Hepatitis C virus (HCV) commonly causes persistent infection, and is one of leading killers worldwide because of result chronic hepatitis, progressive liver fibrosis and cirrhosis, and hepatocellular carcinoma (1, 2). Currently, no vaccine against HCV is available, and pegylated interferon-α alone or in combination with ribavirin is the only standard treatment for patients chronically infected with HCV. However, the therapy is not well tolerated, expensive and only partially effective (3–5). Even with the successful development of HCV subgenomic replicon (6) and infectious virus model JFH1 (Japanese fulminant hepatitis 1) (7), the mechanisms underlying HCV persistence and pathogenesis have been still incompletely understood and urgently need to be further clarified.

HCV, belonging to the family Flaviviridae, is a single-stranded, positive-sense RNA virus with a genome about 9.5 kb in length. The viral transcript initially produces a large polyprotein followed by proteolytic cleavage to form 10 mature proteins (C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (8). Among these viral proteins, nonstructural protein 5A (NS5A) has received extensive attention. It has been found that NS5A contained an interferon sensitivity-determining region with the relationship to interferon therapeutic efficacy (9, 10). Subsequent researches have proved that NS5A was a pleiotropic and membrane-associated viral phosphoprotein (11) that regulated broad aspects of viral life cycle, such as replication (12–15), assembly (16, 17), and release (18, 19). NS5A also modulated various cell events including cell cycle (20), proliferation (21), and antiviral immunity (22), which coordinated host cell environment for virus persistence and pathogenesis. Furthermore, apoptotic deregulation is usually related to persistent viral infection. HCV NS5A has been found to inhibit apoptosis via binding to the PI3K p85 subunit (23), the pro-apoptotic protein Bim1 (24) and p53 (25). Interestingly, Mankouri et al. (26) recently discovered that HCV NS5A inhibited the pro-apoptotic K+ channel through suppressing p38 MAPK phosphorylation. Our previous data have revealed that HCV NS5A interacted with the cellular protein FKBP38, which resulted in repressing apoptosis in NS5A-Huh7 cells and HCV subgenomic replicon cells (27). However, the detailed mechanisms...
for how the NS5A–FKBP38 association led to apoptotic resistance remained unclear.

FKBP38 is a noncanonical member of the FK506-binding proteins (FKBPs) family of immunophilins and lacks the ability to bind to FK506 (28). Shirane and Nakayama (29) reported that FKBP38 targeted Bcl-2 and Bcl-xL to mitochondria and inhibited apoptosis. Their following research found that FKBP38 tethered the 26 S proteasome to mitochondria (30), suggesting that FKBP38 may regulate proteasome-mediated functions. Most importantly, recent studies showed that FKBP38, depending on nutrient and growth factor availability, might act as an intrinsic antagonist for the mammalian target of rapamycin (mTOR) activity via binding to mTOR (31, 32). These data indicated that FKBP38 was an important pleiotropic protein and might participate in numerous cell activities.

mTOR is an evolutionarily conserved serine/threonine protein kinase, and it functions as a central signaling molecule involved in a wide array of cellular processes, such as cell survival, proliferation, and metabolism. Some DNA viruses have been discovered to manipulate the mTOR pathway for viral replication and survival (33). In the present research we investigated whether HCV also hijacked the mTOR pathway for HCV persistence and pathogenesis. Our results here demonstrated that HCV NS5A activated mTOR and inhibited apoptosis, which was a process dependent on NS5A–FKBP38 association. Further analyses found that the NS5A-mediated mTOR activation and apoptosis repression were due to impairing the mTOR-FKBP38 binding, as NS5A competed with mTOR for binding to FKBP38. Our data collectively prove that HCV-encoded viral protein up-regulates the mTOR pathway to block apoptosis and imply that NS5A may contribute not only to HCV persistence but also to the development of HCV-related diseases such as hepatocellular carcinoma.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Full-length NS5A (amino acids 1–447, genotype 1b) and truncated mutant (amino acids 1–213 deleted) cDNAs were cloned from myc-His-NS5A/pcDNA3.1 (27) and inserted into pCMV-myc or pcDNA3.1-FLAG vector with EcoRI and XhoI sites, and the generated plasmids were designed as myc-NS5A, myc-Δ1, and FLAG-NS5A. Full-length HA–FKBP38/pcDNA3.0 (amino acids 1–413) plasmid was described previously (27). The FKBP38 truncated mutant (HA–Δ3×TPR/pcDNA3.0, amino acids 202–351 deleted) was a kind gift of Keiichi I. Nakayama (Kyushu University). myc-FKBP38/pCMV, GST–FKBP38/pGEX-4T-1, and GST–Δ3×TPR/pGEX-4T-1 were constructed with EcoRI and XhoI sites by PCR using HA–FKBP38/pcDNA3.0 and HA–Δ3×TPR/pcDNA3.0 plasmids as templates, respectively.

