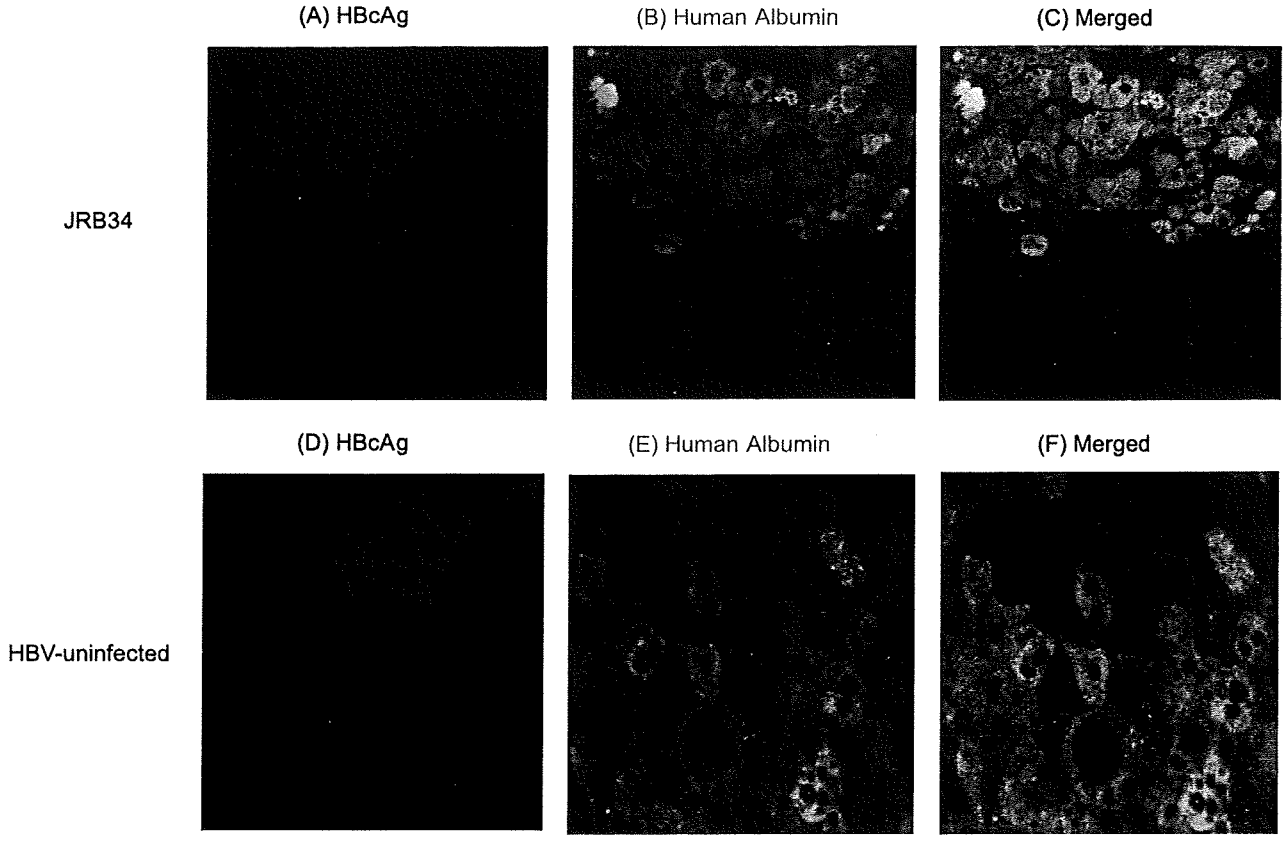

FIG. 7. (A and B) Immunofluorescent staining of a frozen liver section of a chimera mouse inoculated with the HBV/J isolate (JRB34). HBcAg is stained in panel A, and human albumin is stained in panel B. (C) Colocalization of HBcAg and human albumin is revealed by double staining. (D to F) HBV-uninfected mouse liver shows that only human albumin is stained.

outside genotype I strains (Fig. 4). Taken together, genotype J is phylogenetically close to gibbon/orangutan genotypes in the entire genome and to genotype C (C4 in particular) in the S and C genes. However, despite observed interchangeable relatedness with gibbon and genotype C/I strains, no strong evidence of recombination was confirmed in the JRB34.

In the sequence of C gene, carboxyl-terminal arginine-rich region, required for binding with HBV DNA, was preserved in JRB34. It had the G1896A stop codon in the precore region that aborts the translation of HBeAg (5, 30) and A1762T/G1764A double mutations in the core promoter that interfere with the transcription of HBeAg by downregulating preC mRNA (28, 45); they are compatible with the $HBeAg^-$ anti-HBe^+ phenotype of the patient from whom JRB34 was isolated. Since the double mutations are detected frequently in HBV DNA sequences from patients with HCC (17, 33), it could be implicated in hepatocarcinogenesis of the patient from whom JRB34 was isolated. It is not certain, however, if precore and core-promoter mutations had existed in HBV transmitted to the patient who is presumed to have been infected 60 years ago. Since amino acid sequences constituting antigenic loops of HBsAg (6) were the same as those of Australian aborigine isolates of C4, they would share antigenic epitopes of HBsAg. The amino acids at codons 122 and 160 were arginine (with G at nt 365) and lysine (with G at nt 479), respectively (27), in agreement with subtype *ayw* of HBsAg from this patient. Five domains (A to E) of DNA polymerase/reverse transcriptase in the P gene were preserved well in HBV/J, and it did not have mutations in the Tyr-Met-Asp-Asp motif in the domain C that determines the sensitivity to lamivudine (2).

How and when the patient contracted infection with HBV/J is not certain. It is very unlikely, however, that he acquired infection in Japan via perinatal or horizontal transmission. There are no wild primates in Okinawa, where the patient was originally from, and the prevalent human HBV genotypes are limited to B (60%), C (39%), and sporadic cases of A (1%) (32). Furthermore, HBV/J was not found among patient's family members who are currently alive (data not shown). The phylogenetic position within open reading frames of JRB34 in between gibbon/orangutan genotypes and human genotype C gives a clue where and when the patient had contracted HBV infection. He was drafted to Borneo during World War II (1939 to 1945); the island in the Southeast Asia is inhabited by gibbons and orangutans and has a local population mainly infected with genotypes B or C. Zoonotic infection of HBV has been previously reported (11, 46), and HBV of genotype E was recovered from a chimpanzee captured in West Africa where this genotype is common. There is a possibility that JRB34 of

genotype J had been transmitted to the study patient in Borneo during the war (38).

