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Interaction between Hepatitis C Virus Core Protein and E1 Envelope Protein

SHIH-YEN LO,[1]† MARK J. SELBY,[2] AND JING-HSIUNG OU[1]*

Department of Molecular Microbiology and Immunology, University of Southern California, Los Angeles, California 90033,[1] and Chiron Corporation, Emeryville, California 94608[2]

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Hepatitis C virus has three structural genes named C, E1, and E2. The C gene encodes the core (capsid) protein and the E1 and E2 genes encode the envelope proteins. In an immunoprecipitation experiment, the E1 protein was found to be precipitated by an anti-core antibody in the presence but not in the absence of the core protein, indicating that the E1 protein can interact with the core protein. This interaction is independent of whether the E1 and the C genes are linked in *cis* or separated in different DNA constructs for expression. The interaction between the core and the E1 proteins is confirmed by the observation that a hybrid protein derived from the core protein and the tissue plasminogen activator is localized in the nucleus in the absence of the E1 protein and in the perinuclear region in the presence of the E1 protein. Deletion-mapping studies indicate that the carboxy-terminal sequences of both the core and the E1 proteins are important for their interaction. Since little E1 sequence is exposed on the cytosolic side of the membrane of the endoplasmic reticulum, the interaction between the core and the E1 proteins most likely takes place in the endoplasmic reticulum membrane. The E2 protein could not be coprecipitated with the core protein by the anti-core antibody in a similar assay and likely does not interact with the core protein. The implications of these findings on the morphogenesis of the hepatitis C virus virion are discussed.

Hepatitis C virus (HCV) belongs to the flavivirus family. This virus is an enveloped, positive-stranded RNA virus with a genome size of approximately 9 to 10 kb. In addition to being the major cause of posttransfusion viral hepatitis, HCV is also causally linked to hepatocellular carcinoma (18). The HCV genome encodes a polyprotein with a length of over 3,000 amino acids. This polyprotein is proteolytically cleaved to at least 10 viral gene products, which are arranged in the order of NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (7, 10, 21).

The C (core) protein is the capsid protein. this protein is separated from its downstream E1 envelope protein sequence by the signal peptidase located in the lumen of the endoplasmic reticulum (ER) (7, 8). Since the amino terminus of the E1 envelope protein has been directly sequenced and identified as amino acid (aa) 192 of the polyprotein sequence (8, 17), the carboxy terminus of the core protein is presumably located at aa 191. A core protein of this size (P21) has been detected in expression studies both in vitro and in vivo (8, 13). Recent studies, however, revealed that this 191 aa core protein (P21) could undergo a secondary cleavage to generate a truncated core protein approximately 173 aa in length (19). This truncated core protein (P19), which lacks the carboxy terminus of the P21 core protein, is the major C gene product produced in mammalian cells in expression studies (12). Besides the P21 and P19 core proteins, a third minor core protein product named P16 was also detected in the expression studies in mammalian cells (12). P16 shares the same amino-terminal sequence with P21 and P19 and is approximately 151 aa in length (12). The function of this P16 core protein in the life cycle of HCV, however, remains unclear. While P21 and P19 are peripherally associated with the ER membrane (12, 19, 21), P16 has been found to localize predominantly in the nuclei of cells (12).

E1 and E2 are the envelope glycoproteins of HCV. Similar to the core protein, E1 and E2 are released from the rest of the polyprotein sequence by signal peptidase cleavage (7, 8). The carboxy termini of E1 and E2 envelope proteins have been identified at aa 383 and aa 746, respectively (8, 16). The p7 protein follows E2 and is sometimes expressed as a carboxy-terminal extension of E2 (11, 16, 22). The next protein, NS2, is a transmembrane protein that possesses an autoprotease activity (6, 10, 20). NS3 to NS5 are nonstructural proteins involved in HCV replication and polyprotein processing.

Despite the cloning of its genome, morphogenic studies of the HCV virion have been hampered by the low levels of HCV particles in the sera of patients and by the lack of a cell culture system for efficient propagation of this virus. Nevertheless, substantial information has been obtained by studying the expression of various HCV genes in mammalian cells and in cell-free systems. Through these studies, it has been found that the core proteins can form homodimers (reference 14 and unpublished observation). These core protein dimers may be the fundamental unit for formation of the HCV capsid particles. E1 and E2 envelope proteins have also been found to interact with each other to form a heterodimer (3, 7, 15, 17). In addition, the E2 envelope protein has also been found to interact with the NS2 protein, indicating that the assembly complex of the HCV virion may be juxtaposed to the replication complex of the virus in cells (15, 22).

Since HCV is an enveloped virus, its maturation may require the interaction between its core protein and envelope proteins. In order to investigate this possibility and to understand the morphogenesis of the HCV virion, we have decided to study the possible interactions between the HCV core protein and the two HCV envelope proteins. Our results indicate that the HCV core protein can interact with the E1 protein but not with the E2 protein. The C-E1 interaction is independent of whether the two genes that encode these proteins are linked in

* Corresponding author.
† Present address: Department of Medical Technology, Tzu-chi College of Medicine, Hualien, Taiwan.

cis or separated in different DNA plasmids for expression. Deletion-mapping studies indicate that the C-E1 interaction requires the carboxy-terminal sequences of both the C and the E1 proteins. Since little, if any, of the E1 protein sequence was found to be exposed on the cytosolic side of the ER membrane, the core and the E1 protein most likely interact with each other in the ER membrane.

