

E6AP Ubiquitin Ligase Mediates Ubiquitylation and Degradation of Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein is a major component of viral nucleocapsid and a multifunctional protein involved in viral pathogenesis and hepatocarcinogenesis. We previously showed that the HCV core protein is degraded through the ubiquitin-proteasome pathway. However, the molecular machinery for core ubiquitylation is unknown. Using tandem affinity purification, we identified the ubiquitin ligase E6AP as an HCV core-binding protein. E6AP was found to bind to the core protein in vitro and in vivo and promote its degradation in hepatic and nonhepatic cells. Knockdown of endogenous E6AP by RNA interference increased the HCV core protein level. In vitro and in vivo ubiquitylation assays showed that E6AP promotes ubiquitylation of the core protein. Exogenous expression of E6AP decreased intracellular core protein levels and supernatant HCV infectivity titers in the HCV JFH1-infected Huh-7 cells. Furthermore, knockdown of endogenous E6AP by RNA interference increased intracellular core protein levels and supernatant HCV infectivity titers in the HCV JFH1-infected cells. Taken together, our results provide evidence that E6AP mediates ubiquitylation and degradation of HCV core protein. We propose that the E6AP-mediated ubiquitin-proteasome pathway may affect the production of HCV particles through controlling the amounts of viral nucleocapsid protein.

Hepatitis C virus (HCV; a single-stranded, positive-sense RNA virus that is classified in the family *Flaviviridae*) is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (5, 26, 45). More than 170 million people worldwide are chronically infected with HCV (41). The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein (5, 54). The polyprotein is cleaved cotranslationally into at least 10 proteins by viral proteases and cellular signalases (6, 10).

The HCV core protein represents the first 1 to 191 amino acids (aa) of the polyprotein and is followed by two glycoproteins, E1 and E2 (6). The core protein plays a central role in the packaging of viral RNA (25, 40); modulates various cellular processes, including signal transduction pathways, transcriptional control, cell cycle progression, apoptosis, lipid metabolism, and the immune response (9, 40); and has transforming potential in certain cells (43). Mice transgenic for the HCV core gene develop steatosis (32) and later hepatocellular carcinoma (31). These findings suggest that HCV core protein plays a crucial role in hepatocarcinogenesis.

Two major forms of the HCV core protein, p21 (mature form) and p23 (immature form), can be generated in cultured cells (60). Cellular signal peptidase cleaves at the junction of the core/E1, releasing the immature form of the core protein from the polypeptide (12, 46). Signal peptide peptidase cleaves just before the signal sequence, liberating the mature form of the HCV core protein at the cytoplasmic face of the endoplasmic reticulum (29). Several different sites have been proposed as potential cleavage sites of signal peptide peptidase, such as Leu-179 (15, 29), Phe-177 (36, 37), Leu-182 (15), and Ser-173 (46). Further processing of the HCV core protein yields a 17-kDa product with a C terminus at around amino acid 152. A truncated form of the core protein, p17, was found in transfected cells (42, 52) and liver tissues from humans with hepatocellular carcinoma (59). The majority of this protein translocates to the nucleus. The C terminus of the core protein is important for regulating the stability of the protein (20, 52).

We previously showed that the C-terminally truncated forms of the core protein are degraded through the ubiquitin-proteasome pathway (52). We found that the mature form of the core protein, p21, also links to a few ubiquitin moieties, suggesting that the ubiquitin-proteasome pathway involves proteolysis of heterologous species of the core protein (52). Overexpression of PA28 (a REG family proteasome activator also known as REG or Ki antigen) enhances the proteasomal degradation of the HCV core protein (30). A recent study has shown that

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PA28 is involved in the degradation of the steroid receptor coactivator 3 (SRC-3) in an ATP- and ubiquitin-independent manner (27). It is still unclear what E3 ubiquitin ligase is responsible for ubiquitylation of the HCV core protein.

E6AP was initially identified as the cellular factor that stimulates ubiquitin-mediated degradation of the tumor suppressor p53 in conjunction with the E6 protein of cancer-associated human papillomavirus types 16 and 18 (14, 48). The E6-E6AP complex functions as a E3 ubiquitin ligase in the ubiquitylation of p53 (49). E6AP is the prototype of a family of ubiquitin ligases called HECT domain ubiquitin ligases, all of which contain a domain homologous to the E6AP carboxyl terminus (13). Interestingly, E6AP is not involved in the regulation of p53 ubiquitylation in the absence of E6 (55). Several potential E6-independent substrates for E6AP have been identified, such as hHR23A, Blk, and Mdm1 (23, 24, 35). E6AP is also a candidate gene for Angelman syndrome, which is a severe neurological disorder characterized by mental retardation (21).

This study aimed to identify endogenous ubiquitin-proteasome pathway proteins that are associated with HCV core protein. Tandem affinity purification and mass spectrometry analysis identified E6AP as an HCV core-binding protein. Here we present evidence that E6AP associates with HCV core protein in vitro and in vivo and is involved in ubiquitylation and degradation of HCV core protein. We propose that an E6AP-mediated ubiquitin-proteasome pathway may affect the production of HCV particles through controlling the amounts of HCV core protein.

MATERIALS AND METHODS

Cell culture and transfection. Human embryonic kidney 293T cells, human hepatoblastoma HepG2 cells, and human hepatoma Huh-7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 50 IU/ml penicillin, 50 g/ml streptomycin (Invitrogen), and 10% (vol/vol) fetal bovine serum (JRH Biosciences) at 37°C in a 5% CO₂ incubator. 293T cells and HepG2 cells were transfected with plasmid DNA using FuGene 6 transfection reagents (Roche). Huh-7 cells were transfected with plasmid DNA using TransIT LT1 transfection reagents (Mirus).

Plasmids and recombinant baculoviruses. MEF tag cassette (containing *myc* tag, the tobacco etch virus protease cleavage site, and FLAG tag) (16) was fused to the N terminus of the cDNA encoding core protein of HCV NIHJ1 (genotype 1b) (1). To express MEF-tagged core protein in mammalian cells, the genome coding for HCV core protein (amino acids 1 to 191) was amplified by PCR using pBR HCV NIHJ1 as a template. Sense oligonucleotide containing a Kozak consensus translation initiation codon and antisense oligonucleotide containing an in-frame translation stop codon were synthesized by PCR. The amplified PCR product was purified, digested with EcoRI and EcoRV, and then inserted into the EcoRI-EcoRV site of pcDNA3-MEF. FLAG-tagged HCV core expression plasmids based upon pCAGGS (34) were described previously (30). To express E6AP and the active-site cysteine-to-alanine mutant of E6AP in mammalian cells, pCMV4-HA-E6AP isoform II and pCMV4-HA-E6AP C-A were utilized (19). The C-A mutation was introduced at the site of E6AP C843. To express E6AP and E6AP C-A under the CAG promoter, the E6AP fragment and the E6AP C-A fragment were amplified by PCR, purified, digested with SmaI and NotI, and blunt ended using a DNA blunting kit (Takara). These PCR fragments were subcloned into pCAGGS.

To make a fusion protein consisting of glutathione *S*-transferase (GST) fused to the N terminus of E6AP in *Escherichia coli*, the E6AP fragment was amplified by PCR and the resultant product was cloned into the SmaI-NotI site of pGEX4T-1 vector (Amersham Biosciences). To express a series of E6AP truncation mutants as GST fusion proteins, each fragment was amplified by PCR and cloned into the SmaI-NotI site of pGEX4T-1. To purify GST core protein efficiently by two-step affinity purification, we fused hexahistidine (His) tag to the C terminus of GST fusion proteins. To bacterially express HCV core (aa 1 to 173) protein as a fusion protein containing N-terminal GST tag and C-terminal

His tag, core fragment was amplified by PCR and the resultant product was cloned into the EcoRI-NotI site of pGEX4T-1 vector. The resultant plasmid was designated pGEX GST-C173HT. To express GST core (1-152)-His and GST-His in *E. coli*, pGEX core (1-152)-His and pGEX-His were constructed similarly. The resultant plasmids were designated pGEX GST-C152HT and pGEX GST-HT, respectively.

To generate recombinant baculoviruses expressing GST-E6AP, GST-E6AP fragment was excised from pGEX E6AP by digestion with SmaI and Tth111I and ligated into the SmaI-Tth111I site of pVL1392 (Invitrogen). To express GST-E6AP C-A, pVLGST-E6AP C-A was constructed similarly. To generate recombinant baculovirus expressing HCV core (aa 1 to 173) protein as a fusion protein containing N-terminal GST tag and C-terminal His tag, GST-C173HT fragment was amplified by PCR using pGEX GST-C173HT as a template, digested with BglII-XbaI, and subcloned into the BglII-XbaI site of pVL1392. To generate recombinant baculoviruses expressing GST-C152HT and GST-HT, cDNA fragments corresponding to GST-C152HT and GST-HT were amplified by PCR and subcloned into pVL1392, respectively. The resultant plasmids were designated pVLGST-C173HT, pVLGST-C152HT, and pVLGST-HT. To generate recombinant baculovirus expressing MEF-tagged E6AP, cDNA fragment encoding MEF-E6AP was subcloned into pVL1392. To express HCV core protein in the TNT-coupled wheat germ lysate system (Promega), HCV core cDNA was inserted in the EcoRI site of pCMVTNT (Promega). The primer sequences used in this study are available from the authors upon request. The sequences of the inserts were extensively verified using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Recombinant baculoviruses were recovered using a BaculoGold transfection kit (Pharmingen) according to the manufacturer's instructions.

Antibodies. The mouse monoclonal antibodies (MAbs) used in this study were anti-hemagglutinin (anti-HA) MAb (12CA5; Roche), anti-FLAG (M2) MAb (Sigma), anti-c-myc MAb (9E10; Santa Cruz), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) MAb (Chemicon), anti-GST MAb (Santa Cruz), anti-ubiquitin MAb (Chemicon), anti-E6AP MAb (E6AP-330) (Sigma), anticore MAb (B2; Anogen), and another anti-core MAb (2H9) (56). Polyclonal antibodies (PABs) used in this study were anti-HA rabbit PAB (Y-11; Santa Cruz), anti-FLAG rabbit PAB (F7425; Sigma), anti-E6AP rabbit PAB (H-182; Santa Cruz), anti-DDX3 rabbit PAB (47), anti-PA28 rabbit PAB (Affiniti), and anti-GST goat PAB (Amersham). Anticore rabbit PAB (TS1) was raised against the recombinant GST core protein.

MEF purification procedure. 293T cells were transfected with the plasmid expressing MEF core by the calcium phosphate precipitation method (4). After the cells were lysed, the expressed MEF core and its binding proteins were recovered following the procedure described previously (16). 293T cells transfected with pcDNA3-MEF core in four 10-cm dishes were lysed in 2 ml of lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (wt/vol) glycerol, 100 mM NaF, 1 mM Na₃VO₄, 1% (wt/vol) Triton X-100, 5 mM ZnCl₂, 2 mM phenylmethylsulfonyl fluoride, 10 g/ml aprotinin, and 1 g/ml leupeptin. The lysate was centrifuged at 100,000 g for 20 min at 4°C. The supernatant was passed through a 5-μm filter, incubated with 100 μl of Sepharose beads for 60 min at 4°C, and then passed through a 0.65-μm filter. The filtered supernatant was mixed with 100 μl of anti-myc-conjugated Sepharose beads for the first immunoprecipitation. After incubation for 90 min at 4°C, the beads were washed five times with 1 ml of TNTG buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% [wt/vol] glycerol, and 1% [wt/vol] Triton X-100), twice with 1 ml of buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% [wt/vol] Triton X-100), and finally once with 1 ml of TNT buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% [wt/vol] Triton X-100). The washed beads were incubated with 10 U of tobacco etch virus protease (Invitrogen) in TNT buffer (100 μl) to release bound protein complexes from the beads. After incubation for 60 min at room temperature, the supernatant was pooled and the beads were washed twice with 70 μl of buffer A. The resulting supernatants were combined and incubated with 12 μl of FLAG-Sepharose beads for the second immunoprecipitation. After incubation for 60 min at room temperature, the beads were washed three times with 240 μl of buffer A, and proteins bound to the immobilized HCV core protein on the FLAG beads were dissociated by incubation with 80 g/ml FLAG peptide (NH₂-Asp-Tyr-Lys-Asp-Asp-Asp-Lys-COOH) (Sigma).

MS/MS. Proteins were separated by 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining. The stained bands were excised and digested in the gel with lysylendoprotease-C (Lys-C), and the resulting peptide mixtures were analyzed using a direct nanoflow liquid chromatography-tandem mass spectrometry (MS/MS) system (33), equipped with an electrospray interface reversed-phase column, a nanoflow gradient device, a high-resolution Q-time of flight hybrid mass spectrometer (Q-TOF2; Micromass), and an automated data analysis system. All the MS/MS

spectra were searched against the nonredundant protein sequence database maintained at the National Center for Biotechnology Information using the Mascot program (Matrixscience) to identify proteins. The MS/MS signal assignments were also confirmed manually.

Expression and purification of recombinant proteins. *E. coli* BL21(DE3) cells were transformed with plasmids expressing GST fusion protein or His-tagged protein and grown at 37°C. Expression of the fusion protein was induced by 1 mM isopropyl- β -D-thiogalactopyranoside at 37°C for 4 h. Bacteria were harvested, suspended in lysis buffer (phosphate-buffered saline [PBS] containing 1% Triton X-100), and sonicated on ice.

Hi5 cells were infected with recombinant baculoviruses to produce GST-C173HT, GST-C152HT, GST-HT, MEF-E6AP, and His-tagged mouse E1 (17). GST and GST fusion proteins were purified on glutathione-Sepharose beads (Amersham Bioscience) according to the manufacturer's protocols. His-tagged proteins were purified on nickel-nitrilotriacetic acid beads (QIAGEN) according to the manufacturer's protocols. MEF-E6AP and MEF-E6AP C-A were purified on anti-FLAG M2 agarose beads (Sigma) according to the manufacturer's protocols.

Immunoblot analysis. Immunoblot analysis was performed essentially as described previously (11). The membrane was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce).

