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Hepatitis C virus non-structural protein NS5A interacts with FKBP38 and inhibits apoptosis in Huh7 hepatoma cells

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Abstract **Hepatitis C virus non-structural protein NS5A plays an important role in viral replication and various cellular events. To gain further insight into the function of NS5A, we screened a human fetal liver cDNA library for its interacting proteins using the yeast two-hybrid system. FKBP38, a 38 kDa immunosuppressant FK506-binding protein, was identified and its interaction with NS5A was confirmed by both in vitro and in vivo. The interaction was mapped to the amino acids 148–236 of NS5A containing a BH domain (Bcl-2 homology domain). Besides, both NS5A and FKBP38 were found to localize in mitochondria and endoplasmic reticulum. Moreover, NS5A stably expressing Huh7 hepatoma cells showed more resistance to apoptosis and such inhibition of apoptosis could specifically be abrogated by depletion of FKBP38 using RNA interference. These results indicate that HCV NS5A inhibits apoptosis through interaction with FKBP38.**

Keywords: Hepatitis C virus; NS5A; FKBP38; Protein–protein interaction; Apoptosis

1. Introduction

Hepatitis C virus (HCV) is a major causative agent for acute and chronic hepatitis, which may often progress to liver cirrhosis and hepatocellular carcinoma (HCC) [1,2]. It is estimated to infect up to 200 million persons worldwide, corresponding to more than 3% of the world population [3]. The current therapy, pegylated interferon, alone or in combination with ribavirin, is still not effective enough especially in patients infected with genotype 1b [4,5]. HCV study was hampered by the lack of an efficient tissue culture system or an adequate small animal model of HCV infection, although the establishment of HCV subgenomic replicons and very recently, the HCV JFH-1 replicons enabling secretion of infectious viral particles has been a great breakthrough in HCV research [6,7]. At present, the mechanism of HCV replication, persistence and pathogenesis is still unclear.

HCV is a member of the positive strand RNA viruses belonging to the family Flaviviridae and contains a genome of approximately 9.5 kb in length, which encodes a large polyprotein precursor of about 3000 amino acids. This precursor polypeptide is then cleaved by both host and viral proteases to produce structural (core, E1, E2, and P7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [8,9]. Among all of the viral proteins, NS5A has been demonstrated to play an important role in viral replication by coordination with cellular and other non-structural proteins to form a membranous web in infected cells [10]. In addition to its role in viral replication, a number of studies have shown that NS5A is a multi-functional protein involved in many cellular events, many of which would ultimately result in viral persistence and pathogenesis. For example, NS5A expression could alter intracellular calcium and reactive oxygen species levels and in turn activate STAT-3 and NF-κB [11,12]. NS5A could inhibit the activity of the mitogenic and stress activated transcription factor activating protein-1 (AP1) by interfering with Ras-ERK pathway [13]. Cell cycle studies using NS5A expressing cell lines suggested the expression of NS5A may lead to a reduced S phase and an increase in the G_2/M phase [14].

Recently, many studies have focused the anti-apoptosis function on single HCV protein since a growing body of evidence has shown interference with apoptosis could prolong the life of infected cells, resulting in enhanced viral replication and a contribution to viral persistence and pathogenesis. Therefore, after infection, the virus has developed distinct strategies to escape or retard apoptosis triggered by the various apoptotic pathways. For example, the NS2 protein of HCV could interact with a liver-specific pro-apoptotic protein CIDE-B and inhibit CIDE-B induced apoptosis [15]. HCV core protein could suppress apoptosis through upregulation of inhibitor of caspase-activated DNase [16]. Among all of the viral proteins, NS5A has been demonstrated to employ multiple strategies to perturb apoptosis. For instance, NS5A could interact with pro-apoptotic molecule Bin1 and Bax, perturb the mitogenic signaling pathway, and protect cells against TNF-α- and p53-mediated apoptosis [17–22]. However, there is still lacking of the overall understanding of the molecular mechanism of NS5A in mediating cell apoptosis.