The origin of genotype J in gibbon/orangutan or human inhabitants in Borneo is not certain but very likely. HBV DNA and/or HBsAg was detected in 26% (55/213) and 20% (58/297) of gibbons and orangutans, respectively, captured in Southeast Asia (38). HBV is also endemic in people living there, with a prevalence of HBsAg at 2 to 8%. There would be high chances for cross-species transmission of HBV where it prevails both in human beings and nonhuman primates. Phylogenetic analysis for close relationship between human and nonhuman HBV genotypes has indicated geographical influence rather than association with particular species (41).

It is remains to be determined whether genotype J and ape-derived strains originate from species-specific convergent evolution of distant strains or whether they have diverged from a single common ancestor sometime in the past and evolved independently thereafter. The validity of cross-species infection or species-specific evolution for genotype J would be verified by sequence analysis of HBV DNA from gibbons and humans living in Borneo. If they turn out to be the same, cross-species infection will be justified. Should genotype J be restricted to human beings, in converse, species-specific infection will be confirmed.

In conclusion, a novel HBV genotype was identified in the Ryukyu isolate and provisionally named genotype J. Phylogenetic analyses over the full-length sequence and open reading frames indicate a close relationship of genotype J with gibbon/orangutan genotypes and human genotype C. The index patient would have been infected with HBV/J while he resided in Borneo inhabited by gibbons and orangutans. Although only one HBV isolate of genotype J (JRB34) has been identified, this may be only the tip of an iceberg. It would be worthwhile to examine the genotype of HBV infecting people and gibbons, as well as orangutans, living in Borneo and neighboring countries for mapping the epidemiology of genotype J and finding any clinical relevance.

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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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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-

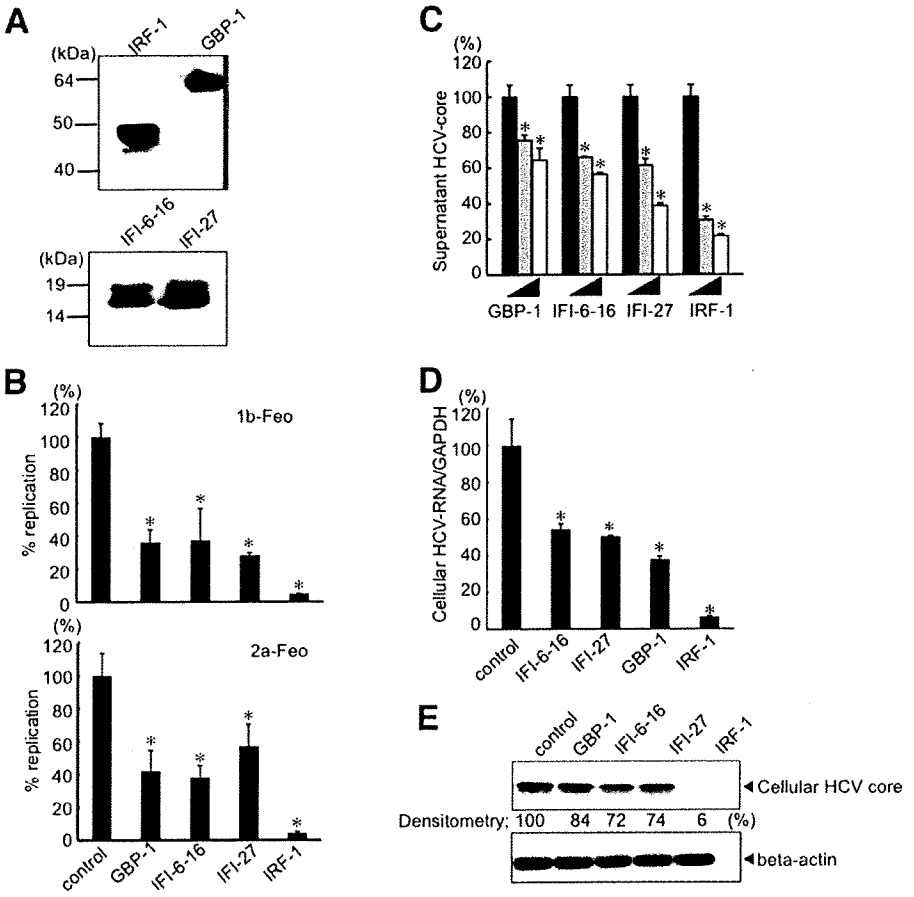

Fig. 1. Suppression of HCV infection and replication by overexpression of ISGs. (A) Western blotting analysis of cells transfected with the ISG-expression plasmids. ISG expression vectors were respectively transfected into HEK-293T cells. The cells were harvested at 48 hours after transfection. Ten micrograms 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

