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IV. 研究成果の刊行物・別冊

Involvement of Creatine Kinase B in Hepatitis C Virus Genome Replication through Interaction with the Viral NS4A Protein[∇]

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Persistent infection with hepatitis C virus (HCV) is a major cause of chronic liver diseases. The aim of this study was to identify host cell factor(s) participating in the HCV replication complex (RC) and to clarify the regulatory mechanisms of viral genome replication dependent on the host-derived factor(s) identified. By comparative proteome analysis of RC-rich membrane fractions and subsequent gene silencing mediated by RNA interference, we identified several candidates for RC components involved in HCV replication. We found that one of these candidates, creatine kinase B (CKB), a key ATP-generating enzyme that regulates ATP in subcellular compartments of nonmuscle cells, is important for efficient replication of the HCV genome and propagation of infectious virus. CKB interacts with HCV NS4A protein and forms a complex with NS3-4A, which possesses multiple enzyme activities. CKB upregulates both NS3-4A-mediated unwinding of RNA and DNA in vitro and replicase activity in permeabilized HCV replicating cells. Our results support a model in which recruitment of CKB to the HCV RC compartment, which has high and fluctuating energy demands, through its interaction with NS4A is important for efficient replication of the viral genome. The CKB-NS4A association is a potential target for the development of a new type of antiviral therapeutic strategy.

Hepatitis C virus (HCV) infection represents a significant global healthcare burden, and current estimates suggest that a minimum of 3% of the world's population is chronically infected (4, 19). The virus is responsible for many cases of severe chronic liver diseases, including cirrhosis and hepatocellular carcinoma (4, 16, 19). HCV is a positive-stranded RNA virus belonging to the family *Flaviviridae*. Its ~9.6-kb genome is translated into a single polypeptide of about 3,000 amino acids (aa), in which the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B reside in the C-terminal half region (6, 34, 44). NS4A, a small 7-kDa protein, functions as a cofactor for NS3 to enhance NS3 enzyme activities such as serine protease and helicase activities. The hydrophobic N-terminal region of NS4A, which is predicted to form a transmembrane α -helix, is responsible for membrane anchorage of the NS3-4A complex (8, 44, 50), and the central region of NS4A is important for the interaction with NS3 (10, 44). A recent study demonstrated the involvement of the C terminus of NS4A in the regulation of NS5A hyperphosphorylation and viral replication (28).

The development of HCV replicon technology several years

ago accelerated research on viral RNA replication (7, 44). Furthermore, a robust cell culture system for propagation of infectious HCV particles was developed using a viral genome of HCV genotype 2a, JFH-1 strain, enabling us to study every process in the viral life cycle (27, 47, 54). RNA derived from genotype 1a, HCV H77, containing cell-culture adaptive mutations, also produces infectious viruses (52). Using these systems, it has been reported that the HCV genome replicates in a distinct, subcellular replication complex (RC) compartment, which includes NS3-5B and the viral RNA (2, 14, 33). The RC forms in a distinct compartment with high concentrations of viral and cellular components located on detergent-resistant membrane (DRM) structures, possibly a lipid-raft structure (2, 41), which may protect the RC from external proteases and nucleases. Almost all processes in viral replication are dependent on the host cell's machinery and involve intimate interaction between viral and host proteins. However, the functional roles of host factors interacting with the HCV RC in viral genome replication remain ambiguous.

To gain a better understanding of cellular factors that are components of the HCV RC and that function as regulators of viral replication, a comparative proteomic analysis of DRM fractions from HCV replicon and parental cells and subsequent RNA interference (RNAi) silencing of selected genes were performed. We identified creatine kinase B (CKB) as a key factor for the HCV genome replication. CKB catalyzes the reversible transfer of the phosphate group of phosphocreatine

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(pCr) to ADP to yield ATP and creatine and is known to play important roles in local delivery and cellular compartmentalization of ATP (48, 51). The findings obtained here suggest that recruitment of CKB to the HCV RC, through CKB interaction with NS4A, is essential for maintenance or enhancement of viral replicase activity.

MATERIALS AND METHODS

Cell lines, antibodies, and reagents. Human hepatoma cell line Huh-7.5.1 (54) was kindly provided by Francis V. Chisari. Cell lines carrying subgenomic replicon RNAs, namely, SGR-N (41) and SGR-JFH1 (23), were derived from the HCV-N (17) and JFH-1 strains (24), respectively. Mouse monoclonal antibodies (MAbs) against HCV NS3 (Chemicon, Temecula, CA), NS4A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), NS5A (Bioscience Resource Project, Sacramento, CA), NS5B (2), FLAG (M2; Sigma-Aldrich, St. Louis, MO), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon), and Flotillin-1 (BD Biosciences, San Jose, CA) and polyclonal antibodies (PAb) against CKB (mouse [Abnova, Taipei, Taiwan], goat [Santa Cruz]), hemagglutinin (HA; Sigma-Aldrich), and FLAG (Sigma-Aldrich) were used. Cyclocreatine (Ccr; also known as 2-imino-1-imidazolidineacetic acid), pCr, and phosphopyruvic acid (pPy) were purchased from Sigma-Aldrich. Recombinant CKB and pyruvate kinase (PK) were obtained from Acris (Herford, Germany) and Calbiochem (San Diego, CA), respectively.

Proteome analysis. RC-rich membrane fractions of cells were isolated as described previously (2, 41). Briefly, cells were lysed in hypotonic buffer. After removing the nuclei, supernatants were treated with 1% NP-40 for 60 min, mixed with 70% sucrose, overlaid with 55 and 10% sucrose, and centrifuged at 38,000 rpm for 14 h. Proteins from membrane fractions were purified by using a 2D Clean-Up kit (GE Healthcare, Tokyo, Japan), followed by labeling with fluorescent dyes: Cy5 for replicon cells, Cy3 for parental cells, and Cy2 for the protein standard containing equal amounts of both cell samples. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was performed using Immobilin DryStrip as the first-dimension gel and 12.5% polyacrylamide gel as the second-dimension gel. The 2D-DIGE images were analyzed quantitatively using the DeCyder software (GE Healthcare). Student *t* test was performed on differences between the tested samples using DeCyder biological variation analysis module. Samples were analyzed in triplicate. The protein spots of interest were excised from the gel, subjected to in-gel digestion using trypsin or lysyl endopeptidase and analyzed by liquid chromatography (MAGIC 2002 System; Michrom Bioresources, Auburn, CA) directly connected to electrospray ionization-ion trap mass spectrometry (LCQ-decaXP; Thermo Electron Corp., Iwakura, Japan). The results were subjected to database (NCBIInr) search by Mascot server software (Matrix Science, Boston, MA) for peptide assignment.

Plasmids. A human CKB cDNA (43; kindly provided by Oriental Yeast Corp., Tokyo, Japan) was inserted into the EcoRI site of pCAGGS, yielding pCAGCKB. To generate expression plasmids for HA-tagged versions of wild-type and deletion mutated CKB, the corresponding DNA fragments were amplified by PCR, followed by introduction into the BglIII site of pCAGGS. A fragment representing the inactive mutant CKB-C283S was synthesized by PCR mutagenesis. To generate FLAG-tagged NS protein expression plasmids, DNA fragments encoding either NS3, NS4A, NS4B, NS5A, or NS5B protein were amplified from HCV strains NIHJ1 (1) and JFH-1 (23) by PCR, followed by cloning into the EcoRI-EcoRV sites of pcDNA3-MEF (20). To generate an HA-tagged NS3 expression plasmid, a fragment encoding NS3 with the HA tag sequence at its N terminus was inserted into pCAGGS.

siRNA transfection. The small interfering RNAs (siRNAs) targeted to CKB (CKB-1 [5'-UAAGACCUCCUGGUGUGGTT-3'] and CKB-2 [5'-CGUCACCCUUGGUAGAGUUTT-3']) and the scramble negative control siRNA to CKB-2 (5'-GGCGUACUAGCUUAUUCGCTT-3') were purchased from Sigma. Cells in a 24-well plate were transfected with siRNA using HiPerFect transfection reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The siRNA sequences for the other genes used in the siRNA screening are available upon request.

HCV infection. Culture media from Huh-7 cells transfected with in vitro-transcribed RNA corresponding to the full-length JFH-1 (47) was collected, concentrated, and used for the infection assay (3).

Quantification of HCV core protein and RNA. To estimate the levels of HCV core protein, aliquots of culture supernatants or of cell lysates were assayed by using HCV Core enzyme-linked immunosorbent assay kits (5). Total RNA was isolated from harvested cells using TRIzol (Invitrogen, Carlsbad, CA). Copy numbers of the viral RNA were determined by reverse transcription-PCR (RT-PCR) (2, 36, 46).

Immunoprecipitation, immunoblot analysis, and immunofluorescence microscopy. The analyses, as well as DNA transfection, were performed essentially as previously described (42). Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, 1 mM calcium acetate). For immunoprecipitation, supernatants of cell lysates were precipitated with anti-FLAG antibody and protein A-Sepharose Fast Flow beads (GE healthcare). For immunofluorescence microscopy, anti-CKB goat PAb and anti-NS4A MAb as primary antibodies and Alexa Fluor 555-conjugated donkey anti-goat immunoglobulin G (Invitrogen) and Alexa Fluor 488-conjugated rabbit anti-mouse immunoglobulin G (Invitrogen) as secondary antibodies were used and observed under an LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Immuno-electron microscopy. Postembedding immunostaining using the colloidal gold-labeling method was performed as described previously (38). Cells were fixed in 4% paraformaldehyde-1% glutaraldehyde at 4°C for 1 h. After dehydration through a graded series of ethanol, cells were embedded in LR White (London Resin Company, London, United Kingdom) and sectioned. After blocking, section grids were incubated with a mixture of anti-NS4A and anti-CKB antibodies at 4°C overnight, followed by treatment with a mixture of 18-nm colloidal gold-conjugated donkey anti-mouse immunoglobulin G and 12-nm colloidal gold-conjugated donkey anti-goat immunoglobulin G antibodies (Jackson ImmunoResearch, West Grove, PA) at 4°C overnight. The sections were stained with uranyl acetate and observed under a transmission electron microscope.

Measurement of CK activity and cellular ATP level. Cells were lysed with passive lysis buffer (Promega, Madison, WI), and CK activities were measured based on Oliver methods (40), in which the activity of converting creatine phosphate and ADP to creatine and ATP was measured. ATP levels in cell lysates were measured by using a CellTiter-Glo luminescent cell viability assay (Promega).