MATERIALS AND METHODS

DNA plasmid constructions. The construction of the DNA plasmids, pCMV-CCEβ, pCMV-RC, and pCMV-RC151, has been previously described (12). pCMV-CCEβ contains the entire coding sequence of the core protein and the E1 envelope protein of the HCV-1 isolate from nucleotides 1 to 1191 (aa 1 to 397). pCMV-RC contains the entire core protein coding sequence of the HCV-RH isolate, and pCMV-RC151 contains the HCV-RH core gene truncated at codon 151. Various C-E1 clones were generated by PCR. The sequences of these C-E1 clones started from the initiation codon of the core protein gene. The 5′ primer contained a *Bsp*HI site to facilitate cloning of the PCR product into pTM1 (4). Various 3′ primers were used to specify the desired carboxy terminus of C-E1, each of which contained a termination codon followed by a *Bgl*II restriction site to be compatible with the *Bam*HI cloning site of pTM1. For expression of E1 alone, the sequence was initiated at aa 173, thereby providing a signal sequence for E1 translocation across the ER membrane. pTM1-E2809 was generated by PCR with primers containing compatible *Nco*I and *Bam*HI sites for cloning in pTM1. Translation from this vector initiated at aa 364 of E1, thereby providing the signal sequence for E2 translocation. The pCMV tpa-core clone was generated from pCMV-tpa-HIV-1 gp120 (2) in which the gp120 sequence was substituted with that of HCV core. pCMV-E1 was generated by PCR and cloned into compatible restriction sites of pRc/CMV (Invitrogen). All clones were verified by DNA sequencing.

Expression and radioimmunoprecipitation of HCV gene products. Details of the experimental procedures for expressing the HCV gene products in CV1 cells, a monkey kidney cell line, have been described (12). Briefly, one 60-mm dish of freshly confluent CV1 cells maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum was infected with a recombinant vaccinia virus carrying the T7 phage RNA polymerase gene (vTf7-3) (5). Two hours postinfection, cells were transfected with 5 μg of DNA plasmid by using $CaPO_4$ precipitation. Sixteen to eighteen hours after transfection, cells were incubated in methionine-free medium for one to two hours and subsequently radiolabeled with [^{35}S]methionine in the same medium (160 μCi/ml) for two to three hours. Cells were lysed with 1 ml of RIPA buffer (50 mM Tris-HCl, pH7.0, 300 mM NaCl, 4 mM EDTA, 0.5% Triton X-100, 0.1% sodium dodecyl sulfate [SDS] and 0.5% sodium deoxycholate) and immunoprecipitated with either 1 μl of human serum isolated from an HCV patient or 1 μl of rabbit anti-core antibody (12). The protein samples were then analyzed on an SDS–13% polyacrylamide gel.

Immunofluorescence staining assay. COS cells growing on a coverslip in a 35-mm petri dish were transfected with 2 μg of plasmid DNA by using the $CaPO_4$ precipitation method. Two days after transfection, cells were fixed with −20°C acetone for 2 min. The primary antibodies used for the double-staining experiments were rabbit anti-core antibody and mouse monoclonal antibody directed against E1 (1:600 dilution). The secondary antibodies used were rhodamine isothiocyanate (RITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:100 dilution).

Microsomal membrane protection experiment. The microsomal membrane protection experiment was performed using our previous procedures (25). Briefly, one 100-mm dish of CV1 cells which had been infected with Tf7-3 was transfected with 20 μg of pCMV-CCEβ plasmid by using the $CaPO_4$ precipitation method. Sixteen to eighteen hours after transfection, cells were labeled with [^{35}S]methionine for three hours by using the procedures mentioned above, rinsed twice with phosphate-buffered saline (PBS), and homogenized in 1 ml of 0.1× TBS (1 mM Tris-HCl, pH7.0, 15 mM NaCl). The cell lysate was briefly centrifuged to remove nuclei and cell debris and divided into three 300-μl aliquots. These three aliquots were treated with 100 μl of PBS, 100 μl (5 mg) of trypsin (BRL-Gibco), and 100 μl of trypsin plus 2 μl of Nonidet P-40 (NP-40) (final NP-40 concentration was 0.5%), respectively, for 1 h at 37°C. The reactions were stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 1 mM. Each sample was then mixed with 600 μl of RIPA buffer and the E1 protein was immunoprecipitated with the human antiserum and subjected to electrophoresis on an SDS–13% polyacrylamide gel.