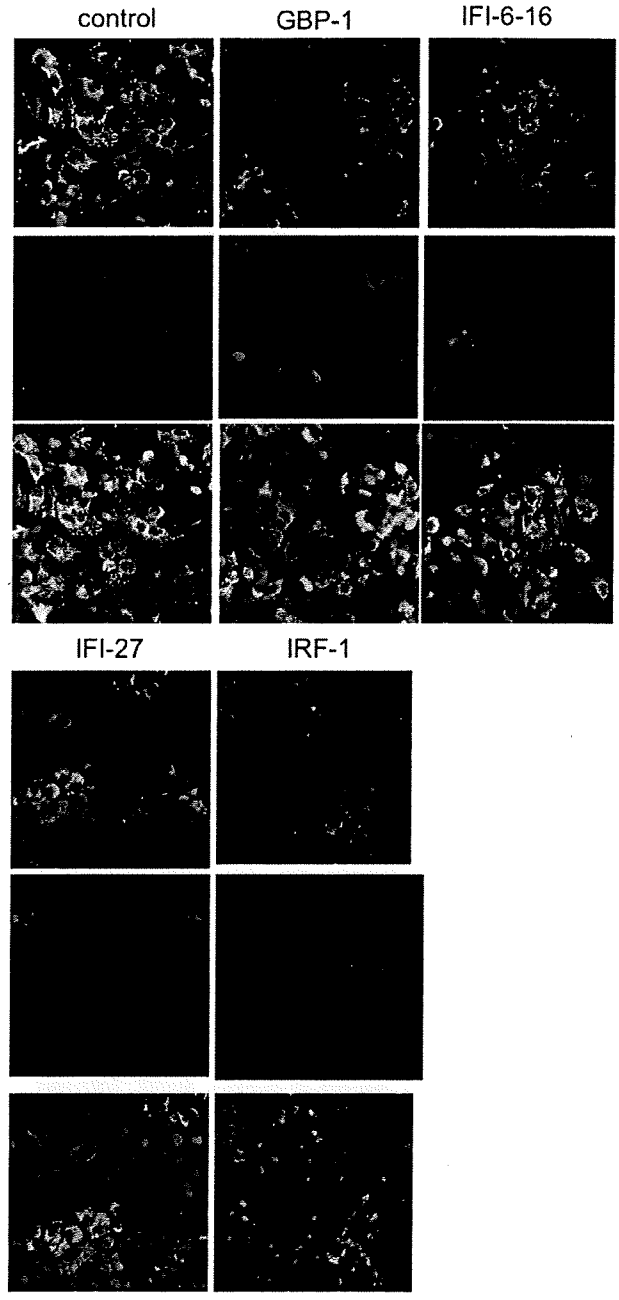

Fig. 2. Suppression of intracellular HCV replication levels by ISG-expression assessed by immunohistochemistry. The ISG-expression plasmid indicated or pcDNA3.1D/V5-His/LacZ as a control was transfected into HCV-JFH1-infected Huh-7.5.1 cells seeded in 18-mm-diameter dishes. At 48 hours after transfection, 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).

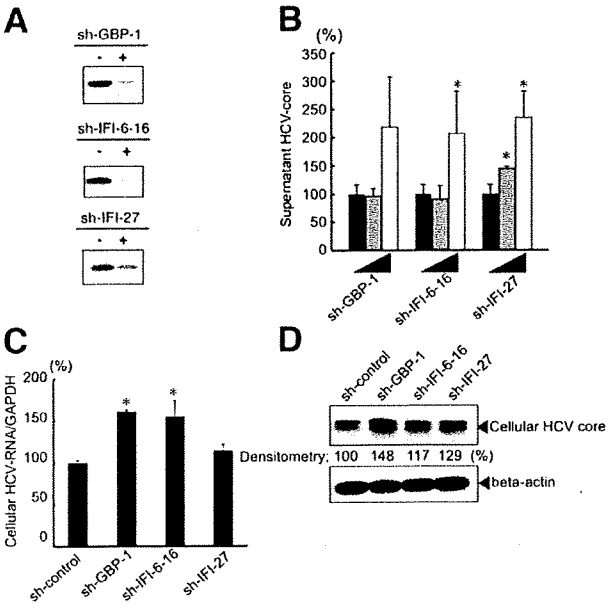

Fig. 3. The effects of shRNA-directed suppression of GBP-1, IFI-6-16, and IFI-27 expression on HCV replication. (A) The ISG-expression plasmids indicated, pcDNA-GBP-1 (upper panel), pcDNA-IFI-6-16 (middle panel), or pcDNA-IFI-27 (lower panel), were cotransfected with shRNA-expressing plasmid, pUC-shRNA-control (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 $\pm$ 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 $\pm$ 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 $\pm$ 88.7%), sh-IFI-6-16 (206.4 $\pm$ 74.5%), or sh-IFI27 (234.2 $\pm$ 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 $\pm$ 1.95% for shRNA-GBP-1, 155.7 $\pm$ 18.6% for shRNA-6-16, 114.6 $\pm$ 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-

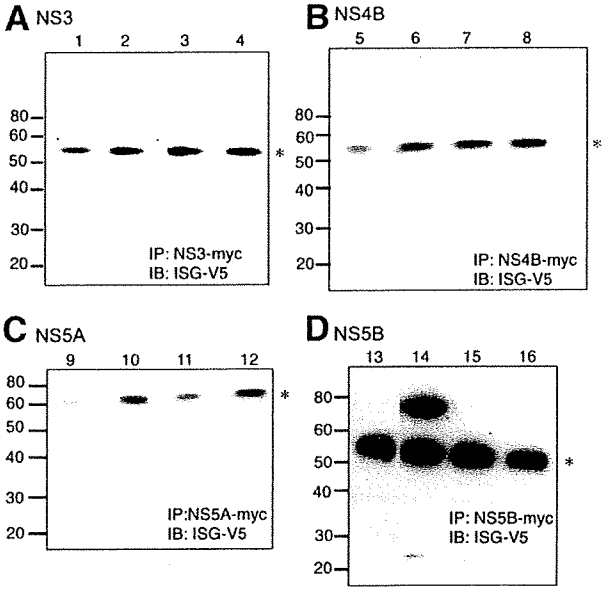

Fig. 4. Interaction of interferon inducible proteins with HCV-NS proteins by immunoprecipitation assay. (A-D) The plasmids indicated expressing myc-tagged HCV-NS protein, NS3 (A), NS4B (B), NS5A (C), or NS5B (D) were cotransfected into HEK-293T with V5-tagged ISG-expression plasmids for GBP-1 (lanes 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.

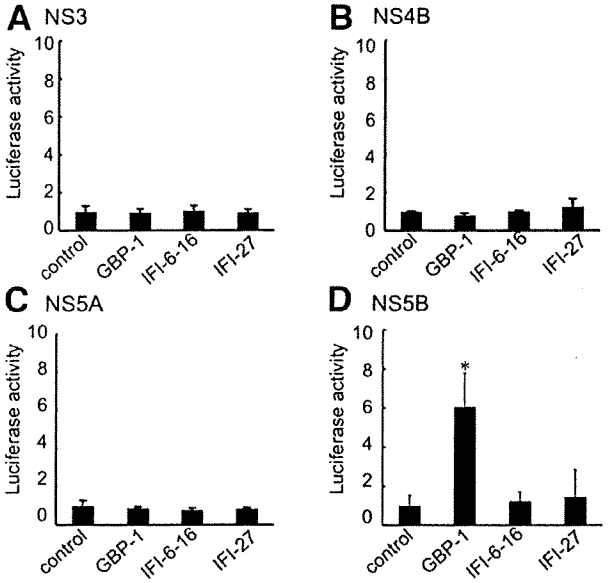

Fig. 5. The analysis of HCV-NS proteins binding ISG proteins by mammalian two-hybrid assay. Interaction of ISG proteins with HCV-NS proteins was confirmed by mammalian two-hybrid assay. The plasmids indicated, pCMV-BD-NS protein NS3 (A), NS4B (B), NS5A (C), or NS5B (D), were cotransfected with pCMV-AD-ISG 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.

