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# Identification of an ATPase Activity Associated with a 71-Kilodalton Polypeptide Encoded in Gene 1 of the Human Coronavirus 229E

GERHARD HEUSIPP, UTE HARMS, STUART G. SIDDELL, AND JOHN ZIEBUHR*

*Institute of Virology, University of Würzburg, Würzburg, Germany*

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**Human coronavirus 229E gene expression involves proteolytic processing of the gene 1-encoded polyproteins pp1a and pp1ab. In this study, we have detected a 71-kDa polypeptide in virus-infected cells that is released from pp1ab by the virus-encoded 3C-like proteinase and that has been predicted to contain both metal-binding and helicase domains. The polypeptide encompasses amino acids Ala-4996 to Gln-5592 of pp1ab and exhibits nucleic acid-stimulated ATPase activity when expressed as a fusion protein with the *Escherichia coli* maltose-binding protein. These data provide the first identification of a coronavirus open reading frame 1b-encoded enzymatic activity.**

The human coronavirus (HCV) 229E gene 1 encodes two large polyproteins, pp1a and pp1ab, with calculated molecular masses of 454 and 754 kDa (9, 10). There is clear evidence that these polyproteins are processed by the virus-encoded 3C-like proteinase, giving rise to individual polypeptides that are involved in viral RNA replication (8, 30). Recently, the putative catalytic residues and the substrate specificity of several coronavirus 3C-like enzymes have been characterized and the proteinase has been detected in virus-infected cells (8, 18–20, 25, 28, 30, 31). In contrast to the increasing amount of information on coronavirus proteinases, little is known about additional enzymatic activities that are associated with the replication of viral RNA. The predicted sequence of four coronavirus pp1ab molecules has allowed for the identification of putative functional domains, including RNA-dependent RNA polymerase (POL), metal-binding (MBD), and NTPase/helicase (HEL) domains (3, 6, 7, 9, 17). However, the polypeptides that exhibit these activities and the enzymatic activities themselves have not yet been properly defined. The present study focuses on the expression and enzymatic activity of the predicted HEL domain of HCV 229E.

Recently, we have detected a 105-kDa open reading frame (ORF) 1b-encoded polypeptide containing a putative RNA-dependent POL domain in HCV 229E-infected cells (8). It has been postulated that during RNA replication and transcription, viral HEL domains closely cooperate with POL domains (6). Thus, to study the expression of the HCV 229E-encoded, putative HEL domain, we produced, first of all, a polyclonal rabbit antiserum (H6) that is specific for the HCV 229E pp1ab amino acids 5012 to 5303. Then, we analyzed lysates obtained from mock-infected or HCV 229E-infected MRC-5 cells at different times after infection by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting (8) using the HEL-specific antiserum. A protein with an apparent molecular mass of 71 kDa was specifically detected in lysates from infected but not from mock-infected cells (Fig. 1). Using this approach, the earliest time point at which the 71-kDa polypeptide could be detected was 5 h after infection. There is no significant change in the amount of the 71-kDa polypeptide detected from 9 to 15 h postinfection.

Previous experiments using *trans* cleavage assays have revealed an HCV 229E 3C-like proteinase cleavage site at the peptide bond Gln-4995-Ala-4996 of pp1ab. In the same experiments, we were able to show that this is the only cleavage site between amino acids 4818 and 5259 in the HCV 229E pp1ab (8). These data, combined with the prediction of cleavage sites flanking the carboxyl terminus of the coronavirus HEL domain (3, 7, 17) led us to anticipate a 3C-like proteinase-mediated cleavage at Gln-5592–Ser-5593 of the HCV 229E polyprotein pp1ab. This cleavage site would also be consistent with the molecular mass of 71 kDa observed for the HCV 229E pp1ab polypeptide described above. To test this prediction, nucleotides 15510 to 18031 of the HCV 229E genomic sequence were amplified by PCR from plasmid pBS-T13A5 (9) and inserted into the T7 expression plasmid pBS-T (8). The resultant plasmid, pS3, was linearized by *Nsi*I, *Spe*I, or *Afl*II, and synthetic mRNAs were generated as described previously (8). Translation of these mRNAs in reticulocyte lysates yielded polypeptides representing the pp1ab amino acids 5074 to 5819, 5074 to 5626, and 5074 to 5580, respectively. All three translation products overlap (in their amino-terminal portion) with the previously analyzed translation product, amino acids 4818 to 5259 (Fig. 2A) (8). After incubation with 1 μg of purified, recombinant 3C-like proteinase (31), the translation products derived from *Nsi*I- and *Spe*I-linearized pS3 were specifically cleaved to produce a clearly identified 58-kDa product. The pS3 *Afl*II-derived translation product remained uncleaved (Fig. 2B, lanes 3 and 6).

