Expression, Characterization, and Immunoreactivities of a Soluble Hepatitis E Virus Putative Capsid Protein Species Expressed in Insect Cells

YIFAN ZHANG,* PATRICK M CATEE, PATRICE O. YARBOUGH, ALBERT W. TAM, and THOMAS FUERST

Genelabs Technologies, Inc., Redwood City, California 94063

Received 23 December 1996/Returned for modification 4 March 1997/Accepted 19 March 1997

The hepatitis E virus (HEV) open reading frame-2 (ORF-2) is predicted to encode a 71-kDa putative capsid protein involved in virus particle formation. When insect Spodoptera frugiperda (Sf9) cells were infected with a recombinant baculovirus containing the entire ORF-2 sequence, two types of recombinant proteins were produced: an insoluble protein of 73 kDa and a soluble protein of 62 kDa. The 62-kDa species was shown to be a proteolytic cleavage product of the 73-kDa protein. N-terminal sequence analysis of the 62-kDa protein indicated that it lacked the first 111 amino acids that are present in the full-length 73-kDa protein. A soluble 62-kDa protein was produced without the proteolytic processing by inserting the coding sequence of amino acids 112 to 660 of ORF-2 in a baculovirus expression vector and using the corresponding virus to infect Sf9 cells. The two recombinant 62-kDa proteins made by different mechanisms displayed immunoreactivities very compatible to each other. The 62-kDa proteins obtained by both proteolytic processing and reengineering demonstrated much higher sensitivities in detecting anti-HEV antibodies in human sera than the antigens made from bacteria, as measured by enzyme-linked immunosorbent assay. The data suggest that the soluble 62-kDa protein made from insect cells contains additional epitopes not present in recombinant proteins made from bacteria. Therefore, the 62-kDa protein may be useful for HEV diagnostic improvement and vaccine development. The reengineered construct allows for the consistent large-scale production of the soluble 62-kDa protein without proteolytic processing.

Hepatitis E virus (HEV) is an RNA virus (26) transmitted enterically, usually through contaminated water. Numerous HEV outbreaks have occurred in developing countries in the last several decades, resulting in tens of thousands of people being infected (3, 24, 32, 43). In most cases, the clinical syndrome of infection is manifested by acute hepatitis, and the disease usually resolves without chronicity. However, the mortality from acute HEV infection ranges from 0.5% for the general population to as high as 20% for pregnant women who become infected (15, 27, 38).

Our understanding of the molecular biology of HEV replication has been hampered by the lack of a tissue culture system for virus replication. However, molecular cloning and sequence analysis of the HEV genome (2, 11, 31, 34) have revealed some possible functions of the three open reading frames (ORFs) of this virus. ORF-1 is predicted to be a poly-cistronic protein containing five domains, including those for methyltransferase and RNA-dependent RNA polymerase (8, 17, 27, 28). ORF-2 is presumed to encode a capsid protein with a predicted molecular size of 71 kDa (2, 11, 31, 34). The function of the small polypeptide of 123 amino acids encoded by ORF-3 is not clear, but the sequence contains an immunodominant epitope recognized by acute- and convalescent-phase sera of infected patients, suggesting a potentially important function (14, 40).

