Differential Subcellular Localization of Hepatitis C Virus Core Gene Products

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The expression of the core gene of two different hepatitis C virus (HCV) isolates was analyzed. In the presence of its downstream E1 envelope protein sequence, two major core protein products with molecular masses of 21 kDa (P21) and 19 kDa (P19) and a minor protein product with molecular mass of 16 kDa (P16) were detected. In the absence of its downstream E1 envelope protein sequence, P21 and P19 remained the major protein products expressed from the core gene of the HCV-RH isolate, whereas P16 became the major protein product of the core gene of the HCV-1 isolate. Analysis of the amino-terminal sequences of P21 and P16 expressed in Escherichia coli revealed that P21 and P16 were co-amino terminal. Deletion-mapping analysis indicated that P16 lacked the carboxy-terminal sequence of P21. Immunofluorescence analysis of the subcellular localization of different HCV core proteins indicated that P21 and P19 displayed a reticular and punctate staining pattern typical of endoplasmic reticulum-associated proteins, while P16 was localized to the nucleus. The distinct subcellular localization of P16 raises the possibility that P16 may have a biological function very different from those of P21 and P19.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus with a genome size of almost 10 kb and belongs to the Flaviviridae family (Francki et al., 1991). HCV is one of the major causes of viral hepatitis and over 50% of the patients infected with HCV become chronic carriers (Kuo et al., 1989). Many of these patients have chronic active hepatitis and will develop liver cirrhosis. This virus is also a major cause of hepatocellular carcinoma (Saito et al., 1990).

The HCV genome has a long open reading frame which encodes a polyprotein with a length of approximately 3010 amino acids (Choo et al., 1991). This polyprotein is processed into at least 10 distinct gene products, which are arranged in the linear order NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Grakoui et al., 1993b). The C gene, which is located at the amino terminus of the polyprotein sequence, encodes the core (capsid) protein. The sequence of the core protein is followed by the sequences encoding E1 and E2 envelope proteins. p7 is located at the carboxy terminus of E2 (Lin et al., 1994). This protein is not always cleaved from the E2 sequence. NS2-NS5 are nonstructural proteins (Failla et al., 1994; Grakoui et al., 1993a; Takamizawa et al., 1991).

The amino-terminal sequence of the E1 protein has been mapped to amino acid 192 (Hijikata et al., 1991) and, thus, the core protein is expected to be 191 amino acids in length. The core protein of this size (P21) has been detected in expression experiments both in vitro (Hijikata et al., 1991; Lo et al., 1994) and in vivo (Harada et al., 1991). The carboxy terminus of the core protein sequence contains two hydrophobic domains (Takamizawa et al., 1991). The first hydrophobic domain is located immediately upstream of the E1 envelope protein sequence and is believed to be the signal sequence for translocation of the E1 envelope protein across the membrane of the endoplasmic reticulum (ER). This domain comprises approximately the sequence from amino acid 170 to amino acid 191. The second hydrophobic domain comprises approximately the sequence from amino acid 121 to amino acid 151. In addition to P21, a core protein approximately 173 amino acids in length (P19) has been detected (Santolini et al., 1994). This protein has apparently lost most of the first hydrophobic domain located between amino acids 170 and 191. The conversion of P21 to P19 in vitro requires the presence of microsomal membranes and is presumably mediated by membrane-associated cellular enzymes (Santolini et al., 1994). Both P21 and P19 are associated with the ER membrane. The role of these two different core protein products in the assembly of the HCV virion, however, remains unclear.

In addition to P21 and P19, we have recently identified a third HCV core protein product approximately 16 kDa in size (Lo et al., 1994). This protein, which we termed P16, is produced by the core protein sequence of the HCV-1 isolate in translation experiments in vitro. The
synthesis of P16 was not detected when the core protein sequence of another HCV isolate, HCV-RH, was used for the in vitro translation experiments (Lo et al., 1994). Further analysis revealed that the synthesis of P16 was due to a single nucleotide substitution which converted codon 9 of the HCV-1 core protein sequence from an arginine codon (Arg-9) to a lysine codon (Lys-9) (Lo et al., 1994). Since fusing a leader sequence to the 5'-end of the HCV core protein coding sequence increases the size of P16 correspondingly, P16 likely resides in the amino terminus of the core gene sequence (Lo et al., 1994).

