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Down-regulation of the internal ribosome entry site (IRES)-mediated translation of the hepatitis C virus: Critical role of binding of the stem-loop IIId domain of IRES and the viral core protein

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Abstract

In a previous study, we observed that hepatitis C virus (HCV) core protein specifically inhibits translation initiated by an HCV internal ribosome entry site (IRES). To investigate the mechanism by which down-regulation of HCV translation occurs, a series of mutations were introduced into the IRES element, as well as the core protein, and their effect on IRES activity examined in this study. We found that expression of the core protein inhibits HCV translation possibly by binding to a stem-loop IIId domain, particularly a GGG triplet within the hairpin loop structure of the domain, within the IRES. Basic-residue clusters located at the N-terminus of the core protein have an inhibitory effect on HCV translation, and at least one of three known clusters is required for inhibition. We propose a model in which competitive binding of the core protein for the IRES and 40S ribosomal subunit regulates HCV translation.

Keywords: Hepatitis C virus; Internal ribosome entry site; Translation; Core protein

Introduction

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Alter and Seeff, 2000; Pawlotsky, 2004). HCV contains approximately 9.6 kb of positive-strand RNA with one open reading frame encoding a precursor polyprotein, which is proteolytically cleaved to produce the mature structural and non-structural proteins of HCV (Choo et al., 1991; Grakoui et al., 1993; Hijikata et al., 1991; Takamizawa et al., 1991). Although HCV exhibits considerable genetic diversity, the 5′ untranslated region (5′UTR) of the viral genome is relatively well conserved among all genotypes.

HCV translation is initiated by a cap-independent mechanism involving an internal ribosome entry site (IRES), comprising nearly the entire 5′UTR of the genome. There is evidence to suggest that the first 12 to 30 nucleotides (nt) of the coding sequence are also important for IRES activity (Hellen and Pestova, 1999; Lu and Wimmer, 1996; Reynolds et al., 1995). The proposed secondary structure of the HCV 5′UTR, thought to contain four major domains (I to IV) (Fig. 1), may be conserved among HCV and related flaviviruses and pestiviruses (Brown et al., 1992; Honda et al., 1999a, 1999b; Zhao and Wimmer, 2001).

Recruitment of the 43S ribosomal complex, containing a small 40S ribosomal subunit, eukaryotic initiation factor (eIF) 3, and a tRNA–eIF2–GTP ternary complex, to mRNA molecules is critical for initiation of eukaryotic protein synthesis. The 40S subunit and eIF3 can bind independently to the HCV IRES (Buratti et al., 1998; Hellen and Pestova, 1999; Kieft et al., 2001; Sizova et al., 1998). However, it appears that interaction between IRES RNA and the 40S

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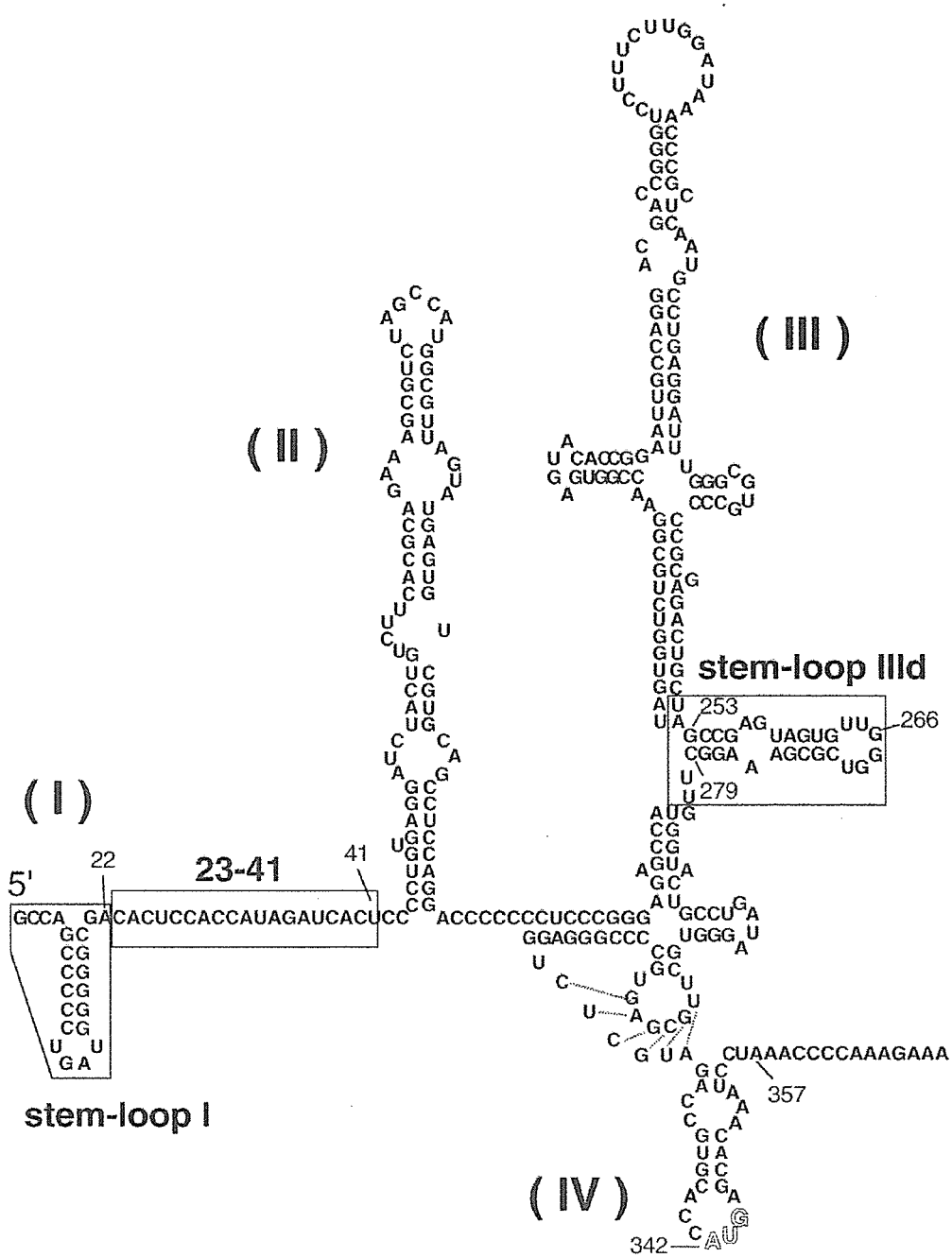

Fig. 1. Predicted secondary structure of the HCV 5′UTR (Honda et al., 1999a, 1999b). The stem-loop I, nt 23–41, and stem-loop IIId domains are highlighted. The initiator AUG codon is shown in the sequence of loop IV. Small numerals indicate the nucleotide positions from the 5′end.

subunit drives formation of the IRES-40S subunit–eIF3 complex since HCV IRES RNA demonstrates similar affinity for the 40S subunit and the 40S–eIF complex (Kieft et al., 2001). Other cellular factors such as La autoantigen (Ali et al., 2000; Ali and Siddiqui, 1997; Isoyama et al., 1999), heterogeneous ribonucleoprotein L (Hahm et al., 1998), poly-C binding protein (Fukushi et al., 2001; Spangberg and Schwartz, 1999), and pyrimidine tract-binding protein (Ali and Siddiqui, 1995; Anwar et al., 2000) also bind to the IRES element and modulate translation.

HCV core protein, which is located at the N-terminus of the viral polyprotein, is a putative nucleocapsid protein given the basic nature of its amino acid (aa) residues and the organization of the HCV genome. HCV core protein can form multimeric complexes, as well as heterodimer complexes with envelope E1 protein (Lo et al., 1996). Physical interaction between the core protein and viral genomic RNA is thought to occur during nucleocapsid formation. The results of several Northwestern analyses suggest that the core protein binds to the 5′UTR of the HCV genome, regardless of the specific RNA sequences involved (Santolini et al., 1994; Hwang et al., 1995; Fan et al., 1999). We previously used both in vivo and in vitro systems to demonstrate that the core protein preferentially binds to positive-stranded viral RNA containing the 5′UTR and

part of the structural protein-coding region (Shimoike et al., 1999). In addition, the core protein has a high affinity for the stem-loop IIId domain of the 5′UTR (Fig. 1) and for (G)-rich nucleotides (Tanaka et al., 2000).

In addition, evidence regarding the importance of the interaction between HCV core protein and HCV RNA in regulating viral translation is accumulating. We previously reported that expression of the core protein down-regulates HCV translation through interaction(s) involving 5′ regions of the viral genome (Shimoike et al., 1999). Although some evidence suggesting inhibition of HCV translation through RNA–RNA interactions, rather than core–RNA interactions, exists (Wang et al., 2000; Kim et al., 2003), several studies indicate that the core protein modulates HCV translation. Specifically, regions of the core protein corresponding to aa 34–44 (Zhang et al., 2002) or aa 1–20 (Li et al., 2003) are important for inhibition of HCV translation. The core protein may down- or up-regulate HCV IRES activity in a dose-dependent manner (Boni et al., 2005).

The aim of the present study was (1) to clarify the nature of interaction between the HCV core protein and the viral IRES element and (2) to gain insight into the relationship between core protein-mediated inhibition of translation and core–IRES interactions using a combination of techniques, including an in vivo reporter assay and in vitro surface plasmon resonance (SPR) analysis.

