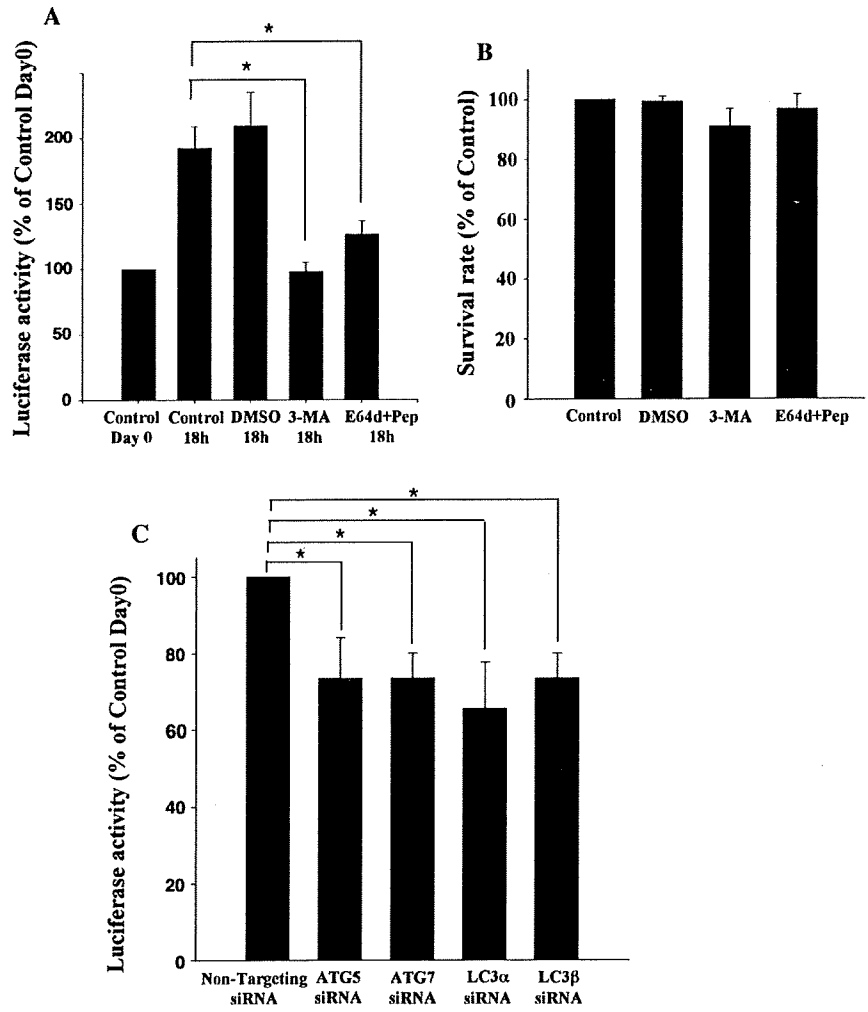

Fig. 2 Inhibition of autophagy suppressed replication of HCV replicon. **a** Cells were treated with 3-methyladenine (3-MA) (10 mM) or a mixture of E64d (1 μg/ml) and pepstatin A (Pep) (1 μg/ml) for 18 h, the levels of replication of HCV replicon were assessed by luciferase assay. **b** Cells were treated with 3-methyladenine (10 mM), mixture of E64d (1 μg/ml) and pepstatin A (1 μg/ml) for 18 h. Cell proliferation reagent WST-1 was added to each well, and the cells were incubated for 1 more hour at 37°C. The absorbance was measured against a background control by microplates reader at 450 nm. The reference wavelength was 650 nm. **c** A combination of four chemically synthesized siRNA duplex molecules targeted to the human ATG5, 7, LC-3α, LC-3β mRNA sequence was transiently transfected into Huh7/Rep-Feo cells using a transfection reagent. siRNA targeted to enhanced green fluorescence protein was used as the control. Forty-eight hours after transfection, levels of HCV replication were analyzed by luciferase assay

Statistical analysis

Differences were compared using ANOVA. Basically P values less than 0.05 were considered as statistically significant.

Results

The inhibition of autophagy suppressed replication of HCV replicon

We counted numbers of autophagosome and autolysosome in cells transduced with HCV replicon Rep-Feo by using electron microscopy. Double membrane vesicles with the morphology of autophagosomes were identified at 2.3 vacuoles/cells in naïve Huh-7 cells, while transfection of HCV replicon increased the number of vacuoles to about fourfold over untransfected Huh-7 cells (Fig. 1a, b). Subsequent treatment of the cells with IFNα (100 U/ml) for 14 days to eliminate HCV replicon substantially decreased the autophagolysosome in cytoplasm of Huh7/Rep-Feo cells (Fig. 1a, b). These observations suggested that HCV replicon induces formation of autophagosomes. To clarify the role of autophagy on the replication of HCV, Huh7/Rep-Feo cells were treated with 3-methyladenine (10 mM) or a mixture of E64d (10 μg/ml) and pepstatin A (10 μg/ml) which inhibited autophagic protein degradation. Replication level of HCV replicon in cells was increased to about twofold after 18 h in control media, however incubation with 3-methyladenine completely blunted increases in replication of HCV replicon. Treatment with 3-methyladenine decreased the number of autophagosomes to about 19% of Huh7/Rep-Feo cells. Furthermore co-incubation with E64d and pepstatin A decreased replication of HCV replicon to about 66% of control (Fig. 2a). Next, WST-1 assay was performed to check the cytotoxicity of these drugs. Treatment with 3-methyladenine or a mixture of

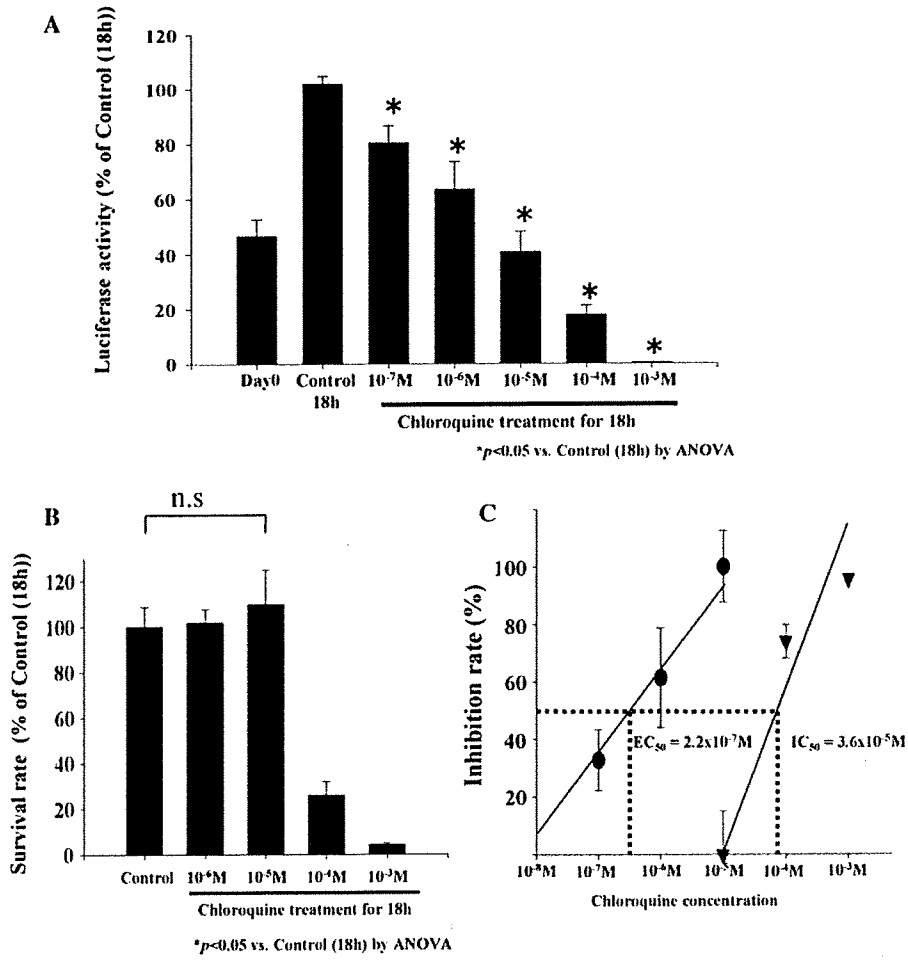

Fig. 3 Effect of chloroquine on inhibition of HCV replication and cell viability. **a** Effect of chloroquine on replication of HCV replicon. Huh-7 Rep/Feo cells were seeded in 48-well plate and incubated with chloroquine (10^{-7}–10^{-3} M) for 18 h. Replication levels of HCV replicon were determined by luciferase assay. Values are shown as percentages of the control cells. [$*P < 0.05$ vs. control (18 h) by ANOVA]. **b** Effect of chloroquine on proliferation of Huh-7 Rep/Feo cell lines in vitro. Cells seeded in 96-well plates were treated with 10^{-6} to 10^{-3}M of chloroquine. After 18 h, effects on cell proliferation were determined by WST-1 assay. [$*P < 0.05$ vs. control (18 h) by ANOVA]. **c** Calculation of EC_{50} and IC_{50}. Concentration of chloroquine inhibiting 50% of the replication of HCV replicon is showed as EC_{50}. IC_{50} is the concentration of chloroquine which inhibits 50% of the cell proliferation of Huh-7 Rep/Feo cells

E64d and pepstatin A did not affect cell viability (Fig. 2b). To clarify the role of autophagy induction in the replication of HCV, we suppressed the induction of autophagy by silencing autophagy-related genes (ATG5, ATG7, LC-3α and LC-3β) by siRNA transfection. Silencing of autophagy-related genes reduced the replication of HCV replicon to about 70% of control (Fig. 2c). Transfection with siRNA of autophagy related genes decreased the number of autophagosomes to about 30% of control. These results indicated that autophagy plays a pivotal role in replication of HCV.

Chloroquine inhibits the replication of HCV replicon

Next, we evaluated the anti-HCV effect of chloroquine, which is a lysosomotropic agent that raises intralysosomal pH and impairs autophagic protein degradation. To assess the effects of chloroquine on the intracellular replication of the HCV replicon, Huh7/Rep-Feo cells were cultured with various concentrations of chloroquine in the medium. The replication of the HCV replicon was increased to about twofold within 18 h in the control media, however, which was suppressed by chloroquine in a dose-dependent manner (Fig. 3a). Next, cytotoxicity of chloroquine was analysed by WST-1 assays. Huh7/Rep-Feo cells treated with chloroquine showed no significant effect on cell viability in doses of lower than 10^{-5} M (Fig. 3b). However, incubation with 10^{-4} M of chloroquine reduced the cell viability to 25% of control. On the basis of the toxicity curve, the IC_{50} of the drug was calculated to be 3.6×10^{-5} M (Fig. 3c). The average EC_{50} of chloroquine was calculated as 2.2×10^{-7} M (Fig. 3c). The replication of HCV replicon was suppressed to nearly 40% of control at 10^{-5} M of chloroquine, which did not affect cell viability. These data indicated that chloroquine efficiently inhibited the replication of HCV replicon in the absence of toxic effect to cells at the concentration of 10^{-5} M. Accordingly, we used 10^{-5} M of chloroquine for the following study.

