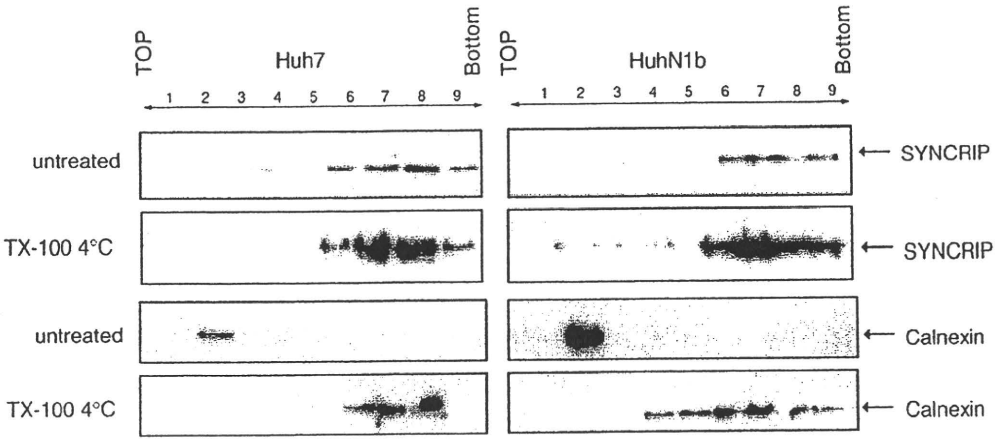

Fig. 1. Membrane flotation assay showed relocalization of SYNCRIP to DRM in HCV replicon cells. Cell lysates were prepared from HCV replicon (HuhN1b) or Huh7 cells by passing through a 25-gauge needle 20 times. Nuclei and unbroken cells were removed by centrifugation at 1000 ×*g* for 5 min in microcentrifuge at 4 °C. The supernatants treated with or without 1% TX-100 at 4 °C for 30 min were fractionated by discontinuous sucrose gradient centrifugation. Fractions were collected from the top, numbered from 1 to 9. Each fraction was concentrated by Centricon YM-30 (Millipore, MA) and immunoblotted by rabbit anti-SYNCRIP antibody or mouse anti-Calnexin antibody, respectively. SYNCRIP was found in both membrane and soluble fractions in the untreated Huh7 and HuhN1b cells, whereas in the HuhN1b cells, some SYNCRIP was localized to the DRM fractions. This phenomenon is not seen in the Calnexin profile.

2003; Mizutani et al., 2000). The nonstructural proteins of HCV are associated with the DRM structures containing Caveolin-2, strongly suggesting that the viral replication complex has properties of lipid rafts (Gao et al., 2004; Mizutani et al., 2000). To determine whether SYNCRIP is in the RNA replication complex, we performed membrane flotation analysis of HCV replicon cells, followed by immunoblotting with anti-SYNCRIP antibody to examine the possible presence of SYNCRIP in the detergent-resistant membrane fractions, where the HCV replication complexes reside. We found that SYNCRIP was present mostly in the cytosolic fractions (fractions 6–9, Fig. 1) in both Huh7 and HuhN1b cells, but a small fraction was associated with the membrane (fractions 2–4, Fig. 1). After treatment with Triton X-100 at 4 °C, some SYNCRIP was still associated with the membrane in HuhN1b cells; in contrast, almost all of SYNCRIP was solubilized in Huh7 cells. Longer exposure of immunoblotting was performed, and SYNCRIP was still not found in the DRM fractions of TX-100 treated Huh7 cells. As a control,

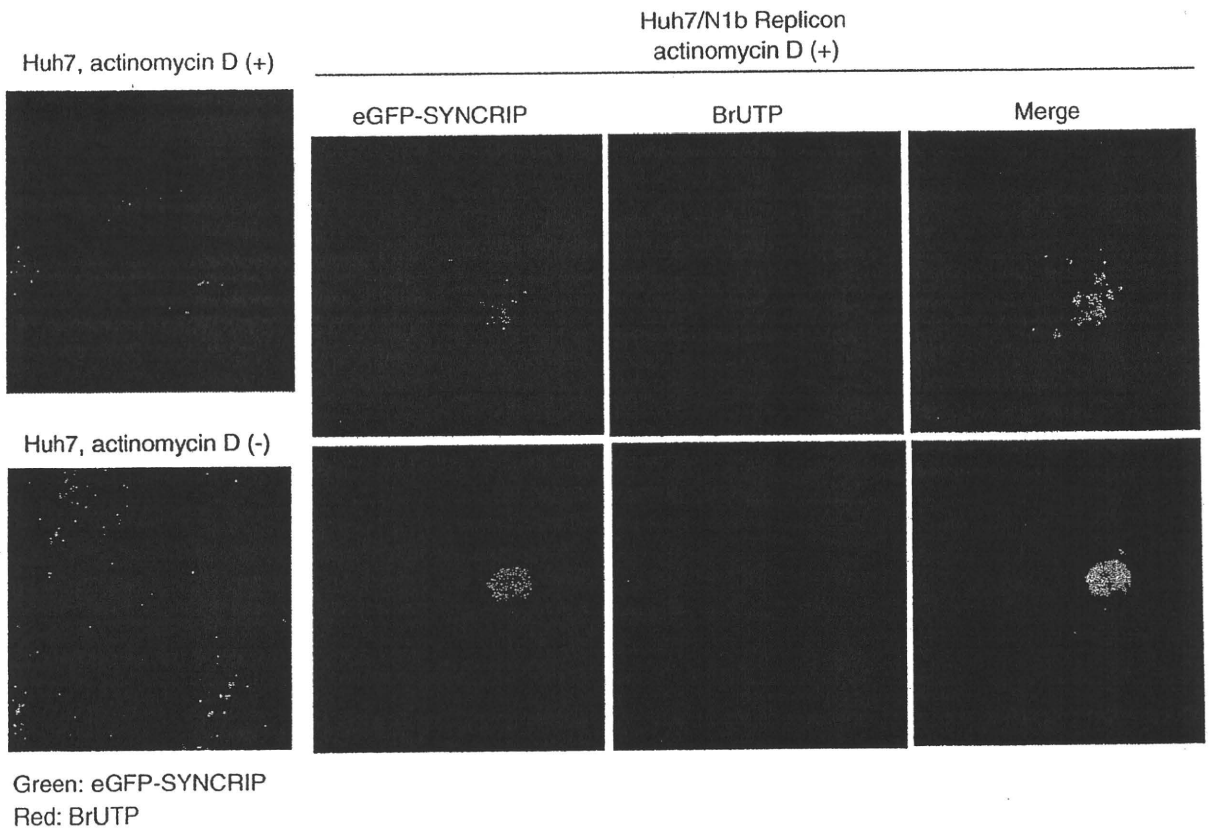

Fig. 2. SYNCRIP colocalization with de novo-synthesized HCV RNA in a HCV replicon cell. peGFP-SYNCRIP was transfected into Huh7 or HCV replicon (HuhN1b) cells by Fugene 6. Two days after transfection, Huh7 or HCV replicon cells were labeled with BrUTP for 15 min after one-hour treatment with Actinomycin D. Actinomycin D treatment inhibited BrUTP incorporation in Huh7 cells (left two panels), but not in HuhN1b replicon cells, where BrU label was detected in the cytoplasm (right 6 panels). Immunofluorescence staining was performed with sheep polyclonal antibody against BrdU (anti-BrdU) followed by Rhodamine-conjugated anti-sheep antibody (Jackson ImmunoResearch). Two different HuhN1b cells are shown, representing two different distribution patterns of BrUTP, as shown previously (Mizutani et al., 2000).

Calnexin, a marker protein of ER membrane, was concentrated exclusively in the membrane fractions (fraction 2–3) in the absence of detergent treatment in both Huh7 and HuhN1b cells (Fig. 1, panel 3). After the cells were treated with cold detergent, Calnexin was redistributed entirely to the soluble fractions, indicating that Calnexin was associated with detergent-soluble membrane, which is a known characteristic of unmodified ER. These data suggested that SYNCRIP is predominantly a cytoplasmic protein, but is relocalized to the DRM fractions (fraction 2–3 after TX-100 treatment) in the HuhN1b replicon cells. The relocalization of SYNCRIP protein, but not Calnexin, to the DRM fraction in the HuhN1b replicon cell indicated that SYNCRIP may be specifically recruited by HCV RNA to the replication complexes, since SYNCRIP binds to HCV RNA (Kim et al., 2004).

