ONLINE METHODS

Study cohorts. From April 2007 to April 2009, samples were obtained from 314 patients with chronic HCV (genotype 1) infection who were treated at 15 multicenter hospitals (liver units with hepatologists) throughout Japan. Each patient was treated with PEG-IFN-α2b (1.5 μg per kg body weight (μg/kg) subcutaneously once a week) or PEG-IFN-α2a (180 μg/kg once a week) plus RBV (600–1,000 mg daily depending on body weight). As a reduction in the dose of PEG-IFN-α and RBV can contribute to a less sustained virological response[21], only patients with an adherence of >80% dose for both drugs during the first 12 weeks were included in this study. HBsAg-positive and/or anti-HIV–positive individuals were excluded from this study.

NVR (seen in ~20% of total treated patients) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pre-treatment baseline value within the first 12 weeks and detectable viremia 24 weeks after treatment. VR was defined as the achievement of SVR or transient TVR in this study; SVR was defined as undetectable HCV RNA in serum 6 months after the end of treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or on completion of the therapy. Of 878 patients with HCV genotype 1 treated by PEG-IFN-α/RBV at 14 hospitals, only 114 (13.0%) met the criteria for NVR in this study. For the GWAS stage of the study, a case-control study was conducted comparing individuals with NVR (82 individuals) and VR (72 individuals). For the replication stage, an independent cohort of samples from 172 Japanese patients with HCV genotype 1, including 50 with NVR and 122 with VR, was obtained from an independent cohort study at Tokyo Medical and Dental University Hospital (Ochanomizu Liver Conference Study Group) and Musashino Red Cross Hospital. Clinical data from the combined cohorts, with a total of 140 SVR, 46 TVR and 128 NVR patients, are shown in **Supplementary Table 4**.

Informed consent was obtained from each patient who participated in the study. The study protocol conforms to the relevant ethical guidelines as reflected in *a priori* approval by the ethics committees of all the participating universities and hospitals.

SNP genotyping and data cleaning. In the GWAS stage, we genotyped 154 Japanese patients with HCV receiving PEG-IFN-α/RBV treatment using the Affymetrix Genome-Wide Human SNP Array 6.0 according to the manufacturer's instructions. After exclusion of 4 NVR samples and 8 SVR samples with QC call rates <95%, the remaining 142 samples were recalled using the Birdseed version 3 software (Affymetrix). The average overall call rate of 78 NVR and 64 VR samples reached 99.46% and 99.46%, respectively. We then applied the following thresholds for QC in data cleaning: SNP call rate ≥95% for all samples, MAF ≥1% for all samples and HWE P value ≥0.001 for VR group[22,23]. A total of 621,220 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots for the SNPs showing $P < 0.001$ in association analyses by comparing allele frequencies in NVR and VR groups were checked by visual inspection. SNPs with ambiguous genotype calls were excluded. **Supplementary Table 5** shows SNPs that might be weakly associated with NVR ($P < 10^{-4}$).

Although the 12 samples noted above were excluded from the GWAS stage by data cleaning, their quality was good enough for the SNP typing in the replication study, and thus they were included in the replication stage. In the subsequent replication stage with high-density association mapping, SNP genotyping in the independent set of 172 patients was completed using the DigiTag2 assay[24] and direct sequencing using the Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). In addition, strongly associated SNPs identified in the GWAS stage were also genotyped for the GWAS samples using the DigiTag2 assay, and the results were 100% concordant to those from the GWAS platform.

Screening for new polymorphisms. To determine possible genomic variants in the region of *IL28B* and its promoter, we sequenced the 3.3-kb region in a total of 48 Japanese patients with HCV (28 NVR and 20 VR). We selected 7 samples from NVR patients who were minor allele homozygotes for 2 SNPs (rs12980275 and rs8099917), 11 samples from NVR and 10 samples from VR heterozygotes, and 10 samples from NVR and 10 samples from VR major allele homozygotes. The sequencing primers were designed using the Visual OMP Nucleic Acid software (**Supplementary Table 6**). PCR was carried using TaKaRa LA *Taq* polymerase (Takara Biochemicals) under the following thermal cycler conditions: stage 1, 94 °C for 1 min; stage 2, 98 °C for 10 s, 68 °C for 15 min, for a total of 30 cycles; stage 3, 72 °C for 10 min. A 50-μl PCR analysis was performed using 2.5 U TaKaRa LA *Taq* with 1× LA PCR buffer II, 0.4 mM dNTP, 10 pmol of each primer and 10 ng of genomic DNA. For sequencing, 7.0 μl of the PCR products were incubated with 3 μl of Exonuclease I/Shrimp Alkali Phosphatase (Takara Biochemicals) first for 90 min at 37 °C and then for another 10 min at 80 °C. Sequencing reactions were performed with the use of a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). After purification with MultiScreen-HV (Millipore) and Sephadex G-50 Fine (GE Healthcare UK Ltd.), the reaction products were applied to the Applied Biosystems 3730 DNA Analyzer.

In the variation screening, three SNPs (rs8103142, rs28416813 and rs4803219) and a few infrequent variations were detected. We then typed these SNPs in all of the 314 patients.

Statistical analysis. The observed association between a SNP and response to PEG-IFN-α/RBV treatment was assessed by χ^2 test with a two-by-two contingency table in three genetic models: allele frequency model, dominant-effect model and recessive-effect model. SNPs on the X chromosome were removed because gender was not matched between the NVR group and the VR group. A total of 621,220 SNPs passed the QC filters in the GWAS stage; therefore, significance levels after the Bonferroni correction for multiple testing were $P = 8.05 \times 10^{-8}$ (0.05/621,220) in the GWAS stage and $P = 0.0031$(0.05/16) in the replication stage. None of the 16 markers genotyped in the replication stage showed deviations from Hardy-Weinberg equilibrium in the VR group ($P > 0.05$).

The inflation factor λ was estimated based on the median χ^2 and revealed to be 1.029 (median) and 1.011 (mean), suggesting that the population substructure should not have any substantial effect on the statistical analysis (**Supplementary Fig. 1**). In addition, the principal component analysis on the 142 patients (78 NVR samples and 64 VR samples) analyzed in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (**Supplementary Fig. 2**).