Results

Effect of the core protein-coding sequence on HCV IRES-initiated translation

Since there is conflicting data regarding the effect of the core protein or the core protein sequence on HCV IRES-directed translation (Shimoike et al., 1999; Zhang et al., 2002; Li et al., 2003; Boni et al., 2005), we sought to determine whether the RNA sequence of the core-coding region inhibits HCV IRES activity in the present experiment. A single substitution replacing A with U at nt 357 was introduced to produce a stop codon near the 5′ end of the region encoding the core protein, as previously described (Wang et al., 2000). This mutant, known as pCAGFS, produces core protein RNA with a single substitution, resulting in a core peptide, five residues in length, encoded by the N-terminal. Western blot analysis was then used to confirm that the core protein is not expressed by HepG2 cells following transfection with pCAGFS (Fig. 2C). RNA molecules transcribed in vitro from two reporter plasmids, HCVLuc and RLuc, expressing firefly luciferase (FL) controlled by the IRES of HCV genotype 1b and *Renilla* luciferase (RL) controlled by a cap-dependent mechanism, respectively, were cotransfected into cells after 48 h of transfection with pCAGFS39 or core-expressing pCAGC191 (Suzuki et al., 2001). Cell lysate samples were prepared 6 h post-reporter transfection and assayed for expression of both luciferases. As shown in Fig. 2A, the translational activity of HCV IRES was reduced in cells expressing the core protein, but not in cells transfected with

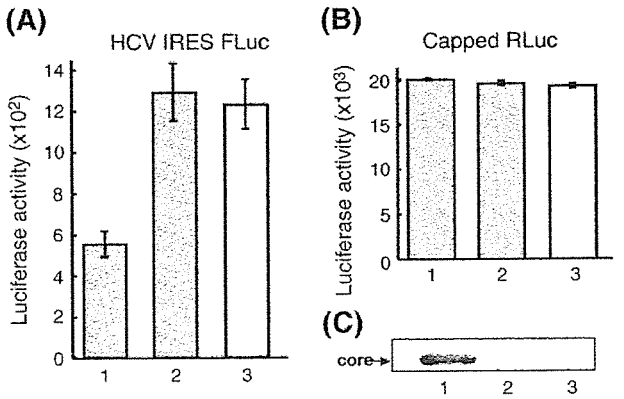

Fig. 2. Effect of the core protein-coding sequence on the translation initiated by HCV IRES. HepG2 cells transfected with pCAGC191 (lane 1), pCAGFS (lane 2), or pCAGGS (lane 3) were cotransfected with reporter RNAs of HCVLuc and the capped RLuc. The activities of both FL (A) and RL (B) were measured by a luminometer. The activities of both FL and RL were determined in at least three independent experiments, each of which conduced with triplicate samples. (C) Western blot analysis of the core protein expressed in the infected cells.

pCAGFS, indicating that the HCV core protein, but not the core-coding sequence, inhibits HCV IRES-directed translation. Transfection with neither core-expressing or non-expressing constructs modulated cap-dependent translation (Fig. 2B).

Effect of partial deletion of the HCV 5′UTR on inhibition of viral IRES-mediated translation by the core protein

In previous studies, we demonstrated that purified HCV core protein binds most efficiently and stably to the stem-loop IIId domain of the 5′UTR of HCV RNA followed by the stem-loop I domain and the region encoding nt 23–41 (Fig. 1; Tanaka et al., 2000). In addition, we revealed that the core protein expressed in HepG2 cells inhibits the IRES-dependent translation of HCV (Shimoike et al., 1999). It can be hypothesized that binding of the core protein to one or more regions of the 5′UTR might inhibit translation. To address this issue, we constructed three reporter plasmids: ΔILuc, Δ23–41Luc, and ΔIIIdLuc, with deletions of domain I (Δ1–22), nt 23–41, and domain IIId (Δ254–278) of the HCV 5′UTR, respectively, also containing the FL gene (Fig. 3A). RNA molecules transcribed from these reporter plasmids in vitro were transfected into HepG2 cells, after which luciferase activity within the cell lysate samples was analyzed. Consistent with previous reports, deletions of domain I (ΔIluc) (Luo et al., 2003; Friebe et al., 2001) or IIId (ΔIIIdLuc) (Jubin et al., 2000) profoundly impaired IRES activity, with a >95% reduction in activity (data not shown), thus demonstrating the importance of these loop structures for HCV translation. Therefore, in the following experiment, we adjusted the dose of each reporter transcript to ensure a consistent level of FL expression.

To investigate the effect of the core protein on translation mediated by wild-type or mutated HCV 5′UTR as described above, cells infected with a recombinant baculovirus carrying

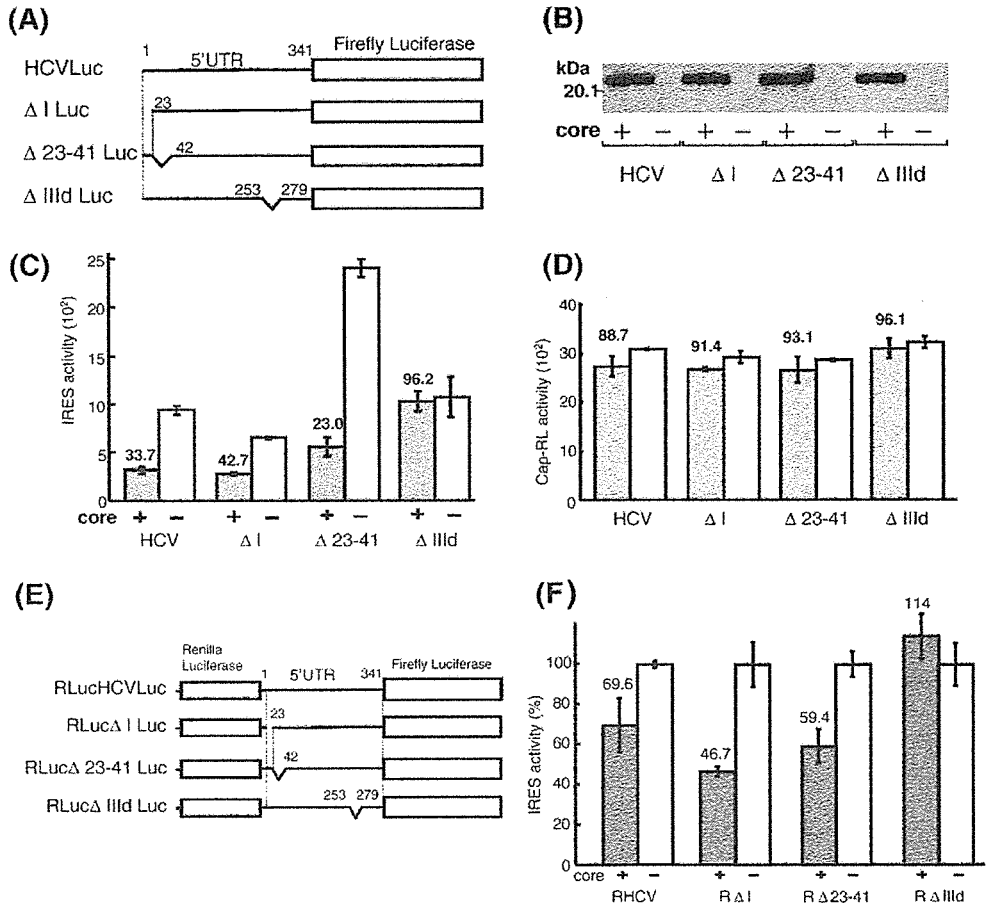

Fig. 3. Effect of deletion mutations in HCV 5′UTR on inhibition of the viral IRES-mediated translation by the core protein. HepG2 cells infected with AcCA39 or AcCAG at a multiplicity of infection of 20 were transfected with monocistronic wild-type (HCVLuc) or deletion mutants (ΔILuc, Δ23–41Luc, ΔIIIdLuc) of reporter RNAs together with the capped RLuc RNA or transfected with bicistronic wild-type (RLucHCVLuc) or deletion mutants (RLucΔILuc, RLucΔ23–41Luc, RLucΔIIIdLuc) of reporter RNAs. The activities of both FL and RL were determined in at least three independent experiments, each of which conduced with triplicate samples. Schematic representation of the monocistronic and bicistronic deletion mutants used in this study is shown in panels A and E, respectively. (B) Western blot analysis of the core protein in each cell lysate in which the luciferase activities were measured. (C) Relative luciferase activities were normalized with those of RLuc. (D) The activities of RLuc in cells cotransfected with RLuc and HCVLuc or deletion mutants are shown. (F) HCV IRES activity was determined by calculating the abundance of Fluc relative to RLuc, with that of each reporter in the absence of the core protein normalized to 100%. Mean values with standard deviations were indicated.

the entire HCV core gene (AcCA39; Shimoike et al., 1999) or an empty vector (AcCAG) were cultured for 2 days, followed by transfection with reporter transcripts, either wild-type HCVLuc (0.1 μg/well), ΔILuc (6 μg/well), Δ23–41Luc (0.2 μg/well), or ΔIIIdLuc (6 μg/well), together with capped RLuc RNA (0.08 μg/well). As indicated in Fig. 3C, expression of the core protein inhibited HCV IRES-mediated translation from ΔILuc and Δ23–41Luc, as well as from HCVLuc, by more than 50%. In contrast, inhibition of translation by the core protein was not observed in cells transfected with ΔIIIdLuc. As shown in Fig. 3D, the expression of neither the core protein nor any of the IRES-directed reporters influenced cap-directed translation. Thus, as previously demonstrated (Shimoike et al., 1999), RL activity was used as an internal control to normalize the efficiency of transfection in the following experiments (Figs. 4 and 6). Western blotting was used to confirm that core protein concentrations within the cell lysate of cells infected with AcCA39 were comparable in the presence of each of the reporter RNA molecules (Fig. 3B). We observed a similar effect of the core protein on HCV IRES activity when equal amounts (6 μg/well) of each HCVLuc, ΔILuc, Δ23–41Luc, or ΔIIIdLuc transcript were transfected (data not shown). These results eliminate the possibility that there is no translational inhibition because the core protein is destabilized in cells transfected with ΔIIIdLuc RNA. We also determined the effect of the core protein on HCV translation initiated from bicistronic reporters: RLucHCVLuc (wild-type), RLucΔILuc (deletion of domain I), RLucΔ23–41Luc (deletion of nt 23–41), and RLucΔIIIdLuc (deletion of domain IIId) (Fig. 3E). Consistent with results obtained from the monocistronic constructs, expression of the core protein showed an inhibitory effect on HCV translation mediated by RLucHCVLuc, RLucΔIluc, or RLucΔ23–41Luc, but not by RLucΔIIIdLuc (Fig. 3F). The capped RL activity from each reporter was similar and was not influenced by expression of the core protein (data not shown). These results suggest that the stem-loop IIId domain of the 5′UTR is important for inhibition of HCV translation by the core protein.