RNA replication assays in permeabilized replicon cells and in vitro. The RNA synthesis assay using permeabilized replicon cells was based on a previously described method (33). Briefly, SGR-JFH1 cells were treated with 5 µg of actinomycin D/ml for 2 h, followed by permeabilization with 50 µg of digitonin/ml for 5 min. The resulting mix was incubated with 500 µM concentrations of ATP, GTP, and CTP; 10 µCi of UTP ([α -³²P]UTP); 50 µg of actinomycin D/ml; and 5 mM pCr with or without 20 U of CKB/ml for 4 h at 27°C. RNA was extracted by using TRIzol and analyzed by 1% formaldehyde agarose gel electrophoresis. The cell-free RNA replication assay was performed as described previously (2).

In vitro helicase assays. Helicase activity on double-stranded RNA (dsRNA) was investigated as described previously (11) with some modifications. The 5' end of the release strand was labeled with [γ -³²P]ATP using T4 polynucleotide kinase (Ambion). The dsRNA substrate was obtained by annealing the labeled RNA with a template strand RNA at a molar ratio of 1:1. The helicase assay mixture contained 5 nM dsRNA, helicase enzyme (80 nM NS3 or NS3-4A [kindly provided by R. De Francesco]), 6 mM ATP, in the presence or absence of 20 U of CKB/ml in an assay buffer (25 mM MOPS-NaOH [pH 7.0], 2.5 mM dithiothreitol, 100 µg of bovine serum albumin/ml, 3 mM MgCl₂, 5 mM pCr, 2.5 U of RNase inhibitor/ml). After the helicase reaction, samples were electrophoresed in a native 8% polyacrylamide gel and autoradiographed.

To determine the effect of PK/pPy system on the helicase activity, PK and pPy were used instead of CKB and pCr. Helicase activity on dsDNA was measured based on homogeneous time-resolved fluorescence quenching using a Trupoint helicase assay kit (Perkin-Elmer, Waltham, MA) according to the manufacturer's instructions.

In vitro protease assay. In vitro HCV protease activity of NS3-4A or NS3 was analyzed by using a SensoLyteHCV protease assay kit (AnaSpec, San Jose, CA) according to the manufacturer's instructions.

RESULTS

Identification of host factors involved in HCV RNA replication by comparative proteomic analysis of DRM fractions and RNAi silencing. To identify host proteins involved in the HCV RC, proteome profiles of the RC-rich membrane fraction in Huh-7 cells harboring subgenomic replicon RNA derived from genotype 1b, N isolate (SGR-N) were compared to those of parental cells by 2D-DIGE. We confirmed that the DRM fraction obtained from SGR-pN cells is functionally active in a

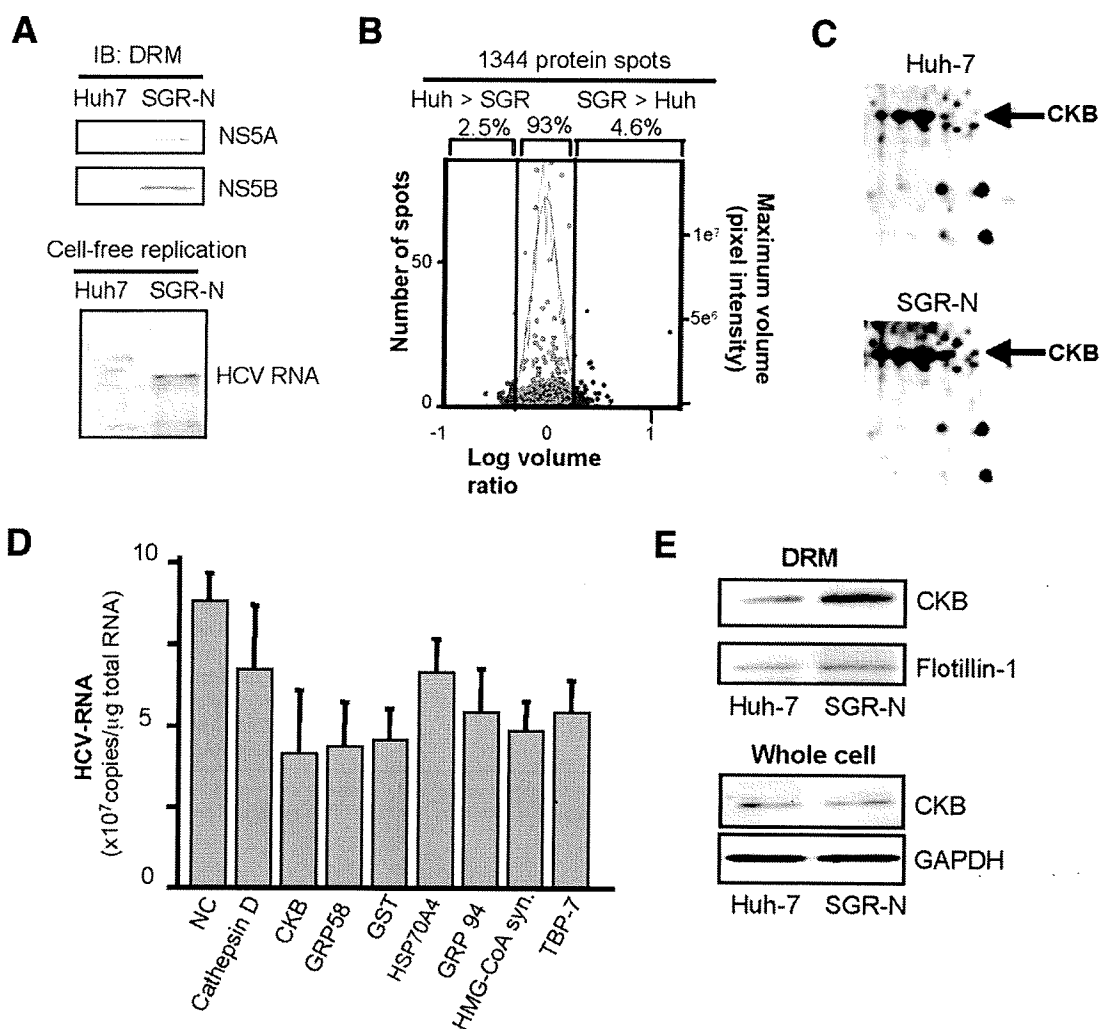


FIG. 1. Comparative proteomic analysis of DRM fractions and RNAi silencing. (A) Preparation of functionally active RC fraction for proteome analysis. DRM fractions obtained from SGR-N cells and parental Huh-7 cells were analyzed by immunoblotting with anti-NS5A and anti-NS5B antibodies (upper panel) and by the cell-free RNA replication assay (lower panel). (B) Histogram representation of proteins detected in 2D-DIGE. Images were analyzed quantitatively by the DeCyder software. The left and right y axis, respectively, indicate the spot frequency and the maximum volume of each spot, given against the log volume ratio (x axis). (C) Comparison of 2D-DIGE maps of proteins from DRM fractions of SGR-N cells and Huh-7 cells. Enlarged 2D-DIGE gel images of regions containing protein spots of CKB (arrows) are shown. (D) Effects of siRNAs of genes selected from comparative proteome analysis on HCV RNA replication. SGR-N cells were transfected with siRNA specific to cathepsin D, CKB (siCKB-1), GRP58, GST, Hsp70 protein 4, GRP94, HMG-coenzyme A synthase, or Tat binding protein 7 or with nontargeting (NC) siRNA. At 48 h posttransfection, total RNA was isolated and HCV RNA levels were assessed by real-time RT-PCR. (E) Enrichment of CKB in the DRM of HCV replicon cells. Equal amounts of DRM fractions from SGR-N and parental Huh-7 cells, or whole-cell lysates from both cells were analyzed by immunoblotting with antibodies against CKB, flotillin-1 or GAPDH.

cell-free replication assay (Fig. 1A). Three independent proteome experiments were performed for a reliable analysis of protein expression. Approximately 1,300 spots were resolved in each gel, and 4 to 5% of the protein spots represented a >2-fold increase in the membrane fraction of replicon cells in each experiment (Fig. 1B). The protein spots that exhibited high reproducibility (an example shown in Fig. 1C) were excised, digested by trypsin or lysyl endopeptidase, and analyzed by mass spectrometry, which identified the corresponding proteins in 27 cases (Table 1). Among the proteins implicated in a variety of functional categories, 10 were involved in protein folding, mainly as chaperones, 7 were metabolic and biosynthesis enzymes including proteins for redox regulation or en-

ergy pathways, 3 were involved in cytoskeleton organization, and 3 proteins were related to cellular processes, mainly proteolysis pathways. The viral NS proteins identified as differentially expressed proteins in the analysis were not listed.

In order to identify host factors involved in HCV replication, we examined the effects on viral RNA replication of transfection of SGR-N cells with siRNAs against genes encoding nine proteins belonging to diverse classes of biological functions (Table 1). Each siRNA reduced the HCV RNA level to 47 to 76% of the level of the siRNA control (Fig. 1D). None of the siRNAs tested exhibited considerable cytotoxicity against the replicon cells, ruling out overt toxicity as a mechanism for inhibition of viral RNA replication. Among the candidate

TABLE 1. Selected proteins that reproducibly increased in the DRM fraction of SGR-N cells^a

Avg ratio	P (Student <i>t</i> test)	Coverage (%)	Protein name	Molecular function	GI no.
5.56	0.04	27	GRP94	Protein folding	15010550
4.99	0.07	47	Hsp60	Protein folding	6996447
3.73	0.07	6	tRNA guanine transglycosylase	Metabolism	30583205
3.56	0.06	23	KIAA0088	Unknown	577295
3.32	0.07	4	Thioredoxin-related protein	Unknown	20067392
3.32	0.13	12	Tat binding protein 1 (TBP-1)	Cellular processes	20532406
3.06	0.14	22	Aldehyde dehydrogenase 1	Metabolism	2183299
3.06	0.14	14	Chaperonin TRiC/CCT, subunit 2	Protein folding	54696794
2.96	0.04	14	Heat shock 70-kDa protein 4 (HSPA4)	Protein folding	6226869
2.96	0.04	29	GRP58	Metabolism/protein folding	2245365
2.94	0.01	37	Mutant β -actin	Cytoskeleton organization	28336
2.65	0.17	33	Glutathione S-transferase (GST)	Catalytic activity	2204207
2.53	0.04	37	Keratin 19	Cytoskeleton organization	6729681
2.46	0.08	6	Heterogeneous nuclear ribonucleoprotein K	Nucleic acid modification	460789
2.45	0.001	13	HMG-coenzyme A synthase	Metabolism	30009
2.4	0.02	31	CKB	Energy pathway/metabolism	180570
2.4	0.02	11	Cathepsin D	Cellular processes	30582659
2.4	0.02	11	C8orf2	Unknown	37181322
2.36	0.1	38	Tropomyosin 4-anaplastic lymphoma kinase fusion protein	Cytoskeleton organization	14010354
2.36	0.1	6	Calreticulin	Protein folding	30583735
2.33	0.01	29	Quinolinic phosphoribosyltransferase	Metabolism	30583301
2.29	0.04	25	Protein disulfide isomerase-related protein 5	Protein folding	1710248
2.29	0.04	16	Tat binding protein 7 (TBP-7)	Cellular processes	263099
2.05	0.11	24	Calumenin	Metabolism	2809324
2.05	0.12	10	TRiC/CCT, subunit 5	Protein folding	24307939
2.03	0.07	20	Hsp90 beta	Protein folding	34304590
2.01	0.07	10	TRiC/CCT, subunit 1	Protein folding	36796

^a The spectra obtained by tandem mass spectrometry were collected using data-dependent mode, and the results were subjected to database (NCBI) search by Mascot server software (Matrix Science, London, United Kingdom) for peptide assignment. Coverage, the ratio of the portion of protein sequence covered by matched peptides to the whole protein sequence. GI no., GenInfo identifier number.

genes examined, we observed a reproducible inhibition of HCV RNA replication by two independent siRNAs targeting CKB (see below).

