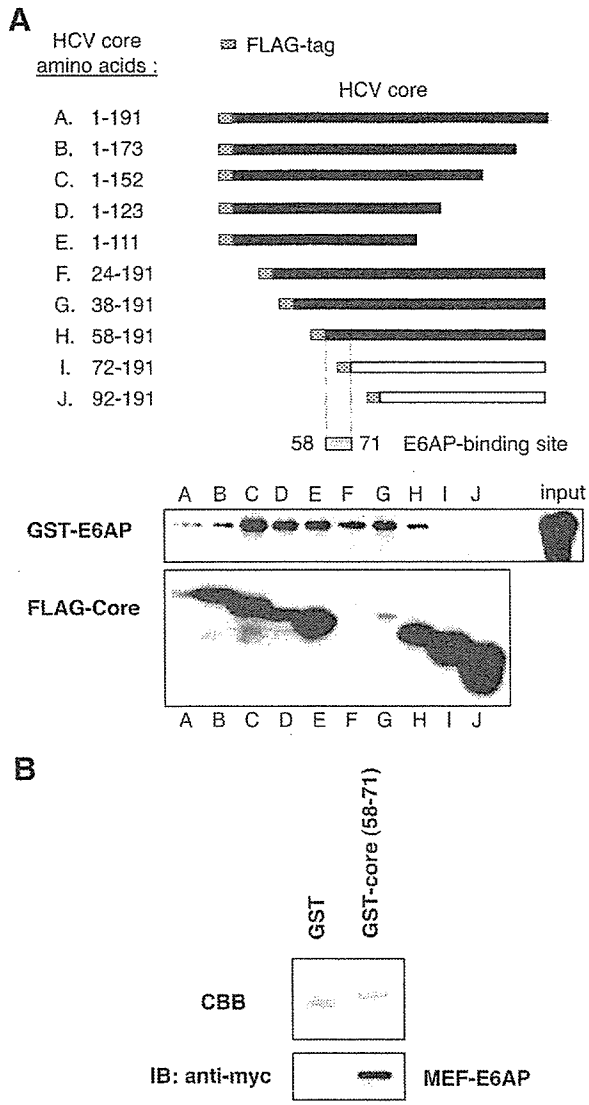

FIG. 3. Mapping of the E6AP binding domain for HCV core protein. (A) In vitro binding of E6AP to HCV core protein. 293T cells were transfected with each plasmid indicated in the upper panel. At 48 h posttransfection, cell lysates were mixed with purified GST-E6AP, immunoprecipitated with anti-FLAG beads, and then immunoblotted 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

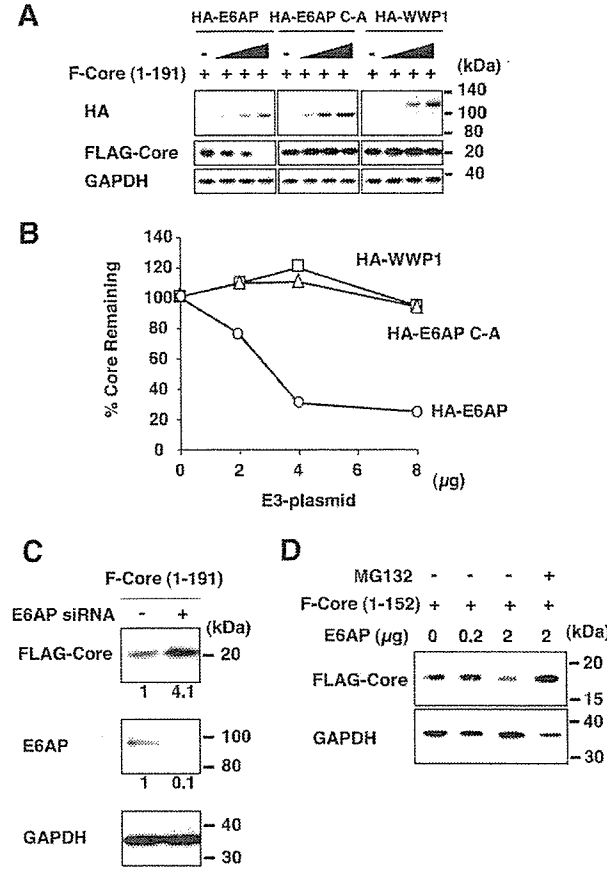

FIG. 4. E6AP decreases steady-state levels of HCV core protein in 293T cells and in HepG2 cells. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG FLAG-core (1–191) along with either pCAG-HA-E6AP, pCAG-HA-E6AP C-A, or pCAG-HA-WWP1 as indicated. At 48 h posttransfection, protein 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 μg 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 μM 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).

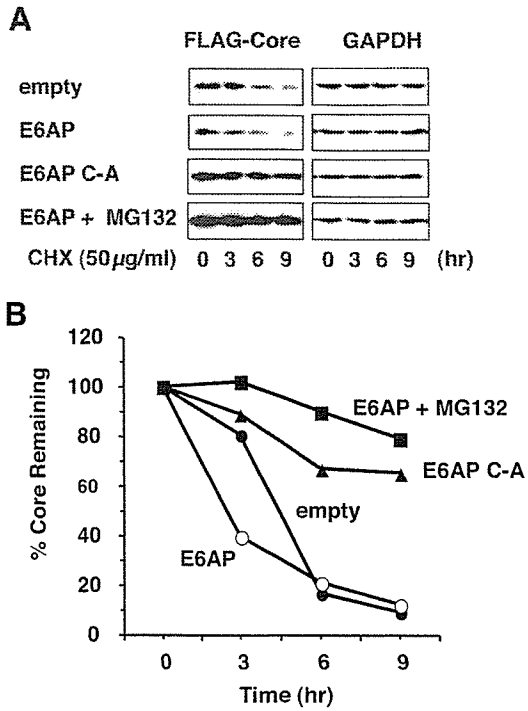

FIG. 5. Kinetic analysis of E6AP-dependent degradation of HCV core protein. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG-FLAG core (1–152) plus 4 μg of empty vector, pCMV-HA-E6AP, or pCMV-HA-E6AP C-A. The cells were treated with 50 μg/ml CHX at 44 h after transfection. 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.

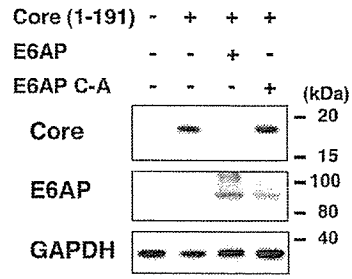

FIG. 6. E6AP promotes degradation of full-length HCV core protein in Huh-7 cells. Huh-7 cells (2×10^5 cells/six-well plate) were transfected with 0.5 μg of pCAG-core (1–191) together with 2 μg of pCMV-HA-E6AP or pCMV-HA-E6AP C-A. At 48 h posttransfection, cells were harvested and analyzed by immunoblotting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

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 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-

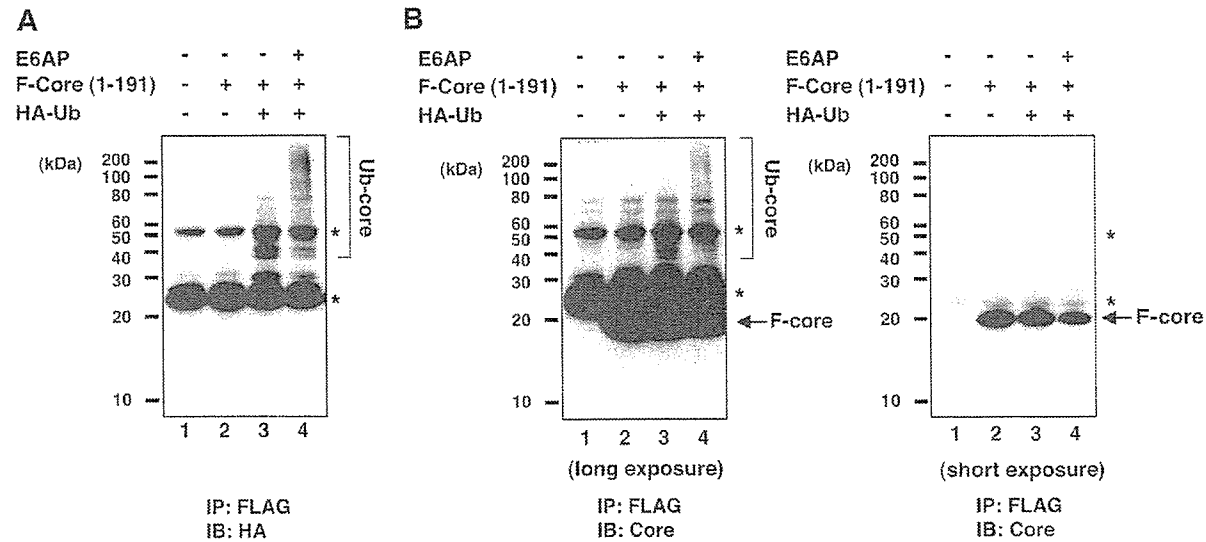

FIG. 7. E6AP-dependent ubiquitylation of HCV core protein in vivo. 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG FLAG-core (1–191) together with 2 μg of plasmid encoding E6AP as indicated. Each transfection also included 2 μg of plasmid encoding HA-ubiquitin. The cell lysates 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