HCV core protein and E6AP binding assays. To map the E6AP binding site on HCV core protein, 2.5 μ g of purified recombinant GST-E6AP expressed in Hi5 cells was mixed with 1,000 μ g of 293T cell lysates transfected with a series of FLAG-tagged HCV core deletion mutants as indicated. The protein concentration of the cells was determined using the bicinchoninic acid protein assay kit (Pierce). The mixtures were immunoprecipitated with anti-FLAG M2 agarose beads (Sigma), and proteins bound to the immobilized HCV core protein on anti-FLAG beads were dissociated with FLAG peptide (Sigma). The eluates were analyzed by immunoblotting with anti-GST PAb. To map the HCV core-binding site on E6AP, GST pull-down assays were performed as described previously (51).

In vivo ubiquitylation assay. In vivo ubiquitylation assays were performed essentially as described previously (57). FLAG-core was immunoprecipitated with anti-FLAG beads. Immunoprecipitates were analyzed by immunoblotting, using either anti-HA PAb or anticore PAb (TS1) to detect ubiquitylated core proteins.

In vitro ubiquitylation assay. For in vitro ubiquitylation of HCV core protein, purified GST-C173HT and GST-C152HT were used as substrates. Purified GST-HT was used as a negative control. Assays were done in 40- μ l volumes containing 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM ATP, 10 mM MgCl₂, 8 μ g of bovine ubiquitin (Sigma), 0.1 mM dithiothreitol, 200 ng mouse E1, 200 ng E2 (UbcH7), and 0.5 μ g each of MEF-E6AP or MEF-E6AP C-A. The reaction mixtures were incubated at 37°C for 120 min followed by purification with glutathione-Sepharose beads and immunoblotting with the indicated antibodies.

siRNA transfection. 293T cells or Huh-7 cells at 3 $\times 10^5$ cells in a six-well plate were transfected with 40 pmol of either E6AP-specific short interfering RNA (siRNA; Sigma) or scramble negative-control siRNA duplexes (Sigma) using HiPerFect transfection reagent (QIAGEN) following the manufacturer's instructions. The siRNA target sequences were as follows: E6AP (sense), 5'-GGGUCUACACCAGAUUGCUTT-3'; scramble negative control (sense), 5'-UUGCGGGUCUAUACCCGATT-3'.

CHX half-life experiments. To examine the half-life of HCV core protein, transfected 293T cells were treated with 50 μ M cycloheximide (CHX) at 44 h posttransfection. The cells at zero time points were harvested immediately after treatment with CHX. Cells from subsequent time points were incubated in medium containing CHX at 37°C for 3, 6, and 9 h as indicated.

Infection of Huh-7 cells with secreted HCV. Infectious HCV JFH1 was produced in Huh-7.5.1 cells (61) as described previously (56). Culture supernatant containing infectious HCV JFH1 was collected and passed through a 0.22- μ m filter. Naive Huh-7 cells were seeded 24 h before infection at a density of 1 $\times 10^6$ in a 10-cm dish. The cells were incubated with 2.5 ml of the inoculum (6.5 $\times 10^3$ 50% tissue culture infectious dose [TCID₅₀]/ml) for 3 h, washed three times with PBS, and supplemented with fresh complete Dulbecco's modified Eagle's medium. Then the cells were transfected with 6 μ g each of pCAGGS, pCAG-HA-E6AP, or pCAG-HA-E6AP C-A by using TransIT LT1 (Mirus). The cells were trypsinized and replated in six-well plates at 1 day postinfection. The culture medium was changed every 2 days. The culture supernatants and the cells were collected at days 3 and 7 postinfection.

Quantitation of HCV RNA and core protein. We quantitated HCV core protein in cell lysate using the HCV core antigen enzyme-linked immunosorbent assay (ELISA) (Ortho-Clinical Diagnostics). Total RNA was extracted from cells

using TRIzol reagent (Invitrogen). To quantitate HCV RNAs, real-time reverse transcription-PCR was performed as described previously (53).

Infectivity assay. The TCID₅₀ was calculated essentially based on the method described previously (28). Virus titration was performed by seeding Huh-7 cells in 96-well plates at 1 $\times 10^4$ cells/well. Samples were serially diluted fivefold in complete growth medium and used to infect the seeded cells (six wells per dilution). Following 3 days of incubation, the cells were immunostained for core with anticore MAb (2H9). Wells that expressed at least one core-expressing cell were counted as positive, and the TCID₅₀ was calculated.

Immunocytochemistry and fluorescence microscopy. Cells on collagen-coated coverslips were washed with PBS, fixed with 4% paraformaldehyde for 30 min at 4°C, and permeabilized with PBS containing 0.2% Triton X-100. Cells were preincubated with BlockAce (Dainippon Pharmaceuticals), incubated with specific antibodies as primary antibodies, washed, and incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin G (ICN Pharmaceuticals, Inc.) and Qdot 565-conjugated goat anti-mouse immunoglobulin G (Quantumdot) as secondary antibody. Then the cells were washed with PBS, counterstained with DAPI (4',6'-diamidino-2-phenylindole) solution (Sigma) for 3 min, mounted on glass slides, and examined with a BZ-8000 microscope (Keyence).

Knockdown of endogenous E6AP in HCV JFH1-infected Huh-7 cells. Naive Huh-7 cells at 10⁶ cells/10-cm dish were inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5 $\times 10^3$ TCID₅₀/ml) and cultured. The cells were replated in a six-well plate at 3 $\times 10^5$ cells/well at day 11 postinfection and transfected with 40 pmol of E6AP siRNA or control siRNA. The culture medium was changed at 24 h after transfection. The cells were harvested at day 2 after transfection, and the intracellular core protein levels were quantitated using the HCV core antigen ELISA. The culture supernatants were collected at day 2 after transfection and assayed for TCID₅₀ determinations.

RESULTS

Identification of E6AP as an HCV core-binding protein. To identify the molecular machinery for HCV core ubiquitylation, we searched for endogenous ubiquitin-proteasome pathway proteins that associated with HCV core protein. HCV core-binding proteins (i.e., MEF core and its binding proteins, recovered from lysed cells) were purified by a tandem affinity purification procedure using a tandem tag (known as MEF tag) (16). Ten proteins were reproducibly detected (Fig. 1A, lane 2), but none were recovered from lysed control cells transfected with empty vector alone (Fig. 1A, lane 1).

To identify the proteins, silver-stained bands were excised from the gel, digested by Lys-C, and analyzed using a direct nanoflow liquid chromatography-MS/MS system. Nine proteins were identified: two known HCV core-binding proteins, human DEAD box protein DDX3 (38) and proteasome activator PA28 (30), and seven potential HCV core-binding proteins. E6AP was identified (Fig. 1A, lane 2) on the basis of five independent MS/MS spectra (Table 1). Immunoblot analyses confirmed the proteomic identification of E6AP, DDX3, PA28, and MEF-core (Fig. 1B to E).

E6AP binding domain for HCV core protein. The E6AP binding domain for HCV core protein was investigated. Figure 2A is a schematic representation of E6AP and known motifs in E6AP. A series of deletion mutants of E6AP as GST fusion proteins were expressed in *E. coli*. GST pull-down assays found that the carboxyl-terminal deletion mutant E6AP (1–517), but not E6AP (1–418) (Fig. 2C, lanes C and D), and the amino-terminal deletion mutant E6AP (418–875), but not E6AP (517–875) (Fig. 2C, lanes J and K), were able to bind to the core protein. The signal was absent when unprogrammed wheat germ extracts (the negative control) were used as a source of proteins (data not shown). GST pull-down assays (Fig. 2B) found that the region from aa 418 to aa 517 is important for binding to the HCV core protein. An assay of the

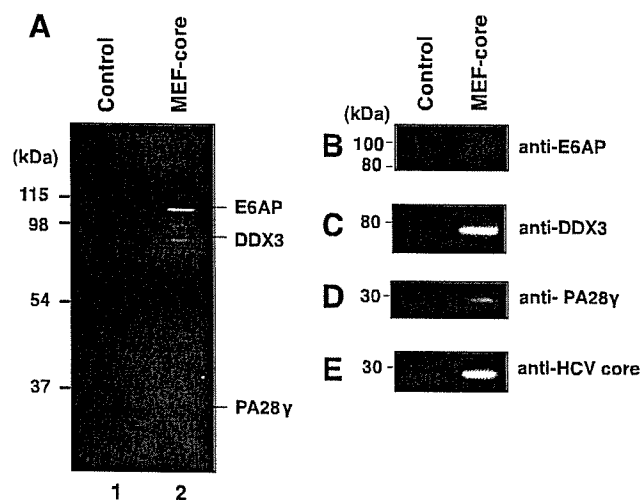


FIG. 1. HCV core protein associates with E6AP in vivo. (A) 293T cells were transfected with pcDNA3-MEF-core or empty plasmid, incubated for 48 h, and then harvested. The expressed MEF-core and binding proteins were recovered using the MEF purification procedure. Proteins bound to the MEF-core immobilized on anti-FLAG beads were dissociated with FLAG peptides, resolved by 9% SDS-PAGE, and visualized by silver staining. Control experiments were performed using 293T cells transfected with vector alone. The positions of E6AP, DDX3, and PA28 are indicated by arrows. (B to E) The proteins detected in panel A were confirmed by immunoblotting with appropriate antibodies: E6AP (B), DDX3 (C), PA28 (D), and MEF-core (E).

ability of GST-E6AP (418–517) to bind to the HCV core protein was confirmatory (Fig. 2C, lane N) and led to the conclusion that the HCV core-binding domain of E6AP was aa 418 to aa 517.

The HCV core-binding domain for E6AP. By use of a panel of HCV core deletion mutants (Fig. 3A), GST-E6AP was found to coimmunoprecipitate with all of the FLAG-core proteins (Fig. 3A, lanes A to H) except FLAG-core (72–191) or FLAG-core (92–191) (Fig. 3A, lanes I and J). No association of control GST protein with any FLAG-core proteins was observed (data not shown). These data suggest that the aa-58-to-aa-71 segment of the HCV core binds to E6AP. The ability of GST-core (58–71) to associate with purified MEF-E6AP confirmed that the core (aa 58–71) was the site for E6AP binding on the HCV core protein (Fig. 3B).

E6AP decreases steady-state levels of HCV core protein in 293T cells and HepG2 cells. One of the features of HECT domain ubiquitin ligases is direct association with their substrates (50). Thus, we hypothesized that E6AP would function as an E3 ubiquitin ligase for the HCV core protein. We as-

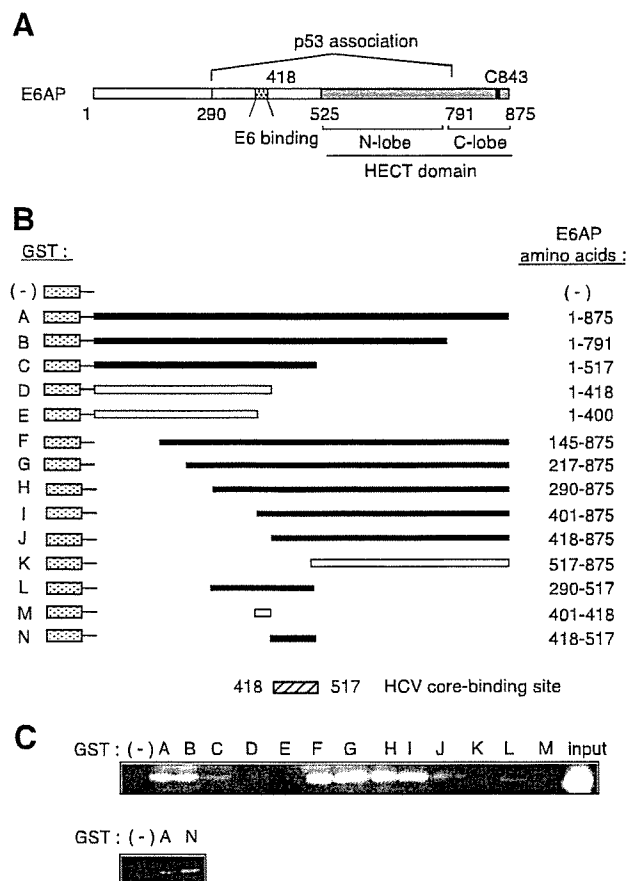


FIG. 2. Mapping of the HCV core-binding domain for E6AP. (A) Structure of E6AP. Shown is a schematic representation of the regions of E6AP isoform II that mediate E6 binding (aa 401 to 418), E6-dependent association with p53 (aa 290 to 791), and the HECT catalytic domain (aa 525 to 875). The catalytic cysteine residue is located at aa 843. (B) Schematic representation of GST-E6AP proteins. GST proteins A through N contain the E6AP amino acids indicated to the right. The shaded region of each represents the GST sequence. Closed boxes represent proteins that are bound specifically to HCV core protein, and open boxes represent those that are not bound. (C) Binding of HCV core protein to GST-E6AP proteins A through N. In vitro-translated core protein (aa 1 to 173) was assayed for association with GST (-) or the GST-E6AP fusion proteins A through N. Association of core protein was detected by immunoblotting with anti-core MAbs.

sessed the effects of E6AP on the HCV core protein in 293T cells. FLAG-core (1–191) together with HA-tagged wild-type E6AP, catalytically inactive mutant E6AP, E6AP C-A (19), or WWP1 (another HECT domain ubiquitin ligase) (22) was introduced into 293T cells, and the levels of the core protein were examined by immunoblotting. The steady-state levels of the core protein decreased with an increase in the amount of E6AP plasmids (Fig. 4A and B). However, neither E6AP C-A mutant nor WWP1 decreased the steady-state levels of the core protein, suggesting that E6AP enhances degradation of the core protein.

To verify the critical need for endogenous E6AP in the core degradation, expression of E6AP was knocked down by siRNA and the expression of the core protein and E6AP was assayed by immunoblotting. Transfection of the E6AP-specific siRNA

TABLE 1. Identification of E6AP by tandem mass spectrometry^a

Peptide <i>m/z</i>	Sequence determined	Residues
720.9	VFSSAEALVQSFR	156–168
922.4	AACSAAMEEDSEASSR	196–213
774.9	MMETFQQLITYK	339–350
1,053.1	ITVLYSLVQGQQLNPYLR	507–524
809.4	EFVISYSDYILNK	712–724

^a The protein was ubiquitin protein ligase E3A (E6AP) isoform 2 (GenBank accession no. NP_000453).

