Hepatitis C Virus Core Functions as a Suppressor of Cyclin-dependent Kinase-activating Kinase and Impairs Cell Cycle Progression*

We investigated how the hepatitis C virus (HCV) core protein affects the cell cycle profile and cell cycle-related molecules by using the HCV core-expressing stable transfectant. Analysis of the cell cycle profile showed that HCV core impaired G1 to S transition. The E2F-mediated transcription, phosphorylation of the retinoblastoma protein, and cyclin-dependent kinase (CDK) 4 and CDK2 activities were suppressed in HCV core-expressing cells. The expression of the G1 phase-related CDK/cyclin complexes and various CDK inhibitors were not substantially affected by expression of HCV core. When influences of HCV core on CDK-activating kinase (CAK) were examined, the expression levels of the CAK components, CDK7, cyclin H, and MAT1, were not affected. However, formation of the ternary CAK complex, CAK activity, and the CDK2 level with activating phosphorylation were inhibited by expression of the HCV core. The direct effect of HCV core on CAK was further assessed in the cell-free system by adding the in vitro translated HCV core protein to the anti-CDK7 immunoprecipitate from the cell. The results showed that HCV core led to dissociation of MAT1 from the CAK complex and suppressed the CAK activity. Furthermore, the binding assay revealed that the HCV core was directed against CDK7. Their interaction occurred mainly in the nucleus by the immunostaining. In conclusion, the HCV core protein interacts with CAK and functions as an extrinsic suppressor of CAK. This may be the molecular basis of HCV core-mediated suppression of cell cycle progression. Our findings suggest a novel mechanism concerning HCV core-mediated alteration in the cell cycle machinery.

Hepatitis C virus (HCV)1 is a major etiologic cause of acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.1 HCV is an enveloped virus with a plus-stranded RNA genome of about 9.5 kb in length. (2) At least 10 HCV proteins are generated from proteolytic processing of a single large polyprotein precursor in the following order, NH2-core-enzyme 1-envelope 2-p7-nonstructural 2-nonstructural 3-nonstructural 4A-nonstructural 4B-nonstructural 5A-nonstructural 5B-COOH (3, 4). A series of studies have revealed that, among HCV proteins, the HCV core protein substantially affects cellular functions, which may be relevant to the pathogenesis of HCV-related liver diseases. Persistent expression of the HCV core has been reported to lead to malignant transformation of the host cell in vitro (5, 6) and in vivo (7). HCV has also been shown to modify the cellular apoptotic cascade under various stimuli (8–11). In addition, the HCV core has been demonstrated to modulate various cellular signal transduction pathways (6, 10, 12–15). Such biological activities of the HCV core to the host cell are thought to be triggered by its direct interaction with cellular proteins. More than 10 HCV core-binding proteins have so far been identified (6, 10, 12–14).

The eukaryotic cell cycle progression is tightly regulated by the cyclin-dependent kinasas (CDKs) and cyclins (reviewed in Refs. 24–28). Various CDK-cyclin complexes work in different phases during cell cycle progression; CDK4/6-cyclin D (cycD) plays a major role in the mid-G1 phase, CDK2-cycE plays a role in the late G1 phase, CDK2-cycA plays a role in the S phase, and CD2 (also termed as CDK1)-cycB plays a role in the G2/M phase. In particular, the retinoblastoma protein pRB is important as a substrate of the CDK-cyclin complexes in the G1 phase. Once, the complexes of CDK4/6-cycD and CDK2-cycE phosphorylate pRB, the transcription factor E2F is activated. Then the E2F enhances transcription of many genes to enter the S phase. The functions of CDK-cyclin complexes are negatively regulated by CDK inhibitors, which are divided into two groups, a CIP/KIP family (p21CIP1, p27KIP1, and p57KIP2) and an INK4 family (p16INK4A, p15INK4B, p18INK4C, and p19INK4B), and p21CIP1 is an especially well known transcriptional target of the tumor suppressor p53 (29).