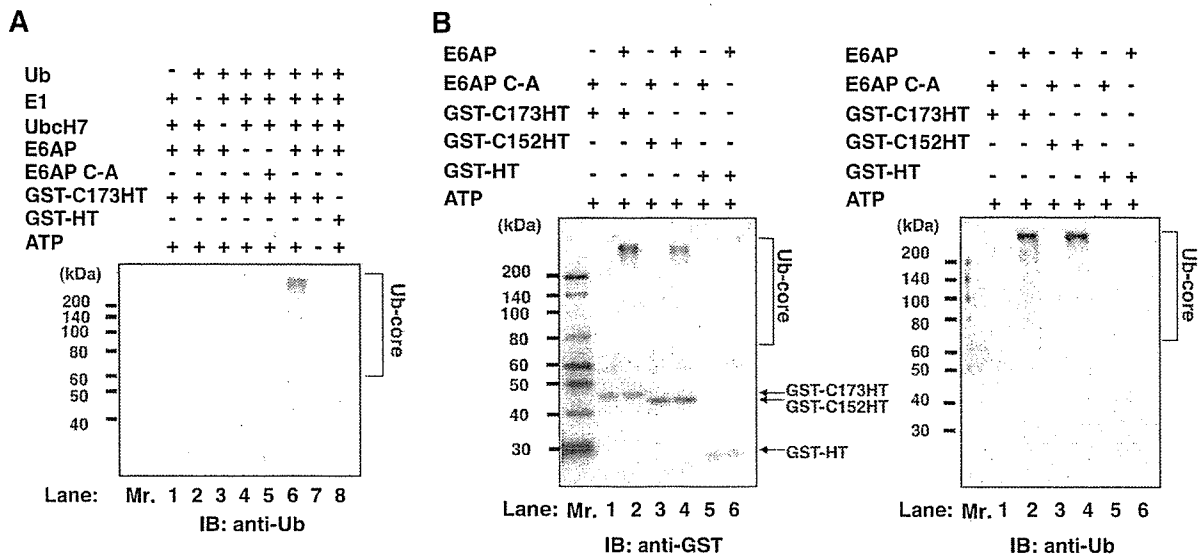

FIG. 8. In vitro ubiquitylation of HCV core protein by recombinant E6AP. For in vitro ubiquitylation of HCV core protein, purified GST-C173HT and GST-C152HT were used as substrates. Purified GST-HT was used as a negative control. Assays were done in 40-μl volumes containing each component as indicated. 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. Knockdown 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. Knockdown 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.

PA28γ was found to interact with HCV core protein in hepatocytes and promote proteasomal degradation of HCV core protein (30). PA28γ, however, has been shown to function

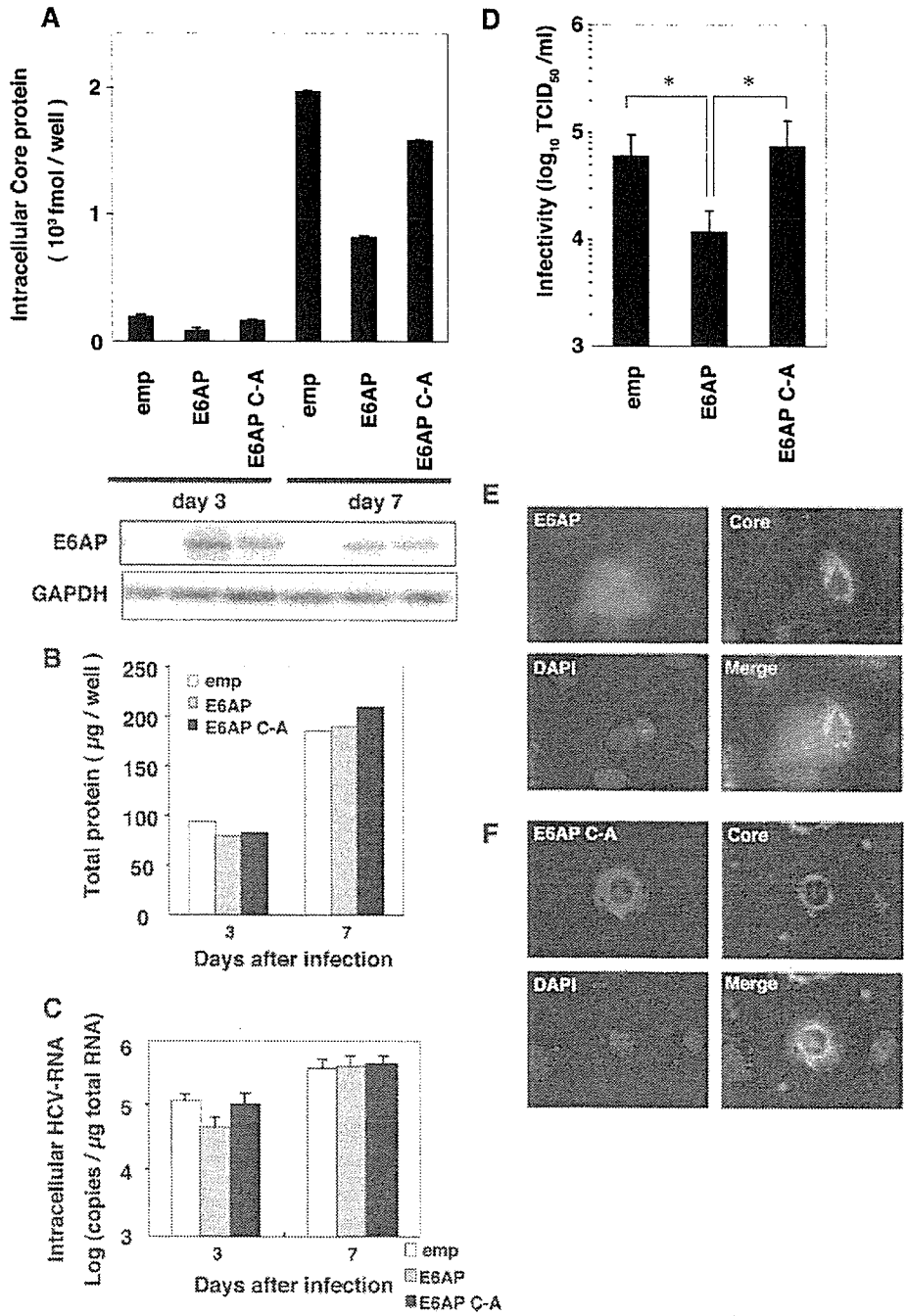

FIG. 9. Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. Naïve Huh-7 cells were seeded as described in Materials and Methods; inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5×10^5 $TCID_{50}$/ml); and transfected with 6 μg 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 $TCID_{50}$ 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.

