Identification and subcellular localization of a 41 kDa, polyprotein 1ab processing product in human coronavirus 229E-infected cells

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The translation products of the human coronavirus (HCV) 229E open reading frames 1a and 1b, the polyproteins 1a and 1ab, are processed by virus-encoded proteinases. One of the key enzymes in this process is a chymotrypsin-like enzyme, the 3C-like proteinase. In this study we have identified an ORF 1b-encoded, 41 kDa processing product in HCV 229E-infected cells by using a monoclonal antibody with defined specificity. We show that this polypeptide is released from polyprotein 1ab by 3C-like proteinase-mediated cleavage of the peptide bonds Gln-6110/Gly-6111 and Gln-6458/Ser-6459. Also, we have investigated the subcellular localization of the 41 kDa processing product. Immunofluorescence microscopy revealed a punctate, perinuclear distribution of the 41 kDa polypeptide in infected cells and an identical subcellular localization was observed for three additional pp1ab-derived polypeptides. In contrast, the virus nucleocapsid protein showed a homogeneous cytoplasmic localization.

The positive-strand RNA genome of the human coronavirus (HCV) 229E comprises approximately 27000 nucleotides. The RNA replicase gene or gene 1, which is located at the 5’ end of the genome, comprises two large, overlapping open reading frames, 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 & Siddell, 1993).

There is clear evidence that the functional polypeptides involved in coronaviral RNA replication are released from the replicase gene polyproteins by extensive proteolytic processing. A key enzyme in this process appears to be the coronavirus 3C-like protease (Ziebuhr et al., 1995; Lu et al., 1995; Tibbles et al., 1996; Grötzinger et al., 1996; Liu et al., 1997). Recently, considerable progress has been made in characterizing the enzymatic properties of the coronavirus 3C-like protease and it appears that the enzyme has a catalytic system which, in many respects, is similar to the picornavirus 3C proteases. Clearly, however, the coronavirus enzyme has several additional, unique features (Liu & Brown, 1995; Lu et al., 1995; Ziebuhr et al., 1995, 1997; Seybert et al., 1997).

For many positive-strand RNA viruses it has been shown that RNA replication involves the formation of large replication complexes that are associated with cellular membranes (Bienz et al., 1990, 1992; Chambers et al., 1990; Froshauer et al., 1988; Barco & Carrasco, 1995). In the case of coronaviruses, however, very little is known about the composition and subcellular localization of the viral replication complex. In previous studies reticular inclusions and double membrane structures that may be involved in viral RNA synthesis have been identified in murine hepatitis virus-infected cells (Dubois-Dalcq et al., 1984) and it has been demonstrated that the putative RNA polymerase and helicase proteins of equine arteritis virus (EAV), a virus that is closely related to coronaviruses, show a perinuclear localization and, most likely, are associated with membranes (Van Dinten et al., 1996).

In this study, we have identified an HCV 229E ORF 1b-encoded, 41 kDa polypeptide in virus-infected cells. We have determined its termini by trans cleavage assays using purified, recombinant 3C-like proteinase. Finally, we show that this polypeptide co-localizes with other pp1ab-derived polypeptides to a perinuclear compartment.

In order to investigate the expression of HCV 229E ORF1b-encoded polypeptides, we have generated a panel of murine monoclonal antibodies (Grötzinger, 1996; Grötzinger et al., 1996), including MAb 10G11. The specificity of MAb 10G11 was analysed by epitope mapping and the antibody was found to recognize three partially overlapping, synthetic peptides that share the amino acid sequence Ser-Leu-Pro-Thr-
Asn-Ile-Ala (Grötzinger, 1996). This sequence corresponds to amino acids 6158 to 6164 of pp1ab. We have used MAb 10G11 to analyse cell lysates obtained from $5 \times 10^5$ HeLa-CD13 cells that had been infected with HCV 229E at an m.o.i. of 10 p.f.u. per cell, or mock infected. The infection of cells, the preparation of cell lysates and the immunoblotting of viral proteins were done as described previously (Grötzinger et al., 1996).