The CDK activity is also controlled by both inhibitory and activating phosphorylations (25, 26). The amino-terminal inhibitory phosphorylation sites (Thr-14 and Tyr-15 in human CDK2) are dephosphorylated by CDC25 phosphatases. On the other hand, phosphorylation of a conserved threonine residue in the T-loop (Thr-160 in human CDK2) is required for full activation of CDKs. The CDK-activating kinase (CAK) undergoes the activating phosphorylation of CDKs. CAK is composed of three components, a catalytic subunit CDK7, a regulatory subunit cycH, and an assembly factor MAT1 (30–32). It has been shown that CAK can phosphorylate all cell cycle-related CDKs, CDC2, CDK2, CDK3, CDK4, and CDK6, at least in the cell-free system (30–34).

Thus far, the effects of HCV core on the cell cycle profile and cell cycle-related molecules have been studied by a few investigators (21, 22, 35). It has been reported that the HCV core can

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1 The abbreviations used are: HCV, hepatitis C virus; CDK, cyclin-dependent kinase; cycD, cyclin D; CAK, CDK-activating kinase; aa, amino acids; GST, glutathione S-transferase; CTD, carboxy-terminal domain; CL2, BNL CL2; IP, immunoprecipitation; MOPS, 4-morpholinepropanesulfonic acid.

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bind to both p53 and p21<sup>CDK1</sup> and that its binding to p53 up-regulates the p53-dependent transcription activity of p21<sup>CDK1</sup> in cultured cells transiently transfected with HCV core-expressing plasmid (21, 22). The stable transfectant with HCV core-expressing plasmid derived from Chinese hamster ovary cells has also been shown to impair the cell cycle regulation accompanied by enhancement of p21<sup>CDK1</sup> expression (35). However, because of unclear mechanisms of HCV core-modulated modulation on the cell cycle-related molecules other than the p53/p21<sup>CDK1</sup> system have not been elucidated. To solve this, we investigated the effects on cell cycle-related molecules caused by persistent expression of the HCV core in cultured murine normal liver cells. In this process, we found that CAK is a novel target of the HCV core and that the HCV core suppresses cell cycle progression by a direct inhibitory effect on CAK assembly and activity.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The HCV core-expressing plasmid pc/3EFANCTH, which was constructed from a mammalian expression vector pc3EFpro, carried the whole HCV core gene and the 5'-part of the envelope 1 gene of a genotype 1b HCV strain (36). Both pc3EFpro and pc3EFANCTH were kindly provided by Dr. T. Wakiya (Department of Microbiology, Tokyo Metropolitan Institute of Medical Science). p2F<sub>core</sub> (1–191) was synthesized from pCMVtag2B (Stratagene) by inserting the HCV core gene (amino acids (aa) 1–191) downstream of the T3 promoter and used for the *in vitro* translation. pTALLUC (Clontech) encoded the luciferase gene driven by the minimal TAL promoter. p2F<sub>core</sub> (Clontech) was a derivative of pTALLUC and possessed a repeated sequence of the E2F-responsive element upstream of the TAL promoter. pCMVβ (Clontech) expressed the β-galactosidase gene driven by the cytomegalovirus promoter. Plasmids p5GEX/hCDK2, p5GEX/hCDK7, p5GEX/hcycH, and p5GEX/hpRB (79–379) were used for the production of human CDK2, CDK7, cycH, and pRB as glutathione S-transferase (GST) fusion proteins. To construct these plasmids, whole coding regions of the cDNAs for CDK2, CDK7, and cycH and the part of the pRB cDNA (corresponding to aa 379–793 of pRB) were obtained by PCR using a cDNA sample from human hepatoma-derived cell lines, Huh-7 (37) (for CDK2) and HepG2 (38) (for pRB), or using the human adult liver cDNA library (Clontech) (for CDK7 and cycH). The cDNA fragments were cloned into the multicloning site of the plasmid p5GEX-1 (Amer sham Biosciences). Plasmid p5GEX/core (1–122) expressed the fusion protein of GST with the truncated HCV core protein (aa 1–122). Plasmid pGCDT produced the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II fused to GST (39), which was a kind gift from Dr. William S. Dynan (Institute of Molecular Biology and Genetics, Medical College of Georgia). p2F<sub>core</sub> (Clontech) was cloned into the pGEX vector, the whole coding region of p2F<sub>core</sub> was amplified by PCR, and the fragment was ligated into the BglII and BamHI sites of pGEX-1 (Amer sham Biosciences). Plasmid p5GEX/core (1–122) expressed the fusion protein of GST with the truncated HCV core protein (aa 1–122). Plasmid pGCDT produced the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II fused to GST (39), which was a kind gift from Dr. William S. Dynan (Institute of Molecular Biology and Genetics, Medical College of Georgia).