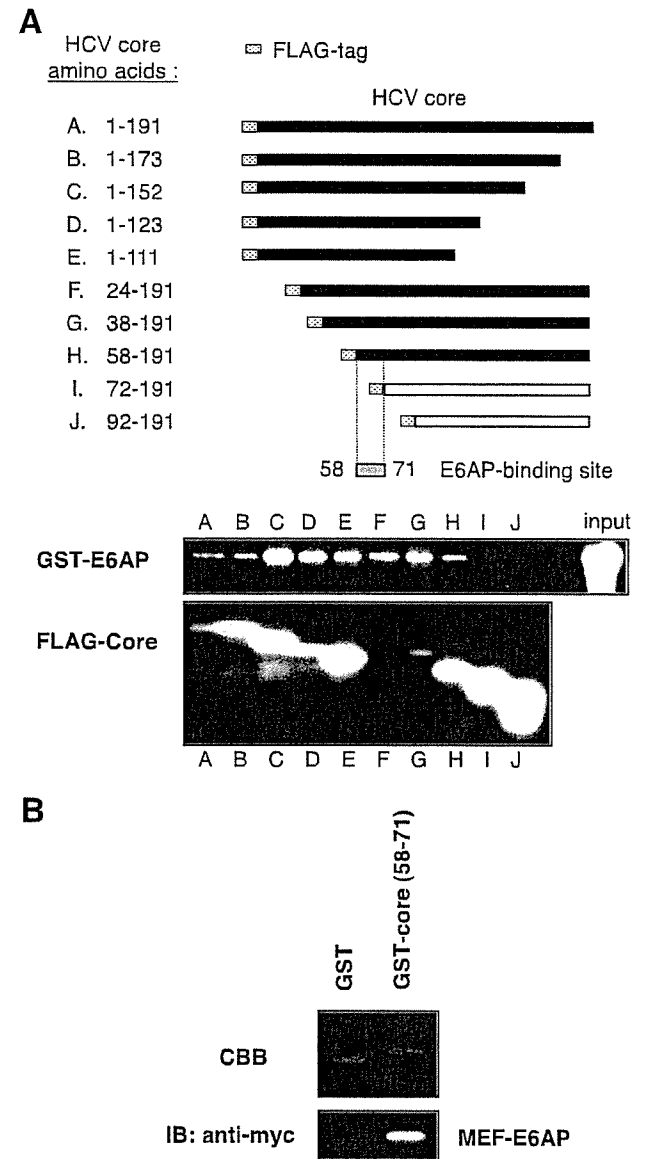


FIG. 3. Mapping of the E6AP binding domain for HCV core protein. (A) In vitro binding of E6AP to HCV core protein. 293T cells were transfected with each plasmid indicated in the upper panel. At 48 h posttransfection, cell lysates were mixed with purified GST-E6AP, immunoprecipitated with anti-GST PAb (middle panel) or anti-FLAG MAb (bottom panel). The last lane (input) represents GST-E6AP used in this assay (middle panel). (B) Binding of GST-core (aa 58 to aa 71) to purified MEF-E6AP. GST served as a negative control for binding. Upper panel, Coomassie blue-stained SDS-PAGE of GST and GST-core (58-71). Lower panel, results of the GST pull-down assay. MEF-E6AP was detected by anti-myc MAb. CBB, Coomassie brilliant blue; IB, immunoblot.

duplex reduced the protein level of E6AP by 90% at 48 h posttransfection (Fig. 4C, middle panel). Immunoblotting revealed a 4.1-fold increase in the level of the core protein in the cells transfected with E6AP siRNA (Fig. 4C, top panel), suggesting that endogenous E6AP plays a role in the proteolysis of the HCV core protein.

Then we examined whether E6AP reduces the steady-state

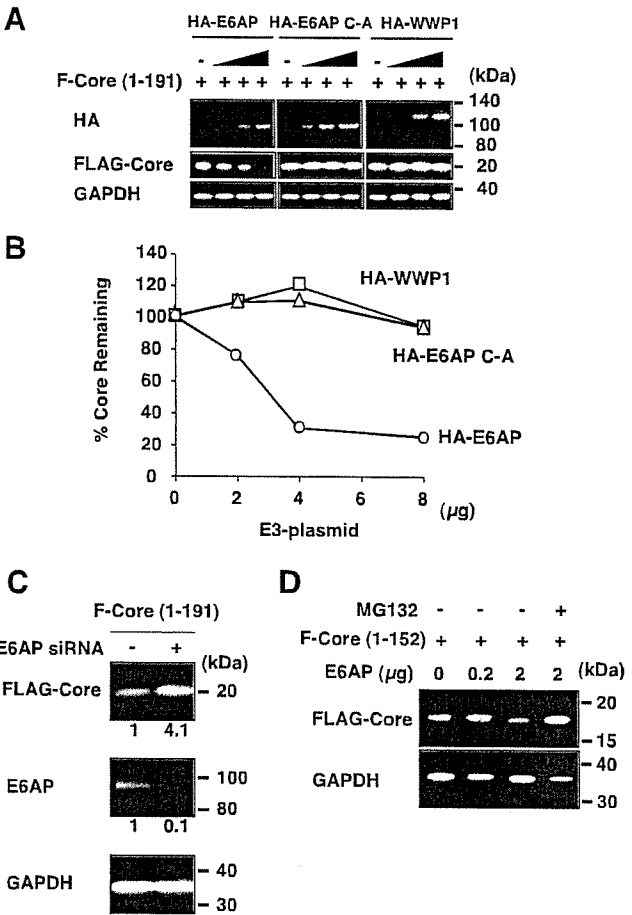


FIG. 4. E6AP decreases steady-state levels of HCV core protein in 293T cells and in HepG2 cells. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μ g of pCAG FLAG-core (1-191) along with either pCAG-HA-E6AP, pCAG-HA-E6AP C-A, or pCAG-HA-WWP1 as indicated. At 48 h posttransfection, protein extracts were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA PAb (top panel), anti-FLAG MAb (middle panel), and anti-GAPDH MAb (bottom panel). (B) Quantitation of data shown in panel A. Intensities of the gel bands were quantitated using the NIH Image 1.62 program. The level of GAPDH served as a loading control. Circles, E6AP; triangles, E6AP C-A; squares, WWP1. (C) Knockdown of endogenous E6AP by siRNA inhibits degradation of HCV core protein in 293T cells. 293T cells (3×10^5 cells/six-well plate) were transfected with 40 pmol of E6AP-specific duplex siRNA (or control siRNA) as described in Materials and Methods. The cells were transfected with 2 μ g of FLAG-core (1-191) expression plasmid and cultured for 24 h, harvested, and analyzed by immunoblotting. Shown is immunoblot detection of FLAG-tagged core protein (top panel), E6AP protein (middle panel), and GAPDH (bottom panel) in control siRNA-treated 293T cells or E6AP-siRNA-treated 293T cells. The relative levels of protein expression were quantitated by densitometry and indicated below in the respective lanes. GAPDH served as a loading control. (D) HepG2 cells (2×10^5 cells/six-well plate) were transfected with pCAG FLAG-core (1-152) along with either empty vector or pCMV E6AP as indicated. The cells were harvested at 44 h posttransfection. Where indicated, cells were treated with 25 μ M MG132 or with dimethyl sulfoxide control 14 h prior to collection. Equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-FLAG MAb (upper panel) or anti-GAPDH MAb (lower panel).

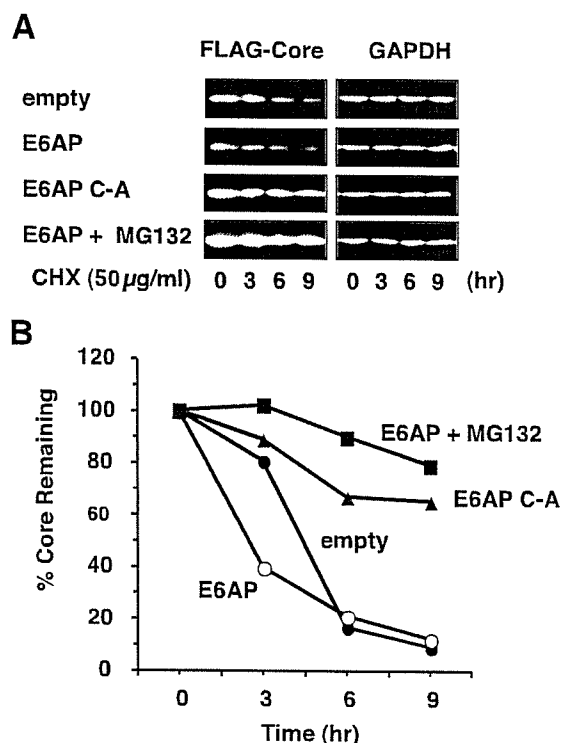


FIG. 5. Kinetic analysis of E6AP-dependent degradation of HCV core protein. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 µg of pCAG-FLAG core (1–152) plus 4 µg of empty vector, pCMV-HA-E6AP, or pCMV-HA-E6AP C-A. The cells were treated with 50 µg/ml CHX at 44 h after transfection. Cell extracts were collected at 0, 3, 6, and 9 h after treatment with CHX, followed by immunoblotting. (B) Specific signals were quantitated by densitometry, and the percent remaining core at each time was compared with that at the starting point. The level of GAPDH served as a loading control. Open circles, E6AP; closed circles, empty plasmid; closed triangles, E6AP C-A; closed squares, E6AP with MG132 treatment. Data are representative of three independent experimental determinations.

levels of the core protein in hepatic cells as well as in 293T cells. Exogenous expression of E6AP resulted in reduction of the core protein in human hepatoblastoma HepG2 cells (Fig. 4D). Treatment of the cells with the proteasome inhibitor MG132 increased the core protein level, suggesting that the core protein was degraded through the ubiquitin-proteasome pathway. These results indicate that E6AP enhances proteasomal degradation of the HCV core protein in both hepatic cells and nonhepatic cells.

Kinetic analysis of E6AP-dependent degradation of HCV core protein. To determine whether the E6AP-induced reduction of the core protein is due to an increase in the rate of core degradation, we performed kinetic analysis using the protein synthesis inhibitor CHX. HCV core protein together with wild-type E6AP or inactive mutant E6AP C-A was expressed in 293T cells. At 44 h after transfection, cells were treated with either 50 µg/ml CHX alone or 50 µg/ml CHX plus 25 µM MG132 to inhibit proteasome function. Cells were collected at 0, 3, 6, and 9 h following treatment and analyzed by immunoblotting (Fig. 5A). Overexpression of E6AP resulted in rapid degradation of the core protein, whereas inactive mutant

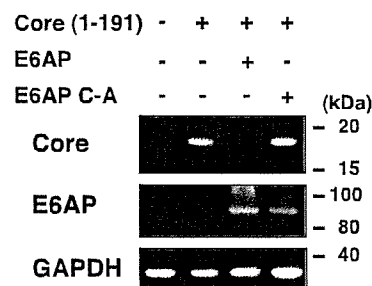


FIG. 6. E6AP promotes degradation of full-length HCV core protein in Huh-7 cells. Huh-7 cells (2×10^5 cells/six-well plate) were transfected with 0.5 µg of pCAG-core (1–191) together with 2 µg of pCMV-HA-E6AP or pCMV-HA-E6AP C-A. At 48 h posttransfection, cells were harvested and analyzed by immunoblotting with anticore MAb (top panel), anti-E6AP PAb (middle panel), or anti-GAPDH MAb (bottom panel).

E6AP C-A increased the half-life of the core protein (Fig. 5B), suggesting that the inactive E6AP inhibited degradation of the core protein in a dominant-negative manner, which is in agreement with previous studies (19, 55). Treatment of the cells with MG132 inhibited the degradation of the core protein (Fig. 5B). Reverse transcription-PCR to determine mRNA levels of the HCV core gene and GAPDH gene found that neither wild-type E6AP nor inactive E6AP changed mRNA levels of the HCV core gene and GAPDH gene (data not shown). These results indicate that E6AP enhances proteasomal degradation of the core protein.

E6AP promotes degradation of the full-length core protein in Huh-7 cells. To determine whether the full-length HCV core protein expressed in hepatic cells is degraded through an E6AP-dependent pathway, human hepatoma Huh-7 cells were transfected with pCAG HCV core (1–191) along with either E6AP or E6AP C-A. To rule out the effects of N-terminal FLAG tag on the core degradation, HCV core protein was expressed as untagged protein. Expression of wild-type E6AP resulted in reduction of the core protein (Fig. 6). On the other hand, HCV core protein was not decreased after transfection of inactive E6AP, indicating that the full-length core protein expressed in Huh-7 cells is also degraded through an E6AP-dependent pathway.