The antibodies used were as follows: phospho-S6K1 (Thr-389), total S6K1, phospho-4EBP1 (Thr-37/46), total 4EBP1, mTOR, cleaved caspase 3, cleaved poly(ADP-ribose) polymerase (PARP), HA tag, myc tag (Cell Signaling); β-actin (Sigma); FKBP38 (R&D Systems); NS5A (Virogen); LY294002 (PI3K inhibitor) and rapamycin (mTOR inhibitor) were purchased from Sigma, and apoptosis inducer staurosporine was obtained from Cell Signaling. Transfection reagents FuGENE 6 and Lipofectamine RNAiMAX were purchased from Roche Applied Science and Invitrogen, respectively.

Construction of Stably NS5A-expressing Cell Lines and Cell Culture—To establish NS5A stably expressing cell lines, Huh7 cells were plated in 60-mm dishes and transfected with pcDNA3.1- FLAG-NS5A or pcDNA3.1-FLAG empty vector. Sixteen hours post-transfection, culture medium was withdrawn, cells were washed with 1× PBS, and fresh medium supplemented with 1000 μg/ml G418 (Invitrogen) was added. Three weeks later individual G418-resistant clones were produced, isolated, and maintained in Dulbecco’s modified Eagle’s containing 500 μg/ml G418 in the presence or absence of 10% fetal bovine serum (Invitrogen). One Huh7 clone stably expressing NS5A (NS5A–Huh7) and another expressing G418-resistant gene (empty vector, neo–Huh7) were chosen for corresponding experiments. HeLa and Huh7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin with or without 10% fetal bovine serum; Huh7 cells harboring the BB7 HCV subgenomic replicon (genotype 1b) as described in Wang et al. (27) were cultured in Dulbecco’s modified Eagle’s supplemented with 0.5 μg/ml blasticidin and 0 or 10% fetal bovine serum.

Western Blotting—After HeLa, Huh7, neo–Huh7, NS5A–Huh7, and HCV replicon cells were transfected with the indicated plasmids for 48 h or followed by other treatments, total cellular proteins were extracted with 2× SDS protein loading buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 10% β-mercaptoethanol, and 0.1% SDS. For phosphorylation detection, cells were lysed in the above lysis buffer supplemented with 0.1 mM sodium vanadate (Na3VO4), 60 mM β-glycerophosphate, 50 mM NaF, and 1× protease inhibitor mixture (Roche Applied Science). After boiling for 8 min, samples were stored at −20°C for use or immediately resolved by 8% SDS-PAGE for 6 h to probe mTOR or 10 and 12% SDS-PAGE for 3 h to probe other indicated proteins. Proteins were then electropherferferred to nitrocellulose membrane (Roche Applied Science), and the membranes were incubated with the corresponding primary antibodies after blocking with 5% nonfat milk in 1× PBS/Tween. Followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), proteins were visualized by an enhanced chemiluminescence system (ECL, PerkinElmer Life Sciences).

Kinase Inhibitor Treatment—Huh7, neo–Huh7, NS5A–Huh7, and HCV replicon cells were treated with 10 μM/liter rapamycin or 50 μM/liter LY294002 for 1 h before cell harvest or apoptosis induction. Cells were subsequently processed for Western blotting, immunoprecipitation, or Hoechst33342 staining.

RNA Interference—For knockdown experiments, neo–Huh7, NS5A–Huh7, and HCV replicon cells were seeded in 12- or 6-well plates overnight with no excess of 30–50% cell confluence, and 100 nm siRNA for NS5A and 80 nm siRNA for FKBP38 or equivalent amounts of control siRNA (siGFP, Invitrogen) were transfected with Lipofectamine RNAiMAX according to the manufacturer’s recommended protocols. The sequences of siRNA duplexes were as follows: siNS5A, 5′-UAA GCA CGG UGU UGA CUG A-3′ and 5′-UCA GUC ACC GUG CAU A-3′; siFKBP38, 5′-CUG GAA CUC ACC AAC AAC
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AAG A-3’ and 5’-UCU UGU GAG GUU CCA G-3’. Forty-eight hours post transfection, cells were subjected to other indicated treatments and analyses. Apoptosis Assays—For detection of caspase 3 and PARP activation, neo-Huh7, NS5A-Huh7, and HCV replicon cells were seeded in a 12-well plate overnight, or Huh7 cells were transfected with indicated plasmids. After serum deprivation for 48 h, apoptosis was induced with 1 μM/liter staurosporine for 4 h in the absence of serum. When indicated, siRNAs for NS5A, FKBP38, and control siRNA were transfected, or rapamycin was added as described above. Cells were lysed in 2 × SDS protein loading buffer and subjected to SDS-PAGE and immunoblotting analysis for cleaved caspase 3 and cleaved PARP.