For the replication study and the high-density association mapping, 16 SNPs were selected from the region of ~40 kb (chr. 9, nucleotide positions 44421319–44461718; build 35) containing the significantly associated SNPs (rs12980275 and rs8099917) in the GWAS stage by analyzing, using Haploview software, LD and haplotype structure based on the HapMap data for individuals of Japanese descent. These SNPs included tagging SNPs estimated on the basis of haplotype blocks, SNPs located within the *IL28B* and *IL28A* genes (rs11881222 and rs576832, respectively) and the significantly associated SNPs identified in the GWAS stage (**Supplementary Table 1**). On the basis of the genotype data from the total of 314 patients in the GWAS stage and replication stages, haplotype blocks were estimated using the four-gamete rule, and three blocks were observed (**Fig. 2**). Association of haplotype with response to PEG-IFN-α/RBV treatment was analyzed using Haploview software.

The logistic regression model was used to assess the factors associated with NVR. STATA 10 (Statacorp LP) was used for all analysis. Age, platelet count, and aminotransferase (ALT) and HCV-RNA levels were applied as continuous variables.

Real-time quantitative RT-PCR for *IL28* gene. A layer of mononuclear cells was collected via Ficoll from peripheral blood. Total RNA was isolated using the RNeasy Mini Kit and the RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript II reverse transcriptase with Oligo $(dT)_{12-18}$ primer (Invitrogen). The relative quantification of the target gene was determined using Custom TaqMan Gene Expression Assays, and the expression of glyceraldehyde-3-phosphate dehydrogenase was used to normalize the gene expression level (Applied Biosystems) according to the manufacturer's protocol. The data were analyzed by the $2[-\Delta\Delta C_t]$ method using Sequence Detector version 1.7 software (Applied Biosystems). A standard curve was prepared by serial tenfold dilutions of

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human cDNA. The curve was linear over 7 logs with a correlation coefficient of 0.998. The specific detection of *IL28B* in real-time PCR is hard to establish, because the nucleotide differences between *IL28A* and *IL28B* consist of only 9 nucleotides scattered throughout the gene. Primers and probes are designed for the *IL28* gene (**Supplementary Table 6**).

URLs. The results of the present GWAS have been registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

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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-

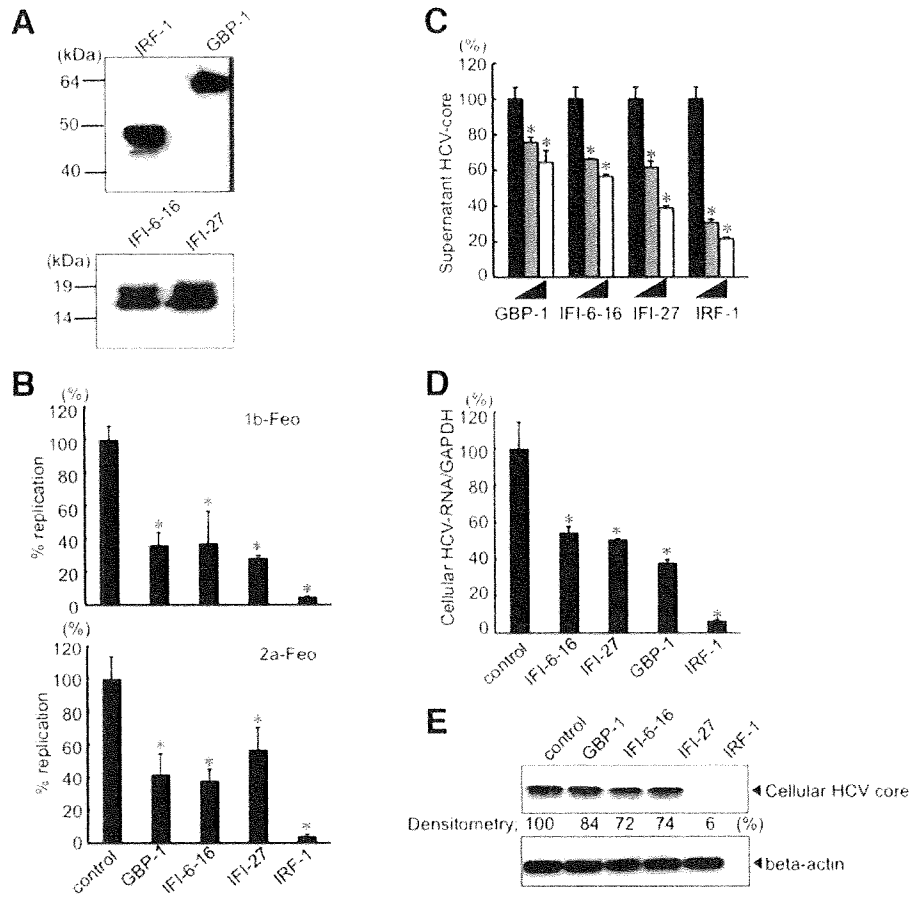

Fig. 1. Suppression of HCV infection and replication by overexpression of ISGs. (A) Western blotting analysis of cells transfected with the ISG-expression plasmids. ISG expression vectors were respectively transfected into HEK-293T cells. The cells were harvested at 48 hours after transfection. Ten micrograms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

