

It is generally difficult to visualize intracellular microstructures and perform antigenic protein localizations using immunogold electron microscopy due to the low resolution and contrast of micrographs. In order to overcome this difficulty, we applied a silver-intensified immunogold labeling method in our experiment (Figs. 5B and C). Using this method, antigen-reactive immunogold particles approximately 20 nm in diameter were observed. Specific immunolabeling of core and E1 protein was detected in the ER or on the ER membranes. Intense immunopositive reactions were also seen on the virus-like particles observed in cytoplasmic vesicles and on ER membranes; however, no such immunolabeling was observed when normal mouse serum was used as a first antibody (data not shown). These results confirm the ultrastructural observations of conventional TEM and suggest that the formation of HCV particles is achieved by budding of the putative core particles at the ER membrane.

#### Infectivity of HCV-LPs depends on E2 glycoprotein

To determine whether HCV-LPs released from RCYM1 cells cultured in the TGP system are infectious, we inoculated naive Huh-7.5.1 cells (Zhong et al., 2005), which are HCV-negative Huh-7.5 (Blight et al., 2002)-derived cells, with a culture supernatant of RCYM1 spheroids. HCV RNAs in the cells at

days 0, 1, 2, 3, and 7 postinoculation were determined by real-time RT-PCR. Fig. 6A shows the kinetics of HCV RNA after the inoculation of HCV-LPs. HCV RNA levels in the infected Huh-7.5.1 cells fluctuated at the indicated times, reaching  $10^3$ – $10^4$  copies/ $\mu$ g of cellular RNA at days 1–7. Immunofluorescence staining 4 days postinoculation revealed that approximately 1% of cells were positive for NS5A protein (Fig. 6B). In contrast, no NS5A-positive cells were detected when the cell supernatant sample obtained from 5 to 15 cell cultured in TGP was used to inoculate Huh-7.5.1 cells (data not shown). These results suggest that HCV-LPs released from TGP-cultured RCYM1 cells are infectious.

To further determine whether viral envelope proteins mediate infection by HCV-LPs, we preincubated HCV-LPs with the anti-E2 monoclonal antibody AP33, which demonstrates potent neutralization of infectivity against HCV pseudoparticles carrying E1 and E2 proteins representative of the major genotypes 1 through 6 (Owsianka et al., 2005), or with patient sera with high titers of HCV neutralization of binding (NOB) antibodies (Ishii et al., 1998), or with anti-FLAG antibody (Fig. 6C). NOB antibodies have the ability to neutralize the binding of E2 protein to human cells (Rosa et al., 1996), and NOB3 and NOB4 were sera obtained from patients who recovered naturally from chronic hepatitis C (Ishii et al., 1998). Intracellular HCV RNA levels were decreased by 43%, 28%,

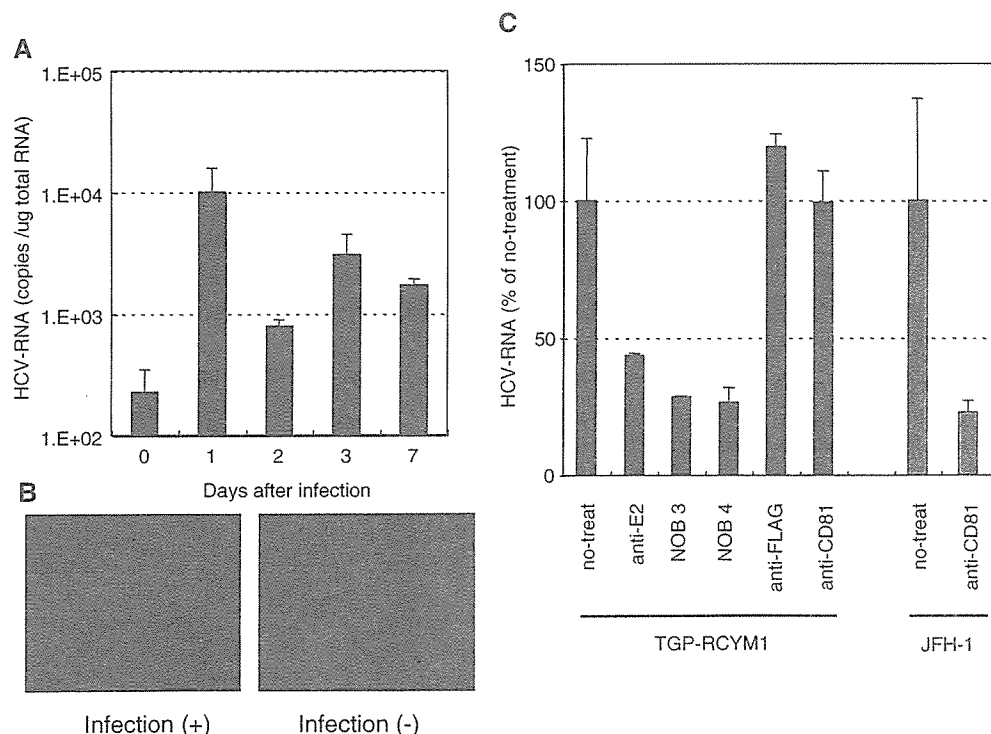


Fig. 6. Infectivity of HCV-LPs secreted from TGP-cultured RCYM1 cells and neutralization of the infection. (A) Kinetics of HCV RNA after the infection of HCV-LPs. Huh-7.5.1 cells were infected with HCV-LPs and harvested at days 0, 1, 2, 3, and 7. HCV RNAs in the cells were determined by real-time RT-PCR. (B) Huh-7.5.1 cells infected with HCV-LPs (upper panel) or without infection (lower panel) were cultured for 4 days, followed by immunostaining with anti-NS5A antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). (C) Huh-7.5.1 cells were infected with HCV-LPs after pretreatment with anti-E2 antibody AP33, neutralization of binding (NOB) antibodies, or anti-FLAG antibody. Anti-human CD81 antibody was preincubated with Huh-7.5.1 cells prior to the infection. Huh-7.5.1 cells were infected with HCV-LPs derived from TGP-cultured RCYM1 cells or JFH1 virus and incubated for 4 days; HCV RNAs in the cells were determined by real-time RT-PCR. The inhibition rate is given as the percentage of the no-treatment controls. Average values with standard deviations in triplicate samples are shown. Closed bars, HCV-LPs secreted from TGP-cultured RCYM1 cells; shaded bars, JFH1 virus.

and 26% in the presence of AP33, NOB3, and NOB4, respectively. No reduction of viral RNA in infected cells was observed following treatment with anti-FLAG antibody. Thus, the present results suggest that viral envelope proteins play a crucial role in the infectivity of HCV-LPs produced by RCYM1 cells cultured in TGP. We further tested anti-CD81 antibody for inhibition of the virus infection in our system. As shown in Fig. 6C, pretreatment of the cells with the anti-CD81 antibody resulted in no inhibition of the intracellular HCV RNA level in the infected cells. In contrast, under the same condition of treatment, the antibody efficiently inhibited the infection of JFH-1 virus, which was produced from the HCV JFH-1 molecular clone as previously described (Wakita et al., 2005; Zhong et al., 2005), suggesting that CD81 has no or little, if any, need for the infection of HCV produced in our system.

#### Potential use of the TGP culture system for HCV production and evaluation of antiviral agents

In a recent report, Lindenbach et al. (2005) found that a cell culture system supporting complete replication of an HCV genotype 2a clone is useful for the evaluation of antiviral drugs. However, to date this complete HCV culture system has not been extended to genotype 1b, which is more frequently detected in patients with hepatitis C and is the most difficult to treat.

We show here the potential utility of the TGP culture of RCYM1 cells for evaluating anti-HCV drugs (Fig. 7). Intracellular HCV RNA levels in TGP-cultured RCYM1 cell spheroids were reduced by 90% after 3 days of culture with 100 IU/ml of IFN- $\alpha$  (Fig. 7A). Likewise, the extracellular HCV particle level, which was calculated using the HCV RNA copy number of the 1.18 g/ml supernatant fraction, was reduced by 89% by IFN- $\alpha$  treatment (Fig. 7B). Moreover, the production of HCV particles was inhibited by treatment with 100  $\mu$ M RBV to the same degree (85%) as intracellular HCV RNA (Fig. 7B).

The level of HCV RNA detected in the 1.04 g/ml fraction of the culture supernatant of the untreated group was approximately one fourteenth of that in the 1.18 g/ml fraction, and the level increased with the addition of IFN- $\alpha$  or RBV (Fig. 7B). Although the mechanism underlying this increase is unknown, a similar phenomenon was observed when several highly cytotoxic agents were evaluated using TGP-RCYM1 cultures (data not shown). It is therefore likely that some cellular proteins associated with HCV RNA are released into the culture supernatant as a result of cell death caused by the moderate cytotoxic effects of IFN and RBV.