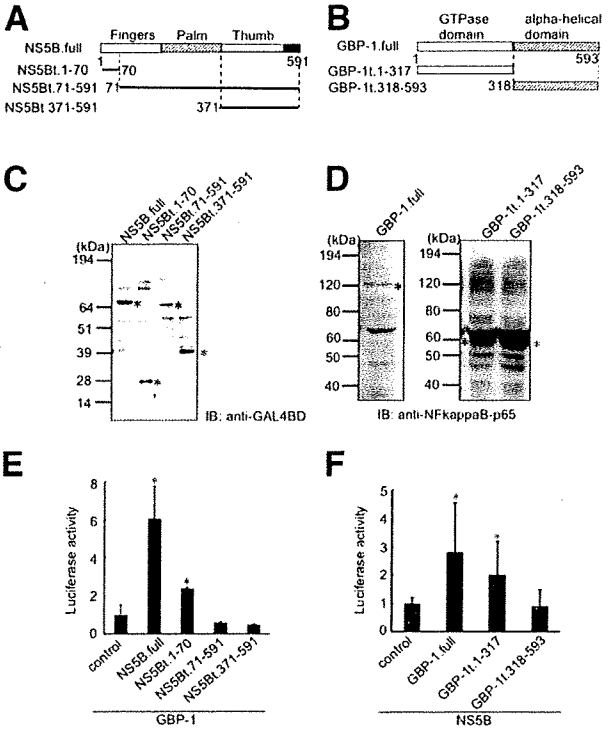

Fig. 6. Analyses of the domains responsible for the interaction between HCV-NS5B and GBP-1. (A) Scheme of full-length and truncated protein in HCV-NS5B. (B) Scheme of full-length and truncated protein in GBP-1. (C) Western blotting analysis of cells transfected with the indicated NS5B truncated protein-expression 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. $*P < 0.05$.

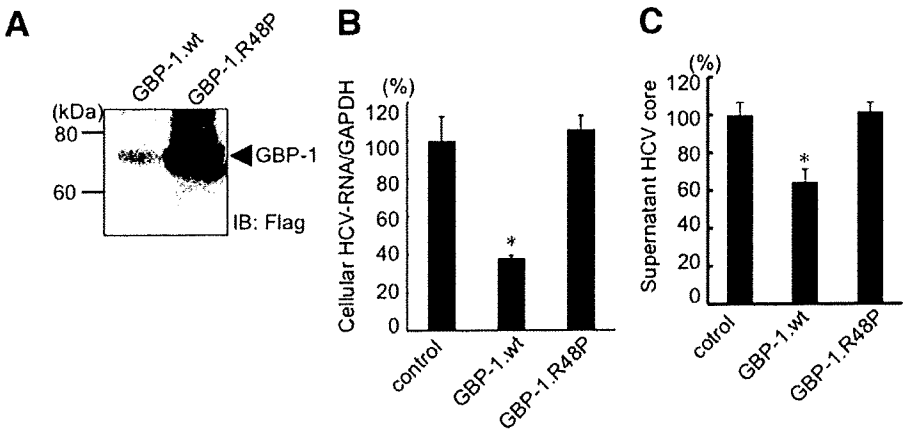

Fig. 7. GTPase activity has an antiviral effect on HCV replication. Western blotting analysis of GBP-1 and GBP-1 mutant, R48P. Plasmids expressing GBP-1 mutant type, R48P, or GBP-1 wildtype were transfected into 293T cells. Cell lysates were harvested at 48 hours after transfection, followed by immunoblotting (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.

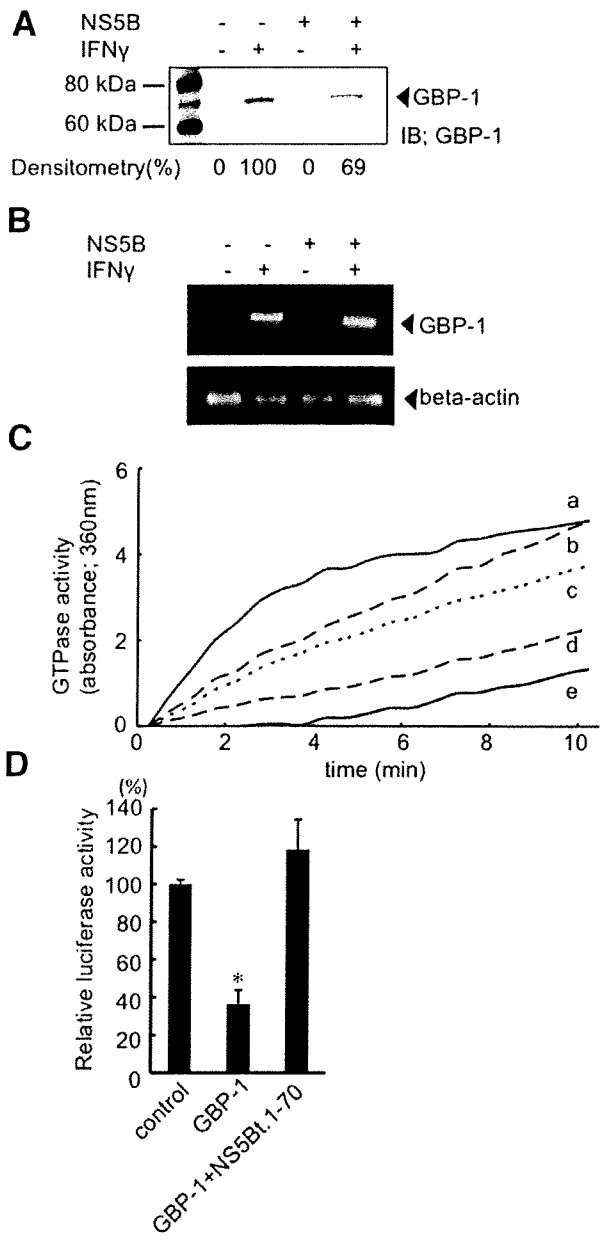

Fig. 8. GTPase activity of GBP-1 was negatively regulated by HCV-NS5B. (A) A GBP-1-expression V5-tagged plasmid was cotransfected into Huh-7 cells with a plasmid expressing myc-tagged-HCV-NS5B and cell lysates were harvested. Cell lysates were immunoprecipitated by anti-myc antibodies. Immunoprecipitated 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 ELIPA 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.

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Bioactivity-guided screening identifies pheophytin a as a potent anti-hepatitis C virus compound from *Lonicera hypoglauca* Miq.

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ABSTRACT

Chronic hepatitis C virus (HCV) infection is a worldwide public issue. In this study, we performed bioactivity-guided screening of the *Lonicera hypoglauca* Miq. crude extracts to find for naturally chemical entities with anti-HCV activity. Pheophytin a was identified from the ethanol-soluble fraction of *L. hypoglauca* that elicited dose-dependent inhibition of HCV viral proteins and RNA expression in both replicon cells and cell culture infectious system. Computational modeling revealed that pheophytin a can bind to the active site of HCV-NS3, suggesting that NS3 is a potent molecular target of pheophytin a. Biochemical analysis further revealed that pheophytin a inhibited NS3 serine protease activity with $IC_{50} = 0.89$ μM. Notably, pheophytin a and IFNα-2a elicited synergistic anti-HCV activity in replicon cells with no significant cytotoxicity. This study thereby demonstrates for the first time that pheophytin a is a potent HCV-NS3 protease inhibitor and offers insight for development of novel anti-HCV regimens.

