

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 \pm 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 μ g (gray bars), or 4 μ g (white bars) of indicated ISG-expression plasmids were transfected. Error bars indicate mean \pm 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 \pm 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-27⁸ 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; $63.7 \pm 7.49\%$, IFI-6-16; $62.6 \pm 19.2\%$, IFI-27; $71.6 \pm 1.22\%$, IRF-1; $95.1 \pm 0.19\%$ / 2a-Feo: GBP-1; $61.9 \pm 12.3\%$, IFI-6-16; $42.9 \pm 7.0\%$, IFI-27; $42.9 \pm 13.2\%$, IRF-1; $95.8 \pm 1.0\%$) (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 ($24.3 \pm 2.90\%$, $35.6 \pm 6.73\%$), IFI-6-16 ($33.9 \pm 0.40\%$, $43.6 \pm 1.14\%$), IFI-27 ($38.5 \pm 3.63\%$, $61.2 \pm 1.28\%$), or IRF-1 ($69.2 \pm 1.89\%$, $78.3 \pm 1.01\%$) 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 ($38.2 \pm 1.39\%$), IFI-6-16 ($54.6 \pm 2.93\%$), IFI-27 ($50.7 \pm 2.81\%$), and IRF-1 ($6.61 \pm 0.341\%$) (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

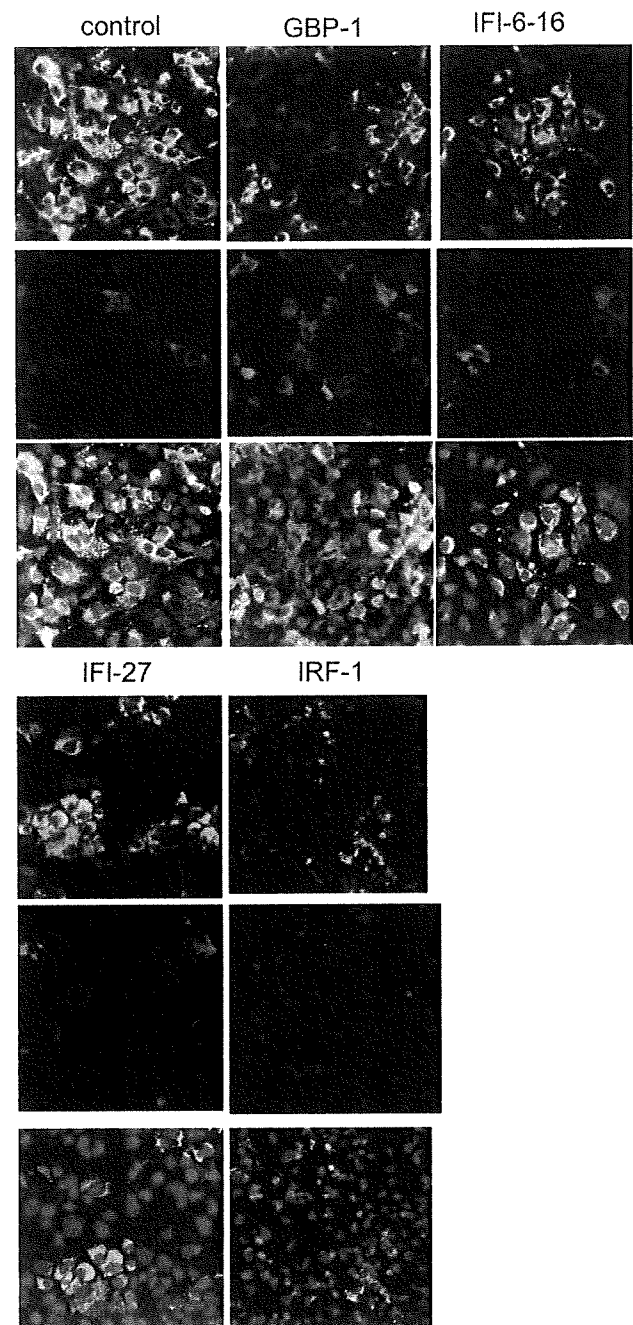


Fig. 2. Suppression of intracellular HCV replication levels by ISG-expression assessed by immunohistochemistry. The ISG-expression plasmid indicated or pcDNA3.1D/V5-His/LacZ as a control was transfected into HCV-JFH1-infected Huh-7.5.1 cells seeded in 18-mm-diameter dishes. At 48 hours after transfection,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

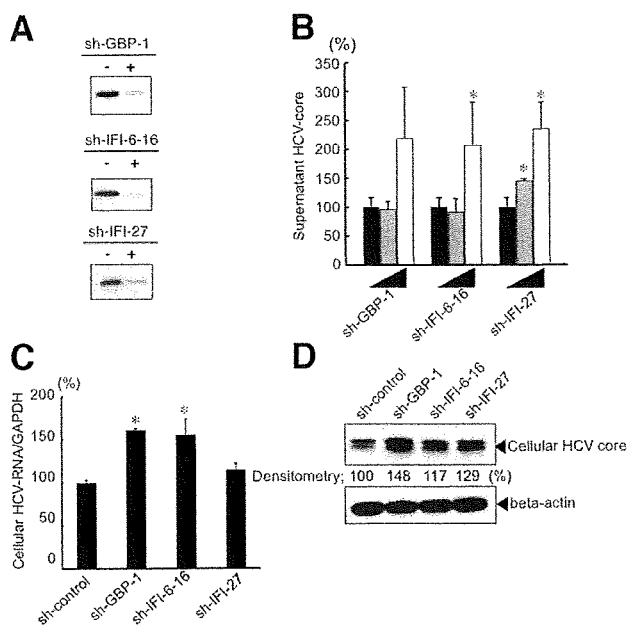


Fig. 3. The effects of shRNA-directed suppression of GBP-1, IFI-6-16, and IFI-27 expression on HCV replication. (A) The ISG-expression plasmids indicated, pcDNA-GBP-1 (upper panel), pcDNA-IFI-6-16 (middle panel), or pcDNA-IFI-27 (lower panel), were cotransfected with shRNA-expressing plasmid, pUC-shRNA-control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

respective shRNAs (Fig. 3A). Next, we transiently transfected shRNA-expression plasmids into HCV-JFH1-infected Huh-7.5.1 cells and performed HCV core antigen assays, real-time RT-PCR, and western blotting to measure HCV-RNA and the protein expression, respectively. Supernatant HCV core protein was significantly and dose-dependently increased by the overexpression of sh-GBP-1 ($218.2 \pm 88.7\%$), 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-