Collectively, these results demonstrate that the HCV production model based on TGP culture is useful for evaluating HCV particle production and the inhibitory effects of anti-HCV drugs.

#### Discussion

In the present report, we describe that HCV-LPs are assembled and released from Huh-7 cells harboring a dicistronic genome-length Con1 HCV RNA in two independent 3D culture systems. The HCV-LPs closely resemble virus-like particles detected in the sera of patients with hepatitis C in terms of both particle size and morphology. The HCV-LPs released into the culture supernatant have a buoyant density of approximately 1.18 g/ml, which is much higher than that of putative HCV particles isolated from patient sera reported previously (Andre et al., 2002; Kanto et al., 1994; Nakajima et al., 1996; Trestard et al., 1998) and slightly higher than the average density of virus particles produced with the JFH-1 isolate (Wakita et al., 2005). One possible explanation is that the HCV particles are highly bound to lipids and low-density lipoproteins in patient sera. In agreement with a recent report (Wakita et al., 2005), our EM examination demonstrated that HCV-LPs are 50–60 nm in diameter and are composed of core-like particles with a diameter of approximately 30 nm that are surrounded by ER-derived E1/

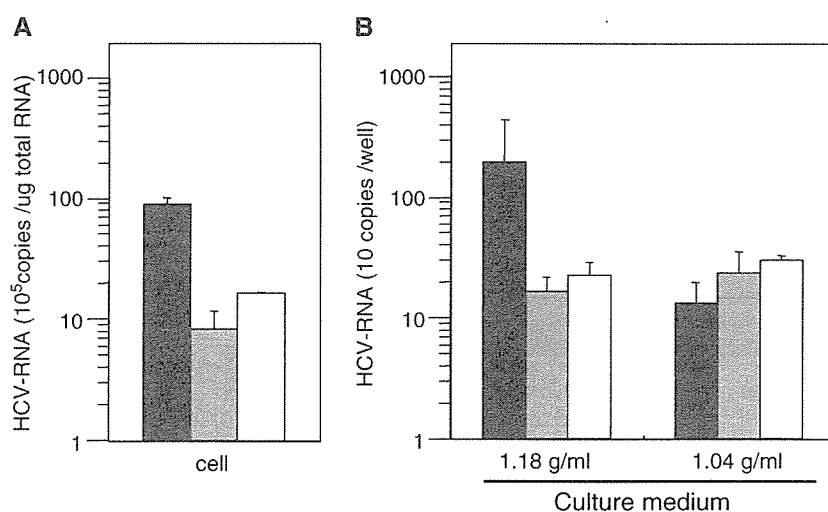


Fig. 7. Inhibition of HCV-LP production by IFN and RBV. TGP-cultured RCYM1 cells were treated with 100 IU/ml IFN- $\alpha$  or 100  $\mu$ M RBV, and HCV RNAs in the cells (A) and in the culture media (B) were then determined. Culture media from each sample were fractionated by sucrose gradient centrifugation and HCV-LP positive (1.18 g/ml) and negative (1.04 g/ml) fractions were assayed. Average values with standard deviations in triplicate samples are shown. Closed bars, no-treatment control; shaded bars, IFN- $\alpha$ ; open bars, RBV.

E2 proteins. These particles are observed at the ER membranes and in dilated cisternae of the ER, suggesting that the interaction of the ER membrane containing HCV envelope proteins with the viral core protein drives the budding process of HCV particles into the ER lumen.

Although studies on the ultrastructure and morphogenesis of HCV-LPs have been conducted using recombinant viral vectors carrying HCV structural protein genes (Baumert et al., 1998; Blanchard et al., 2002, 2003), the present study provides the first visual evidence of assembly and budding of HCV particles in a heterologous expression system in which a full-length viral genome is replicating and the viral particles are secreted into the culture medium. We also demonstrated that the HCV-LPs produced in our 3D culture system are infectious and that their infection is prevented by the monoclonal antibody AP33 directed against E2 (Owsianka et al., 2005) as well as by NOB antibodies (Ishii et al., 1998), which are sera of patients naturally resolving from chronic hepatitis C and exhibiting neutralizing activity. This result is consistent with the recent demonstration that E2 is required for the infectivity of JFH-1 virus (Wakita et al., 2005). It has been shown that CD81 interacts with E2 (Pileri et al., 1998) and that anti-CD81 antibodies or a soluble CD81 fragment block the infection of Huh-7 cells with either pseudotyped retroviral particles, JFH-1 virus or J6/JFH1 chimera (Lindenbach et al., 2005; Netski et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Inconsistent with these studies, however, we found that anti-CD81 antibody did not inhibit the virus infection in our system. Although CD81 is considered to represent an important component in HCV entry, there are several other candidate cellular receptors for HCV (Bartosch and Cosset, 2006) and a study has demonstrated that *in vitro* binding of HCV to hepatoma cell lines was not inhibited by the anti-CD81 antibody (Sasaki et al., 2003).

In a previous report (Aizaki et al., 2003), we describe the production and release of infectious HCV particles from a human hepatocellular carcinoma-derived cell line, FLC4, using RFB culture in two experiments: inoculation of cells with infectious plasma from an HCV carrier and transfection of cells with viral RNA transcribed from the full-length cDNA of genotype 1a, which is known to infect chimpanzees. These findings prompted us to use the RFB system to create a culture model of HCV production based on genome-length dicistronic viral RNA, which has not been found to produce viral particles in standard monolayer cultures. As expected, HCV-LPs were produced and secreted into the medium during RFB culture of RCYM1 cells, whereas virus production was not observed in the conventional monolayer culture of RCYM1 cells. The presence of the viral envelope protein(s) on the HCV-LPs obtained in the RFB culture was strongly suggested from their density analysis with and without NP40 treatment.

We also created another 3D environment supportive of RCYM1 culture using TGP, a chemically synthesized biocompatible polymer which has a sol-gel transition temperature, thus enabling us to culture cells three-dimensionally in the gel phase at 37 °C and to harvest them in the sol phase at 4 °C, without enzyme digestion (Yoshioka et al., 1994). In contrast to other matrix gels made from conventional natural polymers and

developed for 3D culture, including matrigel (Kleinman et al., 1986), collagen gel (Lawler et al., 1983), and soft agar, TGP has several advantages that allow us to investigate the functional characteristics of epithelial cells, their tissue-like morphology, and their potential clinical applications. The use of 3D culture materials other than TGP requires treatment with appropriate digestive enzymes or heating to collect cells grown as spheroids from the culture media, and the matrices may damage the cultured cells to some extent. Thus, it is difficult to keep the viable cells in a functionally and structurally intact. In addition, because matrigel and collagen gel are made from animal or tumor tissue, the possibility that certain pathogens or unidentified factors might influence cell function cannot be excluded. In the present study, we found that Huh-7 and RCYM1 cells formed an organized structure of spheroids after 7–10 days of culture in TGP, and that HCV-LPs were assembled and released from RCYM1 spheroids, as observed in RFB culture. It can be ruled out that HCV-LPs, RNA, and core protein detected in the TGP culture supernatant are released by damaged and/or broken cells because neither digestive enzymes nor heating is used in the culture procedures and no cell damage has been observed in the cultures.

It remains to be clarified why HCV particles were produced from Huh-7 cells harboring the genome-length dicistronic HCV RNA more efficiently in the 3D cultures than in the monolayer cultures. However, this might be related to the fact that directional protein transport in hepatocytes occurs more readily in 3D culture. EM examination demonstrated that, in the RFB and TGP culture systems, human hepatoma cells, such as Huh-7, FLC4, and FLC5 cells, self-assemble into spheroids with possible polarized morphology in which microvilli develop on the cell surface and channels resembling bile canaliculi and junction structures are created in the intercellular spaces (Aizaki et al., 2003; Iwahori et al., 2003). In contrast, human hepatoma cells adhere when grown on a plastic surface, growing as a flat monolayer without exhibiting the characteristics of polarized epithelium. In general, the interaction of viruses with polarized epithelia in the host is one of the key steps in the viral life cycle. A variety of viruses, especially enveloped viruses, mature and bud from distinct membrane domains of the host cells (Compans, 1995; Garoff et al., 1998; Schmitt and Lamb, 2004; Takimoto and Portner, 2004). For example, several respiratory viruses, such as influenza virus, parainfluenza virus, rhinovirus, and respiratory syncytial virus, are released preferentially from the apical surface. Conversely, other viruses egress from the basolateral membrane; these include vesicular stomatitis virus, Semliki Forest virus, vaccinia virus, and certain retroviruses. Thus, it is likely that more organized intracellular trafficking pathways exist in the 3D culture of Huh-7-derived cells, thereby driving the assembly and release of HCV.