From these experiments we were able to conclude that the 3C-like proteinase-mediated cleavage occurs in a region between amino acids 5580 and 5626, which would be consistent with the use of the predicted cleavage site at Gln-5592–Ser-5593. However, we were not able to be more precise because, as is evident in Fig. 2B, it was difficult to clearly identify the carboxyl-proximal cleavage products of the reaction. Also, the apparent molecular masses of both the primary translation products and the cleavage products were slightly higher than calculated. Therefore, to unambiguously define the position of the cleavage site, we chose to express the HCV 229E pp1ab amino acids 5500 to 5771 as a fusion protein with the maltose-

* Corresponding author. Mailing address: Institute of Virology, University of Würzburg, Versbacher Strasse 7, 97078 Würzburg, Germany. Phone: 49-931-2013966. Fax: 49-931-2013934. E-mail: j.ziebuhr@rzbox.uni-wuerzburg.de.

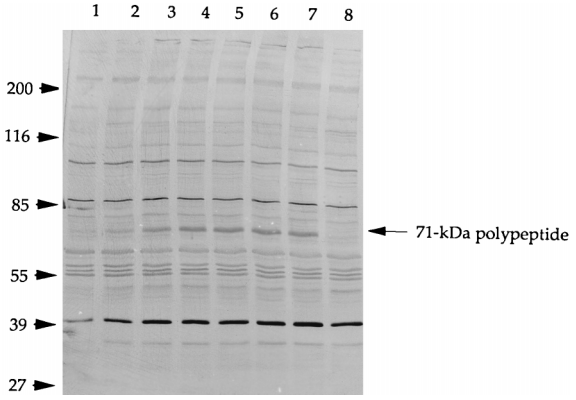


FIG. 1. Detection of a 71-kDa, ORF 1b-encoded polypeptide in HCV 229E-infected cells. Cell lysates from mock-infected (lane 8) and HCV 229E-infected (lanes 1 to 7) HeLa-CD13 cells ($5 \times 10^5$) were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using the MBD-HEL-specific antiserum H6. Lysates were obtained at 3 (lane 1), 5 (lane 2), 7 (lane 3), 9 (lane 4), 11 (lane 5), 13 (lane 6), and 15 (lanes 7 and 8) h postinfection. Molecular mass markers and the 71-kDa, pp1ab polypeptide are indicated.

binding protein (MBP) of *Escherichia coli* (bacterial expression plasmid pMal-cHEL) (Fig. 2A), an approach that has proven useful for the determination of other HCV 229E 3C-like proteinase cleavage sites (8). The purified fusion protein was incubated with recombinant 3C-like proteinase, and a specific, albeit inefficient, cleavage of the substrate was observed (data not shown). The amino-terminal sequence of the carboxyl terminal, 19-kDa cleavage product was determined by automated Edman degradation and found to be Ser-Glu-Ser-Ser-Cys-Gly-Leu, representing amino acids 5593 to 5599 of HCV 229E pp1ab.