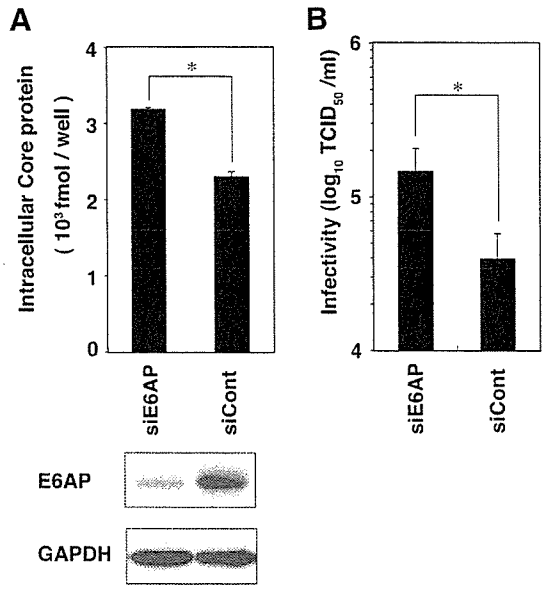

FIG. 10. E6AP silencing leads to an increase in the level of intracellular HCV core protein and supernatant infectivity titer in HCV-infected Huh-7 cells. (A) HCV JFH1-infected cells were replated in a six-well plate at 3×10^5 cells/well and transfected with 40 pmol of E6AP siRNA or control siRNA. The 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 $TCID_{50}$ 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 PA28γ-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. PA28γ was found to colocalize with HCV core protein in the nucleus. Functional differences may exist between the E6AP-dependent pathway and the PA28γ-dependent pathway in the stability control of HCV core protein. The functional role of the E6AP-dependent pathway and the PA28γ-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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Negative regulation of intracellular hepatitis C virus replication by interferon regulatory factor 3

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Background. Interferon regulatory factor (IRF)-3 plays an important role in initiating cellular interferon-stimulated gene-mediated antiviral responses. In the present study, we evaluated the effects of IRF-3 expression and activation on intracellular hepatitis C virus (HCV) replication using an HCV replicon system. ***Methods.*** An HCV replicon was constructed that expressed a neomycin-selectable chimeric firefly luciferase reporter protein. A small interfering (si) RNA oligonucleotide directed against IRF-3 mRNA was designed and synthesized. A eukaryote expression plasmid vector was constructed that expressed IRF-3 mRNA under control of the cytomegalovirus early promoter/enhancer. To evaluate transcriptional activity of the interferon-stimulated genes, a reporter vector was used that expressed firefly luciferase under control of the interferon-stimulated response element (ISRE). ***Results.*** The baseline expression of IRF-3 did not significantly differ between cells with and without expression of the replicon. Transfection of an IRF-3 expression plasmid into the cells raised the ISRE-luciferase activities. The increase of ISRE activity was significantly more potent in the replicon-expressing cells than in cells without replicon expression. Concomitantly, the overexpression of IRF-3 suppressed HCV replication levels. In contrast, siRNA knockdown of IRF-3 suppressed ISRE activity by 38% ± 2%. Interestingly, the suppression of IRF-3 resulted in a significant increase of HCV replication, by up to twofold, depending on the IRF-3 suppression levels. ***Conclusions.*** IRF-3 negatively regulated intracellular HCV replication, and was partially activated in cells that expressed the HCV replicon. Thus, IRF-3 is a key molecule controlling HCV replication through modulation of host interferon gene responses.

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Introduction

Hepatitis C virus (HCV) is a worldwide health-care problem causing a spectrum of liver disease ranging from an asymptomatic carrier state to liver cirrhosis and hepatocellular carcinoma.[1] Currently available anti-HCV treatments are based on high-dose administration of a major antiviral cytokine, interferon (IFN)-α. However, even with the most efficient regimen of pegylated interferon in combination with ribavirin, almost half of all cases are refractory to the treatment and fail to eradicate the virus.[2] Without the IFN therapy, HCV is associated with persistent infection and replication in the liver in spite of intact host immune systems; these features lead us to speculate that HCV escapes from or attenuates host antiviral responses.

Type I IFN plays a central role in eliminating viruses not only in therapeutic applications but also as a na-tural cellular antiviral defense mechanism.[3,4] IFNs mediate antiviral responses by inducing expression of interferon-stimulated genes (ISGs), including those encoding 2,5-oligoadenylate synthetase, double-stranded RNA-dependent protein kinase R, and MxA proteins, resulting in the degradation of cellular RNA, general repression of protein synthesis, and apoptotic cell death.[5] Also, a DNA microarray analysis of chimpanzee liver experimentally inoculated with HCV revealed that expression of ISGs, including those encoding cytokines and chemokines, was the principal reaction during the course of the viral infection and its clearance and that a considerable proportion of the genes were inducible by IFNs.[6]

The expressional control of ISGs is directed by receptor-mediated stimuli of type I IFNs.[7] Binding of

the IFNs onto their receptors activates receptor-associated janus kinases, which phosphorylate signal transducer and activator of transcription (STAT) 1 and STAT2. The phosphorylated STATs, 1 and 2, recruit IFN regulatory factor (IRF)-9 to form a complex with IFN-stimulated gene factor-3, which translocates to the nucleus, binds the IFN-stimulated response element (ISRE) located in the promoter/enhancer region of ISGs, and activates expression of ISGs.[3,4,8]

Other than by type I IFNs, expression of ISGs is controlled by binding ISRE with other molecules, including IRF-1, IRF-3, and IRF-7. Among them, IRF-3 is a transducer of virus-mediated signaling and plays a critical role in the induction of cellular antiviral responses.[8–11] IRF-3, which is ubiquitously expressed in the cytoplasm, is subjected to phosphorylation by virus infection, double-stranded RNA, and bacterial lipopolysaccharides. The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus, and predominantly activates expression of the IFN-β gene and certain ISGs.[4,12,13]

The IRF-3-mediated IFN pathway might be a target of viruses to counteract antiviral responses and to promote virus replication in the infected cells. Ebola virus, bovine diarrhea virus, and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins.[14–16] In the case of HCV, some reports suggest interaction of virus proteins with the cellular IFN system. The viral NS5A protein has been reported to interfere with cellular IFN signaling.[17] It has recently been reported that HCV NS3/4A fusion protein blocks virus-induced activation of IRF-3.[18] Taken together, these findings indicate that IRF-3 is not only a key molecule of cellular innate immune responses but also might be a target of antiviral strategies. However, the mechanisms of IRF-3 activation by HCV infection in hepatocytes have not been explored yet, nor have the effects of the activated IRF-3 on HCV been satisfactorily studied.

An HCV subgenomic replicon is an in vitro model that simulates cellular autonomous replication of HCV genomic RNA. The development of the replicon system has partly overcome the problem of a lack of HCV replication models.[19] Replication of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs,[20–22] suggesting intact IFN receptor-mediated cellular responses. However, in the absence of the exogenous interferon, persistent and high-level expression of the replicon has caused us to speculate that intracellular virus-induced antiviral responses become attenuated or malfunction as a result of the expression of viral proteins. We have previously reported that the baseline activity of ISG expression is substantially decreased in cells expressing replicon and that this decrease is partly attributable to the transcriptional suppression of IRF-1.[23] In the present study, we extended our observations by investigating the effects of IRF-3 expression and activation on HCV replication.

Materials and methods

Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO, USA) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 300 μg/ml G418 (Wako, Osaka, Japan).

HCV replicon constructs and transfected cell lines

An HCV subgenomic replicon plasmid, pHCVIbneodelS (designated pRep-N), was derived from an infectious HCV clone, HCV-N, genotype Ib.[22] The replicon pRep-N was reconstructed by replacing the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotransferase genes (pRep-Feo, Fig. 1).[24,25] RNA was synthesized from pRep-N and pRep-Feo using T7-polymerase (Promega, Madison, WI, USA) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/Rep-N and Huh7/Rep-Feo, respectively). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A, and NS5A protein expression levels and with the replicon RNA expression levels.