The efficient production of HCV in 3D cultures could also be due to the reduction of HCV RNA replication and/or translation in 3D cultures as compared to those in monolayer cultures. RNA replication and/or translation of HCV replicons in Huh-7 cells are highly dependent on host cell growth (Pietschmann et al., 2001). In the present study, we found that the slow growth of spheroids resulted in reduced expression of HCV protein and

viral RNA in 3D-cultured RCYM1 cells compared to that in monolayer cultures containing similar cell numbers. The doubling time of cells grown in TGP or RFB culture was approximately twice that observed in monolayer culture. Although it is possible that amino acid substitutions of culture-adaptive mutations contribute to interference with virus production, another possibility might be that in cases of certain HCV clones, higher expression of the viral proteins leads to their misfolding, thereby precluding the formation of virus particles.

Complete cell culture systems for HCV have recently been developed (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) using a genotype 2a isolate, JFH-1, obtained from a Japanese patient with fulminant hepatitis (Date et al., 2004; Kato et al., 2001, 2003). Unlike many other HCV isolates, JFH-1-based subgenomic replicons do not require culture-adaptive mutations for efficient RNA replication (Kato et al., 2003). Transfection of Huh-7 cells with the full-length JFH-1 genome or a chimeric genome using JFH-1 and J6 results in the efficient production of infectious HCV (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). This newly established HCV culture system is undoubtedly useful for a variety of HCV studies; however, these systems rely on the JFH-1 replicase (NS3 to 5B) and little is known about the reasons that this particular isolate permits efficient HCV production. Virus yield in the 3D systems presented here is significantly lower than that in systems based on JFH-1; it seems that 0.1–1 copies of HCV RNA/cell/day are generated and assembled into viral particles. The ratio of viral RNA to the core protein in these fractions is approximately  $10^5$  RNA copies/1 fmol of the core. Although only moderate production of HCV particles is observed in 3D culture of RCYM1 cells, this is the first study to demonstrate the production of infectious HCV particles derived from genotype 1b, which is highly prevalent worldwide and is thought to present a higher risk of developing hepatocellular carcinoma and/or cirrhosis than infections with other HCV types (Bruno et al., 1997; Silini et al., 1996). The findings of the present study may also suggest that an extremely high efficiency of viral replication, such as that observed in the case of JFH-1 isolate, is not needed to produce HCV particles in 3D cultures of Huh-7 cells. Heller et al. (2005) report HCV virion production in a culture transfected with the genomic cDNA of genotype 1b; however, the infectivity of the virus particles remains to be determined. More recently, it was shown that chimeric HCV containing structural proteins of genotypes 1a, 1b, or 3a was produced from fusion of the core to the p7 or NS2 region with downstream nonstructural regions of JFH1 clone, but that intergenotypic chimeras frequently yielded lower titers of infectious HCV compared to JFH1 or J6/JFH1 chimera (Pietschmann et al., personal communication). The 3D culture system described in the present study might be a helpful method of increasing the efficiency of assembly and release of intergenotypic chimeric HCV.

In summary, we found that the expression of dicistronic genome-length Con1 HCV RNA of genotype 1b in 3D-cultured Huh-7 cells yields infectious virus particles, and we demonstrated the usefulness for producing HCV particles of two 3D culture systems based on RFB and TGP, in which

human hepatoma cells can assemble into spheroids with potentially polarized morphology. HCV morphogenesis occurs in a complex cellular environment in which host factors may either enhance or reduce the assembly and budding process. The culture system described here will allow us to further study viral morphogenesis and the biophysical properties of HCV particles, and it provides a new tool for the future development of anti-HCV drugs.

## Materials and methods

### *Cell lines bearing dicistronic HCV RNAs*

To generate a stable cell line harboring genome-length dicistronic HCV RNA, we electroporated  $10^7$  Huh-7 cells with 50  $\mu$ g of the RNA transcribed from a plasmid pFKI389neo/core-3'/NK5.1 (Pietschmann et al., 2002). The cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 0.5 mg/ml G418 (Promega). After stringent selection for 3 weeks, a fast-growing clone was isolated and designated as RCYM1. A Huh-7-derived cell line, 5–15, harboring a subgenomic replicon (Lohmann et al., 1999) was also used.

### *3D cell cultures*

The RFB system (Able, Japan) was manipulated as described previously (Aizaki et al., 2003) with minor modifications. Briefly, the RFB column, being filled with 4 ml of porous carrier beads made from polyvinyl alcohol, seeded with  $1 \times 10^7$  of RCYM1 or 5–15 cells. The cells were cultured in ASF104 medium (Ajinomoto, Japan) supplemented with 4 g/l of D-glucose, 2% fetal calf serum, and 0.5 mg/ml of G418 (Promega). TGP (Mebiol Gel MB-10; Mebiol, Japan) was supplied as a lyophilized form and its aqueous solution was prepared before use as previously described (Hishikawa et al., 2004; Nagaya et al., 2004; Yoshioka et al., 1994). Briefly, TGP in a flask was dissolved in 10 ml of the culture medium and was maintained at 4 °C overnight. To prepare HCV particles, we suspended  $5 \times 10^6$  cells of RCYM1 in 10 ml of TGP solution and aliquots were poured into a multi-well plate. Upon warming to 37 °C, the TGP solution quickly turned into a gel form, and 3 volumes of the culture medium were added to cover the gel. To recover spheroid cells and the culture supernatant after cultivation, we subjected the cultured plate to a temperature of 4 °C for 10 min to dissolve the gel. In order to separate spheroid cells from the culture medium, we subsequently centrifuged the TGP culture diluted with the overlaid culture medium at  $1000 \times g$  for 5 min.

### *Sucrose density gradient centrifugation*

The culture medium collected from the RFB or TGP was centrifuged at  $8000 \times g$  for 50 min to remove all cellular debris, after which the supernatant was centrifuged at 25,000 rpm at 4 °C for 4 h with an SW28 rotor (Beckman). The precipitant was suspended in 1 ml of TNE buffer [10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 100 mM NaCl] and was then layered on top of continuous 10–60% (wt/vol) sucrose gradient in TNE buffer,

followed by centrifugation at 35,000 rpm at 4 °C for 14 h with an SW41E rotor (Beckman). Fractions (1 ml each) were collected from the top of the tube (12 fractions in total). The density of each fraction was determined by the weight of 100  $\mu$ l of the fraction. For NP40 treatment, 0.5 ml of the TNE-suspended sample as described above was supplemented with 10  $\mu$ l of RNase inhibitor (Takara, Japan) and 5  $\mu$ l of 1M DTT, which was diluted by adding NP40 solution to a final concentration of 0.2%. After incubation at 4 °C for 20 min, the sample was fractionated by discontinuous 10–60% sucrose gradient centrifugation.

#### *Quantitation of HCV RNA and core protein*

Total RNA was extracted from cells and from the culture medium using TRIZOL (Invitrogen) and a QIAamp Viral RNA Mini spin column (Qiagen), respectively. Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems), as described previously (Aizaki et al., 2004; Suzuki et al., 2005). HCV core antigen within cells and culture medium was measured by immunoassay (Ortho HCV-Core ELISA Kit; Ortho-Clinical Diagnostics), following the manufacturer's instructions.

#### *Western blot analysis*

The protein concentration of cells recovered from monolayer or 3D cultures was determined by BCA Protein Assay Kit (Pierce). Aliquots of samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Japan) using a semidry blotter. After overnight incubation at 4 °C in blocking buffer (Dainippon Pharmaceuticals, Japan) with 0.2% Tween 20, the membranes were incubated with appropriately diluted anti-HCV core (Anogen) and anti-NS5A (Austral Biologicals) monoclonal antibody, followed by incubation with horseradish peroxidase conjugated anti-mouse immunoglobulin G (Cell Signaling). The blots were then washed and developed with enhanced SuperSignal West Pico Chemiluminescent Substrate (Pierce).

#### *Immunocytochemistry*

For NS5A staining, infected cells cultured on collagen-coated coverslips were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde at 4 °C for 30 min, followed by permeabilization with PBS containing 0.2% TritonX-100. After preincubation with BlockAce (Dainippon Pharmaceuticals), the samples were stained using mouse anti-NS5A antibody and rhodamine-conjugated goat anti-mouse IgG (ICN Pharmaceuticals) as the first and second antibodies, respectively.