CKB participates in HCV RNA replication and the propagation of infectious virus. CKB is a brain-type creatine kinase isoenzyme and is also detected in a variety of other tissues, including human liver (32). Steady-state levels of CKB in the DRM fraction, as well as in whole-cell lysate of SGR-N cells were compared to those from parental cells by Western blotting. The CKB level in the DRM fraction of replicon cells was higher than that in parental cells (Fig. 1E), confirming the results of the proteome analysis described above. In contrast, the CKB level in whole cells was similar in both cells (Fig. 1E). These results suggest participation of posttranslational modification, such as translocation to the DRM fraction, of CKB in replicon cells.

Figure 2A shows the inhibitory effect on HCV RNA replication of CKB siRNA; siCKB-2, the sequence of which does not overlap with the sequence of siCKB-1 used in the above siRNA screening (Fig. 1D). Transfection with siCKB-2 effectively decreased the cellular level of CKB enzymatic activity (data not shown), as well as the abundance of CKB protein (Fig. 2A), and resulted in 60% reduction in the viral RNA level in SGR-N cells compared to the cells treated with control siRNA. This inhibitory effect of siRNA on HCV RNA abundance was also observed in JFH-1-derived subgenomic replicon (SGR-JFH1) cells. The viral RNA level in the cells transfected with siCKB-2 decreased by 50% compared to the control (Fig. 2A). We also tested the CKB mutant, CKB-

C283S, in which Cys at aa 283, near the catalytic site, has been replaced with Ser (Fig. 3A) and which is known to be enzymatically inactive and to work in a dominant-negative manner (22, 29). As expected, overexpression of CKB-C283S resulted in a reduction in HCV RNA replication in SGR-N cells (Fig. 2B). We obtained a similar result in SGR-JFH1 cells, as described below (Fig. 3E).

To further examine the involvement of CKB in HCV RNA replication, we tested the effect of Ccr, a substrate analogue and possible inhibitor for CK in either SGR-N, SGR-JFH1 (Fig. 2C), or Huh7 cells transiently replicating luciferase-subgenomic replicon (data not shown). We found dose-dependent inhibition of HCV RNA replication but no observed effect on total cellular levels of protein and ATP (Fig. 2D) in the replicon setting used.

We next examined whether the knockdown of CKB or treatment with Ccr would abrogate the production of HCVcc. At 72 h posttransfection with siCKB-2, the HCV core level in cells infected with HCVcc was significantly reduced (Fig. 2E). Treatment of the infected cells with Ccr at various concentrations also reduced the intracellular and supernatant core level and subsequently decreased HCVcc production (Fig. 2F). These results demonstrate that suppression of the HCV RNA replication by the siRNA-mediated knockdown of CKB or treatment with CKB inhibitor leads to reduction of the production of infectious virus.

CKB interacts with HCV NS4A. Having established a role for CKB in HCV RNA replication, we then tried to determine to how CKB influences the HCV life cycle. It has been re-

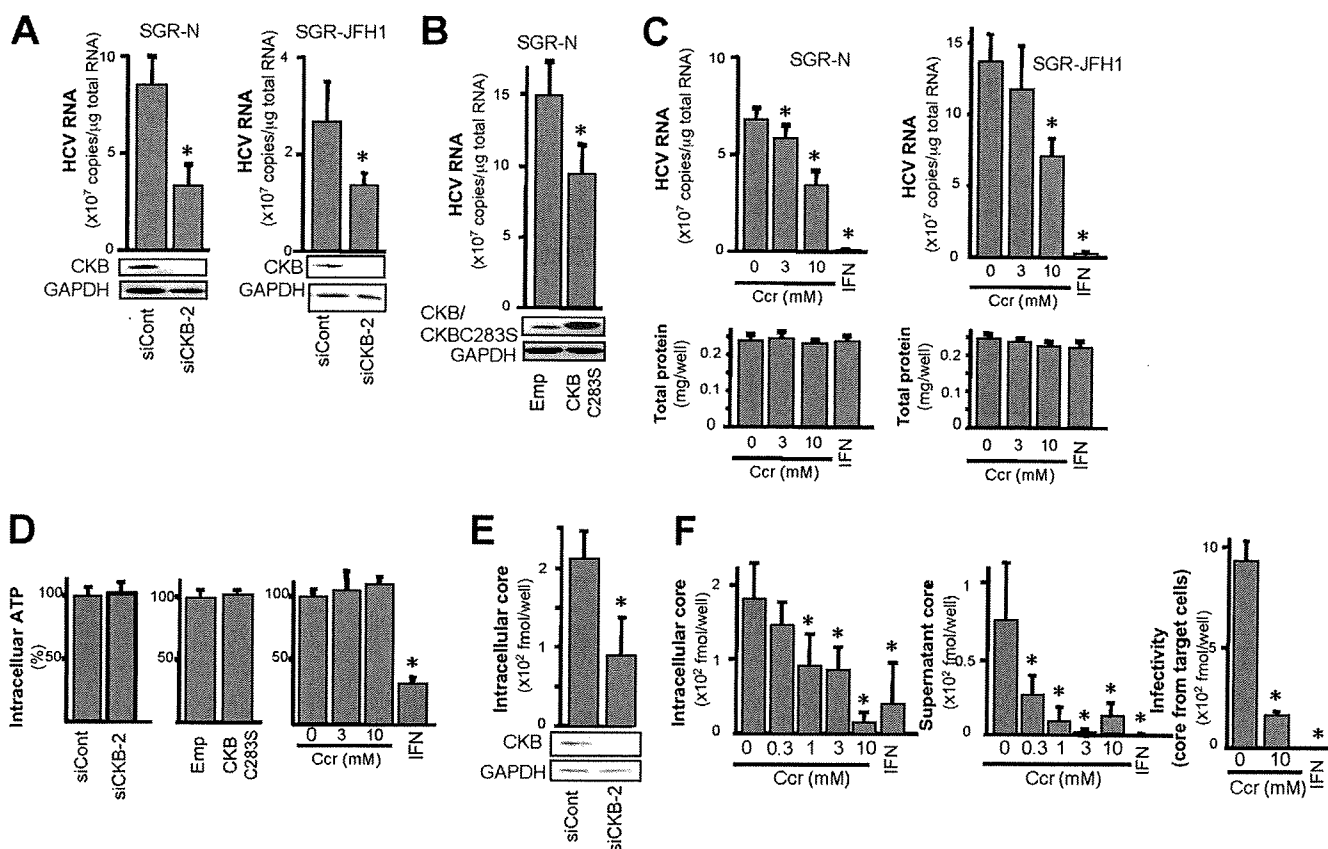


FIG. 2. Involvement of CKB in HCV replication. (A and E) Knockdown of endogenous CKB in SGR-N and SGR-JFH1 cells (A) or HCVcc-infected cells (E). Cells were transfected with siRNA against CKB (siCKB-2) or control siRNA (siCont) and were harvested at 72 h posttransfection. Real-time RT-PCR for HCV RNA levels and immunoblotting for CKB and GAPDH were performed. (B) SGR-N cells were transfected with pCAGCKB-C283S or empty vector, and HCV RNA levels and expression of CKB and CKB-C283S were determined 72 h posttransfection. SGR-N and SGR-JFH1 cells (C) or HCVcc-infected cells (F) were treated with Ccr at various concentrations for 72 h, followed by quantification of HCV RNAs and total cellular proteins. ATP levels (D) were determined after transfection with siCKB-2, pCAGCKB-C283S, or treatment with Ccr for 72 h in SGR-N cells. The ATP levels in the cells transfected with negative control siRNA (left), empty vector (middle), and no treatment (right) were set at 100%, respectively. (F) HCVcc-infected cells were treated with Ccr, and the viral core protein levels in cells (left) and supernatants (middle) were determined at 72 h postinfection. Collected culture supernatants were inoculated into naive Huh-7.5.1 cells after the removal of Ccr. After 72 h, the core proteins in cells were determined (right panel). All data are presented as averages and standard deviation values for at least triplicate samples. *, *P* < 0.05 against control such as transfection with siCont (A and E) or empty vector (B) or nontreatment (C, D, and F).

ported that interaction of CKB with some cellular proteins is required for local availability of CKB activity and local generation of ATP (22, 29). To examine the possible interaction of CKB with HCV NS proteins, HA-tagged CKB (HA-CKB) was coexpressed with FLAG-tagged NS proteins (NIHJ1 strain), followed by immunoprecipitation with an anti-FLAG antibody. CKB was shown to specifically interact with NS4A. No or little interaction was observed between CKB and either NS3, NS4B, NSSA, or NSSB (Fig. 3B). CKB-NS4A interaction was also found with the JFH-1 strain (Fig. 3C).

To identify the CKB region required for the interaction with NS4A, various deletion mutants of CKB were generated (Fig. 3A). An immunoprecipitation assay indicated that NS4A was coimmunoprecipitated with either a full-length CKB, a C-terminal deletion (aa 1 to 357), an N-terminal deletion (aa 297 to 381), or CKB-C283S, but not with aa 1 to 296, aa 1 to 247, or aa 1 to 184 (Fig. 3D, upper middle panel). Further, internal deletions of CKB (CKBdel297-357 and CKB-C283Sdel297-357) failed to interact with NS4A (Fig. 3D, lower panel), sug-

gesting that aa 297 to 357 of CKB are important for its interaction with NS4A. It is noted that the expression of CKB aa 297 to 357 in cells was undetected, presumably due to its misfolding and/or instability. To verify a role for CKB-NS4A interaction in HCV RNA replication, we further determined the effect of expression of either CKB-C283S or its internal deletion lacking aa 297 to 357 (CKB-C283Sdel297-357) on viral replication in SGR-JFH1 cells. As expected, the HCV RNA level was significantly decreased by CKB-C283S, whereas this effect was not observed by CKB-C283Sdel297-357 (Fig. 3E).