Cured Huh7 cells

To establish cured Huh7 cells (cHuh7), from which replicon RNA was eliminated, Huh7/Rep-Feo was treated

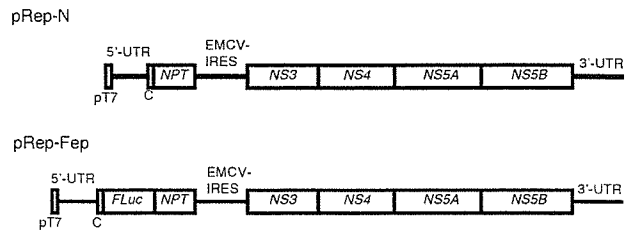

Fig. 1. Structures of the hepatitis C virus (HCV) replicon plasmids. *UTR*, untranslated region; *pT7*, T7 promoter; *C*, truncated HCV core region (nucleotides 342–377); *EMCV*, encephalomyocarditis virus; *Fluc*, firefly luciferase gene; *NPT*, neomycin phosphotransferase gene; *NS3*, *NS4*, *NS5A*, and *NS5B*, genes that encode HCV nonstructural proteins

with 100 U/ml of IFN-α for 14 days. The absence of replicon RNA was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) and by the loss of resistance to G418.[23]

Small interfering RNAs

Three small interfering RNAs (siRNAs) directed against IRF-3 were synthesized: siRNA1 (5′-gug gga gac agg acg cug cTT-3′), siRNA2 (5′-gcc aga cac cuc ucc gga cTT-3′), and siRNA3 (5′-ggu ugu gcc cac gug ccu cTT-3′). A control siRNA was used as previously described (5′-ucg ggg cac ugc uag auc cTT-3′).[24]

Plasmids

The expression plasmid vector pcDNA3.1-IRF-3 expresses the human IRF-3 open reading frame, which was cloned from human hepatocyte mRNA by RT-PCR using primers IRF-3/5′ (5′-CAC CAT GGG AAC CCC AAA GCC ACG GAT CCT-3′) and IRF-3/3′ (5′-GCT CTC CCC AGG GCC CTG GAA ATC CAT G-3′). The PCR product was inserted into the pcDNA3.1 TOPO vector (Invitrogen, Carlsbad, CA, USA) as instructed, and the nucleotide sequence was confirmed. The plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. The plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. pRL-CMV (Promega), which expressed *Renilla* luciferase protein, was used for correction of transfection efficiency.

Transient transfection

DNA and siRNA transfection was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. To perform reporter assays to determine the effect of IRF-3 on ISRE in the cells, a total of 5×10^4 Huh7, cHuh7, and Huh7/Rep-N cells were subcultured onto 24-well plates the day before transfection. A total of 100 ng of pISRE-TA-Luc and various amounts of pcDNA3.1-IRF-3 with empty vector and 0.1 ng of pRL-CMV, to a total mass of DNA of 400 ng, were transfected by using 2 µl of Lipofectamine 2000.

Western blotting

Cytoplasmic and nuclear fractions of cell lysates were prepared as described elsewhere.[26] The purity of the cytoplasmic and nuclear fractions was monitored by immunoblotting using an antibody directed against a nuclear protein, USF-2 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Twenty micrograms of protein was separated using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) or anti-His antibodies (Invitrogen), and detected by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche).

Immunocytochemical staining

Cells seeded onto tissue culture chamber slides were washed with phosphate-buffered saline (PBS) and fixed with 99% cold acetone for 10 min. After rinsing with PBS, cells were incubated with an anti-IRF-3 antibody at a dilution of 1/500 or an anti-His antibody at a dilution of 1/200 in PBS/3% goat serum. After 3 h, cells were washed three times with PBS, and incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibodies. Cells were then washed and mounted with VectaShield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Fluorescence microscopy was carried out with an Olympus BX50.

Luciferase reporter assays

Luciferase activity was measured by luminometer (Lumat LB9501; Promega) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were done in triplicate, and the results were expressed as means ± SD.

MTS assays

To evaluate cytotoxicity, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

Statistical analyses

Statistical analyses were performed using an unpaired, two-tailed Student's *t* test; *P* values less than 0.05 were considered statistically significant.

Results

Expression level of IRF-3 in cells with and without HCV replication

First, we evaluated the expression levels of endogenous IRF-3 in Huh7 cells with or without expression of the HCV replicon. Western blotting analysis showed no sig-

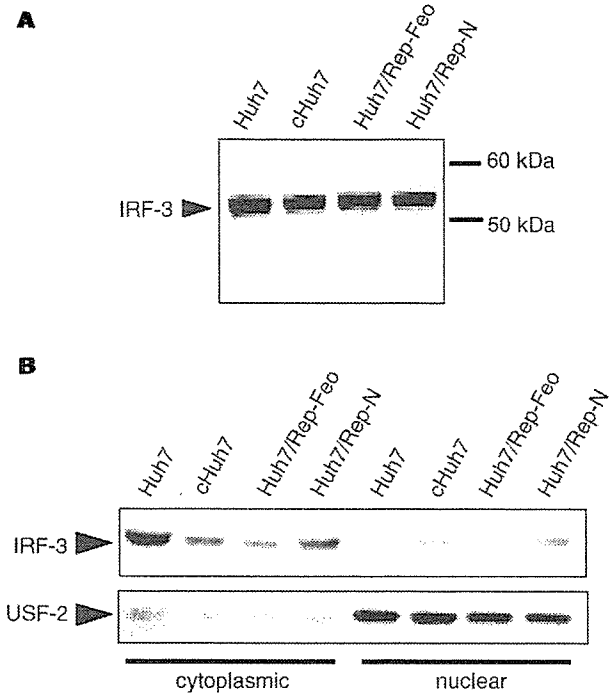

Fig. 2A,B. Expression of endogenous interferon regulatory factor-3 (*IRF-3*) in cells with and without expression of the HCV replicon. **A** Western blotting. Whole cell lysates from Huh7 and Huh7/Rep-Feo were prepared. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting, the expression of IRF-3 protein was detected by a monoclonal anti-IRF-3 antibody. **B** To detect IRF-3 translocated to the nucleus, we prepared the cytoplasmic and the nuclear fractions of cell lysates from naïve Huh7, Huh7/rep-Feo, Huh7/Rep-N, and cured Huh7 (*cHuh7*) cell lines, and detected IRF-3 expression by Western blotting. The purity of cellular fractionation was tested by immunoblotting for USF-2. The differences in the IRF-3 expression levels were due to different yields from the preparation of cytoplasmic and nuclear fractions. However, the ratio of nuclear to the respective cytoplasmic IRF-3 remained equal among the cell lines tested

nificant difference in expression levels of IRF3 between Huh7 and Huh7/Rep-Feo (Fig. 2A). Similarly, levels of IRF-3 mRNA were not significantly different between Huh7 and Huh7/Rep-Feo.

IRF-3, once activated by site-specific phosphorylation, translocates into the nucleus. To examine the nuclear translocation of the activated IRF-3, we prepared nuclear and cytoplasmic fractions of cell lysates from naïve Huh7, cured Huh7, and two Huh7 that expressed the replicon, Huh7/Rep-N and Huh7/Rep-Feo. Western blotting of the cell fractions showed that most IRF-3 protein was localized in the cytoplasm in each cell line, and that there was no obvious increase in nuclear IRF-3 in any of the cell lines, nor were there differences in the nuclear IRF-3 levels between cells with and without the HCV replicon (Fig. 2B). Similarly, the immunocytochemistry analysis showed that IRF-3 was mainly localized in the cytoplasm, and there were no differences in the patterns of IRF-3 staining (data not shown).

Overexpression of IRF-3 and effects on ISRE activity and HCV replication

Because IRF-3 is a strong inducer of ISGs on activation, a slight change in the IRF-3 activation level could affect ISRE enhancer activity. Thus, we examined ISRE reporter activities of cells with and without the replicon, and evaluated the effects of IRF-3 by overexpression. Transfection of an IRF-3 expression plasmid, pcDNA-IRF-3, resulted in expression of 6xHis-tagged IRF-3, which was confirmed by Western blotting using an anti-IRF-3 antibody (Fig. 3A) and an anti-His antibody (Fig. 3B). Immunohistochemistry showed cytoplasmic expression of the transfected IRF-3 (Fig. 3C). Cotransfection of pcDNA-IRF-3 with an ISRE-luciferase reporter plasmid, pISRE-TA-luc, into Huh7, cHuh7, and Huh7/Rep-N cell lines resulted in a significant increase of ISRE activity in cells in which IRF-3 was overexpressed (Fig. 3D). Interestingly, the cell line expressing the replicon, Huh7/Rep-N, showed a significantly higher ISRE induction ratio by IRF-3 overexpression than naïve Huh7 or cured Huh7 (28.7-fold vs. 8.9- or 11.7-fold, $P < 0.01$), suggesting partial activation of IRF-3 in the replicon-expressing cells. Concomitantly with the ISRE activation, transfection of pcDNA-IRF-3 into Huh7/Rep-Feo resulted in a significant decrease of internal luciferase activities to $19.6 \pm 1.8\%$ of control, indicating suppression of cellular HCV replication by IRF-3 overexpression (Fig. 3E). MTS assays of the IRF-3 transfected cell lines showed no obvious effects on cell growth or viability, indicating that these effects of IRF-3 overexpression were not due to nonspecific effects or to cytotoxic cell death (Fig. 3F).