Hepatitis C virus (HCV) belongs to a member of *Flaviviridae* and is a worldwide infectious pathogen causing chronic hepatitis that can progress further to hepatocellular carcinoma [1]. The current therapeutic protocol for HCV infection consists mainly of interferon (IFN) in combination with ribavirin that usually accompanies with strong side effects and moderate successful rate [2,3]. Hence, there is an urgent need to find new regimens to increase the efficacy of anti-HCV therapy.

Natural products metabolized from plants represent desirable sources for novel therapeutic compounds. Both *Lonicera hypoglauca* Miq. and *Lonicera japonica* Thunb are widely used as Jinyinhua in traditional Chinese medicine. Although they have similar geographic distribution, obviously various characteristics are observed [4]. Studies of the phytochemistry and bioactivity of Jinyinhua have mostly focused on *L. japonica* (Japanese honeysuckle) that has been reported to possess properties of anti-inflammation, anti-angiogenic, and anti-nociceptive activities [5,6]. However, the cognate *L. hypoglauca* has barely been studied.

Recently, the subgenomic HCV replicon cells have been developed for mechanistic study of HCV replication [7,8]. In the present study, HCV replicon cells were used to explore whether the extracts from *L. hypoglauca* elicit any anti-HCV activity. We found that *L. hypoglauca* contains an active phytocompound pheophytin a that exhibits strong anti-HCV activity. The inhibition of NS3 protease activity accounts mainly for the anti-HCV activity of pheophytin a. Furthermore, the combination of pheophytin a with INFα-2a elicits synergistic anti-HCV activity with no considerable cytotoxicity. The significance of these findings is discussed.

Materials and methods

Cell culture and viability assay. The subgenomic HCV replicon cells (a kind gift from Professor J.-H. James Ou, University of Southern California), the Huh7/Rep-Feo subgenomic replicon cells containing a luciferase reporter gene, and the Huh7.5 cells (a kind gift from Professor Charles Rice, The Rockefeller University, NY) were cultured as described [9–11]. Cell viability was determined

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by trypan blue exclusion and MTS assay as described by the manufacturer (Promega).

Replicon cell-based assay (Western blot, luciferase, and RT-PCR assay). For HCV replicon cell-based bioactivity-guided screening, the extracts or compounds isolated from *L. hypoglauca* were ectopically applied to the replicon cells for 48 h. The expression of HCV viral proteins was determined by Western blot analysis as described previously [12]. On the other hand, the Huh7/Rep-Feo cells (2×10^5 cells) were seeded in a 6-well plate. At 8 h after seeding, the tested compound was added and incubated for a total of 120 h. The cells were then subjected to luciferase activity assay using the Bright-Glo luciferase assay system (Promega). The IC_{50} was defined as the concentration of compound at which the luciferase activity in the replicon cells was reduced by 50%.

For real-time RT-PCR analysis, total RNA was amplified using the forward primer HCV-F (5′-TGCGGAACCGGTGAGTACA-3′) and the reverse primer HCV-R (5′-CTTAAGGTTTAGGATTCGTGCTCAT-3′) in the presence of SYBR Green I Master Mix (Applied Biosystems). For internal control, RT-PCR was performed using the forward primer β-actin-F (5′-TCACCCACACTGTGCCCATCTACG-3′) and the reverse primer β-actin-R (5′-CAGCGGAACCGCTCATTGCCAATG-3′). The reaction condition was 1 cycle of 48 °C for 30 min., 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min.

Infectious HCV particles production and infection inhibition assay. The production of infectious HCV particles (HCVcc) was performed using the plasmid PFL-J6/JFH (a kind gift from Professor Charles Rice, The Rockefeller University) as described [13]. For infection inhibition assay, 100 μl of HCVcc-containing supernatant (5×10^3 foci forming units) was added to Huh7.5 cells and incubated for 4 h. The virus-containing supernatant was then removed and fresh medium with or without the tested compound was added and incubated for a total of 72 h. The cells were fixed and stained by anti-Core antibody (Affinity BioReagents) and the infectious foci were counted by the fluorescence microscopy.

Extraction. Leaves and stems of *L. hypoglauca* were collected from the Da-kang area of Taichung County in central Taiwan. The species were identified and voucher specimens (*YHT001* (TCF)) were deposited at the Herbarium of the Department of Forestry, National Chung Hsing University, Taiwan. The preparation and purification of crude extracts were performed as mentioned (Supplemental methods).

Molecular modeling of the pheophytin a-HCV NS3/4A complexes. The model of pheophytin a in complex with the HCV-NS3/4A protease was constructed by docking pheophytin a to the crystallographic structure of 1b strain of the HCV-NS3/4A protease (PDB code 1DY8) [14]. Molecular modeling was performed as mentioned (Supplemental methods).

NS3 serine protease activity assay. The NS3 serine protease activity assay was conducted by the FRET-based, SensoLyte™ HCV protease assay kit (AnaSpec). Briefly, HCV-NS3/4A protease was mixed with the tested compound in the assay buffer. After 15 min incubation at room temperature, 50 μl of FRET peptide substrate solution was added and mixed well. For kinetic reading, the fluorescence intensity was measured immediately and continuously at Ex/Em = 490 nm/520 nm. The data was recorded every 5 min for a total of 120 min.

Synergy analysis. To determine whether the effect for the combination of pheophytin a with INFα-2a (Roche) was synergistic, additive or antagonistic, the luciferase-based HCV replicon assay was performed and analyzed according to the classical isobologram analysis [15].

Statistical analysis. The Student's *t* test was used to evaluate the difference between the test sample and solvent control. $p < 0.05$ was considered statistically significant.

Results

Lonicera hypoglauca exhibits potent anti-viral activity in HCV replicon cells

In this study, HCV replicon cells were used to perform bioactivity-guided screening to explore whether the extracts from *L. hypoglauca* elicit any anti-HCV activity. The replicon cells were treated with various concentrations of EtOH-soluble extract (LH-crude) from *L. hypoglauca*. LH-crude caused a dose-dependent inhibition of NS5A expression, a long half-life HCV protein, without affecting cell viability and cell growth (Fig. 1A). Subsequent tracking of LH-crude revealed that the ethyl acetate-soluble fraction (LH-EA) but not the H_2O-soluble fraction (LH-water) was most active (Fig. 1B).