RESULTS

Interaction between HCV E1 and core proteins in *cis*. In order to investigate whether the E1 envelope protein could interact with the core protein, we linked the C-E1 coding sequence of the HCV-1 isolate to the T7 phage promoter. A second DNA plasmid was also constructed for expressing only

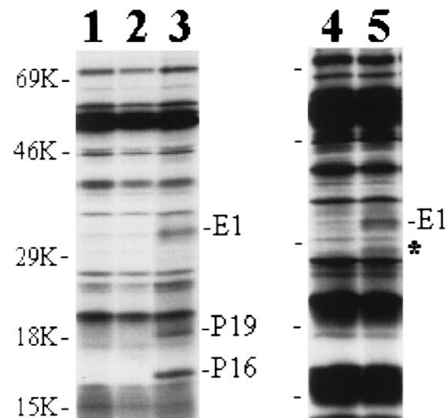

FIG. 1. Coprecipitation of the HCV E1 envelope protein with the core proteins. The HCV E1 (lanes 2 and 5), the C-E1 gene (lane 3) or the control parental plasmid vector pRc/CMV (Invitrogen) (lanes 1 and 4) was transfected into CV1 cells which had been infected with the vaccinia virus vTf7-3. Cells were labeled with [^{35}S]methionine and the cell lysates were radioimmunoprecipitated with either a rabbit anti-core antibody (lanes 1 to 3) or an antiserum isolated from an HCV patient (lanes 4 and 5). The protein samples were then subjected to electrophoresis on an SDS–13% polyacrylamide gel. The asterisk marks the location of a minor 28-kDa E1 protein band. The signal of this E1 protein band varied in different experiments. As shown in lane 5, E1 could be expressed in the absence of most of its preceding core gene sequence. This protein could be precipitated by the human antiserum (lane 5) but not by the rabbit anti-core antibody (lane 2). The E1 protein could also be precipitated by the anti-core antibody in the presence of the P19 and P16 core proteins (lane 3). Molecular size markers are on the left.

the E1 envelope protein. In this second DNA plasmid, the E1 coding sequence plus its preceding signal sequence located at the 3′ end of the core gene sequence was linked to the T7 phage promoter (21a). Thus, in this second plasmid, most of the core gene sequence has been deleted. These two DNA constructs were separately transfected into CV1 cells that had been previously infected with a vaccinia virus carrying the T7 RNA polymerase gene. The transfected cells were then radiolabeled with [^{35}S] methionine and lysed for immunoprecipitation by using a rabbit anti-core antibody previously prepared in our laboratory (12). As shown in Fig. 1, lane 2, no specific signal could be detected if the E1 envelope protein was expressed by itself. This result indicates that the E1 envelope protein does not cross-react with our anti-core antibody. To ensure that the E1 protein was faithfully expressed in this experiment, an aliquot of the same cell lysate used for the experiment shown in Fig. 1, lane 2, was also used for immunoprecipitation with an antiserum isolated from an HCV patient. In our previous experience, we have found that this human antiserum could immunoprecipitate the HCV envelope proteins but not the core proteins (data not shown). As shown in Fig. 1, lane 5, a major 31-kDa protein band and a minor 28-kDa protein band were precipitated by this human antiserum. These two protein bands, which had also been observed before, represent two different glycosylation forms of the E1 envelope protein. Interestingly, if the C-E1 gene was used for the expression studies, the E1 envelope protein was coprecipitated with the core protein by the anti-core antibody. As shown in Fig. 1, lane 3, two major core protein species with sizes of 19 kDa and 16 kDa were precipitated by the antibody. This result is consistent with our previous finding which indicated that the HCV-1 core gene could express both P19 and P16 core proteins (12). In addition to the core proteins, the 31-kDa E1 envelope protein was also precipitated. Since we have demonstrated that the E1 envelope protein does not cross-react with

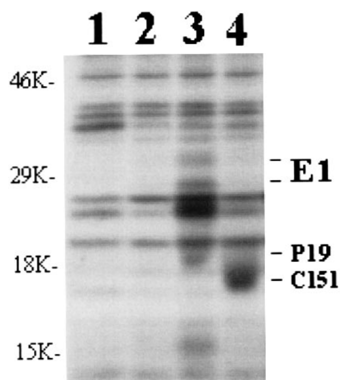

FIG. 2. Interaction between the E1 protein and the P19 core protein in *trans*. CV1 cells grown to confluence on a 60-mm dish were infected with the vaccinia virus vTf7-3 and then transfected with 5 μg of the central pRc/CMV plasmid (lane 1), 2.5 μg each of pRc/CMV and pTM-E1 (lane 2), 2.5 μg each of pCMV-RCβ and pTM-E1 (lane 3), or 2.5 μg each of pCMV-RC151 and pTM-E1 (lane 4). Cells were rabiolabeled with [^{35}S]methionine and the cell lysates were immunoprecipitated with the anti-core antibody. The locations of E1, P19, and the truncated core protein (C151) are marked. Note that E1 was coprecipitated with P19 but not with the core protein truncated at codon 151. pTM-E1 contains the E1 gene of the HCV-1 isolate. Similar results were also obtained if the E1 gene of the HCV-RH isolate was used for the experiment (data not shown). Molecular size markers are on the left.