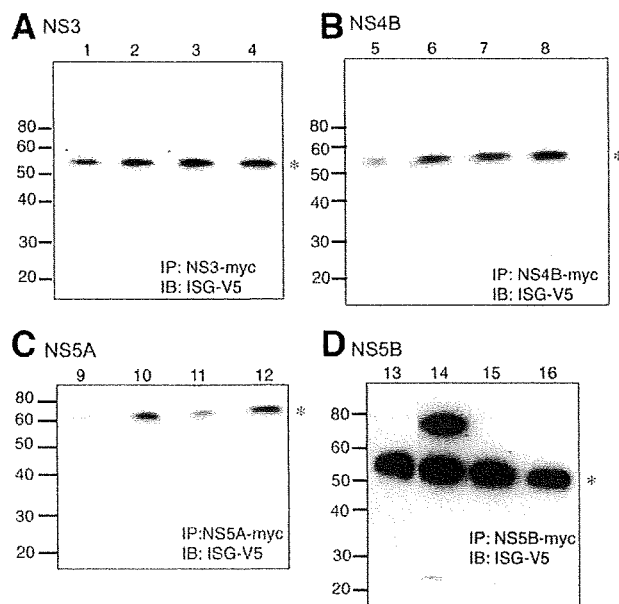


Fig. 4. Interaction of interferon inducible proteins with HCV-NS proteins by immunoprecipitation assay. (A-D) The plasmids indicated expressing myc-tagged HCV-NS protein, NS3 (A), NS4B (B), NS5A (C), or NS5B (D) were cotransfected into HEK-293T with V5-tagged ISG-expression plasmids for GBP-1 (lanes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

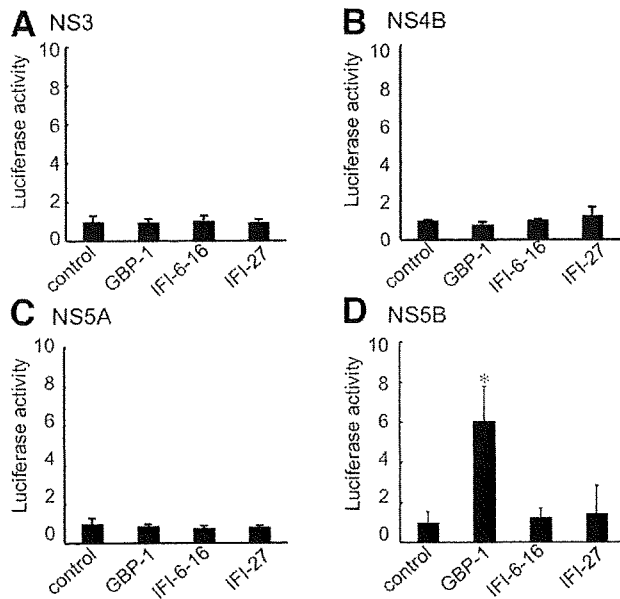


Fig. 5. The analysis of HCV-NS proteins binding ISG proteins by mammalian two-hybrid assay. Interaction of ISG proteins with HCV-NS proteins was confirmed by mammalian two-hybrid assay. The plasmids indicated, pCMV-BD-NS protein NS3 (A), NS4B (B), NS5A (C), or NS5B (D), were cotransfected with pCMV-AD-ISG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

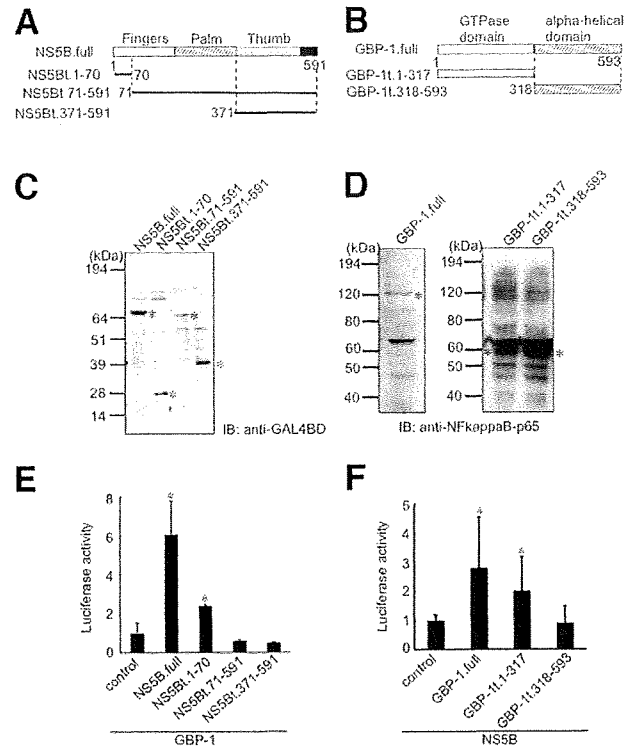


Fig. 6. Analyses of the domains responsible for the interaction between HCV-NS5B and GBP-1. (A) Scheme of full-length and truncated protein in HCV-NS5B. (B) Scheme of full-length and truncated protein in GBP-1. (C) Western blotting analysis of cells transfected with the indicated NS5B truncated protein-expression 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.t.1-317, and pCMV-AD-GBP.t.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- κ B. (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 \pm SD. * $P < 0.05$.