E6AP mediates ubiquitylation of HCV core protein in vivo. To determine whether E6AP can induce ubiquitylation of HCV core protein in cells, we performed in vivo ubiquitylation assays. 293T cells were cotransfected with FLAG-core (1–191) and either E6AP or empty plasmid, together with a plasmid encoding HA-tagged ubiquitin to facilitate detection of ubiquitylated core protein. Cell lysates were immunoprecipitated with anti-FLAG MAb and immunoblotted with anti-HA PAb to detect ubiquitylated core protein (Fig. 7A). Only a little ubiquitin signal was observed on the core protein in the absence of cotransfected E6AP (Fig. 7A, lane 3). In contrast, coexpression of E6AP led to readily detectable ubiquitylated forms of the core protein as a ladder and a smear of higher-molecular-weight bands (Fig. 7A, compare lane 3 with lane 4). Immunoblot analysis with anticore PAb confirmed that FLAG-core proteins were immunoprecipitated (Fig. 7B, lanes 2 to 4, short exposure) and that higher-molecular-weight bands con-

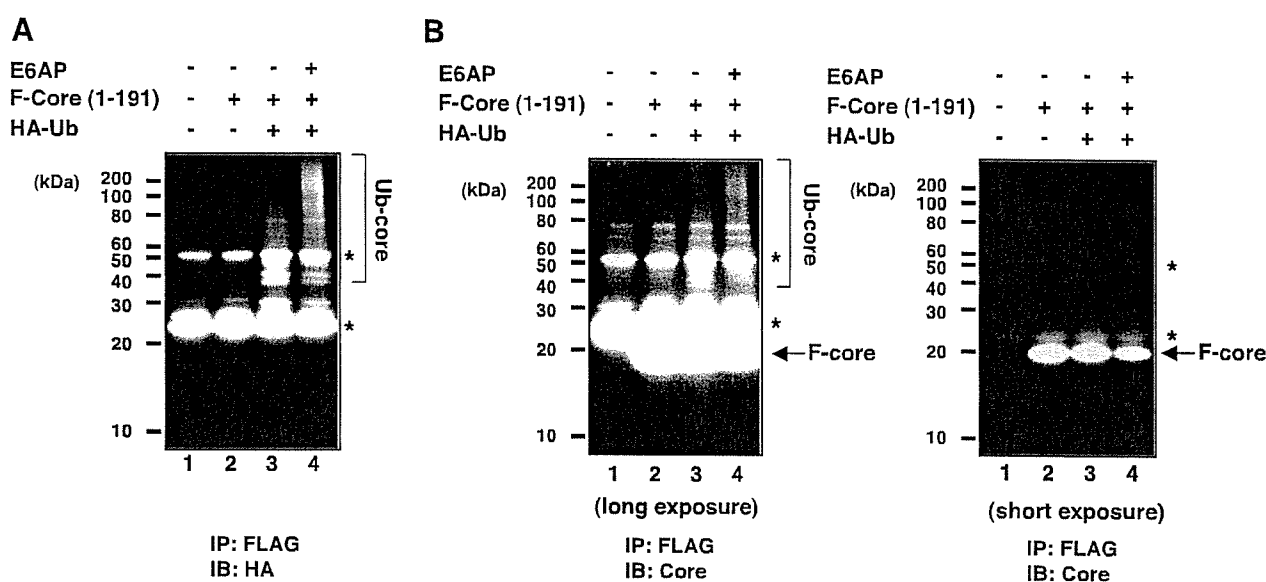


FIG. 7. E6AP-dependent ubiquitylation of HCV core protein in vivo. 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μ g of pCAG FLAG-core (1-191) together with 2 μ g of plasmid encoding E6AP as indicated. Each transfection also included 2 μ g of plasmid encoding HA-ubiquitin. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb (A) or anticore PAb (B). A shorter exposure of the core blot shows immunoprecipitated FLAG-core protein (B, right panel). A longer exposure of the core blot shows the presence of a ubiquitin smear (B, left panel). Asterisks indicate cross-reacting immunoglobulin light chain or heavy chain. Arrows indicate FLAG-core. IB, immunoblot; IP, immunoprecipitation.

jugated with HA-ubiquitin were indeed ubiquitylated forms of the core protein (Fig. 7B, lanes 3 and 4, long exposure).

E6AP mediates ubiquitylation of HCV core protein in vitro. To rule out the possibility that E6AP contributes to core protein degradation by inducing degradation of inhibitors of core turnover, we determined whether E6AP functions directly as a ubiquitin ligase by testing the ability of purified MEF-E6AP to mediate in vitro ubiquitylation of the purified recombinant HCV core protein. HCV core protein was expressed as a fusion protein containing N-terminal GST tag and C-terminal His tag and purified as described in Materials and Methods. GST-C173HT (aa 1-173) and GST-C152HT (aa 1-152) (see Materials and Methods) were used to determine whether the mature core protein and the C-terminally truncated core protein are targeted for ubiquitylation in vitro. The validity of this assay was established by demonstrating that E6AP but not E6AP C-A induced ATP-dependent ubiquitylation of GST-core protein. When in vitro ubiquitylation reactions were carried out either in the absence of MEF-E6AP or in the presence of MEF-E6AP C-A, no ubiquitylation signal was detected (Fig. 8A, lanes 4 and 5). However, inclusion of purified MEF-E6AP in the reaction mixture resulted in marked ubiquitylation of GST-C173HT (Fig. 8A, lane 6), while no ubiquitylation was observed in the absence of ATP (Fig. 8A, lane 7). No signal was detected when GST-HT was used as a substrate (Fig. 8A, lane 8). The higher-molecular-weight species of GST-core proteins were reactive with both anti-ubiquitin MAb (Fig. 8B, right panel, lanes 2 and 4) and anti-GST MAb (Fig. 8B, left panel, lanes 2 and 4). Both GST-C152HT and GST-C173HT were polyubiquitylated by E6AP in vitro (Fig. 8B), indicating that both the C-terminally truncated core and the mature core are polyubiquitylated by E6AP in vitro. These results revealed

that E6AP directly mediated ubiquitylation of HCV core proteins in an ATP-dependent manner.

Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. We used a recently developed system for the production of infectious HCV particles using the HCV JFH1 strain (28, 56, 61) to examine whether E6AP can promote degradation of HCV core protein expressed from infectious HCV. E6AP-dependent core degradation was assessed in Huh-7 cells inoculated with the culture supernatant containing HCV JFH1. Levels of HCV core protein were detectable at day 3 postinfection and increased with time. Immunofluorescence staining for the core protein indicated that the percentage of HCV core-positive cells in the Huh-7 cells was almost 100 at day 7 postinfection. Transfection efficiency was 50 to 60% as measured with GFP-expressing plasmid. At day 7 postinfection, exogenous expression of E6AP reduced the intracellular core protein level by about 60% compared to the empty plasmid-transfected control cells (Fig. 9A). Inactive E6AP had little effect on the core protein levels. Total protein levels in the cells (Fig. 9B) and intracellular HCV RNA levels (Fig. 9C) did not change after transfection of wild-type E6AP or inactive E6AP. The immunofluorescence study revealed that HCV core protein was variably detected and the intensity of core staining was reduced in the cells staining positive for wild-type E6AP compared with neighboring cells staining negative for E6AP (Fig. 9E). Using inactive E6AP revealed colocalization of the core protein and E6AP in the perinuclear region (Fig. 9F) of HCV-infected cells. These results suggest that E6AP enhanced degradation of HCV core protein expressed from infectious HCV. Then we titrated HCV infectivity in the culture supernatant at day 7 postinfection by limiting

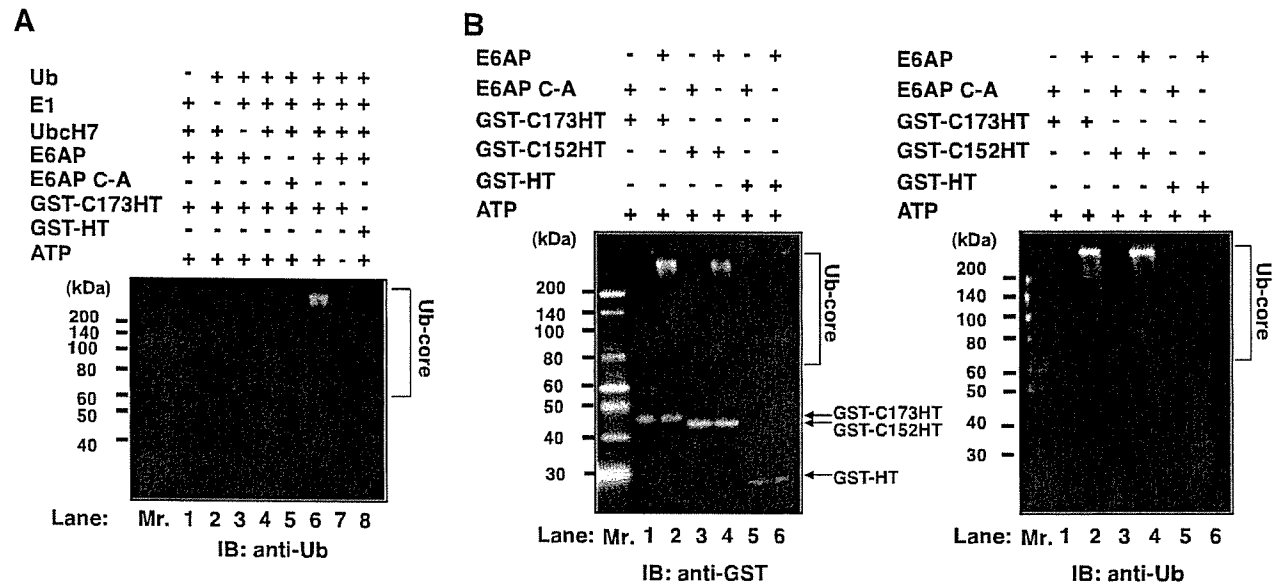


FIG. 8. In vitro ubiquitylation of HCV core protein by recombinant E6AP. For in vitro ubiquitylation of HCV core protein, purified GST-C173HT and GST-C152HT were used as substrates. Purified GST-HT was used as a negative control. Assays were done in 40- l volumes containing each component as indicated. The reaction mixture is described in Materials and Methods. The reaction was carried out at 37°C for 120 min followed by purification with glutathione-Sepharose beads and analysis by immunoblotting with the indicated antibodies. Arrows indicate GST-C173HT, GST-C152HT, and GST-HT, respectively. Ubiquitylated species of GST-core proteins are marked by brackets. IB, immunoblot.

dilution assays. Exogenous expression of E6AP reduced the supernatant infectivity titer, whereas inactive E6AP had no effect on its infectivity titer (Fig. 9D), suggesting that the E6AP-dependent ubiquitin proteasome pathway affects the production of HCV particles through downregulation of the core protein.

E6AP silencing increases the levels of intracellular HCV core protein and supernatant infectivity titers in HCV-infected Huh-7 cells. Finally, to further validate the role of E6AP in HCV production, expression of endogenous E6AP was knocked down by siRNA and the HCV infectivity titers released from HCV JFH1-infected cells were examined. Knock-down of E6AP by siRNA led to an increase in intracellular core protein levels (Fig. 10A) and supernatant HCV infectivity titers (Fig. 10B). Taken together, our results suggest that E6AP mediates ubiquitylation and degradation of HCV core protein in HCV-infected cells, thereby affecting the production of HCV particles.

DISCUSSION

HCV core protein is a major component of viral nucleocapsid, plays a central role in viral assembly (25, 40), and contributes to viral pathogenesis and hepatocarcinogenesis (9). Therefore, it is important to clarify the molecular mechanisms that govern the cellular stability of this viral protein. We have previously reported that processing at the C-terminal hydrophobic domain of the core protein leads to efficient polyubiquitylation of the core protein (52). In this study, we identified E6AP as an HCV core-binding protein and showed that HCV core protein interacts with E6AP in vivo and in vitro, that E6AP enhances ubiquitylation and degradation of the mature core protein as well as the C-terminally truncated core protein, and that HCV core protein expressed from infectious HCV is

degraded via E6AP-dependent proteolysis. HCV core protein and E6AP were found to colocalize in the cytoplasm, especially in the perinuclear region. Moreover, exogenous expression of E6AP reduces intracellular core protein levels and supernatant HCV infectivity titers in HCV-infected Huh-7 cells. Knock-down of endogenous E6AP by siRNA increases intracellular core protein levels and supernatant infectivity titers in HCV-infected cells. These findings suggest that E6AP mediates ubiquitylation and degradation of HCV core protein, thereby affecting the production of HCV particles.

HCV core protein interacts with E6AP through the region of the core protein between aa 58 and aa 71. These 14 amino acids are highly conserved, with the first nine amino acids (PRGRRQPIP) present in the core protein of all the HCV genotypes (3). This result suggests that E6AP-dependent degradation of HCV core protein is common to all HCV genotypes and plays an important role in the HCV life cycle or viral pathogenesis. Our data indicated that HCV core proteins of genotypes 1b and 2a are subjected to proteolysis through an E6AP-mediated degradation pathway. We are currently examining whether E6AP promotes degradation of HCV core proteins of other genotypes.

Studies in addition to ours have reported that other HCV proteins, such as NS5B (8), the unglycosylated cytosolic form of E2 (39), NS2 (7), and F protein (58), are degraded through the ubiquitin-proteasome pathway. These studies suggest that the ubiquitin-proteasome pathway plays a role in the HCV life cycle or viral pathogenesis. To our knowledge, the present study is the first to demonstrate that the ubiquitin-proteasome pathway affects the HCV life cycle.