Next, we conducted the following assay to determine the synergistic inhibitory effect of chloroquine to IFNα on HCV replication. Treatment with chloroquine for 18 h

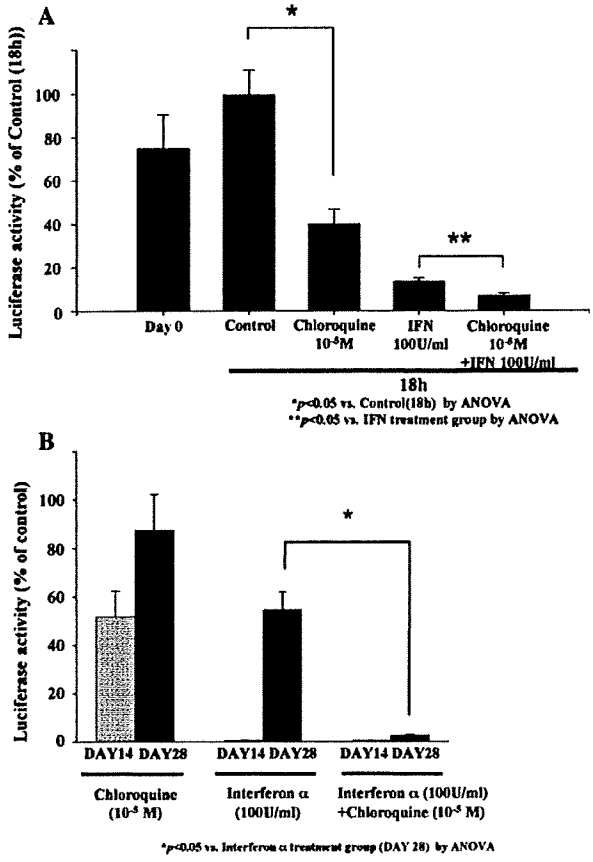

Fig. 4 Combination effect of chloroquine with IFNα on HCV replication. **a** Huh-7 Rep/Feo cells were treated with chloroquine (10^{-5} M) and/or IFNα (100 U/ml) for 18 h. Values are shown as percentages of the control cells [$*P < 0.05$ vs. control (18 h) by ANOVA, $**P < 0.05$ vs. IFNα treatment group by ANOVA]. **b** Assessment of re-propagation of HCV replicon after long term treatment of chloroquine and/or IFNα. Huh-7 Rep/Feo was incubated with chloroquine (10^{-5} M) and/or IFNα (100 U/ml) for 7 days, then drugs were removed from the medium and incubation continued for another 21 days. Luciferase assay was performed at the 7th and 21st days from cessation of drugs. Values are shown as percentages of the control cells [$*P < 0.05$ vs. IFNα treatment group (day 28) by ANOVA]

resulted in a significant decrease of HCV replicon to about 40% of control. On the other hand, incubation with IFNα for 18 h inhibited the replication of HCV replicon to the levels about 15% of controls as expected. However, co-incubation with 100U/ml of IFNα and 10^{-5} M of chloroquine further decreased HCV replication significantly (Fig. 4a).

To determine whether long-term chloroquine treatment inhibits post-treatment re-propagation of HCV replicon, we followed up luciferase activity of the cells at the 7th and 21st days after 7 days of treatment with chloroquine and/or IFNα (Fig. 4b). In HCV replicon cells treated by chloroquine, luciferase activities recovered to 53 and 88% on 7 and 21 days after cessation of treatment. In cells that were treated by IFNα, luciferase activity maintained background level for 7 days post-treatment. However, it reappeared in 21 days. In sharp contrast, co-incubation with IFNα and chloroquine for 7 days suppressed HCV replication for the extensive period up to 21 days, even in the absence of these drugs (Fig. 4b).

Anti-HCV effect of chloroquine independent of IFN signaling pathway

IFN-inducible double-stranded RNA-activated protein kinase R (PKR) plays a key antiviral role against hepatitis C virus [26, 27]. To elucidate the mechanisms of the inhibitory effect of chloroquine on HCV replication, phosphorylated PKR (P-PKR) was evaluated by western blotting analysis. P-PKR was detectable in cells treated with IFNα after 24 h; this increase in P-PKR expression peaked at 24 h after IFNα treatment and was reduced at 48 h (Fig. 5a). In contrast, P-PKR was not observed in cells treated with chloroquine at any time point.

Chloroquine blunts autophagic proteolysis in cells transfected with HCV replicon

It is reported that chloroquine disrupts lysosomal function, preventing effective autophagic protein degradation, leading to the accumulation of ineffective autophagosomes [28]. Therefore, we investigated if chloroquine led to the accumulation of autolysosomes as a result of suppression of proteolysis. We performed electron microscopic investigation to evaluate quantities of autophagosomes and autolysosomes. Ultrastructural analysis identified 0.94 ± 0.1 vacuoles/100 μm^2 of autolysosomes in control cells; however, treatment with chloroquine increased the number of autolysosomes dramatically to about 13-fold over control (Fig. 5b). Furthermore, the molecular form of LC3 protein of the cells, which is a component of autophagosomes, was examined by western blot analysis to ensure that chloroquine treatment leads to the accumulation of autophagosomes and autolysosomes. As shown in Fig. 5c, immunopositive protein bands for LC3-I and LC3-II forms were clearly evident in control cells. After chloroquine treatment, LC3-II expression increased at 4 h (Fig. 5c) to about threefold over control without enhancing LC3-I expression, and at 8 h (Fig. 5c) LC3-II expression was further enhanced. Finally, we evaluated turnover of the long-lived protein leucine, which was mainly degraded by autophagy. Huh7/Rep-Feo cells were labeled with [^{14}C]leucine for 24 h, and degradation of [^{14}C]leucine in cells treated with or without chloroquine was measured. Chloroquine treatment decreased degradation of leucin to 76% of control, indicating that chloroquine blunts degradation of proteins via an autophagic

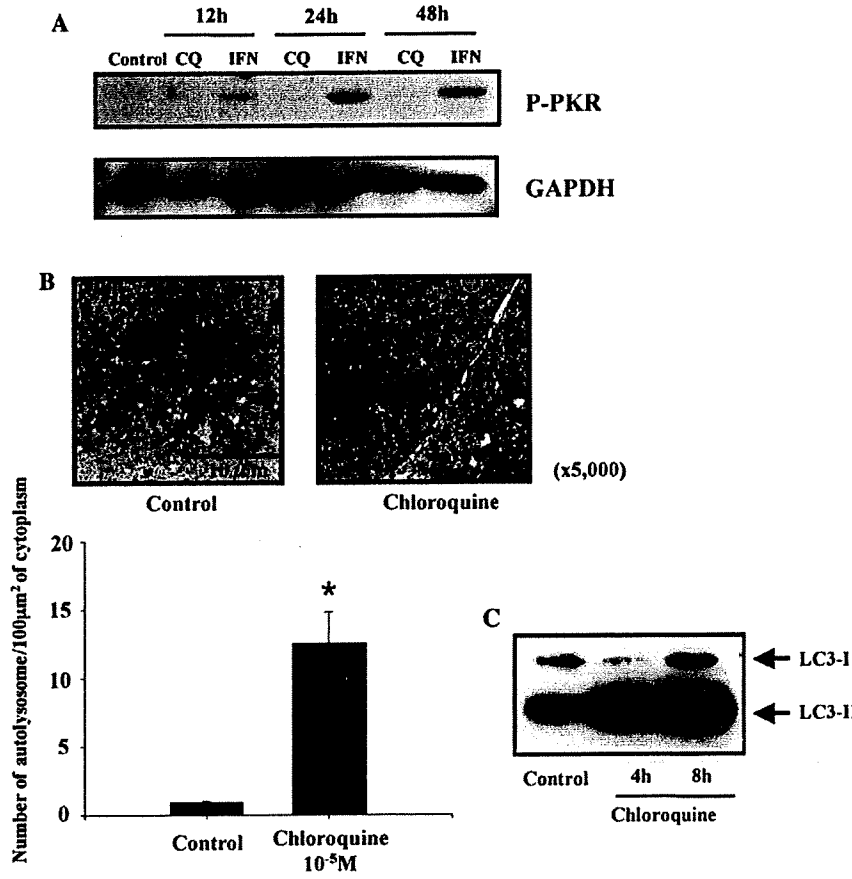

Fig. 5 Chloroquine suppresses autophagic protein degradation, not interferon pathways. **a** Cells were treated with 10^{-5} M of chloroquine (CQ) or 100 U/ml of IFNα for 24–48 h. Phosphorylation of PKR was assessed by western blot analysis. GAPDH was used as loading control. **b** Ultrastructural analysis showing the effect of chloroquine on the number of autolysosomes. Huh-7/Rep-Feo cells were incubated with chloroquine for 18 h. Autolysosomes were identified as the double membrane vesicles (arrow heads) of cytoplasm in Huh-7 Rep/Feo. The number of autolysosomes in 100 μm^2 of cytoplasm was counted by using transmission electron microscopy. Data represent mean ± SEM of individual preparations from pictures (*$P < 0.05$ vs. control by ANOVA). **c** Western blot analysis of LC3 in Huh7 Rep/Feo. The lysate of Huh7 Rep/Feo treated with chloroquine for 4–8 h were immunoblotted with LC3. GAPDH was used as loading control

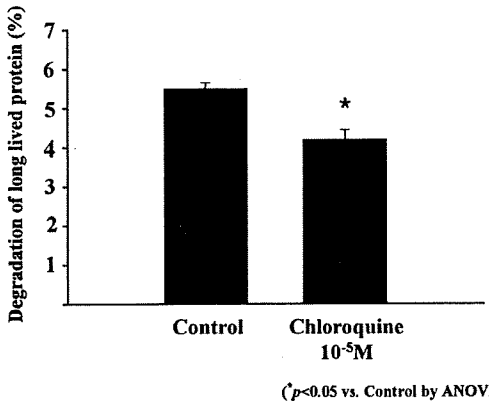

Fig. 6 Turnover of long lived protein. Huh-7 Rep/Feo cells were labeled with [^{14}C]leucin for 4 h, then degradation of long-lived protein in chloroquine treated cells was measured as described in Materials and Methods. The percentage of protein degradation was calculated by dividing the amount of acid-soluble radioactivity in the medium at that time by the amount of acid-precipitable radioactivity present in the cells at time zero. Data are mean ± SEM of value of triplicate in each group (*$P < 0.05$)

pathway (Fig. 6). These results demonstrate that chloroquine-induced the accumulation of autolysosomes was due to disruption of autophagic proteolysis.