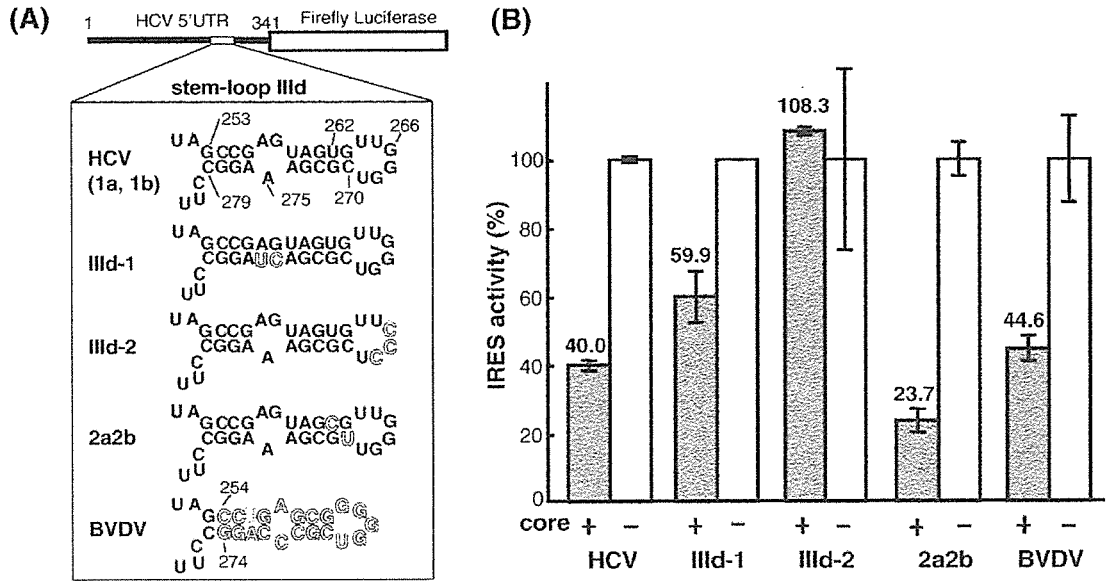

Fig. 4. Mutational analysis of the stem-loop IIId domain. (A) Schematic representation of the predicted secondary structures of the IIId domain of mutated reporters used in this study. (B) The core protein and luciferase reporters are expressed as described in the legend to Fig. 3, except reporters indicated. HCV IRES activity was determined and presented as described in the legend to Fig. 3C.

Mutational analysis of the stem-loop IIId domain

To further investigate the functional role of the stem-loop IIId domain (nt 253–279) in core protein-mediated inhibition of HCV translation, we engineered stem-loop IIId domains with the following four mutations (Fig. 4A): (1) IIId-1Luc, in which the A at nt 275 was changed to UC, thus forming a double-stranded structure instead of a bulge loop in the IIId, (2) IIId-2Luc, in which the GGG triplet (nt 266–268) was changed to a CCC triplet within the loop of stem-loop IIId, (3) 2a2bLuc, in which the U at nt 262 and the C at nt 270 were changed to C and U, respectively, thus changing the genotype to 2a/2b, and (4) BVDVLuc, in which the stem-loop IIId (nt 254–274) sequence was changed to that of bovine viral diarrhea virus (BVDV)-1. Cells that did or did not express the core protein were transfected with each of the above described reporter RNA transcripts, after which luciferase activity was measured. As shown in Fig. 4B, IIId-2Luc, containing a mutation of the GGG triplet of the apical loop, demonstrated no inhibition of HCV IRES-mediated translation by the core protein, whereas IIId-1Luc, containing a mutation within the bulge loop structure, showed only a marginally reduced inhibitory effect of the core protein. We previously demonstrated that HCV core protein binds most efficiently to (1) the stem-loop IIId domain, compared to other structural domains of the 5′UTR, and to (2) G octamer (G_8), as opposed to A_8, C_8, and U_8, using a quantitative SPR method (Tanaka et al., 2000). Thus, the results obtained here suggest that the apical loop is a critical recognition site for translational inhibition by the core protein. It is likely that the inhibitory activity of the core protein on HCV IRES-mediated translation is related to its efficiency of RNA binding.

We also observed the core protein to exert an inhibitory effect on translation directed by either 2a2bLuc or BVDVLuc, similar to that observed with wild-type HCVLuc, involving a 5′UTR sequence of genotype 1. Since the IIId domain sequence of HCVLuc is conserved among genotypes 1, 3, 4, and 5 and since that of 2a2bLuc is shared with genotype 6, it appears that inhibition of HCV translation by the core protein is independent of the viral genotype and occurs in most HCV isolates. Sequence alignment of HCV and various pestiviruses showed that, although the primary nucleotide sequence of the IIId domain exhibits considerable variability, the predicted secondary structure of the domain is highly conserved among these viruses as reviewed previously (Rijnbrand and Lemon, 1999). Furthermore, the GGG triplet followed by U at the apical loop and one bulge loop in the domain are well conserved among HCV and pestiviruses. These suggest that the nucleotide sequence of the apical loop, particularly the GGG triplet, is more important than the stem-structure sequence of the IIId domain for core protein-mediated translational inhibition.

Relationship between translational inhibition and ability of the core protein to bind to the IIId domain within the 5′ UTR

To investigate the relationship between inhibition of HCV translation by the core protein and ability of the core protein to bind to IIId RNA, we prepared two biotinylated oligo RNA molecules, IIId-1 and IIId-2 (nt 251–282), containing identical mutations in the bulge and apical loops of their IIId domains as the mutated reporters IIId-1Luc and IIId-2Luc, respectively (Fig. 4A). These mutant or wild-type oligo RNA (IIId-wt) molecules were then coupled to streptavidin-coated sensor chips and allowed to bind to purified recombinant core protein. The results of subsequent SPR analysis using a BIAcore biosensor are shown in Fig. 5. The core protein was observed to bind to IIId-1 RNA as efficiently as to IIId-wt RNA,

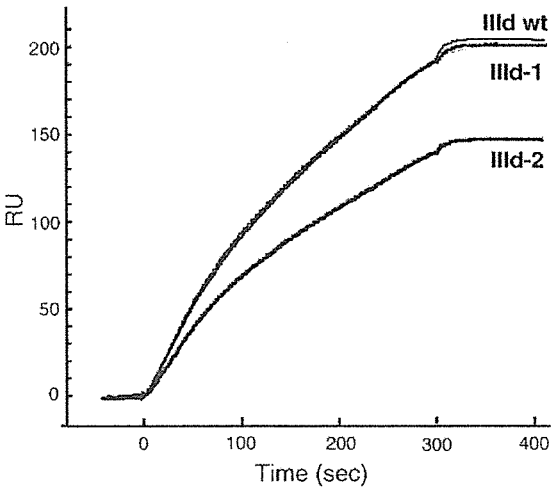

Fig. 5. Binding of the core protein to oligo RNAs corresponding to the mutated IIId domains. The real time binding between the core protein and wild-type (IIId wt) or mutants (IIId-1 and IIId-2) of the stem-loop IIId was examined. Biotinylated oligonucleotides were immobilized of the streptavidin pre-coated sensor chips followed by being exposed to 40 μl of the solution containing the core protein (4 μg/ml) with a flow rate of 8 μl/min. The sample flow was stopped, and the buffer washout began at 300 s. The amounts of immobilized synthetic oligonucleotides, IIId-wt, IIId-1, and IIId-2 were 211.0, 206.9, and 212.4 resonance units, respectively.

suggesting that RNA mutations disrupting the bulge loop structure have little or no effect on binding of the core protein. In contrast, a marked reduction in binding affinity of the core protein for mutant IIId-2 RNA was observed. As a negative control, we found that the core protein does not bind to oligo RNA corresponding to IIIe or IIIf domain (data not shown; Tanaka et al., 2000). It is likely that the apical loop sequence and/or the GGG triplet are important for RNA binding of the core protein, which is consistent with prior observations suggesting that the core protein binds to G-stretch sequence(s) with high affinity.

Combined with the data shown in Fig. 4B, the inhibitory effect of the core protein on HCV IRES activity correlates well with its ability to bind to wild-type and mutated IIId RNA. In light of the observation that the IIId domain is important for IRES activity and from suggestion that the domain IIId interacts with 40S (Otto et al., 2002; Jubin et al., 2000; Lukavsky et al., 2000; Spahn et al., 2001), the HCV core protein may inhibit viral IRES-dependent translation by preventing required interactions between RNA molecules and the 40S by binding to the IRES sequence including the apical loop of the IIId domain.