NS4A is a 54-residue small protein composed of three domains: the N-terminal membrane anchor, the central domain responsible for interacting with NS3, and the C-terminal acidic domain. To define the portion in NS4A responsible for its interaction with CKB, we constructed three NS4A deletion mutants, each separately expressing one of the NS4A domains, with a FLAG tag (Fig. 3F). CKB proved to interact with the central domain, aa 21 to 39, of NS4A, which is involved in

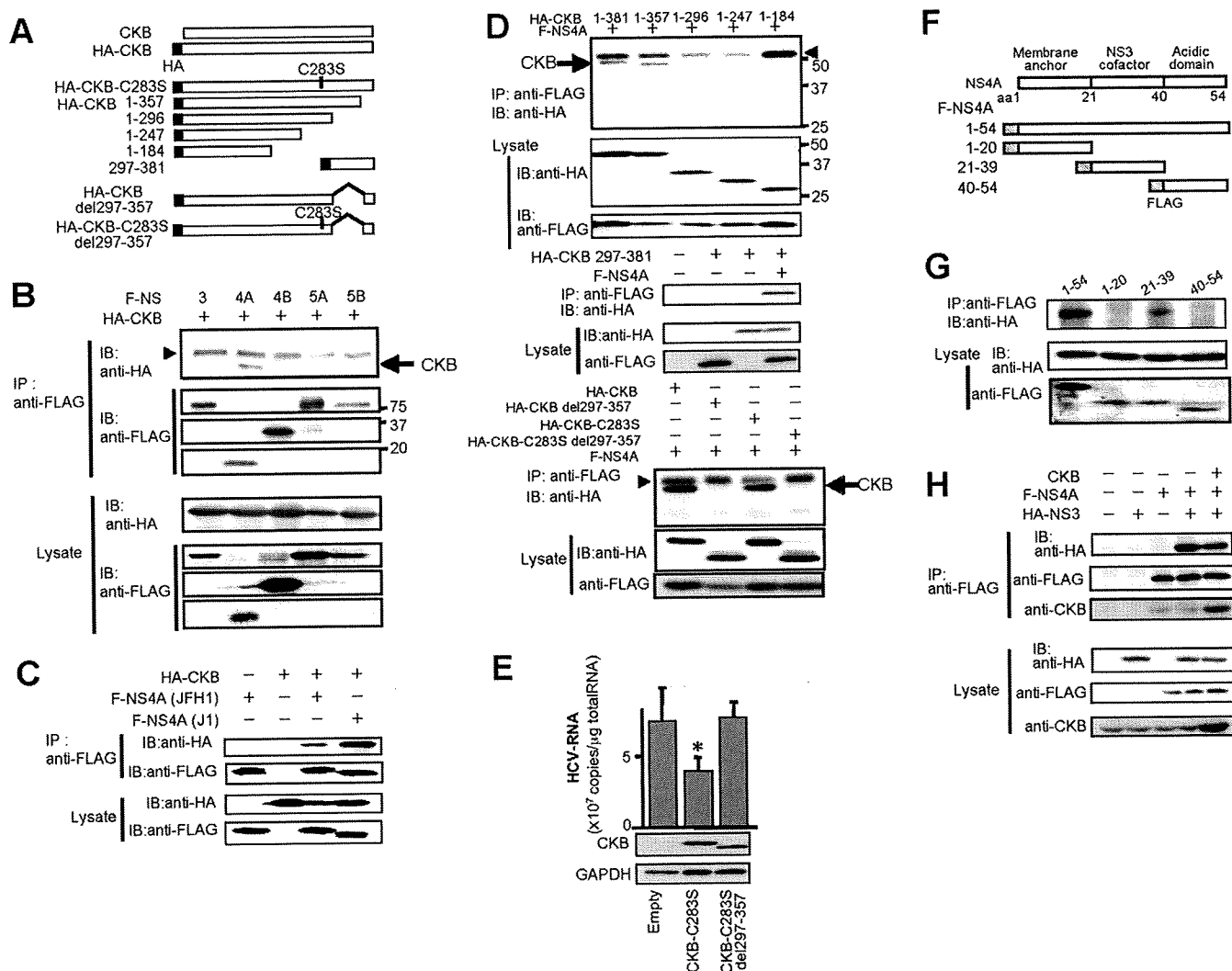


FIG. 3. CKB interacts with HCV NS4A. (A) Structures of CKB constructs used in the present study. A full-length wild-type CKB without an epitope tag (CKB) or with an N-terminal HA tag (HA-CKB), HA-CKB with deletions (aa 1 to 357, aa 1 to 296, aa 1 to 247, aa 1 to 184, and aa 297 to 381 and del297-357), CKB mutant at the catalytic site, Cys-283 (CKB-C283S) or CKB-C283S lacking aa 297 to 357 (CKB-C283Sdel297-357) are shown. HA-CKB was coexpressed with FLAG-tagged versions of each NS protein of strain NIHJ1 (B) or with NS4A of strain JFH-1 (C) in 293T cells and immunoprecipitated (IP) with an anti-FLAG antibody. Immunoprecipitates were subjected to immunoblotting (IB) with anti-HA or anti-FLAG antibody. (D) Each CKB deletion mutant was coexpressed with FLAG-NS4A in 293T cells. Immunoprecipitates were analyzed by immunoblotting. Arrow, CKB; arrowhead, immunoglobulin heavy chain. (E) SGR-JFH1 cells were transfected with the expression plasmid for CKB-C283S, CKB-C283Sdel297-357 or empty vector. At 72 h posttransfection, HCV RNA levels and the expression of CKB and CKB-C283S were determined by real-time RT-PCR and immunoblotting with anti-HA antibody, respectively. For HCV RNA quantitation, data are indicated as averages and standard deviations ($n = 3$). *, $P < 0.05$ against the empty vector control. (F) Structure of NS4A and NS4A constructs. FLAG-tagged NS4A (aa 1 to 54) or its truncated mutants (aa 1 to 20, aa 21 to 39, or aa 40 to 54) are shown. (G) Each NS4A deletion mutant was coexpressed with HA-CKB and analyzed as described above. (H) FLAG-NS4A was coexpressed with HA-NS3 or HA-NS4 and CKB, followed by immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were analyzed by immunoblotting with anti-HA, anti-FLAG or anti-CKB antibody.

formation of the NS3-NS4A complex (Fig. 3G). We therefore investigate whether NS3-NS4A interaction is affected in the presence of CKB and found that exogenous expression of CKB has no influence on NS3-NS4A interaction, and a putative NS3-NS4A-CKB complex was detected in the coimmunoprecipitation analysis (Fig. 3H). Collectively, these results strongly suggest that CKB plays a key role in HCV RNA replication via interaction with NS4A.

Subcellular localization of CKB and NS4A in cells replicating HCV RNA. CKB is distributed throughout cells but is mainly localized in the perinuclear area (31), whereas NS4A is

predominantly localized at the endoplasmic reticulum and mitochondrial membranes (37). We examined the possible subcellular colocalization of CKB and NS4A in SGR-N cells by immunofluorescence staining (Fig. 4A). CKB tended to gather in the perinuclear area of HCV replicating cells and was partially colocalized with NS4A in the area, sharing a dotlike structure. To further analyze the subcellular compartments in which CKB and NS4A coexist, we used double-labeling immunoelectron microscopy on SGR-N cells using antibodies against CKB and NS4A, with secondary antibodies coupled to 12- and 18-nm gold particles, respectively. One fraction of

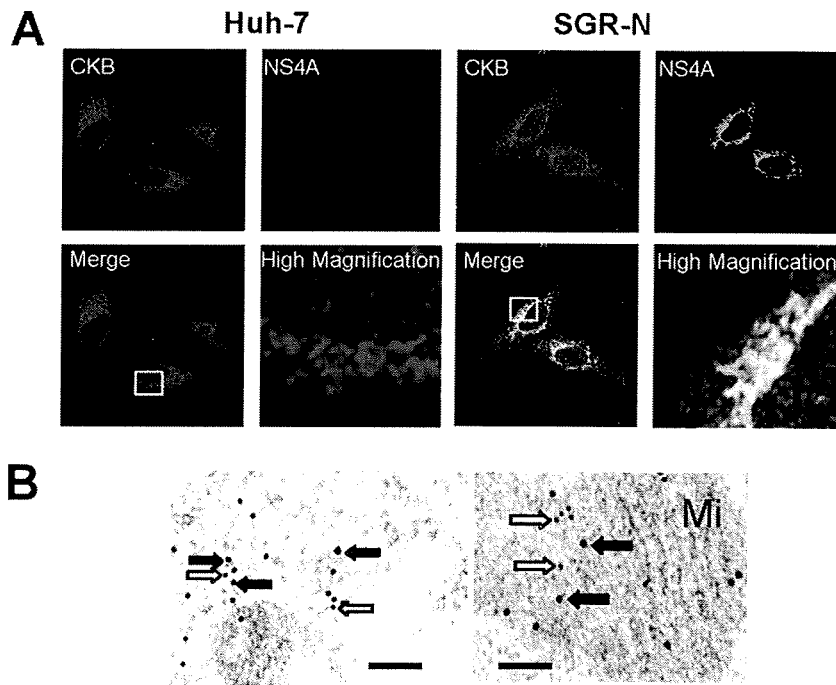


FIG. 4. Colocalization of CKB with HCV NS4A. (A) Indirect immunofluorescence analysis. The primary antibodies used were anti-CKB goat PAb (red) and anti-NS4A MAb (green). Merged images of red and green signals are shown. High-magnification panels are enlarged images of white squares in the merge panels. (B) Immunoelectron microscopic localization of CKB and NS4A. SGR-N cells were double-immunolabeled for CKB (12-nm gold particles; white arrows) and for NS4A (18-nm gold particle; gray arrows). Mi, mitochondria. Bars, 200 nm.

CKB colocalized with NS4A in the cytoplasmic electron-dense regions, presumably derived from altered or folded membrane structures (Fig. 4B, left panel) and mitochondria (Fig. 4B, right panel).