Discussion

Previous reports have disclosed that autophagy plays a pivotal role on the replication of several RNA viruses [10–12]. Our present results demonstrate that autophagy is induced by transfection of HCV replicon and is reduced by deletion of replicon due to IFNα (Fig. 1a, b). These results suggest that autophagy is induced in the presence of HCV replication in its host cells. However, the role of autophagy in the pathogenesis of HCV is largely unclear. We found that the inhibition of autophagosome formation and autophagic proteolysis blunt the replication of genotype 1b subgenomic HCV replicon (Fig. 2a, c). Sir et al. [13] reported that inhibition of autophagy also reduced the replication of the JFH1-based full length genotype 2a genome. Therefore, the utilization of autophagy on viral replication is shown by HCV strains across different genotypes.

On the other hand, not only a silencing of autophagic gene but also pharmacological inhibition of autophagic proteolysis possesses anti-HCV effects (Fig. 2a, c). However, treatment with both chloroquine and the mixture of E64d and pepstatin induced the accumulation of

autophagosomes in cytoplasm. Therefore, it is likely that HCV does not utilize the double membrane structure as the localization of the viral replication formation. These results support the hypothesis that protein degradation due to autophagy is important for HCV replication.

Chloroquine is a well-known inhibitor of autophagic protein degradation and is often used as an anti-malarial agent. Moreover, the anti-viral effect of chloroquine on other RNA viruses has been already reported in clinical trials [15, 16]. In our results, chloroquine inhibits the intracellular replication of an HCV replicon in a dose-dependent manner (Fig. 3a). This antiviral effect of chloroquine was clearly not due to cytotoxic effects (Fig. 3b). Moreover, chloroquine possesses a synergistic effect with IFNα on HCV replication (Fig. 4a). Although IFNα possesses strong anti-HCV effects, re-propagation of HCV replicon was observed after 3 weeks following 7 days of treatment with IFNα. Interestingly, co-incubation with IFNα and chloroquine for 7 days prevented re-propagation of HCV replicon (Fig. 4b). Chloroquine is a lysosomal weak base that is known to affect acid vesicles leading to dysfunction of several proteins [29]. It was demonstrated that disruption of lysosomal function impairs maturation of viruses through inhibiting the low-pH dependent proteases in trans-Golgi vesicles in HIV and the SARS coronavirus infection in vitro [15, 29]. However, little is understood about the mechanism of its antiviral effect. In previous reports, various drugs which possess inhibitory effects on the replication of HCV and have a synergistic action with IFNα have been proposed as new therapeutic agents to treat HCV. Some of them have proved to exhibit their anti-HCV effects through augmentation of IFN-induced antiviral gene responses [30, 31]. However, the anti-HCV effect of chloroquine was not associated with activation of one of IFN receptors signaling molecule PKR (Fig. 5a). Our results showed chloroquine induced the accumulation of ineffective autophagosomes in cytoplasm of Huh7/Rep-Feo cells (Fig. 5b) and inhibited the degradation of long-lived protein leucine (Fig. 6). These findings imply that chloroquine effectively impairs the function of autophagy in our experiment. These results indicated that chloroquine is a new anti-HCV agent that targets the autophagic proteolysis.

Previous reports have shown that chloroquine possesses anti-viral effects on various RNA viruses. Its best-studied effects are those against HIV replication, which are being tested in clinical trials [17, 18]. HCV co-infection is common in HIV-positive patients in USA and Europe [32, 33]. Since HIV infection accelerates the progression of HCV-related liver disease, treatment of HCV is generally recommended. However, co-infected patients have a greater risk of antiretroviral therapy-associated hepatotoxicity than patients with HIV only [34]. Moreover, treatment with ribavirin is believed to increase the risk of anemia in patients taking the HIV drug zidovudine [35]. A clinical study designed for HIV patients showed the safety and efficacy of chloroquine used for long terms up to 48 weeks [36]. Therefore, the combination therapy of interferon and chloroquine is, possibly, a hopeful therapy for HCV–HIV co-infected patients. Since chloroquine is known as one of the inexpensive drugs, therefore, chloroquine might provide a new effective, safe and economical therapeutic option for patients with HCV. In conclusion, autophagic proteolysis might be a new therapeutic target on the replication of HCV.

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Antiviral Effects of the Interferon-Induced Protein Guanylate Binding Protein 1 and Its Interaction with the Hepatitis C Virus NS5B Protein

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Interferons (IFNs) and the interferon-stimulated genes (ISGs) play a central role in antiviral responses against hepatitis C virus (HCV) infection. We have reported previously that ISGs, including guanylate binding protein 1 (GBP-1), interferon alpha inducible protein (IFI)-6-16, and IFI-27, inhibit HCV subgenomic replication. In this study we investigated the effects of these ISGs against HCV in cell culture and their direct molecular interaction with viral proteins. HCV replication and virus production were suppressed significantly by overexpression of GBP-1, IFI-6-16, or IFI-27. Knockdown of the individual ISGs enhanced HCV RNA replication markedly. A two-hybrid panel of molecular interaction of the ISGs with HCV proteins showed that GBP-1 bound HCV-NS5B directly. A protein truncation assay showed that the guanine binding domain of GBP-1 and the finger domain of NS5B were involved in the interaction. Binding of NS5B with GBP-1 inhibited its guanosine triphosphatase GTPase activity, which is essential for its antiviral effect. Taken together, interferon-induced GBP-1 showed antiviral activity against HCV replication. *Conclusion:* Binding of the HCV-NS5B protein to GBP-1 countered the antiviral effect by inhibition of its GTPase activity. These mechanisms may contribute to resistance to innate, IFN-mediated antiviral defense and to the clinical persistence of HCV infection. (HEPATOLOGY 2009;50:1727-1737.)

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality.[1] Hepatitis C is characterized by persistent infection of the liver, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Type-I interferon (IFN) plays a central role in eliminating viruses, not only by way of therapeutic applications[2] but also as a natural cellular antiviral mechanism.[3,4] Interferons are produced naturally in response to virus infection and to cellular exposure to IFN itself. Binding of the IFNs to their receptors activates the Jak-STAT pathway to form a complex with IFN-stimulated gene factor-3 (ISGF3), which translocates to the nucleus, binds the IFN-stimulated response element (ISRE) located in the promoter/enhancer region of the IFN-stimulated genes (ISGs), and activates expression of ISGs.

HCV subgenomic replicons constitute *in-vitro* models that simulate cellular autonomous replication of HCV

Abbreviations: CLEIA, chemiluminescence enzyme immunoassay; Fluc, firefly luciferase; GBP-1, guanylate binding protein 1; GTPase, guanosine triphosphatase; HCV, hepatitis C virus; IFN, interferon; IgG, immunoglobulin G; ISG, interferon-stimulated gene; ISGF3, IFN-stimulated gene factor-3; IRF-1, interferon regulatory factor 1; ISRE, IFN-stimulated response element; NF-κB, nuclear factor-kappaB; NS, nonstructural.

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Received March 12, 2009; accepted July 24, 2009.

Supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare, the Japan Health Sciences Foundation, and the National Institute of Biomedical Innovation.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23195

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

genomic RNA. Replication of the HCV replicon can be abolished by treatment with small amounts of type-I and type-II IFNs.[5,6] These findings suggest that various molecules encoded by the ISGs have antiviral activities against HCV replication. We have reported previously that the baseline activities of ISG expression are substantially decreased in cells expressing HCV replicon and that this decrease is partly attributable to the transcriptional suppression of interferon regulatory factor 1 (IRF-1).[7] We performed expressional screening of ISGs to investigate their antiviral effects against HCV replication and showed that guanylate binding protein 1 (GBP-1), interferon alpha inducible protein (IFI)-6-16, and IFI-27 had novel activities against cells harboring an HCV replicon.[8] In this study we investigated the antiviral effects and molecular mechanism of GBP-1, IFI-6-16, and IFI-27 on HCV-JFH1-infected cells.

Materials and Methods

Cells and Cell Culture. Huh7, Huh7.5.1, and 293T cells were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. To maintain cell lines carrying the HCV replicon (Huh7/Rep-Feo cells), G418 (Nacalai Tesque, Kyoto, Japan) was added to the culture medium to a final concentration of 500 μg/mL.

HCV Replicon Constructs and Transfection. The HCV replicon plasmids, which contain Rep-Feo, were derived from the HCV-N strain, pHC1bneo/delS (1b-Feo) and HCV-JFH1 strain, pSGR-JFH1 (2a-Feo).[6,9] These constructs express a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase. The replicon RNA synthesis and transfection have been described (Huh7/Rep-1bFeo, Huh7/Rep-2aFeo).[10,11]

HCV Cell Culture System. A plasmid, pJFH1-full,[12] which encodes the full-length HCV-JFH1 sequence, was linearized and used as a template for synthesis of HCV RNA using the RiboMax Large Scale RNA Production System (Promega, Madison, WI). After DNaseI (RQ-1, RNase-free DNase, Promega) treatment, the transcribed HCV RNA was purified using ISOGEN (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh-7.5.1 cells were washed twice, and 5×10^6 cells were suspended in Opti-MEM I (Invitrogen, Carlsbad, CA) containing 10 μg of HCV RNA, transferred into a 4-mm electroporation cuvette, and subjected to an electric pulse (1,050 μF and 270 V) using the Easy Ject system (EquiBio, Middlesex, UK). After electroporation, the cell suspension was left for 5 minutes at room temperature and then incubated under normal culture conditions in a 10-mm diameter cell culture dish. The transfected cells were split every 3 to 5 days. The culture supernatants were subsequently transferred onto uninfected Huh-7.5.1 cells.

Construction of Plasmids Expressing ISGs and Analysis of Their Effect on HCV Subgenomic and Genomic Replication. We constructed plasmids expressing GBP-1, IFI-6-16, IFI-27, and IRF-1. The full-length human ISGs were amplified by polymerase chain reaction (PCR) from Huh7 cells and cloned into pcDNA3.1D/V5-His-TOPO (pcDNA4/TO/myc-his for IRF-1) (Invitrogen) to yield the mammalian expression construct, pcDNA-ISG. The ISG-expression plasmid, pcDNA-ISG, was transfected into Huh7/Rep-1bFeo or Huh7/2aFeo cells, and the replication level of the HCV replicon was analyzed by luciferase assay. A plasmid, pcDNA3.1D/V5-His/lacZ (Invitrogen), was used as a control plasmid vector for mock transfection.

Another plasmid, pcDNA-ISG, was transfected into HCV-JFH1 cell culture systems. Forty-eight hours after transfection the culture supernatants, total cellular RNA, and protein, which were used for quantification of HCV core antigen, were harvested.