In order to investigate the functions of various HCV core proteins, we have studied the expression of HCV core proteins in CV1 cells, a cell line derived from monkey kidney. Our results revealed that a small amount of P16 can also be produced by the core gene carrying the Arg-9 codon. In addition, our results obtained from in vitro translation experiments and from sequencing of P16 and P21 expressed in Escherichia coli indicate that P16 and P21 are co-amino terminal. Our subcellular localization experiments revealed that, in contrast to P21 and P19, which are associated with the ER membranes, P16 is a nuclear protein. This distinct subcellular localization of P16 raises the possibility that it may have a function different from those of P21 and P19.

**MATERIALS AND METHODS**

**Construction of DNA plasmids**

pET-RC and pET-CC. The isolation of HCV-RH and HCV-1 cDNA clones has been described previously (Choo et al., 1991; Lo et al., 1994). The core protein coding sequence of either HCV-RH or HCV-1 isolate was isolated from the cDNA clones by the polymerase chain reaction (PCR) using the primers 5' AGA CCG TGC CAT ATG AGC ACG AAT 3' (sense) and 5' GAG TGG CGG AAT TCT TAG GCC GAA 3' (antisense). After blunt-ending the PCR product was cloned into the BamHI (blunt-ended) and Ndel site of a pET3a-derived vector (Rosenberg et al., 1987). The resulting plasmid pET-RC and pET-CC contained the complete core protein coding sequence (a.a. 1–191) of the HCV-RH and the HCV-1 isolate, respectively. All of the HCV cDNA clones derived from PCR were sequenced to ensure that no nucleotide mutations were created.

pCMV-RC and pCMV-CC. The HCV core protein coding sequence as well as 94 nt of its upstream 5' untranslated sequence were isolated by PCR using the primers 5' CTG GCC CCC GCA AGC TTT CTA G 3' (sense) and 5' GAG TGG CGG AAT TCT TAG GCC GAA 3' (antisense). The PCR product was blunt-ended with the Klenow fragment, digested with HindIII, and inserted into the XbaI (blunt-ended)–HindIII site of pRC/CMV vector (Invitrogen). pCMV-RC and pCMV-CC contained the entire core protein coding sequence (a.a. 1–191) of the HCV-RH and the HCV-1 isolate, respectively.

pCMV-RCEβ and pCMV-CCEβ. The core protein coding sequence and its downstream E1 envelope protein sequence were isolated by PCR using the sense primer 5' ACA TTT GCT TCT GAC ACA ACT GTG TTC ACT AGC AAC CTC AAA CAG ACA CCA TGA GCA CGA AT 3' and the antisense primers 5' TCA TTG CAG TTC AGG GC 3' (HCV-RH) or 5' GAC AAA TCT AGA CAC AG 3' (HCV-1). For the HCV-RH isolate, the PCR product was cloned into the Smal site of pUC18 to generate pUC-RCEβ. The HCV sequence was reisolated from pUC-RCEβ with HindIII and EcoRI double digestion and inserted into the NotI–HindIII site of pRC/CMV. The resulting plasmid pCMV-RCEβ contained the entire coding sequence of the HCV-RH core protein and its downstream E1 envelope protein from nucleotide 1 to 1292 (a.a. 1–430). For the HCV-1 isolate, the PCR product was digested with XbaI and cloned directly into the HindIII (blunt-ended)–XbaI site of pRC/CMV. This plasmid contained the entire coding sequence of the HCV-1 core protein and its downstream E1 envelope protein from nucleotide 1 to 1191 (a.a. 1–397). Both pCMV-RCEβ and pCMV-CCEβ also contained partial E2 envelope protein sequence. We have noticed in the past that the length of the E2 sequence did not affect the expression of the core protein (data not shown). In pCMV-RCEβ and pCMV-CCEβ, the 5' untranslated sequence of HCV had been replaced with the 5' untranslated sequence of human α-globin mRNA (Liebhaber et al., 1980). We have found that the α-globin mRNA leader sequence could increase the translation efficiency of the HCV sequence both in vitro and in vivo. This leader sequence has no effect on the expression pattern of the core gene.