**Cell Culture and Protein Extraction**—A murine normal liver cell line, BNL CL2 (CL2) (40), and a human hepatoma cell line, HepG2 (38), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml of streptomycin sulfate, 100 units/ml of penicillin G, and 0.25 μg/ml of amphotericin B at 37 °C in a 5% CO<sub>2</sub>. Three independent clones of the HCV core-expressing cells (designated as CL2 core-I, -II, and -III) and the negative control cells (mock) were established from the CL2 cells as described elsewhere (15, 41). The luciferase activity was normalized for transfection efficiency based on the result of β-galactosidase assay. To determine the E2F-mediated transcription activity, the ratio of the fold activity in transfection with the p2F<sub>core</sub> to that in transfection with pTALLUC was calculated. All of the assays were done in quadruplicate, and the values were expressed as the means ± S.D.

**Northern Blot Analysis**—For Northern blot analysis, total cellular RNA was extracted from CL2 mock and core cells using a TRIZOL reagent (Invitrogen) based on the guanidine-isothiocyanate method. The poly(A)<sup>+</sup> RNA was selected from 50 μg of the total cellular RNA with an oligo(dT) column (Roche Applied Science). The sample was electrophoresed, transferred to a nylon membrane (Hybond N; Amer sham Biosciences), and hybridized to the cDNA probe. After washing, the membrane was autoradiographed. The membrane was dehydrized by boiling in 0.5% SDS and further used for the hybridization to detect β-actin mRNA as a loading control (data not shown).

**Antibodies**—An antibody to pRB was obtained from Pharmingen. Antibodies to CDK4, CDK6, cyclD1, CDK2, cyclE, cyclA, CDC2, cyclB1, p53, p21<sup>WAF1</sup>, p27<sup>KIP1</sup>, CDK7, cycH, and MAT1 were purchased from Santa Cruz. An antibody to phosphorylated CDK2 at the Thr-160 residue (p<sup>Thr-160</sup>-CDK2) came from Cell Signaling. An antibody to GST was from Amersham Biosciences. A mouse monoclonal antibody to HCV core (45) was kindly provided by Dr. T. Wakiya (Department of Microbiology, Tokyo Metropolitan Institute of Medical Science).

**Immunofluorescence Staining and Confocal Microscopy**—The CL2 core cells were plated on a two-well chamber slide. One day after seeding, the cells were fixed with 3% paraformaldehyde, 2% sucrose in phosphate-buffered saline for 30 min and permeabilized with ice-cold methanol for 3 min. After the blocking reaction with 10% fetal calf serum for 30 min at room temperature, the cells were incubated at 4 °C for 14 h with two primary antibodies, a mouse monoclonal HCV core antibody, and a rabbit polyclonal CDK7 antibody. Bound primary antibodies were revealed by incubation for 30 min at room temperature with Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) and Cy3-conjugated anti-rabbit IgG (Jackson Immunoresearch). Finally, a coverslip was mounted in the mounting medium (Vectorshield; Vector Laboratories) with 4',6-diamidino-2'-phenylindole-dihydrochloride. The cells were examined by the confocal microscope analysis using a Radiance 2100 BLD system (Bio-Rad). Colocalization of green and red signals in a single pixel produces yellow, whereas separated signals remain green or red.