#### *Electron microscopy*

To visualize HCV-LPs secreted into the medium, we concentrated and adsorbed sucrose density fractions prepared

as described above onto carbon-coated grids for 1 min. The grids were stained with 1% uranyl acetate for 1 min and examined under a Hitachi H-7600 transmission electron microscope. To prepare thin sections of HCV-LPs, we prefixed precipitated HCV-LPs in 2% glutaraldehyde–0.1 M cacodylate buffer at 4 °C overnight, followed by three rounds of washing with 0.1 M cacodylate buffer. The samples were then postfixed in 2% osmium tetroxide at 4 °C for 2 h, dehydrated in a graded series of ethanol solutions followed by propylene oxide, and embedded in a mixture of EPON 812, dodecyl succinic anhydride (DDSA), methyl nadic anhydride (MNA), and 2,4,6-tri (dimethylaminomethyl) phenol (DMP-30) at 60 °C for 2 days. Thin sections (80 nm) were stained with uranyl acetate and lead citrate. For electron microscopy of RCYM1 cells cultured in TGP, the cells were prefixed in 2% glutaraldehyde–0.1 M cacodylate buffer at 4 °C for 1 h and washed three times with 0.1 M cacodylate buffer, followed by postfixation in 2% osmium tetroxide for 3 h. After dehydration in a graded series of ethanol solutions and propylene oxide, the cells were embedded in a mixture of Epoxy 812, DDSA, MNA, and DMP-30 at 60 °C for 2 days. Thin sections (60–80 nm) were stained with 2% uranyl acetate.

#### *Immunoelectron microscopy*

HCV-LP samples were adsorbed on formvar-carbon grids and then floated for 30 min on a drop of BlockAce. Diluted anti-E2 mouse antibody was then applied for 1 h. After three rounds of washing, diluted anti-mouse IgG conjugated with 5-nm gold particles was applied for 1 h, and the grids were then stained with 1% uranyl acetate. In order to perform immunoelectron microscopy of TGP cultures using silver-intensified immunogold labeling, we fixed the cells in 4% paraformaldehyde–0.1% glutaraldehyde with 0.15 M HEPES buffer at 4 °C, followed by incubation with either anti-core rabbit antibody or anti-E1 mouse antibody overnight. After several washings, anti-rabbit or anti-mouse secondary antibody coupled with 1.4-nm-diameter gold particles (Nanoprobes) was applied overnight. The samples were then washed and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 3 h, followed by enlargement of the gold particles with an HQ-Silver Enhancement Kit (Nanoprobes). For double staining with anti-E1 and anti-core antibodies, the cells were fixed in 7% paraformaldehyde–0.25 M sucrose in 0.03% picric acid–0.05 M cacodylate buffer at pH 7.4. Ten-nanometer gold particle-coupled anti-rabbit and 5-nm gold particle-coupled anti-mouse antibodies were used as secondary antibodies.

#### *Assays for the infectivity of HCV-LPs and neutralization of the infection*

Cell supernatant from 3D-cultured RCYM1 cells was centrifuged at 8000  $\times$  g for 50 min to remove all cellular debris, after which the supernatant was centrifuged at 25,000 rpm at 4 °C for 4 h with an SW28 rotor. The precipitant was suspended in 0.2–0.5 ml of ASF104 medium and the aliquot containing approximately  $1 \times 10^5$  HCV RNA copies was used as each inoculum. Huh-7.5.1

cells (provided by Dr. F. V. Chisari, The Scripps Research Institute) (Zhong et al., 2005), which were seeded at a density of  $10^4$  cells/well in a 48-well plate 24 h before infection. The inocula were incubated for 3 h, followed by 3 rounds of washing with PBS and the addition of complete medium. For the kinetics assay, cells were harvested 0, 1, 2, 3, and 7 days after infection and the amount of intracellular HCV RNA was quantified as described above. Infection with HCV-LP was determined after 4 days by immunofluorescence staining for HCV NS5A. In the neutralization assay, the HCV-LP samples were incubated with the anti-E2 antibody AP33 (Owsianka et al., 2005) at 10  $\mu$ g/ml (kindly provided by Dr. A. H. Patel, University of Glasgow, UK), with the human sera with high titers of NOB antibodies NOB3 and NOB4 (Ishii et al., 1998), or with anti-FLAG antibody (Sigma) at 10  $\mu$ g/ml for 1 h at 37 °C prior to infection. Anti-human CD81 antibody (BD Pharmingen) at 10  $\mu$ g/ml was preincubated with Huh-7.5.1 cells for 1 h at 37 °C, followed by being washed with PBS three times. HCV-LP derived from TGP-cultured RCYM1 cells or JFH1 virus was incubated with these cells, as mentioned above. JFH1 virus was prepared from pJFH1 (Wakita et al., 2005), which contains the full-length cDNA of JFH1 isolate and was kindly provided by T. Wakita (Tokyo Metropolitan Institute for Neuroscience, Japan), as described (Wakita et al., 2005). The cells were harvested 4 days after infection and neutralizing activity was assessed by quantifying the amount of intracellular HCV RNA as described above.

#### Assay for anti-HCV-LP production

At the initiation of the 3D culture of RCYM1 cells ( $5 \times 10^5$  in 1 ml TGP), 100 IU/ml IFN- $\alpha$  (Sumiferon 300; Sumitomo Pharmaceuticals, Japan), or 100  $\mu$ M RBV (MP Biomedicals, Germany) were added and the cells were cultured for 5 days. Culture media were harvested and fractionated by sucrose density centrifugation as described above. Total RNAs were extracted from aliquots of 1.18 g/ml (HCV-LP positive) and 1.04 g/ml (HCV-LP-negative) fractions, followed by quantification of viral RNA.

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## Proteomic Profiling of Lipid Droplet Proteins in Hepatoma Cell Lines Expressing Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein has been suggested to play crucial roles in the pathogenesis of liver steatosis and hepatocellular carcinomas due to HCV infection. Intracellular HCV core protein is localized mainly in lipid droplets, in which the core protein should exert its significant biological/pathological functions. In this study, we performed comparative proteomic analysis of lipid droplet proteins in core-expressing and non-expressing hepatoma cell lines. We identified 38 proteins in the lipid droplet fraction of core-expressing (Hep39) cells and 30 proteins in that of non-expressing (Hepswx) cells by 1-D-SDS-PAGE/MALDI-TOF mass spectrometry (MS) or direct nanoflow liquid chromatography-MS/MS. Interestingly, the lipid droplet fraction of Hep39 cells had an apparently lower content of adipose differentiation-related protein and a much higher content of TIP47 than that of Hepswx cells, suggesting the participation of the core protein in lipid droplet biogenesis in HCV-infected cells. Another distinct feature is that proteins involved in RNA metabolism, particularly DEAD box protein 1 and DEAD box protein 3, were detected in the lipid droplet fraction of Hep39 cells. These results suggest that lipid droplets containing HCV core protein may participate in the RNA metabolism of the host and/or HCV, affecting the pathogenesis and/or virus replication/production in HCV-infected cells.

**Key words:** ADRP, DEAD box protein, hepatitis C virus, lipid droplet, TIP47.

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; MS, mass spectrometry; DNLC, direct nanoflow liquid chromatography; HRP, horseradish peroxidase; ADRP, adipose differentiation-related protein; DDX1, DEAD box protein 1; DDX3, DEAD box protein 3.

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis (1, 2). Persistent HCV infection, which occurs in more than 70% of infected patients, is strongly associated with the development of liver steatosis, which involves the accumulation of intracellular lipid droplets, cirrhosis, and hepatocellular carcinomas (HCC) (3, 4). Since more than 170 million people in the world are currently infected with HCV (1), and there is no cure that is completely effective, understanding the mechanism by which HCV induces serious liver diseases is one of the most important global public health issues. HCV, a member of the *Flaviviridae* family, possesses a single-stranded, positive-sense RNA genome of ~9.6 kb (5). The HCV genome has a single open reading frame that codes for a large precursor polyprotein of ~3,000 amino acids that is processed into at least 10 individual proteins by host and viral proteases (6).

HCV core protein, the product of the N-terminal portion of the polyprotein, generated upon cleavage at the endoplasmic reticulum by signal peptidase and signal peptide

peptidase (7, 8), forms the nucleocapsid of an HCV virion (9). Interestingly, in addition to its function as a structural protein, the core protein exhibits activities leading host cells to lipogenic and malignant transformation *in vitro* (10–12). Moreover, transgenic mice expressing HCV core protein developed liver steatosis and HCC (13, 14), suggesting an important role of the core protein in these diseases. Many studies have shown that HCV core protein substantially affects various cellular regulatory processes, such as gene transcription (15–17) and signal transduction pathways (12, 18–23), and interacts with a variety of host proteins (12, 18, 19, 22, 24–34), but it is not clear what activities/molecules are practically relevant to the pathogenesis of HCV (core)-derived liver steatosis and HCC. Extensive screenings for genes/proteins exhibiting differences in cellular expression by cDNA microarray (35–40) or proteome analysis (41, 42) have also been tried for HCV-related HCC. Although various genes/proteins were identified, further studies are required to identify the molecules eventually involved in the pathogenesis of HCV-related HCC.