Role of basic-residue clusters within the core protein in inhibition of HCV translation

The amino-terminal portion of the core protein is able to bind to viral nucleic acids (Santolini et al., 1994). This region contains three clusters of arginine- and lysine-rich sequences (aa 5–13, 38–43, and 58–71). To investigate the role of these basic-residue clusters in inhibition of HCV translation by the core protein, we constructed a series of core mutants, in which lysine and arginine residues within one or more of the basic-residue clusters of the core protein were substituted with alanine residues, as depicted in Fig. 6A. Two days after transfection with either wild-type (pCAGC191) or core mutant (pCAGC191m1–m7) constructs, the cells were cotransfected with HCVLuc and capped-RLuc RNA. As indicated in Fig. 6B, core mutants containing alanine substitutions within one or two clusters (C191m1, m2, m3, m4, m5, and m6) retained the ability to inhibit HCV IRES-mediated translation, similar to the wild-type core protein. However, a core mutant with alanine substitutions involving all three clusters, C191m7, demonstrated little to no inhibition of translation. Expression of the core protein in each transfectant was determined by Western blotting (Fig. 6C), and none of the mutants influenced cap-dependent translation (data not shown). These results

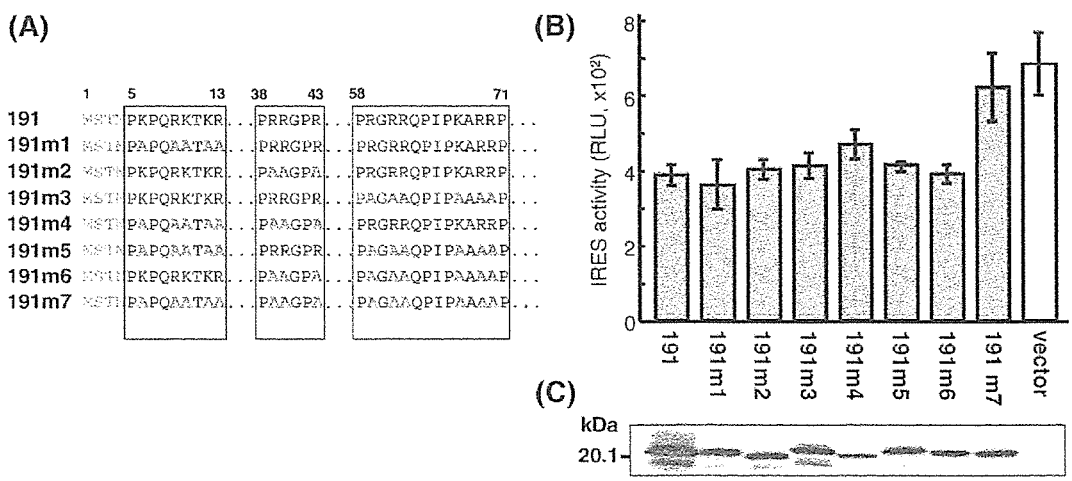

Fig. 6. A role of basic-residue clusters within the core protein in inhibition of the HCV translation. (A) Schematic representation of the mutated core proteins substituted in three basic aa clusters. Lysine or arginine residues substituted with alanine in the clusters are shown with outlined letters. (B) Two days after the transfection with either wild-type (191) or mutated (191m1–m7) core-expressing constructs, HepG2 cells were further cotransfected with HCVLuc and capped-RLuc RNAs. Relative luciferase activities (RLU) were determined as described in Materials and methods and the legend to Fig. 3. (C) The amounts of the wild-type and mutant core proteins expressed in HepG2 cells are shown by Western blotting.

suggest that all three basic-residue clusters of the core protein can mediate inhibition of HCV translation and that at least one cluster is required for inhibition.

Discussion

In this study, we investigated the mechanism by which the core protein modulates HCV IRES activity using an in vivo reporter assay and SPR technology. We demonstrated the importance of a stem-loop IIId domain, spanning nt 253–279, in core protein-mediated inhibition of HCV IRES-mediated translation. In a previous study, we demonstrated preferential binding of the core protein to domain IIId of the 5′UTR followed by domain I and a region spanning nt 23–41 (Fig. 1), upon examining 10 oligonucleotides corresponding to various structured domains of the viral 5′UTR (Tanaka et al., 2000). The core protein did not have an inhibitory effect on translation directed by mutated IRES lacking the IIId domain. However, translation initiated by IRES mutants with deletions of domain I or nt 23–41 was significantly inhibited by the core protein to a similar extent as wild-type IRES-mediated translation (Fig. 3B). Further mutational analysis was then used to determine whether specific IIId nucleotide sequences were important for inhibition of translation by the core protein. We determined that the GGG triplet (nt 266–268) within the IIId apical loop was most critical for core protein-mediated inhibition (Fig. 4B). Combined with the results of SPR analysis (Fig. 5), the data presented here suggest that inhibition of HCV IRES-directed translation by the core protein depends on the binding efficiency of the core protein for the viral IRES element.

Domain III, which is composed of six distinct regions containing stem-loop structures, forms the core of the HCV IRES and is essential for viral translation. Previous studies suggest that domain III plays a role in recruiting the 40S ribosomal subunit and eIF3 by direct interaction with stem-loops IIId/e/f and IIIb, respectively, even though the 40S subunit makes multiple interactions with the IRES and also binds to stem-loop II and the pseudoknot domain of the IRES element (Kieft et al., 2001; Kolupaeva et al., 2000; Sizova et al., 1998). Stem-loop IIId is a highly conserved region within domain III in most HCV isolates, consisting of two double-stranded helical elements separated by a 3-nt internal asymmetric loop with a 6-nt hairpin loop at the distal end of each helical region. IRES sequence deletions, including deletion of stem-loop IIId, as well as point mutations, inhibit binding of the 40S subunit and IRES function (Rijnbrand et al., 1995; Honda et al., 1996; Kieft et al., 1999). Specifically, substitution mutations of the GGG triplet within the IIId apical loop region produce significant loss of IRES activity, as well as alterations in RNA folding, indicating that the GGG triplet is a critical region for HCV translation (Kieft et al., 1999; Jubin et al., 2000). In addition, antisense 2′-*O*-methyloligonucleotides targeted to the IIId domain are known to compete with the 40S subunit for binding and to inhibit viral translation (Tallet-Lopez et al., 2003). Moreover, the secondary structure of the IIId domain is important for binding of the S9 ribosomal protein (Odreman-Macchioli et al., 2000). Consistent with these observations, we also observed that deletion of the IIId domain (ΔIIIdLuc), or a G-to-C substitution within the GGG triplet (IIId-2Luc), significantly reduced IRES activity.

Although the sequence of the IIId domain is highly conserved, sequence polymorphism of the helical region exists among the six major genotypes. With regard to nt 262 and nt 270 of the IIId domain, genotypes 1, 3, 4, and 5 of HCV encode U (nt 262) and C (nt 270), respectively. On the other hand, genotypes 2 and 6 encode C (nt 262) and U (nt 270), respectively. We observed that translation directed by the IRES sequence of genotypes 2 and 6 (2a2bLuc) was more efficient than that directed by the IRES sequence of genotypes 1, 3, 4, and 5 (HCVLuc) (Fig. 4B). Previous studies also demonstrated differences in the efficiency of IRES activity among different HCV genotypes and suggest that the 5′UTR of genotype 2(b) has the most marked IRES activity (Tsukiyama-Kohara et al., 1992; Kamoshita et al., 1997; Collier et al., 1998). Thus, sequence polymorphism involving the helical region of IIId might explain the observed variability in IRES activity when comparing the 5′UTR sequences of different HCV genotypes. Expression of the core protein inhibits HCV translation directed by 2a2bLuc to a similar or same extent as that directed by HCVLuc. This finding suggests that inhibition of viral translation by the core protein commonly occurs during the HCV life cycle and is not limited to certain genotypes. The deletion of the 5′-proximal stem-loop domain I (ΔILuc) significantly reduced IRES activity (data not shown), although the ability of the core protein to inhibit translation was retained (Fig. 3B). Published data regarding the role of domain I in inhibition of HCV translation are not consistent. Some researchers suggest that the 5′-proximal region containing domain I is not essential for HCV IRES activity (Honda et al., 1996; Kamoshita et al., 1997). However, other researchers suggest that this stem-loop element is required for optimal IRES-mediated HCV translation (Friebe et al., 2001; Fukushi et al., 1994; Luo et al., 2003). We compared HCV IRES activity mediated by monocistronic and bicistronic reporters with deletion of domain I and found that an inhibitory effect of the domain I deletion observed from the bicistronic reporter was less evident than that from the monocistronic one: the reduction in IRES activity caused by the deletion was 95% and 40% for the monocistronic and bicistronic constructs, respectively. Although similar trends were observed in the previous studies using cultured cells (Friebe et al., 2001; Luo et al., 2003; Kamoshita et al., 1997), in vitro transcription/translation studies demonstrated that the translational efficiency of the reporters deleted with domain I is higher than that of the wild-type (Honda et al., 1996; Kamoshita et al., 1997). It may be likely that differences in (1) gene constructs such as monocistronic and bicistronic reporters and (2) host cell conditions influence such inconsistent observations.

HCV core protein is highly basic, especially its N-terminal half, and it is thought to encapsulate the viral genome within a viral nucleocapsid. The RNA-binding domain of the core protein has been mapped to 75 aa residues within the N-terminal, in which three clusters of highly arginine/lysine-rich sequences are well conserved among HCV isolates (Santolini et

al., 1994). We previously observed preferential binding between the core protein and positive-stranded HCV RNA spanning the 5′UTR and part of the structural-protein coding region (nt 1–2327) (Shimoike et al., 1999). In this study, we demonstrated the importance of three basic aa residue clusters within the N-terminal region of the HCV core protein for its inhibitory effect on viral IRES activity. At least one cluster is required for inhibition of translation by the core protein. Previous studies with a series of deletion mutants suggest that aa 34–44 (Zhang et al., 2002) or aa 1–20 (Li et al., 2003) within the core protein are crucial for inhibition of translation initiated by HCV IRES. To investigate the contribution of these basic-residue-rich domains within the core protein to inhibition of viral translation, we employed substitution mutagenesis of the full-length core protein in order to reduce the occurrence of conformational changes in the core protein due to the introduction of mutations.