PA28 was found to interact with HCV core protein in hepatocytes and promote proteasomal degradation of HCV core protein (30). PA28, however, has been shown to function

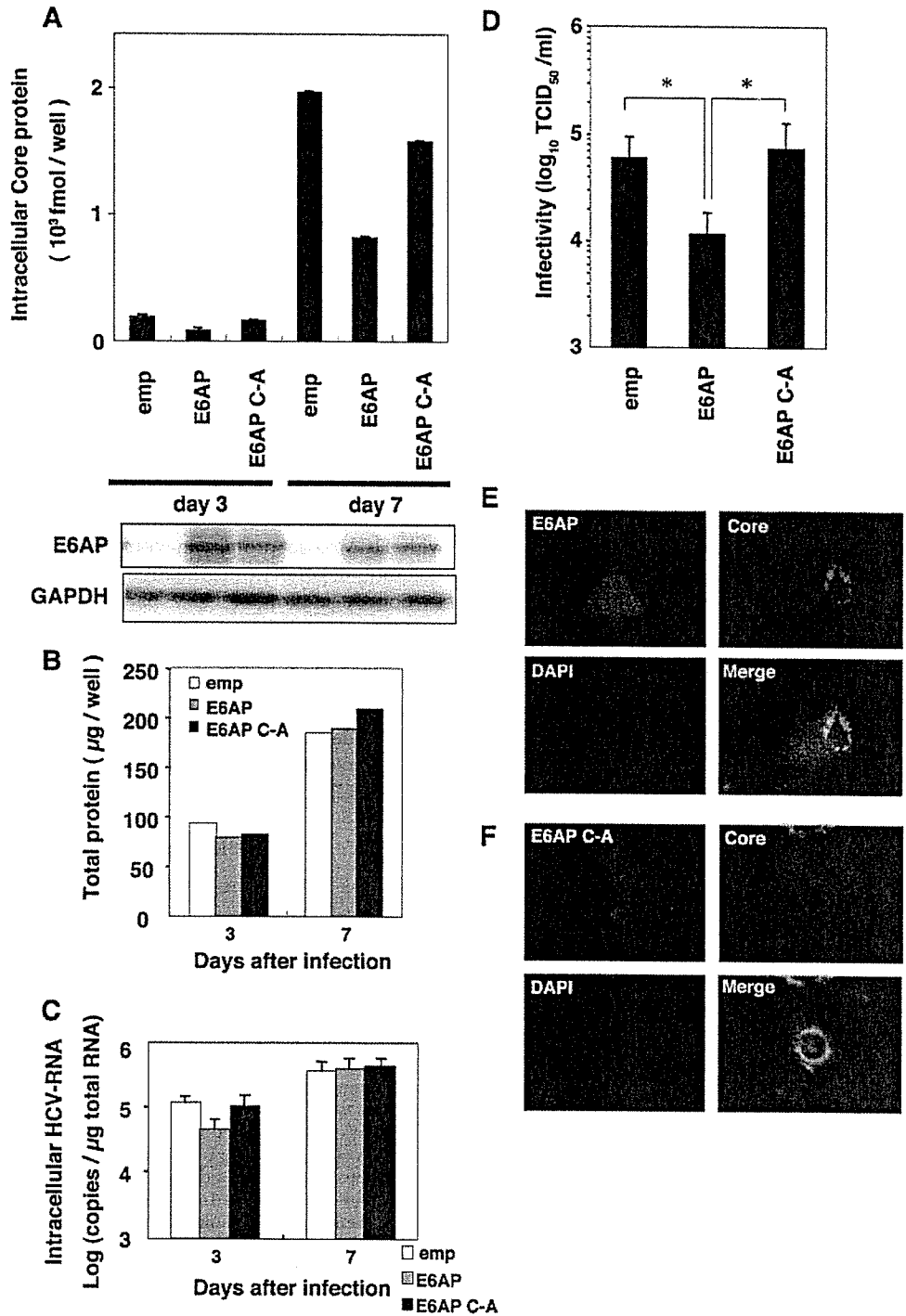


FIG. 9. Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. Naïve Huh-7 cells were seeded as described in Materials and Methods; inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5×10^3 TCID₅₀/ml); and transfected with 6 μ g of empty plasmid, pCAG-HA-E6AP, or pCAG-HA-E6AP C-A. The culture supernatant and the cells were collected at days 3 and 7 postinfection. (A) Intracellular HCV core protein levels. (B) Levels of total protein. (C) Levels of intracellular HCV RNA in HCV-infected Huh-7 cells. Data represent the averages of three experiments with error bars. (D) Supernatant infectivity titers. At day 7 postinfection, culture supernatants were collected and assayed for TCID₅₀ determinations. The difference between empty vector and E6AP or between E6AP and E6AP C-A was significant ($P < 0.05$, Student's *t* test). (E and F) HCV JFH1-infected Huh-7 cells were transfected with either MEF-E6AP plasmid or MEF-E6AP C-A plasmid, grown on coverslips, fixed, and processed for double-label immunofluorescence for HCV core and MEF-E6AP (E) or MEF-E6AP C-A (F). Anticore MAb (2H9) and anti-FLAG PAb were used as primary antibodies. Nuclei were visualized by staining the cells with DAPI. All the samples were examined with a BZ-8000 microscope. Representative images of individual cells are shown with merge images. emp, empty vector.

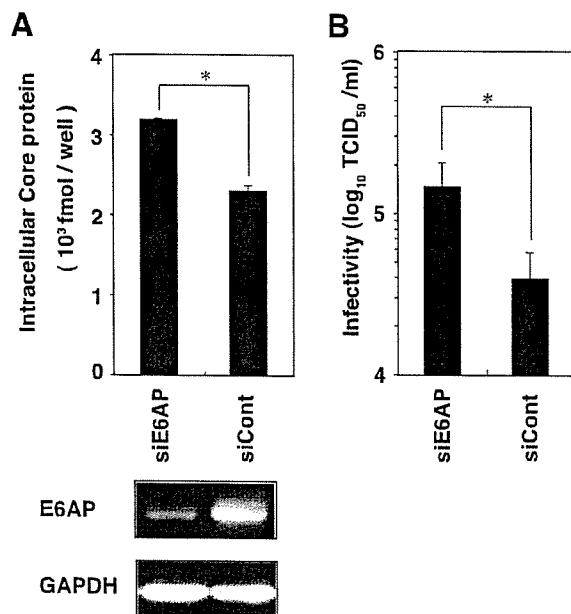


FIG. 10. E6AP silencing leads to an increase in the level of intracellular HCV core protein and supernatant infectivity titer in HCV-infected Huh-7 cells. (A) HCV JFH1-infected cells were replated in a six-well plate at 3×10^5 cells/well and transfected with 40 pmol of E6AP siRNA or control siRNA. The culture medium was changed at 24 h after transfection. The cells were harvested at day 2 after transfection, and the intracellular core protein levels were quantitated using the HCV core antigen ELISA. Equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-E6AP Mab or anti-GAPDH Mab. (B) Culture supernatants were collected at day 2 after transfection and assayed for TCID₅₀ determinations. For both panels, the difference between E6AP siRNA and control siRNA was significant ($P < 0.05$, Student's *t* test).

in a ubiquitin-independent, ATP-independent, and 20S proteasome-dependent pathway (27). There have been reports that several cellular factors, such as p53 (2), p73 (2), and RPN4 (18), are degraded through two alternative pathways, the ubiquitin-dependent 26S proteasome-dependent pathway and the ubiquitin-independent 20S proteasome-dependent pathway. Here we provide evidence that E6AP mediates ubiquitylation of HCV core protein. Still unclear is whether the PA28-dependent pathway requires polyubiquitylation of HCV core protein. HCV core protein is predominantly localized in the cytoplasm, especially at the endoplasmic reticulum membrane, on the surface of lipid droplets, and on mitochondria and mitochondrion-associated membranes (51). In HCV JFH1-infected cells, HCV core was found to localize in the cytoplasm and frequently to accumulate in the perinuclear region and the lipid droplets (44). Our results indicated that E6AP colocalized with HCV core protein especially in the perinuclear region. PA28 was found to colocalize with HCV core protein in the nucleus. Functional differences may exist between the E6AP-dependent pathway and the PA28-dependent pathway in the stability control of HCV core protein. The functional role of the E6AP-dependent pathway and the PA28-dependent pathway remains to be elucidated.

The HCV core-binding region of E6AP was mapped to the region between aa 418 and aa 517. The multicopy maintenance protein 7, Mcm7, interacts with E6AP through a short motif,

termed the L2G box (aa 412 to 414), that lies within the E6 binding site of E6AP (23). Our data indicated that the E6 binding region containing the L2G motif is not required for interaction between HCV core protein and E6AP (Fig. 2C, lane M).

We propose here that E6AP may affect the production of HCV particles through controlling the amounts of HCV core protein. This mechanism may contribute to persistent infection. The E6AP binding domain of the core protein resides in the RNA-binding domain and binding domains for many host factors (40). These factors may affect the binding between E6AP and HCV core protein, resulting in control of E6AP-dependent core degradation. Another possibility is that HCV core protein may affect the normal function of E6AP, thereby contributing to pathogenesis. It will be intriguing to investigate whether HCV core protein has any effect on E6AP-dependent degradation of host factors. The other intriguing possibility is that HCV core-E6AP complex may function as an E3 ligase-like E6-E6AP complex to target host factors for proteasomal degradation and contribute to viral pathogenesis.

In conclusion, we have demonstrated that E6AP interacts with HCV core protein in vitro and in vivo and mediates ubiquitin-dependent degradation of the core protein, leading to downregulation of HCV particles. We propose that the E6AP-mediated ubiquitin-proteasome pathway may play a role in affecting the production of HCV particles through controlling the amounts of viral nucleocapsid protein. Identification of the specific E3 ubiquitin ligase may contribute to gaining a better understanding of the biology of the HCV life cycle as well as molecular details of the ubiquitin-dependent degradation of HCV core protein.

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Oligomerization of Hepatitis C Virus Core Protein Is Crucial for Interaction with the Cytoplasmic Domain of E1 Envelope Protein

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Hepatitis C virus (HCV) contains two membrane-associated envelope glycoproteins, E1 and E2, which assemble as a heterodimer in the endoplasmic reticulum (ER). In this study, predictive algorithms and genetic analyses of deletion mutants and glycosylation site variants of the E1 glycoprotein were used to suggest that the glycoprotein can adopt two topologies in the ER membrane: the conventional type I membrane topology and a polytopic topology in which the protein spans the ER membrane twice with an intervening cytoplasmic loop (amino acid residues 288 to 360). We also demonstrate that the E1 glycoprotein is able to associate with the HCV core protein, but only upon oligomerization of the core protein in the presence of tRNA to form capsid-like structures. Yeast two-hybrid and immunoprecipitation analyses reveal that oligomerization of the core protein is promoted by amino acid residues 72 to 91 in the core. Furthermore, the association between the E1 glycoprotein and the assembled core can be recapitulated using a fusion protein containing the putative cytoplasmic loop of the E1 glycoprotein. This fusion protein is also able to compete with the intact E1 glycoprotein for binding to the core. Mutagenesis of the cytoplasmic loop of E1 was used to define a region of four amino acids (residues 312 to 315) that is important for interaction with the assembled HCV core. Taken together, our studies suggest that interaction between the self-oligomerized HCV core and the E1 glycoprotein is mediated through the cytoplasmic loop present in a polytopic form of the E1 glycoprotein.

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis C, leading to steatosis, cirrhosis, and hepatocellular carcinoma. It is estimated that over 170 million people are infected with HCV worldwide (5, 18, 37). HCV is an enveloped single-stranded plus-sense RNA virus in the *Hepacivirus* genus of the family *Flaviviridae*, which also includes the flaviviruses and pestiviruses (36). The genome of HCV encodes a polyprotein of approximately 3,000 amino acids which is cotranslationally and posttranslationally processed to generate at least 10 viral proteins (12). The structural proteins, the core and E1 and E2 envelope glycoproteins, are encoded in the N-terminal portion of the polyprotein, and the nonstructural proteins, thought to be required for replication of the viral genome, are encoded in the C-terminal region (11). The core protein, which interacts with viral RNA (47) to form the nucleocapsid, is liberated from the N terminus of the polyprotein by signal peptidase cleavage in the downstream E1 protein (at position 191), and the C-terminal transmembrane region of the core protein (residue 164 to 191) is further cleaved at residues 177 or 179 by the signal peptide peptidase (16, 43). The remaining hydrophobic region of the core protein (domain II; residues 119 to 174) has been shown to affect the efficiency of signal peptide peptidase cleavage and the intracellular localization of core protein (14, 44). Although the C-terminal transmembrane

region of core protein and E1 were reported to interact with each other within the intramembrane space (25), the central hydrophobic region from residues 119 to 152 within domain II was also suggested to be responsible for the interaction between core and E1 (27).

Recently, in vitro replication of a JFH1 clone of HCV genotype 2a derived from a patient with fulminant hepatitis C was reported in a cell line that had been cured of its HCV replicon by treatment with interferon (23, 50, 51). However, this reverse genetics system is limited to the JFH-1 clone of genotype 2a and specific cell lines. Robust and reliable in vitro replication of other major genotypes of HCV such as genotypes 1a and 1b has yet to be developed. So far, biological functions of HCV envelope proteins have been characterized by using recombinant envelope proteins expressed in vitro, HCV-like particles produced in insect cells, and the pseudotyped virions based on vesicular stomatitis virus and retroviruses (8). The HCV polyprotein precursor must be specifically threaded through the membrane of the endoplasmic reticulum (ER) to undergo maturation to form the mature envelope glycoproteins (7). In the polyprotein, the C-terminal regions of E1 and E2 each contain a membrane-spanning domain as well as the hydrophobic signal peptide of the downstream viral protein (E2 and p7, respectively). These domains form hairpin structures that pass through the membrane twice, to allow processing by signal peptidase in the ER lumen. Upon signal peptidase cleavage, the C termini are thought to translocate into the cytoplasm to generate the type I membrane topology of the mature glycoproteins. The mature E1 and E2 glycoproteins

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remain noncovalently associated, interacting in part through their C-terminal transmembrane domains, which also mediate retention of the E1-E2 complex in the ER. Based on this model of membrane topology, the HCV envelope glycoproteins possess little or no cytoplasmic region. However, a physical association between E1 and the cytosolic core protein has been reported (25, 27), suggesting that the E1 glycoprotein is able to expose a cytoplasmic domain of sufficient length to interact with the core. In addition, the presence of the core protein has been shown to affect the folding of E1 (32).

We have previously suggested that the E1 glycoprotein may adopt a polytopic (double membrane-spanning) topology that coexists with the dominant type I form (35). In this study, we provide genetic evidence for a polytopic form of the E1 glycoprotein and for exposure of a centrally located cytoplasmic domain. Furthermore, we show that the cytoplasmic region of the polytopic form of E1 is required for interaction with amino acid residues 72 to 91 of the core protein.

MATERIALS AND METHODS

Cell culture. 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 2 mM L-glutamine, penicillin, and streptomycin and supplemented with 10% fetal bovine serum.