To identify the active component in LH-EA with anti-HCV activity, LH-EA was separated into 20 fractions (LH-EA-1 to -20) by

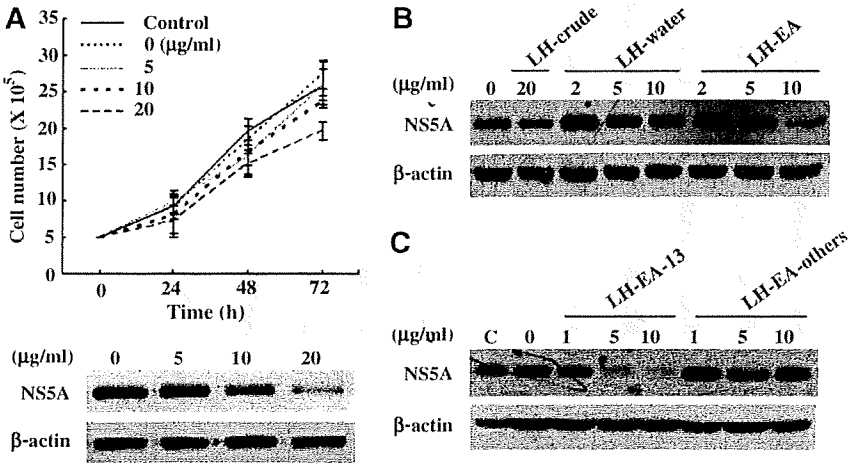

Fig. 1. *Lonicera hypoglauca* exhibits potent anti-viral activity in HCV replicon cells. (A) HCV replicon cells were treated with the indicated concentrations of EtOH extract (LH-crude) from *L. hypoglauca* and viable cells were determined by the trypan blue exclusion analysis. The data represented the means ± SD. ($n = 3$, upper panel). The cell lysates (48 h post-treatment) were subjected to Western blot analysis using the anti-NS5A antibody (lower panel). (B, C) HCV replicon cells were treated with the indicated concentrations of LH-crude, EtOAc-soluble fraction (LH-EA), H_2O-soluble fraction (LH-water), the fraction 13 of LH-EA (LH-EA-13), and the pool of other LH-EA fractions (LH-EA-others), respectively. The cell lysates (48 h post-treatment) were subjected to Western blot analysis using the anti-NS5A antibody. The expression of β-actin was used for the control of equal protein loading. C:No-treated, control replicon cells.

chromatography. Each fraction was further evaluated for their anti-HCV activity using the same replicon cell-based assay. As shown in Fig. 1C, the fraction of LH-EA-13 exhibited the strongest anti-HCV activity.

Pheophytin a reduces HCV protein expression in replicon cells and the infectivity of HCVcc

To understand which compound exhibits anti-HCV activity, the LH-EA-13 fraction was purified by HPLC to obtain compound **1**. The compound **1** molecular formula was determined to be $C_{55}H_{74}N_4O_6$ (MW = 887.23) by fast atom bombardment mass spectrometry. The ^{1}H NMR spectrum of compound **1** presented 1.68 (t, J = 8 Hz, 3H), 1.79 (d, J = 7.2 Hz, 3H), 2.16 (m, 1H), 2.31 (m, 1H), 2.45 (m, 1H), 2.59 (m, 1H), 3.23 (s, 3H), 3.39 (s, 3H), 3.67 (s, 3H), 3.68 (q, J = 8 Hz, 2H), 3.85 (s, 3H), 4.19 (d, J = 9.2 Hz, 1H), 4.50 (dq, J = 7.2, 5.2 Hz, 1H), 6.17 (dd, J = 11.6, 1.6 Hz, 2H), 6.24 (s, 1H), 6.28 (dd, J = 18.0, 1.6 Hz, 2H), 7.99 (dd, J = 18.0, 11.6 Hz, 1H), 8.54 (s, 1H), 9.38 (s, 1H), and 9.51 (s, 1H). The ^{1}H NMR spectrum of compound **1** was identical to the spectrum of pheophytin a which has been identified by Ina and his coworker [16]. The structure of pheophytin a was shown in Fig. 2A. The purity of pheophytin a was estimated to be greater than 99.5% from the ^{1}H NMR spectrum and HPLC analysis.

We then used compound **1** (pheophytin a) to confirm its anti-HCV activity using HCV replicon cells and Huh7/Rep-Feo cells. Pheophytin a did not affect the cell viability of these cells (Fig. 2B left panel, Fig. 2C upper panel). However, it was more potent than the crude extracts in inhibiting NS5A expression in replicon cells (Fig. 2B right panel) and luciferase expression in Huh7/Rep-Feo cells (Fig. 2C lower panel). Pheophytin a also significantly inhibited replicon cells HCV RNA accumulation (Fig. 2D) and the infectivity of HCVcc (Fig. 2E, $p < 0.05$). The calculated IC_{50} was 4.97 μM. These data thereby unveil pheophytin a as the active component of *L. hypoglauca* with anti-HCV activity.

Computational molecular modeling reveals the interaction of pheophytin a with the active site of HCV-NS3 protease

NS3 protease is an attractive target for development of antiviral agent. To gain more chemical insight for the molecular mechanism involved in the anti-HCV activity of pheophytin a, computational molecular modeling was performed by docking pheophytin a onto the active site of HCV NS3/4A. The best predicted binding mode was illustrated in Fig. 3A and B. The amino acids HIS57, LYS136, SER139, and ALA155 were involved in the formation of four hydrogen bonding with pheophytin a. In addition, the amino acids SER42, GLY137, LYS136, VAL132, SER133, CYS159, PHE154,