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

Real-Time Reverse Transcription (RT)-PCR Analysis. Total cellular RNA was isolated using ISOGEN (Nippon Gene). Two micrograms of total cellular RNA were used to generate complementary DNA (cDNA) from each sample using SuperScript II (Invitrogen) reverse transcriptase. Expression of messenger RNA (mRNA) was quantified using the TaqMan Universal PCR Master Mix and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers have been described.[13]

Quantification of HCV Core Antigen in Culture Supernatants. Culture supernatants of JFH1-RNA transfected Huh-7.5.1 cells were collected on the days indicated, passed through a 0.45-μm filter (MILLEX-HA, Millipore, Bedford, MA), and stored at −80°C. The concentrations of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Synthetic Short Hairpin RNA (shRNA) and shRNA-Expression Plasmid. ISG-directed shRNA expression vectors (pUC19-shRNA-ISG) were designed and constructed as described.[11] Briefly, oligodeoxyribonucleotides encoding shRNA sequences were synthesized and cloned just downstream of the human U6 promoter in the

plasmid pUC19. To avoid problems of structural instability of DNA strands arising from the tight palindrome structure during transcription of shRNA, several point mutations were introduced into the sense strand of the shRNA sequences, which retained fully the silencing activity of the shRNA.[11] Sequences of the shRNAs are shown in Supporting Table 1.

Construction of Plasmids Expressing Full-Length and Truncated HCV-NS Proteins. Expression plasmids of HCV-NS3, NS4B, NS5A, and NS5B were constructed by inserting PCR-amplified fragments encoding each HCV-NS protein into pcDNA4/TO/myc-his (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, and pcDNA-NS5B, respectively). The plasmids, which expressed truncated HCV-NS5B proteins, were generated by insertion of various fragments amplified by PCR using pcDNA-NS5B into pcDNA4/TO/myc-his.

Immunoprecipitation Assay. Plasmids expressing HCV-NS protein (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, or pcDNA-NS5B) and plasmids expressing ISG (pcDNA-GBP-1, pcDNA-IFI-6-16, or pcDNA-IFI-27) were cotransfected into HEK-293 T cells. Forty-eight hours after transfection, cellular proteins were harvested and immunoprecipitation assay was performed using an Immunoprecipitation Kit according to the manufacturer's protocol (Roche Applied Science, Mannheim, Germany). The immunoprecipitated proteins were analyzed by western blotting.

Mammalian Two-Hybrid Assay. Mammalian two-hybrid assay (Stratagene, La Jolla, CA) is a method for detecting protein-protein interactions in vivo in mammalian cells. In this assay a gene encoding the HCV-NS protein (NS3, NS4B, NS5A, and NS5B) was fused to the DNA-binding domain of the yeast protein GAL4 (a bait plasmid, pcCMV-BD), whereas another gene (ISG; GBP-1, IFI-6-16, and IFI-27) was fused to the transcriptional activation domain of the mouse protein nuclear factor-kappaB (NF-κB) (an acceptor plasmid, pCMV-AD). These two-hybrid constructs are cotransfected into Huh-7 cells with a reporter plasmid encoding the firefly-luciferase gene. If the ISG protein and HCV-NS protein interact, they create a functional transcriptional activator by bringing the activation domain into close proximity with the DNA-binding domain; this can be detected by expression of the luciferase reporter gene. The ISG-encoding site was inserted into a plasmid, pCMV-AD, by cloning from a plasmid, pcDNA-ISG (pCMV-AD-ISG). The HCV-NS protein gene, which was subcloned from a plasmid, pcDNA-NS protein, was also inserted into a plasmid, pCMV-BD (pCMV-BD-NS protein). The plasmids pCMV-AD-ISG and pCMV-BD-NS protein were cotransfected with a reporter plasmid, pFR-luc encoding Fluc into Huh-7 cells. Cellular proteins were harvested after 48 hours and luciferase assays were performed.

Immunohistochemistry. Huh7.5.1 cells infected with HCV-JFH-1 were seeded onto 18-mm round microcover glasses (Matsunami, Tokyo, Japan). After transfection of plasmids expressing ISG, pcDNA-ISG, Huh7.5.1 cells were fixed with cold acetone. The cells were incubated with the primary antibodies for 1 hour at 37°C and with Alexa Flour 488 goat antimouse immunoglobulin G (IgG) antibody and Alexa 568 donkey antigoat IgG antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Cells were mounted with VectaShield Mounting Medium and DAPI (Vector Laboratories, Burlingame, CA) and visualized with fluorescence microscopy (BZ-8000, Keyence, Osaka, Japan).

The Establishment of a Mutant Form of GBP-1. In order to introduce mutations into GBP-1, the full-length human ISGs were amplified by PCR from a human liver cDNA library (Invitrogen) and cloned into pCMV-Tag Epitope Tagging Mammalian Expression Vectors; pCMV-GBP-1 (Stratagene). The following mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-Change II Site-Directed Mutagenesis Kit; Stratagene): R48P; pCMV-GBP1.R48P.

Guanosine Triphosphatase (GTPase) Assay. Cellular proteins were harvested from Huh7 cells 48 hours after transfection of a plasmid expressing HCV-NS5B, pcDNA-NS5B, or a plasmid for mock transfection, pcDNA3. A GTPase assay was performed to examine GTPase activity of cellular proteins using GTPase ELIPA kits (Cytoskelton, Denver, CO). The assay is based on an absorbance shift (340 to 360 nm) that occurs when 2-amino-6-mercapto-7-methylpurine ribonucleoside is catalytically converted to 2-amino-6-mercapto-7-methyl purine in the presence of inorganic phosphate (Pi). The reaction is catalyzed by purine nucleoside phosphorylase. One molecule of inorganic phosphate will yield one molecule of 2-amino-6-mercapto-7-methyl in an essentially irreversible reaction. Thus, the absorbance at 360 nm is directly proportional to the amount of Pi generated in the reaction.

Transient Transfection. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

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

Western Blot Analysis. Western blotting was performed as described.[10] Briefly, 10 μg of total cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto a poly-

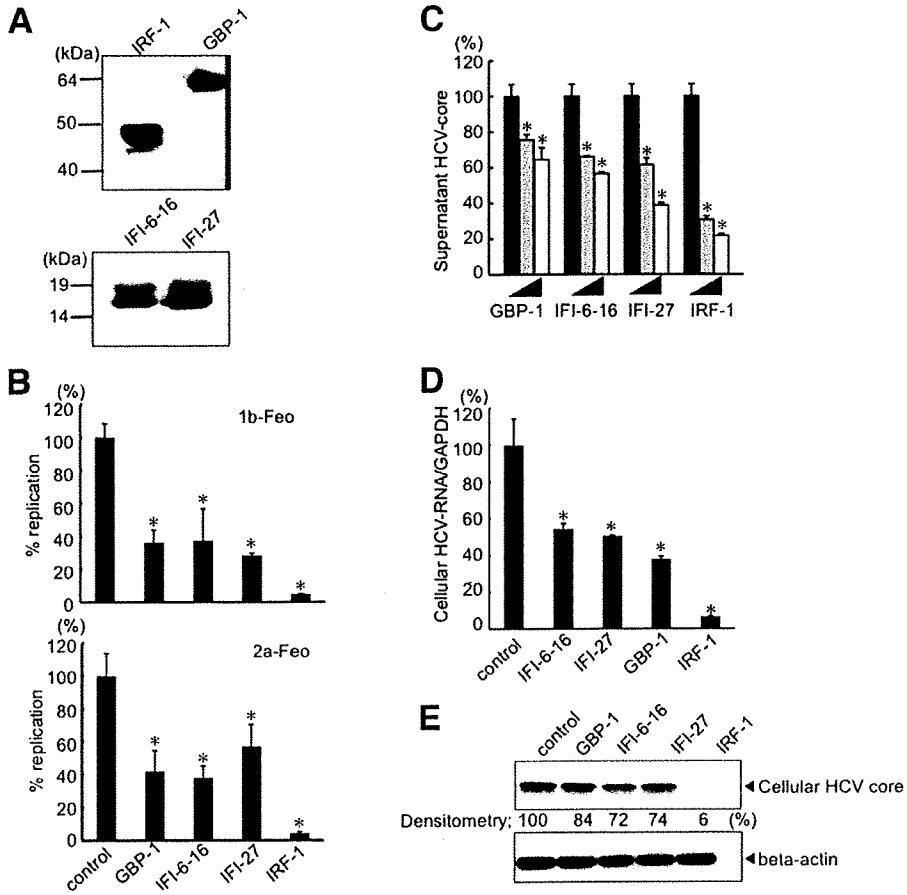

Fig. 1. Suppression of HCV infection and replication by overexpression of ISGs. (A) Western blotting analysis of cells transfected with the ISG-expression plasmids. ISG expression vectors were respectively transfected into HEK-293T cells. The cells were harvested at 48 hours after transfection. Ten micrograms of cell lysate were separated by SDS-PAGE and blotted onto a nylon membrane. The membrane was immunoblotted with anti-myc antibodies or anti-V5 antibodies. (B) The indicated ISG-expression plasmids were transfected into Huh7/Rep-1bFeo or Huh7/Rep-2aFeo cells. Luciferase activities were measured 48 hours after transfection. IRF-1 expression plasmid was used as a positive control.[7] The values are displayed as percentages of luciferase activities relative to that of HCV replicon cells transfected pcDNA3.1D/V5-His/LacZ (control). Error bars indicate mean ± SD. $*P < 0.05$. (C) The indicated plasmids expressing ISG protein were transfected into Huh-7.5.1 cells infected with HCV-JFH1. The culture supernatant of HCV-JFH1 infected Huh-7.5.1 cells was collected 48 hours after transfection and the levels of HCV core antigen in the culture supernatant were measured. The values are displayed as percentage of core antigen relative to that of culture supernatant from infected Huh-7.5.1 cells transfected with pcDNA3.1D/V5-His/LacZ (control). Control plasmids (black bars), 2 μg (gray bars), or 4 μg (white bars) of indicated ISG-expression plasmids were transfected. Error bars indicate mean ± SD. $*P < 0.05$. (D) Total RNA and protein from HCV-JFH1-infected Huh-7.5.1 cells transfected with ISG-expression plasmids were extracted 48 hours after transfection. Total cellular RNA was quantified by real-time RT-PCR. The values are displayed as percentage of HCV RNA relative to that of infected Huh-7.5.1 cells transfected with pcDNA3.1D/V5-His/LacZ (control). Error bars indicate mean ± SD. $*P < 0.05$. (E) Western blotting analysis was performed to assess intracellular suppression of HCV replication. Ten micrograms of harvested cell lysates were subjected to western blotting using anti-HCV core antibodies. The values indicated percentage of densitometry of HCV core protein relative to protein of cells transfected with pcDNA3.1D/V5-His/LacZ (control).

vinylidene fluoride (PVDF) membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL Western Blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK).

Statistical Analyses. Statistical analyses were performed using Student's t test; P values of less than 0.05 were considered statistically significant.