As is shown in Fig. 1, using MAb 10G11, a polypeptide with an apparent molecular mass of 41 kDa could be detected in HCV 229E-infected, but not in mock-infected cells. The molecular mass of the infection-specific polypeptide is consistent with a 3C-like proteinase-mediated cleavage of HCV 229E pp1ab at the peptide bonds Gln-6458 and Ser-6459. These positions are in agreement with the known substrate specificity of the HCV 3C-like proteinase (Ziebuhr et al., 1997) and correspond to positions that have been predicted to occur in the 3C-like proteinase-mediated processing of the related infectious bronchitis virus pp1ab (Gorbalenya et al., 1989). Nevertheless, to confirm the position of these cleavages, we have done cleavage assays utilizing the 

trans 
cleavage activity of purified, recombinant HCV 229E 3C-like proteinase (Ziebuhr et al., 1995, 1997; Grötzinger et al., 1996).

The primers 5’ CGCGGATCCCTTTAATTGTTCCCTAC 3’ and 5’ CCGGAATGATTGAAACTCGAGGTGATG 3’ were used to amplify nucleotides 18549–20731 of the HCV 229E genomic sequence from plasmid pBS-T5B5 (Herold et al., 1993). The PCR product was digested with BamHI and EcoRI and ligated to the BamHI/EcoRI-digested T7 expression plasmid pBS-T (Grötzinger et al., 1996). The resultant plasmid, pS5, was either digested with EcoRV or with XhoI and capped mRNAs were synthesized by in vitro transcription. In vitro translations were then done in rabbit reticulocyte lysate (Promega/Serva) in the presence of $[^35]S$-methionine. These translations produced polypeptides representing HCV 229E pp1ab amino acids 6087–6758 and 6087–6337, respectively.

The trans cleavage assays with in vitro-translation substrates were done as described previously (Grötzinger et al., 1996) except that 1 µg of 3C-like proteinase was used. After incubation with purified, recombinant 3C-like proteinase, the 81 kDa translation product (pp1ab amino acids 6087–6758) was specifically cleaved into polypeptides of approximately 79, 43, 41 and 38 kDa (Fig. 2, lane 3). In the case of the carboxyl-terminally truncated substrate of 32 kDa (pp1ab amino acids 6087–6337) only one cleavage product with an apparent molecular mass of 30 kDa could be detected after incubation with proteinase (Fig. 2, lane 6). Taken together, these data lead us to conclude that at least two cleavage sites must be present in the region between pp1ab amino acids 6087–6758. The data are also consistent with the interpretation that one cleavage site is located approximately 2 kDa from the amino terminus of both substrates and the second site must be located between amino acids 6337 and 6758 of pp1ab.

To define the cleavage sites precisely we therefore expressed, in Escherichia coli TB-1 cells, the HCV 229E pp1ab amino acids 5981–6602 and 5981–6290, respectively, as fusion proteins with the maltose-binding protein (MBP) of E. coli. The resultant fusion proteins, MBP-41/1 and MBP-41/2, were purified and incubated with recombinant 3C-like proteinase as described previously (Grötzinger et al., 1996). As expected, the fusion protein substrates were cleaved specifically and the identities of the cleavage products were verified.
Coronavirus polyprotein 1ab processing

(a)

Coronavirus polyprotein 1ab processing

(b)

Fig. 2. For caption see facing page.
Fig. 3. Immunofluorescence analysis of the subcellular localization of pp1ab-derived polypeptides in HCV 229E-infected cells. MRC-5 cells infected with HCV 229E (b, d, f, h and j) or mock infected (a, c, e, g and i) were stained with rabbit antiserum 1720 (a and b); rabbit antiserum K17 (c and d); rabbit antiserum H6 (e and f); murine MAb 10G11 (g and h) and murine MAb NG12 (i and j).
by immunoblotting using MBP antiserum (New England Biolabs) or MAb 10G11 (data not shown). The amino-terminal sequences of the carboxyl-terminal, 22 kDa cleavage product of the MBP-41/1 fusion protein and the 25 kDa cleavage product of the MBP-41/2 fusion protein were determined by automated Edman degradation as described previously (Grötzinger et al., 1996) and found to be Ser-Ala-Glu-Trp-Lys and Gly-Leu-Glu-Asn-Ile, respectively, representing amino acids 6459–6463 and 6111–6115 of the HCV 229E pp1ab. We conclude that the intracellular polypeptide of 41 kDa encompasses amino acids 6111–6458 of pp1ab. The calculated molecular mass of this peptide sequence is 38.8 kDa, which corresponds well with the molecular mass of the processing product observed in virus-infected cells. It is also interesting to note that these results indicate that the HCV 229E 3C-like proteinase is able to cleave Q/G peptide bonds. All other HCV 229E 3C-like proteinase cleavage sites identified so far have been Q/A or Q/S dipeptides.