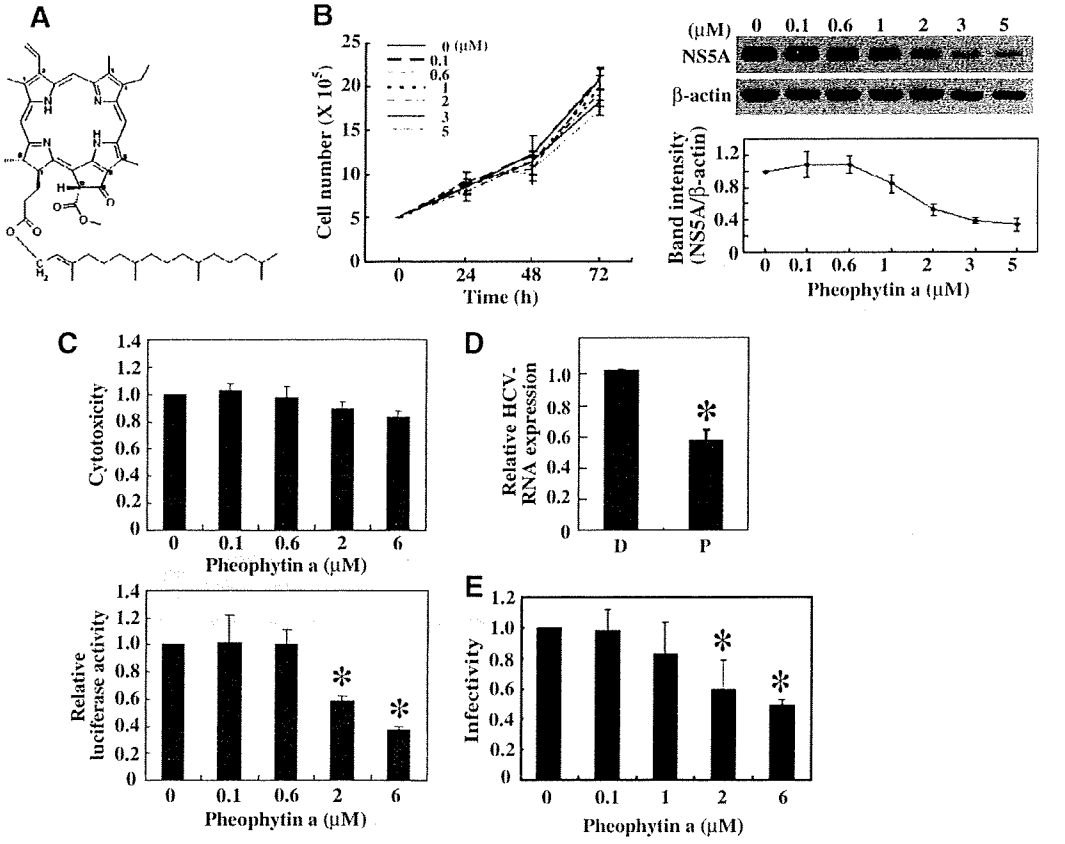

Fig. 2. Pheophytin a inhibits HCV expression in the subgenomic HCV replicon cells and HCVcc. (A) Chemical structure of pheophytin a. (B) HCV replicon cells were treated with the indicated concentrations of pheophytin a and the viable cells were determined by the trypan blue exclusion analysis (left panel). The cell lysates (48 h post-treatment) were subjected to Western blot analysis using the anti-NS5A antibody and the expression of β-actin was used for the control of equal protein loading (right panel). The band intensity of NS5A versus β-actin was determined and the ratios were plotted against the concentration of pheophytin a. (C) Huh7/Rep-Feo cells were treated with the indicated concentrations of pheophytin a. The cell toxicity was determined by MTS assay (upper panel) and the HCV gene expression was determined by luciferase activity assay (lower panel). (D) HCV replicon cells were treated with 6 μM of pheophytin a for 96 h and the total RNA was subjected to real-time RT-PCR assay. The relative HCV-RNA level for pheophytin a- (P) and DMSO-treated (D) control cells was shown. (E) The HCVcc-infected Huh7.5 cells were treated with various concentration of pheophytin a. The cells were stained by the anti-Core antibody and the relative infectivity for the indicated treatment compared to the control treatment cells was shown. $^*p < 0.05$ when compared to the control replicon cells. The data represented the mean ± SD (n = 3).

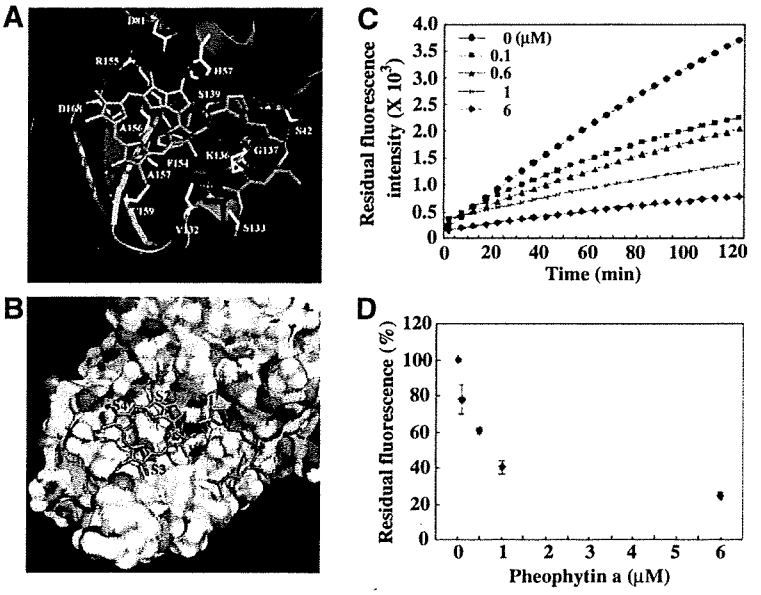

Fig. 3. Computational molecular models and molecular analysis for the interaction between pheophytin a and HCV NS3/4A. (A, B) The computational molecular models of pheophytin a in the active site of the HCV NS3/4A were shown as cartoon and surface in (A) and (B), respectively. The carbon atoms in pheophytin a were colored in magenta. The side-chains of the NS3 amino acid residues within 5 Å radius centered on pheophytin a were shown explicitly. The carbon atoms in NS3 were shown in gray. Nitrogen and oxygen were colored in blue and red, respectively. Trace of the NS3 backbone structure was shown as a blue tube. The secondary structure elements were shown as a ribbon drawing and the important amino acid residues involved in pheophytin a binding were labeled. The green and yellow dotted lines represented tentative donor–acceptor pairs of the hydrogen bonds and the hydrophobic interactions between pheophytin a and NS3, respectively. (C, D) HCV-NS3/4A protease (5 ng/well) was mixed with the indicated concentrations of pheophytin a and the *in vitro* protease activity was determined as described in "Materials and methods". The fluorescence signal was recorded every 5 min (C). The percentage of residual activity at 60 min after initiation of reaction was calculated and was plotted against the concentration of pheophytin a (D). The data represented the means ± SD ($n = 3$).

ALA156, ASP168, and ARG155 also contributed to the hydrophobic interactions and binding with pheophytin a. An important noncovalent hydrogen bond interaction was formed between the hydroxyl group of SER139 of the NS3 catalytic triad and the acyl oxygen in the ester group of pheophytin a. Furthermore, the S1, S2, S3, and S4 sites of NS3 were probably occupied by the porphin group of pheophytin a, and the S′ sites other than S1′ were possibly occupied by a phytol group of pheothytin a. The particular hydrophobic interaction was formed between a phytol group of pheophytin a and a long nonpolar chain of four carbon atoms of LYS136. These data suggest that pheophytin a may have a functional interplay with HCV-NS3.