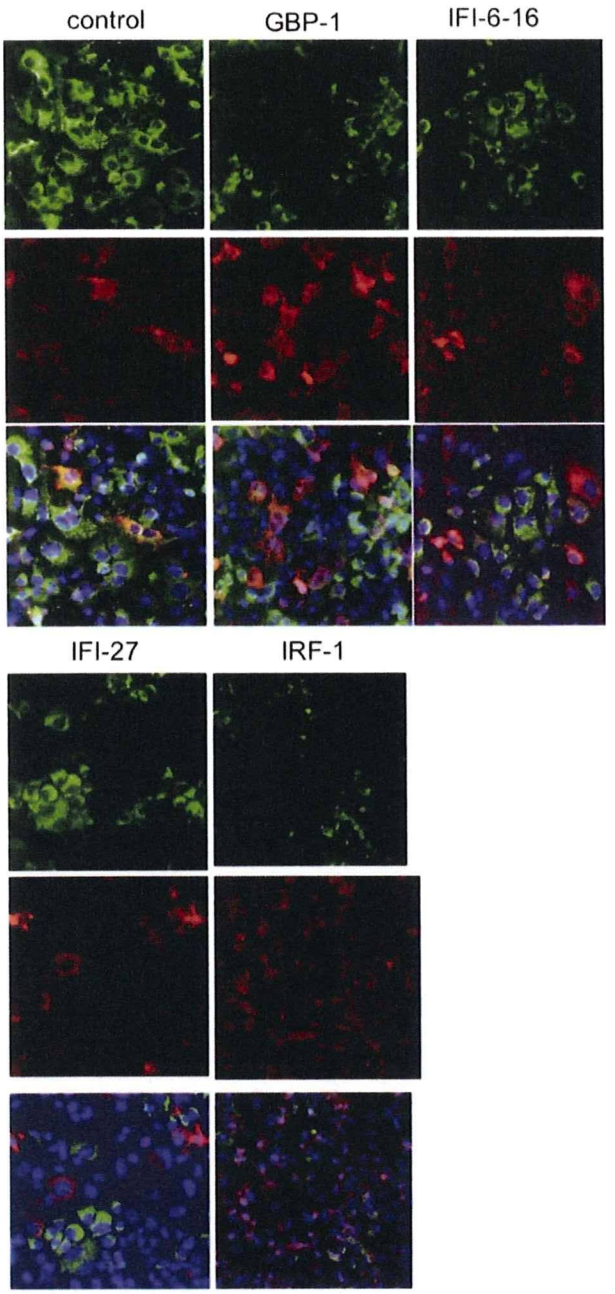

Fig. 2. Suppression of intracellular HCV replication levels by ISG-expression assessed by immunohistochemistry. The ISG-expression plasmid indicated or pcDNA3.1D/V5-His/LacZ as a control was transfected into HCV-JFH1-infected Huh-7.5.1 cells seeded in 18-mm-diameter dishes. At 48 hours after transfection,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

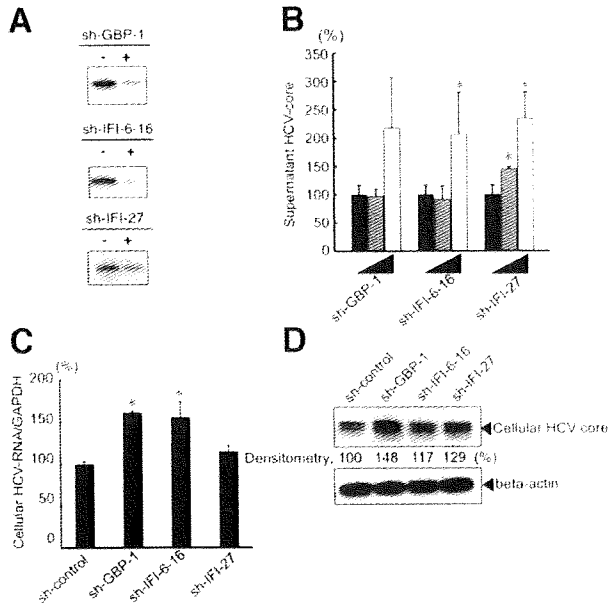

Fig. 3. The effects of shRNA-directed suppression of GBP-1, IFI-6-16, and IFI-27 expression on HCV replication. (A) The ISG-expression plasmids indicated, pcDNA-GBP-1 (upper panel), pcDNA-IFI-6-16 (middle panel), or pcDNA-IFI-27 (lower panel), were cotransfected with shRNA-expressing plasmid, pUC-shRNA-control (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-

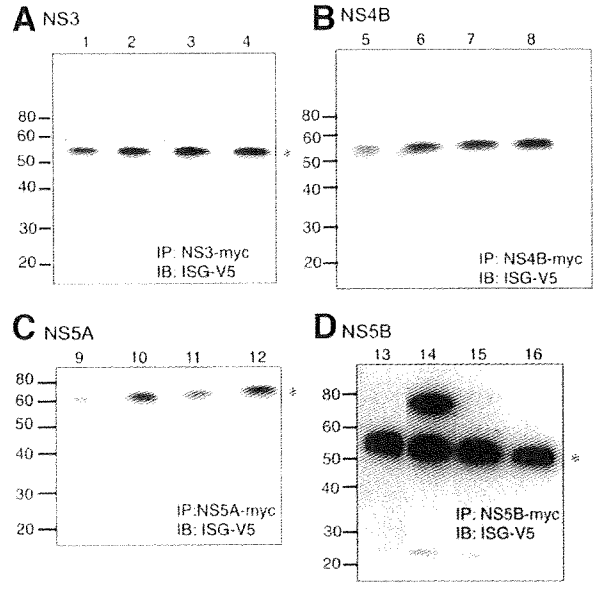

Fig. 4. Interaction of interferon inducible proteins with HCV-NS proteins by immunoprecipitation assay. (A-D) The plasmids indicated expressing myc-tagged HCV-NS protein, NS3 (A), NS4B (B), NS5A (C), or NS5B (D) were cotransfected into HEK-293T with V5-tagged ISG-expression plasmids for GBP-1 (lanes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

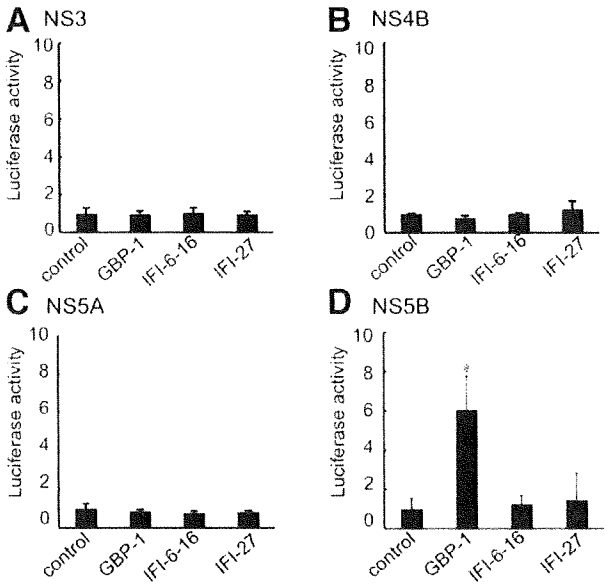

Fig. 5. The analysis of HCV-NS proteins binding ISG proteins by mammalian two-hybrid assay. Interaction of ISG proteins with HCV-NS proteins was confirmed by mammalian two-hybrid assay. The plasmids indicated, pCMV-BD-NS protein NS3 (A), NS4B (B), NS5A (C), or NS5B (D), were cotransfected with pCMV-AD-ISG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