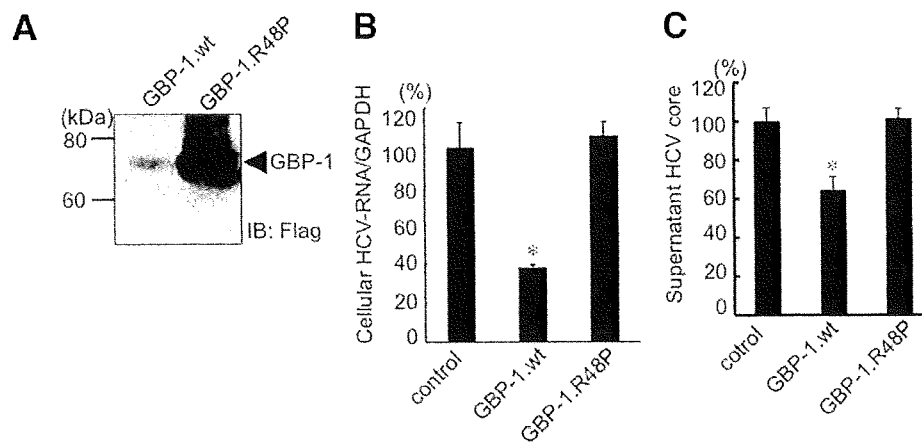


Fig. 7. GTPase activity has an antiviral effect on HCV replication. Western blotting analysis of GBP-1 and GBP-1 mutant, R48P. Plasmids expressing GBP-1 mutant type, R48P, or GBP-1 wildtype were transfected into 293T cells. Cell lysates were harvested at 48 hours after transfection, followed by immunoblotting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

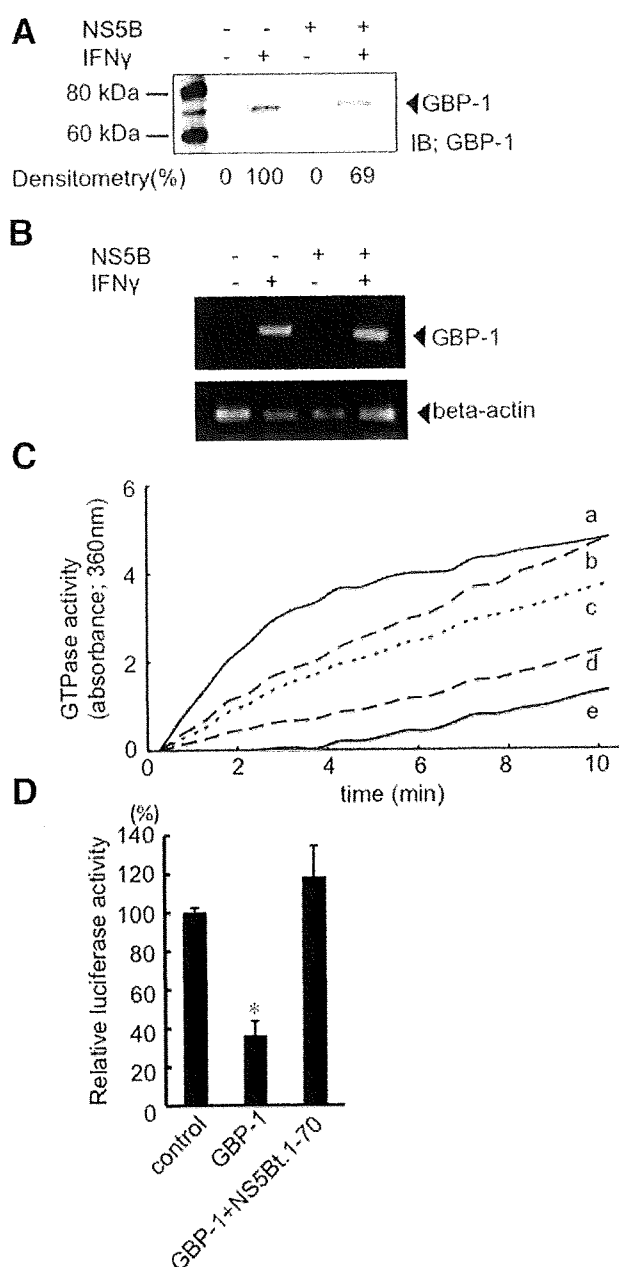


Fig. 8. GTPase activity of GBP-1 was negatively regulated by HCV-NS5B. (A) A GBP-1-expression V5-tagged plasmid was cotransfected into Huh-7 cells with a plasmid expressing myc-tagged-HCV-NS5B and cell lysates were harvested. Cell lysates were immunoprecipitated by anti-myc antibodies. Immunoprecipitated 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 \pm 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- κ B.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 diarrhoea 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 NS3A 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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THE ISOMERASE ACTIVE SITE OF CYCLOPHILIN A IS CRITICAL FOR HCV REPLICATION

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

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ABSTRACT

Cyclosporine A (CsA) and non-immunosuppressive cyclophilin (Cyp) inhibitors such as Debio 025, NIM811 and SCY-635 block hepatitis C virus (HCV) replication in vitro. This effect was recently confirmed in HCV-infected patients where Debio 025 treatment dramatically decreased HCV viral load, suggesting that Cyps inhibitors represent a novel class of anti-HCV agents. However, it remains unclear how these compounds control HCV replication. Recent studies suggest that cyclophilins (Cyps) are important for HCV replication. However, a profound disagreement currently exists as to the respective roles of Cyp members in HCV replication. In this study, we analyzed the respective contribution of Cyp members to HCV replication by specifically knocking down their expression by both transient and stable small RNA interference (sRNAi). Only the CypA knockdown drastically decreased HCV

replication. The re-expression of an exogenous CypA escape protein, which contains escape mutations at the sRNA1 recognition site, restored HCV replication, demonstrating the specificity for the CypA requirement. We then mutated residues, which reside in the hydrophobic pocket of CypA where proline-containing peptide substrates and CsA bind, and which are vital for the enzymatic or the hydrophobic pocket binding activity of CypA. Remarkably, these CypA mutants fail to restore HCV replication, suggesting for the first time that HCV exploits either the isomerase or the chaperone activity of CypA to replicate in hepatocytes and that CypA is the principal mediator of the Cyp inhibitor anti-HCV activity. Moreover, we demonstrated that the HCV NS5B polymerase associates with CypA via its enzymatic pocket. The study of the roles of Cyps in HCV replication should lead to the identification of new targets for the development of alternate anti-HCV therapies.