Substantial progress has been made in characterization of recombinant antigens for HEV diagnostics and vaccine development. Recombinant proteins from ORF-2 of HEV have been shown to be reactive by Western blotting (immunoblotting) and enzyme-linked immunosorbent assay (ELISA) with human sera from well-defined cases of hepatitis E and with sera from primates experimentally infected with HEV (7, 10, 25, 37, 40). Immunodominant epitopes in ORF-2 and ORF-3 have been included in commercially available diagnostic ELISAs for HEV (5, 6, 13, 14, 40, 41). The establishment of an animal model of HEV infection, in cynomolgus macaques (4, 20, 33, 35), made the evaluation of vaccine candidates possible. In a previous study, immunization of cynomolgus monkeys with a truncated ORF-2 from HEV strain Burma expressed in Escherichia coli, trpE-C2, elicited a protective immune response against homologous wild-type HEV challenge (25); however, virus shedding was not prevented when vaccinated animals were challenged with a heterologous strain of HEV. In a subsequent study, a recombinant ORF-2 protein species produced in insect cells showed promising results after single and double vaccination in offering protection from HEV disease (36, 37). We have concentrated on the expression of HEV capsid antigen in eukaryotes such as in insect cells using recombinant baculovirus (21, 29) since many viral immunological epitopes such as those from the viral capsid and outer membrane are highly conformational and may require posttranslational modifications such as glycosylation (1, 12, 16, 18, 19). In this report, we describe our effort to fully characterize the recombinant HEV ORF-2 protein species produced in insect cells. Expression of the full-length ORF-2 under different conditions could produce two distinct species, an insoluble 73-kDa protein and a soluble 62-kDa protein that was dependent on a proteolytic cleavage process. The 62-kDa species demonstrated improved sensitivity in anti-HEV immunoglobulin M (IgM) detection, suggesting that it may be useful for early HEV diagnostics and vaccine development as well. A strategy for efficiently expressing this soluble immunore-
active protein without proteolytic processing was developed, making large-scale production and purification possible.

**MATERIALS AND METHODS**

**Insect cells.** Spodoptera frugiperda (Sf9) suspension cells were a generous gift from Robert Lanford of Southwest Foundation for Biomedical Research (San Antonio, Tex.). The Sf9 monolayer cell line was kindly provided by Sergei A. Tsianos of the National Institutes of Health (Bethesda, Md.). Both cell lines were maintained at 27°C in Grace's insect medium (catalog no. 11605-011; Gibco/BRL) supplemented with 5% fetal bovine serum and 50 µg of gentamicin (catalog no. 15710-015; Gibco/BRL) per ml. In addition, 0.1% Phoronic F-68 (catalog no. 40401-016; Gibco/BRL) was used in suspension cell cultures.

**Recombinant baculoviruses.** Recombinant baculovirus ORF-2- rAcNPV expressing the entire ORF-2 of the HEV Burman strain of HEV was constructed as described previously (10). For the construction of the recombinant baculovirus vBiiiH-62k for the recombinant 62-kDa (r62-kDa) protein, a 224-bp DNA fragment containing nucleotides 5480 to 5684 of the ORF-2 from the Burman strain of HEV, plus restriction endonuclease sites and a protein translation initiation codon, was synthesized by PCR with an HEV cDNA plasmid template, pBIII- ORF-2 (30), and two oligonucleotide primers, GGG-GGG-GAT-CCA-TAT-G GC-GGT-CGC-TCC-GGC-CCA-TAC-CCC-G and ATT-AGA-AGC-TT C-CTG-GGC-CAT-TAT-ATG. BamHI and Hindlll restriction endonuclease sites were incorporated into the primers to facilitate subsequent plasmid constructions. The PCR product was digested with BamHI/HindIII and was ligated to a baculovirus expression vector, pBlueBacIII (Invitrogen, San Diego, Calif.), that had been digested with the same restriction endonuclease to form the plasmid pZY1. A 1.5-kbp Hindlll DNA fragment, corresponding to the 3' portion of ORF-2 ORF-2, was excised from the plasmid pBIIIH-ORF-2 and was inserted into the plasmid pZY1 at the Hindlll site to form the final baculovirus transfer vector pBIII-62k. The vector was recombined into the genome of a wild-type baculovirus to form the recombinant baculovirus vBIII-H-62k.

**Protein purification.** For purification of the 73-kDa full-length ORF-2 protein, pellets of Sf9 baculovirus cultures infected with the recombinant baculovirus ORF-2-rAcNPV were resuspended in PBS containing protease inhibitors. The cell lysate was subjected to centrifugation in a microcentrifuge at 4°C for 15 min. The supernatant and pellet fractions were separately denatured, followed by sonication with the BICINE-SDS buffer. Dithiothreitol was added to the solution, which was then heated and diluted in a refolding buffer containing 50 mM glycine, 10% glycerol, 5 mM (each) reduced and oxidized glutathione, and 1 g of polyethylene glycol 3500 per liter. The solution was allowed to air oxidize overnight and was concentrated.