pCMV-RC151. This plasmid contained the HCV-RH core protein sequence from a.a. 1–151. This truncated HCV-RH core protein sequence was isolated by PCR using the sense primer 5' ACA TTT GCT TCT GAC ACA ACT GTG TTC ACT AGC AAC CTC AAA CAG ACA CCA TGA GCA CGA AT 3' and the antisense primer 5' TGG TCT AGA CTA CAG GCC CTC GGT AGC 3'. The PCR product was digested with XbaI and inserted into the HindIII (blunt-ended)–XbaI site of pRC/CMV. The 5' untranslated sequence of HCV in this plasmid has been replaced with the 5' untranslated sequence of human α-globin mRNA.

pCMV-RC127. This plasmid contained the HCV-RH core protein sequence from a.a. 1–127. This truncated HCV-RH core protein sequence was isolated by PCR using the sense primer 5' CGT GCC CCC GCA AGC TTT CTA G 3' (sense) and the antisense primer 5' GTC TGC CTA G 3'. The PCR product was digested with HindIII and inserted into the XbaI (blunt-ended)–HindIII site of pRC/CMV. This plasmid contained the truncated HCV-RH core protein sequence as well as 94 nucleotides of its upstream 5' untranslated sequence.
Amino-terminal sequencing of HCV core proteins expressed in E. coli

The E. coli BL21(DE3) cells transformed by pET-RC, pET-CC, or the control plasmid pET3a were grown to the stationary phase. A 5-ml culture of these stationary phase cells was inoculated into a fresh 500-ml culture and grown to OD 0.6. IPTG was then added to the culture to a final concentration of 1 mM. After a further incubation at 37°C for 3 hr, the E. coli cells were pelleted by centrifugation. The pellet was dissolved in Laemmli buffer, boiled, and loaded on an SDS-PAGE gel. After electrophoresis, P21 and P16 core proteins were passively eluted from the gel in 30 mM NH₄HCO₃ and 0.1% SDS. The protein was acetone precipitated with the method of Konigsberg and Henderson (1983). The sequence analysis was performed by Edman degradation using Applied Biosystems 470A and 473A protein sequencers.

The HCV core proteins prepared this way were also used for preparation of antibodies in rabbits for Western blot analysis.

Carboxy-terminal truncation experiments

The HCV-1 core gene sequence was truncated by PCR. The truncation at a.a. 164 was conducted using these two primers: 5’ CGT GCC CCC GCA AGC TTG CTA G3’ (sense) and 5’ GTT CCC GGA TCC ATA GTT CAC3’ (antisense). The truncation at a.a. 179 was conducted using the same sense primer and the following antisense primer: 5’ GCA AGA GAG GGA TCC CAG AAG GAA3’

Similarly, the truncation at a.a. 193 was conducted using the same sense primer and the following antisense primer: 5’ GGA GTT GGA TCC TTG GTA GGC CGA3’. The PCR products were inserted into the plasmid vector pRc/CMV. The plasmids were linearized with BamH1 and used for RNA synthesis using the T7 RNA polymerase. The synthesized RNA was then translated using rabbit reticulocytes using our previous procedures (Lo et al., 1994).

Expression of HCV core protein in CV1 cells

CV1 cells were maintained in Dulbecco’s modified Eagle’s medium (DME) containing 5% fetal bovine serum (BSA). The cells were infected with a recombinant vaccinia virus carrying the T7 phage RNA polymerase gene (Fuerst et al., 1986). During infection, cells were incubated in DME containing 1% FBS. Two hours postinfection, cells were transfected with DNA plasmids using the DOTAP reagent (Boehringer-Mannheim) in DME containing 5% FBS. Sixteen to 18 hr after transfection, the cells were lysed with Laemmli buffer and the samples were electrophoresed on an SDS-PAGE gel for Western blot analysis using the rabbit antibody directed against the HCV core proteins expressed in E. coli.