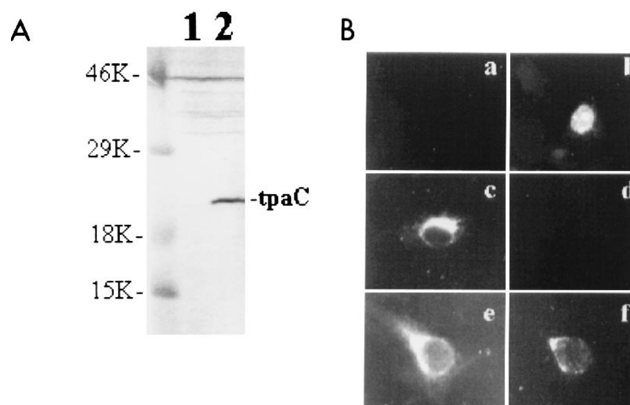

FIG. 3. Colocalization studies of tpa-core and E1 proteins. (A) Expression of the tpa-core fusion protein in COS cells. COS cells were seeded in a 60-mm dish, maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, and transfected with 5 μg of pCMV-tpa-core by $CaPO_4$ precipitation. Cells were lysed 48 h posttransfection and the cell lysate was Western blotted (immunoblotted) with anti-core antibody. Lane 1, cells transfected with a control plasmid; lane 2, cells transfected with pCMV-tpa-core. tpaC indicates the location of the tpa-core fusion protein. Molecular size markers are on the left. (B) Immunofluorescence staining experiment. Details of the procedures are described in Materials and Methods. All cells were double-stained with the rabbit anti-core antibody and mouse monoclonal anti-E1 antibody as primary antibodies and RITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG as secondary antibodies. Panels a, c, and e, FITC staining for the E1 protein; panels b, d, and f, RITC staining for the core protein. Panels a and b, cells transfected with 1.5 μg of the control pRc/CMV plasmid and 0.5 μg of pCMV-tpa-core; panels c and d, cells transfected with 1.5 μg of pCMV-E1 and 0.5 μg of pRc/CMV; panels e and f, cells transfected with 1.5 μg of pCMV-E1 and 0.5 μg of pCMV-tpa-core.

our anti-core antibody, the recovery of the E1 protein by the anti-core antibody must be due to the interaction of the E1 protein with the core protein.

Interaction between HCV E1 and core proteins in *trans*. In the expression studies shown in Fig. 1, the C-E1 coding sequence was linked in *cis*. We have also investigated whether the E1 and core proteins can still interact with each other when these two genes are separately expressed. The E1 expression plasmid used in Fig. 1 was cotransfected with the HCV-RH core gene or a control plasmid vector into CV1 cells. In contrast to the HCV-1 core gene, which expresses both P19 and P16 core proteins, the HCV-RH core gene expresses predominantly the P19 core protein in mammalian cells (12). As shown in Fig. 2, while no E1 protein was precipitated by the anti-core antibody in the absence of the core gene (lane 2), the 31-kDa and the 28-kDa E1 envelope proteins were coprecipitated with P19 by the anti-core antibody (lane 3). Thus, this result indicates that the gene encoding the E1 protein sequence does not have to be linked in *cis* to the gene encoding the core protein sequence for the C-E1 interaction to occur. In addition, it also indicates that P19 alone was sufficient for this interaction. In the above experiments, the HCV-1 E1 protein gene was used for the coexpression studies. Similar results were also obtained with the HCV-RH E1 gene (data not shown). Thus, the interaction between P19 and E1 is conserved between different HCV isolates.

To investigate which domain of P19 may be required for interaction with the E1 envelope protein, we truncated the HCV-RH core gene sequence at codon 151 for the coexpression studies. The truncated core protein expressed would lack the carboxy-terminal sequence of P19, have a nuclear localization, and be structurally similar to the P16 core protein expressed by the HCV-1 isolate (12). As shown in Fig. 2, in contrast to the P19 core protein, the E1 envelope protein could not be coprecipitated with this truncated HCV-RH core protein (lane 4). This result indicates that the interaction between P19 and E1 requires the carboxy-terminal sequence of the P19 core protein. Furthermore, it also indicates that the coprecipitation of the E1 protein observed in Fig. 1 was mediated by P19 and not by P16.

Colocalization studies of the core protein and the E1 envelope protein in cells. To confirm that the core protein could indeed interact with the E1 envelope protein, we have also performed an immunofluorescence double-staining experiment. Since both P19 and E1 proteins are associated with the ER, and P16 does not interact with E1, it was not possible to use either P19 or P16 for the colocalization studies. We have recently found that if the signal sequence of the tissue plasminogen activator (tpa) is linked to the amino terminus of the P19 core protein, the resulting protein product will be localized predominantly to the nuclei of cells. Thus, this protein was used for studying the possible interaction between the core protein and the E1 envelope protein. As shown in Fig. 3A, expression of this tpa-core protein hybrid gene led to the production of a 22-kDa protein. This protein is localized predominantly to the nucleus with an apparent enrichment in the nucleoli (Fig. 3B, panel a). This subcellular localization pattern is similar to that of the P16 core protein previously observed (12). In contrast, if the tpa-core fusion protein was coexpressed with the E1 envelope protein in cells, a substantial amount of the tpa-core protein was found to colocalize with the E1 envelope protein to the perinuclear region (Fig. 3B, panels e and f). This result is in support of the immunoprecipitation results shown in Fig. 1 and 2 and indicates that the P19 core protein can interact with the E1 envelope protein.