For Hoechst33342 staining, neo-Huh7 and NS5A-Huh7 cells plated on the glass coverslips were maintained in a 6-well plate with Dulbecco’s modified Eagle’s and serum-starved for 48 h. After a 1-h rapamycin treatment, apoptosis was induced with 1 μM/liter staurosporine for 8 h in the absence of serum. When indicated, siRNAs for FKBP38 and control siRNA were transfected. The monolayer cells were gently washed with 1 × PBS, fixed with 3.5% paraformaldehyde at room temperature, and permeabilized with 0.1% Triton X-100 in 1 × PBS and then followed by 8 μg/ml Hoechst33342 staining for 30 min. The treated cells were observed under the fluorescence microscope (Olympus IX71). Cells with bright, condensed nuclei were considered as apoptotic cells.

GST Pulldown Analysis—GST, GST-FKB38, and GST-FKB38-Δ3 × TPR fusion proteins were expressed in Escherichia coli BL21 strains and purified with glutathione-agarose beads. For producing NS5A- and mTOR-containing lysates, HeLa and Huh7 cells transfected with pCMV-myc-NS5A, or NS5A-Huh7 cells and HCV subgenomic replicon cells were maintained in 10-cm dishes with or without serum for 48 h. Then cells were lysed in 1 ml of buffer including 0.3% CHAPS, 40 mM HEPES (pH 7.4), 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture. Pre-clearance with 50 μl of 50% slurry of glutathione-agarose beads at 4 °C for 2 h was followed by centrifugation at 14,000 × g for 2 min. The resultant supernatants were divided into three parts; 50 μl of supernatant was mixed with 50 μl of 2 × SDS protein loading buffer, boiled, and stored at −20 °C as the input, and the remaining supernatants were equally added into two 1.5-ml tubes containing 50 μl of glutathione-agarose beads and equal amounts of precleared GST or GST fusion proteins. After incubation at 4 °C for 2 h, the samples were centrifuged for 5 min at 3000 × g, and the supernatants were discarded. The beads were washed 3 times with wash buffer containing 0.3% CHAPS, 50 mM HEPES (pH 7.4), 40 mM NaCl, 2 mM EDTA followed by centrifugation at 3000 × g for 5 min. The supernatants were discarded, and equal volumes of 2 × SDS protein loading buffer were added into beads. Samples were boiled for 8 min and subjected to SDS-PAGE and immunoblotting for detecting (myc-) NS5A, mTOR, and Coomassie staining for detecting GST, GST-FKB38, and GST-Δ3 × TPR fusion proteins.

Co-immunoprecipitation Analysis—Huh7, neo-Huh7, NS5A-Huh7, and HCV replicon cells were plated in 10-cm dishes for 48 h with or without serum supplement. When specified, the indicated plasmids were transfected using FuGENE 6, or rapamycin was treated. For co-immunoprecipitation of myc-NS5A and HA-FKB38, Huh7 cells were lysed in 1 ml of buffer containing 1% Triton X-100, 40 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture. To perform mTOR-involved co-immunoprecipitation, the cell lysis and washes were performed as described above (see “GST Pulldown Analysis,” above). Cell lysates were rotated at 4 °C for 40 min for complete cell lysis then centrifuged at the maximal speed for 20 min to discard cell debris. The supernatants were precleared with 40 μl of protein A/G plus-agarose beads (Santa Cruz) at 4 °C for 1 h or overnight, then centrifuged at 5000 × g for 5 min to discard beads. The supernatants were divided into three parts; 50 μl of supernatant was mixed with 50 μl of 2 × SDS protein loading buffer, boiled, and stored at −20 °C as the input, and the rest supernatants were separated equally into two 1.5-ml tubes. Specific primary antibodies or equal amounts of normal control IgG (Santa Cruz) were added, and the mixtures were rotated at 4 °C for 2 h followed by incubation with 40 μl of protein A/G plus-agarose beads for another 2 h. The mixtures were centrifuged at 3000 × g for 5 min to discard supernatants. The beads were washed 3 or 4 times with corresponding wash buffer followed by centrifugation at 3000 × g for 5 min to discard supernatants. Equal volumes of 2 × SDS protein loading buffer were added into beads, and samples were boiled for 8 min and subjected to Western blotting as described above.

Immunofluorescence Staining and Confocal Laser Scanning Microscope Assay—neo-Huh7, NS5A-Huh7, and HCV replicon cells grown on glass coverslips were maintained in 12-well plates with or without 10% fetal bovine serum. After incubated for 48 h, cells were washed with 1 × PBS and fixed with 4% paraformaldehyde for 15 min. The fixed cells were permeabilized with 0.2% Triton X-100 for 10 min and blocked with 3% bovine serum albumin for 1 h. Cells then were incubated with anti-FKB38 and anti-mTOR antibodies (1: 100) at 4 °C overnight followed by staining for 2 h with Alexa Fluor 488-conjugated anti-mouse secondary antibody (1: 200) and Cy3-conjugated anti-rabbit secondary antibody (1: 300). Cells were washed with 1 × PBS/1: 4 Tween, mounted on glass slides, and visualized by a confocal laser scanning microscope (Leica, TCS-SP2). The expressions of endogenous NS5A, FKB38, mTOR, and β-actin were detected by Western blotting as described above.