SYNCRIP colocalized with de novo synthesized RNA in HCV replicon cells

Previous studies on HCV replicon cells have shown that the newly synthesized HCV RNA and the viral nonstructural proteins colocalized with each other on the distinct speckle-like structure in the cytoplasm of the replicon cells (Gosert et al., 2003; Mizutani et al., 2000). To examine whether SYNCRIP is associated with HCV RNA synthesis in the speckle structures, BrUTP labeling was performed in HCV replicon cells transfected with peGFP-SYNCRIP (a gift from Dr. Mizutani, The University of Tokyo). Briefly, 2 days after transfection of peGFP-SYNCRIP, BrUTP was transfected into actinomycin D-pretreated cells (Kanestrom et al., 1998). Immunofluorescence staining with sheep anti-BrdU polyclonal antibody (Biodesign, ME) was then performed (Kanestrom et al., 1998). Under this condition, all of the BrU-label represents HCV RNA since the cellular transcription is inhibited by actinomycin D treatment. The BrU-labeled RNA was present either in distinct speckle-like structures or in large spherical particles in the cytoplasm of the replicon cell (Fig. 2), consistent with our previous report (Mizutani et al., 2000). These two patterns probably represent two different states of viral RNA synthesis. No BrU-labeled RNA was found in Huh7 cells without an HCV replicon. SYNCRIP was also localized in the cytoplasm in Huh7 cells without HCV replicon, but in a

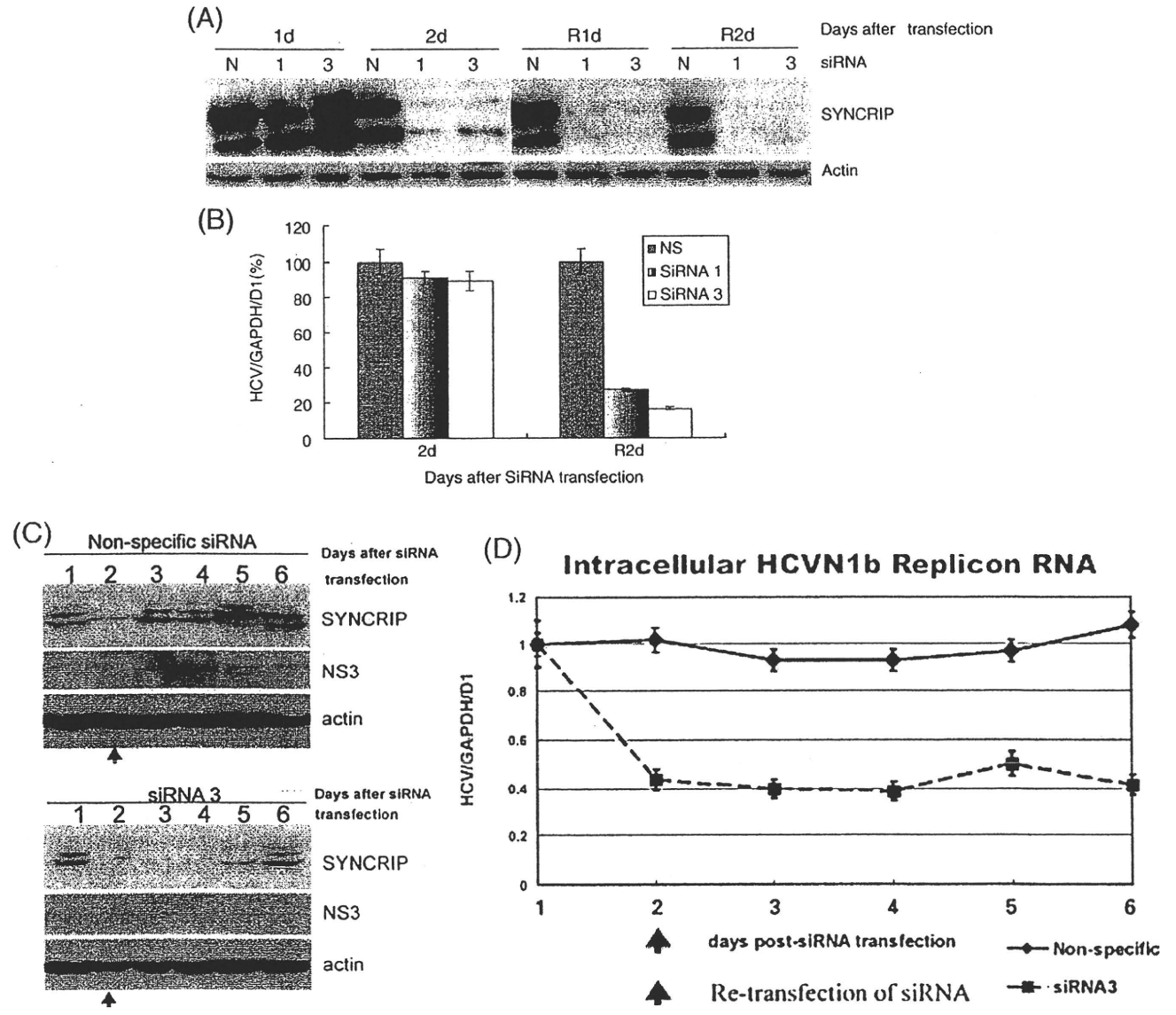

Fig. 3. SYNCRIP knock-down by siRNA in HCV replicon cells. The siRNAs against SYNCRIP (siRNA 1 and 3) (Choi et al., 2004b) or nonspecific siRNA were transfected into HCV replicon cells. Two days after the first transfection, each siRNA was re-transfected into the same cells to ensure complete knock-down of SYNCRIP. Endogenous SYNCRIP protein levels were monitored by immunoblotting (A), and HCV RNA levels were detected by realtime RT-PCR (B). (C), immunoblotting of SYNCRIP and NS3 expression in SYNCRIP siRNA knock-down replicon cells. (D), intracellular replicon RNA level was examined by realtime RT-PCR. R1d and R2d, one or 2 days after re-transfection of siRNA. siRNA 1 and 3, two different clones of siRNA; N, nonspecific siRNA.

more diffuse pattern than that of BrU label in HCV replicon cells. It was found that eGFP-SYNCRIP was partially colocalized with BrU-labeled RNA in the replicon cells (Fig. 2), indicating that only a portion of SYNCRIP was recruited to the HCV RNA replication site. This finding is consistent with the fractionation profile, which showed that SYNCRIP is primarily a cytosolic protein and that only a portion of SYNCRIP is relocalized to the DRM fractions in the replicon cells (Fig. 1). This phenomenon was also observed with PTB in the replicon cell (Aizaki et al., 2006), in which only a small portion of PTB was relocalized to the cytoplasm, whereas the majority remained in the nucleus. These results suggested that a portion of SYNCRIP is localized to the HCV replication complex, implying that SYNCRIP is involved in HCV RNA replication.

In vivo knock-down of SYNCRIP suppressed HCV replication

To determine the biological role of SYNCRIP in HCV RNA replication, we monitored HCV RNA levels in HCV replicon cells in which the endogenous SYNCRIP was knocked down with the RNA interference method (Aizaki et al., 2006; Wagner and Garcia-Blanco, 2002). HuhHyg replicon cells were transfected with either SYNCRIP-specific (siRNA 1 and 3) (Choi et al., 2004b) or nonspecific (NS) siRNA. Protein analysis by immunoblotting was performed with rabbit polyclonal anti-SYNCRIP antibody, and HCV RNA level was monitored by using Taqman quantitative realtime RT-PCR (Gao et al., 2004). The cells transfected with SYNCRIP siRNA showed a significant reduction of the endogenous SYNCRIP by day 2 post-transfection (Fig. 3A). One day after re-transfection with the same siRNAs respectively, almost no endogenous SYNCRIP could be detected (R1d, Fig. 3A). Correspondingly, SYNCRIP siRNA-transfected HCV replicon cells showed a 70–80% reduction of HCV RNA by day 2 after re-transfection with the siRNA as compared to that in the cells transfected with the nonspecific siRNA (R2d, Fig. 3B). The lag time of more than 1 day between the drop of SYNCRIP and that of HCV RNA was probably due to the relative stability of the HCV RNA.

HuhN1b replicon cells were also used to confirm the result obtained using HuhHyg replicon cells. SYNCRIP knock-down was achieved by siRNA transfection, and the viral protein and RNA levels were examined in a time-course study. While the nonspecific siRNA did not significantly affect SYNCRIP expression, the expression levels of SYNCRIP were dramatically decreased after the transfection of specific siRNA against SYNCRIP (siRNA 3) (Fig. 3C). Correspondingly, a 50% decrease in intracellular replicon RNA were detected from day 2 p. t. (Fig. 3D). The decrease in NS3 expression was also detected in siRNA 3-transfected cells; however, the viral protein was not decreased until 4 days after siRNA transfection (Fig. 3C). These results suggest that SYNCRIP affects both HCV RNA translation and RNA replication, but exerts these effects through different mechanisms.

