or in the presence of r3CLpro (0.5–4.5 min.). Molecular mass markers are to the left of the gel and location of cleavage products are noted to the right of the gel.

Table 1. Inhibition r3CLpro activity

Inhibitor	Inhibitor class	Minimal inhibitory concentration (mM)	Inhibition
Leupeptin	Serine/ cysteine	0.2	+
PMSF	Serine	0.1	+
Zinc Chloride	Cysteine	3.0	+
E64	Cysteine	2.0	+
EDTA	Metallo/ Thiol		–
1,10 phenanthroline	Metallo		–

4.3. Inhibition of r3CLpro Activity

We examined the sensitivity of r3CLpro to inhibition by proteinase inhibitors (Table 1). Substrate was incubated with r3CLpro for 1 hr. at 30°C in the presence of several different classes of proteinase inhibitors. The serine proteinase inhibitor PMSF was the most active in blocking cleavage of substrate, but r3CLpro activity also was inhibited by leupeptin, albeit requiring high concentrations. $ZnCl_2$ has been shown to be a specific inhibitor of cysteine proteinases, and was able to inhibit r3CLpro in our assays.

The irreversible cysteine proteinase inhibitor E64 inhibited cleavage activity of r3CLpro, similar to results obtained in virus infected cells and during *in vitro* translation of 3CLpro containing precursors. EDTA and metalloproteinase inhibitor 1,10 phenanthroline did not affect activity, even at high concentrations (not shown) indicating that metal ions are not critical for activity. Together these results suggest some differences in the activity and specificity of MHV 3CLpro in comparison with other proteinases in this family.

5. DISCUSSION

In this report we have demonstrated that the active 3CLpro can be overexpressed in *E. coli* and that that partially purified proteinase is able to cleave substrate. A similar result has been reported for MHV-JHM (Seybert *et al.*, 1997). However, the 229E proteinase was tested against a small synthetic peptide, whereas we have demonstrated activity at both flanking cleavage sites in the polyprotein context. Our results are also consistent with those we previously reported for 3CLpro expressed *in vitro* or in virus-infected cells; specifically that the "mature" 3CLpro has no requirement for membranes. We were surprised at the efficiency of cleavage of substrate, since autoproteolytic liberation of mature proteinases in other virus systems is a regulated process in which precursors play a role in polyprotein processing as well as in other replication functions. However, our results may represent the final steps in activity of 3CLpro, with amplification of processing by mature 3CLpro. In order to address this more carefully, it will be necessary to express r3CLpro along with both flanking domains to assess the degree of cleavage. This may be a challenge in *E. coli*, because of the profoundly hydrophobic nature of these domains (Pinon *et al.*, 1997).

We have previously demonstrated that addition of the irreversible cysteine proteinase inhibitor E64 to virus infected cells results in rapid shutoff of MHV RNA synthesis and virus replication. Our demonstration that r3CLpro is also inhibited by E64 corrobo-

rates our previous results *in vitro* and *in cyto*. Other cysteine substituted chymotrypsin-like proteinases such as the 3Cpro of the picornaviruses are not inhibited by E64. Thus the sensitivity to E64 may represent a significant difference in MHV 3CLpro mechanism. The requirement for high concentrations of E64 and leupeptin may reflect the amount of proteinase used in our assays. Since we have only partially purified the enzyme, we did not quantitate precise amounts added to the assays. Kinetic analyses using highly purified r3CLpro and defined peptide substrates will be necessary to determine the inhibitor profile with more precision.

The partially purified r3CLpro is active at two defined cleavage sites. The availability of the recombinant enzyme will allow us to define other cleavage sites in the gene 1 polyprotein, and will also allow us to pursue structure function analyses and define the kinetics of this novel proteinase.

ACKNOWLEDGMENTS

This work was supported in part by NIH grant RO1-AI 26603 from the National Institutes of Allergy and Infectious Diseases.

REFERENCES

Denison, M. R. and Perlman, S., 1986, Translation and processing of mouse hepatitis virus virion RNA in a cell-free system, *J. Virol* **60**: 12–8.

Denison, M. R., Zoltick, P. W., Hughes, S. A., Giangreco, B., Olson, A. L., Perlman, S., Leibowitz, J. L., and Weiss, S. R., 1992, Intracellular processing of the N-terminal ORF 1a proteins of the coronavirus MHV-A59 requires multiple proteolytic events, *Virology* **189**: 274–84.

Dong, S. and Baker, S. C., 1994, Determinants of the p28 cleavage site recognized by the first papain-like cysteine proteinase of murine coronavirus, *Virology* **204**: 541–9.

Hughes, S. A., Bonilla, P. J., and Weiss, S. R., 1995, Identification of the murine coronavirus p28 cleavage site, *J. Virol* **69**: 809–13.

Lee, H.-J., Shieh, C.-K., Gorbalenya, A. E., Koonin, E. V., LaMonica, N., Tuler, J., Bagdzhadhzyan, A., and 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.

Lu, Y., Lu, X., and Denison, M. R., 1995, Identification and characterization of a serine-like proteinase of the murine coronavirus MHV-A59, *J. Virol* **69:** 3554–9.

Pinon, J., Mayreddy, R., Turner, J., Khan, F., Bonilla, P., and Weiss, S., 1997, Efficient autoproteolytic processing of the MHV-A59 3C-like proteinase from the flanking hydrophobic domains requires membranes, *Virology* **230**: 309–322.

Seybert, A., Ziebuhr, J. and Siddell, S. G., 1997, Expression and characterization of a recombinant murine coronavirus 3C-like proteinase, *J. Gen. Virol.*