INTRODUCTION

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

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

and 4 and -5.91 ± 1.11 IU/mL in patients with genotype 2 and 3 at week 4 (16). The Debio 025 findings are critical because they suggest that Cyp inhibitors represent a novel class of anti-HCV agents. However, it remains unclear how these compounds control HCV replication.

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

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

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

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

EXPERIMENTAL PROCEDURES

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

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

Small RNA Interference Knockdown. Annealed duplex siRNA oligonucleotides contained a 3'-dTdT overhang (Quagen). siRNA target sequences were: AAGGGTTCCTGCTTTCACAGA for CypA; AAGGTGGAGAGACCAAGACA for CypB; GTGACATCACTGGAGATG for CypC; AACCTGCTAAATTGTGCGTTA for CypD; and AATTCTCCGAACGTGTCACGT for control. Cells were transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen). For effect of siRNA Cyp knockdown on HCV RNA replication, cells were transfected with siRNA Cyp and then retransfected 24 h later. An HIV-1-based lentiviral vector was used to express all Cyp shRNA as described previously (24). The Cyp target sequences are the same as

those indicated just above. Lentiviral particles production and transduction was conducted as described previously (24). Generation of stable Cyp-knockdown cell lines was obtained after 3 weeks under puromycin (1 μ g/mL) selection. All cell lines were tested for mycoplasma contamination, which may nonspecifically interfere with HCV replication. To restore CypA expression in Huh7 CypA knockdown cells, a CypA cDNA bearing silent mutations that render it non-targetable by the CypA shRNA was subcloned into pcDNA3 with Hind3 and NotI sites to generate pcDNA3-resistant wild-type (WT) CypA, which contains an N-terminal HA tag. Using pcDNA3-resistant wild-type (WT) CypA as template, we engineered two plasmids carrying either the H126Q (pcDNA3-resistant H126Q CypA) or the R55A (pcDNA3-resistant R55A CypA) mutation in the hydrophobic pocket of CypA that disallows its isomerase activity (25).

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

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

the GFP gene and pseudotyped with the vesicular stomatitis virus G envelope (VSVG) protein) (generous gift from C. Aiken and D. Gabuzda). Forty-eight hours post-infection, intracellular GFP levels were quantified by FACS. HSV-1 infection: Cyp-knockdown Huh7 cells lines were infected with HSV-1-GFP (K26GFP virus encoding the GFP gene) (generous gift from P. Desai). Forty-eight hours post-infection, intracellular GFP levels were quantified by FACS. Dengue infection: Cyp-knockdown Huh7 cells lines were infected with Dengue-2 (Dengue-2 16681) (generous gift from R. Kinney). Dengue-2 infection was examined using an intracellular FACS staining assay (IFSA). Briefly, IFSA was conducted as described previously (27-28) with minor modifications. Three days post-infection, cells were washed, trypsinized, resuspended in PBS at 1×10^6 /ml and fixed with 0.2% paraformaldehyde in PBS for 30 min on ice. Cells were washed, permeabilized in PBS containing 0.2% Tween for 15 min at 37°C, washed and resuspended in FACS buffer (PBS containing 2% FBS). For intracellular staining, cells (10^5) were incubated for 30 min at 4°C with 10 µg/ml of isotype controls, mouse monoclonal anti-Dengue capsid 9A7 IgG (TSRI, Antibody Core Facility). Cell permeabilization was confirmed by staining cells with mouse anti-tubulin IgG (Santa Cruz Biotechnologies). Cells were washed, incubated with secondary phycoerythrin (PE)-conjugated anti-mouse IgG (10 µg/ml) for 30 min at 4°C, washed again, resuspended in PBS, fixed in 2% paraformaldehyde and stored at 4°C until FACS analysis. HCV replication: Cyp-knockdown Huh7 cells lines were electroporated with 10 µg of *in vitro*-transcribed genomic Con1 RNA. Seven days post-transfection, HCV infection was quantified by measuring intracellular NS5B levels by IFSA using anti-NS5B IgG (Alexis Biochemicals).

CypA Binding to HIV-1 Gag in Hepatocytes. CypA-HIV-1 Gag interaction was studied by examining the incorporation of host CypA into budding HIV-1 particles by Western blot. Briefly, HIV-1 particles were transiently expressed in Huh7 cells by genejuice (EMD Biosciences) transfection with a mixture of 5 µg

of proviral HIV-1 (NL4.3) DNA together with 5 µg of CypA plasmids (pcDNA3-resistant WT, H126Q and R55A). Viral supernatants, harvested 48 h post-transfection, were filtered through a 0.2-µm-pore-size filter to remove cellular debris, pelleted through a sucrose cushion, standardized for HIV-1 capsid content by p24 ELISA (Alliance, PerkinElmer), resuspended in 2x sodium dodecyl sulfate loading buffer, and subjected to Western blotting with antibodies directed against the HA tag.

Co-immunoprecipitations. Parental Huh7 cells (3 million) were co-transfected with NS5B-myc (5 µg DNA) and wild-type CypA-HA or H126Q CypA-HA (5 µg DNA) plasmids in the presence or absence of Debio 025 (2 µM). Three days post-transfection, cells were collected and lysed. Cell lysates (1 mL) were pre-cleared for 1 h with 20 µL of agarose beads. Co-immunoprecipitation procedures were conducted according to the manufacturer's instructions (Pierce HA Tag IP/Co-IP Kit). Bound material was eluted and analyzed by Western blotting using anti-HA and anti-myc IgG (Santa Cruz Biotechnology).