It has been reported that SYNCRIP interacts with HCV RNA fragment spanning nt 342 to 374, corresponding to the N-terminus of the core protein-coding region (Kim et al., 2004). Since this region is immediately downstream to the neomycin-phosphotransferase gene in the HCV replicon, it is possible that the observed involvement of SYNCRIP in the HuhN1b replicon cells was due to the possible effects of SYNCRIP on the expression of phosphotransferase. To rule out this possibility, we further examined the role of SYNCRIP in the replication of HCV full-length RNA (pHCV-1b-hyb) without the neomycin phosphotransferase gene. Huh7 cells were first transfected and re-transfected with SYNCRIP-specific siRNA to knock-down the endogenous SYNCRIP protein; 1 day after re-transfection of siRNA, the cells were transfected with the replication-competent full-length HCV RNA (HCV-1b). The SYNCRIP expressions and NS3 levels in siRNA-transfected cells were examined by immunoblotting. SYNCRIP expression was significantly decreased in cells transfected with specific siRNA (siRNA 1 or 3) (Fig. 4A). Correspondingly, NS3 expression was also affected by the specific SYNCRIP siRNA; in siRNA 1- and siRNA 3-transfected cells, NS3 was detected in the first 2 days post-transfection of HCV full-length RNA, but became undetectable thereafter, whereas in the nonspecific siRNA-transfected cells, NS3 was detected up to 4 days after HCV RNA transfection (Fig. 4A). Total intracellular HCV RNA was determined at various days by quantitative realtime RT-PCR. In the cells transfected with the nonspecific siRNA, HCV RNA titer gradually increased during the first 72 h post-transfection, in agreement with the published report (Choi et al., 2004a) (Fig. 4B). In contrast, in the cells transfected with the SYNCRIP-specific siRNA, HCV RNA titer decreased steadily over the same period of time (Fig. 4B). Although NS3 was detected in the first 2 days after HCV RNA transfection in SYNCRIP-knocked down cells (Fig. 4A), there was no sign of HCV replication (Fig. 4B). The intracellular HCV RNA level normalized by NS3 expression level was shown in Fig. 4C. Regardless the NS3 expression detected in SYNCRIP knock-down cells, HCV RNA titer constantly decreased after full-length HCV RNA transfection. There was a slight increase in intracellular HCV RNA level at day 5 post-transfection of HCV full-length RNA, probably due to the increase in SYNCRIP protein level. This result suggested that endogenous SYNCRIP is directly involved in HCV replication, but not through the suppression of the expression of neomycin phosphotransferase gene. These results combined indicate that SYNCRIP is involved in HCV replication by affecting either HCV RNA replication or translation, or both.

SYNCRIP inhibited HCV RNA replication in vitro

The siRNA knock-down approaches showed that once SYNCRIP protein level was decreased, the HCV RNA titer would be correspondingly decreased. Since SYNCRIP has been shown to be directly involved in HCV translation (Kim et al., 2004), the inhibition of HCV RNA replication in SYNCRIP-knock-down cells may have resulted from the indirect effect of inhibition of translation; namely, the viral NS protein synthesis was inhibited, and thereby viral RNA synthesis was decreased.

To distinguish the effect of SYNCRIP on RNA replication from that on translation, we designed experiments to separate viral RNA replication from viral translation. We employed an *in vitro* replication assay using crude membrane fractions of the HCV subgenomic replicon cells (Ali et al., 2002; Gao et al., 2004), after the endogenous SYNCRIP had been knocked down by the siRNA approach. We also performed *in vitro* RNA replication assay after SYNCRIP was depleted with the anti-SYNCRIP antibody from the cell lysates.

Immunoblotting showed that the amount of SYNCRIP in the HuhN1b replicon cells was substantially reduced by the specific siRNA treatment for 2 days (Fig. 5A). At this time, the amount of NS5A was only partially reduced. Cell lysates from siRNA-transfected replicon cells were treated with TX-100 at 4 °C for 30 min and fractionated by sucrose gradient centrifugation to isolate DRM fraction (Aizaki et al., 2006; Ali et al., 2002). The DRM fractions from these cell lysates were then used for *in vitro* replication assay. The HCV RNA synthesis was detected as single band of ^{32}P-labeled RNA. The result showed that there was no detectable RNA replication activity at all in the DRM fractions from the SYNCRIP siRNA-transfected replicon cells when compared with those from the non-transfected or nonspecific siRNA-transfected replicon cells (Fig. 5A). Since there was still a significant amount of NS5A remaining in the siRNA-transfected cells, the total lack of the *in vitro* replication activity in SYNCRIP knocked-down replicon cells suggested a direct role of SYNCRIP in HCV RNA replication.

We further performed an immunodepletion experiment to remove SYNCRIP form the DRM fraction and assessed the effects on HCV RNA replication *in vitro*. For immunodepletion, the DRM fractions from replicon cell lysates were incubated with a rabbit anti-SYNCRIP polyclonal antibody to deplete the endogenous SYNCRIP from the lysate. After incubation, samples were used for cell-free synthesis of

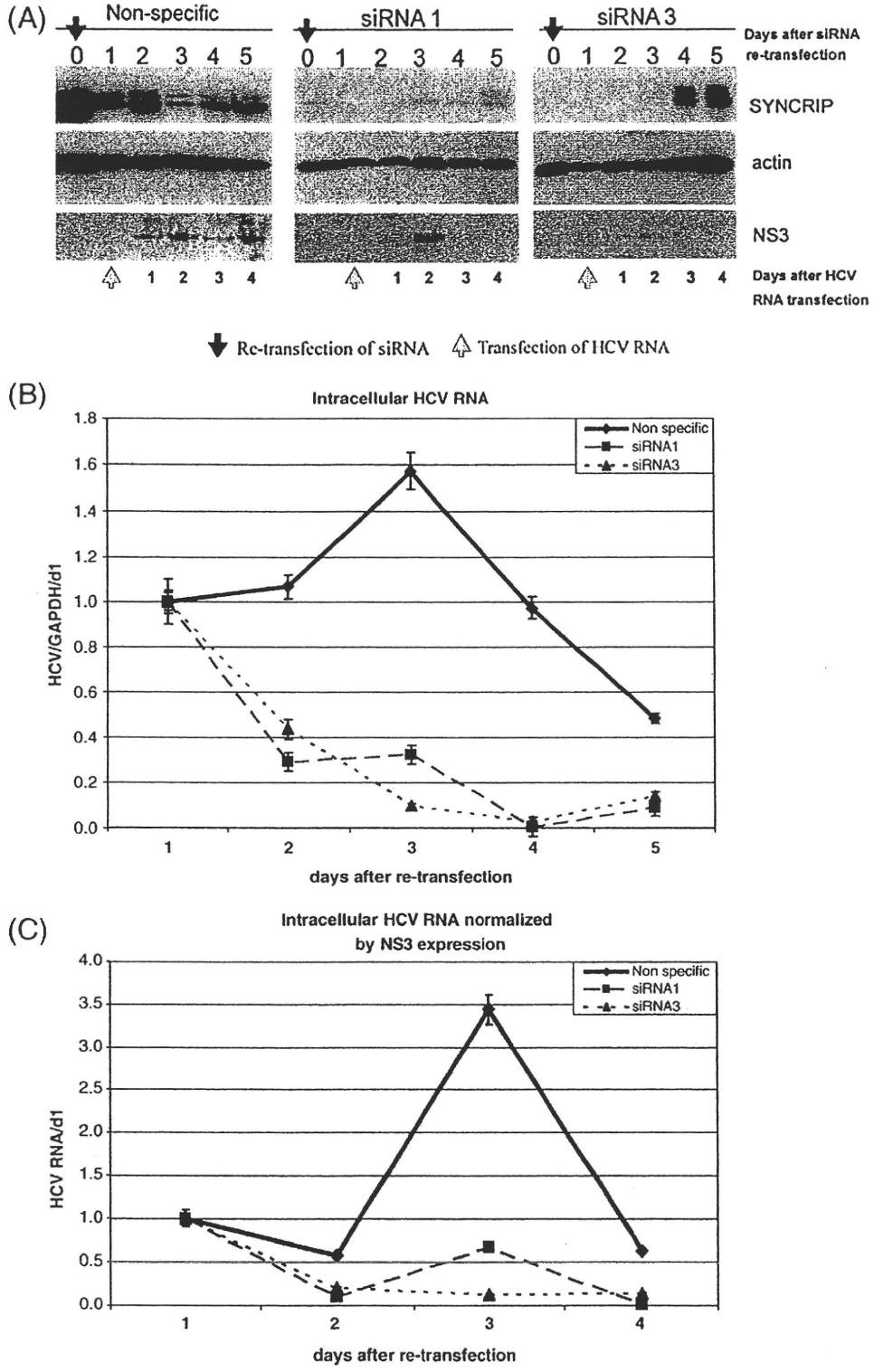

Fig. 4. Deficiency of full-length HCV replication in SYNCRIP knock-down Huh7 cells. The siRNAs against SYNCRIP (siRNA 1 and 3) or nonspecific siRNA were transfected into Huh7 cells twice to knock-down endogenous SYNCRIP level as described in Fig. 3. One day after re-transfection of siRNA, *in vitro* synthesized full-length HCV-1b-hyb RNA was transfected to siRNA-transfected Huh7 cells respectively. (A), immunoblotting of endogenous SYNCRIP after siRNA re-transfection, and NS3 expression levels at various days after HCV RNA transfection. (B), intracellular HCV RNA levels determined by quantitative RT-PCR, and (C), intracellular HCV RNA levels normalized by NS3 expression levels. Huh7 cells were transfected with SYNCRIP-specific or nonspecific siRNA as in (A). One day after siRNA transfection, the replication-competent full-length HCV RNA was transfected into the cells, and HCV RNA levels were detected by realtime RT-PCR on different days after the HCV RNA transfection. The relative amounts of HCV RNA are expressed as in Fig. 3B.