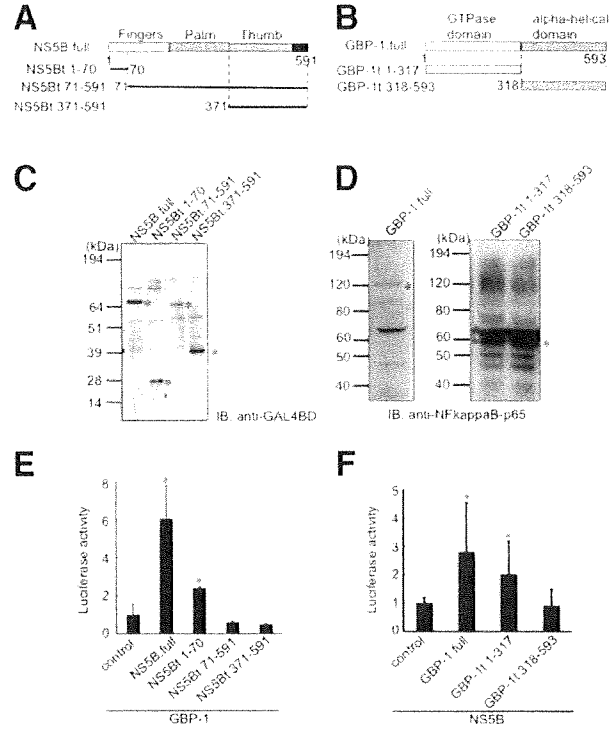

Fig. 6. Analyses of the domains responsible for the interaction between HCV-NS5B and GBP-1. (A) Scheme of full-length and truncated protein in HCV-NS5B. (B) Scheme of full-length and truncated protein in GBP-1. (C) Western blotting analysis of cells transfected with the indicated NS5B truncated protein-expression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

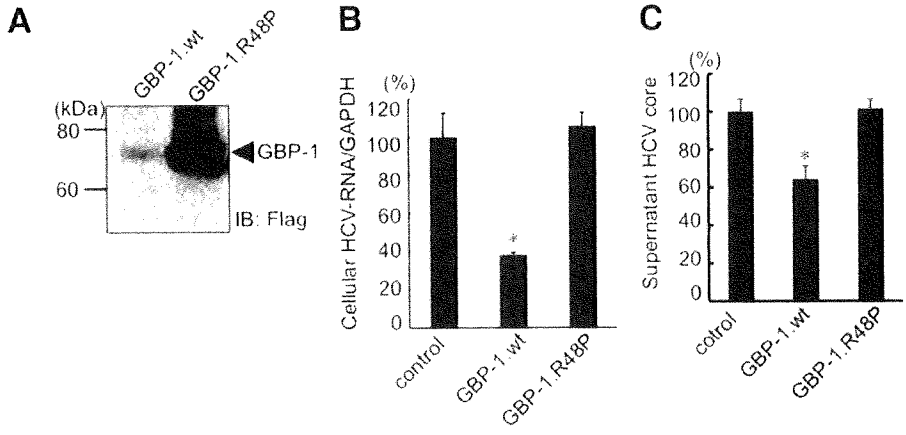

Fig. 7. GTPase activity has an antiviral effect on HCV replication. Western blotting analysis of GBP-1 and GBP-1 mutant, R48P. Plasmids expressing GBP-1 mutant type, R48P, or GBP-1 wildtype were transfected into 293T cells. Cell lysates were harvested at 48 hours after transfection, followed by immunoblotting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

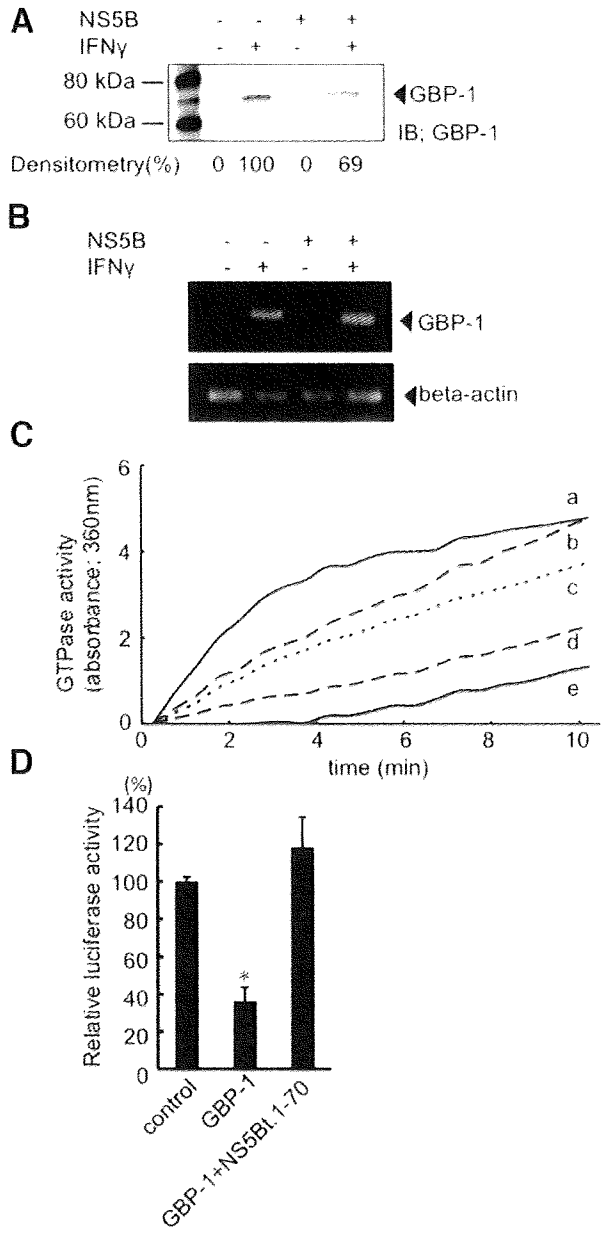

Fig. 8. GTPase activity of GBP-1 was negatively regulated by HCV-NS5B. (A) A GBP-1-expression V5-tagged plasmid was cotransfected into Huh-7 cells with a plasmid expressing myc-tagged-HCV-NS5B and cell lysates were harvested. Cell lysates were immunoprecipitated by anti-myc antibodies. Immunoprecipitated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

Two flavonoids extracts from *Glycyrrhizae radix* inhibit *in vitro* hepatitis C virus replication

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Aim: Traditional herbal medicines have been used for several thousand years in China and other Asian countries. In this study we screened herbal drugs and their purified compounds, using the Feo replicon system, to determine their effects on *in vitro* HCV replication.