In host cells, HCV core protein is distributed mainly in lipid droplets and the endoplasmic reticulum (7, 10, 43–46), in which the core protein is predicted to exert its significant biological/pathological functions. In this study, we thus focused on HCV core protein and lipid droplets, and

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performed comparative targeted proteomic analysis of the lipid droplet proteins in HCV core-expressing and non-expressing hepatoma cell lines using two strategies: conventional 1-D-SDS-PAGE/MALDI-TOF mass spectrometry (MS) and automated high-throughput direct nanoflow liquid chromatography (DNLC)-MS/MS. We found prominent differences in the protein compositions of lipid droplets between HCV core-expressing and non-expressing hepatoma cell lines.

#### MATERIALS AND METHODS

**Cell Lines**—The human hepatoma HepG2 cell line constitutively expressing HCV core protein (Hep39) was established as described previously (47). Another HepG2 cell line transfected with expression vector pcEF321swxneo without the HCV core protein insert (Hepswx) was used as a mock control (47). Both cell lines were plated on collagen-coated dishes (Asahi Techno Glass, Tokyo, Japan) and maintained in the normal culture medium [DMEM supplemented with 10% fetal bovine serum, 100 units/ml Penicillin G, 100 µg/ml streptomycin sulfate, and 1 mg/ml G418 (Sigma, St. Louis, MO, USA)] under a 5% CO<sub>2</sub> atmosphere at 37°C.

**Lipid Droplet Preparation**—Hepswx and Hep39 cells were seeded at  $4 \times 10^6$  cells/dish (150 mm, inner diameter) in 25 ml of normal culture medium and cultured for one day. For efficient formation of lipid droplets by cells, cholesterol (final 20 µg/ml) and oleic acid (final 400 µM)/fatty acid-free BSA (final 60 µM) complex, prepared as stock solutions of 5 mg/ml cholesterol in ethanol and 10 mM oleic acid/1.5 mM BSA in PBS, respectively, were added to the medium. Each cell line was further incubated for 48–72 h at 37°C. For proteomic analysis of lipid droplet proteins, confluent monolayers of Hepswx and Hep39 cells in fifteen cell culture dishes (150 mm, inner diameter) were harvested by scraping and pelleted by centrifugation (200 × *g* for 5 min at 4°C). After being washed with PBS three times, each cell pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose and Complete™, EDTA-free (Roche, Mannheim, Germany) to achieve a final volume equal to five times the volume of the cell pellet (*i.e.* a 20% cell suspension). The cell suspension was homogenized with a ball-bearing homogenizer (48), and then centrifuged at 800 × *g* for 5 min at 4°C. One milliliter of each post-nuclear supernatant fraction was layered under 2 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl (TN-buffer). After centrifugation at 100,000 × *g* for 60 min at 4°C, the lipid droplet fraction, *i.e.* the distinct white band on the top of the preparation, was collected with a pipetman. The floating lipid droplet fraction was diluted with 3.5 ml of TN-buffer and then re-purified by centrifugation (100,000 × *g* for 30 min at 4°C). This washing step was repeated three times. Lipid droplets in the floating fractions in both cells were enriched up to more than 500-fold compared with those in the total cell lysates as estimated by their protein contents. The amounts of lipid droplets isolated from Hepswx and Hep39 cells were nearly the same. The purified lipid droplet fractions (~0.1 mg of protein per ml) were stored at -80°C until use. The purity of the lipid droplet fractions was verified by microscopic and immunoblot (Fig. 1) analyses. Adipose differentiation-related protein (ADRP), a

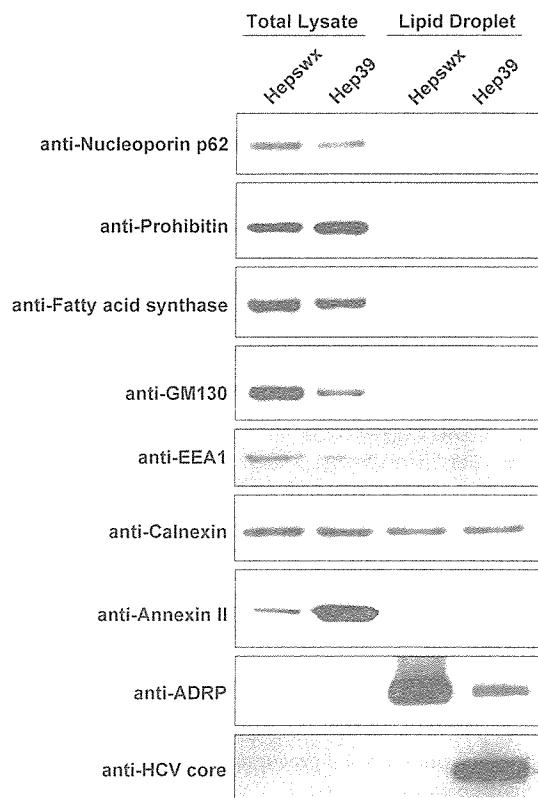


Fig. 1. Immunoblot analysis of lipid droplet fractions in Hepswx and Hep39 cells using antibodies against various organelle markers. Total cell lysates and lipid droplet fractions (1.5 µg of protein per lane in the case of anti-ADRP; 5 µg of protein per lane in others) from Hepswx and Hep39 cells were analyzed by immunoblotting with the indicated antibodies.

known lipid droplet protein, was significantly enriched in the lipid droplet fractions of Hepswx and Hep39 cells (Fig. 1). Other organelle marker proteins, such as nucleoporin p62 for the nucleus, prohibitin for the mitochondria, fatty acid synthase for the cytoplasm, GM130 for the Golgi apparatus, EEA1 for the early endosome, or annexin II for the plasma membrane, were not detected in the lipid droplet fractions of either cells (Fig. 1). Small amounts of calnexin, a marker of the endoplasmic reticulum, which is a major organelle, were detected in the lipid droplet fractions of both cells to a similar extent. Although we did not detect calnexin in the lipid droplet fractions by proteomic analysis (see Tables 1 and 2), the lipid droplet fractions of both cells could be contaminated with a small amount of endoplasmic reticulum.

**1-D-SDS-PAGE/MALDI-TOF MS Analysis**—The lipid droplet fraction (30 µg protein) of each cell line was fractionated in a 10% SDS-polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue. The protein bands were excised from the gel and subjected to in-gel trypsin digestion. The tryptic peptide mixtures were analyzed by MALDI-TOF MS as described previously (49). Prior to MALDI-TOF MS analysis, the peptide mixtures were desalted using C18 Zip Tips (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The peptide data were collected in the reflection mode and with positive polarity, using a saturated solution of

Table 1. Lipid droplet proteins identified in Heps wx and Hep39 cells by means of 1-D-SDS-PAGE/MALDI-TOF MS.

Protein	Molecular mass (kDa) (calc.)	Accession No.	SDS band No. <sup>a</sup>	
			Heps wx	Hep39
PAT family proteins				
Adipose differentiation-related protein (ADRP)	48.1	34577059	5	21
Cargo selection protein/TIP47	47.0	20127486		22
Lipid metabolism				
Acyl CoA synthetase long chain family member 3	80.4	42794752	4	18
Cytochrome <i>b</i> <sub>5</sub> reductase	34.3	4503327	9	26
Lanosterol synthase	83.3	4808278	4	18
NAD(P)-dependent steroid dehydrogenase-like; H105e3	41.9	8393516	8	25
Retinal short-chain dehydrogenase/reductase retSDR2	33.0	7705905	10	27
Cytosolic phospholipase A <sub>2</sub>	85.2	1352707		14
Rab GTPases				
Rab1A	22.7	4758988	13	30
Rab1B	22.2	23396834	13	30
Rab5C	23.5	38258923	11	28
Rab7	23.5	34147513	12	29
RNA metabolism/binding				
DEAD box protein 1 (DDX1)	82.9	6919862		16
DEAD box protein 3 (DDX3)	73.2	3023628		18
HC56/gemin 4	118.8	10945430		15
Other/unknown proteins				
BiP protein	70.9	14916999	3	17
CGI-49 protein	46.9	7705767	6	23
Heat shock protein gp96 precursor	90.2	15010550	2	14
Ancient ubiquitous protein 1	41.4	31712024	7	
Major vault protein	99.3	15990478	1	
Apoptosis-inducing factor-homologous mitochondrion-associated inducer of death	40.5	13543964		24
KIAA0887 protein	52.4	4240263		21
Protein disulfide-isomerase [EC 5.3.4.1] ER60 precursor	56.7	1085373		20
Transport-secretion protein 2.1	57.7	9663151		19
HCV core protein	20.6	974345		31

<sup>a</sup>Band numbers correspond to those in Fig. 2.