HCV RNA. The results showed that the treatment with anti-SYNCRIP antibody inhibited the replication activity in an antibody concentration-dependent manner, whereas a control anti-Ig antibody did not inhibit any activity at the same or an even higher antibody concentration (Fig. 5B and data not shown). As a control, anti-VAP33 (VAP-A) antibody also inhibited HCV RNA replication, similar to the previous result (Hamamoto et al., 2005), whereas anti-Calnexin antibody did not.

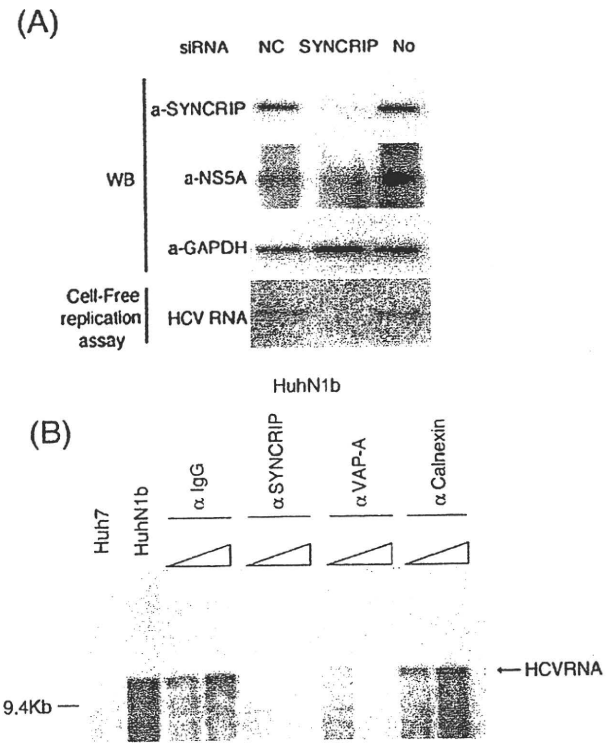

Fig. 5. siRNA-knock-down or immunodepletion of SYNCRIP inhibited HCV replication activity in *in vitro* replication assay. (A) SYNCRIP was knocked down by transfection two times with specific siRNA in HCV replicon cells as in Fig. 3. SYNCRIP, NS5A and GAPDH protein expression were determined by immunoblotting. Cell-free RNA replication assays using the DRM fraction isolated from the cell lysates, as described in Material and methods, were performed. ^{32}P-CTP-labeled HCV RNA product was detected by autoradiography after separation by agarose gel electrophoresis. NC, nonspecific siRNA control; SYNCRIP, SYNCRIP-specific siRNA; No, no siRNA transfection. (B) Partially purified lysates of HCV replicon cells were incubated with anti-IgG, anti-SYNCRIP, anti-VAP-A (VAP-33), or anti-Calnexin antibodies. Then samples were incubated with α-^{32}P-CTP in a cell-free RNA-dependent RNA polymerase assay. The RNA product was separated by formaldehyde agarose gels and identified by autoradiography.

These results combined suggested that SYNCRIP is directly involved in HCV RNA replication, in addition to its role in regulating the translation of HCV RNA. Since SYNCRIP is colocalized with the newly synthesized HCV RNA, it is likely that SYNCRIP is a part of the HCV RNA replication complex and participates in viral RNA synthesis.

Discussion

Our results show that SYNCRIP can modulate HCV RNA replication. It has been previously reported that SYNCRIP can also enhance HCV IRES-dependent translation (Kim et al., 2004). Thus, similar to PTB and La autoantigen, SYNCRIP has dual functions in HCV life cycle. This may be a common characteristic of HCV RNA-binding proteins.

In our study, the relocalization of SYNCRIP to the DRM fractions in HCV replicon cells indicates that SYNCRIP is associated with the RNA replication complex, which is localized in this membrane fraction. It is interesting to note that the distribution of Calnexin was slightly different between the control and the replicon cells; there was some shift of Calnexin toward the lighter sucrose gradient fractions, probably cased by the alteration of cellular membrane structures and the associations of HCV NS proteins to ER membrane structure in HCV replicon cells (Egger et al., 2000; El-Hage and Luo, 2003; Gosert et al., 2003; Mottola et al., 2002). Nevertheless, SYNCRIP was clearly localized in the DRM fraction, whereas Calnexin was not.

The immunodepletion experiments in the current and previous studies have shown that antibody against PTB, hVAP-A, hVAP-B, and SYNCRIP can inhibit HCV RNA replication activities specifically. Previous studies have suggested that the HCV RNA replication complexes are protein complexes with the newly-synthesized RNA being contained within (Yang et al., 2004), and that subtilisin protease treatment could disrupt the replication complexes. However, it was also reported that the HCV replication complexes were resistant to proteinase K treatment at room temperature (Aizaki et al., 2004; Quinkert et al., 2005). The latter study suggested that the HCV replication complexes were very compact, and therefore the accessibility of immunoglobulin to the specific protein target in the replication complex may be limited. However, the ability of these antibodies to inhibit HCV RNA replication suggested that those complexes may not be so compact and are accessible by immunoglobulin molecules under these conditions.

The genome of positive-stranded RNA viruses, such as HCV, poliovirus, and coronavirus, serve as a template for both translation and the synthesis of negative-strand RNA, the latter of which is, in turn, the template for synthesizing more positive-strand RNA. The positive-strand RNA can also be packaged to form new viral particles. Since the same positive-strand RNA can participate in different steps of the viral life cycle, the temporal or spatial regulation is very important. It is likely that the regulation is through RNA–protein interactions. When in complex with specific RNPs, the RNA can be utilized specifically in different steps. With limited numbers of genes in the viral genome, the regulation likely requires the participation of various host factors interacting with the viral RNA or viral proteins.

There are many known host and viral RNA-binding proteins that can facilitate positive-strand RNA replication, such as Tat protein binding to the TAR structure in HIV1 RNA (Dingwall et al., 1989; Wagner and Garcia-Blanco, 2002) and poly(rC)-binding protein binding to the cloverleaf structure of poliovirus RNA (Blyn et al., 1996; Gamarnik and Andino, 2000). However, not so many RNA-binding proteins have been reported to have dual functions in viral RNA replication and translation. Recently, La and PTB, are found to regulate both RNA replication and translation of HCV, probably as a result of their ability to bind to HCV RNA (Aizaki et al., 2006; Domitrovich et al., 2005). Host factors with dual-regulatory functions may play important roles in switching the RNA from translation to replication or replication to translation. For example, PCBP regulates translation–replication switch in poliovirus life cycle (Back et al., 2002; Gamarnik and Andino, 1998). It was reported previously that stem–loop I and II are critical for HCV RNA replication, and stem–loop II, III, and IV are important for HCV RNA translation (El-Hage and Luo, 2003; Fukushi et al., 2001; Qi et al., 2003). La autoantigen was shown to bind to loop IV of HCV 5′NTR (Ali and Siddiqui, 1997). Although there is no evidence that La binds to stem–loop I or II, La can, nevertheless, regulate HCV replication (Domitrovich et al., 2005). Similarly, SYNCRIP was reported to bind at nt 342 to 374 (Kim et al., 2004), a region essential for HCV IRES-driven translation but not HCV replication. Yet we found significant positive regulatory effect of SYNCRIP in HCV RNA replication. It is possible that the binding of SYNCRIP to HCV RNA alters the secondary structure of the RNA or recruits other required factors to facilitate the assembly of the replication complex.