Deletion mapping of the E1 protein sequence required for interaction with the core protein. To identify the E1 protein sequence that may be required for interaction with the core protein, we performed a deletion-mapping experiment. The HCV-1 C-E1 sequence was truncated at aa 380, 370, 360, 350, and 330 and transfected into CV1 cells. The E1 envelope

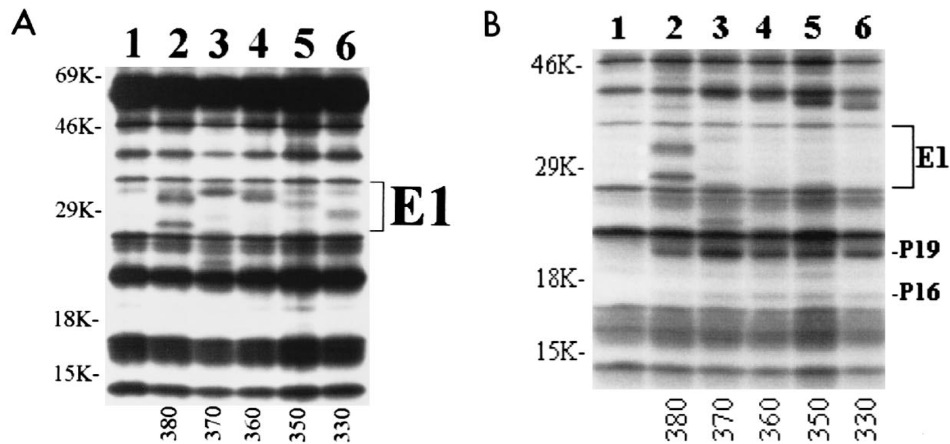

FIG. 4. Deletion mapping of the E1 sequence that interacts with the core protein. The HCV-1 C-E1 sequence was truncated at the codons indicated under the gels and transfected into CV1 cells that had been infected with the vaccinia virus carrying the T7 RNA polymerase gene. Cells were radiolabeled with [^{35}S]methionine and immunoprecipitated with human antiserum (A) or rabbit anti-core antibody (B). Lanes 1, the control pRc/CMV vector; lanes 2 through 6, the C-E1 gene truncated at the indicated aa position. Molecular size markers are on the left.

proteins expressed in CV1 cells were then analyzed by radioimmunoprecipitation by using a human antiserum. As shown in Fig. 4A, all the DNA constructs were able to express their respective truncated E1 envelope proteins. Similar to the results shown in Fig. 2, the C-E1 sequence truncated at aa 380 expressed the 31-kDa and the 28-kDa E1 envelope proteins (lane 2). The C-E1 sequence truncated at aa 370 also expressed an E1 protein, which migrated more slowly than the 31-kDa E1 envelope protein. This reduction of the electrophoretic mobility was due to the removal of the hydrophobic carboxy-terminal sequence of the E1 protein (21a). The truncation of the E1 envelope protein sequence at aa 360, 350, and 330 led to a sequential reduction of the E1 protein size in the gel. All the truncated E1 proteins were expressed at similar levels, indicating that these truncations did not have a significant effect on the expression of the E1 proteins in CV1 cells. An aliquot of the same cell lysates was then subjected to immunoprecipitation by using the anti-core antibody. As shown in Fig. 4B, while the E1 protein truncated at aa 380 was coprecipitated with the core proteins with an efficiency similar to that of the full-length E1 envelope protein shown in Fig. 1 and 2, the E1 protein truncated at aa 370 was coprecipitated poorly with the core proteins by the anti-core antibody. The precipitation efficiency of the E1 protein truncated at aa 370 was approximately 10% of that of the E1 protein truncated at aa 380. This result indicates that the sequence between aa 370 and 380 is required for optimal interaction between the E1 protein and the P19 core protein. As shown in Fig. 4B, further truncation of the E1 protein sequence at aa 360 and beyond resulted in the reduction of the E1 protein signal to a nondetectable level.

Topology of the E1 envelope protein in the ER membrane. To further understand how the E1 protein interacts with the P19 core protein, we performed a microsomal membrane protection experiment to examine the topology of the E1 protein in the ER membrane. Cells expressing the E1 protein were radiolabeled with [^{35}S]methionine, lysed with a hypotonic buffer, treated with either a control buffer or a buffer containing trypsin, and immunoprecipitated with human antiserum. If the E1 protein is partially exposed in the cytosolic side of the ER membrane, it will be sensitive to trypsin digestion. On the other hand, if the E1 protein is localized in the ER membrane or lumen, then it will be protected by the ER membrane from trypsin digestion. As shown in Fig. 5, the treatment with trypsin did not significantly reduce the size or the amount of the E1 envelope proteins (lanes 2 and 4), indicating that the E1 protein resides mostly, if not entirely, in the ER membrane and/or lumen. The protection of the E1 protein from trypsin digestion by the ER membrane was lost if the cell lysates were simultaneously treated with the detergent NP-40 to solubilize the ER membranes (lane 6). Therefore, P19 most likely interacts with the E1 protein in the ER membrane because little E1 sequence is exposed in the cytosol and P19 has been found to be peripherally associated with the ER membrane.