**Western Blot Analysis and Immunoprecipitation**—For Western blot analysis, the total cellular protein was fractionated by SDS-polyacrylamide gel electrophoresis and blotted onto a membrane. After blocking of the membrane, the membrane was incubated with the primary antibody, followed by further incubation with a secondary antibody. Finally, the immune complex was detected by an enhanced chemiluminescent assay (Super Signal; Pierce). As for the IP reaction, 250–500 μg of the total cellular protein was preclared with protein A-Sepharose beads (Amersham Biosciences) at 4 °C for 1 h. After centrifugation, the supernatant was incubated with the specific antibody at 4 °C for 1–2 h. Then the beads were added, and the sample was further incubated at 4 °C for 1 h. After the extensive washing, the product was used for Western blot analysis and the kinase assay. In some experiments, the binding reaction of the IP product with the *in vitro* translation product was carried out at 4 °C for 3 h, prior to the subsequent experiments. The *in vitro*
translation was conducted using the TNT T3 coupled rabbit reticulocyte lysate system (Promega).

**Synthesis for GST Fusion Proteins and Binding Assay**—To construct various GST fusion proteins, the *Escherichia coli* BL21 (Stratagene) was transformed with p5GEX/hCDK2, p5GEX/hCDK7, p5GEX/hcycH, p5GEX/hp53(379–793), p5GEX/core(1–122), pGCDT, and the empty plasmid p5GEX-1. The bacteria were grown in the medium containing 100 μg/ml of ampicillin sodium, and the proteins were expressed by the addition of isopropyl-1-thio-β-D-galactopyranoside. After sonication of the bacterial pellet, the proteins were purified with glutathione-Sepharose 4B beads (Amersham Biosciences). For the binding assay, ~5 μg of the various GST fusion protein or GST protein (negative control) was bound to the beads and incubated with 150 μg of the cellular lysate or 10 μl of *in vitro* translated protein at 4 °C for 2 h. After the extensive washing, the bound protein was used for Western blotting.

**Kinase Assay**—For the kinase assay, 250–500 μg of the total cellular protein was immunoprecipitated with CDK4, CDK2, and CDK7 antibodies as above. After extensive washing, the precipitate was subjected to the kinase assay in the presence of 12.5 mM MOPS, 7.5 mM MgCl2, 0.5 mM EGTA, 20 mM β-glycerophosphate, 1 mM NaF, 1 mM sodium vanadate, 5 mM dithiothreitol, 100 μM ATP, and 10 μCi of [γ-32P]ATP in a total volume of 30 μl. Also added as a substrate was 2 μg of the GST-pRB protein for the CDK2 kinase assay, the histone H1 (Calbiochem) for the CDK2 kinase assay, the GST-CDK2 fusion protein for the CAK assay, or the GST-CDT kinase protein for the CDT kinase assay. The reaction was carried out at 30 °C for 30 min. After the elution, the supernatant was fractionated by SDS-PAGE, and the gel was dried and autoradiographed. As for the CAK assay, the kinase reaction was carried out without [γ-32P]ATP, and the phosphorylated product was detected by Western blotting using a pT160 antibody. All kinase assays were carried out with scaling up by 1.3–1.5-fold.

**Statistical Analysis**—Statistical analysis was performed using Student’s nonpaired *t* test as appropriate. *p* values less than 0.05 were considered to be statistically significant.

**RESULTS**

**HCV Core Protein Impairs G1/S Transition in the Cell Cycle through Suppression of CDK4 and CDK2 Activities**—Fig. 1A shows the cellular growth curve of CL2 mock and core cells. Cell growth was significantly suppressed in the CL2 core cells, compared with the mock cells. Their cell cycle profiles were then assessed by flow cytometry (Fig. 1B). In the mock cells, ~30% of the cells were in the G0/G1 fraction, whereas in the CL2 core cells, more than 60% of cells were in this phase. The cells representing S and G2/M phases were decreased by expression of the HCV core. The effect of the HCV core on the E2F-mediated transcription activity was next studied (Fig. 2A). In the cotransfection experiment using the CL2 cells, cotransfection of the HCV core-expressing plasmid pc/3EFpro was compared with that of the negative control plasmid, pc/3EFpro. Significant reduction in E2F-mediated transcription by HCV core was also seen in the experiment using the CL2 mock and core cells. When the phosphorylation status of pRB was compared between CL2 mock and core cells (Fig. 2B), expression of HCV core caused a decrease of the hyperphosphorylated pRB.
and an increase of the hypo-phosphorylated pRB. As for CDK4 and CDK2 kinase activities (Fig. 2C), both kinase activities were substantially lower in the CL2 core cells than in the mock cells. Thus, persistent expression of the HCV core impaired the G1/S transition in the cell cycle, which may be due to the decreased CDK4 and CDK2 activities and the subsequently occurring inhibition of pRB phosphorylation and E2F-mediated transcription.