The mechanism of switching between translation and replication of HCV RNA is still unclear; conceivably, it may be regulated by these dual-function proteins which are involved in both replication and translation. It will be interesting to determine in the future whether the relative ratio of these proteins may trigger the switch.

Materials and methods

Cells

Huh7 cells were grown at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids. Huh7N1b and HuhHyg replicon cells

harboring an HCV subgenomic replicon RNA derived from the HCV-N strain (Guo et al., 2001) were grown in the same medium containing 0.5 mg/ml of G418 or 100 μg/ml of Hygromycin (Mizutani et al., 2000).

Antibodies and drugs

The primary antibodies used for the analyses in this study were sheep anti-BrdU polyclonal antibody (BioDesign, ME), mouse anti-BrdU monoclonal antibody (Caltag, CA), anti-Calnexin monoclonal antibody (Abcam, MA), anti-GS27 monoclonal antibody (Abcam, MA). Brefeldin A and Nocodazole were purchased from Sigma, and Actinomycin D was from Fisher. The polyclonal anti-SYNCRIP antibody was generated in rabbits by peptide (amino acid 140 to 152) injection (Mizutani et al., 2000).

Labeling and immunofluorescence staining of de novo-synthesized viral RNA

Labeling of de novo-synthesized viral RNA, immunofluorescence staining and confocal microscopy were modified from the previously described procedures (Kanestrom et al., 1998). Briefly, Huh7 or replicon cells were plated on 8-well chamber slides at a density of 1×10^4 cells per well. Two days after seeding, cells were incubated with actinomycin D (10 μg/ml) for 1 h to inhibit cellular RNA synthesis. Subsequently, 2 mM of bromouridine triphosphate (BrUTP) was transfected into cells at 4 °C for 15 min using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals, IN). The cells were washed with phosphate-buffered saline (PBS) twice and cultured at 37 °C for different incubation durations with DMEM supplemented with 10% FBS. After incubation, cells were washed twice with PBS and subsequently fixed by 4% formaldehyde for 1 h at 4 °C. For permeabilization, the cells were treated with 0.1% Triton X-100 (TX-100) (Sigma-Aldrich, St. Louis, MO) in PBS supplemented with 1% FBS for 30 min at room temperature. Primary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and incubated with cells for 1 h at room temperature. After three washes in PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated or Rhodamine-conjugated secondary antibodies diluted at a 1:100 with PBS containing 5% BSA for 1 h at room temperature. The cells were then washed three times in PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

Membrane flotation, detergent solubilization assay

The membrane flotation assay was performed as previously described (Mizutani et al., 2000). Briefly, cells were first lysed in 1 ml of hypotonic buffer [10 mM Tris–HCV (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$] and passed through a 25-gauge needle 20 times. Nuclei and unbroken cells were removed by centrifugation at 1000 g for 5 min in microcentrifuge at 4 °C. Cell lysates were then mixed with 3 ml of 72% sucrose in low-salt buffer [LSB, comprising 50 mM Tris–HCl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$] and overlaid with 4 ml of 55% sucrose in LSB, followed by 1.5 ml of 10% sucrose in LSB. The sucrose gradient was centrifuged at 38,000 rpm in a Beckman SW41 Ti rotor for 14 h for 4 °C. After centrifugation, 1-ml fractions were taken from the top of the gradient, and each was added 1.7 ml of LSB to dilute sucrose and concentrated by being passed through a Centricon YM-30 filter unit (Millipore, Bedford, MA). One half of each sucrose gradient fraction was separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. After blocking, the membrane was incubated with the primary antibody for 1 h at 37 °C, followed by the appropriate species-specific horseradish peroxidase conjugate, for an additional 1 h at 37 °C. Bound antibody was detected by the ECL-plus system (Amersham, Piscataway, NJ).

Transfection of siRNAs and HCV full-length RNA

The siRNAs against SYCRIP are 19-nt sequences located at nt 189–107 and nt140–1438, respectively, of SYNCRIP open reading frame (ORF) and were synthesized by Integrated DNA Technologies, Inc. (Coralville, IW). siRNAs were designed to target two different sites of the human SYNCRIP gene (5′-CUAUCGUGGUGGAUAUGAAGATT-3′, and 5′-AGACAGUGAUCUCUCUCAUGUTT-3′) chosen with the siRNA target finder software from Ambion (http://www.ambion.com/techlib/misc/siRNA_finder.html) (Choi, Mizutani, and Lai, 2004b). Replicon or Huh7 cells were grown in 10% FBS-DMEM without antibiotics. For transfection, cells were plated to a density of 10^5 cells per well in a 24-well plate on day 1. Three microliters of a 20-μM stock of siRNA duplex was mixed with 47 μl of Opti-MEM (Invitrogen, CA) on day 2. In a separate tube, 3 μl of Lipofectamine 2000 (Invitrogen, CA) was resuspended in 12 μl of Opti-MEM, followed by incubation at RT for 7 min. The two mixtures were combined and allowed to sit at RT for 25 min. After the incubation, 35 μl of Opti-MEM was added and the 100 μl mixture was directly added to the well containing 500 μl of growth medium. On day 3, cells were trypsinized and split into a well of the 12-well plate. On day 4, cells were re-transfected using 6 μl of siRNA with 6 μl of Lipofectamine 2000. On day 5, cells were harvested either for Western blot analysis or for RNA isolation.

pHCV-1bhyb, which contains a full-length HCV RNA hybrid sequence of genotype 1a and 1b under the control of T7 polymerase promoter, has been described previously (Choi et al., 2004a). Full-length HCV RNA was *in vitro* transcribed through T7 promoter to obtain a positive-sense HCV RNA of about 9.6 kb. HCV full-length RNA was then transfected into cells 1 day after siRNA re-transfection with Mirus Trans-IT mRNA transfection reagents (Mirus Bio, WI). Briefly, 1 μl of Booster reagent and 1 μl of Trans-IT reagent were added into 100 μl OPTI-MEM sequentially, followed by 1.5 μg of *in vitro* transcribed HCV RNA. The mixture was incubated for 3 min at RT and added into each well of a 12-well plate containing 1 ml of fresh DMEM supplemented with 10% FBS. Cellular RNA was isolated from each well at 0 to 4 days after HCV RNA transfection.

Cell-free replication assay and immunodepletion experiment

Cell lysate of replicon or control Huh7 cells were prepared by a modified protocol (Ali, Tardif, and Siddiqui, 2002). The cells grown in 100-mm-diameter dishes were washed with cold washing buffer (150 mM sucrose, 30 mM HEPES [pH 7.4], 33 mM ammonium chloride, 7 mM KCl, 4.5 mM magnesium acetate), followed by treatment with lysolecithin buffer (250 μg/ml of washing buffer) for 2 min. Three milliliters of washing buffer were added to each culture plate. The buffer was removed by aspiration. The cells were collected by scraping in 120 μl of incomplete replication buffer (100 mM HEPES [pH 7.4]; 50 mM ammonium chloride; 7 mM potassium chloride; 1 mM spermidine; 1 mM [each] ATP, GTP, and UTP; 10 μM CTP), transferred to a new tube, and lysed gently by pipetting 15 times. The cell suspension was centrifuged at 1600 rpm in a microcentrifuge for 5 min at 4 °C.

For immunodepletion experiment, 40 μl of cytoplasmic fraction (supernatant) obtained as above was treated with 1% Nonidet P-40 (NP-40) (Boehringer Mannheim, Quebec, Canada) at 4 °C for 1 h and incubated with 0.1 μg or 1.0 μg of the indicated antibody with an adjusted amount of PBS at 4 °C for 4 h with rotation. After incubation, sample was incubated with ^{32}P-CTP (30 μCi; 800 Ci/mmol), 10 μg of actinomycin D per ml, and 800 U of RNase inhibitor per ml (Promega Corporation, Wis.) for 3 h at 30 °C. Extraction of RNA from the total mixture was performed with the TRI Regent (Molecular Research Center, Inc., Cincinnati, OH). The RNA were precipitated and eluted in 10 μl of RNase-free water. The replication products were analyzed by gel electrophoresis on 1% formaldehyde agarose gel.

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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 (Biodesign, Saco, ME), 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 (PAbs) 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 (Herfold, 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 Immobiline 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 (NCBInr) 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 BglII 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′-UAAGACCUUCCUGGUGUGGTT-3′] and CKB-2 [5′-CGUCAC CCUUGGUAGAGUUTT-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).