**Expression of two HEV ORF-2-derived antigens.** Rabbit anti-HEV antiserum. Expression of two HEV ORF-2-derived antigens, SG3 and 3-2(B), containing the C-terminal 327 and 48 amino acids, respectively, was described previously (41). SG3 was synthesized as a glutathione S-transferase fusion protein, while the 3-2(B) sequence was fused to a bacterial β-galactosidase gene. Polyclonal rabbit anti-sera were then generated by using purified antigens by hyperimmunization of the animals (30).

**SDS-PAGE and immunoblotting.** To prepare protein samples for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), approximately 2 x 10^6 baculovirus-infected Sf9 cells were collected by centrifugation in a microcentrifuge and were resuspended in 150 µl of phosphate-buffered saline (PBS). The cells were lysed by sonication. The cell lysate was subjected to centrifugation in a microcentrifuge at 4°C for 15 min. The supernatant and pellet fractions were separately denatured, followed by sonication with the BICINE-SDS buffer. Dithiothreitol was added to the solution, which was then heated and diluted in a refolding buffer containing 50 mM glycine, 10% glycerol, 5 mM (each) reduced and oxidized glutathione, and 1 g of polyethylene glycol 3500 per liter. The solution was allowed to air oxidize overnight and was concentrated.

**RESULTS**

**Expression of HEV ORF-2 in insect cells.** Our initial goal was to fully characterize the HEV capsid (ORF-2) protein species expressed in insect cells. Sf9 suspension cultures were infected with a previously described recombinant baculovirus expressing ORF-2 from the HEV Burman strain (10), and the cell cultures were maintained for 1 to 7 days. Infected cells were lysed by sonication in the presence of PBS, followed by a centrifugation process to separate soluble from insoluble material. Both of which were analyzed by SDS-PAGE (Fig. 1A). A prominent protein with a molecular size of approximately 73 kDa was observed by Coomassie blue staining of the insoluble fraction of the samples obtained from 2 to 7 days postinfection. This protein was later confirmed to be the full-length ORF-2 protein by tryptic peptide analysis (data not shown). The 73-kDa protein was stable over the course of infection, because no apparent degradation was observed by SDS-PAGE analysis of sequential samples (Fig. 1A).

It was previously reported, however, that several ORF-2-related smaller proteins were observed when an Sf9 monolayer cell line was infected with a recombinant baculovirus containing the full-length ORF-2 from HEV Pakistani strain SARS-4 (37). To examine the effect of different viral strains and cell lines on the formation of ORF-2 proteins, the relevant Sf9 monolayer cell line was infected with the recombinant baculovirus used in the SDS-PAGE analysis whose results are presented in Fig. 1A. A doublet of soluble proteins of approximately 62 kDa (Fig. 1B, lower arrow) was detected late in the infection by Western blot analysis with an antiserum against ORF-2. In addition, a 110-kDa β-galactosidase polypeptide...
from the baculovirus vector was also detected since the antiserum was made against the HEV 3-2(B) epitope (see Materials and Methods section) fused to a β-galactosidase gene. Comparison of serial samples obtained from 2 to 7 days postinfection showed a progressive decrease in the amount of the 73-kDa protein (Fig. 1B, upper arrow), coupled with a progressive increase in the formation of the 62-kDa protein. This observation suggested that the 62-kDa protein was generated by proteolytic processing of the 73-kDa protein.

**Proteolytic cleavage of the 73-kDa protein in vitro.** We hypothesized that the soluble p62-kDa protein might preserve some epitopes which were otherwise destroyed by detergent solubilization of the insoluble recombinant proteins made from bacteria and insect cells as well. To evaluate the stability of the p62-kDa protein for purification, we developed an in vitro cleavage assay. Cell lysates were prepared from monolayer cultures 5 days postinfection, followed by incubation for various times at 22°C before each aliquot was analyzed for the presence of soluble p62-kDa protein (Fig. 2). Progressive disappearance of the insoluble 73-kDa protein coupled with accumulation of the typical soluble p62-kDa protein doublet was readily observed. The conversion was proportional to the time of incubation. The p62-kDa protein was fairly stable because no further degradation products were observed after 5 h of incubation. This stability was important for us to develop a protein purification protocol later (23).