Interaction between the E2 envelope protein and the core protein. As part of our effort to understand the morphogenesis of the HCV virion, we have also investigated whether the core protein could interact with the E2 envelope protein by performing a coprecipitation experiment. The HCV sequence from aa 364 to aa 809 was used for the expression study. This sequence contains the E2 signal sequence derived from the carboxy terminus of E1 and the entire coding sequence of E2

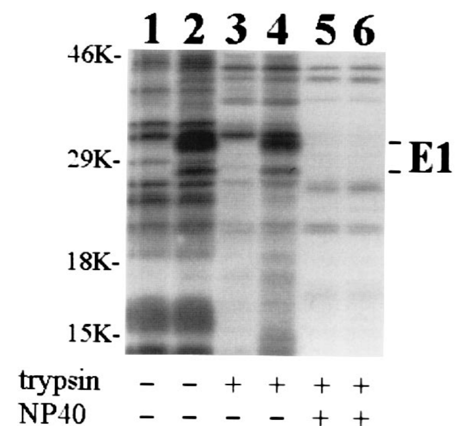

FIG. 5. Microsomal membrane protection experiments for assaying the topology of E1 in the ER membrane. Details of the experimental procedures are described in Materials and Methods. CV1 cells transfected with the control pRc/CMV plasmid (lanes 1, 3, and 5) or pCMV-CCEβ (lanes 2, 4, and 6) were radiolabeled with [^{35}S]methionine and lysed with a hypotonic buffer. The cell lysates were treated with PBS (lanes 1 and 2), trypsin (lanes 3 and 4), or trypsin plus NP-40 (lanes 5 and 6). The reactions were stopped with 1 mM phenylmethylsulfonyl fluoride, and the samples were immunoprecipitated with the human antiserum and analyzed on a 13% acrylamide gel. Molecular size markers are on the left.

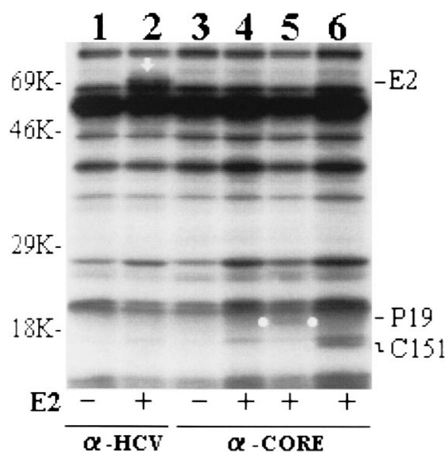

FIG. 6. Analysis of the interaction between the E2 envelope protein and the core proteins. Confluent CV1 cells growing in a 60-mm dish were transfected with 5 μg of the control pRc/CMV plasmid (lanes 1 and 3), 2.5 μg each of pRc/CMV and pTM1-E2809 (lanes 2 and 4), 2.5 μg each of pCMV-RC and pTM1-E2809 (lane 5), or 2.5 μg each of pCMV-RC151 and pTM1-E2809 (lane 6). Cells were radiolabeled with [^{35}S]methionine and immunoprecipitated with the human antiserum (α-HCV) (lanes 1 and 2) or the rabbit anti-core antibody (α-CORE) (lanes 3 to 6). The arrow indicates the 70-kDa E2 protein band and the dots highlight the location of the P19 core protein band. Molecular size markers are on the left.

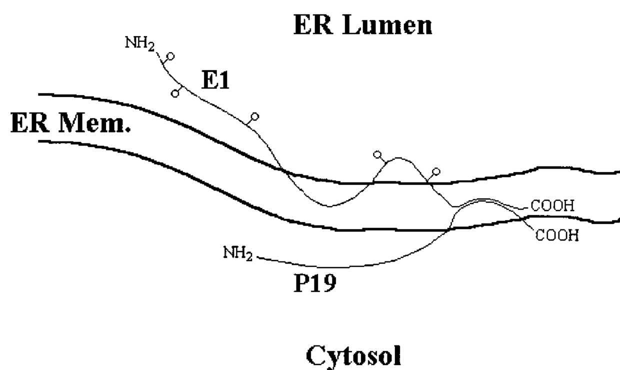

FIG. 7. A possible model for the interaction between the P19 core protein and the E1 envelope protein. The polysaccharide chains are indicated by the lollipops. There are five putative glycosylation sites, located at aa 198, 211, 236, 307, and 327, in the HCV-1 E1 protein sequence. The core protein and the E1 protein likely interact with each other through their hydrophobic domains embedded in the ER membrane (ER Mem.). In this figure, the P19 core protein is shown to interact with the C-terminal hydrophobic domain of the E1 protein. It is possible that the core protein may also interact with the internal hydrophobic domain (aa 260 to 290) of E1.

and p7. As shown in Fig. 6, a 70-kDa E2 protein could be precipitated by the human antiserum (lane 2). This protein size is similar to what was reported before for the E2 protein (7, 16, 22). This E2 protein could not be precipitated by the anti-core antibody either in the absence (lane 4) or in the presence of the P19 (lane 5) or the P16 core protein (lane 6). Thus, unlike E1, E2 does not appear to interact with the core protein on the basis of this assay.