**HCV Core Protein Does Not Influence the Expression Levels of G1 Phase-related CDKs, Cyclins, and CDK Inhibitors**—The expression levels of various CDKs and cyclins were studied in CL2 mock and core cells. When the G1 phase-related CDKs and cyclins were examined (Fig. 3A), the levels of CDK4, CDK6, cycD1, CDK2, and cycE were not affected by expression of the HCV core. Also, the expression level of cycE was not substantially decreased by HCV core (Fig. 3B). As for the expression levels of cycA, CDC2, and cycB1 (Fig. 3C), the level of cycB1 did not differ between CL2 mock and core cells. When the expression levels of tumor suppressor p53 and its transcriptional target p21CIP1 (29) were examined in CL2 mock and core cells, the p53 expression did not differ between them (Fig. 3D, top panel). The CL2 core cells showed slightly lower expression levels of p21CIP1 mRNA than the mock cells (Fig. 3E), although its protein level was not much affected by HCV core (Fig. 3D, middle panel). Also, the p27KIP1 expression was not different between CL2 mock and core cells (Fig. 3D, bottom panel). When the expression levels of proteins belonging to an INK4 family were determined by Western blot analyses, p16INK4A, p15INK4B, and p18INK4C were faintly detected in CL2 mock and core cells with no substantial differences between them. The p19INK4D expression did not differ between CL2 mock and core cells (data not shown). According to these results, HCV core suppressed the expression levels of cycA and CDC2, which play a role in the S and G2/M phases, but did not influence the expression levels of G1 phase-related CDKs, cyclins, and CDK inhibitors. The reduced expression of cycA and CDC2 may occur as a secondary effect of the decreased E2F-mediated transcription, because these two genes are known to be regulated by E2F (46, 47).

**HCV Core Expression Results in Suppression of CAK Assembly and Activity**—We further investigated the level of activating phosphorylation in CDK2 and the degrees of CAK expression, assembly, and activity in CL2 mock and core cells. The level of pT160-CDK2 was significantly lower in the CL2 core cells than in the mock cells (Fig. 4A). For the expression levels of CAK components, CL2 core cells expressed the same levels of CDK7, cycH, and MAT1 as the mock cells (Fig. 4B). For the complex formation of CAK examined by the IP/Western blot analysis, the level of the CDK7-cycH complex did not differ between CL2 mock and core cells (Fig. 4C, top panel). When the ternary form of CAK (CDK7-cycH-MAT1 complex) was investigated by means of the IP reaction using the mixture of CDK7 and cycH antibodies followed by the immunoblot using a MAT1 antibody, CL2 core cells displayed a lower level of ternary CAK complex formation than the mock cells (Fig. 4C, bottom panel). A similar result was also observed in the experiment with the IP reaction using a CDK7 or cycH antibody alone followed by the immunoblot using a MAT1 antibody (data not shown). The kinase assays using the GST-CDK2 substrate (CAK assay) and...
the GST-CTD substrate (CTD kinase assay) were further carried out (Fig. 4). Both CAK and CTD kinase activities were suppressed in the CL2 core cells, compared with the mock cells. These findings indicate that persistent expression of HCV core may inhibit the ternary CAK complex formation, resulting in suppression of CAK activity. We also examined CDC25A expression by Western blotting and the tyrosine-phosphorylated CDK2 level using the IP reaction with a CDK2 antibody, followed by immunoblotting with a phosphorylated tyrosine antibody pY20 in CL2 mock and core cells. The expression level of CDC25A was not different between these cells. The tyrosine-phosphorylated CDK2 was below the detection limit in both CL2 mock and core cells (data not shown). According to these results, HCV core protein may not affect dephosphorylation of CDKs, although the CDC25A activity was not directly assessed in this study.