Results

GBP1, IFI-6-16, and IFI-27 Suppress HCV Subgenomic Replicon and Virus Replication in Cell Culture. First, we assessed the effects of GBP-1, IFI-6-16, and IFI-27[8] on replication of HCV genotype 1b and 2a replicons.[9-11] Transfection of the expression plasmids for IRF-1, GBP-1, IFI6-16, and IFI-27 resulted in expression of corresponding proteins (Fig. 1A). Each ISG-expression

plasmid was transfected into cells harboring HCV genotype 1b or 2a replicons that expressed Fluc and luciferase assays were performed. Expression of GBP-1, IFI-6-16, and IFI-27 resulted in significant reduction of HCV replication (1b-Feo: GBP-1; 63.7 ± 7.49%, IFI-6-16; 62.6 ± 19.2%, IFI-27; 71.6 ± 1.22%, IRF-1; 95.1 ± 0.19% / 2a-Feo: GBP-1; 61.9 ± 12.3%, IFI-6-16; 42.9 ± 7.0%, IFI-27; 42.9 ± 13.2%, IRF-1; 95.8 ± 1.0%) (Fig. 1B).

We next evaluated whether these ISGs affected the replication of HCV-JFH1 in cell culture. Each ISG-expression plasmid was transfected into HCV-JFH1-infected Huh-7.5.1 cells. The secretion of HCV particles was measured using an HCV core antigen assay. HCV core protein in the supernatant, as well as the cellular fraction, was significantly decreased by the overexpression of GBP-1 (24.3 ± 2.90%, 35.6 ± 6.73%), IFI-6-16 (33.9 ± 0.40%, 43.6 ± 1.14%), IFI-27 (38.5 ± 3.63%, 61.2 ± 1.28%), or IRF-1 (69.2 ± 1.89%, 78.3 ± 1.01%) in a dose-dependent manner (Fig. 1C). Real-time RT-PCR showed that the expression level of HCV RNA was significantly suppressed by plasmids expressing GBP-1 (38.2 ± 1.39%), IFI-6-16 (54.6 ± 2.93%) IFI-27 (50.7 ± 2.81%), and IRF-1 (6.61 ± 0.341%) (Fig. 1D). In Fig. 1E, levels of intracellular HCV core protein were decreased but less than those of HCV-RNA, which might be attributable to the longer half-life of HCV core protein than that of HCV-RNA. To visualize the suppressive effects of ISG on HCV in cell culture, we performed immunohistochemistry on the HCV-JFH1 cell culture system after transfection of the ISG plasmids using anti-HCV core antibodies and anti-ISG protein antibodies. As shown in Fig. 2, HCV core protein expression was markedly decreased in cells in which the ISG transgene was overexpressed. As a control, a LacZ expression plasmid was transfected into the same cells and HCV core protein expression did not decrease. Taken together, GBP-1, IFI-6-16, and IFI-27 showed direct antiviral effects on HCV genomic replication and also particle formation and secretion from cultured cells.

The Effects of Knockdown of GBP-1, and IFI-6-16, and IFI-27 on the HCV-JFH Cell Culture System. We subsequently investigated the effects of suppression of GBP-1, IFI-6-16, and IFI-27 expression on the HCV-JFH1 cell culture system. To conduct the study we used shRNA expression-plasmids, pUC19-shRNA-GBP-1, pUC19-shRNA-6-16, and pUC-shRNA-IFI-27, which expressed shRNA that targeted the corresponding genes. The shRNA-expressing plasmids were cotransfected with plasmids expressing the respective target genes into Huh7 cells. Western blotting showed that the expression levels of the target proteins were significantly suppressed by the

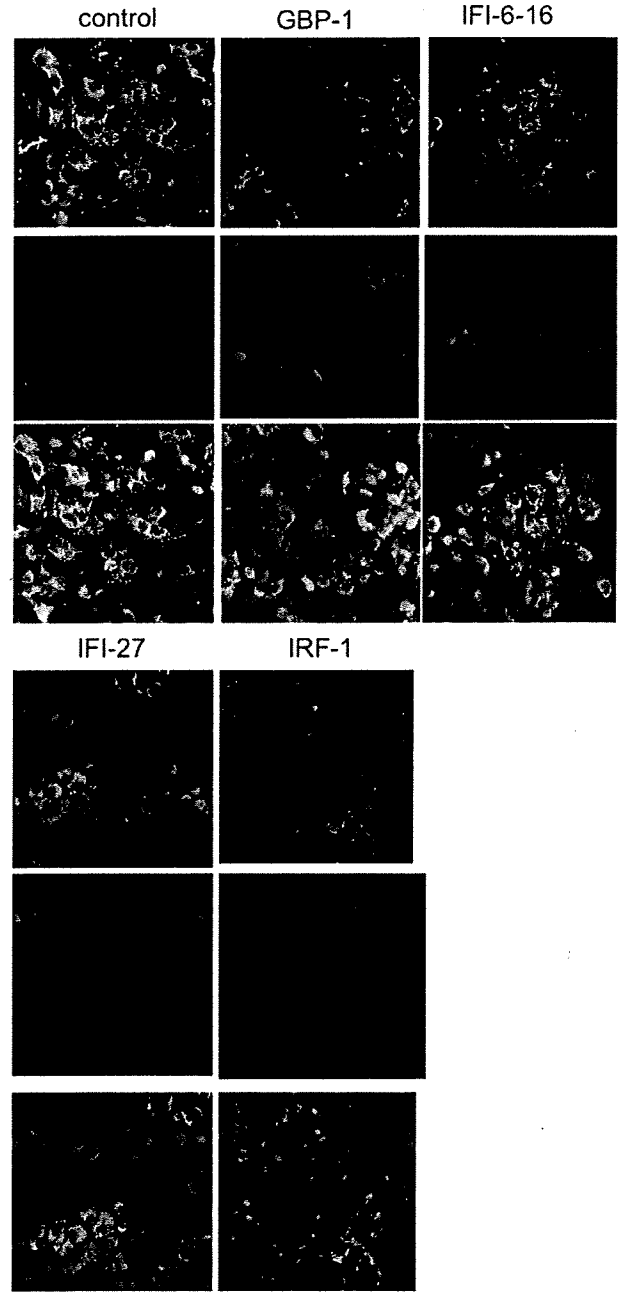

Fig. 2. Suppression of intracellular HCV replication levels by ISG-expression assessed by immunohistochemistry. The ISG-expression plasmid indicated or pcDNA3.1D/V5-His/LacZ as a control was transfected into HCV-JFH1-infected Huh-7.5.1 cells seeded in 18-mm-diameter dishes. At 48 hours after transfection, cells were incubated with anti-HCV core antibodies and anti-V5 antibodies (anti-myc antibodies for IRF-1) followed by Alexa Flour 488 goat antimouse IgG antibodies and Alexa 568 donkey antigoat IgG antibodies. Nuclei were stained with DAPI. Representative immunofluorescence images derived from a number of experiments are shown as three images of a single focal plane of Huh-7.5.1 cells, showing HCV core antigen (green), ISG (red), DAPI staining (blue), and the superimposed images (merge).

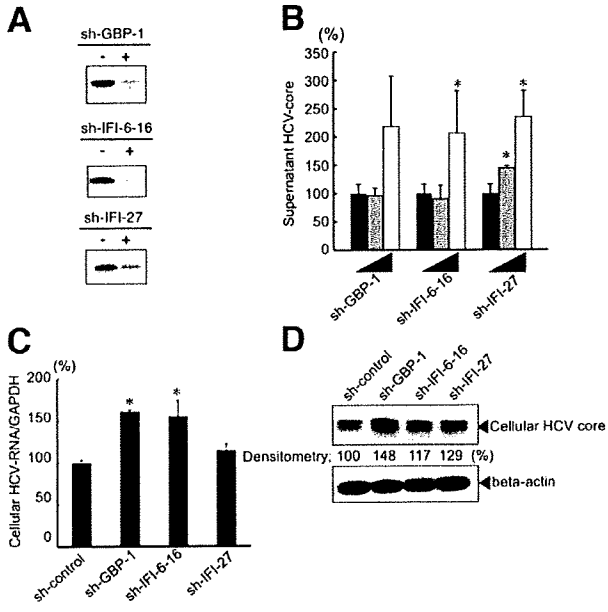

Fig. 3. The effects of shRNA-directed suppression of GBP-1, IFI-6-16, and IFI-27 expression on HCV replication. (A) The ISG-expression plasmids indicated, pcDNA-GBP-1 (upper panel), pcDNA-IFI-6-16 (middle panel), or pcDNA-IFI-27 (lower panel), were cotransfected with shRNA-expressing plasmid, pUC-shRNA-control (indicated with a minus on each panel), or pUC-shRNA-ISG (indicated with plus on each panel); pUC-shRNA-GBP-1, pUC-shRNA-IFI-6-16, pUC-shRNA-IFI-27 into Huh7 cells. Cells were harvested at 48 hours after transfection and western blotting was performed using anti-V5 antibodies. (B) The indicated plasmid expressing shRNA was transfected into Huh-7.5.1 cells infected with HCV-JFH1. Forty-eight hours after transfection the levels of HCV core antigen were measured in the culture supernatant. The values are shown as percentage of HCV core antigen in culture supernatant relative to transfection with control plasmid, pUC-shRNA-control. Control plasmid (black bars), 1 μg (gray bars), or 2 μg (white bars) of indicated shRNA-expression plasmids were transfected. Error bars indicate mean ± SD. $^*P < 0.05$. (C) As in (B), transfection with an shRNA-expression plasmid was performed. At 48 hours after transfection total cellular RNA was extracted, followed by real-time RT-PCR. Error bars indicate mean ± SD. $^*P < 0.05$. (D) Cell lysates were harvested and western blotting analysis was performed using anti-HCV core antibodies followed by antimouse IgG secondary antibodies. The values are displayed as the percentage of chemiluminescence relative that of transfection with the control plasmid, pUC-shRNA-control.

respective shRNAs (Fig. 3A). Next, we transiently transfected shRNA-expression plasmids into HCV-JFH1-infected Huh-7.5.1 cells and performed HCV core antigen assays, real-time RT-PCR, and western blotting to measure HCV-RNA and the protein expression, respectively. Supernatant HCV core protein was significantly and dose-dependently increased by the overexpression of sh-GBP-1 (218.2 ± 88.7%), sh-IFI-6-16 (206.4 ± 74.5%), or sh-IFI27 (234.2 ± 46.3%) (Fig. 3B). In cells that were transfected with sh-GBP-1, sh-IFI-6-16, and sh-IFI27, expression of HCV and particle formation were significantly increased (161.1 ± 1.95% for shRNA-GBP-1, 155.7 ± 18.6% for shRNA-6-16, 114.6 ± 7.13% for shRNA-IFI-27; Fig. 3C). We also found by western blotting analysis that expression of HCV core protein was increased by the shRNA (Fig. 3D).