**Purification of the p62-kDa protein and its N-terminal sequence analysis.** The soluble p62-kDa protein from monolayer cells was purified for N-terminal sequence analysis. The final purified p62-kDa protein from a Poros HQ/F column was run on a protein gel, shown in lane 2 of Fig. 3A; it ran as a single band on this low-resolution gel. The purity of the p62-kDa protein estimated by Coomassie blue staining was at least 95%.

The purified p62-kDa protein was subjected to electrophoresis, and both bands of the doublet were transferred to a polyvinylidene difluoride membrane for N-terminal sequencing by Edman degradation. The details are described in the Materials and Methods section. (A) Coomassie blue staining of the p62-kDa protein after Poros HQ/F column purification. The DNA sequence which encodes from Ala-112 to the carboxyl end of ORF-2 was incorporated at the 5′ end to ensure the proper start of translation. After transcription and four rounds of plaque purification, viral stocks and cell lysates were prepared from 10 individual plaque isolates. Western blot analysis was performed to screen for the viral plaques that could produce a soluble 62-kDa protein (r62-kDa protein) in suspension cultures at the highest level (Fig. 4). Nine of 10 viral isolates produced a soluble 62-kDa doublet similar to that of the p62-kDa protein, indicating that the synthesis of the soluble 62-kDa protein was not dependent on the proteolytic processing. The solubility of the r62-kDa protein ranged from 50 to 100%, depending on the level of expression in each isolate. One isolate was chosen for amplification for further studies.

**Comparative immunoreactivities of insect cell-produced ORF-2 proteins.** The immunoreactivities of baculovirus-expressed ORF-2 were detected by immunoblotting with anti-3-2(B) antiserum and showed two bands, a 73-kDa protein (Fig. 1B, upper arrow) and a p62-kDa protein (lower arrow). The presence (+) or absence (−) of protease inhibitors in the preparations of cell lysate is indicated.

**Construction of a recombinant baculovirus that expresses amino acids 112 to 660 of ORF-2 in S99 suspension cultures.** The DNA sequence which encodes from Ala-112 to the carboxyl end of ORF-2 was incorporated in a baculovirus expression vector, pBlueBacIII. The corresponding recombinant virus vBIII-62k was constructed as described in the Materials and Methods section. A methionine codon was incorporated at the 5′ end to ensure the proper start of translation. After transcription and four rounds of plaque purification, viral stocks and cell lysates were prepared from 10 individual plaque isolates. Western blot analysis was performed to screen for the viral plaques that could produce a soluble 62-kDa protein (r62-kDa protein) in suspension cultures at the highest level (Fig. 4). Nine of 10 viral isolates produced a soluble 62-kDa doublet similar to that of the p62-kDa protein, indicating that the synthesis of the soluble 62-kDa protein was not dependent on the proteolytic processing. The solubility of the r62-kDa protein ranged from 50 to 100%, depending on the level of expression in each isolate. One isolate was chosen for amplification for further studies.

**Comparative immunoreactivities of insect cell-produced ORF-2 proteins.** The immunoreactivities of baculovirus-expressed ORF-2 were detected by immunoblotting with anti-3-2(B) antiserum and showed two bands, a 73-kDa protein (Fig. 1B, upper arrow) and a p62-kDa protein (lower arrow). The presence (+) or absence (−) of protease inhibitors in the preparations of cell lysate is indicated.
pressed ORF-2 proteins were examined in an ELISA format with HEV-seropositive human serum samples (Tables 1 to 3). An E. coli-produced HEV ORF-2 antigen (SG3) containing 332 amino acids of the C terminus of ORF-2 (41) was included for comparison. This protein was previously observed to contain broadly immunoreactive epitopes. In reactions in which equal molar amounts of HEV antigens were coated onto ELISA plates, greater reactivity was seen with the p62-kDa protein than with the SG3 antigen or the 73-kDa protein (Table 1). The limited quantities of the human sera in this panel prevented us from further defining the relative reactivities of each antigen by an endpoint titer assay, but results of this type of assay are presented in Table 3. The 62-kDa protein reacted with low-level antibodies to HEV in some sera that would have gone undetected by using the SG3 antigen (Table 1). The difference in sensitivity between the two antigens was generally more significant in anti-HEV IgM reactions (Table 3). Despite the lower immunoreactivities seen with the 73-kDa protein, we could demonstrate that the freshly purified 73-kDa protein preparations could have increased immunoreactivities sometimes close to that seen with the p62-kDa protein. Freezing and thawing, however, greatly reduced the immunoreactivities of the refolded 73-kDa protein, although the protein remained intact, as examined by Coomassie blue staining. This temperature lability was observed only with the 73-kDa protein.