RESULTS

HCV NS5A Activates the mTOR Pathway Independent of PI3K-AKT—We initially investigated whether HCV NS5A could regulate mTOR signal pathway. The reason for our focusing on the effect of HCV NS5A on the mTOR pathway is due to the extensive involvement and multiple functions of NS5A in numerous signal pathways (11). Additionally, because the mTOR pathway is controlled by nutrients and growth factors, S6K1 and 4EBP1 are two well characterized substrates downstream of mTOR (34), we thus performed experiments in the presence or absence of serum in cell culture to determine the phosphorylation status of S6K1 at Thr-389 and 4EBP1 at Thr-
37/46, which served as markers for mTOR activity. As shown in Fig. 1A, the phosphorylation of S6K1 and 4EBP1 in NS5A-transfected Huh7 cells under serum-starved conditions was significantly higher than those in mock- or empty vector-transfected cells. And this activation was in time- and dose-dependent manners (Figs. 1, B and C). Interestingly, NS5A overexpression under sufficient serum-supplied condition did not affect the phosphorylation levels of S6K1 and 4EBP1 (Fig. 1A). More physiologically, the much higher phosphorylation of S6K1 and 4EBP1 were also observed in NS5A-Huh7 and HCV replicon cells without serum supplement, as compared with low basal levels of phosphorylation in neo-Huh7 cells (Fig. 1D).

These results indicated that NS5A activated the mTOR pathway.

To test if activation of the mTOR pathway by NS5A was specific, mTOR kinase inhibitor rapamycin was treated in neo-Huh7, NS5A-Huh7, and HCV replicon cells, or NS5A expression was knocked down using specific siNS5A in neo-Huh7 and NS5A-Huh7 cells. As expected, rapamycin and siNS5A abolished the phosphorylation increases of S6K1 and 4EBP1 (Figs. 1, D and E). A previous report showed that NS5A bound to PI3K p85 subunit, thus activating PI3K and Akt (35), both of which are upstream components of mTOR (33). Therefore, we used specific kinase inhibitor LY294002 for PI3K to probe if the mTOR pathway activation by NS5A was achieved through the PI3K and Akt. As shown in Fig. 1F, LY294002 could obviously inhibit S6K1 and 4EBP1 phosphorylation in NS5A-Huh7 cells with the presence of serum. This further confirmed the results in Fig. 1A that NS5A did not activate the mTOR pathway under sufficient serum-fed condition. However, in the absence of serum, LY294002 was unable to down-regulate the increased phosphorylation of S6K1 and 4EBP1 as compared with DMSO-treated NS5A-Huh7 cells. In control neo-Huh7 cells with or without serum culture, LY294002 markedly suppressed S6K1 and 4EBP1 phosphorylation. Accordingly, these results implied that NS5A specifically activated the mTOR pathway independent of PI3K and Akt.

**HCV NS5A Targets FKBP38 to Activate mTOR**—Our above data suggested that the mTOR pathway activation by NS5A was not via PI3K and Akt; we next asked which component in the mTOR pathway was targeted by NS5A. Because of our previous finding that NS5A interacted with FKBP38 (27) and the recent discovery that FKBP38 was a new member in the mTOR pathway and an intrinsic antagonist for mTOR activity (31, 32), we thus investigated whether the mTOR pathway activation by NS5A was dependent on the NS5A-FKBP38 association. For this purpose, plasmids encoding wild type NS5A, wild type FKBP38, a NS5A mutant without FKBP38-binding region (NS5A-H9004), or a FKBP38 mutant without the NS5A binding region (FKBP38-H9004) (27, 36) (Fig. 2A) was transfected alone or cotransfected into Huh7 cells.
without serum addition. The results showed that wild type FKBP38 remarkably decreased the phosphorylation levels of S6K1 and 4EBP1 compared with those of empty vector-transfected cells (Fig. 2B); this was consistent with previous reports (31, 32). Cotransfection of wild type NS5A and FKBP38 significantly promoted S6K1 and 4EBP1 phosphorylation, whereas cotransfection of wild type NS5A and FKBP38-Δ3×TPR or cotransfection of NS5A-Δ1 and wild type FKBP38 did not promote the phosphorylation of S6K1 and 4EBP1, which were presented at similar levels to wild type FKBP38-overexpressing cells (Fig. 2B).

Meanwhile, we found the elevated phosphorylation of S6K1 and 4EBP1 in wild type NS5A- and FKBP38-cotransfected cells were abrogated by rapamycin treatment (Fig. 2C), which was in line with the results presented in Fig. 1D. To exclude the possibility that inhibition of S6K1 and 4EBP1 phosphorylation by rapamycin in wild type NS5A- and FKBP38-cotransfected cells was because rapamycin impaired the binding of NS5A to FKBP38, co-immunoprecipitation experiments were archived in Huh7 cells with or without rapamycin treatment. As shown in Fig. 2D, rapamycin did not affect the interaction of NS5A and FKBP38.