Immunofluorescence analysis

CV1 cells infected with the recombinant vaccinia virus and transfected with the HCV core protein expression plasmid were fixed with −20°C acetone for 2 min. The rabbit anti-core, diluted 1:200 in PBS containing 1% BSA, 0.05% sodium azide and 0.02% saponin, was used as the primary antibody. The secondary antibody used was rhodamine-conjugated goat anti-rabbit.

RESULTS

Expression of the HCV core proteins in CV1 cells

The HCV sequence containing the core gene and its downstream E1 envelope protein gene was placed under the expression control of the T7 phage promoter. The resulting DNA plasmid was then transfected into CV1 cells which had previously been infected with recombinant vaccinia virus carrying the T7 phage RNA polymerase gene. The expression of the core protein was then analyzed by Western blot. As shown in Fig. 1A, lane 1, expression of the core gene of the HCV-1 isolate, which carried the Lys-9 codon, led to synthesis of a major core protein product approximately 20 kDa and a minor core protein product 16 kDa. The 20-kDa protein band comigrated with the HCV core protein truncated at amino acid 172 and is presumably mostly P19.
envelope protein sequence, P16 became the major HCV-1 core gene products.

Amino-terminal sequencing of HCV core proteins

We have previously found that linking a leader sequence to the 5'-end of the HCV-1 core gene coding sequence would increase the size of P16 correspondingly (Lo et al., 1994). This result indicated that the sequence of P16 most likely initiated from the 5'-end of the HCV-1 core protein coding sequence. To further investigate this possibility, we decided to directly determine the amino-terminal sequences of both P16 and P21. In order to obtain a sufficient amount of the core protein for the sequencing experiment, we used E. coli as the host for expressing the core proteins. As shown in Fig. 3A, similar to our previous in vitro translation results and the expression results obtained from mammalian cells (Fig. 2), a protein slightly larger than the 18-kDa molecular weight marker was expressed from the HCV-RH core protein sequence. This protein had an electrophoretic mobility similar to P21 synthesized in vitro (data not shown) and is presumed to be P21. Lack of P19 expression in E. coli might be due to the absence of eukaryotic enzymes required for the conversion of P21 to P19 (Santolini et al., 1994). In contrast, a 16-kDa protein (P16) was the predominant core protein expressed from the HCV-1 sequence. Both P21 and P16 were purified by gel electrophoresis and subjected to amino-terminal sequencing. As shown in Table 1, the sequences of both P21 and P16 initiated from the second codon of their respective

(data not shown). However, as will be discussed below, P21 was also often detected in our expression experiments. The above result is consistent with our previous in vitro studies which indicated that P19/P21 and P16 could both be expressed from the HCV core gene sequence in the presence of its downstream E1 envelope protein sequence (Lo et al., 1994). The expression of the HCV-RH core gene, which carried the Arg-9 codon, was also analyzed. As shown in Fig. 1A, lane 2, this expression led to predominant synthesis of P19/P21. Interestingly, a small but detectable amount of a 16-kDa protein was also detected when the expression level of the HCV-RH core proteins in CV1 cells was elevated (Fig. 1A, lane 3). This protein, which had an electrophoretic mobility similar to that of P16, is likely the P16 homologue (Fig. 1B). Thus, the expression of P16 is not limited to the HCV core gene that carries the Lys-9 codon, although apparently this Lys-9 codon significantly enhances the expression of P16.

The expression of the HCV core gene was also analyzed in the absence of its downstream E1 envelope protein sequence. As shown in Fig. 2, lane 1, the expression of the HCV-RH core gene by itself led to the predominant synthesis of P21 and P19. On the other hand, the expression of the HCV-1 core gene by itself led to the predominant synthesis of P16 (Fig. 2, lane 4). These results are consistent with our previous data obtained from in vitro translation studies (Lo et al., 1994). The P16 core protein had an electrophoretic mobility similar to that of an HCV-RH core protein truncated at amino acid 151 (Fig. 1, lane 3). Thus, P16 of both the HCV-1 and HCV-RH isolates expressed in CV1 cells are estimated to be about 151 amino acids.