Intermolecular Interaction of the Three ISGs and HCV Nonstructural Proteins. It has been reported that HCV structural and nonstructural (NS) proteins interact with various host proteins. ISGs such as LMP7, PKR, and 2′,5′-oligoadenylate synthase (25OAS) also are known to interact with HCV NS proteins.[14,15] We performed immunoprecipitation assays to study the direct intermolecular interaction of HCV proteins and ISGs. Plasmids expressing individual HCV-NS proteins were cotransfected with the ISG-expression plasmids into HEK-293T cells. Cellular proteins were harvested 48 hours after transfection and subjected to immunoprecipitation assay. As shown in Fig. 4, we found that GBP-1 bound to HCV-NS5B. The molecular interactions between the NS proteins and ISGs were verified by the mammalian two-hybrid assay. We transfected fusion gene plasmids that express bait and each HCV NS protein together with, as targets, the three ISGs individually cotransfected into cells with reporter plasmids. Only GBP-1 and NS5B showed a significantly increased luciferase activity that was induced by bait-target interaction (Fig. 5). These re-

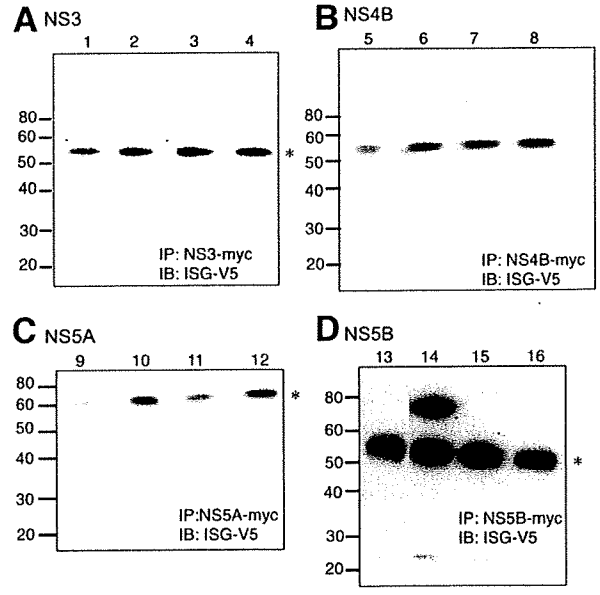

Fig. 4. Interaction of interferon inducible proteins with HCV-NS proteins by immunoprecipitation assay. (A-D) The plasmids indicated expressing myc-tagged HCV-NS protein, NS3 (A), NS4B (B), NS5A (C), or NS5B (D) were cotransfected into HEK-293T with V5-tagged ISG-expression plasmids for GBP-1 (lanes 2, 6, 10, 14), IFI-6-16 (lanes 3, 7, 11, 15), IFI-27 (lanes 4, 8, 12, 16), or mock vector (lanes 1, 5, 9, 13). Cell lysates were harvested at 48 hours after transfection and were immunoprecipitated using anti-myc antibodies. Immunoprecipitated proteins were detected by western blot analysis using anti-V5 antibodies. Asterisks indicate Ig heavy chain.

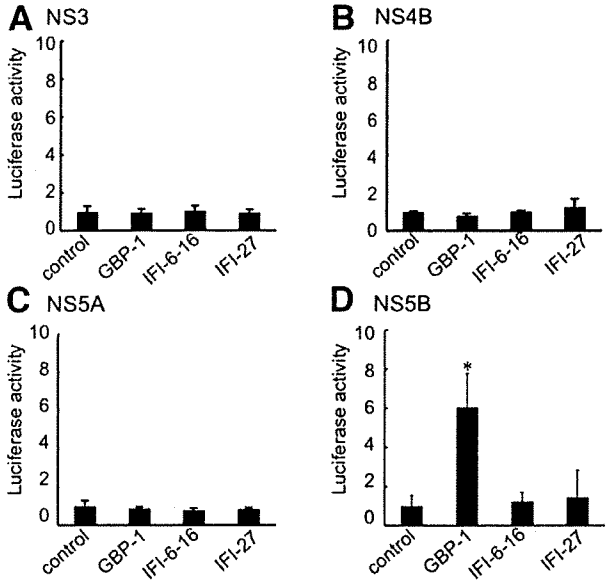

Fig. 5. The analysis of HCV-NS proteins binding ISG proteins by mammalian two-hybrid assay. Interaction of ISG proteins with HCV-NS proteins was confirmed by mammalian two-hybrid assay. The plasmids indicated, pCMV-BD-NS protein NS3 (A), NS4B (B), NS5A (C), or NS5B (D), were cotransfected with pCMV-AD-ISG and reporter plasmid, pFR-luc into Huh7 cells. Cells were harvested at 48 hours after transfection and luciferase activities were measured. The luciferase activities were displayed as relative to those in which empty plasmid, pCMV-AD, was transfected. Significantly higher luciferase activities suggest positive molecular interaction between the bait (BD) and the acceptor (AD) proteins. Values are displayed as means ± SD. $*P < 0.05$.

sults indicated a significant and specific molecular interaction between NS5B and GBP-1.

The Analysis of the Domains of Interaction in HCV-NS5B and GBP-1. Having shown a molecular interaction between GBP-1 and HCV-NS5B, we carried out protein truncation assays. To define interacting regions of GBP1 and NS5B, we constructed expression plasmids for the truncated proteins according to the reported functional domains, which were the GTPase and alpha-helical domains for GBP-1 and the finger, palm, and thumb domains for NS5B (Fig. 6A,B).[16,17] In Fig. 6C,D, plasmids expressing truncated proteins of NS5B or GBP-1 were transfected into HEK-293T cells. Cell lysates were used in western blotting to confirm expression of truncated proteins. These plasmids expressing truncated proteins were transfected into cells and monitored by the mammalian two-hybrid assay. After 48 hours of transfection, luciferase assays were performed to monitor molecular interaction. As shown in Fig. 6E, a significantly higher luciferase activity was obtained when the cells were transfected with full-length GBP-1 and a truncated NS5B that contained amino acids (aa) 1 though 70, which corresponded to the finger domain. Truncation of GBP-1 showed that the GTPase domain (aa 1 through 317) and full-length NS5B gave significantly higher luciferase activities (Fig. 6F). These results showed that the NS5B-finger domain and the GTPase domain of GBP-1 were the regions of mutual molecular interaction.

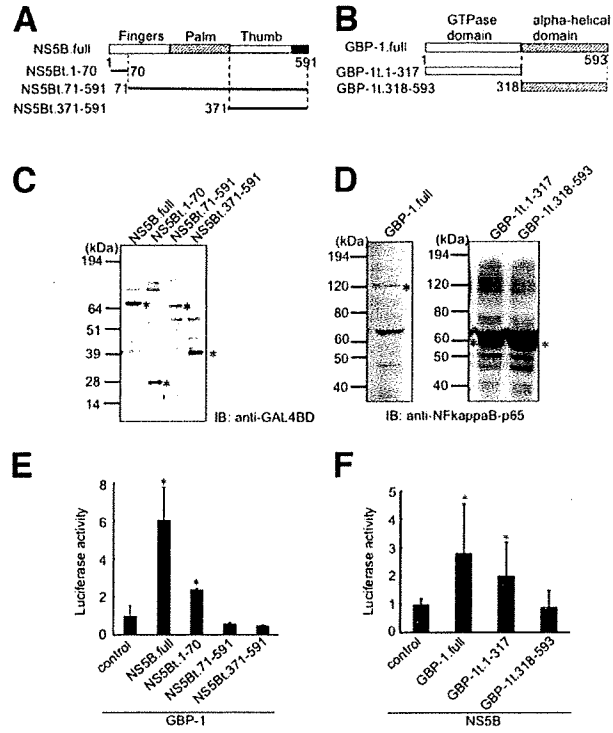

Fig. 6. Analyses of the domains responsible for the interaction between HCV-NS5B and GBP-1. (A) Scheme of full-length and truncated protein in HCV-NS5B. (B) Scheme of full-length and truncated protein in GBP-1. (C) Western blotting analysis of cells transfected with the indicated NS5B truncated protein-expression plasmids (pCMV-BD-NS5B.full, pCMV-BD-NS5Bt.1-70, pCMV-BD-NS5Bt.71-591, and pCMV-BD-NS5Bt.371-591). Expression vectors were respectively transfected into HEK-293T cells. The cells were harvested at 48 hours after transfection. Ten micrograms of cell lysate were separated by SDS-PAGE and blotted onto a nylon membrane. The membrane was immunoblotted with anti-GAL4BD antibodies. Asterisks indicate full-length or truncated NS5B proteins that were fused to the DNA-binding domain of the yeast protein GAL4. (D) Western blotting analysis of cells transfected with the indicated GBP-1 truncated protein-expression plasmids (pCMV-AD-GBP.full, pCMV-AD-GBP-1t.1-317, and pCMV-AD-GBP-1t.318-593). As in (C), transfection and western blotting were performed. Primer antibodies for immunoblotting were used anti-NFkappaB-p65. Asterisks indicate full-length or truncated GBP-1 proteins that were fused to the transcriptional activation domain of the mouse protein NF-κB. (E) Plasmids expressing HCV-NS5B full or HCV-NS5B truncated proteins were transfected into Huh7 cells with plasmid, pCMV-AD-GBP-1, and an Fluc-expression plasmid. The luciferase activities are displayed as relative to those in which empty plasmid, pCMV-AD, was transfected. Significantly higher luciferase activities suggest positive molecular interaction between the bait (BD) and the acceptor (AD) proteins. (F) Plasmids expressing truncated GBP-1 proteins were cotransfected with plasmid, pCMV-BD-NS5B, and a plasmid expressing Fluc into Huh7 cells. Luciferase activities were measured after 48 hours. Values are displayed as means ± SD. $*P < 0.05$.

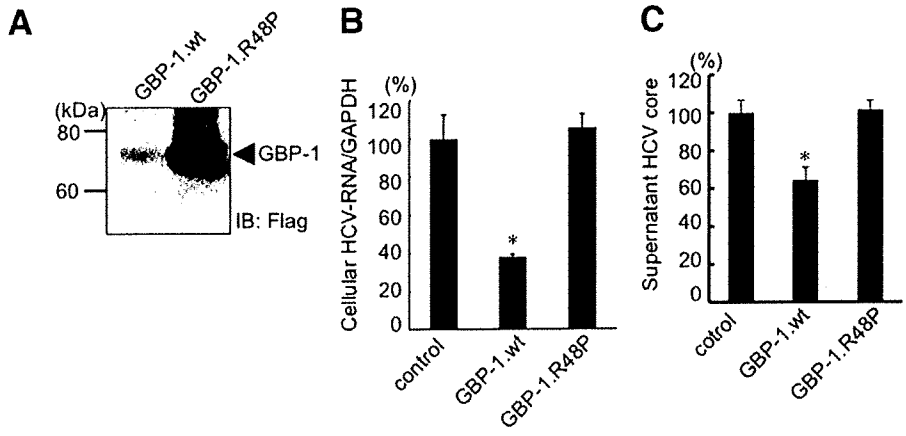

Fig. 7. GTPase activity has an antiviral effect on HCV replication. Western blotting analysis of GBP-1 and GBP-1 mutant, R48P. Plasmids expressing GBP-1 mutant type, R48P, or GBP-1 wildtype were transfected into 293T cells. Cell lysates were harvested at 48 hours after transfection, followed by immunoblotting (A). The indicated plasmid was transfected into Huh-7.5.1 infected HCV-JFH1. Culture supernatants and cellular RNA were harvested at 48 hours after transfection. HCV core antigens were measured in culture supernatants (B) and HCV RNA was amplified by real-time RT-PCR (C). Values are displayed as means ± SD. $*P < 0.05$.