To gain further insight into the function of NS5A, we carried out yeast two-hybrid analysis using a NS5A isolate with

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Abbreviations: HCV, hepatitis C virus; NS, non-structural protein; FKBP38, 38kDa FK506-binding protein; HCC, hepatocellular carcinoma; GFP, green fluorescent protein; HA, influenza hemagglutinin; aa, amino acid

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genotype 1b from an interferon-resistant patient to screen a human fetal hepatocyte cDNA library. In this work, we identified a 38 kDa immunosuppressant FK506-binding protein 38 kDa FK506-binding protein (FKBP38) as a new NS5A interacting protein. FKBP38 was previously reported to interact with anti-apoptotic molecule Bcl-2, target Bcl-2 into the mitochondria and inhibit apoptosis [23]. The interaction between NS5A and FKBP38 was confirmed by coimmunoprecipitation and confocal microscopic assays in vitro and in vivo. Further study showed the segment of NS5A containing BH1 domain was essential for association with FKBP38. Immunofluorescence staining showed both NS5A and FKBP38 were found to reside in mitochondria and endoplasmic reticulum. To further explore the role of NS5A in cell apoptosis, NS5A stably expressing Huh7 hepatoma cells was constructed in which Bcl-2 expression was deficient. Apoptosis assay showed that, compared to control cells, it was more resistant to apoptosis. More importantly, such resistance to apoptosis could be specifically abrogated by suppression of endogenous FKBP38 using RNA interference. These results uncover a novel interaction between NS5A and FKBP38 conferring the anti-apoptotic function to NS5A.

2. Materials and methods

2.1. Construction of NS5A

The cloning of full-length NS5A from HCV genotype 1b patient was done as described [24] and the full-length NS5A was used as template for construction of NS5A truncated mutants by PCR amplification with appropriate sequence specific primers and ExTaq polymerase (Takara).

2.2. Yeast two-hybrid assays

The Yeast AH109 reporter strain transformed with plasmid $pGBKT_7$ containing the full-length NS5A was used to screen the human fetal liver MATCHMAKER Gal4 cDNA library (Clontech) using the reporter genes HIS, ADE and MEL. The cDNA library was screened as described in the manufacturer's protocol (Clontech). The positive clones that contained cDNA encoding putative NS5A-interacting proteins were sequenced and subjected to BLAST analysis. For mapping the interacting domain of NS5A, a series of truncated mutants of NS5A and full-length FKBP38 were cotransformed into AH109 yeast strains respectively and selected on Leu/Trp/His/Ade deficient plates.

2.3. In vitro binding assay

The full-length cDNA of FKBP38 was cloned into $pcDNA_3$/HA vector. The plasmids $pGBKT_7$-NS5A and $pcDNA_3$/HA-FKBP38 were used as templates in protein translation using the TNT quick coupled transcription/translation system (Promega). [^{35}S] Methionine (Amersham)-labeled NS5A and HA-FKBP38 were incubated with monoclonal anti-HA antibody (Babco) in lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride), followed by adsorption to BSA blocked protein A/G plus-agarose (Santa Cruz). The beads were washed thrice with lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% NP-40). The antibody–protein complexes were then resolved in 12% SDS–PAGE and subjected to autoradiography.

2.4. Cell culture

Cos7 cells (African Green Monkey kidney cells) and human hepatoma cell line Huh7 cells were propagated in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The BB7 HCV subgenomic replicons (genotype 1b) were obtained from Dr. Charles M. Rice (The Rockefeller University, New York). Huh-7 cells were transfected with HCV subgenomic replicon RNA and maintained as described [25]. Huh-7 clone R cells harboring autonomously replicating HCV replicon RNA were used in related experiments. For NS5A stably expressing cell line, Huh7 cells were transfected with $pcDNA_{3.1}$/myc-His-NS5A or empty vectors using Fugene6 (Roche). Sixteen hours post transfection, medium was changed and supplemented with 0.5 g/l of neomycin (Gibco/BRL). After culturing in selection medium for three weeks, the individual neomycin-resistant clones were isolated. One clone expressing the neomycin resistance gene (vector) and two clones expressing NS5A (5AC1 and 5AC2) were selected for the further study.

2.5. Immunoprecipitation and immunoblotting analysis

To establish a system for validating the interaction in mammalian cells, Cos7 cells were cotransfected with $pcDNA_3$/HA-FKBP38 and $pcDNA_{3.1}$/myc-His-NS5A or their empty plasmids, or HCV subgenomic replicons were transfected with $pcDNA_3$/HA-FKBP38 or pCMV/HA-FKBP51(Kindly gift from Dr. David F. Smith, Johnson Research Center, Mayo Clinic Scottsdale), respectively. Forty-eight hours post transfection, cells were lysed in 1 ml of lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerol phosphate, 0.1 mM sodium orthoranadate, 0.1 mM NaF, 0.1 mM benzamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). For endogenous immunoprecipitation, 1×10^7 HCV subgenomic replicons were lysed directly. The lysates were pre-cleaned with protein A/G plus-agarose by rotating at 4 °C for 2 h. The indicated antibody was then added into the supernatant in the presence of 40 μl fresh protein A/G plus-agarose and incubated at 4 °C overnight. The immunoprecipitates were separated by SDS–PAGE, followed by immunoblotting analysis using appropriate antibody. For immunoblotting analysis, equal quantity of cell lysates was separated by 10% SDS–PAGE. The proteins were electroblotted onto the nitrocellulose membrane (Roche) and incubated with the indicated primary antibody, after incubated with the horseradish peroxidase-conjugated secondary antibody (Santa Cruz), the antibody–antigen complexes were visualized using chemiluminescence reagent (Perkin–Elmer).