Although an increasing body of evidence shows involvement of the core protein in translational regulation, there are conflicting data regarding the exact mechanism by which this occurs. In contrast to studies describing direct inhibition of HCV translation by expression of the core protein (Shimoike et al., 1999; Zhang et al., 2002; Li et al., 2003), a recent report suggests that the core protein modulates HCV IRES function in a dose-dependent manner, with low amounts of the core protein producing up-regulation and greater amounts resulting in down-regulation (Boni et al., 2005). The core protein does not only inhibit translation initiated by the HCV IRES, but also cap-dependent translation and translation initiated by encephalomyocarditis virus (EMCV) IRES (Li et al., 2003). In an earlier study, neither cap- nor EMCV IRES-dependent translation were inhibited by expression of the core protein (Shimoike et al., 1999). Other studies suggest that the core protein-coding sequence, but not the core protein itself, modulates HCV IRES function, through a long-range RNA–RNA interaction (Wang et al., 2000; Kim et al., 2003). In the present experiment, however, down-regulation of HCV IRES-directed translation by the core protein-coding RNA sequences was eliminated by introducing a base-substitution mutation into the N-terminus of the core sequence in order to create a termination codon (Fig. 2). These contradictory findings might be due to different experimental conditions, such as the use of different reporter systems and host cells, as well as different levels of core protein in the assays used. To investigate the effect of the core protein on HCV IRES-dependent translation, we employed in vivo RNA transfection of monocistronic reporter constructs because HCV IRES is located at the 5′ end of the viral genome, and not internally, thus making it unnecessary to use a bicistronic reporter. Concerning bicistronic contexts, the possibility that the first cistronic sequence might influence IRES regulation directed by the second cistronic gene cannot be excluded. There is evidence to suggest that differences in translational regulation by the core protein might exist among different cell lines, including HepG2, Huh-7, and CV-1 cells (Wang et al., 2000; Li et al., 2003). We also observed differences between HepG2 and Huh-7 cells in terms of ability of the core protein to inhibit HCV IRES- and cap-dependent translation, which was not observed in Huh-7 cells (data not shown), as previously reported (Wang et al., 2000). Such cell-type specific effects might be related to differences in core protein expression since core protein expression by the recombinant baculovirus AcCA39 seems to be less abundant in Huh-7 cells, compared to HepG2 cells (data not shown). It is also possible that a cell-specific factor(s) are involved in translational regulation by the core protein. Thus, some interaction(s) between the highly ordered HCV IRES structure and/or the core protein and related host factors are likely cell-type-specific. Our previous report showed difference in the translation efficiency mediated by HCV IRES among human liver-derived cell lines, although the effect of the core protein on their translation was not determined (Aoki et al., 1998).

We performed the gel mobility shift assay to demonstrate the inhibition of the interaction between the HCV 5′UTR and the ribosome 40S subunit (40S). The complexes between purified 40S and the radiolabeled HCV 5′UTR (nt 1–330) were detected, and the amount of this band was decreased in the core protein-dose-dependent manner. In this condition, the core–5′UTR complex was competed with a non-labeled oligo RNA corresponding to IIId domain, but not with oligo RNAs of domain IV. However, the complex between the core protein and the 5′UTR was detected around the wells of the gel. To our knowledge, there has been no published data that in the gel mobility shift assay the core–5′UTR complex runs into the gel. Although these findings may support the idea that the core protein directly prevents binding of 40S to the HCV IRES, direct biochemical probing of the proposed interaction must wait for the advances in the protein chemistry of the HCV core protein.

Finally, based on the results of the present study and the existing literature, we propose a model of down-regulation of HCV translation mediated by the core protein (Fig. 7). In HCV-infected cells, the virus uncoats and releases its genomic RNA, which serves as a template for protein translation. Highly folded secondary and tertiary RNA elements in the 5′UTR function as *cis* signals for interaction with the 40S subunit and eIF3 during the initial process of HCV IRES-dependent translation. The high affinity interaction between HCV IRES and the 40S subunit is thought to be important for recruitment of the 43S particle to viral RNA, and the stem-loop IIId domain is a prerequisite for this interaction. Since the core protein binds most efficiently to the IIId domain in the HCV IRES element, it is relevant to note that the core protein may prevent an essential RNA–40S interaction by blocking the IIId domain, thereby reducing the viral translation efficiency. At an early stage of the HCV replication cycle, translation of the viral genome yields a polyprotein, which is subsequently processed to yield individual mature proteins. At a certain point, enough core protein is available to inhibit HCV translation by competing with the 40S subunit for IRES binding. Cells in which HCV translation is negatively controlled may have reduced levels of core protein due to its degradation by the ubiquitin/proteasome pathway (Suzuki et al., 2001; Moriishi et al., 2003), thereby decreasing the inhibitory effect of core protein. Thus, the core protein may

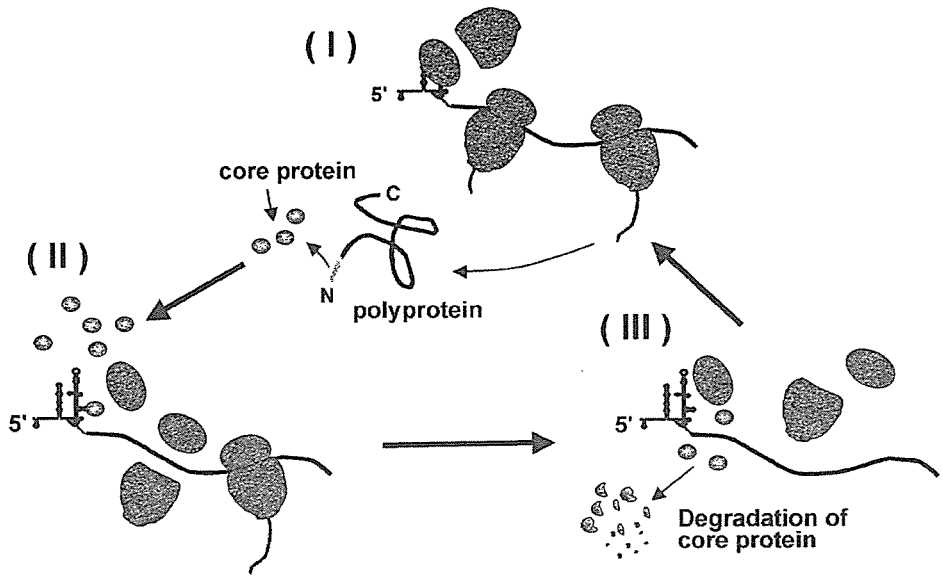

Fig. 7. Model for the regulation of HCV translation mediated by the core protein. Step 1: HCV translation is initiated through recognition of the 40S subunit and eIF3 by the IRES RNA tertiary structure. The viral polyprotein is expressed and processed into matured proteins, resulting in generation of the core protein. Step 2: The expressed core protein binds to the stem-loop IIId in the 5′UTR and inhibits the viral translation by competing with 40S subunit for binding to the IRES. Step 3: The reduced translational efficiency results in decreasing the levels of HCV proteins and replication. Degradation of the core protein through the ubiquitin/proteasome pathway may also contribute to reducing the amounts of the core protein in cells. A low concentration of the core protein possibly leads to recovery of the translational efficiency.

contribute, through its competitive interaction with the IRES IIId domain, to virus persistence by maintaining a low level of HCV replication.

Materials and methods

Plasmid construction

pT7ΔloopILuc (termed ΔILuc in this report), a 271-nt fragment containing a T7 promoter followed by nt 23–249 from the 5′ terminus of the HCV genome (clone NIHJ1; genotype 1b) (Aizaki et al., 1998), was amplified by PCR using pT7HCVLuc (HCVLuc) (Shimoike et al., 1999) as a template and primers *Hin*dIIIT7S (5′-CCCAAGCTTTAATACGACTCACTATACACTCCACCATAG-3′) and *Nhe*IAS (5′-CTAGCTAGCAGTCTCGCGCGGGG-3′). The PCR product was digested with both *Hin*dIII and *Nhe*I and ligated with a 5.3-kbp *Hin*dIII–*Nhe*I fragment of pT7HCVLuc. pT7Δ23–41Luc (Δ23–41Luc) was made using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) in order to introduce a deletion of nt 23–41 from the 5′ terminus. The primers used for PCR were 23–41S (5′-CCTAGATTGGGGGCGACCCCTGTGAGGAAC-3′) and the 23–41 AS complement (5′-GTTCCTCACAGGGGTCGCCCCCAATCAGG-3′), and pT7HCVLuc was used as a template.

pT7ΔIIIdLuc (ΔIIIdLuc) was made by digestion of pT7HCVLuc with *Nhe*I and *Stu*I, thereby generating 30-bp (corresponding to the stem-loop IIId region), 1.8-kbp, and 3.8-kbp fragments. After this, the 1.8-kbp and 3.8-kbp fragments were isolated, blunt-ended, and then ligated.

pT72a2bLuc (2a2bLuc), pT7BVDVLuc (BVDVLuc), pT7IIId-1Luc (IIId-1Luc), and pT7IIId-2Luc (IIId-2Luc) were made as follows. pT7HCVLuc was partially digested with *Stu*I. A 5.6-kbp fragment was isolated and completely digested with *Nhe*I. The resulting fragment was ligated with annealed two partially complementary oligonucleotides with the following *Nhe*I and *Stu*I sites: 5′-CTAGCCGAGTAGTGTTGGGTCGCGACTAGG-3′ and 5′-CCTAGTCGCGACCCAACACTACTCGG-3′ for IIId-1,5′-CTAGCCGAGTAGTGTTCCCTCGCGAAAGG-3′ and 5′-CCTTTCGCGAGGGAACACTACTCGG-3′ for IIId-2, 5′-CTAGCCGAGTAGCGTTGGGTTGCGAAAGG-3′ and 5′-CCTTTCGCAACCCAACGCTACTCGG-3′ for 2a2b, and 5′-CTAGCCTGAGCGGGGGTCGCCCAGG-3′ and 5′-CCTGGGCGACCCCCGCTCAGG-3′ for BVDVLuc (the underlined nucleotides were substituted for the wild type nucleotides; see Fig. 4A).