Effect of IRF-3 siRNA oligonucleotides on ISRE-luc and HCV Feo-replicon cells

To investigate the effects of suppression of IRF-3 synthesis on HCV replication, three synthetic siRNAs, siRNA1, siRNA2, and siRNA3, were used. Western blotting showed that transfection of each siRNA into Huh7 cells resulted in a decrease of the IRF-3 protein level, by 39.5%, 57.8%, and 37.4%, respectively. To study the effects of IRF-3 suppression on HCV replication, siRNAs were transfected into Huh7/Rep-Feo cells, and a luciferase assay was done after 4 days of transfection. The siRNAs upregulated HCV replication to various extents (Fig. 4A). Thus, we used siRNA3, which was the most efficient, for the following assays. Cotransfection of ISRE-TA-Luc with siRNA3 or a

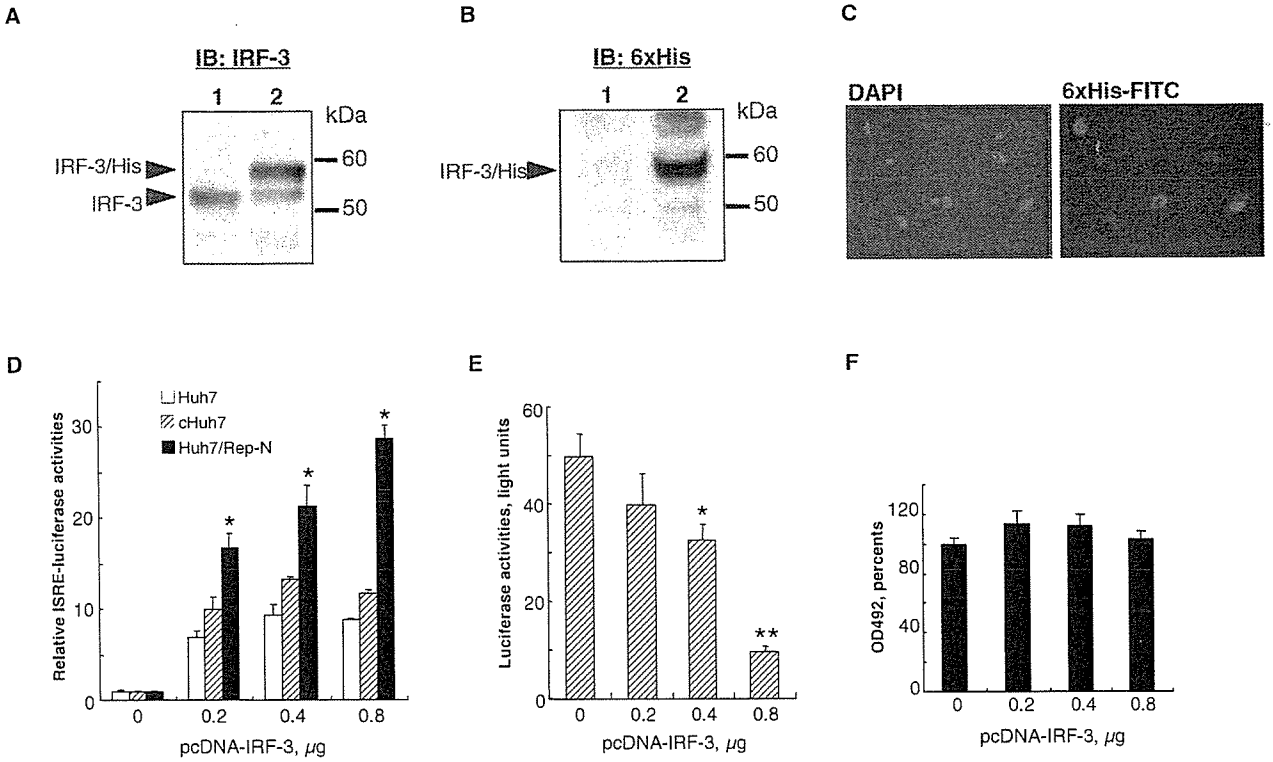

Fig. 3A–F. Effects of IRF-3 overexpression on the regulation of interferon-stimulated response element (*ISRE*) activity and HCV replication. An IRF-3-expression plasmid, pcDNA-IRF-3, was transfected into Huh7 cells (*lane 2*), and Western blotting analyses were performed using monoclonal anti-IRF-3 antibody (**A**) and anti-His antibody (**B**). Untransfected Huh7 is shown as a control (*lane 1*). **C** Fluorescence microscopy. The pcDNA-IRF-3 was transfected into Huh7 cells, and the cells were stained with 4′,6-diamidino-2-phenylindole (*DAPI*) and with anti-His antibody followed by fluorescein isothiocyanate (*FITC*)-labeled secondary antibody. The figure shows DAPI staining for nuclei (*left panel*) and transgenic IRF-3 expression (*right panel*). Magnification, ×40. **D** Effects of transgenic IRF-3 expression on ISRE reporter activity. The pcDNA-IRF-3 and ISRE-TA-luc reporter plasmids were cotransfected into Huh7 (*white bars*), cured Huh7 (*gray bars*), and Huh7/Rep-N cells (*black bars*), and luciferase activities were measured 24 h after the transfection. *Error bars* denote means + SD (*$P < 0.01$ relative to Huh 7 cells and cured Huh7 cells; Student-Newman-Keuls test). **E** Effects of transgenic IRF-3 expression on the levels of HCV replication. The indicated amounts of pcDNA-IRF-3 were mixed with empty pcDNA plasmid to adjust the total amount of DNA, mixed with Lipofectamine 2000, and transfected into Huh7/Rep-Feo cells seeded onto 24-well culture plates, and the luciferase activities were measured 24 h after transfection. *Error bars* denote means + SD (*$P = 0.006$; **$P = 0.0001$ relative to transfection with the empty vector). **F** MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays. The indicated amounts of pcDNA-IRF-3 were transfected into Huh7/Rep-Feo cells with the same conditions described above, and MTS assays were performed. *Error bars* indicate means + SD

control siRNA into Huh7 and Huh7/Rep-N resulted in significant suppression of ISRE-luciferase activities, by 50% in both Huh7 cells and Huh7/Rep-N (Fig. 4B). To study the relation between the suppression level of IRF-3 by siRNA3 and HCV replication, siRNA3 was transfected into Huh7/Rep-Feo cells, and a luciferase assay was done after 2 days of transfection. siRNA3 upregulated HCV replication in a dose-dependent manner (Fig. 4C).