2.6. Immunofluorescence staining and confocal microscopic assay

Cos7 cells seeded in a six-well chamber with coverslips were cotransfected with plasmids $pcDNA_3$/HA-FKBP38 and either $pEGFPC_2$-NS5A or $pEGFPC_2$ by calcium–phosphate method. Forty-eight hours post transfection, cells were fixed with 2% formaldehyde and permeabilized with 0.2% Triton X-100 in PBS containing 1% fetal calf serum. Samples were then incubated with mouse anti-HA monoclonal antibody at 4 °C overnight. After being washed thrice with PBS containing 1% fetal calf serum, samples were incubated at 37 °C for 1 h with a rhodamine-conjugated anti-mouse secondary antibody. After further wash, coverslips were finally mounted on glass plates and cells were observed using confocal laser scanning microscope (Leica, TCS-SP2). For staining of endogenous FKBP38 and NS5A, HCV subgenomic replicons were fixed, permeated and incubated with anti-NS5A and anti-FKBP38N1 antibodies (kindly gift of Michiko Shirane). After incubated with corresponding secondary FITC- or Rhodamine-conjugated antibodies, cells were visualized with confocal laser scanning microscope. For mitochondrial staining, HCV subgenomic replicons were stained with 50 nM Mito Tracker Orange CMTRos (Molecular Probes) for 45 min at 37 °C and then fixed and incubated with anti-NS5A or anti-FKBP38 antibody. For endoplasmic reticulum colocalization, HCV subgenomic replicons were fixed and stained with anti-FKBP38 antibody and either endoplasmic reticulum specific protein disulfide isomerase (PDI) antibody or anti-β-actin antibody, or HCV subgenomic replicons were transfected with $pEGFPC_2$-NS5A and stained with either anti-PDI or anti-β-actin antibody and immunofluorescent staining was carried out as described above.

2.7. Subcellular fractionation analysis

The subcellular fractionation analysis was carried out as described [23]. Briefly, HCV subgenomic replicons were washed with PBS, resuspended in CFS buffer (10 mM HEPES–NaOH (pH 7.2), 0.22 M mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM sodium pyruvate, 0.1 mM PMSF and 1 mM dithiothreitol) and homogenized by aspiration through a 22-gauge needle. The homogenate was centrifuged at $750 \times g$ for 5 min, and the

resulting postnuclear supernatant was centrifuged for 10 min at $10000 \times g$ to obtain a pellet highly enriched in mitochondria and a postmitochondrial supernatant as the cytosolic fraction.

2.8. Induction and detection of apoptosis

For induction of apoptosis, NS5A stably expressing Huh7 cell lines and control cell line were deprived of serum for 4 h and then exposed to staurosporine (1 μM; 8 h, Sigma) or cycloheximide (100 μg ml^{-1}; 24 h, Sigma). Apoptosis was evaluated by FACScan flow cytometer using Cell Quest software. The percentage of apoptosis was quantitated from sub-G1 events and data were presented as the means + S.D. Morphological changes in the nuclear chromatin in cells undergoing apoptosis were detected by staining with the DNA binding fluorochrome DAPI. Cells growing on coverslips were transfected with siRNA and then treated with staurosporine. After being washed twice with PBS and fixed by incubation in 2% formaldehyde for 30 min, cells were incubated with DAPI for 5 min and observed by fluorescence microscope. To detect the 85 kDa cleaved PARP product of the cells undergoing apoptosis, 40 μg of total proteins were extracted and subjected to immunoblotting analysis using anti-PARP antibody (Sigma). The content of β-actin was used as internal control.