$\alpha$ -cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile and 0.1% trifluoroacetic acid as the matrix. Spectra were obtained using a Voyager DE-STR MALDI-TOF mass spectrometer (PE Biosystems, Foster City, CA, USA). Internal calibration was performed with adrenocorticotrophic hormone, fragment 18–39 (Sigma), and bradykinin fragment (Sigma). The data base-fitting program MS-Fit available at the WWW site of the University of California, San Francisco ([prospector.ucsf.edu/ucsfhtml3.4/msfit.htm](http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm)) was used to interpret the MS spectra of protein digests (50).

**DNLC-MS/MS Analysis**—The lipid droplet fraction (10  $\mu$ g protein) of each cell line was first delipidated by chloroform-methanol extraction as originally described (51). Two volumes of chloroform and 1 volume of methanol were mixed with 0.8 volume of the lipid droplet fraction. Then, 1 volume of chloroform and 1 volume of water were added to the mixture, and the mixture was vortexed for 30 s, and centrifuged at 10,000  $\times g$  for 5 min at room temperature. The resulting organic (lower) phase was removed. The aqueous (upper) phase and interface, containing all the lipid droplet proteins, was lyophilized. The delipidated lipid droplet proteins were digested with endoproteinase Lys-C, and the resulting peptides were analyzed by DNLC-MS/MS as described (52, 53).

**Cell Fractionation**—All manipulations were performed at 4°C or on ice. After being washed with PBS, confluent monolayers of Heps wx and Hep39 cells were harvested by scraping and pelleted by centrifugation (200  $\times g$ , 5 min). The precipitated cells were homogenized with a ball-bearing homogenizer in 10 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, and Complete™, EDTA-free. After centrifugation of the lysate at 800  $\times g$  for 5 min, the cytosolic fraction (100,000  $\times g$  supernatant) and membrane fraction (100,000  $\times g$  precipitate) were separated from the post-nuclear supernatant fraction by centrifugation at 100,000  $\times g$  for 60 min. The membrane fraction was resuspended in TN-buffer and then re-purified twice by centrifugation. The protein concentrations of these preparations were determined with BCA protein assay reagents (Pierce Biotechnology, Rockford, IL, USA) using BSA as a standard.

**Immunoblot Analysis**—Equivalent amounts of proteins from Heps wx and Hep39 cells were separated in a 10 or 12.5% SDS-polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were blocked overnight at 4°C or 30 min at room temperature in TBS containing 0.1% Tween 20 and 5% skim milk. The blots were probed with a mouse

Table 2. Lipid droplet proteins identified in Hepswx and Hep39 cells by means of DNLC-MS/MS.

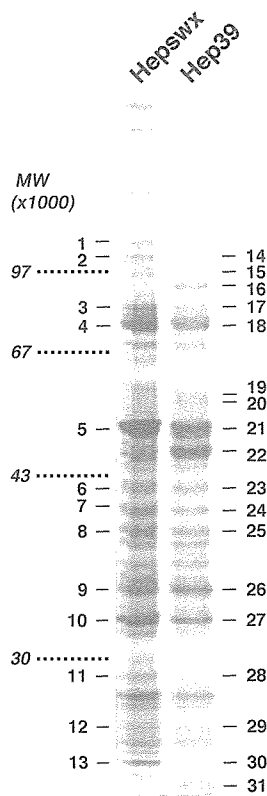
Protein	Accession No.	Molecular mass (kDa) (calc.)	Matched peptide sequence	
			Hepswx <sup>a</sup>	Hep39 <sup>a</sup>
<b>PAT family proteins</b>				
Adipose differentiation-related protein (ADRP)	34577059	48.1	+ TITSVAMTSALPIIQK DAVTTTIVTGAK EVSDSLLTSSK	+ TITSVAMTSALPIIQK DAVTTTIVTGAK EVSDSLLTSSK
Cargo selection protein / TIP47	20127486	47.0	+ VSGAQEMVSSAK	+ VSGAQEMVSSAK
<b>Lipid metabolism</b>				
Acyl-CoA synthetase long-chain family member 3	42794752	80.4	+ VLSEAAISASLEK	+ ELTELARK
Cytochrome <i>b</i> <sub>5</sub> reductase	4503327	34.2	+ DILLRPELEELRNK	+ SNPIIRTVK
Gastric-associated differentially-expressed protein YA61P	6970062	14.9	+ AIGLVVPSLTGK	+ AIGLVVPSLTGK
Retinal short-chain dehydrogenase/reductase retSDR2	7705905	33.0	+ HGLEETAAK	+ FDAVIGYK
Sterol carrier protein 2-related form, 58.85K	86717	58.8	+ LQNLQLQPGNAK	+ LQNLQLQPGNAK
Acyl-CoA synthetase long-chain family member 4	4758332	74.4	+ SDQSYVISFVVPNQK	
Fatty acid binding protein 5	4557581	15.2	+ ELGVGIALRK	
Hydroxysteroid (17-beta) dehydrogenase 4	4504505	79.7		+ NHPMTPEAVK
<b>Rab GTPases</b>				
Rab1A	4758988	22.6	+ <sup>b</sup> QWLQEIDRYASENVNK RMGPGATAGGAEK	+ RMGPGATAGGAEK
Rab1B	23396834	22.1	+ <sup>b</sup> QWLQEIDRYASENVNK	+ RMGPGAASGGERPNLK
Rab7	34147513	23.5	+ NNIPYFETSAK	+ ATIGADFLTK
Rab18	20809384	22.9	+ HSMLFIEASAK	+ ILIIGESGVGK
Rab10	12654157	22.5	+ LLLIGDSGVGK	
Rab11	4758986	24.5	+ VVLIGDSGVGK	
Rab8	539607	23.6		+ IRTIELDGK
<b>RNA metabolism/binding</b>				
DEAD box protein 1 (DDX1)	6919862	82.4		+ FGFFGGGTGK
DEAD box protein 3 (DDX3)	3023628	73.2		+ GVRHTMMFSATFPK
IGF-II mRNA-binding protein 3	30795212	63.7		+ EGATIRNITK
Ribosomal protein L29	14286258	17.8		+ AQAAAPASVPAQAPK
<b>Other/unknown proteins</b>				
Apoptosis-inducing factor homologous mitochondrion-associated inducer of death	13543964	40.5	+ EVTLIHSQVALADK	+ EVTLIHSQVALADK
BiP protein	14916999	72.3	+ SQIFSTASDNQPTVTIK	+ VYEGERPLTK
Hypothetical protein DKFZp586A0522.1	7512845	28.2	+ LQHIQAPLSWELVRPH- IYGYAVK	+ RELFSNLQEFAGPSGK
Prolyl 4-hydroxylase, beta subunit	20070125	57.1	+ VHSFPTLK	+ AEGSEIRLAK
Ancient ubiquitous protein 1	31712024	41.4	+ GTQSLPTASASK	
Heat shock protein gp96 precursor	15010550	90.2	+ FAFQAEVNRMMK	
Hypothetical protein FLJ21820	11345458	37.3	+ DIYGLNGQIEHK	
Molecule possessing ankyrin repeats induced by lipopolysaccharide	38173790	78.1	+ CLIQMGAAVEAK	
Ubiquitin-conjugating enzyme E2G 2, isoform 1	15079469	18.6	+ RLMAEYK	
CGI-49 protein	7705767	46.9		+ AGGVFTPGAAFSK
DILV594	37182139	31.4		+ RELFSQIK
Hypothetical protein DKFZp564F0522.1—human (fragment)	7512734	33.1		+ ILRTSSGSIREK
Hypothetical protein HSPC117	7657015	55.2		+ EQLAQAMFDHIPVGVGSK
Tumor protein D52-like 2 isoform e	40805860	22.2		+ TQETLSQAGQK
Vesicle amine transport protein 1	15679945	41.9		+ VVTYGMANLLTGPK

<sup>a</sup>+, detected. <sup>b</sup>This peptide sequence is present in both Rab1A and Rab1B.

monoclonal anti-ADRP antibody (PROGEN Biotechnik GmbH, Heidelberg, Germany) (1:25), a guinea pig polyclonal anti-TIP47 antibody (PROGEN Biotechnik GmbH) (1:250), a mouse monoclonal anti-HCV core protein antibody (Anogen, Ontario, Canada) (1:1,000), a mouse monoclonal anti-DDX1 antibody (Pharmingen, San Diego, CA, USA) (1:500), or a rabbit polyclonal anti-DDX3 antibody (antibody custom-made by Invitrogen, CA, USA) (1:500) for 90 min at room temperature. The blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (BIO-RAD), HRP-conjugated goat anti-mouse IgG (BIO-RAD), or HRP-conjugated goat anti-guinea pig IgG (ICN Pharmaceuticals, Aurora, OH, USA) at 1:2,000 dilution for 60 min. Detection of immunoreactive proteins was performed with an ECL system (Amersham Biosciences Corp., Piscataway, NJ, USA).