In view of the low efficiency with which the Gln-5592–Ser-5593 cleavage site was used in the *trans* cleavage assay described above, we felt it was necessary to do an additional peptide-based cleavage assay. Thus, a 15-mer peptide containing the Gln-5592–Ser-5593 cleavage site (Glu-Ile-Thr-Met-Thr-Asp-Leu-Gln-Ser-Glu-Ser-Ser-Cys-Gly-Leu; pp1ab amino acids 5585 to 5599) was synthesized by solid-phase chemistry and purified by high-pressure liquid chromatography (HPLC) on a reversed-phase $C_{18}$ silica column (Jerini Bio-Tools, Berlin, Germany). The identity and homogeneity of the peptide were confirmed by mass spectrometry and analytical reversed-phase chromatography. The peptide substrate (1 mM) was incubated with 1 μM recombinant 3C-like proteinase (31) in a buffer containing 20 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (BIS-TRIS)-HCl, pH 7.0, at 25°C for 20 min. The reaction products were analyzed by HPLC as previously described (25). As Fig. 3 shows, the peptide substrate is cleaved very rapidly; after 20 min of incubation, the conversion of the substrate is complete.

In summary, these data allow us to conclude that the putative HCV 229E HEL domain is expressed as a 71-kDa polypeptide encompassing 597 amino acids. This polypeptide is released from pp1ab by the HCV 229E 3C-like proteinase, and it contains both HEL and MBD domains. These findings support previous predictions of Gorbalenya et al. (7). Interestingly, the peptide bond between Gln-5277 and Gly-5278 of pp1ab does not appear to be cleaved by the 3C-like proteinase. The corresponding dipeptide in the transmissible gastroenteritis virus pp1ab sequence has been predicted to represent a 3C-like proteinase cleavage site (3), and it will be interesting to determine if HCV 229E and transmissible gastroenteritis virus differ in this respect.