The r62-kDa protein from the reengineered construct performed nearly identically to the processed one or the p62-kDa protein in detecting HEV IgG (Table 2), suggesting the conformational and immunological similarities of the two proteins. A small subset of HEV-positive human sera were serially diluted and assayed to further define sensitivity of the r62-kDa antigen (Table 3). The endpoint ELISA showed that the r62-kDa protein exhibited increased sensitivity, sometimes up to 30-fold, of anti-HEV IgM detection, compared to the sensitivities of detection of currently used HEV antigens such as SG3. This improvement could also be confirmed with sera from experimentally infected primates (data not shown).

**DISCUSSION**

Our objectives in this study were threefold: (i) to fully characterize the HEV ORF-2 protein species expressed in insect cells, (ii) to identify the most promising candidate in terms of immunoreactivity, and (iii) to design a process to express this candidate protein in a large-scale format for diagnostic assays and vaccine development. Several laboratories have reported expression of antigenic HEV ORF-2 proteins in insect cells. However, results have varied with respect to the size and solubility of recombinant proteins. In the present study, we observed two ORF-2 protein products in SF9 cells of different origins: the insoluble 73-kDa protein produced in a suspension cell line and the soluble p62-kDa protein from another cell line grown primarily as monolayers. Why was the 73-kDa protein cleaved (in vivo) in one cell line but not in the other is yet to be determined. However, we have determined that (i) the protease(s) for the cleavage of the 73-kDa protein to the 62-kDa protein came from baculovirus rather than host cells and (ii) a switch between suspension and monolayer growth in both cell lines had no effect on the size of the recombinant protein (data not shown). Therefore, the source of cell line is apparently very important for this cleavage.

**TABLE 1. Comparative ELISA analysis of human HEV-seropositive samples with three antigens, the SG3 antigen and the p62-kDa and the 73-kDa proteins**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anti-HEV response (OD_{490}) of the following antibodies to the indicated antigen:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SG3 antigen p62-kDa protein</td>
<td>SG3 antigen 73-kDa protein</td>
<td>p62-kDa protein 73-kDa protein</td>
</tr>
<tr>
<td>Borneo S89</td>
<td>0.237 2.527 0.356</td>
<td>0.895 2.579 1.077</td>
<td></td>
</tr>
<tr>
<td>MBOQ283</td>
<td>0.123 0.892 0.147</td>
<td>2.602 2.771 2.558</td>
<td></td>
</tr>
<tr>
<td>Sudan 54</td>
<td>0.532 2.652 0.754</td>
<td>2.159 2.717 2.310</td>
<td></td>
</tr>
<tr>
<td>Som 053</td>
<td>1.342 2.560 1.283</td>
<td>1.930 2.386 1.539</td>
<td></td>
</tr>
<tr>
<td>FVH26</td>
<td>0.077 0.319 0.069</td>
<td>0.379 1.353 0.571</td>
<td></td>
</tr>
<tr>
<td>Som 458</td>
<td>0.279 1.525 0.248</td>
<td>0.206 2.441 0.257</td>
<td></td>
</tr>
</tbody>
</table>

*a All three antigens were subjected to freezing and thawing. For details see Materials and Methods section.
*b OD_{490}, optical density at 490 nm.