Methods: We screened herbal drugs and their purified extracts for the activities to suppress hepatitis C virus (HCV) replication using an HCV replicon system that expressed chimeric firefly luciferase reporter and neomycin phosphotransferase (Feo) genes. We tested extracts and 13 purified compounds from the following herbs: *Glycyrrhizae radix*; *Rehmanniae radix*; *Paeoniae radix*; *Artemisiae capillari spica*; and *Rhei rhizoma*.

Results: The HCV replication was significantly and dose-dependently suppressed by two purified compounds, isoliquiritigenin and glycycoumarin, which were from *Glycyrrhizae radix*. Dose-effect analyses showed that 50% effective concentrations were 6.2 ± 1.0 μg/mL and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively. The MTS assay did not show any effect on cell growth and viability at these effective concentrations, indicating that the effects of the two compounds were specific to HCV replication. These two compounds did not affect the HCV IRES-dependent translation nor did they show synergistic action with interferon-alpha.

Conclusion: Two purified herbal extracts, isoliquiritigenin and glycycoumarin, specifically suppressed *in vitro* HCV replication. Further elucidation of their mechanisms of action and evaluation of *in vivo* effects and safety might constitute a new anti-HCV therapeutics.

Key words: hepatitis C virus, herbal drugs, replicon

INTRODUCTION

HEPATITIS C VIRUS (HCV) infects 170 million people worldwide and is characterized by chronic liver inflammation and fibrogenesis leading to end-stage liver failure and hepatocellular malignancy.[1,2] The difficulty in eradicating HCV is attributable, in part, to limited treatment options against the virus. Currently, combination therapy using pegylated interferon-alpha (IFN) and ribavirin has been used worldwide.[3–5] The success rates, however, are almost half of patients treated. Furthermore, these therapies carry a significant risk of serious side effects. Thus, the development of alternative therapeutic agents against HCV is our high priority goal.

We have reported an HCV subgenomic replicon that expresses chimeric luciferase reporter "Feo" protein.[6] This Feo replicon supports stable and high levels of autonomous HCV RNA replication in transfected cells. Furthermore, the level of luciferase correlates well with levels of HCV RNA production, so that luciferase can be used as a reliable surrogate marker for HCV replication. This chimeric reporter replicon system has contributed the discovery of novel anti-HCV substances such as cyclosporins,[7–9] short interfering RNA,[10,11] interferon-gamma[12] and HMG-CoA reductase inhibitors.[13,14]

Traditional herbal drugs have been used for several thousand years in China and other Asian countries. Although these pharmacological activities are not fully

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Table 1 List of herbal drugs and their purified extracts

Herbal drug	Purified compound
Glycyrrhizae radix	Isoliquiritigenin
	Glycycoumarin
	Isoliquiritin
	Licuroside
Paeoniae radix	Paeoniflorin
	1,2,3,6-tetra-O-galloyl-β-D-glucose
Rhei Rhizoma	Rhein 8-O-β-glucoside
Rehmanniae radix	Acteoside
	Martynoside
	Isoacteoside
Artemisiae capillari spica	Demethoxycapillarisin
	3,4-di-o-galloylquinic acid
	Acteosyringone

characterized, they also have been safely used for many clinical conditions in Japan. For example, Sho-saiko-to (TJ-9; Xiao-Chae-Hu-Tang in Chinese), an oral medicine, which consists of seven herbal components (*Bupleuri radix, Pinelliae tuber, Scutellariae radix, Ginseng radix, Grycyrrhhizae radix, and Zingiberis rhizoma*),[15] has been clinically used for the treatment of chronic viral liver disease. It has been reported to regulate the cytokine production system in patients with hepatitis C[16] and to prevent the development of HCC in patients with non-B cirrhosis.[17] *Glycyrrhizin*, the major component of *Glycyrrhizae radix* (licorice), has also been used for the treatment of chronic hepatitis in Japan, known to have an alanine transaminase-lowering effect.[18,19] Despite the clinical effects of these herbal drugs, they did not suppress the HCV replication *in vitro*.[15]

In the present study, we applied the Feo replicon system to screen the herbal drugs and their purified compounds for their effects on *in vitro* HCV replication. Here, we show that two purified compounds from the herbal extracts specifically and substantially suppressed HCV replication.

MATERIALS AND METHODS

Purified compounds (Table 1)

THIRTEEN COMPOUNDS WERE purified from five herbal drugs: *Glycyrrhizae radaix; Rhemanniae radix; Paeoniae radix; Artemisiae Capillari Spica;* and *Rhei Rhizoma* (Table 1; Tsumura, Tokyo, Japan). These extracts were prepared at concentrations of 5 mg/mL in dimethyl sulfoxide (DMSO), then stored at −20°C until use. Recombinant human interferon (IFN) alpha-2b was obtained from Schering-Plough (NJ, USA).

Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma, MO, USA) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in a medium containing 200 μg/mL G418 (Wako, Osaka, Japan).

HCV subgenomic replicon construct

An HCV subgenomic replicon plasmid, pHCV1bneo-delS,[20] was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotrasnferase (pRep-Feo) (Fig. 1a). RNA was synthesized from pRep-

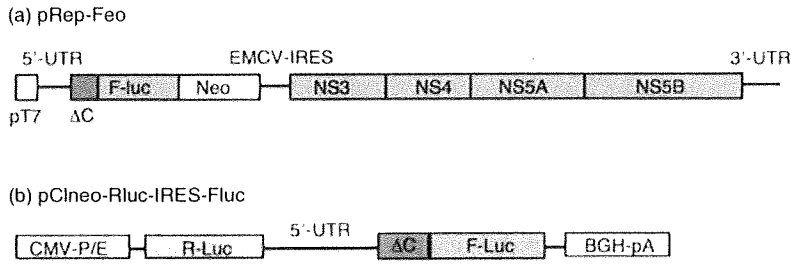

Figure 1 HCV subgenomic replicon and reporter plasmid constructs. (a) An HCV subgenomic replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene comprising the firefly luciferase (Fluc) and Neo, which we designated as "Feo"[6]. NS, nonstructural region; pT7, T7 promoter; 3′ UTR, 3′ untranslated region. (b) A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV-IRES-mediated translation efficiency . The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-internal ribosome entry site (IRES)-mediated initiation, was stably transfected into Huh7 cells.

Feo and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established.[10,21]

HCV-IRES reporter construct

A plasmid, pCIneo-Rluc-IRES-Fluc, was used to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Fig. 1b).[22] The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-IRES-mediated initiation, was stably transfected into Huh7 cells. After culture in the presence of G418, Huh7/CRIF cells were established.[11] Activities of the HCV-IRES-mediated translation were measured by culture of Huh7/CRIF cells in the presence of drugs and by dual luciferase assays after 48 h.