**HCV Core Protein Directly Dissociates MAT1 from CAK Complex and Suppresses CAK Activity**—As the next step, the direct binding of HCV core to CAK was further studied. The total cellular proteins from murine CL2 and human HepG2 cells were immunoprecipitated with a CDK7, cycH, or MAT1 antibody, and each precipitate was blotted with an antibody to the HCV core (aa 1–191) with the IP product. The in vitro translation product from the empty plasmid pCMVtag2B was also used as a negative control. The eluted product by Western blotting (data not shown), indicating that the inhibitory effect on CAK assembly and activity may not have been due to an excess amount of HCV core. Furthermore, when the in vitro translated hepatitis B virus surface protein was used for this assay instead of the HCV core, the CAK activity was not suppressed by the addition of this protein (data not shown). Our findings strongly suggest that HCV core may dissociate MAT1 from the CAK complex through possible direct interaction with the particular CAK component, resulting in the suppression of CAK activity.

**HCV Core Protein Interacts with CDK7**—The direct binding of HCV core to CAK was further studied. The total cellular protein from the CL2 core-I cells was immunoprecipitated with a CDK7, cycH, or MAT1 antibody, and each precipitate was blotted with an antibody to the HCV core (Fig. 6A). In all cases using antibodies to CDK7, cycH, and MAT1 on the IP reaction, the three CAK components were precipitated efficiently. Nevertheless, HCV core was detected in the precipitates using CDK7 and cycH antibodies but not in that using a MAT1 antibody. This indicates that the HCV core may be directed against either CDK7 or cycH and that the interaction of the HCV core with the CDK7-cycH complex may induce dissociation of MAT1, as suggested above. To clarify the direct target molecule of HCV core, the in vitro GST fusion protein binding assay was carried out. The truncated HCV core protein (aa 1–122) fused to GST was used for this assay, because the full length of the HCV core fusion protein could not be obtained because of poor solubility. Total cellular proteins from murine CL2 and human HepG2 cells were incubated with the purified truncated HCV core protein, and the bound complex was checked by Western blotting using an antibody to CDK7 or cycH. The bound complex included the detectable level of

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**Fig. 4.** Expression, assembly, and activity of CAK in CL2 mock and core cells. A, cellular proteins from CL2 mock and core cells were blotted with an antibody to pT160-CDK2. B, the expression levels of CDK7, cycH, and MAT1 were examined in CL2 mock and core cells by Western blotting. C, the CDK7-cycH complex and the CDK7-cycH-MAT1 (ternary CAK) complex were detected in CL2 mock and core cells by the IP/Western blot analysis. D, cellular proteins from CL2 mock and core cells were precipitated with a CDK7 antibody, and the precipitate was used for the kinase reaction with the GST-CDK2 fusion protein (for the CAK assay, upper panel) or the GST-CTD fusion protein (for the CTD kinase assay, lower panel) as a substrate. IB, immunoblot.
amounts of HCV core. Then the sample was used for the CAK assay, as followed by the binding reaction. The product was then blotted with antibodies to CDK7, cycH, and MAT1. B, cellular proteins from CL2 (left panel) and HepG2 (right panel) cells were precipitated with a CDK7 antibody, and the precipitate was subjected to the binding reaction with the in vitro translation product containing the various amounts of HCV core. Then the sample was used for the CAK assay, as shown in Fig. 4D.

CDK7, whereas cycH was not detected in both cases using CL2 and HepG2 cellular proteins (Fig. 6B). Furthermore, human CDK7 and cycH fused to GST were synthesized and used for the binding reaction together with the in vitro translated full length of the HCV core protein (aa 1–191). HCV core was detected in the bound complex with GST-CDK7 but not in that with GST-cycH (Fig. 6C). Taken together, the HCV core protein could bind directly to both murine and human CDK7 proteins, whereas its binding to cycH may be indirect. Also, the region within aa 1–122 of HCV core was important for interaction with CDK7.