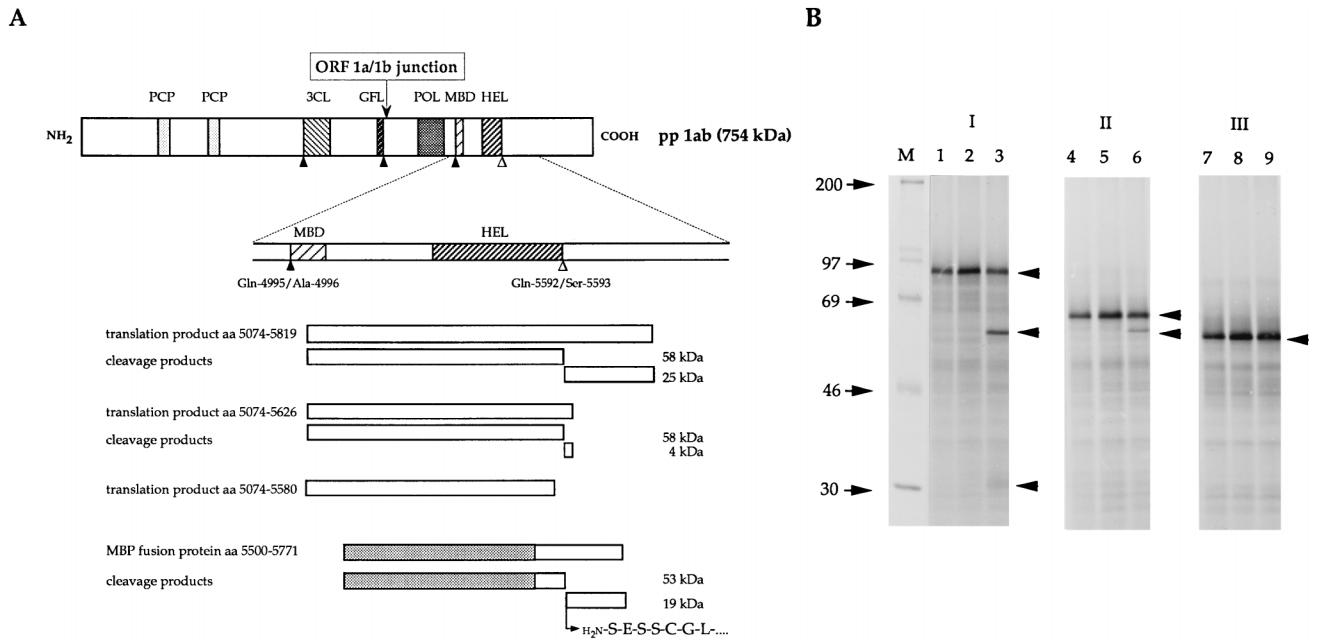


FIG. 2. *trans* cleavage assays with in vitro-translated or bacterially synthesized substrates containing the predicted HEL domain. (A) Putative functional domains within the HCV 229E pp1ab (7, 9) are shown. The 3C-like proteinase-mediated cleavages identified to date (8, 30) are designated by solid triangles and the predicted cleavage releasing the carboxyl terminus of the putative HEL domain (7) is represented by an open triangle. The substrate proteins and cleavage products are indicated with their calculated molecular masses. Additionally, the amino-terminal sequence of the 19-kDa, MBP-cHEL cleavage product is shown in the single-letter code. Abbreviations used: PCP, papain-like cysteine proteinase; 3CL, 3C-like proteinase; GFL, growth factor/receptor-like domain; aa, amino acid. (B) In vitro translation products of mRNAs derived from plasmid pS3 DNA. Shown are pp1ab amino acids 5074 to 5819 (lanes 1 to 3), amino acids 5074 to 5626 (lanes 4 to 6), and amino acids 5074 to 5580 (lanes 7 to 9), without incubation (lanes 1, 4, and 7) or after incubation with buffer (lanes 2, 5, and 8) or buffer containing recombinant 3C-like proteinase (lanes 3, 6, and 9). The primary translation products and cleavage products are indicated, and molecular weight markers (in thousands) (catalog no. CFA 626; Amersham) are shown.

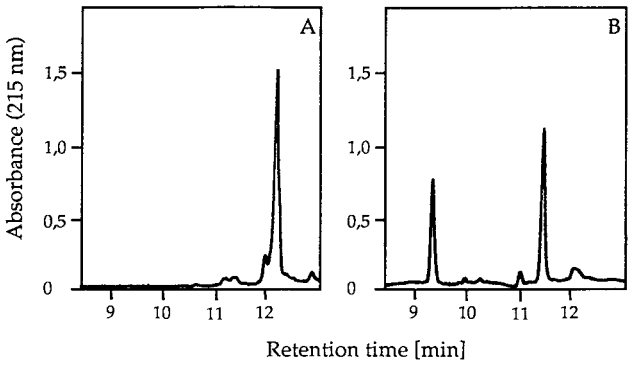


FIG. 3. Cleavage of the synthetic peptide Glu-Ile-Thr-Met-Thr-Asp-Leu-Gln-Ser-Glu-Ser-Ser-Cys-Gly-Leu by recombinant HCV 229E 3C-like proteinase. The peptide substrate (1 mM), representing amino acids 5585 to 5599 of pp1ab and containing one predicted Gln-Ser cleavage site (7), was incubated with 1 μM 3C-like proteinase for 20 min at 25°C. The substrate proteolysis was analyzed by reversed-phase HPLC as described previously (25). (A) Peptide with buffer; (B) peptide with buffer containing 3C-like proteinase.

On the basis of the comparative sequence analysis, it has been predicted that both NTPase and helicase activities will be associated with pp1ab or its derivatives (3, 7, 9, 17). However, to date, these enzymatic activities have not been demonstrated for any coronavirus ORF 1b-encoded protein. Thus, having determined the termini of the 71-kDa polypeptide, we sought an abundant source of the HCV 229E HEL polypeptide for biochemical studies. The coding sequence of the 71-kDa HEL polypeptide (pp1ab amino acids 4996 to 5592) was amplified by PCR from plasmid pBS-T13A5 (9). The downstream primer contained a translation stop codon and an *Xba*I restriction site. The PCR product was treated with T4 DNA polymerase, phosphorylated with polynucleotide kinase, digested with *Xba*I, and ligated with *Xmn*I/*Xba*I-digested pMal-c2 DNA (New England Biolabs, Schwalbach, Germany), resulting in the bacterial expression plasmid pMal-HEL. The expression in *E. coli* TB1 cells and the purification of the MBP-HEL fusion protein by amylose affinity chromatography were done essentially as described previously for the HCV 229E 3C-like proteinase (11, 30). Thereafter, the fusion protein was purified further by chromatography on a Superdex 200-pg column (Pharmacia-Biotech, Freiburg, Germany) run under isocratic conditions with 20 mM Tris-HCl (pH 7.3), 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol. As a control, a fusion protein encoded by plasmid pMal-c2 (consisting of MBP and the N-terminal portion of the *E. coli* β-galactosidase [MBP–β-Gal]) was expressed and purified under the same conditions. However, in this case, the final gel filtration step was omitted.

Upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG), TB1[pMal-HEL] bacteria expressed an MBP-HEL polypeptide with an apparent molecular mass of 113 kDa. TB1[pMal-c2] cells produced an MBP–β-Gal polypeptide of approximately 51 kDa (Fig. 4A, lanes 1, 2, 5, and 6). As expected, the 113-kDa MBP-HEL polypeptide reacted in Western blot assays with the HEL-specific H6 antiserum, whereas both the MBP-HEL and the MBP–β-Gal polypeptides were recognized by an MBP-specific antiserum (New England Biolabs) (data not shown). Growth of the bacteria at 25°C resulted in soluble fusion proteins that could be enriched by amylose affinity chromatography (Fig. 4A, lanes 3 and 7). The MBP-HEL fusion protein was found to be less stable than the MBP–β-Gal fusion protein, and to remove degradation products from the MBP-HEL protein, we introduced an additional size exclusion chromatography (Fig. 4A, lane 4). Finally, we sought

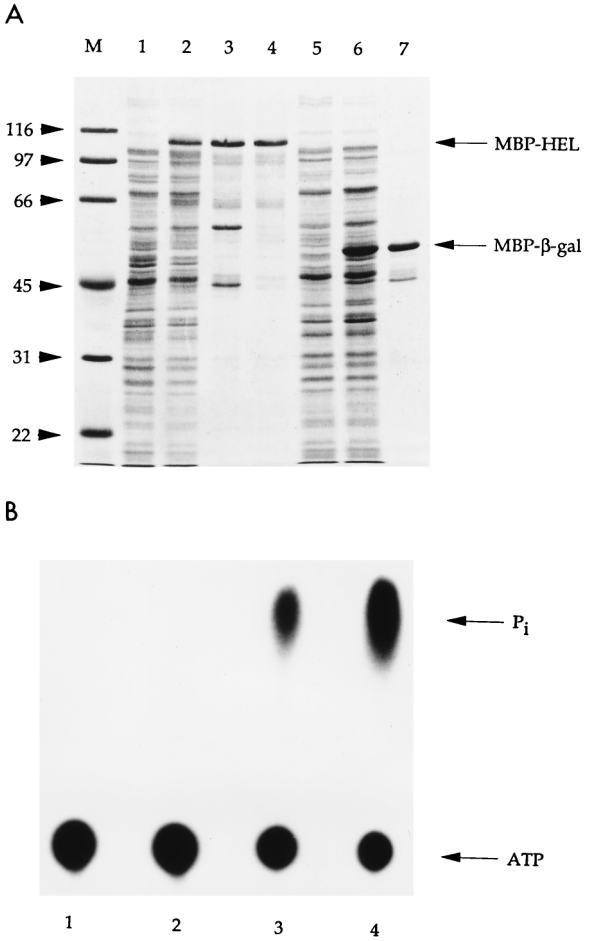


FIG. 4. Expression, purification, and ATPase activity of the MBP-HEL fusion protein. (A) Aliquots taken at each step of the purification were analyzed on an SDS–12.5% polyacrylamide gel, and the proteins were stained with Coomassie brilliant blue. Lanes: M, protein molecular mass markers (with masses, in kilodaltons, indicated on the left); 1 and 2, cleared lysates of noninduced (lane 1) and IPTG-induced (lane 2) TB1 bacteria with the expression plasmid pMal-HEL; 3, pooled peak fractions from the amylose affinity chromatography of the MBP-HEL fusion protein; 4, pooled peak fractions from the Superdex 200-pg chromatography of the MBP-HEL fusion protein; 5 and 6, cleared lysates of non-induced (lane 5) and IPTG-induced (lane 6) TB1 bacteria with the expression plasmid pMal-c2; 7, pooled peak fractions from the amylose affinity chromatography of the MBP–β-Gal fusion protein. The positions of the MBP-HEL and MBP–β-Gal fusion proteins are indicated by arrows. (B) The determination of ATPase activity was performed by the [γ-$^{32}$P]ATP–thin-layer chromatography assay described in the text. Reaction aliquots were separated by using 0.15 M formic acid–0.15 M LiCl (pH 3.0) as the liquid phase and analyzed by phosphorimaging. Incubations were with buffer only (panel 1), 0.3 μg of MBP–β-Gal fusion protein (panel 2), 0.3 μg of MBP-HEL fusion protein (panel 3), and 0.3 μg of MBP-HEL fusion protein supplemented with 0.5 mM poly(A) (panel 4). The positions of ATP and $P_i$ are indicated.