2.9. Depletion of FKBP38 by RNA interference

One scramble siRNA as a negative control and four candidate siRNA duplexes targeting the ORF of FKBP38 were obtained from Shanghai GENECHEM Co., Ltd: siRNA1: 5′-AAGAGUGGCUGGACAUUCUGG-3′, siRNA2: 5′-GCCAAAGTGGACATGACGT-3′, siRNA3: 5′-CAGACAACATCAAGGCTCT-3′, siRNA4: 5′-CTGGAACCTTCCAACAAGA-3′. 200 nM siRNA were transfected into one well of a 24-well plate using oligotransfectamine (Invitrogen) with no more than 50% of the cell confluence. Forty-eight hours post transfection, cells were harvested and the content of FKBP38 was assayed by immunoblotting. The efficiency of inhibition was confirmed by three independent experiments.

3. Results

3.1. HCV NS5A physically associates with FKBP38 in vitro

To identify the cellular proteins interacting with NS5A, full-length NS5A with genotype 1b from an IFN treatment-resistant patient was used to screen a human fetal liver cDNA library. After second round screening, several clones encoded a 38 kDa immunosuppressant FK506-binding protein FKBP38. To verify the result from yeast two-hybrid screening, full-length NS5A and tagged protein HA-FKBP38 were translated in vitro in the presence of [^{35}S] methionine (Fig. 1A, lanes 1 and 2) and in vitro binding assay was then performed. As shown in Fig. 1A, NS5A could be pulled down with HA-FKBP38 in the presence of anti-HA antibody (Fig. 1A, lane 3), while no protein could be detected in the absence of HA-FKBP38