Luciferase assays and measurements of antiviral activity

Huh7/Rep-Feo cells were cultured with various concentrations of herbal extracts or compounds. Levels of HCV replication were quantified by internal luciferase assay after 48 h of culture. Luciferase activities were quantified using a luminometer (Promega, WI, USA) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means ± SD as percentages of the controls. The 50% effective concentrations (EC50) were calculated using probit method. The determination of EC50 was performed three times, and presented as mean ± SD in each compound.

Realtime RT-PCR analysis

Total cellular RNA was extracted from cultured cells or liver tissue using ISOGEN (Nippon Gene, Tokyo, Japan). Two μg of total cellular RNA was used to generate cDNA from each sample using the SuperScript II reverse-transcriptase (Invitrogen, CA, USA). The replicon RNA expression levels were measured using the Applied Biosystems 7500 Fast Realtime PCR System (Applied Biosystems, CA, USA) and QuantiTect SYBR Green PCR Kit (QIAGEN, CA, USA). Sequences of a pair of primers has been described elsewhere.[23]

Northern blottings

Expression of HCV subgenomic RNA was detected as previously reported.[24] Total cellular RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Fifteen micrograms of the total cellular RNA was electrophoresed on a 1.0% denaturing agarose-formaldehyde gel and was transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech, Sweden). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for beta-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche, Germany) and visualized using a Fluoro-Imager (Roche).

Western blottings

Western blotting was done as reported previously.[24] Thirty micrograms of total cell lysate was separated using NuPAGE 4–12% Bis-Tris gels (Invitrogen, CA, USA) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche). The antibodies used were anti-NS5A (BioDesign, ME, USA), anti-core (provided by Dr. Wakita), and anti-beta-actin antibodies (Sigma).

MTS assays

To evaluate cell viability, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

HCV-JFH1 virus cell culture

An *in vitro* transcribed HCV-JFH1 RNA[25] was transfected into Huh7.5.1 cells.[26] Naïve Huh7.5.1 cells were subsequently infected by culture supernatant of the JFH1-RNA transfected Huh-7.5.1 cells, and subjected to culture in the presence of drugs. Culture medium was collected serially and HCV core antigen was measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen; Ortho-Clinical Diagnostics, Tokyo, Japan). Cellular virus expression was measured by the Western blotting using anti-core antibodies.[27]

Statistical analyses

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

RESULTS

Suppression of HCV replication by purified herbal extracts, isoliquiritigenin and glycycoumarin

TO SCREEN THE herbal drugs and these purified extracts (Table 1) for their antiviral effects against HCV replication, Huh7/Rep-Feo cells were cultured with various concentrations of 5 herbal extracts; *Glycyrrhizae radaix, Rhemanniae radix, Paeoniae radix, Artemisiae capillari spica, and Rhei rhizoma*, and 13 compounds purified from these herbal extracts. Levels of HCV replication were quantified by internal luciferase assay after 48 h. None of the herbal extracts showed any effects on HCV replication (data not shown). On the other hand, among the 13 purified compounds, isoliquiritigenin and glycycoumarin, which were purified from *Glycyrrhizae radix*, suppressed replication of HCV replicon in a dose-dependent manner. The EC50s were 6.2 ± 1.0 and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively (Figs 2a,3a). The MTS assay did not show any effect on cell growth and viability (Fig. 2b), indicating that the antiviral action of the two compounds is not due to cytotoxic or antiproliferative effects. Huh7/Rep-Feo cells were cultured with various concentrations of isoliquiritigenin and glycycoumarin, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. After addition of each compounds, suppressive effect of the HCV replicon lasted for 48 h in a dose and time-dependent manner (Fig. 3b).

Realtime-RT-PCR and Western blotting analyses

In the realtime RT-PCR analysis and Northern blot analyses, levels of the replicon RNA decreased in a dose-dependent manner following treatment with isoliquir-

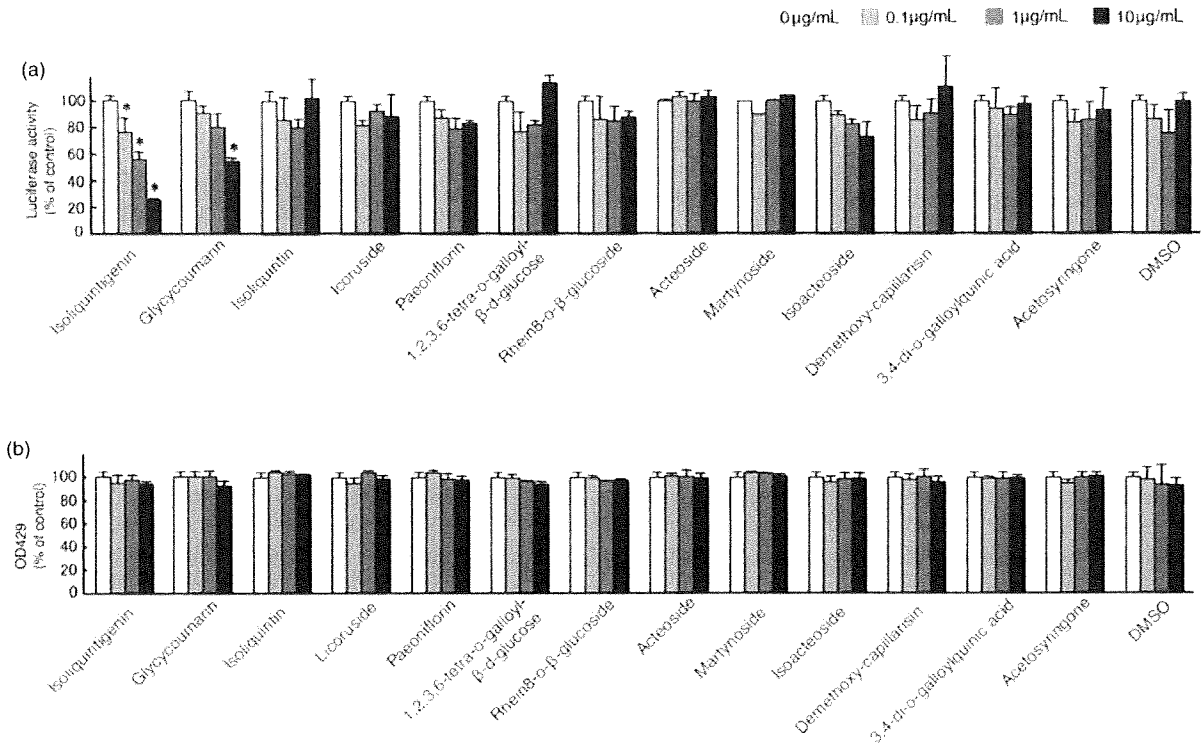

Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Asterisks indicate p-values of less than 0.05. (b) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of 13 compounds indicated. Error bars indicate mean ± SD.