pRLucHCVLuc, pRLucΔ23–41Luc, and pRLucΔIIIdLuc: 2.6-kb fragments were amplified by PCR using pT7HCVLuc, pT7Δ23–41Luc, and pT7ΔIIIdLuc as template DNAs, respectively, and primers *Xba*I 5′endS (5′-GCTCTAGAGCCAGCCCCCGATTGGGGGCGA) and *Xba*I 3′endAS (5′-GCTCTAGAACTAGTGGATCCGGAT). The PCR products were digested with *Xba*I and ligated with a 3.3-kb *Xba*I fragment of pRL-null Vector (Promega, Madison, WI).

pRLucΔILuc: 2.6-kb fragment was amplified by PCR using pT7ΔILuc as a template DNA and primers *Xba*IloopIS (5′-GCTCTAGACACTCCACCATAGATCACCCCC) and *Xba*I 3′endAS. The PCR product was digested with *Xba*I and ligated with a 3.3-kb *Xba*I fragment of pRL-null Vector.

pCAGC191 (Suzuki et al., 2001) carries nt 329–914, containing the entire HCV coding region of the core protein of clone HCV J1 (Aizaki et al., 1998), controlled by the CAG promoter. pCAGFS contains a frame shift mutation, involving substitution of A with T at nt 357, to make a stop codon (TAA)

(refer to Fig. 1). Only the first five residues (MSTNP) of the core protein are translated from this plasmid. To create a series of mutated core-expressing constructs: pCAGC191m1, -m2, -m3, -m4, -m5, -m6, and -m7, alanine substitutions were introduced into the basic-residue clusters of the core protein by PCR mutagenesis with primers containing base alterations, as described previously (Suzuki et al., 2005). The PCR products were then cloned into pCR2.1 (Invitrogen Corp., Carlsbad, CA) and verified by DNA sequencing. Individual cDNAs were excised and inserted separately into pCAGGS. The primer sequences used in these constructions are available from the authors upon request.

Cells

A human hepatocellular carcinoma cell line, HepG2, was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) containing 50 μg/ml of Gentamycin (Biological Industries Ltd., Israel) and supplemented with 10% fetal calf serum.

RNA preparation

The reporter plasmids were linearized by digestion with adequate restriction enzymes, and the resulting DNA fragments were used as templates for in vitro transcription. HCVLuc and a series of HCVLuc mutants were linearized by digestion with *Xho*I. pRL-null (Promega, Madison WI) was linearized by *Xba*I digestion. pRLucHCVLuc and a series of pRLucHCVLuc mutants were linearized by *Bam*HI digestion. An in vitro transcription kit, MEGAscript (Ambion, Austin, TX), was used for RNA synthesis, during which reaction mixtures containing 1 μg of DNA template and 2 μl of T7 enzyme mix were incubated at 37 °C for 2 h. For capped RNA synthesis, linearized pRL-null, pRLucHCVLuc, and a series of pRLucHCVLuc mutants were used as templates, and 2 μl of each ATP, CTP, and UTP (7.5 mM), as well as 1 μl of GTP (7.5 mM) and 1 μl of cap homologue m7G (5′) ppp (5′) G (7.5 mM; Ambion), was used. The reaction mixtures were subsequently treated twice with 2 U of DNase I at 37 °C for 20 min followed by EDTA (25 mM) and lithium chloride (3.75 M) to terminate the reaction. Capped mRNA synthesized contained 11 nucleotides at 5′UTR and no poly(A) tail.

Transfection

For DNA transfection, 100 μl of Opti-MEM (Invitrogen Corp.) and 4 μl of TransIT-LT1 reagent (Mirus Corp., WI) were mixed and incubated at room temperature for 5 min followed by the addition of 2 μg of each plasmid expressing core protein, mutant core protein, or empty vector followed by incubation for a further 15 min. For RNA transfection, synthesized reporter RNA and 2.5 μl of Tfx-20 (Promega) were mixed in 100 μl of Opti-MEM and incubated for 15 min prior to transfection. One day prior to DNA transfection, cells (2.5×10^5) were seeded into a 12-well plate. The transfection mixture described above was added to the cells in 500 μl of Opti-MEM medium after the cells were washed twice with 500 μl of Opti-MEM.

Luciferase assay

The cells infected or transfected with a recombinant baculovirus or plasmid carrying the entire HCV core gene (AcCA39 or pCAGC191) or an empty vector (AcCAG or pCAGGS) were cultured for 2 days followed by transfection with reporter RNA, either HCVLuc (0.1 μg/well), ΔILuc (6.0 μg/well), Δ23–41Luc (0.2 μg/well), ΔIIIdLuc (6.0 μg/well), IIId-1 (0.1 μg/well), IIId-2 (6.0 μg/well), 2a2bLuc (0.1 μg/well), or BVDVLuc (0.1 μg/well), along with capped RL RNA (0.08 μg/well). After 6 h of incubation, FL and RL activities were determined using the Dual-Luciferase Reporter Assay System (Promega), as previously described (Aoki et al., 1998; Shimoike et al., 1999). Luminescent signals were measured with a TR717 luminometer (Applied Biosystems Japan Ltd., Tokyo, Japan).

Western blot analysis

Expression of HCV core protein was detected by Western blotting, as previously described (Shimoike et al., 1999). Briefly, protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Tokyo, Japan) after separation by SDS-PAGE. After blocking, the membranes were probed with a polyclonal antibody against glutathione-*S*-transferase core (aa 1–191) fusion protein, at a 1:100 dilution.

SPR experimental procedure

To prepare the core protein, insect Tn5 cells were infected with a recombinant baculovirus Ac39. The core protein was partially purified from the cell lysate, as previously described (Tanaka et al., 2000). Interactions between the core protein and synthetic RNA oligonucleotides were examined by SPR analyses with BIAcore 2000 (Biacore K.K., Tokyo, Japan). The SPR experimental procedure was as previously described (Tanaka et al., 2000). Briefly, a biotinylated oligonucleotide spanning nt 251–282 (IIId-wt) and mutant IIId domains (IIId-1 and IIId-2) (Fig. 4A) were synthesized followed by immobilization on streptavidin pre-coated sensor chips. Forty microliters of solution containing the core protein (4 μg/ml) was injected onto the sensor chip surface at a flow rate of 8 μl/min. The sample flow was stopped, and buffer washout started at 5 min post-injection.

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

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

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

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

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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 PA28γ (a REG family proteasome activator also known as REGγ or Ki antigen) enhances the proteasomal degradation of the HCV core protein (30). A recent study has shown that

PA28γ 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 *h*omologous to the *E6*AP *c*arboxyl *t*erminus (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 μg/ml streptomycin (Invitrogen), and 10% (vol/vol) fetal bovine serum (JRH Biosciences) at 37°C in a 5% CO_2 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 *Trans*IT LT1 transfection reagents (Mirus).

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

To make a fusion protein consisting of glutathione *S*-transferase (GST) fused to the N terminus of E6AP in *Escherichia coli*, the E6AP fragment was amplified by PCR and the resultant product was cloned into the SmaI-NotI site of pGEX4T-1 vector (Amersham Biosciences). To express a series of E6AP truncation mutants as GST fusion proteins, each fragment was amplified by PCR and cloned into the SmaI-NotI site of pGEX4T-1. To purify GST core protein efficiently by two-step affinity purification, we fused hexahistidine (His) tag to the C terminus of GST fusion proteins. To bacterially express HCV core (aa 1 to 173) protein as a fusion protein containing N-terminal GST tag and C-terminal His tag, core fragment was amplified by PCR and the resultant product was cloned into the EcoRI-NotI site of pGEX4T-1 vector. The resultant plasmid was designated pGEX GST-C173HT. To express GST core (1–152)-His and GST-His in *E. coli*, pGEX core (1–152)-His and pGEX-His were constructed similarly. The resultant plasmids were designated pGEX GST-C152HT and pGEX GST-HT, respectively.

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

Antibodies. The mouse monoclonal antibodies (MAbs) used in this study were anti-hemagglutinin (anti-HA) MAb (12CA5; Roche), anti-FLAG (M2) MAb (Sigma), anti-c-*myc* MAb (9E10; Santa Cruz), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) MAb (Chemicon), anti-GST MAb (Santa Cruz), anti-ubiquitin MAb (Chemicon), anti-E6AP MAb (E6AP-330) (Sigma), anticore MAb (B2; Anogen), and another anti-core MAb (2H9) (56). Polyclonal antibodies (PAbs) used in this study were anti-HA rabbit PAb (Y-11; Santa Cruz), anti-FLAG rabbit PAb (F7425; Sigma), anti-E6AP rabbit PAb (H-182; Santa Cruz), anti-DDX3 rabbit PAb (47), anti-PA28γ rabbit PAb (Affiniti), and anti-GST goat PAb (Amersham). Anticore rabbit PAb (TS1) was raised against the recombinant GST core protein.