To confirm these results, special siRNA for FKBP38 was transfected into neo-Huh7, NS5A-Huh7, and HCV replicon cells. The results (Fig. 2, E and F) showed that FKBP38 knockdown obviously elevated S6K1 and 4EBP1 phosphorylation in neo-Huh7 cells, which was consistent with previous report (31). Furthermore, NS5A-Huh7 and HCV replicon cells with knockdown of FKBP38 expression still maintained higher levels of S6K1 and 4EBP1 phosphorylation than those in control siRNA-treated cells. Also, rapamycin treatment alone or a combined treatment of rapamycin and FKBP38 knockdown remarkably inhibited S6K1 and 4EBP1 phosphorylation in neo-Huh7, NS5A-Huh7, and HCV replicon cells. These results further supported that NS5A activated mTOR by preventing FKBP38 from binding to mTOR. Taken together, these data suggested that NS5A upregulated mTOR activity via FKBP38, because this mTOR activation was dependent on NS5A-FKBP38 interaction.

**HCV NS5A Suppresses Cell Apoptosis via the mTOR Pathway**—The mTOR pathway is a central mediator involved in broad spectra of cellular processes, such as cell survival, proliferation, and metabolism (33). Several reports show that mTOR activation reduced cell apoptosis (37, 38). We, therefore, hypothesized that the NS5A-activated mTOR pathway might inhibit apoptosis. To investigate this possibility, we examined the effect of rapamycin treatment or NS5A knockdown on the sensitivity of neo-Huh7 and NS5A-Huh7 cells to apoptosis induced by staurosporine in the absence of serum. We found that the levels of cleaved caspase 3 and cleaved PARP in NS5A-Huh7 cells without rapamycin pretreatment were much lower than those in NS5A-Huh7 cells with rapamycin pretreatment and those in neo-Huh7 cells with or without rapamycin pretreatment (Figs. 3, A and B), which indicated that NS5A inhibited apoptosis via the mTOR pathway.

To validate these results, we adopted two additional methods, Hoechst33342 staining and NS5A knockdown, to monitor
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apoptosis in the same cell types with the same treatment as above. As demonstrated in Fig. 3C, the number of apoptotic cells in NS5A-Huh7 cells without rapamycin pretreatment was fewer than those in NS5A-Huh7 cells with rapamycin pretreatment and those in neo-Huh7 cells with or without rapamycin pretreatment. Fig. 3D showed that in parallel to the high sensitivity of neo-Huh7 cells with various treatments to apoptosis, rapamycin pretreatment or NS5A knockdown restored the sensitivity of NS5A-Huh7 cells to apoptosis, characterized by obvious increases of cleaved caspase 3 and cleaved PARP, whereas NS5A-Huh7 cells without rapamycin pretreatment and NS5A knockdown were highly resistant to apoptosis, evidenced by extremely low levels of cleaved caspase 3 and cleaved PARP. These data further demonstrated that NS5A specifically suppressed apoptosis via the mTOR pathway.

HCV NS5A Suppresses Apoptosis by Impairing the FKBP38 and mTOR Interaction—Because our above results proved that the mTOR pathway activation by NS5A was dependent on NS5A-FKB38 binding, we examined if NS5A-mediated apoptotic inhibition via the mTOR pathway was also dependent on the binding of NS5A to FKB38. As presented in Fig. 4A, when myc-NS5A or HA-FKB38 was co-expressed with HA-Δ3×TPR or myc-ΔI in Huh7 cells, the levels of caspase 3 and PARP activation were increased regardless of rapamycin treatment. However, when myc-NS5A and HA-FKB38 were co-expressed without rapamycin treatment, the levels of cleaved caspase 3 and PARP were decreased. In these cells, rapamycin treatment recovered the high level of cleaved caspase 3 and PARP.

Additionally, the levels of cleaved caspase 3 and PARP in siFKBP38-treated neo-Huh7 cells were much lower than those in control siRNA-treated, rapamycin-treated, or a combination treatment of rapamycin and siFKBP38 cells (Fig. 4, B, D, and E). NS5A-Huh7 or HCV replicon cells transfected with siFKBP38 had an obvious resistance to apoptosis induced by staurosporine, characterized by extremely low levels of cleaved caspase 3 and PARP. In contrast, rapamycin treatment alone or a combined treatment of rapamycin and siFKBP38 led to high levels of cleaved caspase 3 and PARP in NS5A-Huh7 or HCV replicon cells (Fig. 4, B, D, and E). Consistently, as shown in Fig. 4C, determined by Hoechst33342 staining, the apoptotic number in neo-Huh7 cells treated with siFKBP38 was greatly lower than that in cells treated with control siRNA, rapamycin, or rapamycin together with siFKBP38. The apoptotic number in NS5A-Huh7 cells treated with siFKBP38 was also significantly lower than that in cells with rapamycin treatment alone or a combined treatment of rapamycin and siFKBP38. These data indicated that apoptotic inhibition exerted by NS5A was through impairing the interaction of FKB38 and mTOR.