Discussion

Persistent virus replication in host cells is the function of the interplay between the cellular antiviral system and the counteraction of the virus to evade host antiviral responses.[17] In our present study, even though IRF-3 expression levels were mostly similar between cells with and without HCV replication (Fig. 2A), overexpression (Fig. 3A–C) and knockdown (Fig. 4A) of IRF-3 were associated with up- and downregulation of ISG expression, as indicated by ISRE reporter activities (Figs. 3D and 4B), and were inversely correlated with HCV subgenomic replication levels (Figs. 3E and 4C). These

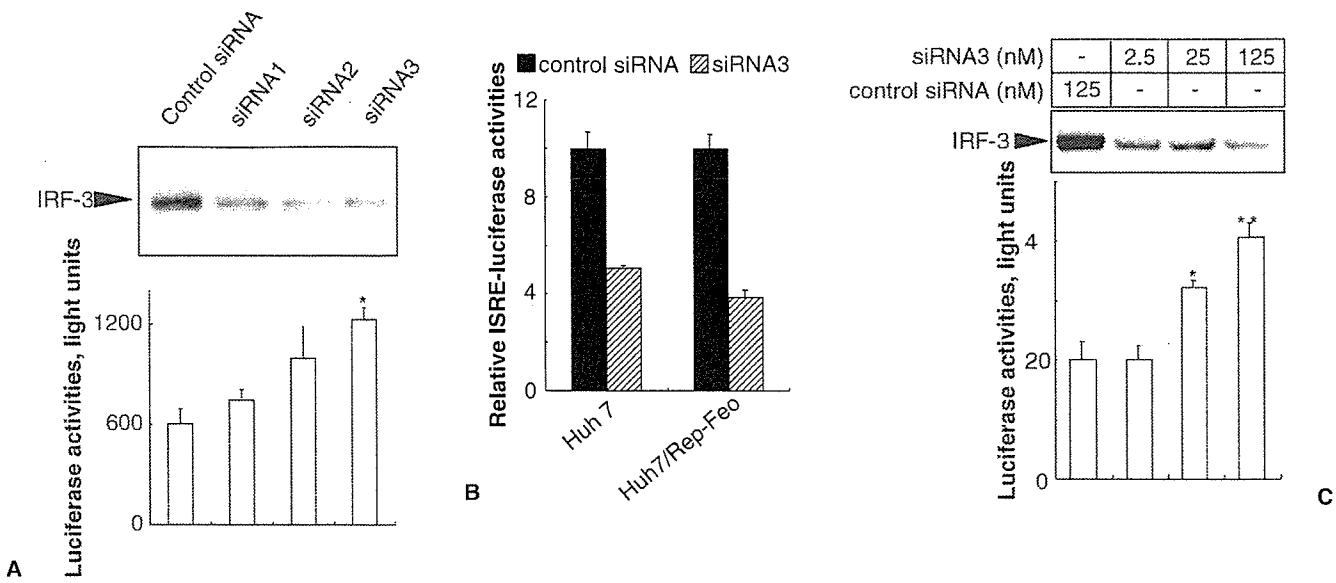

Fig. 4A–C. Suppression of IRF-3 expression by siRNA and effects on ISRE activity and HCV replication. **A** Suppression of endogenous IRF-3 expressions by IRF-3-directed siRNAs. Three siRNAs, siRNA1, siRNA2, and siRNA3, were transfected into Huh7 cells, and IRF-3 were detected by Western blotting using monoclonal IRF-3 antibody. Transfection of the three siRNAs substantially inhibited expression of IRF-3 protein. Three siRNAs and a control siRNA were transfected into Huh7/Rep-Feo cells that was plated onto 24-well plates. Graph: luciferase activities of the corresponding samples. *Error bars* denote means + SD (*$P = 0.0007$ relative to transfection with a control siRNA). Suppression of IRF-3 by siRNA increased HCV replication. **B** Effects of an IRF-3-directed siRNA, siRNA3, on ISRE-reporter activity. The siRNA and a control siRNA were individually transfected with pISRE-TA-luc reporter plasmid into Huh7 and Huh7/Rep-Feo cells, and luciferase activities were measured 24 hours after transfection. *Error bars* denote means + SD (*$P < 0.001$ relative to transfection with a control siRNA). **C** The correlation between the suppression level of IRF-3 by siRNA and HCV replication. The indicated amounts of siRNA3 or a control siRNA were transfected into Huh7/Rep-Feo cells, which were plated onto 24-well plates. The Western blotting shows dose-dependent suppression of IRF-3. *Graph*: luciferase activities of the corresponding samples. *Error bars* denote means + SD (*$P = 0.003$; **$P = 0.0007$ relative to transfection with a control siRNA)

results are consistent with a previous report that IRF-3 mediates expression of the antiviral gene via ISRE.[12] Furthermore, the replicon-expressing cells showed a significantly higher rate of ISRE activation than naïve or cured cells when IRF-3 was overexpressed (Fig. 3D). These results suggest different activation levels of the IRF-3-mediated pathway by replication of the HCV subgenome. Because IRF-3 is a strong inducer of interferon-β production,[4,12,13] it is possible that the effects of IRF-3 on HCV replication were predominantly mediated by interferon production, which led to activation of ISRE-dependent transcription. Collectively, our results suggest that replication of the HCV subgenome was closely correlated with expression and activation levels of IRF-3 and that IRF-3 was a key cellular factor controlling ISRE-regulated ISG expression and cellular antiviral responses.

Foy et al.[18] reported that the HCV NS3/4A fusion protein substantially blocks phosphorylation and nuclear translocation of IRF-3 by experimental virus infection. We have also confirmed that double-stranded RNA-induced activation of the IRF-3 pathway was abolished in cells expressing the HCV replicon (unpublished data). Our present results reinforce the reports that IRF-3 is a key molecule of the cellular innate immune responses against HCV and that it may constitute a target of antiviral strategies.

Although our findings suggest activation of the cellular IRF-3-IFN pathway along with HCV replication, there are still unsolved questions: Which molecule is the sensor of HCV? Which viral component is the target of the detection? How is the triggered signal transduced to the IRF-3 activation leading to IFN responses? Studies of the virus-induced IFN signaling pathway are making progress in the discovery and elucidation of these issues. Several molecules have been recently identified that are involved in innate immune responses against various pathogens, including viruses: toll-like receptor (TLR) families, which recognize viral components of double- or single-stranded RNAs and lipoproteins;[27] two kinases, IKKe and TBK1, which catalyze phosphorylation of IRF-3;[28,29] and TRIF,[30] which mediates Myd88-independent TLR signaling. TLR3 has been reported to recognize double-stranded RNA and to activate IRF3-

mediated IFN signal transduction, suggesting that TLR3 could be a candidate receptor for innate immune responses against viruses.[8,11] However, our preliminary studies have shown that treatments with polyinosinic polycytidylic acid [poly(I-C)] and lipopolysaccharides, which are ligands of TLR3 and TLR4, respectively, have no effect on cellular ISRE activities or on HCV subgenomic replication (data not shown), a part of which is consistent with the findings of previous studies.[31] More recently, a DExD-box helicase, RIG-I, has been identified as a cytoplasmic receptor molecule that recognizes double-stranded RNA.[32] Speculatively, unknown molecules may recognize HCV genomic replication in cells and activate the IRF-3-mediated antiviral pathway.

Because our present study was based on the HCV subgenomic replicon system, which expresses only viral nonstructural proteins and not structural proteins, our results may have limited implication for the association between HCV infection and the innate immune system. Moreover, Huh7 cells, which are the host of the HCV replicon, are of human hepatoma origin.[33] Most hepatomas arise from chronic viral hepatitis and liver cirrhosis.[1] Although little information is available on what Huh7 cells were derived from, it is possible that these cells have been primed by past HCV infection, which could modify the cellular innate immunity continuously. To address these possibilities, further investigation using other cell lines that stably support HCV replication may be warranted.

Although in vitro HCV replication is highly sensitive to exogenous IFN, in clinical settings, a majority of HCV-infected patients are resistant to IFN treatments.[2] Our results suggest that an IRF-3-mediated innate immune system response might be activated by HCV infection in hepatocytes. This initial reaction in the host cells against the virus may determine the activities of the cellular and humoral immune responses that follow, and the clinical course of the infection thereafter. At present, few reports correlate clinical features with the function of IRF-3 in the HCV-infected liver. Castelruiz et al.[33] have reported that patients with chronic hepatitis C show a significant increase in IFN-β mRNA in liver tissue. Thus, one of our next objectives is to elucidate how the innate immune system participates in the whole clinical process of HCV infection, and whether individual differences in the innate immune response influence clinical features.

In conclusion, our results demonstrate that IRF-3 negatively regulates HCV replication in vitro, possibly through IRF3-mediated ISG expression pathways. Therefore, IRF-3 might be a key molecule not only as a mediator of the host antiviral responses against HCV but also as a potential therapeutic target to control HCV replication.