Immunoelectron 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-^{32}P]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-^{32}P]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 $MgCl_2$, 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-N cells is functionally active in a

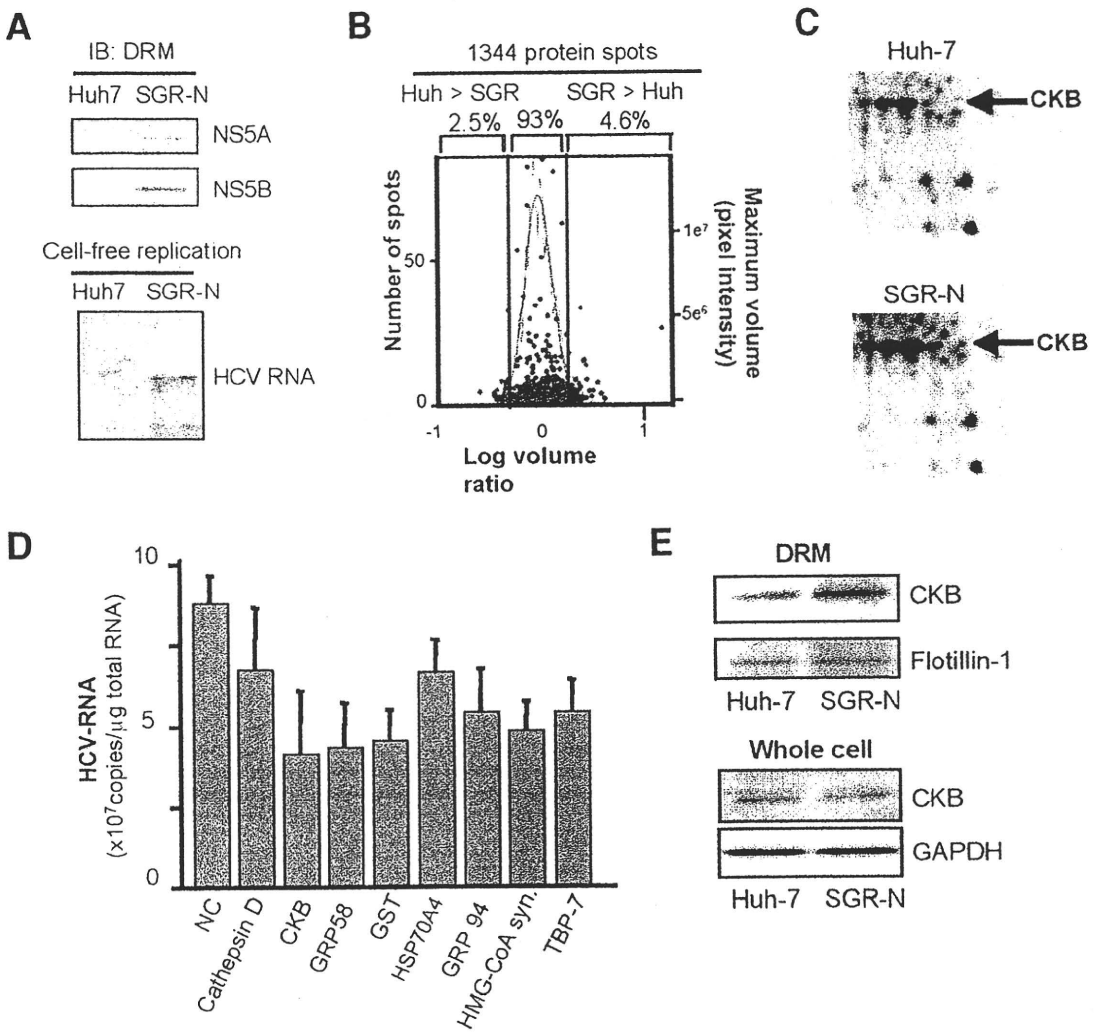

FIG. 1. Comparative proteomic analysis of DRM fractions and RNAi silencing. (A) Preparation of functionally active RC fraction for proteome analysis. DRM fractions obtained from SGR-N cells and parental Huh-7 cells were analyzed by immunoblotting with anti-NS5A and anti-NS5B antibodies (upper panel) 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, flotilin-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 energy 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 *t* 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	Quinolinate 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 (NCBInr) 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-

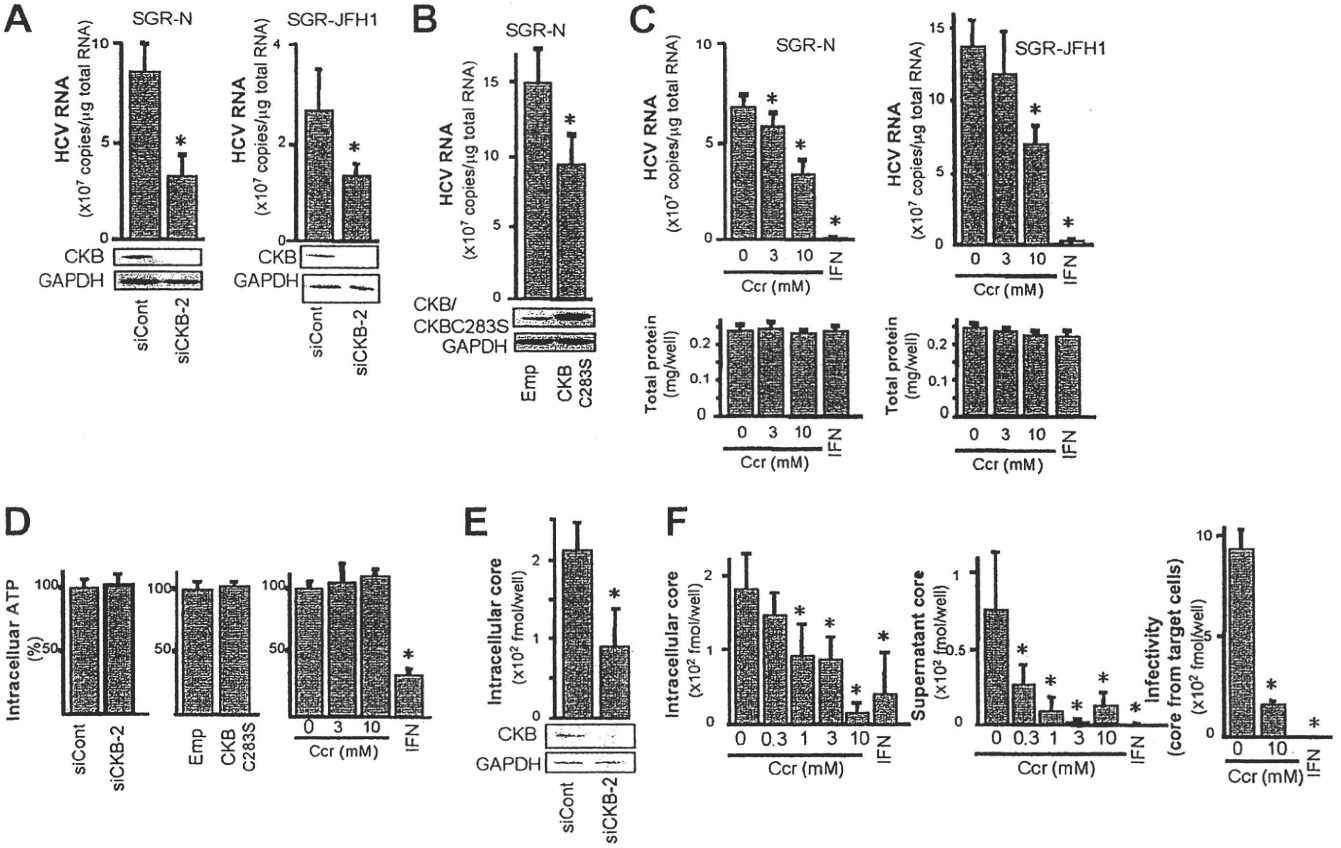

FIG. 2. Involvement of CKB in HCV replication. (A and E) Knockdown of endogenous CKB in SGR-N and SGR-JFH1 cells (A) or HCVcc-infected cells (E). Cells were transfected with siRNA against CKB (siCKB-2) or control siRNA (siCont) and were harvested at 72 h posttransfection. Real-time RT-PCR for HCV RNA 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, NS5A, or NS5B (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), suggesting 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