CKB enhances functional HCV replicase and NS3-4A helicase. NS4A is known to mediate membrane association of the NS3-4A complex and to function as a cofactor in NS3 enzyme activity. To understand the mechanism(s) underlying positive regulation of HCV RNA replication through CKB via its interaction with NS4A, we first investigated whether CKB modulates NS3-4A helicase activity. NS3-4A helicase is a member of the superfamily-2 DexH/D-box helicase, which unwinds RNA-RNA substrates in a 3'-to-5' direction. During RNA replication, the NS3-4A helicase is believed to translocate along the nucleic acid substrate by changing its protein conformation, utilizing the energy of ATP hydrolysis (9). We then tested the effect of CKB on RNA- or DNA-unwinding activity using purified recombinant full-length NS3 and NS3-4A complex (12). As shown in Fig. 5A (left middle panel), both NS3 and NS3-4A helicase activity unwound dsRNA substrate most efficiently when CKB, ATP, and pCr were added to the reaction mixture. The enhancing effect of CKB was observed in the presence of pCr but not in the absence of it, suggesting that catalytic activity of CKB is important for its effect on the HCV helicase activity. Similar results were obtained from the DNA helicase assay using dsDNA substrate (Fig. 5B). To address the specificity of the stimulation by the CKB/pCr system, effects of PK and pPy, which are also involved in the ATP generation, were determined (Fig. 5A, right panels). Exogenous PK and pPy at the same concentrations as those of CKB and pCr

used in the study exhibited no effect on the HCV helicase activity.

The effect of CKB on NS3-4A serine protease activity, which is considered to be ATP-independent, was also assessed in an *in vitro* protease assay using the purified viral proteins as mentioned above (Fig. 5C). As expected, NS3-4A complex exhibited significantly higher activity than NS3 alone; however, CKB did not affect the protease activities of NS3 or NS3-4A.

Finally, we investigated loss and gain of function of CKB in HCV replicase activity, which requires high-energy phosphate, in the context of semi-intact replicon cells. Miyanari et al. (33) reported that the function of the active HCV RC can be monitored in permeabilized replicon cells treated with digitonin. Thus, permeabilized replicon cells in the presence or absence of exogenous CKB were incubated with [α - 32 P]UTP to detect newly synthesized RNA. As indicated in Fig. 5D, an ~8-kb band corresponding to HCV subgenomic RNA was most abundant in cells in the presence of exogenous CKB, ATP and pCr. The enhancing effect of CKB was observed in the presence but not in the absence of pCr, suggesting that catalytic activity of CKB is important for its effect on the replicase activity. As for the RNA helicase assay, exogenous PK and pPy did not enhance the replicase activity (data not shown). HCV replicase activity in permeabilized cells to which we had introduced siCKB-2 was diminished compared to that in siRNA control-treated cells. Interestingly, the replicase activity in the CKB-depleted cells was recovered by the addition of CKB. Thus, our findings suggest that CKB functions as a key regulator of HCV genome replication by controlling energy-dependent viral enzyme activities.

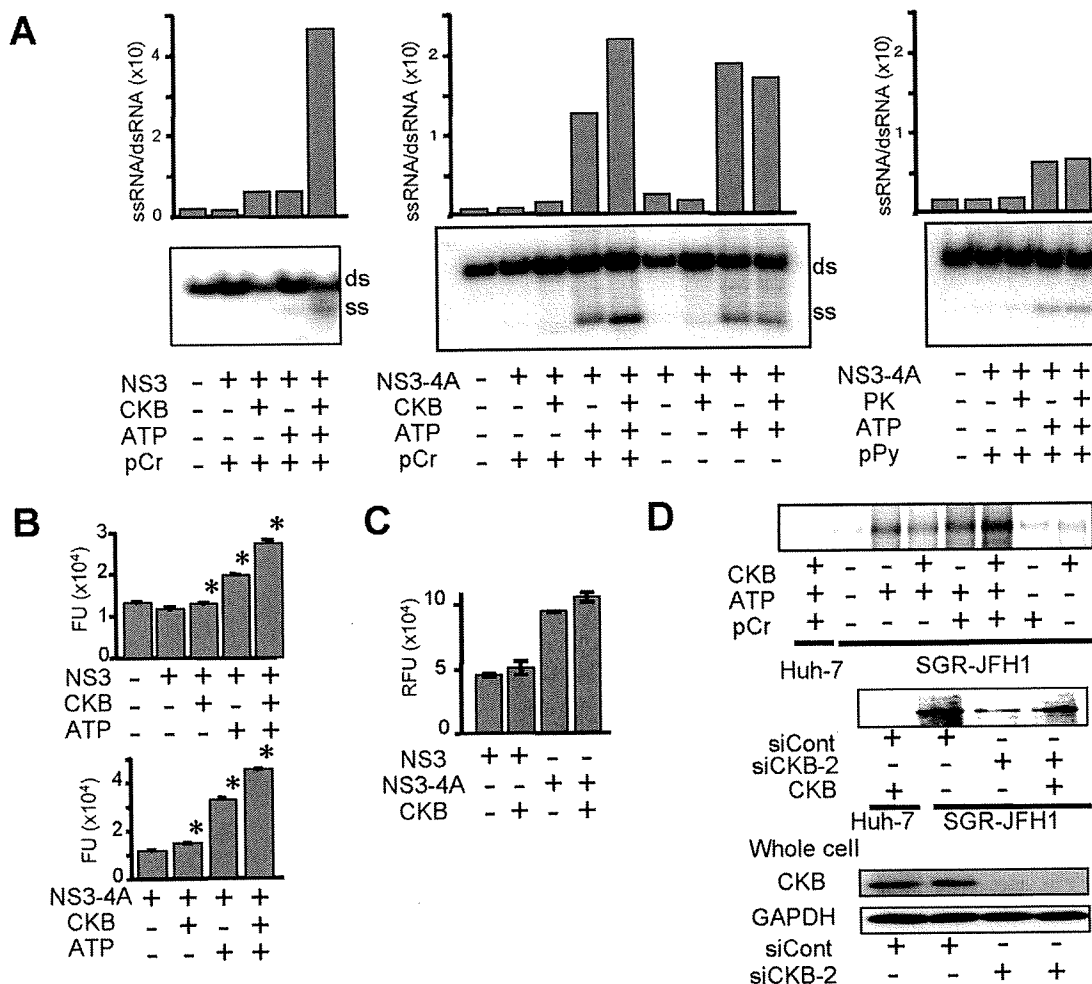


FIG. 5. CKB enhances NS3-4A helicase and HCV replicase activities. (A) In vitro RNA helicase activity of NS3-4A or NS3 was determined by detecting unwound single-strand RNA (ss) derived from the partially dsRNA substrate (ds). Band intensities corresponding to unwound products and those to dsRNA substrates were determined by ImageQuant 5.2 (Molecular Dynamics), and the ssRNA/dsRNA ratios were calculated. The results are representative of three similar experiments. (B) In vitro DNA helicase activity of NS3-4A or NS3 was analyzed by using a commercially available kit. The data represent averages and standard deviations ($n = 3$). *, $P < 0.05$ against the value without supplementation of CKB and ATP. (C) The in vitro HCV protease activity of NS3-4A or NS3 in the presence or absence of CKB was analyzed. Error bars represent standard deviations ($n = 3$). (D) Replicase activity in permeabilized replicon cells. The upper panel shows the activity for synthesis of HCV subgenomic RNA in the digitonin-permeabilized SGR-JFH1 cells with or without supplementation of CKB was measured. The middle panel shows results for SGR-JFH1 or Huh-7 cells that were transfected with siCKB-2 or siCont and permeabilized at 72 h posttransfection. The permeabilized cells with or without supplementation of CKB were subjected to the replicase assay. The lower panel shows the immunoblotting results for whole-cell lysates of siRNA-transfected cells.

DISCUSSION

Viral replication requires energy and macromolecule synthesis, and host cells provide the viruses with metabolic resources necessary for their efficient replication. Thus, it is highly likely that interaction of viruses with host cell metabolic pathways, including energy-generating systems, contributes to the virus growth cycle. In the regulation of HCV genome replication, the functions of the viral NS proteins that comprise the RC might be regulated by association in individual host cell factors. For example, hVAP-A and -B function as cofactors of modulating RC formation via interacting with NS5A and NS5B (13, 18). Cyclophilin B is involved in stimulating viral RNA binding activity via interacting with NS5B (49). FKBP8 (39) and hB-ind1 (45) play an important role in recruiting Hsp90 to

RC via interacting with NS5A. However, the association of viral protein(s) with the cellular energy-generating system to directly regulate the activity of the RC has not been well understood.

In the present study, the accumulation of CKB, an ATP-generating enzyme, in the HCV RC-rich membrane fraction of viral replicating cells and its importance in replication of the HCV genome and production of infectious virions have been demonstrated. Enzymatic analyses with semi-intact replicon cells and purified NS3-4A protein revealed that CKB enhances the functional replicase and helicase of HCV. Its enhancing effect was observed in the presence of pCr but not in its absence, suggesting that the catalytic activity of CKB is important for enhancing the replicase and

helicase activities. Moreover, we clearly detected a CKB-NS4A complex using anti-tag antibodies in cotransfection experiments, but the endogenous complex could not be immunoprecipitated from cells expressing only endogenous levels of CKB, probably because of the inefficiency of the available antibodies. Further, a deletion of the NS4A-interacting region within an inactive mutant of CKB (CKB-C283S) resulted in the loss of its dominant-negative effect on HCV replication.

Creatine kinase, an evolutionarily conserved enzyme, is known to be critical for the maintenance and regulation of cellular energy stores in tissues with high and rapidly changing energy demands (48). In mammals, three cytosolic and two mitochondrial isoforms of CK, which share certain conserved regions, are expressed (35). The brain-type CK, CKB, plays a major role in cellular energy metabolism of nonmuscle cells, reversibly catalyzing the ATP-dependent phosphorylation of creatine and, hence, providing an ATP buffering system in subcellular compartments of high and fluctuating energy demand (21, 29). CKB is overexpressed in a wide range of tumor tissues and tumor cell lines, including hepatocellular carcinoma (32), and is used as a prognostic marker of cancer.

Although CK and creatine phosphate have been supplemented to *in vitro* replicase assays of some RNA viruses (15, 33), understanding of CKB function in the virus life cycle has been limited. One study indicated that the CK substrate analog, Ccr, exhibits antiviral activity against several herpesviruses but not influenza viruses or vesicular stomatitis virus (26). We have demonstrated here that HCV genome replication is downregulated by either treatment with Ccr, siRNA-mediated knockdown of CKB, or the exogenous expression of CKB-C283S. Coimmunoprecipitation experiments revealed that the essential domain within NS4A for the interaction with CKB is the NS4A central domain, aa 21 to 39, which is also responsible for NS3-4A complex formation. However, the NS3-4A interaction was not impaired by overexpression of CKB, and CKB was found to be able to form a complex with NS3-4A (Fig. 3H). Since CKB does not directly interact with NS3 (Fig. 3A), it is likely that NS3-4A-CKB association occurs through two interactions of NS3-4A and NS4A-CKB. We examined whether the formation of the ternary complex affects HCV enzymatic activities, possibly through conformational changes in the viral proteins, and found that CKB has no influence on NS3-4A protease activity (Fig. 5C). With regard to helicase activity, the effect of CKB on RNA unwinding activity by NS3-4A was similar to the effect of NS3 alone in the presence of ATP (Fig. 5A). It is conceivable that interaction with CKB causes no or little global change in the NS3-4A conformation and does not affect the viral helicase and protease activities.