HCV NS5A Interferes with the Interaction of mTOR with FKB38 in Vitro—Recent researches have shown that FKB38 bound to mTOR and suppressed mTOR activity under nutrient-deprived conditions (31, 32). Based on these data, we proposed that NS5A may disrupt the mTOR-FKB38 binding, which thus activated mTOR to inhibit apoptosis as described above. To validate this proposal, we performed GST pulldown experiments using E. coli BL21-expressed GST-FKB38 and GST-Δ3×TPR, mammalian cell-expressed NS5A, and endogenous mTOR (the available mTOR plasmids encoding full-length and 289-kDa mTOR protein were not successfully expressed). The results showed that GST-FKB38 could not pull down mTOR in NS5A-overexpressed HeLa and Huh7 cells in the absence of serum, whereas GST-FKB38 could still pull down mTOR in empty vector-transfected cells in the absence of serum (Figs. 5, A and B). No mTOR was pulled down by GST-FKB38 in empty vector-transfected cells with 10% serum supplement, which was consistent with a previous report (31). Similarly, GST-FKB38 was unable to pull down mTOR in NS5A-Huh7 and HCV replicon cells in the absence of serum; under these conditions, FKB38 pulled down NS5A (Fig. 5C). However, in neo-Huh7 cells without serum addition, GST-FKB38 retained the ability to pull down mTOR. In neo-Huh7 cells with 10% serum supplement, mTOR was not pulled down by GST-FKB38 (Fig. 5C). These data suggested that NS5A impaired the mTOR-FKB38 binding via interacting with FKB38.

Based on the above results, we proposed that FKB38 mutant with the NS5A-binding region deleted (FKBP38-Δ3×TPR) should rescue the binding of mTOR to FKB38 in the presence of NS5A. To test this hypothesis, GST-Δ3×TPR and GST-FKB38 were used to perform a GST pulldown experiment in NS5A-Huh7 and HCV replicon cells in the absence of serum. As shown in Fig. 5D, GST-Δ3×TPR, which was unable to bind to NS5A, could reversibly pull down mTOR, but wild
type GST-FKB38 with binding to NSSA failed to pull down mTOR. Taken together, these results confirmed the notion that NSSA impaired the mTOR-FKB38 binding by associating with FKB38.

HCV NSSA Interferes with the Interaction of mTOR with FKB38 in Vivo—To validate the GST pulldown results, we further analyzed the binding among NSSA, FKB38, and mTOR using co-immunoprecipitation assays. As shown in Fig. 6A, in the absence of serum, HA-FKB38 could not co-immunoprecipitate with mTOR when it was co-expressed with myc-NSSA in Huh7 cells, whereas HA-FKB38 co-immunoprecipitated with mTOR in the absence of myc-NSSA overexpression. There was no detectable HA-FKB38-mTOR binding observed in cells overexpressing HA-FKB38 alone or in combination with myc-NSSA with serum addition, and serum had no effect on the HA-FKB38-myc-NSSA binding (Fig. 6A). To investigate whether mTOR directly bound to NSSA, special mTOR antibody or Myc-tag antibody was utilized to perform another two co-immunoprecipitation experiments. The results showed that mTOR could not co-immunoprecipitate with myc-NSSA when myc-NSSA was expressed alone or in combination with HA-FKB38 in serum-starved Huh7 cells (Fig. 6B), nor did myc-NSSA co-immunoprecipitate with mTOR in myc-NSSA- and HA-FKB38-cotransfected Huh7 cells under serum deprivation conditions (Fig. 6C). These results suggested that no direct or indirect binding occurred between mTOR and NSSA.

Furthermore, when myc-ΔI and HA-FKB38 were co-expressed or myc-NSSA and HA-Δ3×TPR were co-expressed in Huh7 cells without serum supplement, mTOR-FKB38 bindings were restored at nearly the same levels as in Huh7 cells transfected with HA-FKB38 alone (Fig. 6D). These data were consistent with GST pulldown results described above (Fig. 5D). Again, there was no mTOR-HA-FKB38 binding found in Huh7 cells expressing wild type myc-NSSA and HA-FKB38 in the absence of serum (Fig. 6D). Additionally, when NSSA interacted with FKB38, the mTOR-FKB38 associations disappeared in NSSA-Huh7 cells as well as HCV replicon cells under serum starvation conditions (Fig. 6E), whereas this interaction remained intact in control neo-Huh7 cells with no serum culture. No mTOR-FKB38 binding was detected in serum-supplied neo-Huh7 cells (Fig. 6E), which was also in accordance with GST pulldown results (Fig. 5C). Collectively,
these data demonstrated that HCV NS5A disrupted the mTOR-FKBP38 interaction by binding to FKBP38 in vivo.