DISCUSSION

HCV is an enveloped virus. Its maturation likely requires the interaction between its core and envelope proteins. To investigate this possibility and to understand the morphogenesis of the HCV virion, we have studied the possible interactions between the HCV core and envelope proteins. Our observation that the E1 envelope protein can be precipitated by anti-core antibody in the presence but not in the absence of the core proteins indicates that the E1 envelope protein can interact with the core protein (Fig. 1). It appears more likely that the E1 and core proteins interact with each other directly rather than indirectly through a cellular protein bridge, because we did not detect coprecipitation of a specific cellular protein in our immunoprecipitation studies. The core-E1 interaction occurs regardless of whether the E1 gene was coexpressed in *cis* or in *trans* with the C gene (Fig. 2). The interaction between the core and E1 proteins was observed with the C and E1 genes of both HCV-1 and HCV-RH isolates, indicating that this interaction is likely a conserved morphogenic event of all HCV viruses.

The interaction between the HCV core protein and the E1 envelope glycoprotein is supported by an immunofluorescence double-staining experiment. It has previously been demonstrated that the P19 core protein is associated with the ER membrane (12, 19, 21, 24). As shown in Fig. 3, linking the signal sequence of tpa to the amino terminus of the P19 core protein sequence produced a 22-kDa fusion protein which was localized predominantly to the nucleus. The subcellular localization of this 22-kDa protein is similar to that of the P16 core protein (12). When this tpa-core fusion protein was coexpressed with the E1 protein, it was found to be colocalized with E1 to the perinuclear region. This observation, which indicates an interaction between this fusion protein and the E1 protein, is in support of the observation that P19 can interact with E1.

The interaction between E1 and the core protein requires the sequence of the core protein located between aa 151 and 173 because P19, which is approximately 173 aa in length, could interact with E1 but the core protein truncated at aa 151 could not (Fig. 2). This sequence may be the domain in the core protein sequence that interacts with E1 or, alternatively, this sequence may be important for the core protein to become associated with the ER membrane for the C-E1 interaction to occur.

In a similar deletion-mapping experiment, truncation of the E1 envelope protein sequence between aa 380 and 383 was not found to affect the C-E1 interaction. On the other hand, truncation of the E1 sequence at aa 370 resulted in the loss of most of the E1 envelope protein signal in the coprecipitation experiment. This result indicates that the sequence between aa 370 and 380 is important for efficient interaction between the core protein and the E1 protein. Since the sequence between aa 370 and 380 is highly hydrophobic, this sequence may be important for proper association of the E1 protein with the ER membrane for optimal C-E1 interaction. Further truncation of the E1 sequence at aa 360 and beyond eliminated entirely the E1 signal in the coprecipitation experiment. This may be due to removal of the E1 domain that interacts with the core protein, the improper folding of the E1 protein after truncation, and/or the loss of association of the E1 protein with the ER membrane.

Since the E1 protein truncated at aa 370 could still interact, albeit inefficiently, with the core protein (Fig. 4), the sequence in E1 that interacts with the core protein likely resides upstream of aa 370. By performing the microsomal membrane protection assay, we found that little E1 sequence was exposed on the cytosolic side of the ER membrane (Fig. 5). This result is consistent with the result obtained from an in vitro translation study (8). Since P19 is peripherally associated with the ER membrane (19), E1 most likely interacts with P19 in the ER membrane. A model of this interaction is illustrated in Fig. 7.

In this model, E1, which has two major hydrophobic domains at aa 262 to 291 and aa 330 to 381 (15, 23), would penetrate the ER membrane twice. The P19 core protein sequence between aa 121 and aa 151, which is also hydrophobic, may interact with one of the two hydrophobic domains of E1 in the ER membrane. Note that this model does not conflict with our observation that the core protein truncated at aa 151 does not interact with E1. The core protein truncated at aa 151 is a nuclear protein and may not have the opportunity to interact with E1, which is membrane associated. If this is indeed the case, then the reason that tpa-P19 was able to interact with E1 may be because the tpa signal sequence would bring P19 to the ER during translation.

Note that as shown in Fig. 4A, when the human antiserum was used for the coprecipitation experiment, no significant amount of the P19 core protein was found to be coprecipitated with the E1 envelope protein truncated as aa 380. The reason for this is unclear. It may be because the E1 epitopes recognized by the human antiserum were masked by P19 and, thus, only those E1 molecules that were not bound by P19 were immunoprecipitated by the antiserum.

In contrast to E1, the E2 envelope protein could not be coprecipitated with either P19 or the truncated P16 core protein by the anti-core antibody in our assays (Fig. 6). This result indicates that E2 most likely does not interact directly with the core protein. E2, however, can indirectly interact with the core protein through its interaction with E1 (3, 7, 15, 17). It is interesting that E2 has also been shown to interact with NS2, which has been shown to interact with NS5A and NS5B (6, 10, 15, 22). NS3 has also been shown to complex with NS4A (1) and to certain degrees with other HCV nonstructural proteins (10). Thus, there are multiple interactions either directly or indirectly between different HCV gene products.