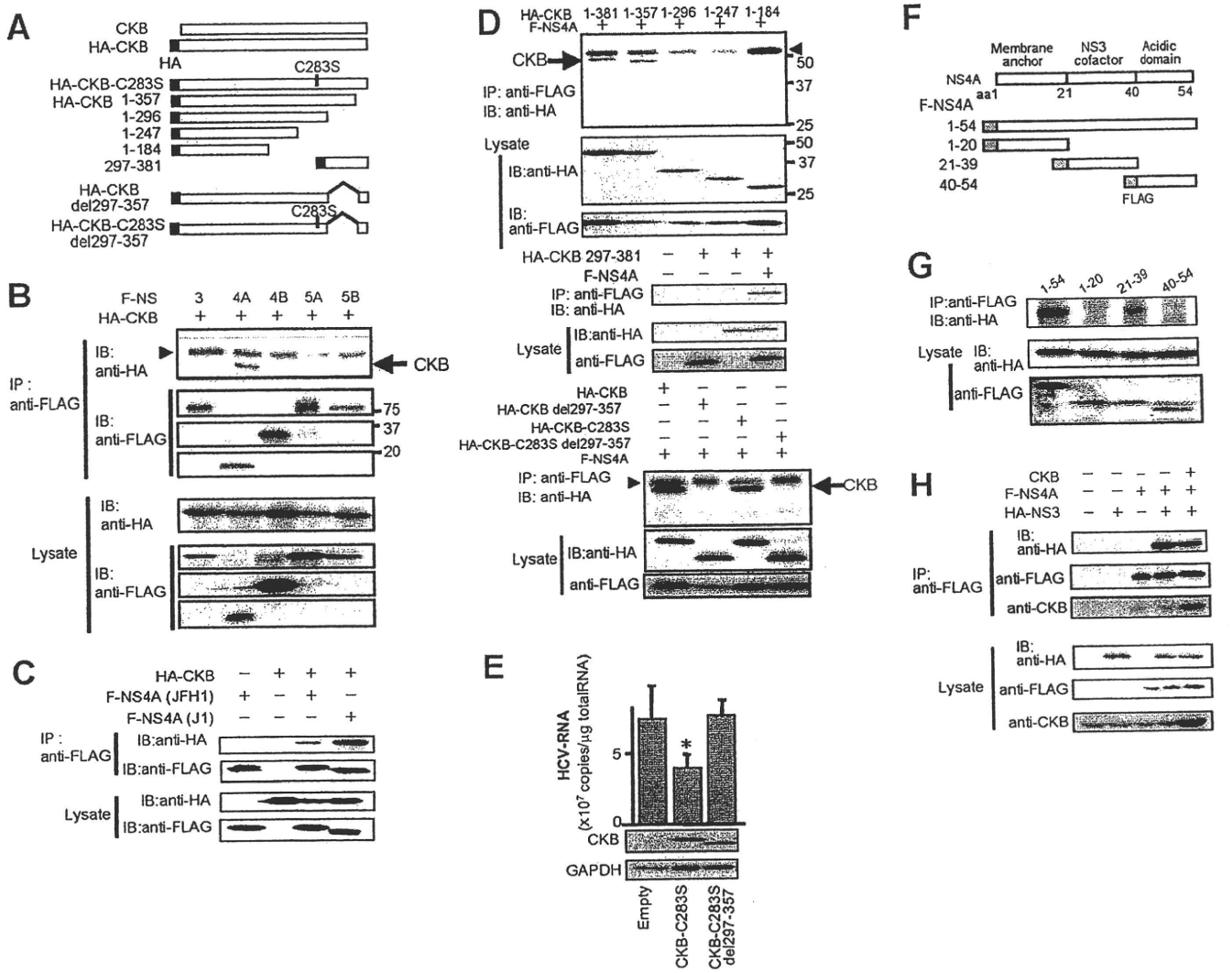

FIG. 3. CKB interacts with HCV NS4A. (A) Structures of CKB constructs used in the present study. A full-length wild-type CKB without an epitope tag (CKB) or with an N-terminal HA tag (HA-CKB), HA-CKB with deletions (aa 1 to 357, aa 1 to 296, aa 1 to 247, aa 1 to 184, and aa 297 to 381 and del297-357), 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-NS3 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

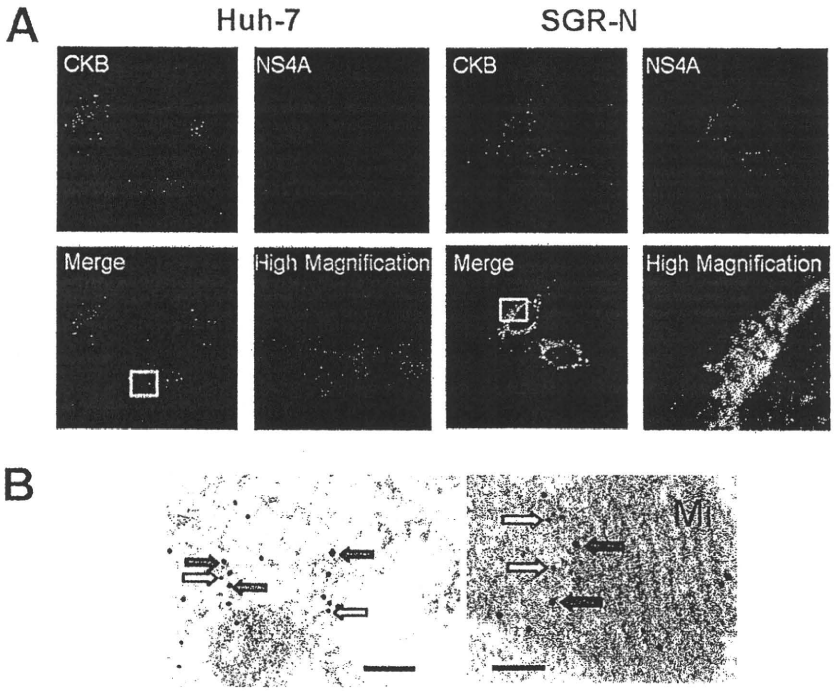

FIG. 4. Colocalization of CKB with HCV NS4A. (A) Indirect immunofluorescence analysis. The primary antibodies used were anti-CKB goat PAb (red) and anti-NS4A MAb (green). Merged images of red and green signals are shown. High-magnification panels are enlarged images of white squares in the merge panels. (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.