RESULTS

Analysis of the Respective Contribution of Cyp Members to HCV Replication by Transient Small RNA Interference. Previous studies suggested that at least three members of the Cyp family – CypA, CypB and CypC – modulate HCV replication (9, 17-18). We thus examined if HCV exploits all these Cyp members or rather a unique Cyp member to efficiently replicate in human hepatocytes. To address this issue, we knocked down each of these Cyps by transient small inhibitory RNA (siRNA) interference and examined the effect of the Cyp knockdown on HCV replication. Specifically, Huh7 cells containing the subgenomic HCV Con1 replicon (genotype 1b) were transfected with siRNAs that target either luciferase (control siRNA), CypA (CypA siRNA), CypB (CypB siRNA), CypC (CypC siRNA) or CypD (CypD siRNA). To avoid siRNA toxicity, cells were washed 24 h post-transfection. Seven days post-transfection, cells were collected and lysed. To ensure that the

siRNA treatments did not non-specifically influence growth and viability of transfected hepatocytes, cells were counted and analyzed for trypan blue uptake 7 days post-transfection. We exclusively analyzed the lysates of cells, which gave numbers of viable cells comparable to those of control siRNA-treated cells. Cell lysates were standardized for protein content and analyzed for Cyp content by Western blot using antibodies directed against CypA, CypB, CypC or CypD.

The expression of each Cyp was profoundly reduced by siRNAs Cyp compared to siRNA control (Figure 1A), demonstrating the efficacy of the siRNA treatments. Moreover, siRNA Cyp treatments were specific since each siRNA Cyp treatment did not alter the expression of the other Cyps (Figure 1A). For example, the siRNA, which targeted CypA did not influence CypB, CypC or CypD expression (Figure 1A). Importantly, transient CypB, CypC or CypD knockdown did not significantly influence HCV protein expression. Indeed, NS5A and NS5B levels in siRNA CypB-, CypC- and CypD-treated hepatocytes were similar to those of control siRNA-treated cells (Figure 1B), suggesting that these Cyps play no or a minor role in HCV protein expression. In sharp contrast, NS5A and NS5B levels were profoundly diminished in hepatocytes treated with the siRNA CypA (Figure 1B), demonstrating that CypA rather than CypB, CypC and CypD, plays a major role in HCV protein expression.

We then asked whether CypA, but not other Cyp members, is also critical for HCV RNA replication. To address this issue, naïve Huh7 cells were electroporated with *in vitro*-transcribed genomic Con1 RNA. One day post-electroporation, cells were transfected with control or siRNA Cyp as described above. Viral RNA replication was monitored for 8 days by analyzing intracellular HCV RNA via reverse-transcription quantitative polymerase chain reaction as described previously (23). We verified that the expression of CypA, CypB, CypC and CypD was knocked down via the Cyp siRNA treatment 8 days post-electroporation (data not shown). We found that the HCV RNA replication in hepatocytes treated with siRNA CypC and CypD was comparable to that in

siRNA control-treated cells, whereas the viral RNA replication in siRNA CypB was only slightly diminished (Figure 1C). In contrast, viral RNA replication was profoundly attenuated in the siRNA CypA-treated cells (Figure 1C). Altogether these data suggest that CypA, but not CypB, CypC and CypD, represents an essential host factor for both HCV RNA replication and HCV protein expression.

Demonstration of the Exclusive Contribution of CypA to HCV Replication by Stable Small RNA Interference.

The introduction of siRNA into cultured cells provides a fast and efficient means of knocking down gene expression. However, siRNA has been shown to be effective for only short-term gene inhibition in certain mammalian cells. Supporting this notion, we found that although Cyp expression was dramatically reduced 5 to 7 days after siRNA transfection, Cyp expression recovered 9 to 10 days post-siRNA transfection (data now shown). Even a slight rebound in Cyp protein expression may interfere with the interpretation of the data. To avoid this issue, we conducted experiments similar to those described above, but using shRNA to stably silence Cyp gene expression. We constructed plasmids encoding shRNA that target CypA, CypB or CypC. As above, we constructed as control, a plasmid that encodes an shRNA that targets luciferase. Plasmids were packaged into HIV-1-based particles pseudotyped with the VSV-G envelope to permit entry into and infection of hepatocytes. The advantage of using the HIV-1 based vector is that the DNA encoding the shRNA will be stably integrated into the host chromosomes of the hepatocytes. Parental Huh7 cells were exposed to the HIV-1-based particles for 24 h, cultured under puromycin selection for 3-4 weeks and analyzed for Cyp content by Western blot. As expected, CypA, CypB and CypC levels in shRNA Cyp-transduced cells were severely reduced compared to control shRNA-treated cells (Figure 2A).

We then tested the Cyp-knockdown (KD) cell lines for their capacities to support HCV RNA replication as described above. Importantly, the HCV RNA replication was only profoundly reduced in the stable CypA-KD cell line (Figure 2B), further suggesting that CypA,

but not CypB, CypC and CypD, is essential for HCV replication. It is important to note that we did not observe differences in growth between the cell lines (data not shown), suggesting that these particular Cyps do not play a significant role in Huh7 cell division and multiplication.

To further demonstrate the specificity of the CypA requirement for HCV replication, CypA-, CypB-, CypC-knockdown and control cell lines were exposed to various viruses including the human immunodeficiency virus type-1 (HIV-1), the herpes simplex virus type-1 (HSV-1), the flavivirus Dengue and HCV. Infectivity was scored by measuring the intracellular levels of GFP for both HIV-1 and HSV-1, levels of capsid for Dengue and levels of NS5B for HCV. GFP levels were significantly reduced in HIV-1-exposed CypA-knockdown cells compared to control, CypB- and CypC-KD cells (Figure 2C, top left panel). This is in accordance with previous studies, which suggest that HIV-1 requires CypA to optimally infect human cells (29-30). All cell lines exposed to HSV-1 or Dengue expressed similar levels of GFP (HSV-1) and capsid (Dengue) (Figure 2C, top right and bottom left panel, respectively), suggesting that HSV-1 and Dengue do not require any of these Cyps to infect human cells. Importantly, NS5B levels were dramatically reduced in the CypA-knockdown cells compared to control, CypB- and CypC-knockdown cells (Figure 2C). This further suggests that HCV, like HIV-1, specifically exploits CypA to infect and replicate in human cells, more specifically in hepatocytes.