In general, translation initiation in eukaryotes includes an ATP-dependent process such as unwinding the secondary structure in the 5'-untranslated region to permit assembly of 48S ribosomal complexes. It was reported, however, that 48S complex formation on the HCV internal ribosome entry site (IRES) has no requirement for ATP hydrolysis (25). In fact, we found that Huh-7 cells with or without gene silencing of CKB exhibited the same level of HCV IRES activity by transfection with IRES-reporter constructs (data not shown).

Collectively, we conclude that CKB is targeted to the HCV RC through its interaction with NS4A and functions as a pos-

itive regulator for the viral replicase by providing ATP. It is likely that the catalytic activity of CKB that associates with the viral RC is important for enhancing the RNA replication. The role of CKB-NS4A interaction in the enhancing effect seems to be limited. Although either knocking down CKB, expression of the dominant-negative mutant of CKB, or Ccr treatment resulted in the reduction of HCV replication (Fig. 2A to C), the total cellular ATP levels were not changed under these conditions (Fig. 2D). This suggests that CKB contributes to enhancing HCV replication through controlling the ATP level in the particular RC compartment. A tight coupling of a fast ATP regeneration and delivery system to the viral RC is advantageous for achieving efficient replication of the viral genome. To our knowledge, the findings presented here provide the first experimental evidence of the involvement of viral protein in recruiting an ATP generating/buffering system to the subcellular compartment for viral genome replication, a site with high-energy turnover. Given that the levels of HCV RNA were not dramatically diminished by the knocking down, dominant-negative mutant or Ccr, CKB may not be absolutely critical for the viral replication. One would argue that energy required for HCV genome replication can be partly complemented from the intracellular ATP pool.

Although there are several isoforms of CK as described above, the most abundant CK species expressed in Huh-7 cells in the present study was CKB, and no other isoenzymes, including mitochondrial CK, were detected by an isoform analysis based on the overlay gel technique (32; data not shown). Thus, the CKB isoenzyme appears to be a key molecule in the energy metabolism of HCV replicating cells. To identify potential HCV RC components, we used a comparative proteome analysis of the DRM fraction in cells harboring HCV subgenomic replicon and the DRM fractions in parental cells and then identified proteins that were more abundant in the fraction of HCV replicating cells. In agreement with similar previously reported approaches using the DRM or lipid raft fraction (30, 53), the functional categories of identified proteins included protein folding or assembly, cell metabolism and biosynthesis, cellular processes, and cytoskeleton organization (Table 1). Interestingly, Mannova et al. found that CKB was upregulated in the fraction of Huh-7 cells carrying the genotype 1b Con1 isolate-derived HCV replicon, as determined using stable isotope labeling by amino acids combined with one-dimensional electrophoresis (30). However, the effect of CKB on regulation of the HCV life cycle was not examined in that study.

In conclusion, CKB interacts with HCV NS4A and is important for efficient replication of the viral genome. Recruitment of CKB to the HCV replication machinery through its interaction with NS4A may have important implications for the maintenance or enhancement of the functional replicase activity in the RC compartment, where high-energy phosphoryl groups are required. A strategy for specific interception of energy supply at the subcellular site of HCV genome replication by disruption of the NS4A-CKB interface may lead to development of a new type of antiviral agent.

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Proteasomal Turnover of Hepatitis C Virus Core Protein Is Regulated by Two Distinct Mechanisms: a Ubiquitin-Dependent Mechanism and a Ubiquitin-Independent but PA28 γ -Dependent Mechanism[∇]

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We have previously reported on the ubiquitylation and degradation of hepatitis C virus core protein. Here we demonstrate that proteasomal degradation of the core protein is mediated by two distinct mechanisms. One leads to polyubiquitylation, in which lysine residues in the N-terminal region are preferential ubiquitylation sites. The other is independent of the presence of ubiquitin. Gain- and loss-of-function analyses using lysineless mutants substantiate the hypothesis that the proteasome activator PA28 γ , a binding partner of the core, is involved in the ubiquitin-independent degradation of the core protein. Our results suggest that turnover of this multifunctional viral protein can be tightly controlled via dual ubiquitin-dependent and -independent proteasomal pathways.

Hepatitis C virus (HCV) core protein, whose amino acid sequence is highly conserved among different HCV strains, not only is involved in the formation of the HCV virion but also has a number of regulatory functions, including modulation of signaling pathways, cellular and viral gene expression, cell transformation, apoptosis, and lipid metabolism (reviewed in references 9 and 15). We have previously reported that the E6AP E3 ubiquitin (Ub) ligase binds to the core protein and plays an important role in polyubiquitylation and proteasomal degradation of the core protein (22). Another study from our group identified the proteasome activator PA28 γ /REG- γ as an HCV core-binding partner, demonstrating degradation of the core protein via a PA28 γ -dependent pathway (16, 17). In this work, we further investigated the molecular mechanisms underlying proteasomal degradation of the core protein and found that in addition to regulation by the Ub-mediated pathway, the turnover of the core protein is also regulated by PA28 γ in a Ub-independent manner.

Although ubiquitylation of substrates generally requires at least one Lys residue to serve as a Ub acceptor site (5), there is no consensus as to the specificity of the Lys targeted by Ub (4, 8). To determine the sites of Ub conjugation in the core protein, we used site-directed mutagenesis to replace individual Lys residues or clusters of Lys residues with Arg residues in the N-terminal 152 amino acids (aa) of the core (C152), within which is contained all seven Lys residues (Fig. 1A). Plasmids expressing a variety of mutated core proteins were generated by PCR and inserted into the pCAGGS (18). Each core-expressing construct was transfected into human embryonic kidney 293T cells along with the pMT107 (25) encoding a Ub

moiety tagged with six His residues (His₆). Transfected cells were treated with the proteasome inhibitor MG132 for 14 h to maximize the level of Ub-conjugated core intermediates by blocking the proteasome pathway and were harvested 48 h posttransfection. His₆-tagged proteins were purified from the extracts by Ni²⁺-chelation chromatography. Eluted protein and whole lysates of transfected cells before purification were analyzed by Western blotting using anticore antibodies (Fig. 1B). Mutations replacing one or two Lys residues with Arg in the core protein did not affect the efficiency of ubiquitylation: detection of multiple Ub-conjugated core intermediates was observed in the mutant core proteins comparable to the results seen with the wild-type core protein as previously reported (23). In contrast, a substitution of four N-terminal Lys residues (C152K6-23R) caused a significant reduction in ubiquitylation (Fig. 1B, lane 9). Multiple Ub-conjugated core intermediates were not detected in the Lys-less mutant (C152KR), in which all seven Lys residues were replaced with Arg (Fig. 1B, lane 11). These results suggest that there is not a particular Lys residue in the core protein to act as the Ub acceptor but that more than one Lys located in its N-terminal region can serve as the preferential ubiquitylation site. In rare cases, Ub is known to be conjugated to the N terminus of proteins; however, these results indicate that this does not occur within the core protein.

To investigate how polyubiquitylation correlates with proteasome degradation of the core protein, we performed kinetic analysis of the wild-type and mutated core proteins by use of the Ub protein reference (UPR) technique, which can compensate for data scatter of sample-to-sample variations such as levels of expression (10, 24). Fusion proteins expressed from UPR-based constructs (Fig. 2A) were cotranslationally cleaved by deubiquitylating enzymes, thereby generating equimolar quantities of the core proteins and the reference protein, dihydrofolate reductase-hemagglutinin (DHFR-HA) tag-modified Ub, in which the Lys at aa 48 was replaced by Arg to prevent its polyubiquitylation (Ub^{R48}). After 24 h of transfection

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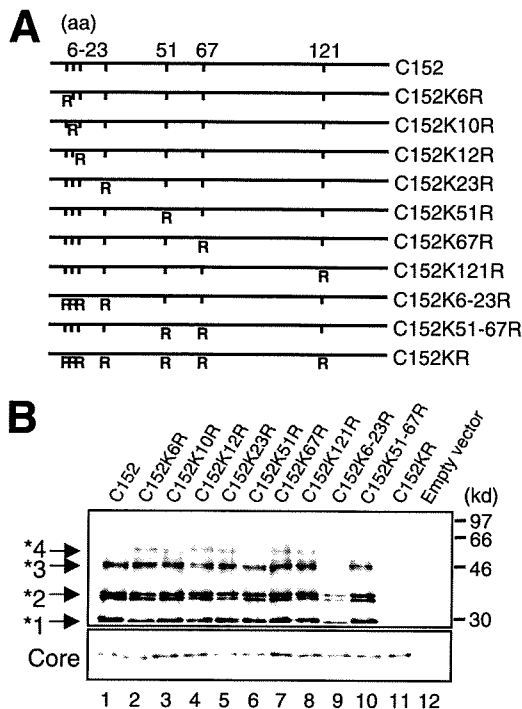


FIG. 1. In vivo ubiquitylation of HCV core protein. (A) The HCV core protein (N-terminal 152 aa) is represented on the top. The positions of the amino acid residues of the core protein are indicated above the bold lines. The positions of the seven Lys residues in the core are marked by vertical ticks. Substitution of Lys with Arg (R) is schematically depicted. (B) Detection of ubiquitylated forms of the core proteins. The transfected cells with core expression plasmids and pMT107 were treated with the proteasome inhibitor MG132 and harvested 48 h after transfection. His₆-tagged proteins were purified and subsequently analyzed by Western blot analysis using anticore antibody (upper panel). Core proteins conjugated to a number of His₆-Ub are denoted with asterisks. Whole lysates of transfected cells before purification were also analyzed (lower panel). Lanes 1 to 11, C152 to C152KR, as indicated for panel A. Lane 12; empty vector.

tion with UPR constructs, cells were treated with cycloheximide and the amounts of core proteins and DHFR-HA-Ub^{R48} at the indicated time points were determined by Western blot analysis using anticore and anti-HA antibodies. The mature form of the core protein, aa 1 to 173 (C173) (13, 20), and C152 were degraded with first-order kinetics (Fig. 2B and D). MG132 completely blocked the degradation of C173 and C152 (Fig. 2B), and C152K6-23R and C152KR were markedly stabilized (Fig. 2C). The half-lives of C173 and C152 were calculated to be 5 to 6 h, whereas those of C152K6-23R and C152KR were calculated to be 22 to 24 h (Fig. 2D), confirming that the Ub plays an important role in regulating degradation of the core protein. Nevertheless, these results also suggest possible involvement of the Ub-independent pathway in the turnover of the core protein, as C152KR is more destabilized than the reference protein (Fig. 2C and 2D).