The GTPase Activity has a Direct Antiviral Effect on HCV. To determine whether the GTPase activity of GBP1 was necessary for the antiviral action, we constructed a mutant GBP-1 expression plasmid in which an aa 48 P for R substitution was introduced in the catalyzing domain to abolish GTPase activity (Fig. 7A).[18] As shown in Fig. 7B,C, the inactivated mutant GBP1 showed no antiviral activity. These results indicated that the GTPase activity is essential for the specific suppression of HCV replication.

HCV-NS5B Inhibits GTPase Activity and Results in Continuous Intracellular Replication of HCV. There are several reports that HCV establishes stable infection and replication in cells by interacting with host proteins and affecting their antiviral, antiproliferative, and apoptotic activities.[14,19] Therefore, we determined whether binding of NS5B to GPB-1 affects protein expression levels or enzymatic functions. As shown in Fig. 8A, transfection of NS5B into cells significantly suppressed IFN-gamma-induced production of GBP-1 proteins. However, there were no significant differences in mRNA expression levels between cells with and without overexpression of NS5B (Fig. 8B). Expression of NS5B showed no significant effect on mRNA and protein expression levels of IFI-6-16 and IFI-27. These findings suggested that NS5B specifically suppresses GBP1 posttranslation. Next, we examined the effects of NS5B on the GTPase activity of GBP1 using a cell-free GTPase assay (Fig. 8C). Baseline cellular GTPase activity was increased by overexpression of GBP-1 or by addition of IFN-gamma. On the contrary, transfection of NS5B substantially suppressed GTPase activities of baseline and also IFN-treated cells.

Finally, we investigated whether NS5B negatively regulate antiviral activity of GBP-1 on HCV. GBP-1-expression plasmid was transfected into HCV replicon cells, Huh7/Rep-1bFeo, together with or without expression plasmid of a truncated NS5B1-70, which lacked enzymatic activity but showed molecular interaction with GBP-1 (Fig. 6E). Luciferase assays showed that the suppressive effect of GBP-1 on HCV was abolished by overexpression of NS5B1-70 (Fig. 8D). These results suggest that NS5B may substantially rescue the inhibitory effect of GBP-1 on HCV replication.

Discussion

Persistence of virus replication in host cells is governed by the cellular antiviral system and by the ability of the virus to evade antiviral responses.[20] In this study we demonstrated previously undescribed antiviral activities of the three ISGs, GBP-1, IFI-6-16, and IFI-27, against HCV-JFH1 replication and release of virus particles (Figs. 1, 2). GBP-1 is a well-described GTPase.[21] A mutation in the GTPase-catalyzing domain completely abolished antiviral activity against HCV (Fig. 7B,C), suggesting that GTPase activity is essential for antiviral action. Furthermore, we have shown specific binding of GBP-1 and HCV-NS5B, involving the GBP-1 guanine-binding domain and the NS5B finger domain (Fig. 6). Interestingly, overexpression of NS5B substantially suppressed the GTPase activity of host cellular GTPases, including GBP-1, and rescued the antiviral action of GBP-1 onto HCV (Fig. 8). Taken together, the interplay between the viral NS5B and host GBP-1 protein might be involved in the persistence of HCV infection and its resistance to IFN.

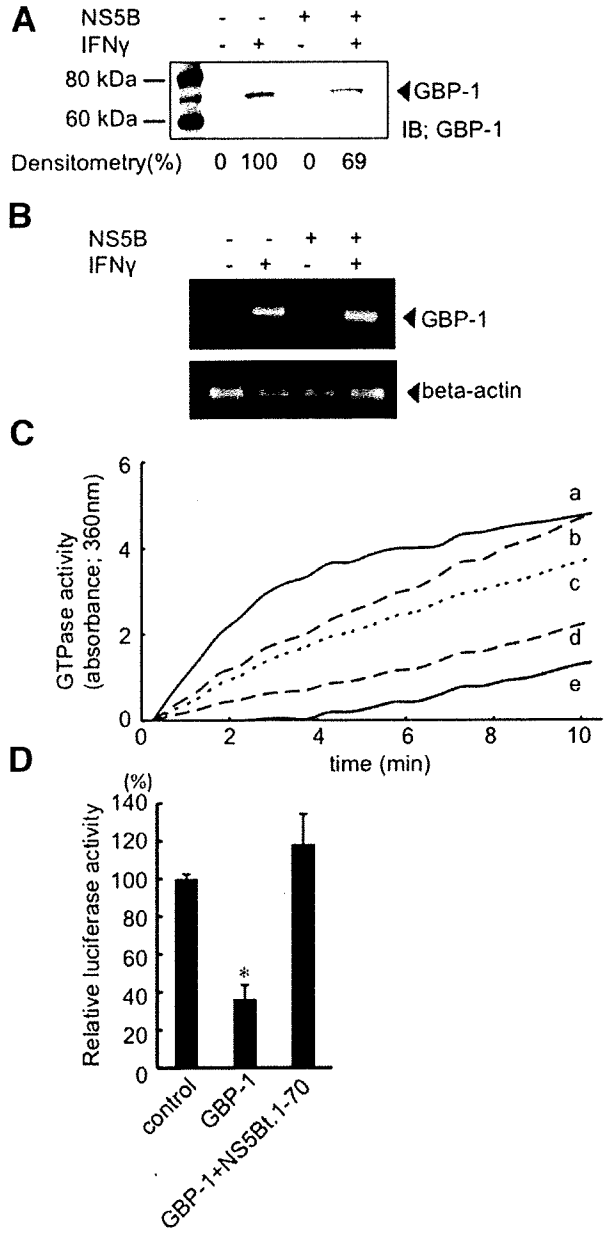

Fig. 8. GTPase activity of GBP-1 was negatively regulated by HCV-NS5B. (A) A GBP-1-expression V5-tagged plasmid was cotransfected into Huh-7 cells with a plasmid expressing myc-tagged-HCV-NS5B and cell lysates were harvested. Cell lysates were immunoprecipitated by anti-myc antibodies. Immunoprecipitated proteins were separated on SDS-PAGE gels, followed by immunoblotting using anti-V5 antibodies. (B) After transfection, total cellular RNA was extracted and amplified by RT-PCR. (C) GTPase activity assays. GTPase activity in cell lysates was measured using a GTPase ELISA Biochem Kit. The indicated graph is displayed as a time course of increase of OD in wavelength (360 nm) for GTPase from the baseline OD. (Graph a, IFN-gamma(+), GBP-1-overexpression and NS5B(−); graph b, IFN-gamma(+) and NS5B(−); graph c, IFN-gamma(−) and NS5B(−); graph d, IFN-gamma(+) and NS5B-overexpression(+); graph e, IFN-gamma(−) and NS5B-overexpression(+)). (D) Effects of HCV-NS5B on antiviral activities in GBP-1. The indicated plasmids expressing control (pcDNA3.1), NS5B truncated protein (NS5Bt.1-70) were cotransfected with GBP-1-expression plasmids into Huh7/Rep-1bFeo cells. Luciferase activities were measured 48 hours after transfection. The values are displayed as percentages of luciferase activities relative to that of HCV replicon cells transfected control. Error bars indicate mean ± SD. $*P < 0.05$.

GBP-1 belongs to a group of GTPase families, including the p47 GBP family, the p65 GBP family, Mx, and very large inducible GTPase.[22-25] The p65 GBP family has five members, GBP-1 to GBP-5.[26-29] There is one report that GBP-1 mediates antiviral effects against vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) in HeLa cells.[30] In our previous study, overexpression of GBP-1 significantly suppressed replication of an HCV subgenomic replicon and suppression of GBP-1 caused the HCV replication level to increase. In HCV-JFH1-infected cells we found that overexpression of GBP-1 significantly reduced the formation of HCV particles and the intracellular replication level of HCV RNA, and that shRNA-directed suppression of GBP-1 resulted in an increase of HCV particles and HCV RNA. These findings suggest that GBP-1 may suppress intracellular HCV replication directly and that the expression of GBP-1 in the absence of IFN treatment may result in inhibition of virus replication. However, little is known about the underlying antiviral mechanisms. Our previous study showed that GBP-1 had no effect on the translational activity of the HCV internal ribosome entry site (IRES) and intracellular signaling pathways such as ISRE, AP-1, GAS, and NF-κB.[8]

GBP-1 has functional homology with MxA, which shows GTPase activity and mediates degradation of cellular RNA, general repression of protein synthesis, and apoptotic cell death.[21] The GTPase domain of GBP-1 is able to bind GMP, GDP, and GTP with similar affinities and to hydrolyze GTP to GMP.[16,31-33] Analyses of the crystal structure of GBP-1 have shown that it has a 36-kDa N-terminal large G (LG) domain (residues 1-317), which retains the main biochemical properties of GBP-1, and an elongated 32 kDa carboxy-terminal alpha-helical domain.[16] In our study we demonstrated that the antiviral activity of GBP-1 required the GTPase activity that is the main biochemical property of GBP-1. We highlight the GBP-1 mutant, GBP-1.R48P, which exhibits no detectable GTP binding or hydrolysis and acts as a dominant negative mutant.[34] Overexpression of GBP-1.R48P had no antiviral effect on HCV replication in cells expressing the HCV subgenomic replicon or replicating HCV-JFH1. These results suggest that the GTPase activity of GBP-1 has an antiviral action against HCV replication. It has been reported that IFNs induced translocation of GBP-1 proteins from the cytoplasm to the Golgi complex.[18] We expect that GBP-1 proteins play some role in inhibiting the HCV life cycle.

It has been reported that some HCV proteins interact with various host proteins. Among the ISGs, LMP7, PKR, and 25OAS interacted with HCV-NS proteins.[14,15,19] We focused on the interaction of ISG pro-

teins with HCV-NS proteins using immunoprecipitation and mammalian two-hybrid assays. We found that GBP-1 bound to NS5B. Furthermore, we revealed that the molecular regions responsible were the LG domain of GBP-1 and the finger domain of NS5B. It was suggested that GBP-1 suppresses the biochemical activity of NS5B, the RNA-dependent RNA polymerase, or that NS5B affected the GTPase of GBP-1 to enable persistent infection by HCV. We performed cotransfection of GBP-1-expression plasmids with plasmids expressing NS5B into Huh7 cells to analyze these interactions. In western blotting analysis we found that the level of GBP-1 protein was reduced by HCV-NS5B. We confirmed that the biochemical properties of GBP-1 proteins were suppressed by NS5B proteins in GTPase assays. These results suggest that HCV-NS5B has a suppressive effect on the GTPase activity of GBP-1, resulting in persistent infection and replication of HCV.