**TABLE 2. Comparison of the immunoreactivities of the r62-kDa, p62-kDa, and the 73-kDa proteins in seropositive samples from regions where HEV is endemic**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anti-HEV IgG response (OD_{490}) to the following:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r62-kDa protein</td>
<td>p62-kDa protein</td>
<td>73-kDa protein</td>
</tr>
<tr>
<td>ABTD80</td>
<td>2.229 2.212</td>
<td>2.212 0.344</td>
<td></td>
</tr>
<tr>
<td>Mex F387</td>
<td>2.635 2.664</td>
<td>2.664 1.069</td>
<td></td>
</tr>
<tr>
<td>ABTD 86</td>
<td>2.399 1.689</td>
<td>1.689 0.309</td>
<td></td>
</tr>
<tr>
<td>MB 2/88</td>
<td>1.961 2.083</td>
<td>2.083 0.724</td>
<td></td>
</tr>
</tbody>
</table>

*a OD_{490}, optical density at 490 nm.

**TABLE 3. ELISA endpoint titers of HEV-seropositive human sera against the SG3 antigen and the r62-kDa and 73-kDa proteins**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anti-HEV titer of the following antibodies to the indicated antigen:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SG3 antigen r62-kDa protein 73-kDa protein</td>
<td>SG3 antigen r62-kDa protein 73-kDa protein</td>
<td></td>
</tr>
<tr>
<td>F387</td>
<td>1:102,400 1:102,400 1:204,800</td>
<td>&lt;1:100 1:160 1:400</td>
<td></td>
</tr>
<tr>
<td>KS</td>
<td>1:25,600 1:102,400 1:102,400</td>
<td>1:100 1:800 1:400</td>
<td></td>
</tr>
<tr>
<td>AZ</td>
<td>1:3,200 1:51,200 1:3,200</td>
<td>1:400 1:12,800 1:3,200</td>
<td></td>
</tr>
</tbody>
</table>

*a See Materials and Methods section for details.
kDa protein to the soluble p62-kDa protein by the proteolytic cleavage suggested that the primary sequence of the p62-kDa protein might be a determining factor for its solubility. Therefore, we were concerned whether (i) modification of the p62-kDa protein sequence by the inclusion of an initiating methionine and (ii) the unique expression of this coding sequence outside the context of the full-length protein would produce a protein with the same desirable physical and immunogenic characteristics shown by the p62-kDa protein. To test this hypothesis, the soluble p62-kDa protein was purified by near homogeneity followed by N-terminal sequencing. The cleavage site that generated the p62-kDa protein from the 73-kDa protein was found between residues Thr-111 and Ala-112 in ORF-2. The truncated ORF-2 was inserted in a baculovirus expression vector to generate the recombinant virus vBIII-62k. Interestingly, the r62-kDa protein produced on the basis of this expression strategy indeed demonstrated all the properties of the 62-kDa protein that was obtained by cleavage. This approach to resolving the problem of insolubility of recombinant proteins produced in insect cells may be extended to other cases in the future.

There are two explanations for the significant increase in the sensitivity of anti-HEV IgM detection in human serum samples with our newly engineered 62-kDa protein compared to that of the 62-kDa protein of ORF-2. The truncated ORF-2 was inserted in a baculovirus expression vector to generate the recombinant virus vBIII-62k. Interestingly, the r62-kDa protein produced on the basis of this expression strategy indeed demonstrated all the properties of the 62-kDa protein that was obtained by cleavage. This approach to resolving the problem of insolubility of recombinant proteins produced in insect cells may be extended to other cases in the future.

In several studies in which more human sera were assayed, we found that the specificity of the r62-kDa protein was not compromised, despite the increased sensitivities of anti-HEV antibody detection (data not shown).

The soluble r62-kDa protein should be suitable for the development of an anti-HEV IgM assay as well as for HEV vaccine development. Indeed, preclinical testing indicated that the r62-kDa antigen was capable of eliciting protective immunity in cynomolgus macaques against a very stringent heterologous challenge with 1,000 to 10,000 cynomolgus monkey 50% infectious doses of the Mexico isolate of HEV (9,39).

ACKNOWLEDGMENTS

We gratefully acknowledge Robert Lanford and Sergei Tsarev for providing Sf9 cells; Q. Min, T. Bird, and E. Garza for technical assistance; and J. Lisfe for critical review of the manuscript.

REFERENCES


30. Tam, A. W. Unpublished data.