We have shown that PA28 γ specifically binds to the core protein and is involved in its degradation (16, 17). Recent studies demonstrated that PA28 γ is responsible for Ub-independent degradation of the steroid receptor coactivator SRC-3 and cell cycle inhibitors such as p21 (3, 11, 12). Thus, we next investigated the possibility of PA28 γ involvement in the deg-

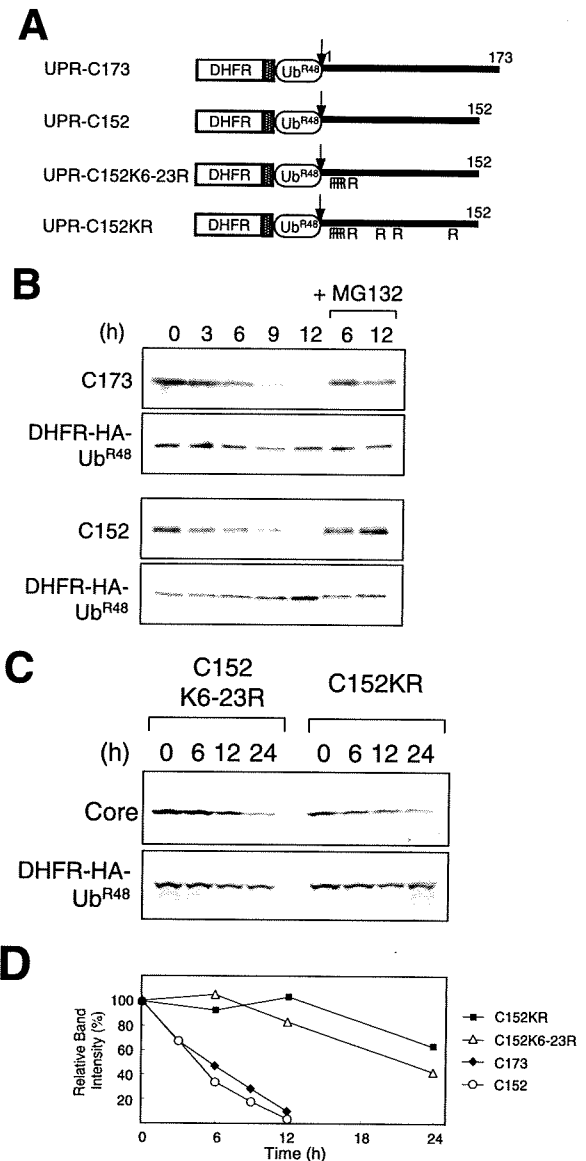


FIG. 2. Kinetic analysis of degradation of HCV core proteins. (A) The fusion constructs used in the UPR technique. Open boxes indicate the DHFR sequence, which is extended at the C terminus by a sequence containing the HA epitope (hatched boxes). Ub^{R48} moieties bearing the Lys-Arg substitution at aa 48 are represented by open ellipses. Bold lines indicate the regions of the core protein. The amino acid positions of the core protein are indicated above the bold lines. The arrows indicate the sites of in vivo cleavage by deubiquitylating enzymes. (B and C) Turnover of the core proteins. After a 24-h transfection with each UPR construct, cells were treated with 50 μ g of cycloheximide/ml in the presence or absence of 10 μ M MG132 for the different time periods indicated. Cells were lysed at the different time points indicated, followed by evaluation via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis using antibodies against the core protein and HA. (D) Quantitation of the data shown in panels B and C. At each time point, the ratio of band intensity of the core protein relative to the reference DHFR-HA-Ub^{R48} was determined by densitometry and is plotted as a percentage of the ratio at time zero.

radation of either C152KR or C152. Since C152KR carries two amino acid substitutions in the PA28 γ -binding region (aa 44 to 71) (17), we tested the influence of the mutations of C152KR on the interaction with PA28 γ by use of a coimmunoprecipi-

tation assay. When Flag-tagged PA28 γ (F-PA28 γ) was expressed in cells along with C152 or C152KR, F-PA28 γ precipitated along with both C152 and C152KR, indicating that PA28 γ interacts with both core proteins (Fig. 3A). Figure 3B reveals the effect of exogenous expression of F-PA28 γ on the steady-state levels of C152 and C152KR. Consistent with previous data (17), the expression level of C152 was decreased to a nearly undetectable level in the presence of PA28 γ (Fig. 3B, lanes 1 and 3). Interestingly, exogenous expression of PA28 γ led to a marked reduction in the amount of C152KR expressed (Fig. 3B, lanes 5 and 7). Treatment with MG132 increased the steady-state level of the C152KR in the presence of F-PA28 γ as well as the level of C152 (Fig. 3B, lanes 4 and 8).

We further investigated whether PA28 γ affects the turnover of Lys-less core protein through time course experiments. C152KR was rapidly destabilized and almost completely degraded in a 3-h chase experiment using cells overexpressing F-PA28 γ (Fig. 3C, left panels). A similar result was obtained using an analogous Lys-less mutant of the full-length core protein C191KR (Fig. 3C, right panels), thus demonstrating that the Lys-less core protein undergoes proteasomal degradation in a PA28 γ -dependent manner. These results suggest that PA28 γ may play a role in accelerating the turnover of the HCV core protein that is independent of ubiquitylation.

Finally, we examined gain- and loss-of-function of PA28 γ with respect to degradation of full-length wild-type (C191) and mutated (C191KR) core proteins in human hepatoma Huh-7 cells. As expected, exogenous expression of PA28 γ or E6AP caused a decrease in the C191 steady-state levels (Fig. 4A). In contrast, the C191KR level was decreased with expression of PA28 γ but not of E6AP. We further used RNA interference to inhibit expression of PA28 γ or E6AP. An increase in the abundance of C191KR was observed with PA28 γ small interfering RNA (siRNA) but not with E6AP siRNA (Fig. 4B). An increase in the C191 level caused by the activity of siRNA against PA28 γ or E6AP was confirmed as well.

Taking these results together, we conclude that turnover of the core protein is regulated by both Ub-dependent and Ub-independent pathways and that PA28 γ is possibly involved in Ub-independent proteasomal degradation of the core protein. PA28 is known to specifically bind and activate the 20S proteasome (19). Thus, PA28 γ may function by facilitating the delivery of the core protein to the proteasome in a Ub-independent manner.

Accumulating evidence suggests the existence of proteasome-dependent but Ub-independent pathways for protein degradation, and several important molecules, such as p53, p73, Rb, SRC-3, and the hepatitis B virus X protein, have two distinct degradation pathways that function in a Ub-dependent and Ub-independent manner (1, 2, 6, 7, 14, 21, 27). Recently, critical roles for PA28 γ in the Ub-independent pathway have been demonstrated; SRC-3 and p21 can be recognized by the 20S proteasome independently of ubiquitylation through their interaction with PA28 γ (3, 11, 12). It has also been reported that phosphorylation-dependent ubiquitylation mediated by GSK3 and SCF is important for SRC-3 turnover (26). Nevertheless, the precise mechanisms underlying turnover of most of the proteasome substrates that are regulated in both Ub-dependent and Ub-independent manners are not well understood. To our knowledge, the HCV core protein is the first

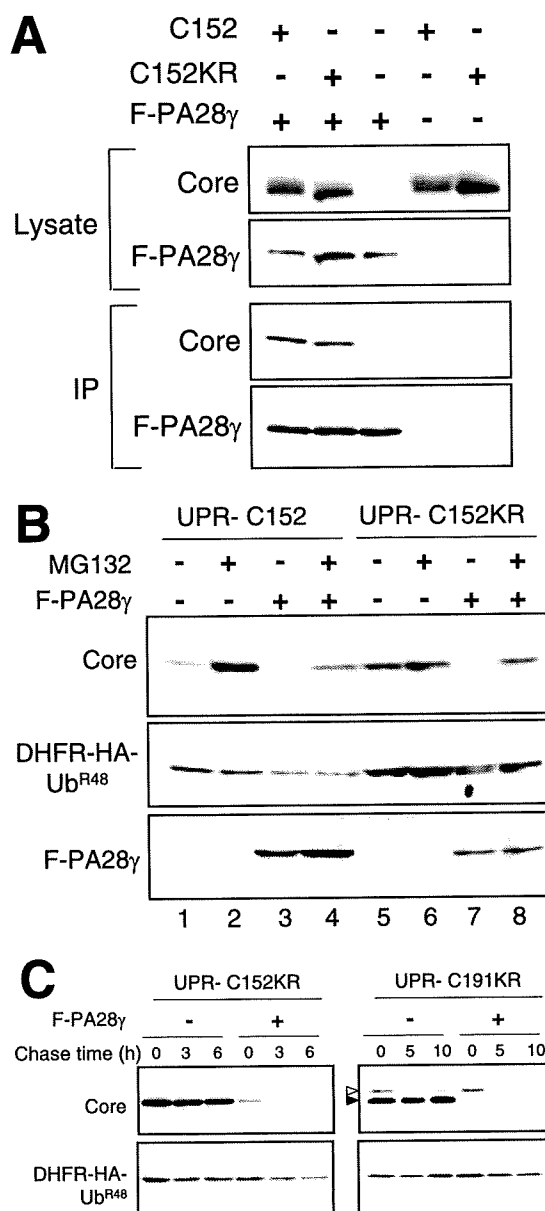


FIG. 3. PA28 γ -dependent degradation of the core protein. (A) Interaction of the core protein with PA28 γ . Cells were cotransfected with the wild-type (C152) or Lys-less (C152KR) core expression plasmid in the presence of a Flag-PA28 γ (F-PA28 γ) expression plasmid or an empty vector. The transfected cells were treated with MG132. After 48 h, the cell lysates were immunoprecipitated with anti-Flag antibody and visualized by Western blotting with anticore antibodies. Western blot analysis of whole cell lysates was also performed. (B) Degradation of the wild-type and Lys-less core proteins via the PA28 γ -dependent pathway. Cells were transfected with the UPR construct with or without F-PA28 γ . In some cases, cells were treated with 10 μ M MG132 for 14 h before harvesting. Western blot analysis was performed using anticore, anti-HA, and anti-Flag antibodies. (C) After 24 h of transfection with UPR-C152KR and UPR-C191KR with or without F-PA28 γ (an empty vector), cells were treated with 50 μ g of cycloheximide/ml for different time periods as indicated (chase time). Western blot analysis was performed using anticore and anti-HA antibodies. The precursor core protein and the core that was processed, presumably by signal peptide peptidase, are denoted by open and closed triangles, respectively.