## RESULTS

**Proteomic Analysis of Lipid Droplets by 1-D-SDS-PAGE/MALDI-TOF MS**—Lipid droplet proteins from control (HCV core non-expressing) Heps wx cells and HCV core-expressing Hep39 cells were separated by 10% SDS-PAGE, and the protein bands were visualized by Coomassie Brilliant Blue staining (Fig. 2). In each cell



**Fig. 2. Signature SDS-PAGE patterns of the lipid droplet fractions of Heps wx and Hep39 cells.** Proteins in the purified lipid droplet fractions (30  $\mu$ g of protein per lane) of Heps wx cells and Hep39 cells were separated in a 10% SDS-polyacrylamide gel, and visualized by Coomassie Brilliant Blue staining. The 31 numbered bands were excised from the gel, subjected to in-gel trypsin digestion, and processed for MALDI-TOF-MS. Molecular weights (MW) are given to the left of the gel.

line  $\sim$ 30 bands were seen. The visible bands (areas) were excised from the gels, trypsinized, and analyzed by MALDI-TOF MS. Among the 31 bands, we identified 25 proteins: 15 proteins in Heps wx cells and 23 proteins in Hep39 cells (Fig. 2 and Table 1). Thirteen of the 25 proteins were detected in both types of cell. The lipid droplet proteins found in both Heps wx and Hep39 cells could be categorized into four groups: (1) PAT family proteins, *i.e.* ADRP and TIP47; (2) multiple molecules involved in lipid metabolism; (3) several Rab GTPases; and (4) other/unknown proteins (Table 1). In addition, Hep39 cells contained another group of proteins involved in RNA metabolism/binding (Table 1).

**Proteomic Analysis of Lipid Droplets by DNLC-MS/MS**—Some protein bands in Fig. 2 could not be identified, probably due to the restricted separation capacity of 1-D-SDS-PAGE (*i.e.* multiple proteins migrating to the same area). We had, however, difficulty in applying 2-DE to the separation of lipid droplet proteins because of their hydrophobic characteristics. We then tried a new LC-based MS strategy. Lipid droplet fractions from Heps wx and Hep39 cells were delipidated and then digested with Lys-C. The resulting peptide mixtures were directly analyzed using a DNLC-MS/MS system (52). We identified 36 lipid droplet proteins: 24 proteins in Heps wx cells and 27 proteins in Hep39 cells (Table 2). Twenty-three lipid droplet proteins were newly identified with this system. Fifteen proteins detected in both cell lines were classified into four categories (Table 2) as in the case of 1-D-SDS-PAGE/MALDI-TOF MS analysis. A group of proteins involved in RNA metabolism/binding was also found only in Hep39 cells (Table 2).

**Proteins Exhibiting Differences in Their Association with Lipid Droplets Due to HCV Core Protein Expression**—SDS-PAGE patterns of lipid droplet proteins were similar but revealed several distinct differences in protein composition between Heps wx and Hep39 cells (Fig. 2). The most remarkable differences were seen in the bands corresponding to PAT family proteins. The amount of ADRP, a major PAT family protein in lipid droplets in the liver (54, 55), and likely to be the most abundant lipid droplet protein in Heps wx cells (Fig. 2, band 5), seemed to be less in HCV core-expressing Hep39 cells (Fig. 2, band 21). On the other hand, TIP47, which is also known to be a PAT family protein in lipid droplets (56, 57), was detected as a major protein only in Hep39 cells (Fig. 2, band 22, and Table 1). To confirm these findings, the contents of ADRP and TIP47 in the lipid droplet fractions of Heps wx and Hep39 cells were examined by immunoblot analysis with specific antibodies. The lipid droplet fraction of HCV core-expressing Hep39 cells showed an apparently lower content of ADRP and a much higher content of TIP47 than the levels in Heps wx cells (Fig. 3).

Next we examined the cellular distributions of ADRP and TIP47 in Heps wx and Hep39 cells by cell fractionation. ADRP was highly concentrated in the lipid droplet fractions of both cells, even though the content in the lipid droplets was much lower in Hep39 cells than in Heps wx cells (Fig. 4). ADRP was not detected in post-nuclear supernatant fractions or in either the cytosol or membrane fractions, probably because of low expression levels in these cells or low affinity of the anti-ADRP antibody we used

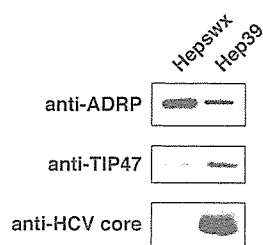


Fig. 3. The lipid droplet fraction of Hep39 cells contains less ADRP, but more TIP47, than Hepswx cells. Lipid droplet fractions (1.5  $\mu$ g of protein per lane) from Hepswx and Hep39 cells were analyzed by immunoblotting with the indicated antibodies.

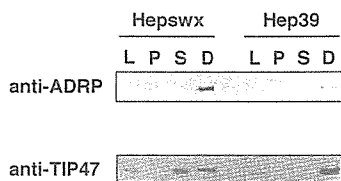


Fig. 4. Subcellular localization of ADRP and TIP47 in Hepswx and Hep39 cells. Hepswx and Hep39 cells were fractionated into post-nuclear supernatant (lane L), 100,000  $\times$  g precipitate (lane P), 100,000  $\times$  g supernatant (lane S), and lipid droplet (lane D) fractions as described in "MATERIALS AND METHODS." Ten micrograms of protein was processed for gel electrophoresis, and then analyzed by immunoblotting with anti-ADRP and anti-TIP47 antibodies.

(Fig. 4). The mRNA expression level of ADRP in Hep39 cells was less than half that in Hepswx cells (data not shown), consistent with the immunoblot data shown in Fig. 4. These results suggest that the lower ADRP content in the lipid droplet fraction of Hep39 cells is due to a low expression level of ADRP. In contrast, Hep39 cells had much more TIP47 in the lipid droplet fraction (Figs. 3 and 4, lanes D), but the cellular TIP47 content of Hep39 cells was not more than that in Hepswx cells (Fig. 4, lanes L). Besides the lipid droplet fraction, the cytosolic fraction of Hepswx cells was found to contain TIP47 at a substantial level, while the cytosolic fraction of Hep39 cells did not (Fig. 4, lanes S). These results indicate that the intracellular distribution of TIP47 shifts drastically from the cytosol to lipid droplets in HCV core-expressing Hep39 cells.

Another obvious difference between Hepswx and Hep39 cells in Fig. 2 is the presence of a specific  $\sim$ 85 kDa band (Fig. 2, band 16) in Hep39 cells, which was identified as DEAD box protein 1 (DDX1), a DEAD box protein family member, by 1-D-SDS-PAGE/MALDI-TOF MS analysis (Table 1). DNLC-MS/MS analysis also supported the existence of DDX1 in the lipid droplet fraction of Hep39 cells (Table 2). In addition, DEAD box protein 3 (DDX3), another DEAD box protein family member, was also detected in the lipid droplet fraction of Hep39 cells by means of the two different strategies used for proteomic analysis (Tables 1 and 2), suggesting that DDX3 is a major lipid droplet protein in Hep39 cells. To verify the association of DDX1 and DDX3 with lipid droplets in Hep39 cells, immunoblot analysis was carried out. Figure 5 shows that DDX1 and DDX3 exist in the lipid droplet fraction of HCV core-expressing Hep39 cells, but not Hepswx cells. These results imply the



Fig. 5. Hep39 cells, but not Hepswx cells, have DDX1 and DDX3 in the lipid droplet fraction. Lipid droplet fractions (0.5  $\mu$ g of protein per lane) in Hepswx and Hep39 cells were analyzed by immunoblotting with anti-DDX1 and anti-DDX3 antibodies.

special pathological functions of DDX1 and DDX3 in lipid droplets in HCV core-expressing cells.

