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SUBSTRATE SPECIFICITY OF THE HUMAN CORONAVIRUS 229E 3C-LIKE PROTEINASE

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1. ABSTRACT

Coronavirus gene expression involves proteolytic processing of the gene 1-encoded polyproteins and a key enzyme in this process is the virus-encoded 3C-like proteinase. In this study, we describe the biosynthesis of the human coronavirus 229E 3C-like proteinase in *Escherichia coli* and the substrate specificity of the purified protein. Using immunofluorescence microscopy, we have also investigated the subcellular localization of the 3C-like proteinase and have found a punctate, perinuclear distribution of the proteinase in virus-infected cells.

2. INTRODUCTION

The human coronavirus (HCV) 229E genome is a positive-strand RNA of approximately 27,000 nucleotides. The replicase gene or gene 1, which is located at the 5' end of the genome, is comprised of two large, overlapping open reading frames (ORFs), ORF 1a and ORF 1b. The upstream ORF, ORF 1a, encodes a polyprotein, pp1a, with a calculated molecular mass of 454 kDa. In vitro studies suggest that the downstream ORF, ORF 1b, is expressed as a fusion protein with the ORF 1a-derived polyprotein pp1a by a process of (-1) ribosomal frameshifting. The fusion protein, pp1ab, has a calculated molecular mass of 754 kDa (Herold et al., 1993; Herold and Siddell, 1993).

There is clear evidence that the functional polypeptides involved in coronaviral RNA replication are released from the replicase gene-encoded polyproteins by extensive proteolytic processing. A key enzyme in this process appears to be the virus-encoded 3C-like proteinase (Liu et al., 1997; Heusipp et al., 1997a, 1997b; Grötzinger et al., 1996; Lu

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

et al., 1996; Tibbles et al., 1996; Ziebuhr et al., 1995; Liu et al., 1994). Recently, considerable progress has been made in characterizing the enzymatic properties of the coronavirus 3C-like proteinase and it appears that the enzyme has a catalytic system which is similar to the picornavirus 3C proteinases. However, the coronavirus enzyme has also several additional, unique features (Ziebuhr et al., 1997; Lu and Denison, 1997; Seybert et al., 1996; Lu et al., 1995; Liu and Brown, 1995; Ziebuhr et al., 1995).

In infected cells, the 3C-like proteinases of mouse hepatitis virus (MHV) A59 and HCV 229E have been identified as polypeptides with apparent molecular masses of 27 kDa and 34 kDa, respectively (Lu et al., 1996; Ziebuhr et al., 1995). However, to date nothing is known about the subcellular localization of the coronavirus 3C-like proteinase.

3. MATERIALS AND METHODS

3.1. Bacterial Expression and Purification of the HCV 229E 3C-Like Proteinase

The construction of plasmid pMalc2–3CL and the bacterial expression of the HCV 229E 3C-like proteinase have been described previously (Ziebuhr et al., 1995). Briefly, a fusion protein, MBP-3CLpro, containing the maltose-binding protein (MBP) of *E. coli* and the 3C-like proteinase domain of HCV 229E (amino acids 2966–3267 of pp1a and pp1ab) was synthesized in bacteria and then purified by amylose-affinity chromatography. The authentic 3C-like proteinase was released from the fusion protein by factor Xa cleavage and the protein mixture was loaded onto a Phenylsepharose HP column (Pharmacia Biotech, Freiburg, Germany) that had been pre-equilibrated with 12.5 mM Bis-Tris-HCl, pH 7.0, 300 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA. After extensive washing of the column, the recombinant 3C-like proteinase was eluted with 12.5 mM Bis-Tris-HCl, pH 7.0, 1mM dithiothreitol, 0.1 mM EDTA. The fractions containing the 3C-like proteinase were pooled and concentrated to 3 mg/ml using Centricon-3 concentrators (Amicon, Beverly, Mass.). The concentrated material was purified further by chromatography on a Superdex 75 pg column (Pharmacia Biotech) run under isocratic conditions with 10 mM Tris-HCl, pH 7.3, 200 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol. Finally, the purified protein was concentrated to 10 mg/ml using Centricon-3 concentrators and stored at -80°C.

3.2. Trans-Cleavage Assays

3.2.1. Peptide Substrates. Synthetic peptides were prepared by solid-phase chemistry and purified by high-performance liquid chromatography (HPLC) on a reversed-phase C_{18} silica column (Jerini Bio-Tools, Berlin, Germany). The identity and homogeneity of the peptides were confirmed by mass spectrometry and analytical reversed-phase chromatography. Cleavage reactions were incubated for 2 h at 25°C and contained 20 mM Bis-Tris-HCl, pH 7.0, 1 μM recombinant 3C-like proteinase and 0.5 mM substrate peptide in a total volume of 20 μl. The reactions were terminated by the addition of 80 μl 0.1% trifluoroacetic acid and the mixture was centrifuged for 5 min at 14 000 x *g* prior to analysis by reversed-phase HPLC on a 3.9 x 150 mm Delta Pak C_{18} column (Waters, Milford, Mass.). Cleavage products were resolved using a 22-min, 5–90% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The absorbance was determined at 215 nm. Under these conditions, the conversion of functional substrate peptides was complete. Nonfunctional substrate peptides did not show detectable hydrolysis under the same conditions.