HCV NS5A Disrupts the Colocalization of FKBP38 and mTOR—Because NS5A impaired the binding of FKBP38 to mTOR, it seemed that NS5A may disrupt FKBP38 and mTOR colocalization. To test this possibility, we conducted confocal microscopy studies. As shown in Fig. 7A, in the absence of serum, FKBP38 obviously colocalized with mTOR in the cytoplasm of neo-Huh7 cells. However, FKBP38 and mTOR separately distributed in cytoplasm of neo-Huh7 and HCV subgenomic replicon cells in the absence of serum. Furthermore, in the presence of serum, FKBP38 and mTOR also independently distributed in neo-Huh7, NS5A-Huh7, and HCV subgenomic replicon cells. The expressions of NS5A, FKBP38, and mTOR were confirmed by Western blotting, and shown in Fig. 7B. These results indicated that NS5A could disrupt the colocalization of FKBP38 and mTOR. The above data were in accordance with GST pulldown and co-immunoprecipitation results and further supported the notion that NS5A disrupted the interaction between mTOR and FKBP38 in the absence of serum.

DISCUSSION

The successful establishment of viral persistent infections requires that viruses tightly regulate the key signal pathways involved in cellular growth, proliferation, and survival. The mTOR signal pathway is such a pathway. Many DNA viruses have been found to regulate the mTOR pathway to enhance their infection and survival in host cells (33). Such viruses survival via controlling cellular translation, ribosomal biosynthesis, and metabolism in a manner-dependent on nutrients and growth factors availability (44).

Our present data demonstrated that in NS5A-overexpressing Huh7, NS5A-Huh7, and HCV subgenomic replicon cells, NS5A up-regulated mTOR activity under serum-starved conditions, characterized by elevating phosphorylation levels of S6K1 and 4EBP1 (Fig. 1). In addition, NS5A knockdown or rapamycin treatment abrogated phosphorylation of S6K1 and 4EBP1 (Fig. 1). This suggested that NS5A specifically elevated mTOR activity under serum-starved conditions, characterized by elevating phosphorylation levels of S6K1 and 4EBP1 (Fig. 1). In addition, NS5A knockdown or rapamycin treatment abrogated phosphorylation of S6K1 and 4EBP1 (Fig. 1).

mTOR is a critical component of the mTOR pathway, and it exists in two distinct complexes termed mTORC1 and mTORC2, respectively, in which mTOR is the catalytic subunit. The more extensively investigated mTORC1 is nutrient- and growth factor-sensitive and highly sensitive to rapamycin. mTOR(C1), through phosphorylating its downstream targets S6K1 and 4EBP1, plays central roles in cellular growth, proliferation, and metabolism in a manner-dependent on nutrients and growth factors availability (44).
ity through PI3K-Akt. Therefore, another component down-stream of PI3K-Akt in the mTOR pathway might be targeted by NS5A. Our previously published results show that the domain I region of NS5A associated with cellular protein FKBP38 (27), which was further confirmed by the claims of another research group that NS5A bound to the 3\times H11003 TPR region of FKBP38 (36).

Recent researches have discovered that FKBP38 was a new member of the mTOR pathway and an intrinsic antagonist for mTOR activity (31). Accordingly, we used NS5A-H9004 and FKBP38-H9003 TPR mutants to reveal that NS5A-mediated mTOR activation was dependent on the binding of NS5A to FKBP38 in Huh7 cells (Fig. 2, A–C). FKBP38 knockdown experiments in NS5A-Huh7 and HCV replicon cells confirmed these results (Fig. 2, E and F). Although the inhibitory activity of FKBP38 on mTOR remains to be conclusively determined (46), our results indicated that FKBP38 actually suppressed mTOR activity in Huh7 cells under serum-starved conditions (Fig. 2, B, E, and F), which was in agreement with the original report (31) and an additional recent study (32). These data provided strong evidence that FKBP38 was an intrinsic inhibitor for mTOR activity (31). Taken together, our data supported that HCV NS5A targeted FKBP38 to activate the mTOR pathway.

Our previous results have identified the existence of an NS5A-FKBP38 interaction (27). As binding of FKBP38 to mTOR inhibited mTOR activity (31), we further examined the interactions among NS5A, FKBP38, and mTOR. GST pulldown experiments suggested NS5A competed with mTOR for its association with FKBP38 (Fig. 5). Co-immunoprecipitation experiments (Fig. 6) solidified this conclusion. Consistently, confocal microscope studies showed that NS5A disrupted the colocalization of FKBP38 and mTOR (Fig. 7). We also found that there was no direct or indirect binding between NS5A and mTOR (Fig. 6). These results clearly demonstrated that up-regulation of mTOR activity by NS5A was due to the impairment of mTOR-FKBP38 binding by the NS5A-FKBP38 association. It was important to note that the sites of FKBP38 responsible for binding to NS5A and mTOR were localized in two distinct regions (31, 36). Therefore, the question arose as to why NS5A, FKBP38, and mTOR were unable to form a triple complex. One possibility was that NS5A interacted with FKBP38 leading to a conformational change in the latter, which thus prevented FKBP38 from interacting with the large 289Kd mTOR protein.