## DISCUSSION

To analyze lipid droplet proteins, we performed proteomic analysis by means of 1-D-SDS-PAGE/MALDI-TOF MS and automated DNLC-MS/MS, and identified 25 and 36 proteins, respectively (Tables 1 and 2). Many more lipid droplet proteins were identified by DNLC-MS/MS, and 22 major proteins separated by 1-D-SDS-PAGE (Fig. 2, bands, 2, 3, 4, 5, 7, 9, 10, 12, 13, 16, 17, 18, 21, 22, 24, 26, 27, 29, and 30) and detected on MALDI-TOF MS analysis were also detected on DNLC-MS/MS analysis. These results indicate that DNLC-MS/MS is a very sensitive and reliable system as well as a high-throughput method. Particularly, DNLC-MS/MS would be a powerful system for exhaustive proteomic analysis of protein mixtures/complexes (up to  $\sim$ 100 proteins) such as lipid droplets.

In our targeted proteomic study, we identified a total of 48 lipid droplet proteins: 30 proteins in control Hepswx cells, 38 proteins in HCV core-expressing Hep39 cells, and 20 proteins in both cell lines. The resident lipid droplet proteins were classified into four groups (Tables 1 and 2), consistent with the recently reported data obtained on proteomic analysis of lipid droplet proteins in other cell lines (58–60). In addition, multiple proteins, such as the sterol carrier protein 2-related form, fatty acid binding protein 5, and apoptosis-inducing factor homologous mitochondrion-associated inducer of death, were newly identified as lipid droplet proteins in this study. These accumulated data obtained on proteomic analysis will be useful for understanding the biogenesis and functions of lipid droplets about which little is yet known.

A prominent effect of the expression of HCV core protein on the composition of lipid droplet proteins was observed among the PAT family proteins, i.e. ADRP and TIP47. HCV core-expressing Hep39 cells contained much less ADRP in the lipid droplet fraction (Fig. 3), probably because of the lower cellular expression level and the lack of induction of expression upon lipid loading (data not shown). In contrast, a substantial amount of TIP47 was associated with the lipid droplet fraction of Hep39 cells (Fig. 3). Perilipin, a structural protein of lipid droplets in adipocytes, ADRP, and TIP47, termed PAT family proteins (61), share extensive amino acid sequence similarity (61–63), suggesting a common biological function in lipid droplet formation. For example, the transition in surface protein composition of lipid droplets from ADRP to perilipin occurs during adipocyte differentiation (64). Thus, TIP47 might replace ADRP

on the lipid droplets in Hep39 cells. Cellular TIP47 was not up-regulated in Hep39 cells, resulting in a reduction of TIP47 in the cytosolic fraction (Fig. 4). Since TIP47, originally identified as having the ability to interact with the mannose 6-phosphate/IGF-II receptor (63), appears to be essential for the endocytic recycling system (65–67), the altered distribution of cellular TIP47 in Hep39 cells could affect intracellular membrane trafficking pathways. Consistent with this assumption, our preliminary results showed that the rate of protein secretion from cells was apparently slower for Hep39 cells than Hepswx cells (unpublished data). Patients chronically infected with HCV (68) and HCV core-transgenic mice (69) exhibit decreased levels of plasma very low density lipoproteins secreted from the liver, also suggesting interference with intracellular membrane trafficking (secretion pathways) by HCV core proteins. We currently speculate that the reduction in cellular ADRP expression mediated by HCV core protein causes the accumulation of TIP47 in lipid droplets as a substitute, and that the resulting depletion of TIP47 in the cytosol could cause the impairment of intracellular membrane trafficking, followed by the cellular accumulation of membrane lipids and consequent lipid droplet formation. Although further studies remain to be done to confirm these possibilities, we suggest that HCV core protein influences not only the biogenesis of lipid droplets but also intracellular membrane trafficking.

Another interesting finding in this study is that Hep39 cells, unlike Hepswx cells, contain DEAD box proteins, DDX1 and DDX3, as major lipid droplet proteins (Figs. 2 and 5). On the basis of the results of studies involving yeast two-hybrid assays, DDX3 has been shown to be able to interact with HCV core protein, and studies involving immunofluorescent microscopy have revealed that DDX3 is distributed in cytosolic spots such as lipid droplets (27, 70, 71). These results, together with our present findings, suggest that DDX3 is associated with lipid droplets via HCV core proteins located on lipid droplets. In addition to DDX1 and DDX3, which possess ATPase/RNA helicase activities (27, 72, 73), several other proteins involved in RNA metabolism/binding, including HC56/gemin 4 and IGF-II mRNA-binding protein 3, were also detected in the lipid droplet fraction of HCV core-expressing Hep39 cells (Tables 1 and 2). Recently Dvorak *et al.* reported that RNA itself can be associated with lipid droplets in human mast cells (74). Taken together, these data strongly suggest that lipid droplets containing HCV core proteins may participate in the RNA metabolism of the host and/or HCV in HCV-infected cells. Furthermore, the findings that DDX1 is overexpressed in cell lines derived from tumors such as retinoblastomas and neuroblastomas (75), and that cellular expression of DDX3 induces anchorage-independent cell growth (76) suggest the involvement of DDX1 and DDX3 in carcinogenesis.

Some groups recently reported profiles of mRNAs up- or down-regulated by expression of the HCV core protein (77–79), but these mRNAs included no molecules identified as lipid droplet proteins in this study. Since lipid droplets are a minor organelle in cells, it might be difficult to detect changes in the mRNA expression levels of lipid droplet proteins. The merits of targeted proteomic study are that it is possible to focus on minor cellular fractions, and also to detect changes in the intracellular distributions

of proteins. Actually, the mRNA expression levels of TIP47, DDX1, and DDX3 did not change in Hep39 cells (data not shown).

We identified many other lipid droplet proteins found in either Hepswx or Hep39 cells, but their biological functions remain mostly unknown (Tables 1 and 2). Elucidation of the biological functions of these proteins will lead to an advanced understanding of the pathogenesis of HCV-derived liver diseases.

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## E6AP Ubiquitin Ligase Mediates Ubiquitylation and Degradation of Hepatitis C Virus Core Protein<sup>∇</sup>

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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 $\gamma$  (a REG family proteasome activator also known as REG $\gamma$  or Ki antigen) enhances the proteasomal degradation of the HCV core protein (30). A recent study has shown that

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PA28 $\gamma$  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 Mcm7 (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  $\mu$ g/ml streptomycin (Invitrogen), and 10% (vol/vol) fetal bovine serum (JRH Biosciences) at 37°C in a 5% CO<sub>2</sub> 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 Tth1111 and ligated into the SmaI-Tth1111 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 BglIII-XbaI, and subcloned into the BglIII-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 pCMVNT (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 $\gamma$  rabbit PAB (Affinit), 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 Na<sub>2</sub>VO<sub>4</sub>, 1% (wt/vol) Triton X-100, 5  $\mu$ M ZnCl<sub>2</sub>, 2 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml leupeptin. The lysate was centrifuged at 100,000  $\times$  g for 20 min at 4°C. The supernatant was passed through a 5- $\mu$ m filter, incubated with 100  $\mu$ l of Sepharose beads for 60 min at 4°C, and then passed through a 0.65- $\mu$ m filter. The filtered supernatant was mixed with 100  $\mu$ l 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  $\mu$ l) 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  $\mu$ l of buffer A. The resulting supernatants were combined and incubated with 12  $\mu$ l of FLAG-Sepharose beads for the second immunoprecipitation. After incubation for 60 min at room temperature, the beads were washed three times with 240  $\mu$ l of buffer A, and proteins bound to the immobilized HCV core protein on the FLAG beads were dissociated by incubation with 80  $\mu$ g/ml FLAG peptide (NH<sub>2</sub>-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- $\beta$ -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  $\mu$ g of purified recombinant GST-E6AP expressed in Hi5 cells was mixed with 1,000  $\mu$ 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- $\mu$ l volumes containing 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM ATP, 10 mM MgCl<sub>2</sub>, 8  $\mu$ g of bovine ubiquitin (Sigma), 0.1 mM dithiothreitol, 200 ng mouse E1, 200 ng E2 (UbcH7), and 0.5  $\mu$ 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'-GGGUC UACACCAGAUUGCUTT-3'; scramble negative control (sense), 5'-UUGCG GGUCUAAUACCGATT-3'.

**CHX half-life experiments.** To examine the half-life of HCV core protein, transfected 293T cells were treated with 50  $\mu$ g/ml 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- $\mu$ 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 [TCID<sub>50</sub>/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  $\mu$ 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 TCID<sub>50</sub> 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 TCID<sub>50</sub> 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  $10^6$  cells/10-cm dish were inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 ( $6.5 \times 10^3$  TCID<sub>50</sub>/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 TCID<sub>50</sub> 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 $\gamma$  (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 $\gamma$ , 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