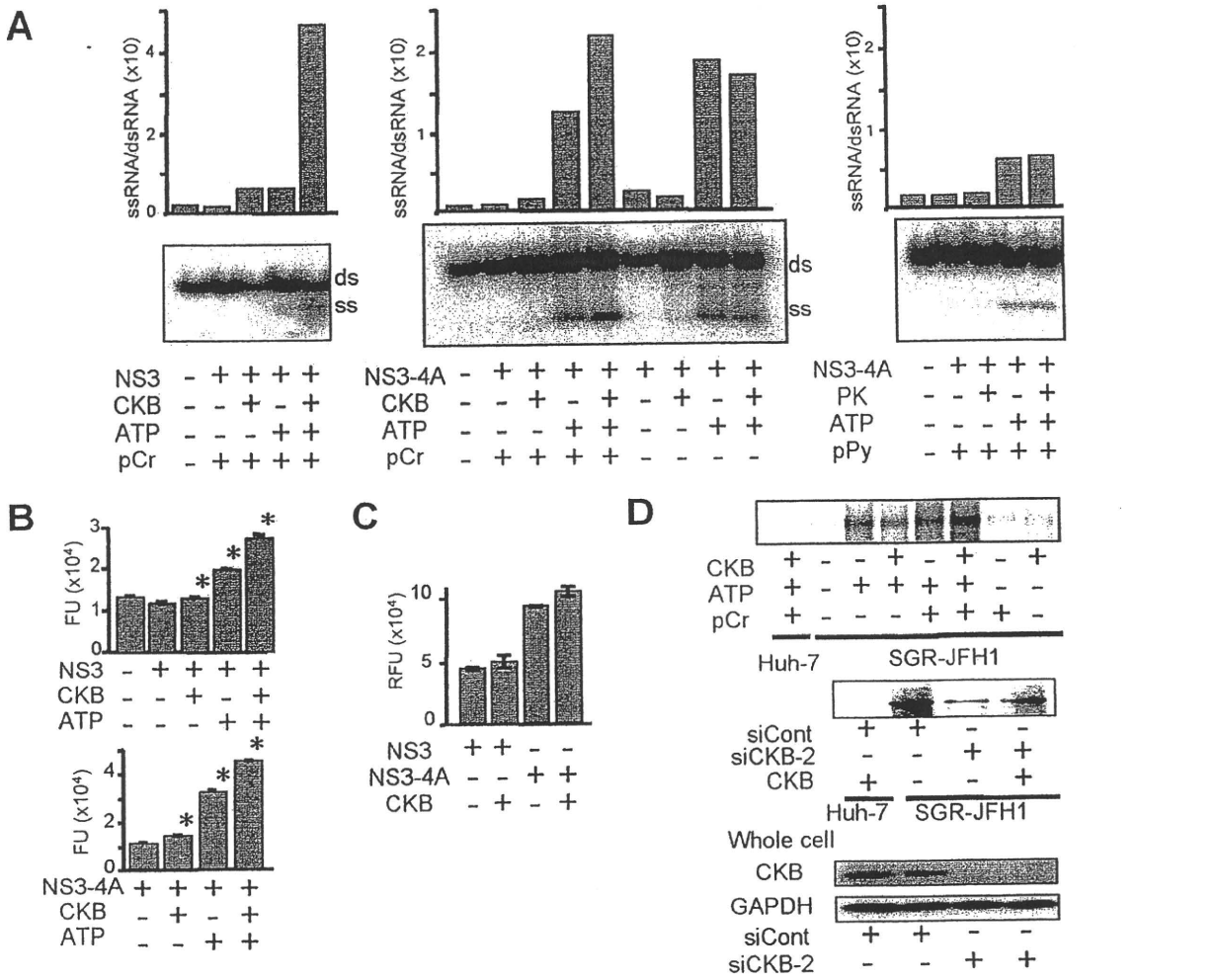

FIG. 5. CKB enhances NS3-4A helicase and HCV replicase activities. (A) In vitro RNA helicase activity of NS3-4A or NS3 was determined by detecting unwound single-strand RNA (ss) derived from the partially dsRNA substrate (ds). Band intensities corresponding to unwound products and those to dsRNA substrates 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 positive 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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Proteomics Analysis of Mitochondrial Proteins Reveals Overexpression of a Mitochondrial Protein Chaperon, Prohibitin, in Cells Expressing Hepatitis C Virus Core Protein

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The hepatitis C virus (HCV) core protein is involved in viral pathogenesis such as oxidative stress induction and lipid metabolism disturbance, and is primarily located in the cytoplasm and endoplasmic reticulum in association with lipid droplets as well as in the mitochondria. To clarify the impact of the core protein on mitochondria, we analyzed the expression pattern of mitochondrial proteins in core protein-expressing cells by two-dimensional polyacrylamide gel electrophoresis. Several proteins related to the mitochondrial respiratory chain or protein chaperons were identified by mass spectrometry. Among the identified proteins with consistently different expressions, prohibitin, a mitochondrial protein chaperon, was up-regulated not only in core-expressing cells but also in full-genomic replicon cells and livers of core-gene transgenic mice. The stability of prohibitin was increased through interaction with the core protein. Further analysis demonstrated that interaction of prohibitin with mitochondrial DNA-encoded subunits of cytochrome c oxidase (COX) was disturbed by the core protein, resulting in a significant decrease in COX activity. *Conclusion:* The HCV core protein affects the steady-state levels of a subset of mitochondrial proteins including prohibitin, which may lead to an impaired function of the mitochondrial respiratory chain with the overproduction of oxidative stress. (HEPATOLOGY 2009;50:378-386.)

The hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which often leads to cirrhosis and, eventually, to the development of hepatocellular carcinoma (HCC). However, the mechanism of hepatocarcinogenesis in HCV infection is not yet fully elucidated. The HCV core protein forms the viral nucleocapsid protein and has various properties that modulate cellular processes in numerous ways. The core protein binds to cellular proteins, suppresses or enhances apoptosis, and modulates the transcription of some host genes.[1] In addition, transgenic mice expressing the core protein develop HCC,[2-4] indicating a direct contribution of the core protein to the pathogenesis of hepatitis C.

The core protein is mostly localized to the endoplasmic reticulum (ER), but we and other groups have shown its localization to the mitochondria in cultured cells and transgenic mice.[2,5,6] In addition, the double structure of mitochondrial membranes is disrupted in hepatocytes of core-gene transgenic mice.[2-4] Evidence suggests that the core protein modulates some mitochondrial functions, including fatty acid β-oxidation, the impairment of which may induce lipid abnormalities and hepatic steatosis. In addition, the mitochondrion is an important source of reactive oxygen species (ROS). In livers of transgenic

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; COX, cytochrome c oxidase; ER, endoplasmic reticulum; Ero1, ER protein endoplasmic oxidoreduction-1; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSP, heat shock protein; IFN, interferon; MnSOD, manganese superoxide dismutase; NS, nonstructural; OST48, oligosaccharyltransferases-48; PDH, pyruvate dehydrogenase; PDI, protein disulfide isomerase; ROS, reactive oxygen species; TFA, trifluoroacetic acid.

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mice harboring the core gene, increased ROS production has been observed.[7-9] A recent study found, by the proteomic profiling of biopsy specimens, that an impairment in key mitochondrial processes, including fatty acid oxidation and oxidative phosphorylation, and in the response to oxidative stress occurs in HCV-infected human liver with advanced fibrosis.[10] Therefore, it is probable that the HCV core protein affects mitochondrial functions because such pathogenesis is observed in both HCV core-transgenic mice and HCV-infected patients.[11-13]

The recent progress in proteomics has opened new avenues for disease-related biomarker discovery. Among proteomics approaches, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a technique for the separation and identification of proteins in a sample by displacement in two dimensions oriented at right angles to one another. This method is generally used as a component of proteomics and is the step used for the isolation of proteins for further characterization by mass spectrometry. 2D-PAGE is particularly useful when comparing two related samples such as healthy and diseased tissue. For example, proteins that are more abundant in diseased tissue may represent novel drug targets or diagnostic markers. In fact, several candidate biomarkers for many human cancers have been identified by this approach.[14] There are, however, tens of thousands of proteins in a cell, differing in abundance over six orders of magnitude. 2D-PAGE is not sensitive enough to detect rare proteins, and hence many proteins are not resolved. Therefore, splitting a sample into different fractions is often necessary to reduce the complexity of protein mixtures prior to 2D-PAGE. For this advantage, Lescuyer et al.[15] performed a 2D-PAGE of human mitochondrial proteins derived from the placenta and identified proteins mainly by peptide mass fingerprinting.

In this study, we performed a 2D-PAGE of mitochondria isolated from HepG2 cells stably expressing the HCV core protein and identified several proteins of different expressions when compared with control HepG2 cells. Among up-regulated proteins in the core-expressing cells, we focused on prohibitin, which functions as a mitochondrial protein chaperon, and found that the core protein interacts with prohibitin and represses the interaction between prohibitin and subunit proteins of cytochrome c oxidase (COX), which may lead to decreases in the expression level of the proteins and in COX activity. These results may explain the pathogenesis of liver disease in HCV infection including ROS induction.

Materials and Methods

Cells and Purification of Mitochondria. Hep39 cells,[16] which stably express the HCV core protein, and control HepG2 cells (Hepswx) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1 mg/mL G418. Mitochondria were purified using Nycodenz (Nycomed Pharma, Zürich, Switzerland) according to the protocols reported by Okado-Matsumoto et al.[17] For transient transfection experiments, HepG2 cells were transfected with a core-expression plasmid using TransIT-LT1 (Mirus Bio, Madison, WI). Huh7 cells harboring HCV genotype 1b full-genomic (RCYM1)[18] or subgenomic replicon (5-15), and livers of 3-month-old core-gene transgenic mice[2] were also used for the analysis.

2D-PAGE. Gel electrophoresis in the first dimension was performed using an immobilized pH gradient gel (Immobiline Dry Strip gel, pH 4-7 linear, 13 cm; GE Healthcare, Uppsala, Sweden). The two-dimensional separation was performed on 12.5%, 14 × 16 cm^2, SDS polyacrylamide gels. After the electrophoresis, gels were silver-stained using a silver staining kit (GE Healthcare) according to the manufacturer's protocols. The stained gels were scanned and electronic images of the gels were analyzed using ImageMaster 2D Elite software (GE Healthcare).

In-Gel Digestion and Matrix-Assisted Laser Desorption Ionization, Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). Protein spots on the gels were excised and a "control" piece was cut from a blank region of the gel and processed in parallel with the sample. In-gel digestion with trypsin was performed as reported.[19] The resulting peptides were concentrated using Zip-Tip C18 (Millipore, Bedford, MA). The peptide mixtures were eluted from Zip-Tip with 75% acetonitrile in 0.1% trifluoroacetic acid (TFA). The matrix (α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% TFA) was deposited on a dried sample target. Then 0.5-μL aliquots of the analyte solution were deposited onto matrix surfaces and the solvent was allowed to evaporate at ambient temperature. The digests were analyzed with a TOF mass spectrometer, PE Biosystems Voyager DE STR MALDI (Foster City, CA).

Database Analysis. For protein identification the measured monoisotopic masses of the peptides were analyzed using MS-Fit provided by UCSF (http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm).

Immunoblotting and Immunoprecipitation. Purified mitochondria were lysed and sonicated in RIPA buffer, then centrifuged at 16,000 rpm for 10 minutes. Protein concentration was determined using a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). The samples were separated by sodium dodecyl sulfate (SDS)-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Japan), then blocked with BlockAce (Snow Brand, To-