Apoptosis is a critically important process for maintaining homeostasis. Its dysfunction has been usually found in persistent viral infections (47) and cancers (48). Several reports have suggested that mTOR activation contributed to cell survival (37, 38). These previous findings were in agreement with our results, as we found NS5A repressed cell apoptosis induced by staurosporine in NS5A-Huh7 and HCV replicon cells under serum-deprived conditions. Moreover, the inhibitory effect was blocked with rapamycin treatment or NS5A knockdown (Fig. 3). Furthermore, there were no differences in apoptosis among control neo-Huh7 cells with or without rapamycin treatment (Fig. 3, B and E). These data implied that rapamycin itself, at the indicated dose and indicated treatment time-course in our cell system, had no impact on apoptosis. Conclusively, HCV NS5A specifically inhibited apoptosis via the mTOR pathway, and our results further confirmed the anti-apoptotic role of NS5A.

![FIGURE 6. HCV NS5A impairs the mTOR-FKBP38 binding in vivo. A, B, C, and D, the indicated plasmids or corresponding empty vectors were transfected into Huh7 cells with or without 10% serum supplement. 48 h post transfection, cells were lysed, and samples were, respectively, immunoprecipitated (IP) with anti-HA (for FKBP38) (A), anti-mTOR (B), anti-myc (for NS5A) (C), or anti-HA (for FKBP38 or FKBP38 mutant Δ3×TPR) (D). Immunoprecipitates were analyzed by Western blot with the indicated antibodies. E, neo-Huh7, NS5A-Huh7, and HCV replicon cells were cultured with or without 10% serum supplement for 48 h, and cells were harvested for immunoprecipitation with anti-FKBP38. The resultant immunoprecipitates were subjected to Western blot analysis with the indicated antibodies.](https://example.com/figure6.png)
Two recent reports indicated that full-length HCV model J6/JFH1 could induce apoptosis of infected Huh7.5 (49, 50). This finding seemed to be inconsistent with our results that NS5A repressed apoptosis. The discrepancy could be explained as follows. J6/JFH1-infected Huh7.5 was an acute model. Viruses replicated themselves at high levels, which thus resulted in cellular stress and apoptosis. However, in chronically HCV-infected patients, viral replication was usually at a low level. This was consistent with one of observations that with the infection time increasing, the viral replication, viral titer, and number of cell apoptosis all decreased (49). Besides, as a full-length HCV virus, J6/JFH1-induced cell apoptosis under acute infection conditions was probably due to a comprehensive effect resulting from all J6/JFH1-encoded viral proteins, as many reports have demonstrated that HCV-encoded proteins such as core, E1, E2, NS2, NS3, NS4A exerted both pro- and anti-apoptotic effect (51–56). Collectively, we predicted that J6/JFH1-induced apoptosis under acute infection condition was a temporarily protective response of host cells. Once established a chronic infection, HCV can utilize various strategies (for example, NS5A regulates mTOR pathway) to inhibit cell apoptosis, which was not only beneficial for viral survival in infected cells but also in line with evolutionary principles for a chronic virus.

Two researches have shown that S6K1 phosphorylated and inactivated pro-apoptotic protein BAD (37, 57). This indicated that NS5A-mediated apoptotic repression via the mTOR pathway might be through S6K1-controlled BAD. As a central signaling pathway in mammalian cells, the mTOR pathway has frequently been found to be deregulated or mutated in cancers (34) and to be regulated by many DNA viruses (33). These findings highlighted its critical roles in viral persistent infections and pathogenesis. In addition to its anti-apoptotic function (29), FKBP38 has been identified as a target gene transcriptionally controlled by tumor suppressor tuberous sclerosis complex (TSC), a component upstream of mTOR. It up-regulated FKBP38 expression to inhibit cell size (58). This suggested that FKBP38 played a role in tuberous sclerosis complex-mediated tumor inhibition. Furthermore, FKBP38 has been suggested to be involved in promoting HCV replication via formation of a triple complex with HCV NS5A and Hsp90 (36). These data suggested that FKBP38 might play important roles in HCV infection and pathogenesis.

Combining with our overall findings summarized in Fig. 8, we can speculate that HCV NS5A-mediated activation of the mTOR pathway via disruption of the mTOR-FKBP38 complex contributed not only to HCV persistent infection but also to the development of HCV-related diseases such as hepatocellular...
carcinoma. Most importantly, anti-HCV drugs targeting NS5A are currently undergoing investigation (59). One such drug has a potent inhibitory effect on viral replication in a phase I clinical trial of chronically HCV-infected patients (60). Therefore, our results also provide support that anti-HCV drugs targeting NS5A are worthy of further development.

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