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Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication

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SUMMARY. Type-I interferons (IFNs) and the interferon-stimulated genes (ISGs) play a major role in antivirus responses against hepatitis C virus (HCV) infection. In this study, we studied expression profiles of ISGs in cells supporting subgenomic HCV replication (Huh7/Rep), and screened their activities to suppress HCV replication. Real-time PCR analyses showed that the expression levels of 23 ISGs were significantly lower in Huh7/Rep than naive Huh7 cells due to transcriptional suppression of the interferon-stimulated response element (ISRE). Furthermore, the expression level of ISGs was also decreased in the cured Huh7 cells in which replicon had been eliminated (cHuh7), indicating adaptation of the cells to support HCV replication by downregulating ISGs. On the other hand, expression of HCV replicon was significantly suppressed by overexpression of several ISGs including PKR, MxA, IRF-9, GBP-1, IFI-6-16, IFI-27, 25OAS and IRF-1. Knock down of GBP-1, IFI-6-16 and IFI-27 by short hairpin RNA resulted in increase of HCV replication. Thus, we conclude that downregulation of ISG expression is required in the host cells supporting HCV replication and that several ISGs directly suppress HCV replication. The search for ISGs that regulate HCV replication may help to elucidate the cellular antiviral defence mechanisms against HCV infection.

Keywords: guanylate binding protein-1, hepatitis C virus, interferon-induced protein 6–16, interferon-inducible protein-27, interferon-stimulated gene, replicon.

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Abbreviations: HCV, Hepatitis C virus; IFN, interferon-alpha; ISG, interferon-stimulated gene; 25OAS, 2′, 5′-oligoadenylate synthetase; MxA, myxovirus resistance 1; PKR, double-stranded RNA-dependent protein kinase R; IFI-56K, interferon-induced protein 56; IRF, interferon regulatory factor; GBP-1, guanylate binding protein-1; IFI-6-16, interferon-induced protein 6–16; IFI-27, interferon-inducible protein 27; ISGF-3, interferon-stimulated gene factor-3; TAP1, transporter ATP-binding cassette, major histocompatibility complex 1; IFP35, interferon inducible protein 35kD; PLSCR1, phospholipid scramblase 1; LMP7-E1, major histocompatibility complex encoded proteasome subunit LMP7-E1; eIF2-alpha, eukaryotic initiation factor-2 alpha; eIF3, eukaryotic initiation factor-3; STAT, signal transducer and activator of transcription; NS5A, nonstructural protein 5A; Fluc, firefly luciferase; ISRE, interferon-stimulated response element; GAS, interferon-gamma activation site; AP1, activator protein 1; NF-kappa B, nuclear factor-kappa B; Rluc, renilla luciferase; shRNA, short hairpin RNA; IRES, internal ribosome entry site.

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INTRODUCTION

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality [1,2]. HCV is characterized by persistent infection in the liver that leads to the development of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Type-I interferon (IFN) plays a central role in eliminating viruses not only as therapeutic applications [3] but also as natural cellular antiviral defence mechanisms [4,5]. DNA microarray analysis of chimpanzee liver experimentally inoculated with HCV revealed that expression of the interferon-stimulated genes (ISGs), including cytokines and chemokines, was the principal reaction during the course of the viral infection and its clearance and that a considerable proportion of the genes were inducible by IFNs [6].

Interferons are naturally produced in response to virus infection, and to cellular exposure to IFN itself [7]. The expressional control of the ISGs is directed by receptor-mediated stimuli of type-I IFNs [8]. Binding of the IFNs onto their receptors activate receptor associated janus kinases, which phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2. The phosphorylated STATs 1 and 2 recruit IFN regulatory factor-9 (IRF-9) to form a

complex of IFN-stimulated gene factor-3 (ISGF-3), which translocates to the nucleus, binds the IFN-stimulated response element (ISRE) located in the promoter/enhancer region of ISGs, and activates expression of ISGs [4,5,9].

Interferons induce expression of a variety of ISGs, several of which show antiviral function by limiting virus replication at multiple points within the replication cycle [7,10]. At least four ISGs have been reported to direct antiviral activity through distinct cellular pathways of translational control; 2′, 5′-oligoadenylate synthetase (OAS), double-stranded RNA-dependent protein kinase R (PKR), myxovirus resistance 1 (MxA), and interferon-induced protein 56 (IFI-56K). Transcriptional induction of 2′, 5′-OAS activates ribonuclease L (RNase L) which leads to translational suppression though the cleavage and subsequent inactivation of 28S rRNA [11]. PKR is activated by viral double-stranded RNA, and catalyses the phosphorylation of eukaryotic initiation factor-2 alpha (eIF2-alpha). The phosphorylated eIF2-alpha blocks translation initiation by reducing the cellular pool of functional eIF2 and by disrupting the critical delivery of methionyl-tRNA to the 40S ribosome [12]. MxA protein results in degradation of cellular RNA, general repression of protein synthesis and apoptotic cell death [13]. IFI-56K binds eukaryotic initiation factor-3 (eIF3) and suppresses translation [10,14]. However, cells in which of PKR, MxA, or 25OAS are knocked down still retain IFN responses to suppress HCV replication, suggesting that there may be unidentified ISGs that show antiviral activities [15].

An HCV replicon system is an *in vitro* model that simulates stable and noncytopathic cellular autonomous replication of HCV genomic RNA [16,17]. There has been a lack of adequate HCV replication models, although this problem has been partially overcome by the introduction of the HCV replicon system. Replication of HCV replicon can be abolished by treatment with small amounts of type-I and type-II IFNs [15,18,19], suggesting intact IFN receptor-mediated cellular responses. In the present study, we analysed the expression profiles of ISGs by using the HCV replicon system which expresses chimeric luciferase reporter protein [20,21], and by using overexpression analysis, we identified several ISGs that suppress HCV replication.

MATERIALS AND METHODS

Cells and cell culture

Huh7 and 293T cells were maintained in Dulbecco's modified minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 10% foetal calf serum at 37 °C under 5% CO_2. To maintain cell lines carrying the HCV replicon (Huh7/Rep or Huh7/Rep-Feo cells), G418 (Nakalai Tesque, Kyoto, Japan) was added to the culture medium at a final concentration of 500 μg/mL.

HCV replicon constructs and transfection

Hepatitis C virus replicon plasmid, pRep-Feo was derived from the HCV-N strain, pHC1bneo/delS [19]. The pRep-Feo expressed chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase [20,21]. The replicon RNA synthesis and transfection have been described (Huh7/Rep-Feo) [17].

Establishment of the cured Huh7 cells

Cured Huh7 cells (cHuh7) were established by eliminating replicon from Huh7/Rep-Feo cells by treatment with 100 U/mL of IFN-alpha for 14 days. Clearance of replicon RNA was confirmed by RT-PCR and by the loss of resistance to G418.

Reverse transcription and Light Cycler-based PCR assay:

Total cellular RNA was extracted from Huh7, Huh7/Rep, and cHuh7 cells using Isogen (Wako, Osaka, Japan). Two micrograms of total cellular RNA was used to generate cDNA from each sample using SuperScript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase. The mRNA expression levels were measured with a Light Cycler PCR and detection system (Roche Applied Science, Indianapolis, IN, USA). Thermocycling was done in a final volume of 10 μL containing 1 μL cDNA sample or calibrator; 1.25 mM $MgCl_2$; 0.5 μM of each primer and 1 μL of LightCycler FastStart DNA Master SYBR Green 1 mix (Roche). Cycle numbers of the logarithmic linear phase were plotted against the logarithm of the concentration of template DNA. The concentrations of DNA in the samples were calculated by comparing the cycle numbers of the logarithmic linear phase of the samples with the external standards. Genes assayed were IP10, IFI-56K, MxA, GBP-1, IFI-6-16, TAP1, 9-27, IFP35, PLSCR1, LMP7-E1 and PKR (Table 1).

Construction of plasmids expressing ISG and the reporter assay

We constructed plasmids expressing 18 ISGs, which were expressed in the liver during acute HCV infection [6] and induced by IFN-alpha treatment of Huh7 cells (Table 2). The full-length human ISGs were amplified by PCR from Huh7, HeLa, or SuperScript cDNA library human liver (Invitrogen) and cloned into pcDNA3.1 or pcDNA3.1D/V5-His-TOPO (Invitrogen) to yield the mammalian expression construct, pcDNA-ISG. Each ISG-expression plasmid, pcDNA-ISG was transfected into Huh7/Rep-Feo cells, and the replication level of HCV replicon was analysed by luciferase assay. A plasmid, pcDNA3.1D/V5-His/lacZ (Invitrogen) was used as a control plasmid vector for mock transfection.