HCV Requires the Isomerase Activity of CypA to Replicate in Human Hepatocytes.

The peptide bond generally exists in two relatively stable isomeric forms: *cis* and *trans* (31). The ribosome synthesizes peptide bonds in the lower energy-state *trans* peptide bond form, which is sterically favored, and whose side chains are 180 degrees opposite each other (32). However, bonds preceding each proline (peptidyl-prolyl bonds) also occur in the *cis* form in both unfolded and native proteins, with the side chains adjacent to each other (33). The isomerization to the *cis* form is required for both de novo protein folding, protein restructuring and refolding processes following cellular

membrane traffic. Spontaneous *cis/trans* isomerization of peptidyl-prolyl bonds is a slow process at room temperature that does not require free energy. Thus, this isomerization represents a rate-limiting step in the refolding of chemically denatured proteins (34).

We thus asked if CypA promotes HCV replication via its isomerase activity. To address this issue, we created a CypA mutant deprived of its isomerase activity. Specifically, we replaced the histidine located at position 126 (H126) in the hydrophobic pocket of CypA by a glutamine, creating the H126Q CypA mutant. Importantly, this mutation diminishes CypA isomerase activity by more than 99% compare to wild-type CypA (25). To determine if HCV requires the isomerase activity of CypA to replicate, we had to transfect the H126Q CypA mutant into the CypA-KD cells and asked if HCV replication can be rescued. In order to introduce the H126Q CypA mutant into the CypA-KD cell line, we had to generate the H126Q mutation into the context of an shRNA-escape CypA plasmid. We thus generated one plasmid encoding the wild-type shRNA-escape CypA and another encoding the H126Q shRNA-escape CypA. CypA-KD cells were transfected with wild-type and H126Q shRNA-escape CypA plasmids and tested for their capacities to support HCV RNA replication as described above. Importantly, the introduction of the wild-type shRNA-escape CypA into the CypA-KD cells restored HCV RNA replication at levels similar to those observed in parental Huh7 cells (Figure 3A). This rescue not only demonstrates the specificity of the CypA knockdown, but it also further demonstrates the importance of CypA in HCV replication. More importantly, the introduction of the H126Q shRNA-escape CypA into the CypA-KD cells did not restore viral RNA replication (Figure 3A). Note that the cellular levels of H126Q CypA were similar to those of wild-type CypA (Figure 3B). Importantly, we obtained similar data for the R55A CypA mutant (data not shown), which is also deprived of isomerase activity (25). These findings strongly suggest that the isomerase activity of CypA is essential for HCV replication in human hepatocytes.

The hydrophobic pocket of CypA does not only contain the residues vital for the

isomerase activity of CypA, it also contains the residues responsible for the binding of CypA to its, to date, unique known viral ligand - the structural polyprotein HIV-1 Gag. One can thus envision that the H126Q mutation fails to rescue HCV activity in hepatocytes because the mutation, not only blocks the enzymatic activity of CypA, but it also precludes CypA binding to its still unidentified HCV ligand. Therefore, we asked if the H126Q mutation, which abolishes the enzymatic activity of CypA, also prevents the binding of CypA to HIV-1 Gag within human hepatocytes. CypA binding to HIV-1 Gag was never examined in hepatocytes. To address this issue, we measured amounts of CypA incorporated via Gag into HIV-1 particles released from human hepatocytes. As controls, hepatocytes were treated with CsA or Debio 025, which, by binding to the hydrophobic pocket of CypA, prevents CypA-Gag interaction (30, 35). Specifically, Huh7 cells were co-transfected with HIV-1 together with wild-type or H126Q CypA-HA in the presence or absence of CsA or Debio 025. Forty-eight post-transfection, both transfected cells and released virions were analyzed for CypA content by Western blotting using anti-HA antibodies.

In the absence of any treatment, HIV-1 particles released from hepatocytes contain significant amounts of wild-type CypA (Figure 3B, top panel), demonstrating that CypA-Gag interactions also occur in human hepatocytes. Virions released from hepatocytes treated with either CsA or Debio 025 contain minimal amounts of CypA (Figure 3B, top panel), suggesting that the two compounds, by binding to the hydrophobic pocket of CypA, interfere with CypA-Gag interactions in hepatocytes. Although the H126Q CypA mutant was efficiently expressed in transfected hepatocytes (Figure 3B, bottom panel), it was not incorporated into released virions (Figure 3B, top panel), suggesting that the H126Q CypA mutation in the hydrophobic pocket of CypA alters both the enzymatic activity of CypA and the binding capacity of CypA to its viral ligand in hepatocytes. Thus, the inability of the H126Q CypA mutant to support HCV replication may arise from either its inability to isomerize peptidyl-prolyl bonds, its inability to bind to its viral or host ligand, or both. It is also important

to note that the inability of the overexpressed isomerase-deficient H126Q CypA mutant to restore HCV replication argues against the possibility that CypA plays a more important role in HCV replication than other Cyp members simply due to its superabundance in a cell. Overexpression of CypB or CypC in the CypA-knockdown cells did not rescue HCV replication either, further supporting the notion that the CypA requirement for HCV is specific (data not shown).

HCV NS5B Polymerase Associates With CypA Via Its Enzymatic Pocket.

A previous study presented data suggesting that CypA binds to NS5B (18). We thus asked if the isomerase pocket of CypA is critical for the interaction between host CypA and the HCV NS5B polymerase. To address this issue, hepatocytes were co-transfected with myc-tagged NS5B and HA-tagged wild-type or H126Q CypA plasmids in the presence or absence of the Cyp inhibitor Debio 025. Three days post-transfection, cells were collected and lysed. Cell lysates were then used for co-immunoprecipitation experiments. First, we confirmed that wild-type CypA associates with HCV NS5B (Figure 4). Importantly, the Cyp inhibitor prevents NS5B-CypA association (Figure 4). Most importantly, we found that the isomerase-deficient CypA, although well expressed in transfected cells, fails to associate with NS5B (Figure 4). This demonstrates for the first time that the enzymatic pocket of CypA is critical for the contact between host CypA and the viral NS5B polymerase.