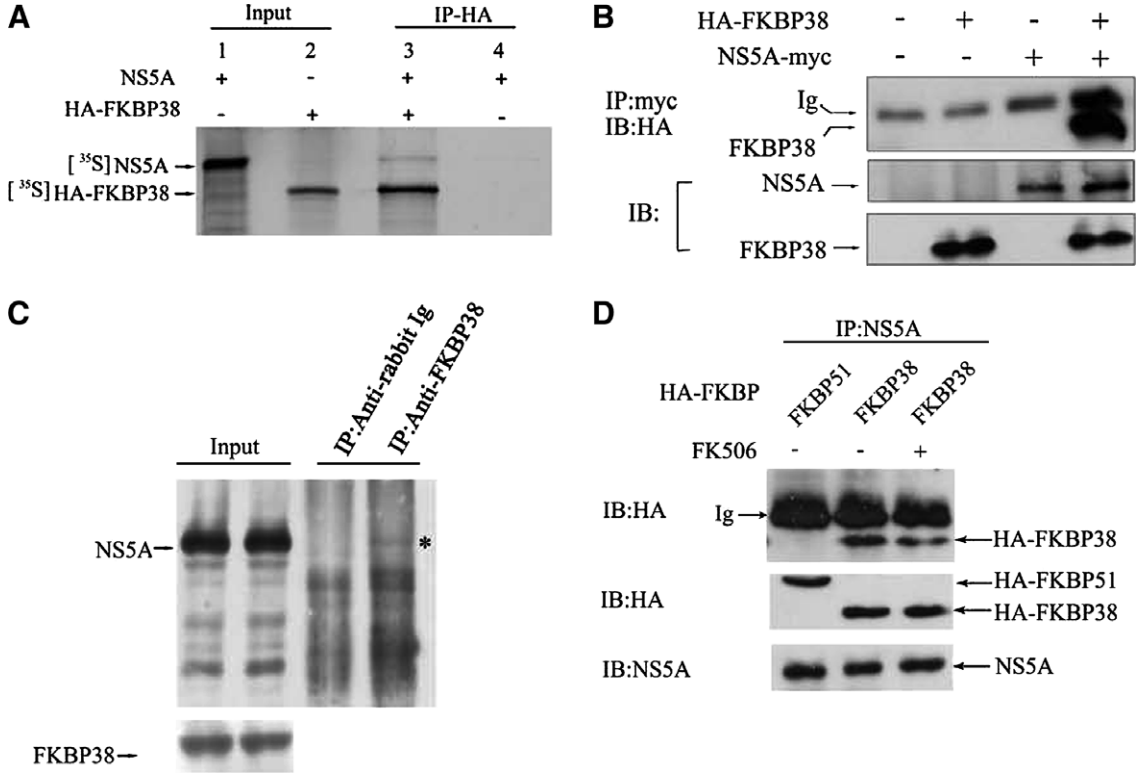

Fig. 1. NS5A associates with FKBP38. (A) In vitro binding assay between NS5A and FKBP38. NS5A and Tagged protein HA-FKBP38 were translated in vitro with [^{35}S] methionine incorporation. After translation NS5A and HA-FKBP38 were immunoprecipitated with monoclonal anti-HA antibody, the antibody–protein complex was resolved in 12% SDS–PAGE and subjected to autoradiography. (B) NS5A interacts with FKBP38 in vivo. Cos7 cells were co-transfected with the expression vectors $pcDNA_3$/HA-FKBP38 and pcDNA3.1/myc-His-NS5A, or the corresponding empty plasmids. Cell lysates were immunoprecipitated with anti-myc antibody, immunoprecipitates were analyzed by immunoblotting using anti-HA antibody. The expression of HA-FKBP38 and NS5A-myc were confirmed by immunoblotting using anti-HA or anti-myc antibody, respectively. (C) HCV subgenomic replicon lysates were incubated with anti-FKBP38 antibody or rabbit normal IgG, the immunocomplexes were then analyzed by immunoblotting using anti-NS5A antibody, the asterisk shows the band of NS5A immunoprecipitated by anti-FKBP38 antibody. (D) HCV subgenomic replicons were transfected with pCMV/HA-FKBP51 or $pcDNA_3$/HA-FKBP38 with or without 1uM FK506, and the immunoprecipitation analysis was done as described above.

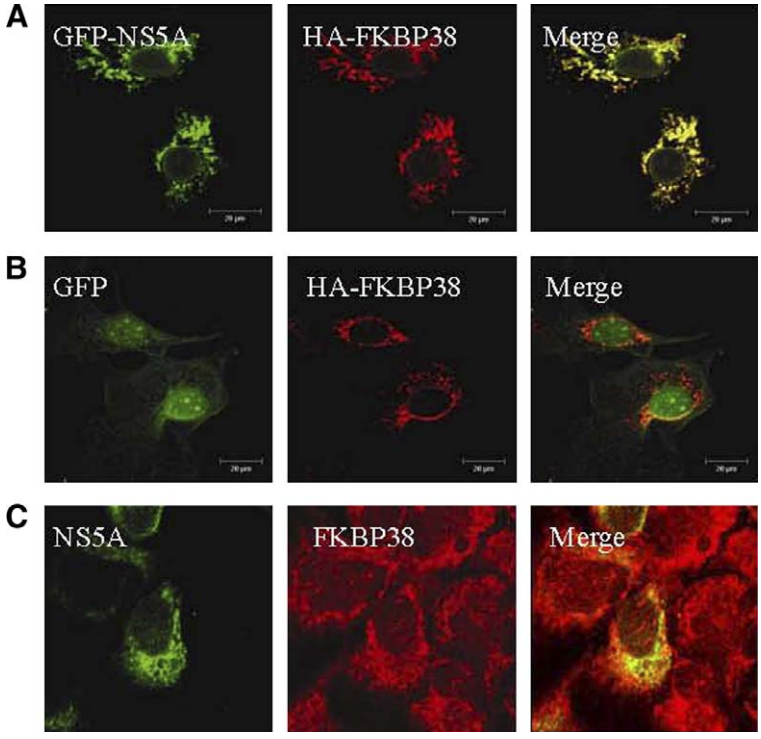

Fig. 2. Confocal microscopic analysis for the association between NS5A and FKBP38. (A and B) Cos7 cells seeded on coverslips were co-transfected with expression vectors encoding HA-FKBP38 with either GFP-NS5A (A) or GFP (control, B). Forty-eight hours post transfection, cells were fixed and immunostained with anti-HA antibody and rhodamine-conjugated secondary antibody, then observed under confocal laser scanning microscope. Images recorded in green (GFP) and red (rhodamine) channels are presented on the left and middle, respectively, and composite images are shown in the right. (C) HCV subgenomic replicons were fixed and immunostained with anti-NS5A and anti-FKBP38 antibodies and corresponding FITC or rhodamine-conjugated secondary antibodies, then cells were observed as described above.

(Fig. 1A, lane 4), indicating that NS5A could directly bind FKBP38 in vitro.

3.2. Interaction of HCV NS5A and FKBP38 in mammalian cells

We then carried out coimmunoprecipitation assay to confirm their association in mammalian cells. Cos7 cells were cotransfected with pcDNA$_3$/HA-FKBP38 and pcDNA$_{3.1}$/myc-His-NS5A plasmids or corresponding empty plasmids. Cell lysates were immunoprecipitated (IP) with anti-myc antibody, and then analyzed by immunoblotting (IB) using anti-HA antibody. As shown in Fig. 1B, HA-FKBP38 could directly associate with NS5A-myc only when both proteins were expressed. To further confirm this result, cell lysates were immunoprecipitated with reciprocal antibodies and same result