3.2.2. Fusion Protein Substrates. Fusion proteins consisting of MBP and various replicase gene-derived peptide sequences were synthesized in *E. coli* TB1 cells and purified by amylose-affinity chromatography as described previously (Grötzinger et. al., 1996; Heusipp et al., 1997a; Heusipp et al., 1997b). In this way, five fusion proteins were purified that contain the following gene 1-encoded amino acids (aa): (i) aa 3934–4237, (ii) aa 4774–5259, (iii) aa 5500–5771, (iv) aa 5981–6602, and (v) aa 5981–6290. These substrate proteins were incubated with recombinant 3C-like proteinase as described by Grötzinger et al. (1996). The cleavage products were separated by SDS-polyacrylamide gel electrophoresis, transferred electrophoretically to polyvinylidene difluoride membranes, and the amino-terminal sequences of the carboxyl-terminal cleavage products were determined by automated Edman degradation as previously described (Ziebuhr et al., 1995).

3.3. Immunofluorescence Assay

MRC-5 cells were grown on coverslips, infected with HCV 229E (multiplicity of infection of 10 pfu per cell), and incubated at 33°C. After 15 h, the cells were fixed for 10 min with 4% paraformaldehyde in PBS. After washing with PBS, the cells were permeabilized with PBS-0.2% Triton X-100 and washed with PBS-1% NP-40. The indirect immunofluorescence assays were carried out with the HCV 229E 3C-like proteinase-specific antiserum K17 (Ziebuhr et al., 1995) at a 1:100 dilution in 10 mM Tris-HCl, 150 mM NaCl, 1% NP-40 containing 5% normal goat serum to reduce the background fluorescence. A FITC-conjugated goat anti-rabbit immunoglobulin G (1:100 dilution, Dianova, Hamburg, Germany) was used as the secondary antibody.

4. RESULTS AND DISCUSSION

4.1. Substrate Specificity of the HCV 229E 3C-Like Proteinase

Recently, considerable progress has been made in characterizing the primary structure of substrates that are recognized and cleaved by the HCV 229E 3C-like proteinase (Ziebuhr et al., 1997; Heusipp et al., 1997a; Heusipp et al., 1997b; Grötzinger et al., 1996, Ziebuhr et al., 1995). In order to substantiate the conclusions that have been drawn from these experiments, we have extended, in the present study, the analysis of putative functional substrates of the HCV 229E 3C-like proteinase. We have used both synthetic peptides and bacterially synthesized fusion protein substrates in the trans-cleavage assays with recombinant 3C-like proteinase. These data, combined with published results on the substrate specificity of the HCV 229E 3C-like proteinase, are summerized in Table 1 and lead us to the following conclusions. The Gln residue in the P1 position is absolutely conserved among all 3C-like proteinase substrates identified to date. The P2 and P1′ positions are occupied by Leu/Ile and Ser/Ala/Gly, respectively. However, as Table 1 also shows, the presence of the conserved Leu/Ile-Gln-Ser/Ala/Gly sequence is not sufficient for cleavage by the 3C-like proteinase: Two peptides containing either Ile-Gln-Ser or Ile-Gln-Gly tripeptides are not cleaved. Clearly, proteolysis is not exclusively determined by the P2 through P1' positions. It is also interesting to note, that the HCV 229E 3C-like proteinase cleaves efficiently an MHV-A59 derived peptide substrate indicating that the substrate specificity of coronavirus 3C-like enzymes is, at least to some extent, conserved.