Thus, our results indicate that P16 could be expressed from the HCV-1 core gene sequence in either the absence or the presence of the downstream E1 envelope protein sequence. In the presence of the downstream E1 envelope protein sequence, P21/P19 was the major core gene product; however, in the absence of the E1

FIG. 2. Expression of the HCV core gene in the absence of its downstream E1 envelope protein sequence in CV1 cells. Lane 1, cells transfected with the HCV-RH sequence (pCMV-RC); lane 2, cells transfected with the control DNA plasmid (pRC/CMV); lane 3, cells transfected with a truncated HCV-RH sequence with a length of 151 amino acids (pCMV-RC151); lane 4, cells transfected with the HCV-1 sequence (pCMV-CC).

FIG. 3. Characterization of HCV core proteins. (A) Expression of HCV core proteins in E. coli. Details of the expression procedures are described in Materials and Methods. The E. coli BL21(DE3) cells transformed by pET-3a (lane 1), pET-RC (lane 2), or pET-CC (lane 3) were grown to the stationary phase. A 5-ml culture of these stationary phase cells was inoculated into a fresh 500-ml culture and grown to OD 0.6. At this time, IPTG was added to the culture to a final concentration of 1 mM. After further incubation at 37°C for 3 hr, the E. coli cells were pelleted by centrifugation. The pellet was dissolved in Laemmli buffer and the solution boiled and loaded on a SDS-PAGE gel. The gel was then stained with Coomassie blue. (B) Truncation analysis of the HCV-1 core protein sequence. The HCV sequence was truncated at different locations by PCR. Details for HCV RNA synthesis using T7 RNA polymerase and for translation using rabbit reticulocyte lysates have been described before (Lo et al., 1994). The arrowhead marks the location of P16.
DIFFERENTIAL LOCALIZATION OF HCV

TABLE 1
Amino-Terminal Sequences of P21 and P16 Core Proteins
Expressed in E. coli

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>HCV-1</td>
<td>M-S-T-N-P-K-P-Q-K_9-K-N-K-R-N-T-N</td>
</tr>
<tr>
<td>P16</td>
<td>X-T-N-P-K-P-Q-K_9-K-N-K-R-N-T-N</td>
</tr>
</tbody>
</table>

*Details of the experimental procedures are described in Materials and Methods. The sequence analysis was performed by Edman degradation using Applied Biosystems 470A and 473A protein sequencers.

The first cycle of Edman degradation released a mixture of several amino acids including serine. Since the identity of the first amino acid was not clear, it was indicated with an X. Note that, based on the sequence obtained, the X residue would correspond to the second amino acid of the core protein coding sequence. The locations of Arg-9 and Lys-9 were marked with the number. The single-letter code for the amino acids is used.

The sequences of HCV-RH and HCV-1 were deduced from their respective nucleotide sequences. The loss of the initiator methionine residue from P21 and P16 may be due to the amino-terminal peptidase activity present in E. coli cells.

Thus, the results shown in Table 1 are consistent with our previous in vitro translation studies and indicate that P21 and P16 are co-amino terminal. If this is indeed the case, then P16 should lack the carboxy-terminal sequence of P21. To investigate this possibility, we have performed the carboxy-terminal truncation experiment. As shown in Fig. 3B, truncation of the carboxy-terminal sequence up to amino acid 164 did not affect the expression of P16. The slower migrating protein band seen in each lane presumably represents the full-length run-off translation product. Thus, this result indicated that P16 indeed lacked the carboxy-terminal sequence of P21. We have attempted to determine the carboxy-terminal sequence of P16 expressed in E. coli by direct sequence analysis. Our results show heterogeneity in the sequence (data not shown). Thus, we have not been able to localize precisely the carboxy terminus of P16. The heterogeneity of the carboxy-terminal sequence of P16 could explain why the P16 core protein expressed in different systems, including the reticulocyte lysates, E. coli and CV1 cells, had similar but not identical electrophoretic mobilities (data not shown).