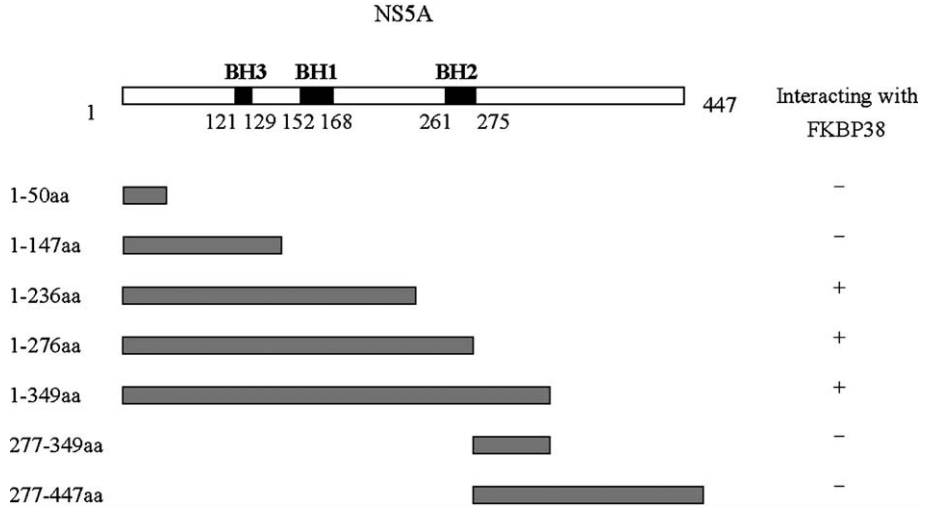

Fig. 3. Mapping of NS5A regions responsible for the association with FKBP38. Schematic representation of the full-length NS5A and three BH domains (Bcl-2 homology domain) within NS5A are indicated. The corresponding truncated mutants of NS5A were constructed and yeast co-transformation assays were performed with truncated mutants of NS5A and full-length FKBP38 plasmid, respectively. The interactions were scored positive (+) or negative (−) based on cell growth and α-galactosidase assay.