Plasmids. A cDNA of E1 glycoprotein was amplified from HCV type 1b strain J1 (1) by PCR using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and inserted between *NheI* and *BamHI* sites of pJW4303, which contains the signal sequence of tissue plasminogen activator and the bovine growth hormone polyadenylation signal (a kind gift from J. M. Mullins), to generate pJW383. For the deletion analysis, the plasmids pJW360 and pJW288 encoding residues 192 to 360 and 192 to 288, respectively, were amplified by PCR and cloned into pJW4303. The plasmids pJW383d1 and pJW383d2, containing deletions in residues 261 to 286 and 289 to 340, respectively, were generated from pJW383 by splicing of overlapping extensions (13, 15) as described previously (44). A cDNA fragment encoding core to E2 proteins of HCV strain J1 was amplified by PCR and cloned into pCAGGS-PUR (28), and glycosylation site mutations in the E1 protein were generated by the method of splicing by overlapping extension. For the yeast two-hybrid assay, pGBKT7HCVCore173 was used as bait, as described previously (38). The gene encoding amino acids 288 to 346 of HCV E1 protein was amplified from cDNA of strain J1 and introduced into *NdeI* and *EcoRI* sites of a pGADT7 vector (Clontech, Palo Alto, CA). In the same way, deletion mutants of core protein encoding residues 1 to 151, 1 to 25, 24 to 173, 38 to 173, 58 to 173, 72 to 173, and 92 to 173 were amplified by PCR and cloned into a pGBKT7 vector. The FLAG sequence was introduced between amino acids 195 and 196 of the cDNA encoding residues 1 to 383 of the HCV polyprotein and replaced Ala²⁹³ with Arg to avoid processing by signal peptidase and spacer amino acids (Gly-Gly-Gly-Ser), and influenza virus hemagglutinin (HA) sequence was added at the C terminus. The resulting cDNA fragment encoding core protein, FLAG tag, E1, and HA tag was cloned into a pcDNA3.1(+) vector and designated Flag-core-E1-HA (see Fig. 2D, below) and used for in vitro transcription and translation. Similarly, the FLAG sequence was introduced into the cDNA encoding residues 151 to 383 of the HCV polyprotein, and the HA sequence was added at the C terminus. The resulting cDNA fragment encoding the C-terminal hydrophobic/transmembrane region of the core protein, FLAG tag, E1, and HA tag was designated Flag-E1-HA (see Fig. 3A, below). The DNA fragments encoding residues 1 to 191 with amino acids 72 to 91 deleted were generated by splicing via overlapping extension and cloned into pCAGGS (Core 72-91) (see Fig. 4A, below) (42). The DNA fragment encoding the cytoplasmic domain of the E1 protein with a C-terminal HA tag was amplified by PCR and introduced at *HindIII* and *SacII* sites of pEGFP-C3. pCAGGS plasmids encoding core to p7 replacing residues 304 to 307, 308 to 311, 312 to 315, 316 to 319, 320 to 323, 324 to 327, or 328 to 331 with Ala were generated by using splicing with overlapping extension (see Fig. 6A, below).

Antibodies. Mouse monoclonal antibody to HA tag (HA11) and anti-FLAG antibody (M2) were purchased from Covance (Richmond, CA) and Sigma, respectively. Mouse monoclonal antibodies to core protein (clones 11-7, 11-10, and 11-14) were gifts from S. Yagi (2). Anti-E1 mouse monoclonal antibody (clone 0726) was prepared by immunization using the membrane fraction of the

CHO L10 cell line, which constitutively expresses HCV envelope proteins (30). Anti-E2 monoclonal antibody (clone 187) was a generous gift from M. Kohara.

Yeast two-hybrid assay. A yeast two-hybrid assay was carried out by using Matchmaker system 3 (Clontech) according to the manufacturer's protocol. The bait vector pGBKT7HCVcore 173 (38) or empty plasmid was transfected into *Saccharomyces cerevisiae* strain AH109 together with the prey vectors, pGADT7-based constructs (see Table 1, below). The yeast cells possessing pGBKT7/p-53 and pGADT7/large T antigen were used as positive controls, while yeast cells possessing pGBKT7 and pGADT7 were the negative controls. These transfected yeast colonies were cultivated on dropout plates lacking Trp, Leu, His, and Ade (test plates) or plates lacking Trp and Leu (control plates) and then incubated at 30°C for 1 week.

Transfection, immunoblotting, and immunoprecipitation. Liposome-mediated DNA transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was described previously (38). Transfected cells were cultured at 2×10^5 cells/well in a six-well plate, harvested 30 to 48 h posttransfection, washed twice with phosphate-buffered saline (PBS), and incubated at 4°C for 30 min in 0.25 ml of lysis buffer (20 mM Tris-HCl [pH 7.4], 135 mM NaCl, 1% Triton X-100, and 10% glycerol supplemented with 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 5 mM Na₂VO₄). After freezing and thawing, lysed cells were centrifuged at 20,000 g for 5 min. The resulting cleared lysate was stored at -80°C prior to use for immunoprecipitation and blotting. Immunoprecipitation was carried out according to the method described previously (44). Briefly, lysates were preincubated at 4°C for 5 h in the lysis buffer with or without 1 mM MgCl₂ and 0.1 mg/ml of yeast tRNA (Sigma) prior to immunoprecipitation. The resulting lysates (0.2 ml) were gently rotated with 1.0 g of anti-FLAG, anti-HA, or mixed mouse monoclonal anti-HCV core antibodies or mouse monoclonal antibody to the E1 protein at 4°C for 3 h with or without 1 mM MgCl₂ and 0.1 mg/ml of yeast tRNA. The immunocomplex was gently rotated at 4°C for 3 h with 10 l of 50% (vol/vol) protein G-Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ) with or without 1 mM MgCl₂ and 0.1 mg/ml of yeast tRNA and then centrifuged at 20,000 g for 30 s. The precipitated beads were washed five times with 0.5 ml of lysis buffer containing or lacking 1 mM MgCl₂ and 0.1 mg/ml of yeast tRNA and then boiled in 50 l of the loading buffer. The boiled samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins in gels were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA) and then blotted with primary antibody and secondary horseradish peroxidase-conjugated antibody. The immunocomplexes on membranes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

Protease protection assay of HCV proteins synthesized by in vitro transcription/translation. A plasmid encoding a FLAG-core-E1-HA protein was transcribed under the control of a T7 promoter by using the RiboMax large-scale RNA production system with Ribo m⁷G cap analog (Promega, Madison, WI). In vitro translation was carried out in the presence of [³⁵S]methionine-cysteine (Amersham, Piscataway, NJ) by using rabbit reticulocyte lysate and canine pancreatic microsomal membrane (Promega). Translated sample was diluted sevenfold with PBS and then mixed with tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Sigma) at a final concentration of 2 g/ml. The mixture was incubated at 30°C for 60 min with or without 0.5% Nonidet P-40, and then soybean trypsin inhibitor (Sigma) was added at a final concentration of 20 g/ml. Digestion products were immunoprecipitated with anti-FLAG antibody.

Indirect immunofluorescence analysis. 293T cells were washed with PBS at 40 h after transfection and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 for 3 min at room temperature and blocked with nonfat milk solution. Cells were incubated with the anti-E1 antibody for 60 min at 37°C and then with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG; TAGO, Burlingame, CA). HCV E1 protein was visualized by fluorescence microscopy (TE300; Nikon, Tokyo, Japan).

Velocity sedimentation with sucrose gradients. Transfected 293T cell were suspended in MNT buffer (20 mM 2-morpholinoethanesulfonic acid, 100 mM NaCl, 30 mM Tris-HCl [pH 8.6], and 0.1% Triton X-100) and then incubated at 4°C for 5 h with or without 0.1 mg/ml of yeast tRNA and 1 mM MgCl₂. Each sample was layered on top of 12 ml of sucrose with a 20 to 60% gradient and then centrifuged in a Beckman SW 41Ti rotor (Beckman Coulter, Tokyo, Japan) at 30,000 rpm for 3 h at 4°C. Centrifuged lysates were collected from the bottoms of the tubes and then concentrated with trichloroacetic acid. After washing with ethanol, concentrated proteins were subjected to SDS-PAGE and immunoblotting.

RESULTS

Prediction of the topology of the E1 protein in the membrane. Although a small fraction of the HCV envelope glycoproteins expressed in 293T cells is translocated onto the plasma membrane (3), the vast majority of E1 is retained in the ER membrane (6). Previously, we showed that both a central hydrophobic region of E1 (residues 260 to 288) and the C-terminal transmembrane domain (residues 360 to 383) are important for ER retention (29). As in the C-terminal hydrophobic region, the amino acid sequence of the central hydrophobic region is highly conserved among HCV isolates (4). To investigate the role of these two hydrophobic regions in the biogenesis of the E1 glycoprotein, we utilized the TMHMM algorithm (19), a computer program trained to identify potential transmembrane helical regions. The algorithm identified both hydrophobic regions as having a high probability of transmembrane helix (Fig. 1A). To examine the function of the hydrophobic regions as transmembrane domains, we constructed a series of deletion mutants in the E1 protein in which one or the other of the hydrophobic segments was absent (Fig. 1B). Mutant E1 glycoproteins were expressed in 293T cells, and the cellular localization of E1 proteins was determined by indirect immunofluorescence analysis (Fig. 1B). The full-length E1 (383) was detected only in permeabilized cells, consistent with its retention in the ER. The 383d2 mutant, which contains both hydrophobic regions but lacks the intervening hydrophilic region (residues 289 to 340), was also detected in the cytoplasm but not on the cell surface as the full-length E1. By contrast, deletion mutants lacking the central (383d1) or C-terminal (288 and 360) hydrophobic domains were detected on the cell surface in nonpermeabilized cells, suggesting that both the central and the C-terminal hydrophobic domains are required for retention of the E1 protein on the ER membrane. If the central hydrophobic domain traverses the ER membrane as predicted by the TMHMM program, the region between positions 288 and 360 would be expected to lie in the cytoplasmic space. Based on this model and on the results with E1 deletion mutants, we suggest that the E1 protein might be able to retain two membrane topologies: the conventional type I topology and a polytopic topology that spans the membrane twice with N and C termini in the ER lumen and an intervening cytoplasmic loop, as reported previously (35) (Fig. 1C). Recently, a similar polytopic form of the fusion glycoprotein of Newcastle disease virus was identified (31).

Mutational analysis of putative N-glycosylation sites of the E1 glycoprotein. To explore the membrane topologies of E1, we examined the utilization of potential glycosylation sites. The E1 protein of HCV strain J1 (1) contains seven N-glycosylation sequence motifs (Asn-X-Ser/Thr) at amino acid positions 196, 209, 233, 234, 250, 305, and 325 (Fig. 2A). The Asn residues at these possible N-glycosylation sites were individually replaced with Gln, and the mutant E1 glycoproteins were expressed as a core-, E1-, or E2-containing polyprotein in 293T cells. In all cases, the mutant polyproteins were expressed and properly processed by signal peptidase and signal peptide peptidase to generate the core, E1, and E2 proteins (Fig. 2B). The mutant E1 proteins displayed distinct glycoforms consistent with changes in glycosylation. The wild-type E1 glycoprotein exhibited a major band of 34 kDa and a minor band of 32 kDa.

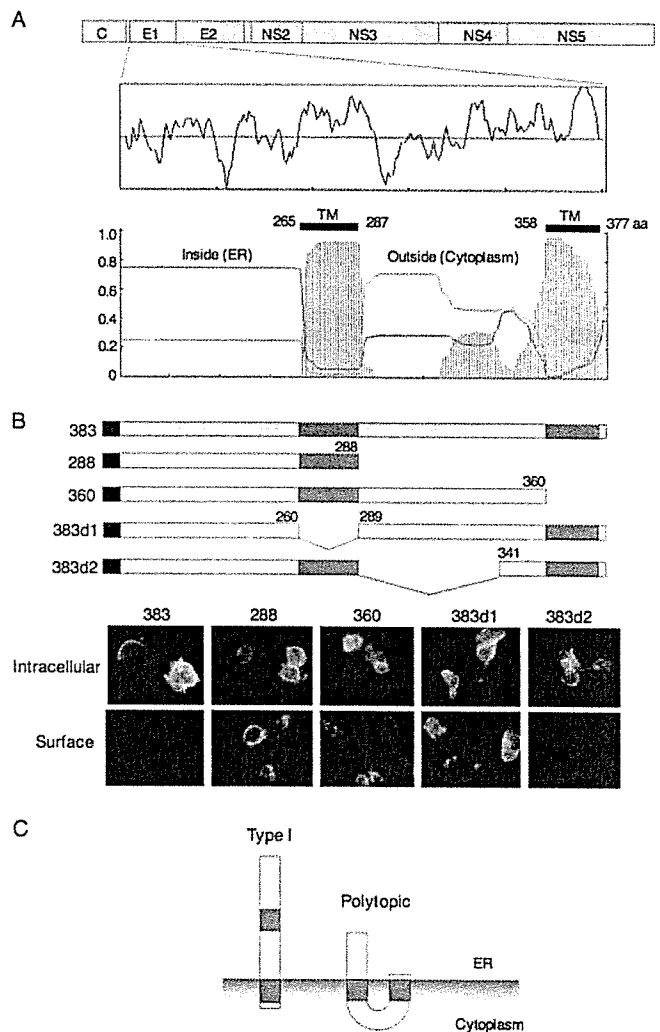


FIG. 1. Prediction of the membrane topology of the E1 protein. (A) Genome structure of HCV and a hydrophobic profile of the amino acid sequence of the E1 protein are shown at the top. The transmembrane helices in the E1 protein were predicted by the TMHMM program (19), and regions of high probability (amino acid residues 265 to 287 and 358 to 377) are indicated. (B) 293T cells transfected with the wild type (383) and deletion constructs were fixed with paraformaldehyde and permeabilized with Triton X-100 (intracellular) or not permeabilized (surface). E1 proteins were visualized with an anti-E1 monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG. (C) Possible topologies of the E1 protein on the ER. (Left) Type I topology model possessing a C-terminal transmembrane region; (right) a polytopic topology that spans the membrane twice, with both N and C termini in the ER lumen and with an intervening cytoplasmic loop.