Subcellular localization of the HCV core proteins

P21 and P19 have previously been reported to be associated with the membranes of the endoplasmic reticulum (Santolini et al., 1994). This result was confirmed by immunofluorescence staining experiment. As shown in Fig. 4a, the core proteins of the HCV-RH isolate expressed in the presence or absence of its downstream E1 envelope protein sequence displayed a reticular and punctate immunofluorescence staining pattern typical of ER-associated proteins. The HCV-1 core proteins also displayed a similar subcellular localization when the gene was expressed in the presence of its downstream E1 envelope protein sequence (Fig. 4b). Interestingly, when the HCV-
1 core gene sequence was expressed in the absence of its downstream E1 envelope sequence in CV1 cells, the core protein was localized predominantly to the nucleus, with an apparent enrichment in the nucleoli (Fig. 4c). Since HCV-1 core gene expressed in the absence of its downstream E1 envelope protein gene sequence will produce predominantly P16 (Fig. 2), this result indicates that P16 is a nuclear protein. This speculation is supported by the observation that the HCV-RH core protein truncated at amino acid 151 is also localized predominantly to the nucleus (Fig. 4e). Note that while P16 is also expressed from the HCV-1 core gene in the presence of the E1 envelope protein sequence, no significant nuclear staining of the core protein was detected in Fig. 4b. This may be due to the reduced level of P16 expression in the presence of the E1 protein sequence and/or the bright cytoplasmic staining signal which obscured the nuclear core protein signal.

As shown in Fig. 4g, the HCV-RH core protein truncated at amino acid 127 also had a similar nuclear localization pattern. This result indicates that, if a nuclear localization signal is present in the HCV core protein sequence, it would likely reside upstream of amino acid 128.

**DISCUSSION**

In this report, we have demonstrated that a truncated HCV core protein of 16 kDa is produced from the HCV-1 core gene sequence. This core protein, which we termed P16, is apparently co-amino terminal with the full-length core protein P21. A protein of a similar electrophoretic mobility is also produced by the core gene of the HCV-RH isolate, albeit at a much lower level. This protein is likely the P16 homologue. Its low level of expression may be the reason why this protein was not detected by other laboratories in the past. In any case, our results confirm our previous observation that the Lys-9 codon in the core gene sequence plays an important role in enhancing the expression of P16 (Lo et al., 1994).

P16 was the major core protein product when the HCV-1 core gene was expressed in the absence of its downstream E1 envelope protein sequence (Fig. 2). However, P21/P19 was the major core gene product when the HCV-1 core gene was expressed in the presence of its downstream E1 sequence (Fig. 1). How the E1 envelope protein sequence affected the expression of the HCV-1 core gene is unclear. It is likely that the E1 envelope protein sequence might have affected the conformation of the nascent core protein during translation and reduced the efficiency of proteolytic processing required for the expression of P16.

While P21 and its derivative P19 display a reticular and punctate cytoplasmic localization typical of ER-associated proteins (Santolini et al., 1994; also see Fig. 4a), P16 displays a nuclear and nucleolar staining pattern (Fig. 4c). Since P19 was estimated to be 173 amino acids in length, the core protein sequence between amino acids 151 and 173 must be very important for membrane association. It is possible that this sequence is important for exposing the second hydrophobic domain located between amino acids 121 and 151 for P19 to become associated with the ER membranes.

It has been reported that the HCV core protein expressed in Huh-7 hepatoma cells is localized predominantly in the cytoplasm 2 days after DNA transfection and in the nucleus 6 days after DNA transfection (Shih et al., 1993). By using Huh-7 cells as the host cells for the expression experiment, we have not been able to observe nuclear localization of the core protein of the HCV-RH isolate even 6 days after DNA transfection. It is likely that the difference in subcellular localization of the HCV core protein observed by the two different laboratories is due to the difference of HCV isolates used in the experiments. It will be interesting to determine whether the nuclear core protein detected by Shih et al. (1993) was predominantly P16.

The observation that P16 may be enriched in the nucleolus is interesting. It has been reported that the HCV core protein can bind to the ribosomes (Santolini et al., 1994). Thus, this nucleolar localization of P16, if it is confirmed, may be due to its ability to bind to ribosomes which are assembled in the nucleolus. The biological function of P16 remains unclear. However, its distinct localization in the nucleus and the nucleolus suggests that it may have a biological function very different from those of P21 and P19.

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