HCV Core Protein Does Not Influence Intracellular Localization of CAK—Finally, we investigated whether HCV core would affect the nuclear localization of CAK components. When the expression levels of CDK7, cycH, and MAT1 in the nuclear fraction were examined (Fig. 7A), they were not different between CL2 mock and core cells. We also investigated the intracellular localization of HCV core and CDK7 proteins in the CL2 core-I cells by immunostaining. As shown in Fig. 7B, the HCV core was expressed mainly in the cytoplasm but was also detected in the nucleus. On the other hand, CDK7 was expressed strongly in the nucleus, although the signal was seen sparsely in the cytoplasm. For the colocalization of both proteins, the HCV core was colocalized with a portion of CDK7 generally in the nucleus. Thus, the HCV core did not influence the nuclear translocation of CAK. The inhibitory effect of the HCV core on CAK may be exerted by its direct interaction with CDK7 in the nucleus.

DISCUSSION

In the present study, we aimed to elucidate the biological properties of the HCV core protein toward cell cycle progression and cell cycle-related molecules in the host cell. The HCV core-expressing stable transfectant, CL2 core, which was established from a murine normal liver-derived cell line (15, 41), was used for this purpose, because cells constitutively expressing the HCV core protein could not be isolated from the human hepatoma-derived cell lines Huh-7 (37), HepG2, and Hep3B (38). These CL2 core cells were shown to possess a high expression level of HCV core compared with the cultured cells of the HCV-infected liver specimen from chronic hepatitis C patients (48), suggesting that, unlike the cultured cells with forced expression of HCV core, the expression level of HCV core may differ among hepatocytes in the HCV-infected liver tissue. According to these, our CL2 core cells were speculated to express the physiological level of HCV core at least in a single cell.
suppression of the CAK activity in the cases of both cellular proteins. These results strongly imply that the HCV core may directly interact with CAK and act as a suppressor of CAK by disrupting the ternary CAK complex in murine and possibly human cells.

The direct interaction of the HCV core with each of the CAK components was further examined. For in vitro interaction of HCV core with CAK in the CL2 core-I cells, as examined by the IP/Western blotting analysis, it was revealed that the HCV core targeted either CDK7 or cycH. This suggests the existence of the HCV core-CDK7-cycH complex in cells. However, when the in vitro binding assay was carried out using the truncated HCV core protein fused to GST, CDK7, but not cycH, was included in the bound complex in both cases using CL2 and HepG2 cellular proteins. These discrepant results may have been due to the different conditions of the two assays. Otherwise, unlike the full length of the HCV core protein, the truncated HCV core protein would disrupt the complex formation of CDK7 and cycH. In either case, our finding indicates that the HCV core may directly bind to both murine and human CDK7 proteins. The result of another binding assay supports this, because the full length of HCV core protein could bind to GST-CDK7 but not to GST-cycH.

As for the intracellular localization of CAK, expression of the HCV core did not affect the nuclear translocation of the CAK components. In the immunofluorescence analysis, the colocalization of HCV core with CDK7 was found to occur mainly in the nucleus. This indicates that only a small portion of the HCV core, which is translocated to the nucleus, may interact with CDK7. It has been demonstrated that the complete loss of CAK results in cell death caused by the inability of cell cycle progression in the previous report using the cultured mat1−/− blastocysts (49). Our CL2 core cells may be able to survive because of incomplete suppression of CAK by the HCV core. According to this, it would be reasonable that only a portion of the HCV core protein is involved in the direct inhibitory effect on CAK.

It has been suggested by a few investigators that the HCV core may considerably affect the p53/p21CIP1 status. It has been reported that the HCV core binds to both p53 and p21CIP1 (21, 22) and impairs the cell cycle regulation in association with the increased expression of p21CIP1 (22, 35). As for the p53 status in the CL2 cells used for this study, it has been shown that the CL2 cells possess the wild-type sequence of p53 gene and do not lose its expression (50). Therefore, the differences in the p53/p21CIP1 status between CL2 mock and core cells were also examined in this study. In contrast to these previous reports, persistent expression of HCV core did not substantially affect the expression levels of p53 and p21CIP1. Thus, HCV core-mediated suppression of cell cycle progression was not found to be responsible for modification of the p53/p21CIP1 status in our CL2 cells. Such conflicting results may be due to the different expression level of the HCV core or different kinds of cultured cells.