DISCUSSION

It is well established that host proteins highly regulate the viral life cycles. Because cellular chaperones and enzymes control the correct folding of host proteins, one could assume that they also control the correct folding of viral proteins. This assumption is now apparently proven to be correct for two prime human pathogens: HIV-1 and HCV. In 1993, Luban et al. using the yeast two-hybrid system identified for the first time the interaction between CypA

and HIV-1 Gag (35). One year later, two independent studies elegantly demonstrated that CypA-Gag interactions are critical for HIV-1 replication in human cells (29). Specifically, Thali et al. showed that CsA, by preventing CypA-Gag interactions, inhibits HIV-1 infection (30). Moreover, Franke et al. showed that the introduction of mutations in the CypA-binding region of Gag also decreases HIV-1 infection of human cells (29). Further supporting the notion that HIV-1 requires CypA to replicate in human cells, several studies demonstrated that CypA-knockout or -knockdown human cells poorly support HIV-1 replication (24, 36-37). Similarly to HIV-1, we present here several lines of evidence that CypA is also required for HCV replication. We showed that both transient and stable small RNA interferences, which specifically target CypA, profoundly hamper HCV RNA replication as well as HCV protein expression. In contrast to previous studies (9, 17), we did not observe a significant contribution to HCV replication of other Cyp members including CypB, CypC and CypD. Although we do not have any clear explanation for these apparent divergent results, one cannot exclude the possibility that the use of different cells, HCV strains or replication systems somehow modulates the respective contribution of Cyp members to HCV replication. It is critical to emphasize that during the course of our study, two independent studies from the Tang (18) and the Bartenschlager (38) laboratories obtained similar results, which convincingly showed that CypA, but not CypB and CypC, is an essential factor for HCV infection. Thus, to date, the findings of three independent studies including ours, all using a stable shRNA knockdown approach, converge to the same conclusion, which is that CypA serves as a major host cofactor for HCV replication. A couple of observations may explain why HCV preferentially exploits CypA rather than CypB and CypC. One is that CypA is 10- and 100-fold more abundant in a cell than CypB and CypC, respectively (22, 39). Another is that CypA, which resides in the cytosol, is more appropriately located to interact with the HCV replication complex than CypB and CypC, which reside in the lumen of the endoplasmic reticulum (22).

Does CypA assist HCV replication as a peptidyl isomerase? To date, an auxiliary and an essential biochemical functions can be attributed to Cyps. The auxiliary function is characterized by polypeptide sequestration using extended catalytic subsites of the enzyme whereas catalysis essentially requires direct participation of active site residues (40). We demonstrated here that when we mutate residues that reside in the hydrophobic pocket of CypA (histidine or arginine in position 126 and R55, respectively) where the proline-containing peptide substrates bind, the resulting CypA mutants fail to restore HCV replication. The simplest hypothesis for the mechanism of action of CypA in the HCV life cycle is that it catalyses a *trans* to *cis* or a *cis* to *trans* isomerization of a peptidyl-prolyl bond in a viral or host protein critical for HCV replication. The observation that Cyp inhibitors (Debio 025, NIM811 and SCY-635), which neutralize PPIase activity, but which are not immunosuppressive, also block HCV replication is consistent with this hypothesis. In *Drosophila melanogaster*, the CypA homolog, called NinaA, forms a stable and specific complex with the Rh1 isoform of rhodopsin. The formation of this complex is essential for the transit of the visual pigment through the endoplasmic reticulum (41-42). Interestingly, an elegant study by Schmid and colleagues showed that a proline serves as a molecular timer in the infection of *Escherichia coli* by the filamentous phage fd (43). The phage infection is activated by the disassembly of two domains of its gene-3-protein, which is located at the phage tip. A proline (Pro213) located in the hinge between the two gene-3-protein domains, serves as a timer for the infective state. The timer is switched on by *cis*-to-*trans* and switched off by the unusually slow *trans*-to-*cis* isomerization of the Gln212-Pro213 peptide bond. Importantly, the switching rate and the phage infectivity are determined by the local sequence surrounding Pro213, and can be tuned by mutagenesis (43). Another hypothesis is that the PPIase activity and the auxiliary polypeptide sequestration function of CypA are both required for its function in HCV replication. Indeed, mutagenesis of NinaA failed to identify a mutant, which distinguishes the isomerase from

the polypeptide sequestration activity of CypA (41). Similarly, we showed here that mutations, which disrupt the isomerase activity of CypA, also disrupt the capacity of CypA to bind HIV-1 Gag in hepatocytes. This is in accordance with the work of Luban and colleagues, who showed that all mutations, which neutralize CypA enzymatic activity, also preclude CypA incorporation into HIV-1 (44).

The mechanisms of action of CypA in HCV replication remain to be unraveled. A current hypothesis is that Cyps by interacting with the nonstructural protein 5B (NS5B), increase the affinity of the polymerase to the viral RNA, and therefore enhances HCV replication (45). This hypothesis is supported by the fact that two studies showed that HCV resistance to CsA (39, 46), resulted in the emergence of mutations in NS5B. Moreover, Yang et al. presented convincing pulldown data showing that CypA binds NS5B (18). These data are in accordance with our co-immunoprecipitation data, which showed that the isomerase pocket of CypA serves as a binding site for the NS5B polymerase. This finding demonstrates for the first time that there is a direct correlation between NS5B binding to the isomerase pocket of CypA and HCV replication. These new findings are important because they suggest that CypA, by catalyzing a *trans* to *cis* or a *cis* to *trans* isomerization of a peptidyl-prolyl bond within NS5B, enhances HCV replication. Interestingly, recent resistance mapping studies suggest that Cyp inhibition may

also act on the HCV NS5A protein (46-48). Thus, further work is required to determine if NS5A, NS5B, both or another viral protein represent(s) the true ligand(s) for CypA.