The 325 mutant was unchanged from the wild-type E1, suggesting that the 325 position is not utilized, presumably due to an unfavorable NWSP motif in the genotype 1a protein (33). The 209, 233/234, and 250 mutants migrated faster than the authentic E1 protein and exhibited two bands of 32 and 30 kDa. The E1 of the 196 mutant was apparently not recognized by the monoclonal antibody directed to the N-terminal region of E1. In the 233 and 234 mutants, glycosylation occurred at the remaining Asn (234 or 233, respectively). These mutants comigrated with the wild-type E1 glycoforms, suggesting that

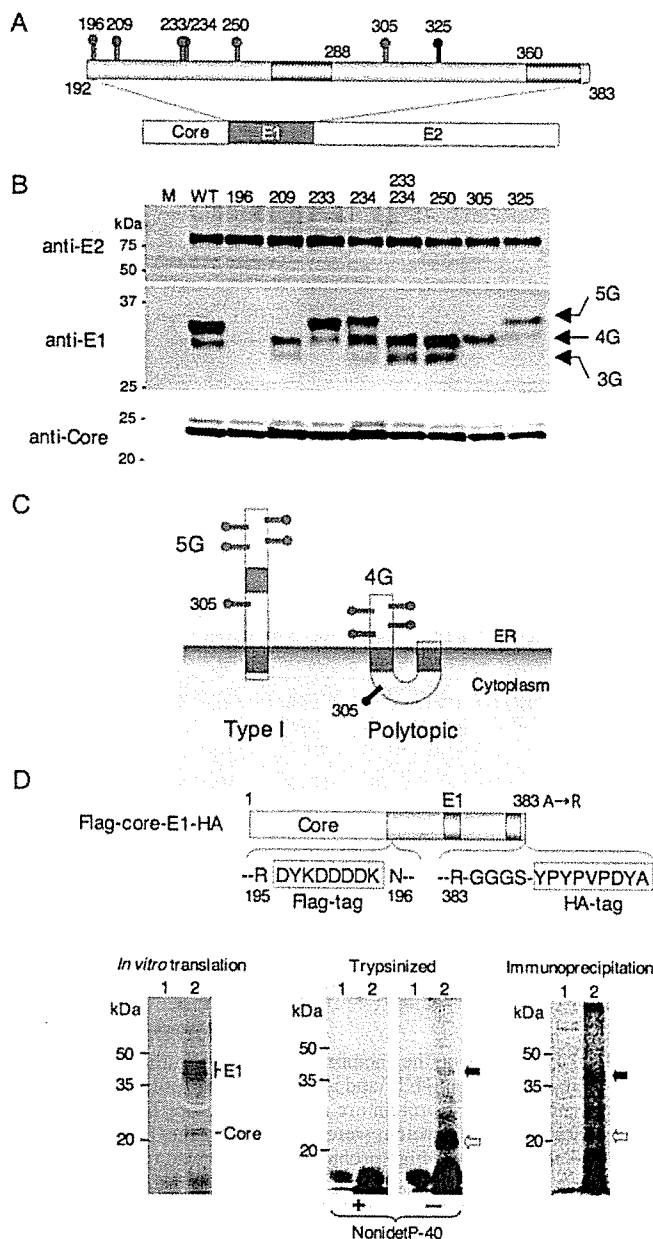


FIG. 2. Mutational analysis of N-glycosylation sites and protease protection assay of the E1 protein. (A) Positions of potential N-glycosylation sites (gray and black spikes) in the E1 protein are shown. (B) Asn residues in the possible N-glycosylation sites in the E1 protein were individually replaced with Gln. Mutant plasmids encoding the core, E1, and E2 polyproteins (A) were expressed in 293T cells, and processed core, E1, and E2 proteins were detected by immunoblotting. (C) Type I and polytopic topology models of E1 proteins bearing carbohydrates at positions of 196, 209, 234, 250, and 305 (5G) and 196, 209, 234, and 250 (4G), respectively. The 305 mutant would exhibit a single band of 4G irrespective of the topologic models. (D) Structure of the FLAG-core-E1-HA construct encoding the HCV core and E1 polyprotein carrying FLAG and HA tags in the N- and C-terminal regions of the E1 protein (top). (Bottom, left) In vitro translation of capped RNA transcribed from the FLAG-core-E1-HA (lane 2) and without RNA (lane 1) in the presence of [³⁵S]methionine-cysteine using rabbit reticulocyte lysate and canine pancreatic microsomal membrane. (Bottom, middle) Translated products of FLAG-core-E1-HA (lane 2) and without RNA (lane 1) were digested with trypsin in the presence () or absence () of 0.5% Nonidet P-40. (Bottom, right) Digestion products were immunoprecipitated with control (lane 1) and anti-FLAG (lane 2) antibody. Black and white arrows indicate protected and digested E1 protein, respectively.

only one or the other of the overlapping motifs can be utilized in the wild-type molecule. Glycosylation in this region was absent in the double mutant (233/234). The existence of two glycoforms of E1 may reflect incomplete and stochastic use of the available glycosylation sites or, alternatively, the presence of two discrete topological forms of E1 protein. For instance, the major band of 34 kDa in the wild-type glycoprotein might correspond to the type I topology form, with glycosylation at 196, 209, 234, 250, and 305 (5G), whereas the minor band of 32 kDa might correspond to the polytopic form of E1, bearing glycans at positions 196, 209, 234, and 250 (4G). In this regard, it is noteworthy that the 305 mutant of E1 exhibited only a single band of 32 kDa. The absence of a second glycoform is consistent with the putative cytoplasmic localization of Asn305 in a polytopic form of E1 (Fig. 2C). Taken together, this mutational analysis provides support to the model in which the HCV E1 glycoprotein is able to exist in either the type I or polytopic form. In the latter form, an extended cytoplasmic domain in E1 would be available to interact with the core protein in the virion.

Protease protection assay of the E1 protein. To confirm the presence of the cytoplasmic domain in the E1 protein, in vitro translation products of the HCV core and E1 polyprotein carrying FLAG and HA tags in the N- and C-terminal regions of the E1 protein, respectively, were digested with trypsin, and the protected portion of the E1 glycoprotein was immunoprecipitated by anti-FLAG antibody. As shown below in Fig. 4D, treatment of the translation products with trypsin in the presence of Nonidet P-40 resulted in complete digestion, and a 22-kDa band (major) and several 35-kDa faint bands were detected in the absence of the detergent. When in vitro-translated HCV core protein was treated similarly, no band was detected, irrespective of the presence of detergent (data not shown); therefore, the protected bands from trypsin digestion were derived from the E1 protein. Although the 22- to 35-kDa bands were specifically immunoprecipitated with anti-FLAG antibody but not with control antibody, the 35-kDa protein corresponding to the type I topology of the E1 protein resistant to trypsin digestion was dominant. This might be due to the difference in the reactivity of the anti-FLAG antibody, which recognizes the intact E1 protein more efficiently than digested ones. These results further support the presence of the polytopic form of HCV E1 glycoprotein, which has a cytoplasmic region together with a type I topology in the ER.

HCV core protein binds to the E1 protein in the presence of tRNA. The HCV core protein undergoes extensive conformational changes upon binding to nucleic acid and self-assembling into nucleocapsid-like particles (20). To investigate the effects of nucleic acid on oligomerization of the core protein, lysates of 293T cells expressing HCV core protein were incubated in the presence or absence of yeast tRNA (20) and subjected to velocity sedimentation in a sucrose gradient. Oligomerized core protein was detected in fractions 1 to 4 in the presence of tRNA but not in those in the absence of tRNA (Fig. 3A). To specifically examine the interaction between HCV core and E1 proteins in the assembly of the nucleocapsid-like particles, we coexpressed the core protein with an E1 protein possessing a FLAG tag near its N terminus and an HA tag at the C terminus (Flag-E1-HA) (Fig. 3B, left). The transfected cells were lysed with Triton X-100, and the E1 protein

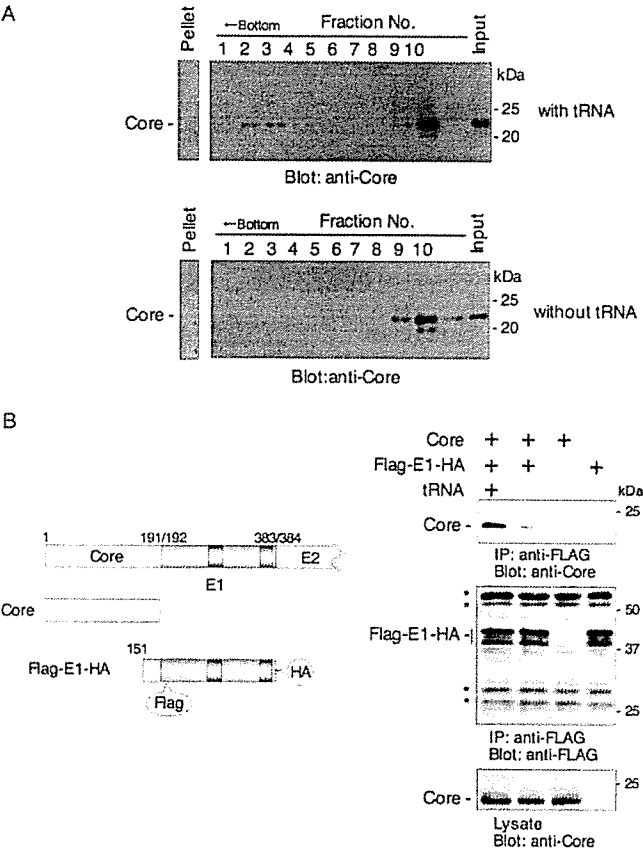


FIG. 3. HCV core protein binds to E1 protein in the presence of tRNA. (A) Cell lysates of 293T cells expressing HCV core protein were subjected to velocity sedimentation with a sucrose gradient in the presence or absence of tRNA. Oligomerized core protein was detected in fractions 1 to 4 in the presence of tRNA but not in those in the absence of tRNA. (B, left) cDNAs used for expression. FLAG-E1-HA encodes FLAG tag after the signal peptide and HA tag after the transmembrane region. (Right) Immunoprecipitation analyses. Cell lysates of 293T cells expressing core and FLAG-E1-HA proteins were immunoprecipitated by anti-FLAG antibody in the presence or absence of tRNA. The asterisks indicate nonspecific bands.

was immunoprecipitated by using an anti-FLAG antibody. Coprecipitation of core protein with E1 was assessed by Western blot analysis using a core-specific monoclonal antibody. Although HCV core protein was clearly coprecipitated with FLAG-E1-HA in the presence of tRNA, little association was seen in the absence of tRNA (Fig. 3B, right). Nonspecific precipitation of the core protein with tRNA was not observed (data not shown). Although a small amount of the intracellular core protein may already associate with viral RNA under the intracellular conditions, a large amount of RNA may be required for oligomerization that is detectable by the sedimentation assay. Together, our results suggest that tRNA facilitates oligomerization of the HCV core protein and potentiates the interaction between the core protein and E1.

The region spanning amino acid residues 72 to 91 in the HCV core protein is crucial for binding to the E1 protein in yeast. The interaction between the HCV core and E1 proteins likely occurs on the cytosolic side of the cell membrane and, thus, presumably involves the posited cytoplasmic loop region

in the polytopic form of the E1 glycoprotein. To investigate the possibility for this specific interaction in cells, core protein lacking the transmembrane region (Core1-173) was examined for interaction with the putative E1 cytoplasmic loop region in a yeast two-hybrid system (Table 1). When Core1-173 was expressed with the E1 cytoplasmic region (residues 288 to 346), the yeast was able to grow on the dropout plate lacking Trp, Leu, His, and Ade, suggesting that the core protein associates with the cytoplasmic loop of the E1 protein in yeast. To determine the region of the HCV core protein responsible for the interaction with the cytoplasmic domain of E1, deletion mutants of the core were tested. Association in the yeast two-hybrid system was seen with Core24-173, Core38-173, Core58-173, Core72-173, and Core1-151 mutants, but not with Core92-173 and Core1-25. Nonspecific interaction of the GAL4 activation domain with these core mutants was not observed. These results suggest that the region spanning from amino acid residues 72 to 91 in the HCV core protein is important for interaction with the cytoplasmic domain of the E1 protein in yeast.

Amino acid residues 72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein in mammalian cells. To examine the involvement of amino acid residues 72 to 91 of the HCV core protein in the interaction with the E1 protein in mammalian cells, FLAG-E1-HA was coexpressed with either a wild-type core or a deletion mutant lacking amino acid residues 72 to 91 (Core 72-91) in 293T cells (Fig. 4A). Cell lysates were incubated with yeast tRNA, and FLAG-E1-HA was immunoprecipitated with anti-FLAG antibody. As shown in Fig. 4B (left), only the wild-type core protein, but not Core 72-91, coprecipitated with E1. Self-oligomerization was also prevented by the deletion in Core 72-91 (Fig. 4B, right). These results suggest that amino acid residues 72 to 91 in the HCV core protein play a crucial role in the interaction with the E1 protein and oligomerization of the core protein.

The E1 cytoplasmic domain interacts with the core protein in mammalian cells and inhibits the interaction with intact E1 protein in trans. To assess the involvement of the E1 cytoplasmic region in the interaction with core protein in mammalian

TABLE 1. Interaction between the core and the E1 cytoplasmic region in yeast

Bait	Growth with prey ^a			
	E1 cytoplasmic loop		No insert	
	Dropout	Control	Dropout	Control
Core1-173				
Core24-173				
Core38-173				
Core58-173				
Core72-173				
Core92-173				
Core1-151				
Core1-25				
No insert				

^a HCV core mutants were expressed as fusion proteins with the DNA binding region by using a bait plasmid. The HCV E1 cytoplasm region was expressed as a fusion protein with an activation domain by using a prey plasmid. Yeast growth was observed in dropout plates lacking Trp, Leu, Ade, and His (dropout) or plates lacking Trp and Leu (control). , growth; , no growth.

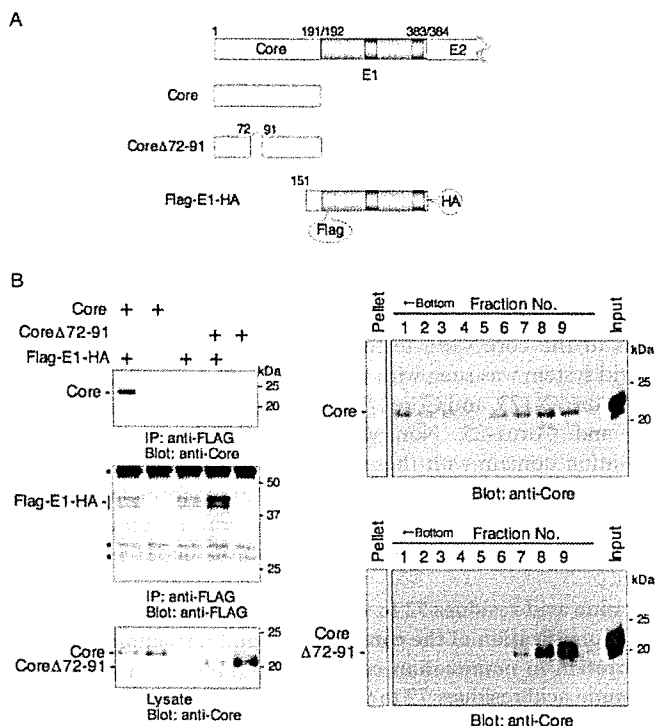


FIG. 4. Amino acid residues 72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein. (A) cDNAs used for expression. Core 72-91 is an HCV core protein carrying a deletion of amino acid residues 72 to 91. (B, left) FLAG-E1-HA was coexpressed in 293T cells with either a wild-type core or Core 72-91, and the interaction was analyzed by immunoprecipitation in the presence of tRNA. The asterisks indicate non-specific bands. (Right) Oligomerization of a wild-type core or Core 72-91 in the presence of tRNA. Wild-type core protein was self-oligomerized, but Core 72-91 was not.

cells, we constructed an enhanced green fluorescent protein (EGFP) fusion protein carrying the E1 cytoplasmic domain followed by an HA tag (EGFP-cdE1-HA) (Fig. 5A). Upon coexpression of EGFP-cdE1-HA with the wild-type core protein in 293T cells, the two proteins could be coprecipitated using anti-HA antibody (Fig. 5B). The mutant Core 72-91 protein was unable to associate with EGFP-cdE1-HA (Fig. 5B). Together, these studies demonstrate that the cytoplasmic loop region of E1 is able to interact with the core protein and that core residues 72 to 91 are required for this association.