The data described above adds further to our understanding of the 3C-like proteinase-mediated processing of pp1ab. From this study, the identification of HCV 229E pp1ab polypeptides in virus-infected cells (Grötzinger et al., 1996; Heusipp et al., 1997) and from the data relating to the substrate specificity of the HCV 229E 3C-like proteinase (Ziebuhr et al., 1995, 1997; Grötzinger et al., 1996; Heusipp et al., 1997), we feel confident in predicting that, beyond those already described, no additional sites for the 3C-like proteinase exist in the ORF 1b-encoded region of pp1ab. Thus, in addition to the 105 kDa polymerase (POL) polypeptide (Grötzinger et al., 1996), the 71 kDa metal-binding/helicase (HEL) polypeptide (Heusipp et al., 1997) and the 41 kDa polypeptide (this study), we predict that two additional processing products of 58 and 34 kDa spanning the regions Ser5593 to Gln6110 and Ser6459 to Lys6758, respectively, are expressed in HCV 229E-infected cells. The production of immunological reagents that would allow us to identify these gene products is in progress.

In the case of coronaviruses there is almost no information on the intracellular localization of gene 1-encoded proteins. Therefore, we decided to use our immunological reagents to determine by immunofluorescence the intracellular localization of the 41 kDa polypeptide and three additional pp1ab-derived polypeptides. To this end, MRC-5 cells were grown on coverslips and infected with HCV 229E (m.o.i. 10 p.f.u. per cell). After 15 h, the cells were washed once with PBS and fixed for 10 min with 4% paraformaldehyde in PBS. After washing with PBS the cells were permeabilized with 0.2% Triton X-100 and washed three times with 1% NP40 in PBS. Indirect immunofluorescence assays with FITC-labelled secondary antibodies were done using standard procedures. The following primary antibodies were used: (i) rabbit antiserum specific for the amino-proximal region of HCV 229E pp1a/pp1ab (amino acids 41–250, antisera IS1720; J. Herold, unpublished), (ii) rabbit antiserum specific for the HCV 229E 3C-like proteinase (antisera K17; Ziebuhr et al., 1995), (iii) rabbit antiserum specific for the HCV 229E metal-binding and helicase domains (antisera H6; Heusipp et al., 1997), (iv) MAb 10G11 specific for the 41 kDa polypeptide encoded in ORF1b (Grötzinger, 1996) and (v) the nucleocapsid protein-specific MAb NG12 (Ziebuhr, 1995). The results obtained are shown in Fig. 3. At 15 h post-infection, we observed a punctate immunofluorescence of the perinuclear region in virus-infected cells using each of the pp1ab-specific antibodies (Figs. 3 a–h). The observed staining pattern was characteristic, although the reagents differed in their sensitivity and some produced a high background of staining even in mock-infected cells. It should be remembered that, as we have previously stated (Ziebuhr et al., 1995), the amounts of replicate gene products in HCV 229E-infected cells are extremely low. This can be clearly seen by comparison with, for example, the virus nucleocapsid protein which revealed a homogeneous cytoplasmic staining pattern (Fig. 3i, j). These data suggest that the coronaviral pp1ab proteins analysed in this study are located in a perinuclear, presumably membranous compartment. This is the first experimental indication that the coronaviral replication complex is membrane associated and involves a combination of gene 1-encoded polypeptides.

Sequence analyses have revealed a high degree of conservation among the coronavirus ORF1b-encoded proteins (Lee et al., 1991; Herold et al., 1993; Eleouet et al., 1995). However, only a few putative functional domains, for example, the RNA polymerase or the metal-binding and helicase domains have been identified so far (Gorbalenya et al., 1989). Interestingly, the peptide sequence of the coronavirus 41 kDa polypeptide described in this paper is highly conserved among corona- and closely related viruses (Gorbalenya & Snijder, 1996) and, as far as we know, is unique to this group of viruses. It will be of particular interest to elucidate the function of this presumably coronavirus-specific polypeptide.

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References


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