MEF purification procedure. 293T cells were transfected with the plasmid expressing MEF core by the calcium phosphate precipitation method (4). After the cells were lysed, the expressed MEF core and its binding proteins were recovered following the procedure described previously (16). 293T cells transfected with pcDNA3-MEF core in four 10-cm dishes were lysed in 2 ml of lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (wt/vol) glycerol, 100 mM NaF, 1 mM Na_3VO_4, 1% (wt/vol) Triton X-100, 5 μM $ZnCl_2$, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 μg/ml leupeptin. The lysate was centrifuged at 100,000 × *g* for 20 min at 4°C. The supernatant was passed through a 5-μm filter, incubated with 100 μl of Sepharose beads for 60 min at 4°C, and then passed through a 0.65-μm filter. The filtered supernatant was mixed with 100 μl of anti-myc-conjugated Sepharose beads for the first immunoprecipitation. After incubation for 90 min at 4°C, the beads were washed five times with 1 ml of TNTG buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% [wt/vol] glycerol, and 1% [wt/vol] Triton X-100), twice with 1 ml of buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% [wt/vol] Triton X-100), and finally once with 1 ml of TNT buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% [wt/vol] Triton X-100). The washed beads were incubated with 10 U of tobacco etch virus protease (Invitrogen) in TNT buffer (100 μl) to release bound protein complexes from the beads. After incubation for 60 min at room temperature, the supernatant was pooled and the beads were washed twice with 70 μl of buffer A. The resulting supernatants were combined and incubated with 12 μl of FLAG-Sepharose beads for the second immunoprecipitation. After incubation for 60 min at room temperature, the beads were washed three times with 240 μl of buffer A, and proteins bound to the immobilized HCV core protein on the FLAG beads were dissociated by incubation with 80 μg/ml FLAG peptide (NH_2-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-COOH) (Sigma).

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

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

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

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

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

HCV core protein and E6AP binding assays. To map the E6AP binding site on HCV core protein, 2.5 μg of purified recombinant GST-E6AP expressed in Hi5 cells was mixed with 1,000 μg 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-μl volumes containing 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM ATP, 10 mM $MgCl_2$, 8 μg of bovine ubiquitin (Sigma), 0.1 mM dithiothreitol, 200 ng mouse E1, 200 ng E2 (UbcH7), and 0.5 μg 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 3×10^5 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 GGUCUAAUCACCGATT-3′.

CHX half-life experiments. To examine the half-life of HCV core protein, transfected 293T cells were treated with 50 μg/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-μm filter. Naïve Huh-7 cells were seeded 24 h before infection at a density of 1×10^6 in a 10-cm dish. The cells were incubated with 2.5 ml of the inoculum (6.5×10^3 50% tissue culture infectious dose [$TCID_{50}$]/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 μg 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 $TCID_{50}$ was calculated essentially based on the method described previously (28). Virus titration was performed by seeding Huh-7 cells in 96-well plates at 1×10^4 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 $TCID_{50}$ 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. Naïve Huh-7 cells at 10^6 cells/10-cm dish were inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5×10^3 $TCID_{50}$/ml) and cultured. The cells were replated in a six-well plate at 3×10^5 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 $TCID_{50}$ 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 PA28γ (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, PA28γ, and MEF-core (Fig. 1B to E).

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

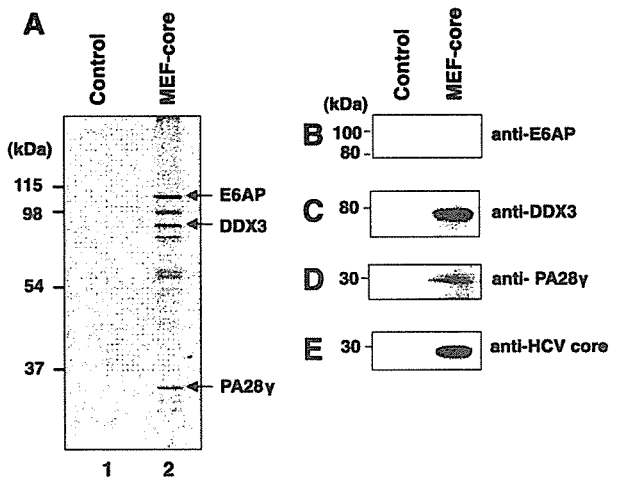

FIG. 1. HCV core protein associates with E6AP in vivo. (A) 293T cells were transfected with pcDNA3-MEF-core or empty plasmid, incubated for 48 h, and then harvested. The expressed MEF-core and binding proteins were recovered using the MEF purification procedure. Proteins bound to the MEF-core immobilized on anti-FLAG beads were dissociated with FLAG peptides, resolved by 9% SDS-PAGE, and visualized by silver staining. Control experiments were performed using 293T cells transfected with vector alone. The positions of E6AP, DDX3, and PA28γ are indicated by arrows. (B to E) The proteins detected in panel A were confirmed by immunoblotting with appropriate antibodies: E6AP (B), DDX3 (C), PA28γ (D), and MEF-core (E).

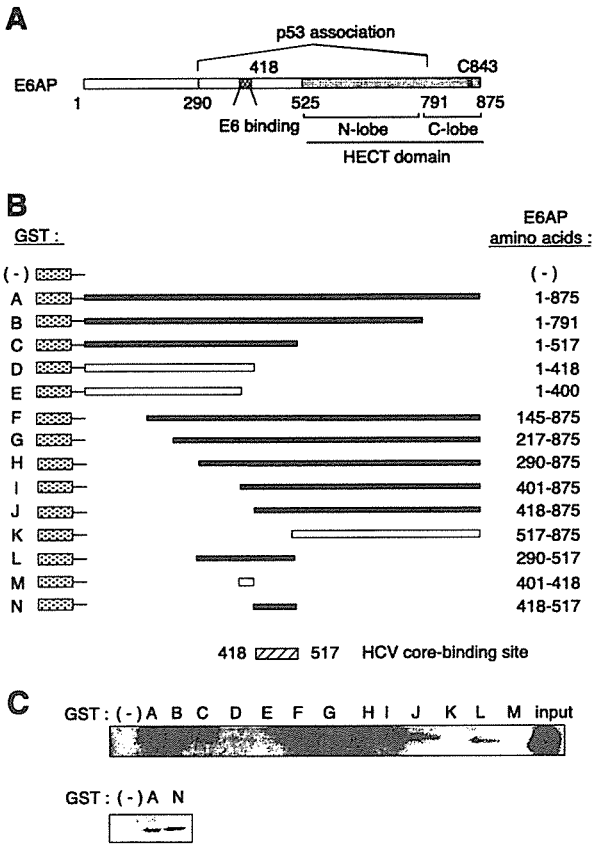

FIG. 2. Mapping of the HCV core-binding domain for E6AP. (A) Structure of E6AP. Shown is a schematic representation of the regions of E6AP isoform II that mediate E6 binding (aa 401 to 418), E6-dependent association with p53 (aa 290 to 791), and the HECT catalytic domain (aa 525 to 875). The catalytic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

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

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

E6AP decreases steady-state levels of HCV core protein in 293T cells and HepG2 cells. One of the features of HECT domain ubiquitin ligases is direct association with their substrates (50). Thus, we hypothesized that E6AP would function as an E3 ubiquitin ligase for the HCV core protein. We assessed the effects of E6AP on the HCV core protein in 293T cells. FLAG-core (1–191) together with HA-tagged wild-type E6AP, catalytically inactive mutant E6AP, E6AP C-A (19), or WWP1 (another HECT domain ubiquitin ligase) (22) was introduced into 293T cells, and the levels of the core protein were examined by immunoblotting. The steady-state levels of the core protein decreased with an increase in the amount of E6AP plasmids (Fig. 4A and B). However, neither E6AP C-A mutant nor WWP1 decreased the steady-state levels of the core protein, suggesting that E6AP enhances degradation of the core protein.

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

TABLE 1. Identification of E6AP by tandem mass spectrometry[a]

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

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

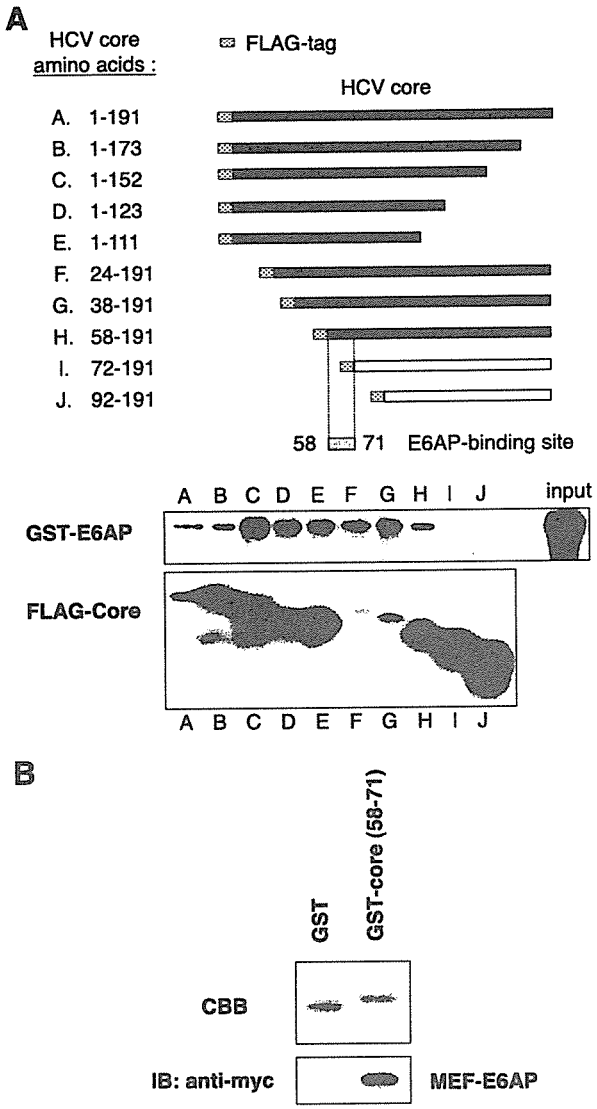

FIG. 3. Mapping of the E6AP binding domain for HCV core protein. (A) In vitro binding of E6AP to HCV core protein. 293T cells were transfected with each plasmid indicated in the upper panel. At 48 h posttransfection, cell lysates were mixed with purified GST-E6AP, immunoprecipitated with anti-FLAG beads,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