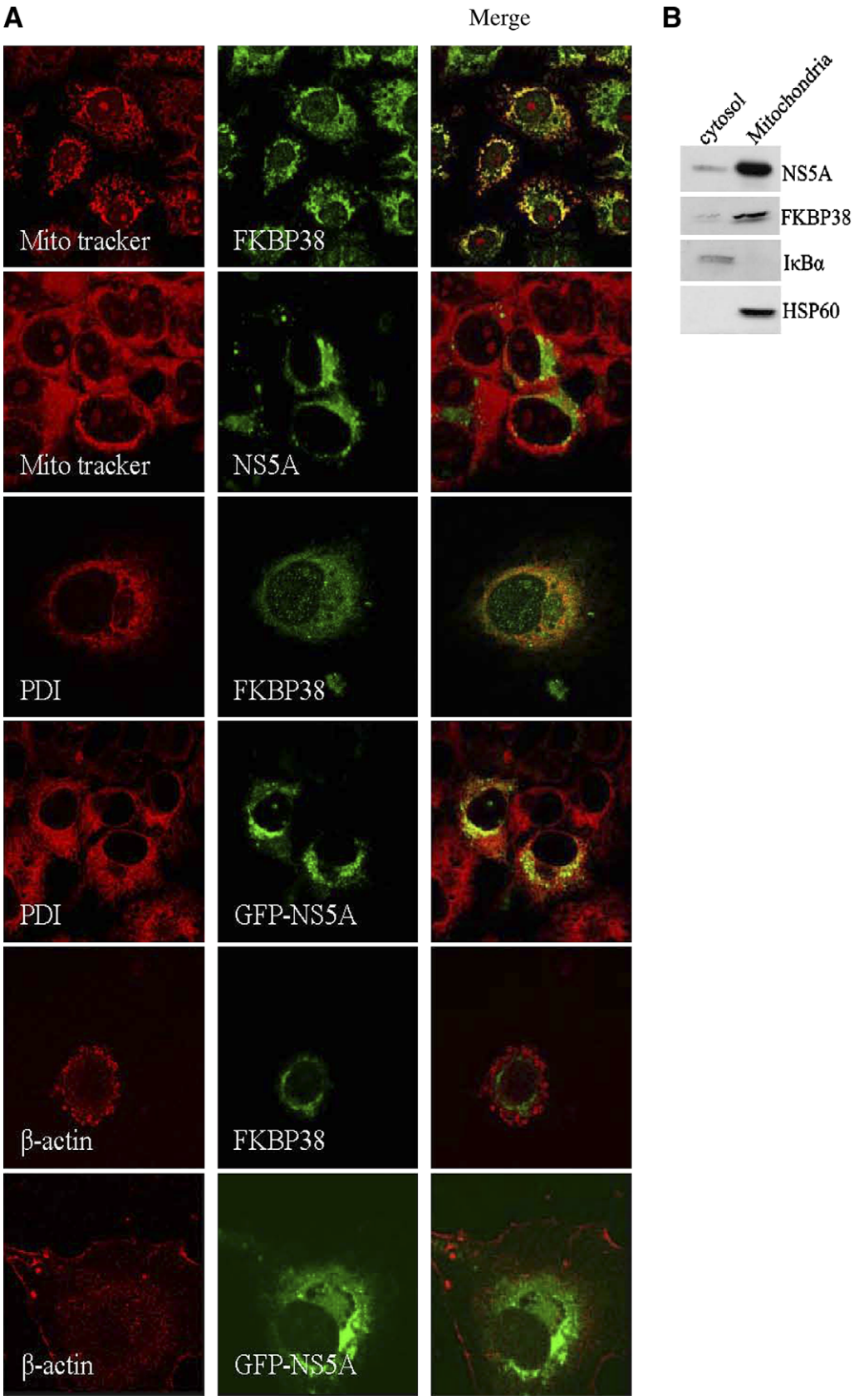

Fig. 4. Colocalization of NS5A and FKBP38 in mitochondria and endoplasmic reticulum. HCV subgenomic replicons were stained with mitochondria specific dye Mito Tracker (red) and incubated with anti-FKBP38 or anti-NS5A antibody and corresponding FITC-conjugated secondary antibodies (green), cells were observed using confocal laser scanning microscope. For endoplasmic reticulum staining, HCV subgenomic replicons were fixed and stained with anti-FKBP38 antibody and either endoplasmic reticulum specific protein disulfide isomerase (PDI) antibody or anti-β-actin antibody, or HCV subgenomic replicons were transfected with $pEGFPC_2$-NS5A and then fixed and stained with anti-PDI or anti-β-actin antibody, cells were observed as described above.

was obtained (data not shown). To detect the endogenous binding in the context of HCV replication, HCV subgenomic replicons harboring autonomously replicating HCV RNA were used. Cell lysates were immunoprecipitated with anti-FKBP38 or rabbit normal IgG and immunoblotting assay was then performed using NS5A antibody to detect NS5A protein in the immunocomplex. As shown in Fig. 1C, NS5A could directly associate with endogenous FKBP38 in HCV subgenomic replicons. Since FKBP38 was a member of FKBP subfamily and acted as an immunosuppressant FK506-binding protein, to detect the specificity of the interaction between NS5A and FKBP38, HCV subgenomic replicons were transfected with pCMV/HA-FKBP51 or $pcDNA_3$/HA-FKBP38 with or without FK506. Results showed that, there was no interaction between NS5A and FKBP51 and the association between NS5A and FKBP38 was not affected in the presence of FK506, indicating such interaction was FK506-independent (Fig. 1D). The above results suggested NS5A could specifically interact with FKBP38 in vivo.

3.3. Colocalization of HCV NS5A and FKBP38 in mammalian cells

To elucidate whether there was a colocalization between NS5A and FKBP38, Cos7 cells were transiently cotransfected with plasmids $pcDNA_3$/HA-FKBP38 and either $pEGFPC_2$-NS5A or $pEGFPC_2$. Cells were assayed by immunofluorescence staining and visualized with confocal laser scanning microscope. As shown in Fig. 2A, GFP-NS5A could completely colocalize with HA-FKBP38 in the cytoplasm (Fig. 2A, right). As a negative control, GFP protein was found to distribute in both cytoplasm and nucleus and no overlap could be detected (Fig. 2B). Further verification in HCV subgenomic replicons was done to detect their localization in the context of viral replication. Both NS5A and endogenous FKBP38 were found to localize in cytoplasm (Fig. 2C, left and middle) and merged together (Fig. 2C, right).

3.4. Mapping the interacting domain within NS5A

To map the precise NS5A domain responsible for binding FKBP38, a series of truncated mutants of NS5A were constructed and their binding activities were determined using yeast two-hybrid analysis. As shown in Fig. 3, FKBP38 interacted with the C-terminal deletion mutant of NS5A (aa 1–236), while another NS5A mutant (aa 1–147) could not, implying the NS5A segment aa 148–236 maybe important in mediating the interaction with FKBP38. It was reported that NS5A contained three Bcl-2 homology domains (BH domain) that were crucial in inhibiting apoptosis (Fig. 3, upper) [20]. Our results suggested that the amino acid residues 148–236 of NS5A containing a BH1 domain may play an important role in mediating the interaction with FKBP38.