Pheophytin a elicits its anti-HCV effect by inhibition of NS3 serine protease activity

To further delineate the effect of pheophytin a on HCV-NS3, the NS3 serine protease activity assay was performed in the presence of pheophytin a. The amount of fluorescence signal was decreased with increasing concentration of pheophytin a in a dose-dependent manner (IC_{50} = 0.89 μM), indicating that pheophytin a exhibits the anti-NS3 serine protease activity (Fig. 3C). Plotting the residual protease activity of reactions containing various concentrations of pheophytin a as percentage of activity in the absence of the compound (expressed as 100%) resulted in a titration curve typical of a binding inhibitor (Fig. 3D).

Combination of pheophytin a with INFα-2a significantly enhances anti-HCV activity without an increase in cytotoxicity

To determine whether pheophytin a and INFα-2a have synergistic inhibitory effect on HCV gene expression, the classic isobologram analysis was performed. A typical isobologram used to measure the drug–drug interaction was shown in Fig. 4A with synergy, additivity, and antagonism presented as concave, linear and convex isoeffective curves, respectively [17]. Our results demonstrated that the curve was below the line representing additive effect, indicating the synergy of the two drugs against the replicon cells (Fig. 4B). In addition, MTS assays did not show any difference in cell survival with the drug concentrations used in this isobologram analysis (data not shown), suggesting that the synergistic effect of pheophytin a and INFα-2a on HCV gene expression is not due to cytotoxicity.

Discussion

The global prevalence of HCV infection averages 3% according to the estimation made by the World Health Organization. Through bioactivity-guided screening, structure–activity relationship, and biochemical analysis, we report herein that HCV-NS3 is a potent molecular target of *L. hypoglauca*-derived pheophytin a. As a result, the viral proteins and RNA expression and the HCV infectivity are diminished. Notably, concomitant treatment of HCV replicon cells with pheophytin a and INFα-2a elicits synergistic effect and enhances anti-HCV activity without compensation of cell survival. This study thereby offers insight to the molecular basis for the anti-HCV activity of *L. hypoglauca* and indicates pheophytin a as a potent adjuvant regimen for INFα-2a therapy in the clinical setting.

Among the HCV nonstructural proteins, NS3-mediated processing of the protein junctions is essential for viral replication and therefore provides an attractive target for development of antiviral agents [18]. In addition to pheophytin a, several studies also discovered natural products with anti-NS3 protease activity. These include nature compounds from Galla Chinese and *Rhodiola kirilowii*

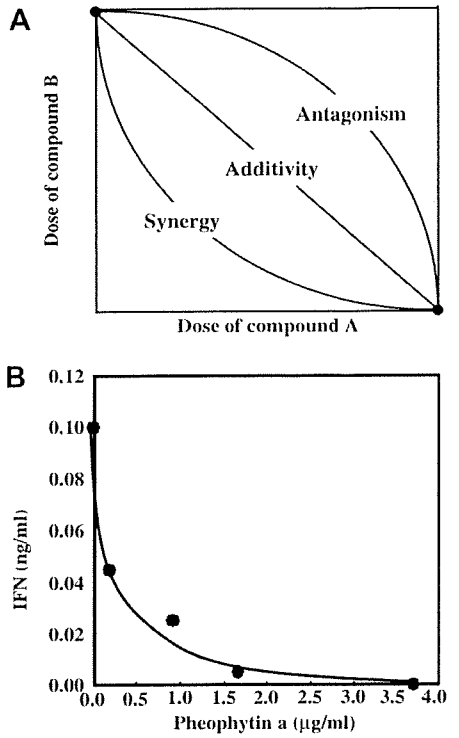

Fig. 4. Pheophytin a and IFNα-2a elicit synergistic inhibitory effect on HCV gene expression. (A) Typical isobologram for analyzing the interaction between two drugs. (B) The isobologram for the combined uses of pheophytin a and IFNα-2a. Luciferase-based replicon cell assays were performed under the indicated treatments. The fixed ratios adjusted by the IC_{50} (FICs) at 50% inhibition were calculated and the FICs for pheophytin a and IFNα-2a were plotted on the *X*-axis and *Y*-axis, respectively.

[19,20]. The potency of pheophytin a appears to be comparable with these compounds and thus pheophytin a is added to a list of natural compounds with potent anti-HCV activity. Nevertheless, pheophytin a represents the only natural compound with the anti-HCV activity directly evaluated by the subgenomic replicon (genotype 1b) and the newly developed HCVcc system.

The mechanism underlying the anti-HCV activity of pheophytin a is also addressed in this study. Laboratory evidence suggests that inhibition of NS3 protease activity accounts mainly for the action of pheophytin a. At first, computational prediction modeling indicates pheophytin a can interact with the active site of NS3 protease. The *in vitro* protease activity assay further demonstrates that pheophytin a is a NS3 protease inhibitor. Notably, using HCV replicon cells for selection of pheophytin a-resistant strains revealed various amino acid substitutions in the NS3 protein coding region (unpublished data). One of the resistant strains carries an NS3 V36A substitution. Similar V36 variant has been found in the telaprevir-treated patient and was thought to loosen binding to Phe^{43} which is part of the S′ substrate-binding pocket of the HCV protease [21]. Hence, inhibition of NS3 activity is pivotal for pheophytin a to elicit its anti-HCV activity.

Recently, natural prevalence of HCV variants with decreased sensitivity to the current use of NS3 protease inhibitors have been found in treatment-naive subjects [22]. For instances, the R155K mutant shows reduced susceptibility to the three protease inhibitors, BILN-2061, ITMN-191 and VX-950 [23]. This raises concern over the potential emergence of these variants as the multidrug-resistant, highly fit mutants in HCV patients treated with protease inhibitors. In contrast to the pure protease inhibitors, pheophytin a not only inhibits NS3 protease activity but also elicits various biological and cellular effects such as the anti-inflammatory activity and the activation of mitogen-activated protein kinase signaling [16,24]. The difference modes of action for pheophytin a may thereby offer advantages in overcoming the drug-resistant variants that is worthy to further investigation.

In conclusion, we demonstrate herein that pheophytin a derived from the extracts of *L. hypoglauca* represents a novel natural compound with strong anti-HCV-NS3 protease activity and little cytotoxicity. This study thereby contributes to our understanding for the anti-HCV activity of pheophytin a and offers new insight for development of novel therapeutic agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.043.

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