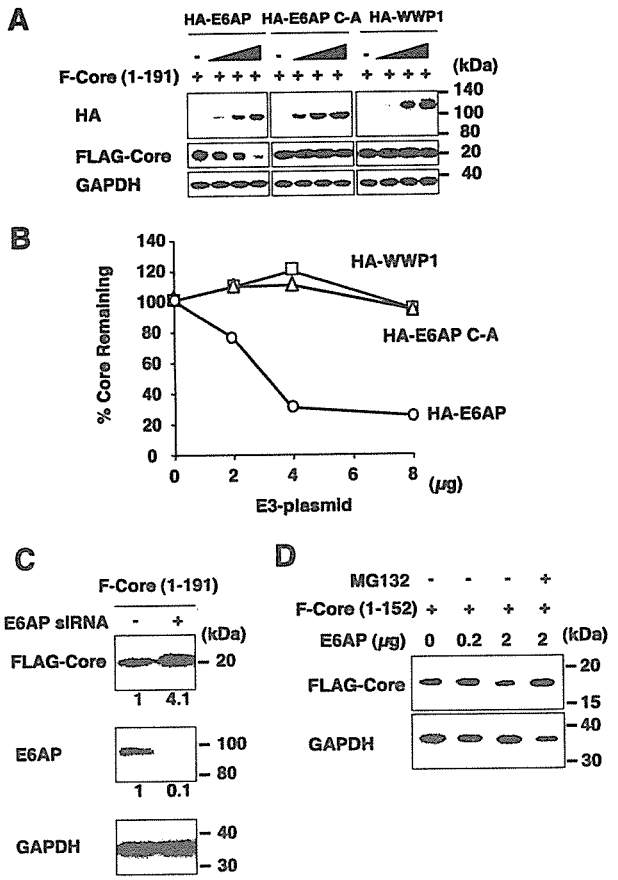

FIG. 4. E6AP decreases steady-state levels of HCV core protein in 293T cells and in HepG2 cells. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG FLAG-core (1–191) along with either pCAG-HA-E6AP, pCAG-HA-E6AP C-A, or pCAG-HA-WWP1 as indicated. At 48 h posttransfection, protein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

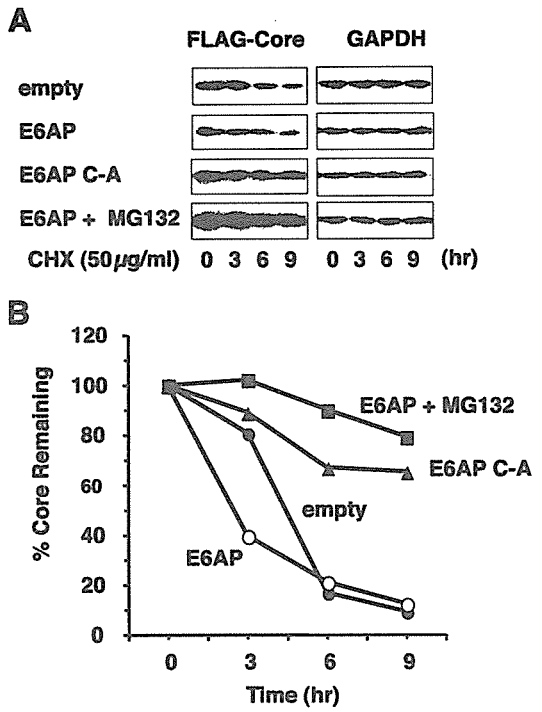

FIG. 5. Kinetic analysis of E6AP-dependent degradation of HCV core protein. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG-FLAG core (1–152) plus 4 μg of empty vector, pCMV-HA-E6AP, or pCMV-HA-E6AP C-A. The cells were treated with 50 μg/ml CHX at 44 h after transfection.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

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

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

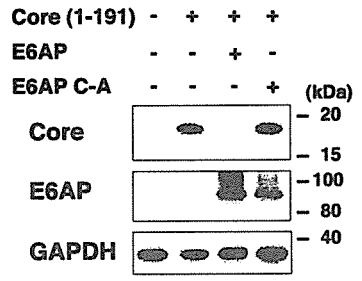

FIG. 6. E6AP promotes degradation of full-length HCV core protein in Huh-7 cells. Huh-7 cells (2×10^5 cells/six-well plate) were transfected with 0.5 μg of pCAG-core (1–191) together with 2 μg of pCMV-HA-E6AP or pCMV-HA-E6AP C-A. At 48 h posttransfection, cells were harvested and analyzed by immunoblotting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

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

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

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

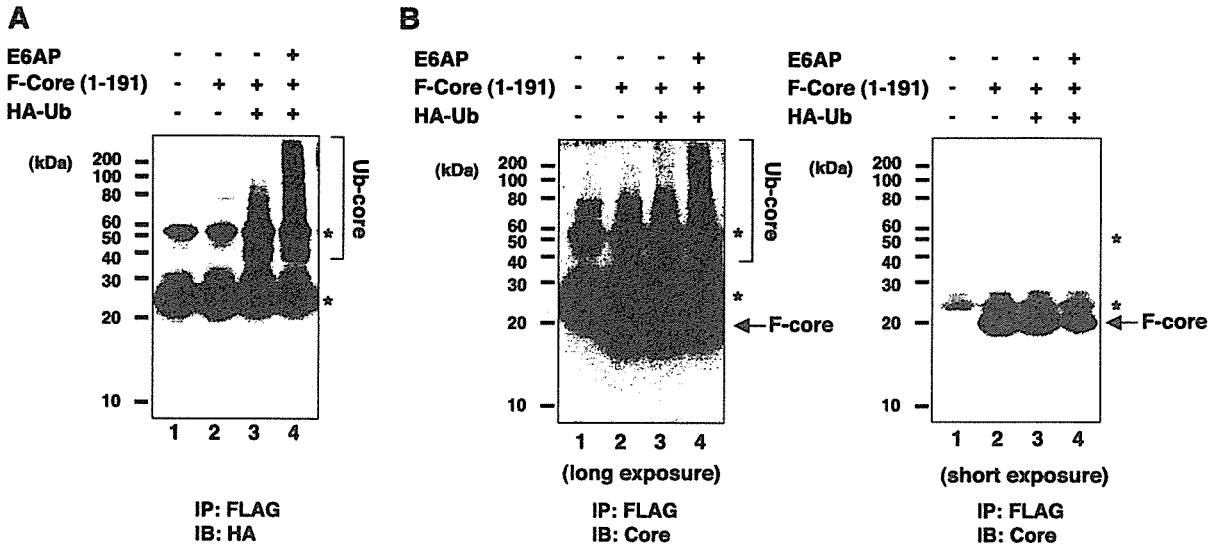

FIG. 7. E6AP-dependent ubiquitylation of HCV core protein in vivo. 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG FLAG-core (1–191) together with 2 μg of plasmid encoding E6AP as indicated. Each transfection also included 2 μg of plasmid encoding HA-ubiquitin. The cell lysates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

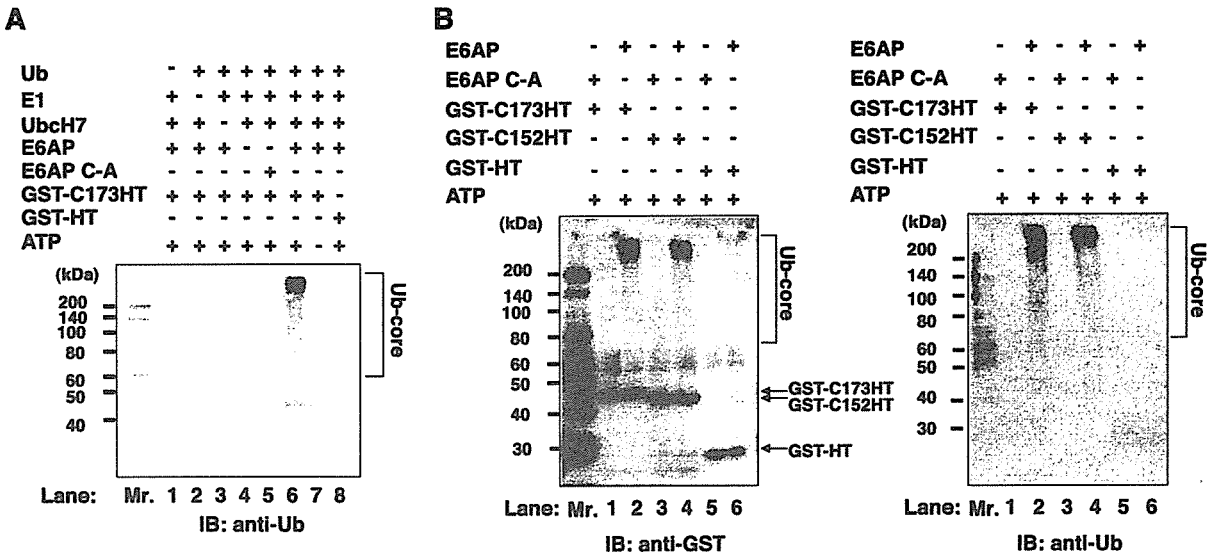

FIG. 8. In vitro ubiquitylation of HCV core protein by recombinant E6AP. For in vitro ubiquitylation of HCV core protein, purified GST-C173HT and GST-C152HT were used as substrates. Purified GST-HT was used as a negative control. Assays were done in 40-μl volumes containing each component as indicated.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