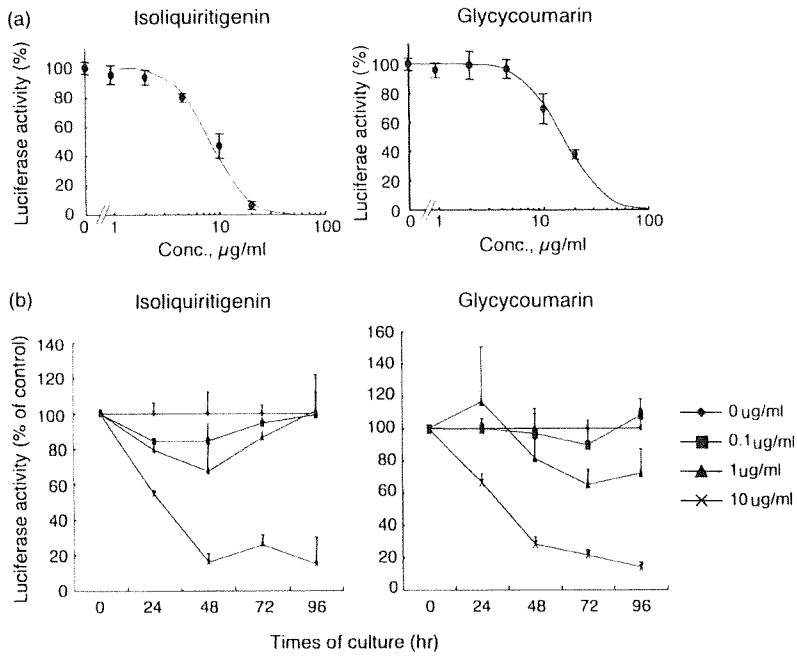

Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritinenin and glycycoumarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycoumarin. Error bars indicate mean ± SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo cells were cultured with the concentrations of isoliquiritigenin and glycycoumarin indicated. The internal luciferase activities were measured at times of culture indicated. Assays were performed in triplicate. Error bars indicate mean ± SD.

itigenin and glycycoumarin (Fig. 4a,b). Similarly, in Western blot analysis, the HCV non-structural protein, NS5A, which was translated from the HCV replicon, decreased by corresponding amounts in response to treatment with isoliquiritigenin and glycycoumarin (Fig. 4c). Densitometric analysis of NS5A protein showed that the intracellular levels of the virus protein in Huh7/Rep-Feo cells correlated well with the luciferase activities.

Absence of synergistic anti-HCV effects of interferon-alpha with isoliquiritigenin or glycycoumarin

To determine whether IFN and these two compounds have a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were cultured with combinations of IFNα-2b and isoliquiritigenin or glycycoumarin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentrations of isoliquiritigenin or glycycoumarin of 0, 0.1, 1, 10 μg/mL, respectively (Fig. 5). The curves did not show synergy of the two compounds and IFN against the HCV replicon. To see whether the action of isoliquiritigenin and glycycoumarin involve interferon-Jak/STAT-ISRE pathway, we conducted ISRE reporter assays. We transfected the p-55C1BLuc plasmid in Huh7 cells and cultured the cells in the presence of isoliquiritigenin or glycycoumarin. After 12 h of incubation, those drugs did not activate ISRE-promoter activities (data not shown). These results suggested that the action of the compounds on the intracellular replication of HCV replicon was independent of the IFN-ISRE pathway.

Isoliquiritigenin and glycycoumarin do not suppress the HCV IRES-dependent translation

We next determined whether these two compounds suppress HCV IRES-dependent translation, we used Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc (Huh7/CRIF; Fig. 1b). Treatment of these cells with isoliquiritigenin or glycycoumarin resulted in no significant change of the internal luciferase activities at concentrations of these two compounds that suppressed expression of the HCV replicon (Fig. 6a). The MTS assay did not show any effect on cell growth and viability at concentrations used in this assay (Fig. 6b).

Isoliquiritigenin and glycycoumarin suppress HCV-JFH1 virus cell culture

The demonstrated inhibitory effects isoliquiritigenin and glycycoumarin on HCV subgenomic replication were validated further by using HCV-JFH1 cell culture system.[25] As shown in Figure 7a, treatment of the cells with the two compounds suppressed time-dependent