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REFERENCES

1. **Bartenschlager, R., V. Lohmann, T. Wilkinson, and J. O. Koch.** 1995. Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. J. Virol. **69:**7519–7528.
2. **Chapman, B. S., R. M. Thayer, K. A. Vincent, and N. L. Haigwood.** 1991. Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. Nucleic Acids Res. **19:**3979–3986.
3. **Dubuisson, J., and C. M. Rice.** 1996. Hepatitis C virus glycoprotein folding: disulfide bond formation and association with calnexin. J. Virol. **70:**778–786.
4. **Elroy-Stein, O., and B. Moss.** 1990. Cytoplasmic expression system based on constitutive synthesis of bacteriophage T7 RNA polymerase in mammalian cells. Proc. Natl. Acad. Sci. USA **87:**6743–6747.
5. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eucaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
6. **Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. A second hepatitis C virus-encoded proteinase. Proc. Natl. Acad. Sci. USA **90:**10583–10587.
7. **Grakoui, A., C. Wychowski, C. Lin, S. M. Feinstone, and C. M. Rice.** 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. **67:**1385–1395.
8. **Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, and K. Shimotohno.** 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc. Natl. Acad. Sci. USA **88:**5547–5551.
9. **Hijikata, M., H. Mizushima, T. Akagi, S. Mori, N. Kakiuchi, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno.** 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. **67:**4665–4675.
10. **Hijikata, M., H. Mizushima, Y. Tanji, Y. Komada, Y. Hirowatari, T. Akagi, N. Kato, K. Kimura, and K. Shimotohno.** 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc. Natl. Acad. Sci. USA **90:**10773–10777.
11. **Lin, C., B. D. Lindenbach, B. M. Pragai, D. W. McCourt, and C. M. Rice.** 1994. Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different termini. J. Virol. **68:** 5063–5073.
12. **Lo, S.-Y., F. Masiarz, S. B. Hwang, M. M. C. Lai, and J.-H. Ou.** 1995. Differential subcellular localization of hepatitis C virus core gene products. Virology **213:**455–461.
13. **Lo, S.-Y,. M. J. Selby, M. Tong, and J.-H. Ou.** 1994. Comparative studies of the core gene products of two different hepatitis C virus isolates: two alternative forms determined by a single amino acid substitution. Virology **199:** 124–131.
14. **Matsumoto, M., S. B. Hwang, N. Zhu, and M. M. C. Lai.** Homotypic interaction and multimerization of hepatitis C virus core protein. Virology, in press.
15. **Matsuura, Y., T. Suzuki, R. Suzuki, M. Sato, H. Aizaki, I. Saito, and T. Miyamura.** 1994. Processing of E1 and E2 glycoproteins of hepatitis C virus expressed in mammalian and insect cells. Virology **205:**141–150.
16. **Mizushima, H., M. Hijikata, S.-I. Asabe, M. Hirota, K. Kimura, and K. Shimotohno.** 1994. Two hepatitis C virus glycoprotein E2 products with different C termini. J. Virol. **68:**6214–6222.
17. **Ralston, R., K. Thudium, K. Berger, C. Kuo, B. Gervase, J. Hall, M. Selby, G. Kuo, M. Houghton, and Q.-L. Choo.** 1993. Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. J. Virol. **67:**6753–6761.
18. **Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Q.-L. Choo, M. Houghton, and G. Kuo.** 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA **87:**6547–6549.
19. **Santolini, E., G. Migliaccio, and N. La Monica.** 1994. Biosynthesis and biochemical properties of the hepatitis C virus core protein. J. Virol. **68:** 3631–3641.
20. **Santolini, E., L. Pacini, C. Fipaldini, G. Migliaccio, and N. La Monica.** 1995. The NS2 protein of hepatitis C virus is a transmembrane polypeptide. J. Virol. **69:**7461–7471.
21. **Selby, M. J., Q.-L. Choo, K. Berger, G. Kuo, E. Glaser, M. Eckart, C. Lee, D. Chien, C. Kuo, and M. Houghton.** 1993. Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. J. Gen. Virol. **74:**1103–1113.

21a. **Selby, M. J., et al.** Unpublished data.

22. **Selby, M. J., E. Glazer, F. Masiarz, and M. Houghton.** 1994. Complex processing and protein:protein interactions in the E2:NS2 region of HCV. Virology **204:**114–122.
23. **Spaete, R. R., D. Alexander, M. E. Rugroden, Q.-L. Choo, K. Berger, K. Crawford, C. Kuo, S. Leng, C. Lee, R. Ralston, K. Thudium, J. W. Tung, G. Kuo, and M. Houghton.** 1992. Characterization of the hepatitis C virus E2/NS1 gene product expressed in mammalian cells. Virology **188:**819–830.
24. **Suzuki, R., Y. Matsuura, T. Suzuki, A. Ando, J. Chiba, S. Harada, I. Saito, and T. Miyamura.** 1995. Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted. J. Gen. Virol. **76:**53–61.
25. **Yeh, C.-T., and J.-H. Ou.** 1991. Phosphorylation of hepatitis B virus precore and core proteins. J. Virol. **65:**2327–2331.