Table 1 List of interferon-stimulated genes analysed by the RT-PCR

Category/gene	Gene accession number
Cytokines/Chemokines	
IP10	X02530
MK	M94250
IL8	NM000584
Antiviral genes	
MxA	M30817
PKR	M35663
GBP-1	NM002053
IFI-56 K	M24594
25OAS	NM003733
Transcription factors	
IFP35	U72882
IRF-9 (ISGF3gamma)	XM033291
STAT1a	M97935
STAF50	X82200
Interferon inducible genes	
IFI-6-16 (G1P3)	BT006850
ISG15	M13755
IFI-27 (ISG12)	X67325
Apoptosis-related genes	
PLSCR1	AF098642
TRAIL	U37518
XAF1	X99699
Proteasome	
LMP7-E1	Z14982
MECL1	X71874
RING4 (TAP1)	X57522
Antiproliferative genes	
9–27	J04164
Immune modulation	
Mac2BP	L13210
Unknown	
RIG-G (IFIT4)	U52513
NP (IFI41,75)	L22342
HCV microtubul	D28915
IRF family	
IRF-1	NM002198
Cytoskeletal	
Beta-actin	X00351

Plasmids for signal transduction and the reporter assays

We analysed the effects of IFN on signal transduction of ISRE, interferon-gamma activation site (GAS), activator protein 1 (AP1), and nuclear factor-kappa B (NF-kappa B). Plasmids, pISRE-TA-Luc, pGAS-TA-Luc, pAP1-TA-Luc, and pNF-kappaB-Luc (Clontech Laboratories, Franklin Lakes, NJ, USA) contained consensus motifs upstream of the firefly luciferase gene. A plasmid, pTA-Luc (Clontech), which lacks the enhancer element, was used for background determination. The reporter plasmid of IFI-56K promoter was constructed. IFI-56K natural promoter (IFI-56K promoter -250-+93; gene accession number; X06559) was cloned from genomic DNA by PCR. The DNA product was inserted into pGL3 basic (Promega, Madison, WI, USA) as instructed (IFI-56K-Luc). Plasmid, pRL-CMV (Promega), which expresses the renilla luciferase protein, was used for normalization of transfection efficiency [22].

Plasmids for HCV-IRES and reporter assay

To measure the effect of ISG-expression on the HCV-IRES-mediated translational efficiency, reporter assays using HCV-IRES-luciferase construct were performed. A plasmid, pClneo.Rluc-IRES-Fluc, expressed bicistronic RNA from which renilla luciferase was translated in a cap-dependent manner and firefly luciferase was translated in an HCV-IRES-dependent manner. A plasmid, pCIneo.Rluc-IRES-Fluc was tranfected with the ISG-expression plasmid, pcDNA-ISG into naive Huh7 cells. Huh7 cells were seeded at 8×10^4 per well in 24-well plates on the day before transfection. A total of 200 ng of pClneo.Rluc-IRES-Fluc were cotransfected with 200 ng of pcDNA-ISG or pUC19-shRNA331 into each well by using 1 μL of lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. At 48 h after transfection, the cell lysates were collected and dual luciferase assays were performed. Plasmid, pUC19-shRNA331, which was directed towards the HCV-5′-untranslated region (UTR) and inhibited translation of HCV, was transfected into naive Huh7 cells as a positive control which suppressed the activity of HCV-IRES.

Synthetic shRNA and shRNA-expression plasmid

The ISG-directed short hairpin RNA (shRNA)-expression vectors (pUC19-shRNA-ISG) were designed and constructed as described previously [21]. Briefly, oligodeoxyribonucleotides encoding shRNA sequences were synthesized and cloned just downstream of human U6 promoter in the plasmid pUC19. To avoid problems due to structural instability of DNA strands arising from the tight palindrome structure to transcribe shRNA, several point mutations were introduced in the sense strand of the shRNA sequences, which fully retained silencing activity of the shRNA [21,23]. Sequences of the shRNAs were as follows: shRNA-IFI-6-16; 5′-TGA AGC CCA GCG CGG GCA GCC CGG CGA CTT TGG AGT CGC CGG GCT GCC CGT GTT GGG CTT TAT TTT TT -3′, shRNA-IFI-27; 5′- CGA TTC CCG CCG CAG TGA AGC CCA TGG CAT TGG TGC CAT GGG CTT CAC TGT GGC GGG AAT TGT TTT TT -3′, and shRNA-GBP-1; 5′-CGA GGC CCG TTG ACC TGG ATG CCT CCT GAC CAA TCA GGA GGC ATC CAG GTT AAC GGG CTT TGT TTT TT -3′. Two control shRNA vectors were used: pUC19-shRNA-control expressed shRNA directed towards an unrelated target, the Machado-Joseph disease gene, and an HCV shRNA directed towards the 5′-untranslated region of HCV genome, pUC19-shRNA331 which significantly suppressed HCV replication [21].

Table 2 Expression profiles of interferon-stimulated genes in naive Huh7, Huh7/Rep and cured Huh7 cells

Gene	Fold induction by IFN†in Huh7	Fold induction by IFN† in Huh7/Rep	Basal expression in Huh7/Rep‡	Basal expression in cHuh7‡
IP10	101.5	7.16	0.13*	0.58*
RIG-G	88.2	–	0.01	0.39
IFI-27	73.1	–	0.72*	–
IFI-56 K	71.8	24.7	0.41*	0.21*
MxA	46.5	12.6	0.49*	0.06*
IRF-9	36.8	–	0.49*	0.65*
GBP-1	14.8	5.51	0.38*	0.68
IFI-6-16	12.1	18.7	0.48*	0.26*
HCV microtubul	10.6	–	0.70	–
RING4	10.2	–	0.89	0.80
STAF50	10.1	–	0.98	–
TRAIL	9.37	–	0.58	–
9-27	9.87	124.6	0.56*	0.49*
IFP35	9.79	6.98	0.70*	0.73*
PLSCR1	6.70	–	0.62*	0.65*
STAT1a	6.33	–	0.92	–
NP	4.75	–	0.40*	–
LMP7	3.26	5.60	0.36*	0.16*
PKR	2.35	–	0.47*	–
Mac2BP	1.57	–	0.63*	0.06*
MECL1	1.34	–	1.50	0.56*
MK	0.99	–	0.70*	0.87
IL8	0.87	1.44	0.22*	0.60*
IRF-1	–	–	0.54*	0.13

IFN, interferon; ISGs, interferon-stimulated gene.
*P-values of <0.05.
†Relative induction levels of ISGs by treating Huh7 or Huh7/Rep cells with 100 U/mL of interferon-alpha.
‡Values represent relative expression levels in comparison with those of naive Huh7 cells.

Luciferase assays

Luciferase activity was measured with a Lumat LM9501 luminometer (Promega) using a Bright-Glo Luciferase Assay System (Promega) or a Dual-Luciferase Reporter Assay System (Promega).

MTS assays

To evaluate cell growth and cell viability, MTS assays were performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega).

Western blot analysis

Western blotting was performed as described [20]. Briefly, 10 μg of total cell lysate was separated by SDS-PAGE, and blotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labelled anti IgG antibody, and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK). Antibodies used were anti-6xHis, anti-V5 (Invitrogen), anti-PKR, anti-IRF-9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NS5A antibodies (kindly provided by Dr Kohara), and anti-beta-actin antibodies (Sigma, St Louis, MO, USA).

Statistical analyses

Statistical analyses were performed using Student's t-test; P-values of <0.05 were considered statistically significant.

RESULTS

Decreased expression levels of ISGs in cells expressing HCV replicon and in the cured Huh7 cells.

Type-I IFNs stimulate the expression of numerous ISGs [24]. We studied a set of these genes, which are overexpressed in the early phase of acute HCV infection, and elimination of virus from the liver of a chimpanzee that had been