To further confirm the specificity of the interaction of the E1 cytoplasmic region with the core protein, we examined the ability of the EGFP-cdE1-HA protein to inhibit the association of the intact E1 protein (in Flag-E1-HA) with the wild-type core protein (Fig. 5C). Expression of EGFP-cdE1-HA but not EGFP-HA competed strongly with the interaction between core and the FLAG-tagged Flag-E1-HA protein. These results suggest that the cytoplasmic loop in the intact E1 glycoprotein can directly bind to HCV core protein. Interestingly, the EGFP-cdE1-HA protein was unable to inhibit this interaction in the context of the intact core and E1 and E2 polyproteins (data not shown), suggesting that expression of the core and E1 proteins in *cis* may prevent subsequent interaction with E1 expressed in *trans*.

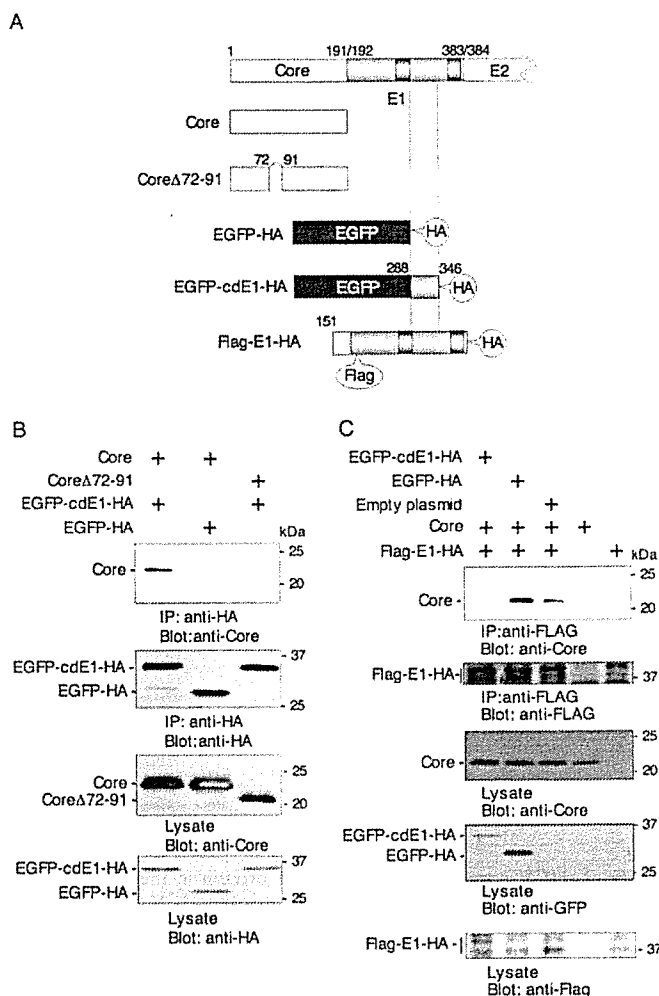


FIG. 5. Interaction of the E1 cytoplasmic loop with the core protein. (A) cDNAs used for expression. EGFP-cdE1-HA is an EGFP fusion protein carrying the E1 cytoplasmic region of amino acid residues 288 to 346 followed by an HA tag. (B) Wild-type core or Core 72-91 was coexpressed with EGFP-cdE1-HA in 293T cells, and their interaction was analyzed by immunoprecipitation. EGFP-cdE1-HA coprecipitated with wild-type core protein, but not with Core 72-91. (C) Inhibition of the interaction of the core protein with FLAG-E1-HA by expression of EGFP-cdE1-HA. Expression of EGFP-cdE1-HA but not of EGFP disrupted the interaction between core and E1 proteins.

Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein. Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes revealed that the region from Gln³⁰² to Pro³²⁸ is highly conserved (Fig. 6A). To determine residues in the E1 cytoplasmic region that are critical for interaction with the core protein, blocks of four residues each in the conserved region were replaced with Ala in the polyprotein (core, E1, E2, and p7) (Fig. 6A). These mutant polyproteins were expressed in 293T cells and immunoprecipitated with anti-core antibody; coprecipitated E1 protein was detected by immunoblotting using an anti-E1 monoclonal antibody (Fig. 6B). The replacement of four amino acid residues, 304 to 307, with Ala in the conserved region of the E1

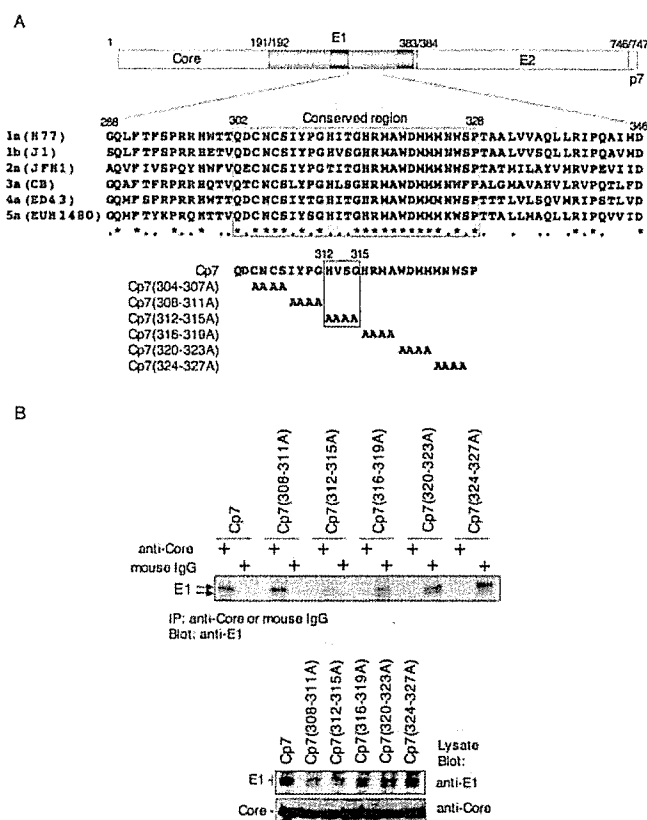


FIG. 6. Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein. (A) Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes (1a, H77 [AF009606]; 1b, J1 [D89815]; 2a, JFH1 [AB047639]; 3a, CB [AF046866]; 4a, ED43 [Y11604]; 5a, EUH1480 [Y13184]). A conserved region from Gln³⁰² to Pro³²⁸ is shown by gray shading. Mutant polyproteins consisting of the core, E1, E2, and p7 proteins with four residues each replaced by Ala in the conserved E1 region were constructed. Four amino acid residues, His³¹², Val³¹³, Ser³¹⁴, and Gly³¹⁵, in the E1 cytoplasmic region of strain J1 and substitution of the amino acids with Ala in Cp7 (312-315A) are indicated by the box. (B) These mutant polyproteins were expressed in 293T cells and immunoprecipitated with anti-core antibody or nonspecific mouse IgG in the presence of MgCl₂ and tRNA. The E1 protein that coprecipitated with the core protein was detected by immunoblotting. The substitution of four amino acid residues, 304 to 307, with Ala in the conserved region of the E1 protein, Cp7 (304-307A), could not be examined due to the low level of expression.

protein could not be examined due to a low level of expression (data not shown). Among the mutant constructs examined, only the substitution at residues 312 to 315, Cp7 (312-315A), markedly diminished association with the core protein (Fig. 6B). These results suggest that this region in the E1 cytoplasmic domain of the J1 strain of HCV (His³¹², Val³¹³, Ser³¹⁴, and Gly³¹⁵) is important for interaction with the core protein.

DISCUSSION

The biogenesis of the transmembrane glycoproteins involves a series of coordinated translation and membrane integration events that are directed by topogenic determinants within the nascent chains and that ultimately lead to the most favored topology for any given polypeptide (24). However, there is an

increasing number of examples of glycoproteins that can assume multiple topological orientations. The large envelope protein of the hepatitis B virus, for instance, has been suggested to adopt distinct topologies that enable the protein to serve in virus assembly as a matrix-like protein and in virus entry as a receptor binding protein (22). An unglycosylated form of the HCV E2 protein has been identified and shown to interact with protein kinase R in the cytosol (45). In Newcastle disease virus, type I and polytopic forms of the fusion protein are present in the same cell, and the polytopic form is suggested to be involved in the membrane fusion event (31).

HCV glycoproteins E1 and E2 were shown to possess transmembrane domains and associate to form noncovalent heterodimers that are statically retained in the ER membrane upon recombinant expression (10, 29, 46). Previously, the E1 protein of genotype 1a was suggested to possess a single C-terminal transmembrane domain, based in part on its utilization of potential glycosylation sites (33) and on a model of the transmembrane domains of the E1 and E2 proteins, in which the C terminus reorients, upon signal peptidase cleavage, from the ER lumen to protrude slightly into the cytoplasm (7). In our study, we have suggested that the E1 protein can also adopt a polytopic topology in which the protein spans the ER membrane twice and includes an intervening cytoplasmic region. In this model, the membrane orientation of the C-terminal transmembrane region is inverted and translocation of the signal peptidase-cleaved C terminus is not required.

Our analysis revealed that the 305 mutant of the 1b genotype expressed by transfection exhibited a single band of 32 kDa, whereas that of genotype 1a expressed by recombinant vaccinia viruses has been reported to contain two bands (33). Although we do not know the reason for this discrepancy, it may relate to differences in the expression systems. HCV proteins expressed by vaccinia virus and Sindbis virus vectors formed disulfide-linked aggregates (9, 11, 34), and coexpression of a large amount of vaccinia viral proteins also may alter the proper processing of the expressed proteins, as suggested by Merola et al. (32). However, further work will be necessary to clarify the reasons for the differences in glycosylation patterns of E1 mutants obtained in the different expression systems.

Mottola et al. analyzed the determinants for ER localization of the E1 protein and showed that the juxtamembrane region of E1, between amino acid residues 290 and 333, was required for ER retention (41). This region lies within the ectodomain of the E1 protein in the type I topology and in the cytoplasmic region of the protein in the proposed polytopic form. ER localization determinants of transmembrane proteins have in general been located either in the cytosolic or in the transmembrane domain, not in the luminal ectodomain, except for the yeast Sec20 protein (41). Therefore, assignment of the ER localization signal to the cytoplasmic region of the E1 protein might further support the possibility of the polytopic topology model. Affinity purification and membrane reconstitution of the E1 protein carrying an affinity tag (S-peptide) in the putative cytoplasmic region are also consistent with this model (35). Together, these findings provide indirect support that the E1 glycoprotein can adopt a polytopic form.

As previously reported (20), oligomerization of the HCV core protein to form nucleocapsid-like particles requires the presence of stem-loop RNA structures, such as those in tRNA.

Here, we have demonstrated that self-assembly of the core protein occurs without envelope protein in the presence of tRNA and that tRNA is required for the association of E1 glycoprotein with the core protein, suggesting that oligomerization of the core protein may be a prerequisite for this interaction during virus assembly. Based on hydrophobicity and the clustering of basic amino acids, the HCV core protein is proposed to possess three domains: the N-terminal basic and hydrophilic region (domain 1; residues 1 to 118), a central basic and hydrophobic domain (domain 2; residues 119 to 174), and the hydrophobic signal sequence for E1 (domain 3; residues 175 to 191) (14). Biophysical characterization of the core protein indicated that the C-terminal residues 125 to 179 were critical for the folding and oligomerization of the core protein (21). Although our mutant HCV polyprotein containing Ala substitutions at residues 312 to 315 in the cytoplasmic region of the E1 protein exhibited a clear reduction in its interaction with the core protein, a substantial amount of residual binding was retained. These results suggest that regions other than the residues from 312 to 315 in the E1 protein are also involved in the interaction with the core protein.

In Semliki Forest virus, the cytoplasmic domain of the E2 glycoprotein, which corresponds to the E1 protein in HCV, has been shown to interact with the capsid protein (26, 49). Assembly of alphaviruses has also been found to require the specific interaction between the C-terminal cytoplasmic domain of the E2 protein and the capsid protein (17). Although the functional significance of the two forms of the HCV E1 protein is still unclear, the E1 cytoplasmic region among different HCV genotypes is well conserved and four amino acid residues, His³¹², Val³¹³, Ser³¹⁴, and Gly³¹⁵ of strain J1, were shown to be important for interaction with the core protein. Although the four amino acid sequences identified in strain J1 of genotype 1b are not strictly conserved among the different HCV genotypes (Fig. 6A), a pattern of polar-hydrophobic-polar-glycine residues can be discerned in all of them. The interaction of the cytoplasmic E1 protein with the core protein may indicate that the polytopic form is a mature E1 protein that is incorporated into virions.

In conclusion, the polytopic topology model of the HCV E1 protein and the interaction of oligomerized core protein with the cytoplasmic region of the E1 protein may provide clues to aid in understanding the biosynthesis and assembly of the HCV structural proteins. HCV core protein is also involved in the development of liver steatosis, type II diabetes mellitus, and hepatocellular carcinoma in transgenic mice (39, 40, 48). A detailed knowledge of the assembly of HCV particles will provide the basis for the development of effective therapeutics for chronic hepatitis C.

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