Although both HIV-1 and HCV exploit the abundant cytosolic CypA, it is likely that the immunophilin acts at distinct steps of their viral life cycles. HIV-1 requires CypA post-entry (early events) (49). It is currently thought that target cell CypA, by interacting with the HIV-1 core delivered into the cytosol of infected cells, protects these cores from an antiviral activity present in human cells (50-53). The identity of the anti-HIV-1 factor counteracted by CypA remains unknown. In contrast to HIV-1, HCV requires CypA before budding (late events). CypA likely does not act on the HCV core, like HIV-1, since HCV replicon (no core expressed) replication is also CypA-dependent. Nevertheless, further studies are required to determine if CypA acts at several steps of the HCV life cycle. Although CypA apparently acts at distinct steps of the HIV-1 and HCV life cycles, one cannot exclude the possibility that CypA via its hydrophobic pocket assists these two prime human pathogens by accomplishing the same task such as peptidyl-prolyl *cis-trans* isomerization or another unknown task. Given that the Cyp inhibitor Debio 025 exhibits a remarkable anti-HCV activity in patients (15-16), it is imperative to understand at a molecular level how this novel class of anti-HCV agents - Cyp inhibitors - block HCV replication.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: Respective Contribution of Cyp Members to HCV Replication. A. Huh7 cells containing the subgenomic HCV Con1 replicon were transfected with an irrelevant control siRNA or siRNAs that target CypA (siRNA CypA), CypB (siRNA CypB), CypC (siRNA CypC) or CypD (siRNA CypD). Cells were washed 24 h post-transfection. Seven days post-transfection, cells were collected and dialysed. To ensure that the siRNA treatments did not non-specifically influence growth and viability of transfected

hepatocytes, cells were counted and analyzed for trypan blue uptake at the time of collection. Cell lysates were standardized for protein content and analyzed for Cyp content by Western blot using antibodies directed against CypA, CypB, CypC or CypD. **B.** Same as A. except that cell lysates were analyzed for HCV protein expression using antibodies directed against NS5A and NS5B. **C.** Naïve Huh7 cells were electroporated with 10 μg of *in vitro*-transcribed genomic Con1 RNA. Twenty-four hours post-HCV RNA electroporation, cells were transfected with siRNA Cyp and then retransfected 24 hours later. At the indicated time points, intracellular HCV RNA was analyzed via reverse-transcription quantitative polymerase chain reaction and presented as genome equivalents (GE) per microgram total RNA.

Figure 2: HCV, Like HIV-1, Requires Host CypA to Fully Replicate in Human Cells. **A.** Naïve Huh7 cells were transduced with an VSVG-pseudotyped HIV-1-based vector containing an irrelevant control shRNA or shRNAs that target CypA, CypB or CypC. Transduced cells were selected for 7 weeks under puromycin (1 $\mu\text{g}/\text{mL}$). Stable cell lines were analyzed for CypA, CypB and CypC content by Western blotting. **B.** Stable Cyp-KD cell lines were electroporated with 10 μg of *in vitro*-transcribed genomic Con1 RNA. At the indicated time points, intracellular HCV RNA was analyzed via reverse-transcription quantitative polymerase chain reaction and presented as genome equivalents (GE) per microgram total RNA. **C.** Top panels. Cyp-KD cells lines were exposed to cell-free VSVG-HIV-1-GFP (left panel) or HSV-1-GFP (right panel) at a multiplicity of infection (MOI) of 0.1. Forty-eight hours post-infection, percentage of GFP-positive cells was quantified by FACS. Bottom left panel. Cells were exposed to cell-free Dengue-2 (MOI of 0.1). Three days post-infection, Dengue-2 infection was quantified by measuring the amounts of intracellular Dengue-2 capsid using an intracellular FACS staining assay (IFSA). Bottom right panel. Cyp-KD cells lines were electroporated with 10 μg of *in vitro*-transcribed genomic Con1 RNA. Seven days post-transfection, HCV infection was quantified by measuring the amounts of intracellular HCV NS5B. Data are expressed in % of infected cells (GFP-, capsid- or NS5B-positive cells) by fixing arbitrary infection of parental cells at 100. Results are representative of three independent experiments.

Figure 3: HCV Requires the Isomerase Activity of CypA to Replicate in Hepatocytes. **A.** Parental or CypA-knockdown Huh7 cell lines were electroporated with 10 μg of *in vitro*-transcribed genomic Con1 RNA. Twenty-four hours post-HCV RNA electroporation, cells were transfected with shRNA-resistant wild-type or H126Q CypA-HA in the presence or absence of the Cyp inhibitors CsA (2.5 μM) or Debio 025 (2 μM). At the indicated time points, intracellular HCV RNA was analyzed via reverse-transcription quantitative polymerase chain reaction and presented as genome equivalents (GE) per microgram total RNA. **B.** The inability of the H126Q CypA mutant to bind to HIV-1 Gag in hepatocytes was examined by measuring amounts of CypA incorporated into released particles. Huh7 cells were co-transfected with HIV-1 together with wild-type or H126 CypA-HA in the presence or absence of Cyp inhibitors CsA (10 μM) or Debio 025 (2 μM). Forty-eight post-transfection, both transfected cells and released virions were analyzed for CypA content by Western blotting using anti-HA antibodies. Cell lysates were standardized for protein content, whereas virions were standardized for HIV-1 capsid content by p24 ELISA.

Figure 4: The HCV NS5B Polymerase Associates With CypA Via Its Enzymatic Pocket. Parental Huh7 cells (3 million) were co-transfected with NS5B-myc (5 μg DNA) and wild-type CypA-HA or H126Q CypA-HA (5 μg DNA) in the presence or absence of the Cyp inhibitor Debio 025 (2 μM). Three days post-transfection, cells were collected and lysed. Cell lysates were pre-cleared with agarose beads. CypA-NS5B association was assessed by co-immunoprecipitation using the Pierce HA tag Co-IP kit. Bound material was eluted and analyzed by Western blotting using anti-HA and anti-myc antibodies. Results are representative of three independent transfections.