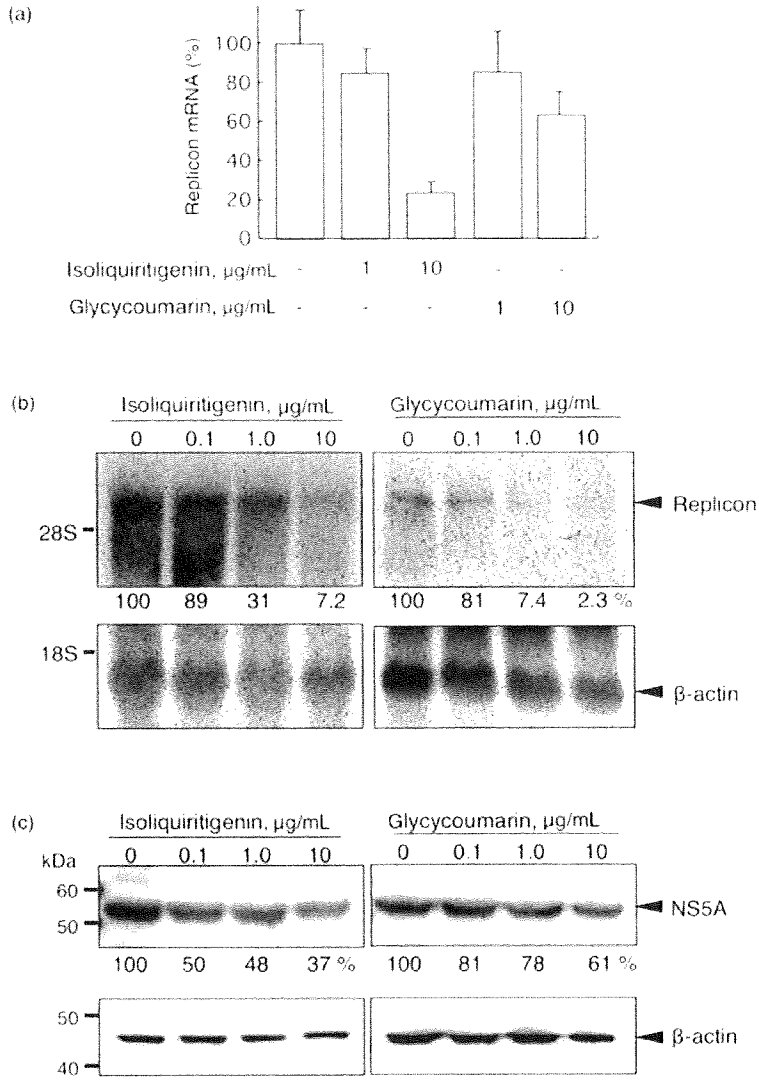

Figure 4 Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycycoumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycycoumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot hybridization. Fifteen micrograms of total cellular RNA was electrophoresed in each lane. The upper part of the membrane containing the hepatitis C virus replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with beta-actin probe. Densitometry for replicon RNA was performed and indicated as percents of drug-negative control. (c) Western blotting. Thirty micrograms of total cellular protein was electrophoresed in each lane. Densitometry of NS5A protein was performed and indicated as percents of drug-negative control.

increase of HCV core antigen in the medium. In all time points, core antigen levels were significantly lower in culture that were treated with isoliquiritigenin and glycycoumarin than the untreated culture. The effect of glycycoumarin was partly reversed on day six probably due to chemical instability of the compound. Consistently, the Western blot showed that the cellular HCV core protein expression was substantially suppressed by treatment with isoliquiritigenin and glycycoumarin (Fig. 7b).

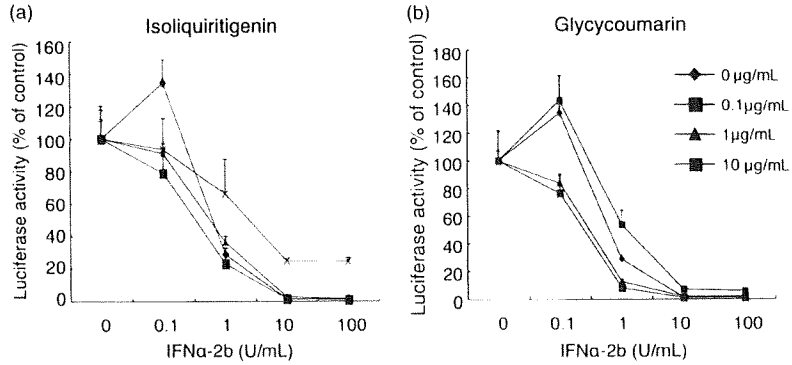

Figure 5 Effects of (a) isoliquiritigenin and (b) glycycoumarin used in combination with interferon(IFN)-α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN-α-2b and isoliquiritigenin or glycycoumarin at concentrations indicated. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Plots of 100% in each curves represent replicon expression levels that were treated with indicated amounts of isoliquiritigenin or glycycoumarin and without IFN.

DISCUSSION

THE PRESENT STUDY demonstrates that two purified herbal extracts, isoliquiritigenin and glycycoumarin, isolated from *Glycyrrhizae radix*, suppress replication of an HCV replicon (Fig. 2). Northern and Western blot analyses reveal that both RNA synthesis and its translation were reduced by the two compounds in dose- and time-dependent manners (Figs 3,4). The two drugs did not show activation of type-I interferon-dependent, ISRE-mediated transcription or synergistic action with interferon-alpha on HCV replication (Fig. 5,6), which suggests that the anti-HCV effects of the compounds are independent of interferon-antiviral mechanisms. Finally, we have demonstrated that the two compounds show inhibitory effects on HCV virus cell cultures (Fig. 7).

Flavonoid is a class of plant pigment, found in wide range of green vegetables and fruits. They are classified into flavon, flavonol, flavanone, flavanol, isoflavone, chalcone, anthocyanin and catechin, according to their molecular structures. Many flavonoids have various biological functions such as antibacterial,[28] antioxidative and anticarcinogeninc activities.[29] Isoliquiritigenin is a simple chalcon derivative and found in licorice and vegetables including shallots and bean sprouts. Isoliquiritigenin has several biochemical activities similar to other flavonoids. It has various biochemical activities such as antioxidative and superoxide scavenging activities,[30] an antiplatelet aggregation effect,[31] an inhibitory effect on aldose reductase activity,[32] estrogenic properties[33] and selective inhibition of H2 receptor-mediated signaling.[34]

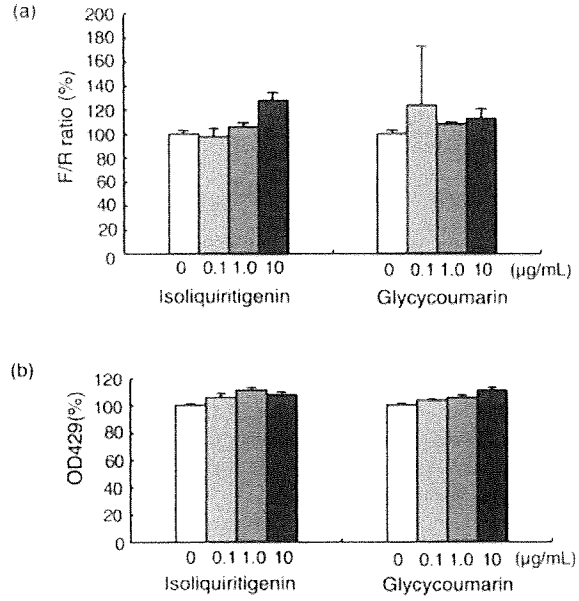

Figure 6 Isoliquiritigenin and glycycoumarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably trasfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated, and dual luciferase activities were measured after 48 h of treatment. Values are displayed as ratios of Fluc to Rluc. Error bars indicated mean ± SD. (b) MTS assay of Huh7/neo-Rluc-IRES-Fluc cells cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated. MTS assays at 48 h after treatment with each drug were performed in triplicate. Error bars indicate mean + SD.