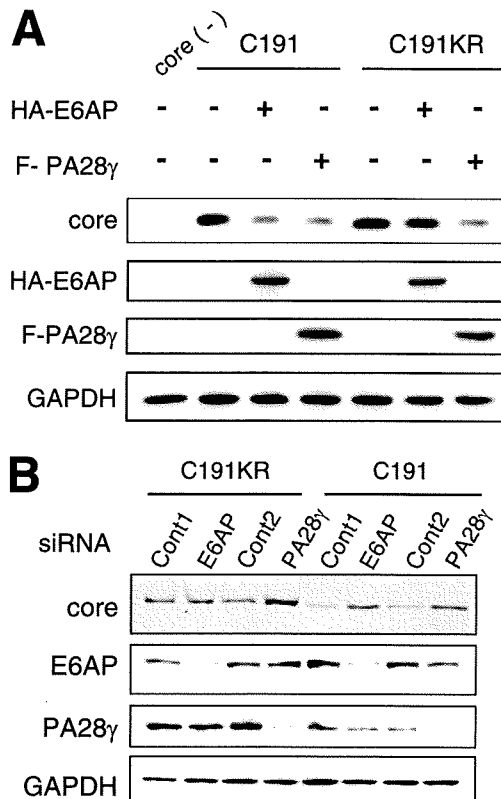


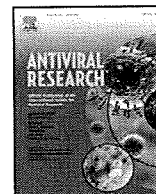
FIG. 4. Ub-dependent and Ub-independent degradation of the full-length core protein in hepatic cells. (A) Huh-7 cells were cotransfected with plasmids for the full-length core protein (C191) or its Lys-less mutant (C191KR) in the presence of F-PA28 γ or HA-tagged-E6AP expression plasmid (HA-E6AP). After 48 h, cells were lysed and Western blot analysis was performed using anticore, anti-HA, anti-Flag, or anti-GAPDH. (B) Huh-7 cells were cotransfected with core expression plasmids along with siRNA against PA28 γ or E6AP or with negative control siRNA. Cells were harvested 72 h after transfection and subjected to Western blot analysis.

viral protein studied that has led to identification of key cellular factors responsible for proteasomal degradation via dual distinct mechanisms. Although the question remains whether there is a physiological significance of the Ub-dependent and Ub-independent degradation of the core protein, it is reasonable to consider that tight control over cellular levels of the core protein, which is multifunctional and essential for viral replication, maturation, and pathogenesis, may play an important role in representing the potential for its functional activity.

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Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin

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ABSTRACT

Ribavirin (RBV), a nucleoside analogue, is used in the treatment of hepatitis C virus (HCV) infection in combination with interferons. However, potential mechanisms of RBV resistance during HCV replication remain poorly understood. Serial passage of cells harboring HCV genotype 2a replicon in the presence of RBV resulted in the reduced susceptibility of the replicon to RBV. Transfection of fresh cells with RNA from RBV-resistant replicon cells demonstrated that the RBV resistance observed is largely replicon-derived. Four major amino acid substitutions: T1134S in NS3, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B region, were identified. Site-directed mutagenesis of these mutations into the replicon indicated that Y2471H plays a role in the reduced susceptibility to RBV and leads to decrease in replication fitness. The results, in addition to analysis of sequence database, suggest that HCV variants with reduced susceptibility to RBV identified are preferential to genotype 2a.

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1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, such as chronic hepatitis, cirrhosis and hepatocellular carcinoma, affecting approximately 170 million people worldwide (WHO, 2000). HCV belongs to the genus Hepacivirus of the family Flaviviridae, and its genome is a single-stranded, positive-sense RNA of 9.6 kb. HCV displays marked genetic heterogeneity and is currently classified into 6 major genotypes and more than 50 subtypes. HCV genotypes have regional distribution and, of those, genotypes 1 and 2 are detected worldwide (Simmonds et al., 2000). Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN- α) in combination with ribavirin (RBV). However, approximately 50% of treated patients infected with genotype 1 do not respond or show only a partial or transient response and treatment is limited by the adverse effects of both agents (Manns et al., 2001; Fried et al., 2002).

HCV replication is associated with a high rate of mutation that gives rise to a mixed and changing population of mutants, known as quasispecies (Martell et al., 1992; Domingo, 1996). The characteristic of HCV may have important implications concerning viral persistence, pathogenicity and resistance to antiviral agents

(Domingo, 1996; Forns et al., 1999; Farci and Purcell, 2000). Most previous studies on the possible relationship between HCV quasispecies and response to chemotherapy have been carried out in HCV genotype 1 patients. In addition, several studies have successfully demonstrated that the HCV subgenomic replicon is derived from genotype 1, which typically contains HCV nonstructural genes placed downstream of the neomycin phosphotransferase gene, in selecting variants resistant to antiviral inhibitors. Two studies have demonstrated the identification of HCV genotype 1 mutants responsible for decreased sensitivity to RBV (Young et al., 2003; Pfeiffer and Kirkegaard, 2005). However, little is known about the generation of genotype 2 isolates resistant to antivirals including RBV, or the molecular mechanisms that confer resistance.

In this study, we report the generation and characterization of HCV genotype 2a replicon variants with reduced susceptibility to RBV. The impacts of major amino acid substitutions observed on RBV susceptibility and viral replication capacity were also examined.

2. Materials and methods

2.1. Compounds

RBV and IFN- α were purchased from MP Biomedicals (Eschwege, Germany) and Daiinippon Sumitomo Pharma (Osaka, Japan), respectively.

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Table 1
Primers used for PCR and nucleotide sequencing.

Region	Primer name	Nucleotide sequence	Position ^a	Polarity
NS3–4A–4B region	PCR primers			
	JF1S	GAAAAACACGATGATACCATG	1756–1776	Sense
	JF1AS	AACCCAGTCCCACACGTC	4650–4633	Antisense
	Sequencing primers			
	JF5S	CACTTTCAGTGACAACAGCA	2322–2341	Sense
	JF6S	CGCCACCGACGCCCTCATGA	3003–3022	Sense
NS5A–NSSB region	Sequencing primers			
	JF4AS	CTGGTCGACAACGGACTGGT	4109–4090	Antisense
	PCR primers			
	JF2S	TGCTCCGGATCCTGGCTC	4612–4629	Sense
	JF2AS	TACCTAGTGTGTGCCGCTCTA	7786–7806	Antisense
	Sequencing primers			
	JF3S	TGAGGTCCATGCTAACAGA	5209–5228	Sense
	JF4S	TCGAGGGGGAGCCTGGAGAT	5870–5889	Sense
JF3AS	GAGTGTCTAACTGTTTCCAG	7220–7200	Antisense	

^a Reference strain: Gene Bank accession no. AB114136.

2.2. Cell culture

The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with MEM non-essential amino acids (Invitrogen) 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO₂ incubator. HCV replicon cells JFH-1/4-1 (Miyamoto et al., 2006), which are Huh-7-derived cells carrying a subgenomic replicon of JFH-1 (Kato et al., 2003) were maintained in the Huh-7 medium as above, supplemented with 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan).

2.3. Quantification of HCV RNA

Total RNA was isolated from harvested cells using Trizol (Invitrogen). Copy numbers of the viral RNA were determined by real-time RT-PCR involving single-tube reactions and performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA), as described previously (Aizaki et al., 2003; Takeuchi et al., 1999).

2.4. Cell viability assay

Cells were seeded at density of 5×10^4 cells/well in 24-well plates and RBV at various concentrations was added on the next day. Cultures were further incubated for 3 days at 37 °C under a humidified 5% CO₂ atmosphere. Cytotoxicity assay was performed by Cell Titer-GLO™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were quantified with LUMATLB 9501 (Berthold Technologies, Bad Wilbad, Germany).

2.5. Isolation and nucleotide sequencing of HCV nonstructural regions from replicon-containing cells

Total cellular RNA was isolated from replicon cells with or without RBV treatment as described above. cDNA synthesis was carried out by using Super Script™ III First-Strand Synthesis System for RT-PCR (Invitrogen) with primer JF1AS for NS3/4B region and JF2AS for NS5AB region. Two cDNA fragments, corresponding to NS3–NS4B and NS5A–NS5B regions, were amplified by PCR using Takara EX Taq DNA polymerase (Takara BIO, Kyoto, Japan) and specific primers (Table 1; Date et al., 2004). PCR products were subcloned into pGEM-T vector (Promega) and inserts were sequenced using QIA prep^R Spin Mini Prep kit (QIAGEN, Tokyo, Japan). Nucleotide sequences were analyzed with the 3100 Avant Genetic Analyzer (PE Applied Biosystems).

2.6. Plasmid constructions

pSGR-JFH1/luc, a subgenomic replicon construct with luciferase reporter derived from HCV genotype 2a JFH-1 isolate was reported previously (Miyamoto et al., 2006). Mutant replicons carrying T1134S, P1969S, V2405A, and Y2471H were created by PCR-based site-directed mutagenesis and cDNA fragments containing the above mutations were inserted into the corresponding sites of pSGR-JFH/luc. All plasmids were confirmed by sequencing the entire PCR-generated inserts. Each mutant is referred to by the original amino acid (one letter code) followed by the residue positions within the complete open reading frame of full-length JFH-1 and the substituted amino acid (one letter code).

2.7. RNA synthesis and transient replication assay

The transient replication assay method was described previously (Kato et al., 2005). Briefly, purified plasmids of pSGR-JFH1/Luc, -JFH1/Luc-T1134S, -JFH1/Luc-P1969S, -JFH1/Luc-V2405A and -JFH1/Luc-Y2471H were linearized with XbaI and were treated with proteinase K and SDS, followed by phenol–chloroform extraction. RNA was synthesized with Ampliscribe™ T7 Transcription Kits (Epicentre BIO Technologies, Madison, WI, USA). Each transcribed RNA (5 µg) was electroporated into 2.5×10^6 of Huh7 cells pulsed at 290 mV, 975 µFD with Gene pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transfected cells were resuspended in growth medium without selection antibiotics and were plated in 24-well plates at 6×10^4 cells per well. Cells were harvested at different time points post-transfection and were lysed in Passive Lysis Buffer (Promega). Luciferase activity in cells was determined using the Luciferase Assay System (Promega).

3. Results

3.1. Selection of replicon variants derived from genotype 2a with reduced susceptibility to RBV

It has been reported that RBV inhibits HCV RNA replication in Huh-7 cells bearing the viral subgenomic replicon RNAs with the EC₅₀ (50% effective concentration) values of 15–225 µM (Zhou et al., 2003; Tanaka et al., 2004; Kato et al., 2005; aus dem Siepen et al., 2007). To select for RBV-associated replicon variants, cells bearing a genotype 2a HCV replicon were serially passed in the presence of 200 µM RBV as well as 1 mg/ml G418. After 20-week treatment, variant cells were then tested for RBV resistance. HCV RNA levels were determined after a 72-h incubation with various concentrations of RBV in the absence of G418, and about 5-fold-reduced susceptibility to RBV was observed in the variant replicon