Table 1. Substrate specificity of recombinant HCV 229E 3C-like proteinase

Substrate[a]	Sequence															Polyprotein
P	K	T	L	I	F	T	L	Q	↓	A	A	F	G	N	A	G	HCV 229E pp1a/1ab
B/P	V	S	Y	G	S	T	L	Q	↓	A	G	L	R	K	M	A	HCV 229E pp1a/1ab
P	Q	M	F	G	V	N	L	Q	↓	S	G	K	T	T	S	M	HCV 229E pp1a/1ab
B	T	C	D	R	T	A	I	Q	↓	S	F	D	N	S	Y	L	HCV 229E pp1a/1ab
B	Y	E	K	S	T	V	L	Q	↓	A	A	G	L	C	V	V	HCV 229E pp1ab
B/P	E	I	T	M	T	D	L	Q	↓	S	E	S	S	C	G	L	HCV 229E pp1ab
B	T	F	T	E	V	N	L	Q	↓	G	L	E	N	I	A	F	HCV 229E pp1ab
B	A	T	F	Y	P	Q	L	Q	↓	S	A	E	W	K	C	G	HCV 229E pp1ab
P	L	C	T	T	S	F	L	Q	↓	S	G	I	V	K	M	V	MHV-JHM pp1a/1ab
P	C	F	L	V	T	K	F	R		R	M	F	G	D	L	S	HCV 229E pp1a/1ab
P	D	S	F	C	K	T	I	Q		S	A	L	S	V	V	S	HCV 229E pp1a/1ab
P	K	Q	R	I	T	T	I	Q		G	P	P	G	S	G	K	HCV 229E pp1ab

[a]P, synthetic peptide; B, bacterially synthesized fusion protein.
The cleavage of a specific peptide bond is indicated by an arrow.

4.2. Intracellular Localization of the HCV 229E 3C-Like Proteinase

In the case of coronaviruses, there is a paucity of information on the intracellular localization of replicase gene-encoded polypeptides. Therefore, we decided to study the intracellular localization of the 3C-like proteinase by immunofluorescence microscopy. MRC-5 cells were infected with HCV 229E at a multiplicity of infection of 10 pfu per cell, and 15 h postinfection we performed an indirect immunofluorescence analysis using the proteinase-specific rabbit antiserum K17 (Ziebuhr et al., 1995). The results of this experiment are shown in Figure 1. We observed a punctate, perinuclear immunofluorescence

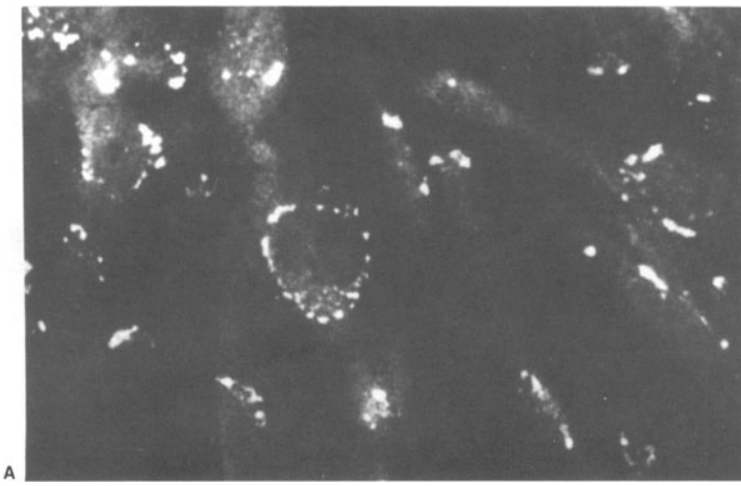

Figure 1. Immunofluorescence analysis of the subcellular localization of the 3C-like proteinase in HCV 229E-infected cells. MRC-5 cells were infected with HCV 229E (panel A) or mock infected (panel B) and analyzed 15 h postinfection.

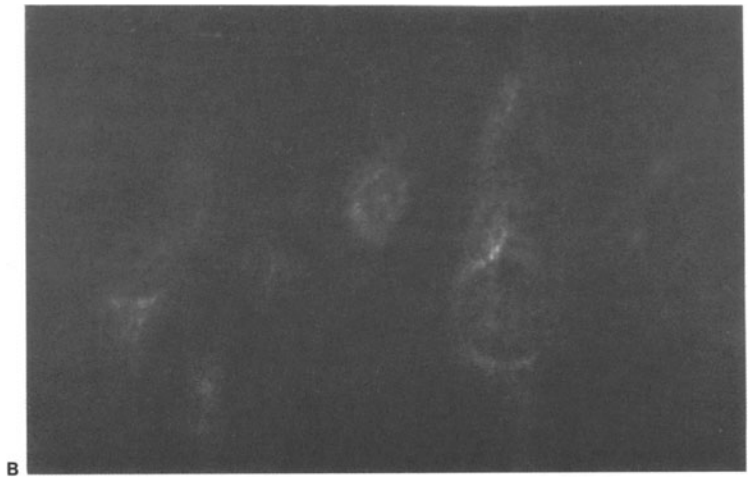

Figure 1B.

in virus-infected cells. A very similar staining pattern was also observed in HCV 229E-infected HeLa-CD13 cells (data not shown). These data provide the first experimental evidence that the coronavirus 3C-like proteinase is located in a perinuclear, presumably membraneous compartment. Interestingly, a similar immunofluorescence pattern has also been reported for the replicase gene-encoded putative helicase and RNA polymerase proteins of the equine arteritis virus (EAV), a virus that is closely related to coronaviruses (van Dinten et al., 1996).

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