CAK components are known to also be parts of the general transcription factor TFIIH and play an essential role not only in the cell cycle progression but also in transcription. TFIIH is composed of nine polypeptides, XPB, XPD, p62, p54, p44, p34, and three CAK components, CDK7, cycH, and MAT1 (51, 52). Biochemical analysis has revealed that CAK exists in three distinct forms in cells. The major form is a free ternary CAK complex, but CAK is also present as a CAK-XPD complex and the nine-subunit “holo” TFIIH (51). CAK phosphorylates the CTD of the largest subunit of RNA polymerase II (53), and phosphorylation of the CTD is believed to initiate promoter clearance and transcription elongation. In this study, the HCV core was found to suppress the CTD kinase activity by CAK.

When the cell growth curve and the cell cycle profile were compared between CL2 mock and core cells, expression of HCV core impaired cell growth and the G1/S transition in the cell cycle. It was also shown that persistent expression of HCV core suppressed CDK4 and CDK2 activities and subsequently inhibited the pRB phosphorylation and E2F-mediated transcription. Thus, HCV core-mediated inhibition of cell cycle progression was based on decreased CDK4 and CDK2 activities. We next carried out an extensive investigation on the factors modulating the CDK activity, which included the expression and complex formation of CDKs and cyclins, the expression levels of various CDK inhibitors, and the phosphorylation/dephosphorylation status of CDKs. It was found that the decrease of CDK2 phosphorylation caused by suppression of CAK activity was only a factor, which was identified as a possible cause of HCV core-mediated inhibition of CDK activity.

In the CL2 core cells, the expression levels of CAK components, CDK7, cycH, and MAT1 were not decreased, but the MAT1 level bound to the CDK7-cycH complex was suppressed, compared with the mock cells. Both CAK and CTD kinase activities were also lower in the CL2 core cells than in the mock cells. To investigate whether HCV core would directly modify the CAK activity, the binding reaction of the anti-CDK7 IP product with the in vitro synthesized HCV core protein was carried out using both murine CL2 and human HepG2 cellular proteins. It is noteworthy that the addition of the HCV core induced the release of MAT1 from the CAK complex and led to suppression of the CAK activity in the cases of both cellular proteins. These results strongly imply that the HCV core may directly interact with CAK and act as a suppressor of CAK by disrupting the ternary CAK complex in murine and possibly human cells.

Abrogation of CAK Complex and Activity by HCV Core Protein

Fig. 7. Intracellular localization of CAK in CL2 mock and core cells. A, the nuclear fraction was obtained from CL2 mock and core cells, and the CDK7, cycH, and MAT1 proteins were detected by Western blotting. B, the CL2 core-I cells were double-stained with the mouse monoclonal HCV core antibody and the rabbit polyclonal CDK7 antibody and analyzed by the confocal microscopic analysis. Images recorded in green (HCV core) and red (CDK7) channels are shown separately in the upper panels, and a composite image is shown in the left lower panel. The right lower panel shows the nuclear staining with 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI). IB, immunoblot.
suggesting that the HCV core may affect basal transcription. Furthermore, TFIIH is an essential factor for nucleotide excision DNA repair in cells (54). It is speculated that the direct effect on cell cycle progression caused by persistent expression of HCV core would strikingly affect this process in the pathogenesis of HCV-related liver diseases.

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Our results conclusively showed that CAK is a novel target of the HCV core protein in the host cell. HCV core can directly bind to CDK7, and its binding causes dissociation of MAT1 from the CDK7-cycH complex and disrupts the stable ternary CAK complex. Such HCV core-mediated inhibition of CAK may prevent the activities of all cell cycle-related CDKs, including CDK2. This may be the molecular basis of HCV core-mediated suppression of cell cycle progression. It has recently been reported that a knock-out of the mat1 gene led to embryonic lethality in mice because of the destabilization of CDK7 and cycH and the inability of cell cycle progression (49). Thus, CAK has been shown to be an indispensable factor to achieve cell cycle progression in vivo. According to this, it may be of great biological significance that the HCV core protein functions as an extrinsic suppressor of CAK in the host cell. In chronic HCV infection, the disease stage progresses with time accompanied by repeated liver cell injury and regeneration. The inhibitory effect on cell cycle progression caused by persistent expression of HCV core would strikingly affect this process in the pathogenesis of HCV-related liver diseases.

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