Several reports suggest that viruses target the IFN system of the host cells to establish replication.[35] The IRF-3-mediated IFN-beta induction pathway could be a target for viruses to counteract antiviral responses and promote their replication. Ebola virus, bovine viral diarrhea virus (BVDV), and influenza A virus interfere with the activation of IRF-3 through the activities of their virus-encoded proteins.[36-38] It was recently reported that the HCV NS34A protease blocks virus-induced activation of IRF-3, possibly by proteolytic cleavage of Cardif.[39,40] We recently reported that the double-stranded RNA-triggered and RIG-I-triggered IFN expression and this blockade was partly mediated by NS4B.[41] These reports suggest that HCV-NS proteins are involved in the persistence of intracellular replication and infection of HCV. In the present study we found that NS5B bound to GBP-1 and blocked its GTPase activity, the main biological property of GBP-1 to cause HCV replication and infection. Accordingly, we suggest that NS5B has RNA-dependent RNA polymerase activity but also an effect on antiviral host defense.

In conclusion, we found novel antiviral effects of GBP-1, IFI-6-16, and IFI-27 on the HCV life cycle. GBP-1 interacted with HCV-NS5B, but NS5B has suppressive activities on GBP-1 to establish persistent infection and intracellular replication.

Acknowledgment: We thank Mr. Satoshi Yamazaki at JST, ERATO, Nakauchi Stem Cell and Organ Regeneration Project for excellent technical assistance.

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JBC Papers in Press. Published on April 20, 2009 as Manuscript M109.007625
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M109.007625

THE ISOMERASE ACTIVE SITE OF CYCLOPHILIN A IS CRITICAL FOR HCV REPLICATION

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Running Title: HCV Exploits the Enzymatic Activity of Cyclophilin A to Replicate in Hepatocytes

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ABSTRACT

Cyclosporine A (CsA) and non-immunosuppressive cyclophilin (Cyp) inhibitors such as Debio 025, NIM811 and SCY-635 block hepatitis C virus (HCV) replication in vitro. This effect was recently confirmed in HCV-infected patients where Debio 025 treatment dramatically decreased HCV viral load, suggesting that Cyps inhibitors represent a novel class of anti-HCV agents. However, it remains unclear how these compounds control HCV replication. Recent studies suggest that cyclophilins (Cyps) are important for HCV replication. However, a profound disagreement currently exists as to the respective roles of Cyp members in HCV replication. In this study, we analyzed the respective contribution of Cyp members to HCV replication by specifically knocking down their expression by both transient and stable small RNA interference (sRNAi). Only the CypA knockdown drastically decreased HCV replication. The re-expression of an exogenous CypA escape protein, which contains escape mutations at the sRNAi recognition site, restored HCV replication, demonstrating the specificity for the CypA requirement. We then mutated residues, which reside in the hydrophobic pocket of CypA where proline-containing peptide substrates and CsA bind, and which are vital for the enzymatic or the hydrophobic pocket binding activity of CypA. Remarkably, these CypA mutants fail to restore HCV replication, suggesting for the first time that HCV exploits either the isomerase or the chaperone activity of CypA to replicate in hepatocytes and that CypA is the principal mediator of the Cyp inhibitor anti-HCV activity. Moreover, we demonstrated that the HCV NS5B polymerase associates with CypA via its enzymatic pocket. The study of the roles of Cyps in HCV replication should lead to the identification of new targets for the development of alternate anti-HCV therapies.

INTRODUCTION

HCV is the main contributing agent of acute and chronic liver diseases worldwide (1). Primary infection is often asymptomatic or associated with mild symptoms. However, persistently infected individuals develop high risks for chronic liver diseases such as hepatocellular carcinoma and liver cirrhosis (1). The combination of IFN alpha and ribavirin that serves as current therapy for chronically HCV-infected patients not only has a low success rate (about 50%) (2), but is often associated with serious side effects (2). There is thus an urgent need for the development of novel anti-HCV treatments (2).

The immunosuppressive drug cyclosporine A (CsA) was reported to be clinically effective against HCV (3). Controlled trials showed that a combination of CsA with IFN alpha is more effective than IFN alpha alone, especially in patients with a high viral load (4-5). Moreover, recent in vitro studies provided evidence that CsA prevents both HCV RNA replication and HCV protein production in an IFN alpha-independent manner (6-10). CsA exerts this anti-HCV activity independently of its immunosuppressive activity because the non-immunosuppressive Cyp inhibitors such as Debio 025, NIM811 and SCY-635 also block HCV RNA and protein production (9, 11-14). Unlike CsA, these molecules do not display calcineurin affinity and specifically inhibit the peptidyl-prolyl cis-trans isomerase (PPIase) Cyps. Most importantly, recent clinical data demonstrated that Debio 025 dramatically decreased HCV viral load (3.6 log decrease) in patients coinfected with HCV and HIV (15). This 14-day Debio 025 treatment (1200 mg orally administered twice daily) was effective against the 3 genotypes (1, 3, and 4) represented in the study. More recently, the anti HCV effect of Debio 025 in combination with peginterferon alpha 2a (peg-IFNα2a) was investigated in treatment unexperienced patients with chronic hepatitis C. Debio 025 (600 mg administered once daily) in combination with peg-IFNα2a (180 μg per week) for four weeks induced a continuous decay in viral load which reached –4.61 ± 1.88 IU/mL in patients with genotypes 1 and 4 and -5.91 ± 1.11 IU/mL in patients with genotype 2 and 3 at week 4 (16). The Debio 025 findings are critical because they suggest that Cyp inhibitors represent a novel class of anti-HCV agents. However, it remains unclear how these compounds control HCV replication.

The fact that several recent studies using small RNA interference (sRNAi) knockdown approaches suggest that Cyps are critical for the HCV life cycle (9, 17-18) strongly implies that there is a direct or indirect link between the CsA- and CsA derivative-mediated inhibitory effect on HCV replication and host Cyps.

The discovery 20 years ago of the first cellular protein showing PPIase activity (19) was entirely unrelated to the discovery of CypA as an intracellular protein possessing a high affinity for CsA (20). It is only a few years later that Fisher and colleagues (21) demonstrated that the 18 kDa protein with PPIase activity and CypA represent a single unique protein. All Cyps contain a common domain of 109 amino acids, called the Cyp-like domain (CLD), which is surrounded by domains specific to each Cyp members and which dictate their cellular compartmentalization and function (22). Bacteria, fungi, insects, plants and mammals contain Cyps, which all have PPIase activity and are structurally conserved (22). To date, 16 Cyp members have been identified and 7 of them are found in humans: CypA, CypB, CypC, CypD, CypE, Cyp40 and CypNK (22).

Although there is a growing body of evidence that Cyps control HCV replication in human hepatocytes, a major disagreement currently exists on the respective roles of Cyp members in HCV replication. One study suggests that CypB, but not CypA, is critical for HCV replication (17), another suggests that CypA, but not CypB and CypC, is critical for HCV replication (18), and a third study suggests that three Cyps - CypA, B and C - are all required for HCV replication (9). Thus, although it becomes evident that Cyps serve as HCV cofactors, their respective contributions and roles in the HCV life cycle remain to be determined. An understanding of the mechanisms that control Cyp inhibitor-mediated anti-HCV effect is imperative because it will provide new alternate anti-HCV therapies and

shed light on the still poorly understood early and late steps of the HCV life cycle.

EXPERIMENTAL PROCEDURES

Cells and Drugs. Huh7 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. CsA (Sigma) was prepared in dimethyl sulfoxide at 10 mg/ml and diluted in tissue culture medium for each experiment to 2.5 μM. Debio 025 (gift from Debiopharm, Lausanne, Switzerland) was prepared in ethanol at 10 mM and diluted further in tissue culture medium to 2 μM for each experiment.

HCV RNA Replication. Ten micrograms of in vitro-transcribed genomic Con1 RNA was electroporated into Huh-7 cells. At the indicated time points, intracellular HCV RNA was analyzed via reverse-transcription quantitative polymerase chain reaction and presented as genome equivalents (GE) per microgram total RNA as described previously (23). The primers for reverse-transcription quantitative polymerase chain reaction were: HCV, 5-ATGGCGTTAGTATGAGTGTC-3 (sense) and 5-GGCATTGAGCGGGTTGATC-3 (antisense); glyceraldehyde 3-phosphate dehydrogenase, 5-GAAGGTGAAGGTCGGAGTC-3 (sense) and 5-GAAGATGGTGATGGGATTTC-3 (antisense).

Small RNA Interference Knockdown. Annealed duplex siRNA oligonucleotides contained a 3'-dTdT overhand (Quiagen). siRNA target sequences were: AAGGGTTCCTGCTTTCACAGA for CypA; AAGGTGGAGAGAGCACCAAGACA for CypB; GTGACATCACCACTGGAGATG for CypC; AACCTGCTAAATTGTGCGTTA for CypD; and AATTCTCCGAACGTGTCACGT for control. Cells were transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen). For effect of siRNA Cyp knockdown on HCV RNA replication, cells were transfected with siRNA Cyp and then retransfected 24 h later. An HIV-1-based lentiviral vector was used to express all Cyp shRNA as described previously (24). The Cyp target sequences are the same as those indicated just above. Lentiviral particles production and transduction was conducted as described previously (24). Generation of stable Cyp-knockdown cell lines was obtained after 3 weeks under puromycin (1 μg/mL) selection. All cell lines were tested for mycoplasm contamination, which may nonspecifically interfere with HCV replication. To restore CypA expression in Huh7 CypA knockdown cells, a CypA cDNA bearing silent mutations that render it non-targetable by the CypA shRNA was subcloned into pcDNA3 with Hind3 and NotI sites to generate pcDNA3-resistant wild-type (WT) CypA, which contains an N-terminal HA tag. Using pcDNA3-resistant wild-type (WT) CypA as template, we engineered two plasmids carrying either the H126Q (pcDNA3-resistant H126Q CypA) or the R55A (pcDNA3-resistant R55A CypA) mutation in the hydrophobic pocket of CypA that disallows its isomerase activity (25).

Western blotting. Parental or subgenomic HCV Con1-containing Huh7 cells (1 million) treated with or without siRNA or shRNA targeting Cyps were trypsinized and washed twice with 10 mL sterile phosphate-buffered saline and lysed for 30 minutes on ice in 100 μL lysis buffer (10 mM NaCl, 10 mM Tris (pH 7.4), 0.5% NP40, 1x protease inhibitors). Lysates were cleared via centrifugation at 14,000 revolutions per minute for 10 minutes in a microcentrifuge. Supernatants (70 μL) were collected and protein concentration of cell lysates measured with a Coomassie-based BioRad kit (BioRad Laboratories, Hercules, CA). Cell lysates were then subjected to Western blotting with antibodies to CypA (26), CypB (Zymed Laboratories), CypC (Protein Tech Group, Inc.), CypD (Calbiochem), NS5A (ViroStat) and NS5B (Alexis Biochemicals). Amido black staining of the membranes confirmed that the loading of samples had been properly normalized. The cellular expression of resistant WT, H126Q or R55A CypA proteins was verified by Western blotting using anti-HA antibodies (TSRI, Antibody Core Facility).

Virus Infection and Replication. HIV-1 infection: Cyp-knockdown Huh7 cells lines were infected with HIV-1-GFP (NL4.3 virus encoding