to release the authentic HCV 229E HEL domain from the MBP-HEL fusion protein by endoproteinase Xa treatment. We observed, however, that factor Xa cleaves within the HEL domain, despite the fact that HEL does not contain the Ile-Glu-Gly-Arg tetrapeptide sequence recognized by the endoproteinase (data not shown). Nevertheless, since many fusion proteins are enzymatically active (23), we decided to test for ATPase activity with the intact fusion protein. The ATPase activity assays were done with 0.3 μg of purified fusion protein in a buffer containing 20 mM HEPES-KOH (pH 7.4), 5 mM magnesium acetate, 1 mM dithiothreitol, 200 μM ATP, and 15 μCi of [γ-$^{32}$P]ATP (3,000 Ci/mmol; Amersham, Braunschweig,

Germany) per ml in a final volume of 40 μl. When indicated, 0.5 mM poly(A) (780-mer of polyadenylic acid; Pharmacia-Biotech) was included in the reaction mixture. The reaction was incubated at 25°C for 20 min and stopped on ice by adding EDTA to a concentration of 100 mM. The samples were analyzed by polyethyleneimine-cellulose thin-layer chromatography with 0.15 M formic acid–0.15 M LiCl (pH 3.0) as the liquid phase. Phosphorimaging of the dried chromatographic plates was used to locate the positions of ATP and $P_i$ and to quantitate the hydrolysis reaction (ImageQuant software; Molecular Dynamics, Sunnyvale, Calif.). Figure 4B shows that the MBP-HEL fusion protein described above is able to efficiently hydrolyze ATP to ADP and $P_i$ (Fig. 4B, panel 3). As expected, the control reactions containing either buffer alone (Fig. 4B, panel 1) or an MBP–β-Gal fusion protein (Fig. 4B, panel 2) did not hydrolyze ATP under the conditions described. From these data, we conclude that the 71-kDa polypeptide encompassing amino acids 4996 to 5592 of the HCV 229E pp1ab has ATPase activity. The NTPase activity of helicase-like proteins is typically stimulated by nucleic acids (12, 14, 16, 26, 27, 29), and the data shown in Fig. 4B demonstrate that this is also true for the ATPase activity of MBP-HEL. The addition of 0.5 mM poly(A) to the reaction mixture enhanced the ATPase activity 2.2-fold ± 0.3-fold (Fig. 4B, panel 4).

In summary, we have identified a 71-kDa polypeptide in HCV 229E-infected cells which exhibits a poly(A)-stimulated ATPase activity in vitro. The intracellular 71-kDa polypeptide contains both MBD and HEL domains. The presence of such additional zinc finger motifs has also been reported for other helicases (13, 21, 22), and it is tempting to believe that, in the case of coronaviruses, the amino-proximal domain might bind nucleic acid and function in conjunction with the helicase domain. The coronavirus helicase has been classified as belonging to the SF1 superfamily (4, 5). To date, no RNA duplex-unwinding activity has been demonstrated for any of the virus-encoded SF1-type enzymes. We hope that the system described here can be used to address the putative helicase function of the 71-kDa polypeptide. In this respect, our first goal will be to test whether additional cellular or viral proteins are required to attain helicase activity in vitro. Cofactor requirements have been reported for numerous helicases, including the eucaryotic translation initiation factor 4B (24), the herpesvirus UL5 helicase (2), and the *E. coli* RecB helicase (1, 15). Eventually, these experiments will help to elucidate the functions of an ATP-dependent helicase activity during the coronavirus replication cycle.

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