3.5. Mitochondrial and endoplasmic reticulum localization of NS5A and FKBP38

Since FKBP38 was previously reported to localize in mitochondria while NS5A was mainly in the endoplasmic reticulum (ER), then the subcellular localization of NS5A and FKBP38 was investigated in HCV subgenomic replicons by comparing the immunostaining of mitochondria, ER specific protein disulfide isomerase (PDI) and β-actin filaments (Fig. 4A). FKBP38 was shown to be colocalized with both mitochondria and ER specific marker protein PDI of the cells, but not with the β-actin, indicating ER was also a subcellular compartment for FKBP38. More importantly, NS5A was also found to be colocalized with mitochondria. However, since the antibodies for NS5A, PDI and β-actin were all monoclonal antibodies, to perform the double-immunofluorescence staining, the HCV subgenomic replicons were transfected with $pEGFPC_2$-NS5A to detect its colocalization with PDI or β-actin. As expected, GFP-NS5A could colocalize with PDI.

To confirm the mitochondrial targeting of NS5A and FKBP38, we carried out subcellular fractionation analysis using HCV subgenomic replicons. Consistent with our immunofluorescence data, both NS5A and endogenous FKBP38 were mainly retained in the mitochondrial fraction containing the mitochondrial protein heat shock protein 60 (HSP60), and little was detected in the cytosolic fraction containing IκBα (Fig. 4B).

3.6. Expression of NS5A in Huh7 hepatoma cells inhibits apoptosis

Since NS5A contained three BH domains and localized in mitochondria while FKBP38 was reported to target Bcl-2 into mitochondria and inhibit apoptosis, to assess the capability of NS5A in regulating apoptosis and exclude the impacts of Bcl-2, we constructed NS5A stably expressing Huh7 hepatoma cells in which Bcl-2 expression was deficient (Fig. 5A). We then chose two NS5A stable cell lines (5AC1 and 5AC2) and one control cell line (Vector) for further investigation (Fig. 5B). Cells were induced with pro-apoptotic drugs staurosporine and cycloheximide, respectively. In our experimental conditions, NS5A stable cells showed more resistance to apoptosis compared to vector cells and cells were more sensitive to

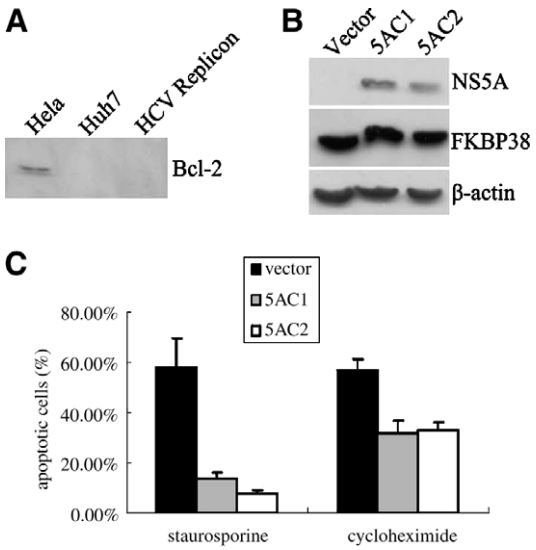

Fig. 5. Anti-apoptotic action of NS5A in Huh7 hepatoma cells. (A) Expression of Bcl-2 in Hela, Huh7 and HCV subgenomic replicons. (B) Characterization of NS5A stably expressing Huh7 cells. Two NS5A stable cell lines (5AC1 and 5AC2) and control cell line (Vector) were lysed and expression of NS5A and FKBP38 was detected by immunoblotting, the content of β-actin as internal control. (C) NS5A stably expressing Huh7 cell lines and its control cell line were deprived of serum for 4 h and then stimulated with staurosporine (1 μM) or cycloheximide (100 μg/ml), respectively. The percentage of apoptotic cells was determined with flow cytometer.

staurosporine than cycloheximide (Fig. 5C). These results showed the function of NS5A in inhibiting apoptosis.

3.7. The anti-apoptosis function of NS5A is through the interaction with FKBP38

In order to investigate whether such anti-apoptotic property of NS5A was through the interaction with FKBP38, RNA interference was carried out to inhibit the expression of endogenous FKBP38. As seen in Fig. 6A, two RNAi sequences (siRNA3 and siRNA4) could significantly suppress FKBP38 expression. We then chose one NS5A stable cell line (5AC2) and one RNAi sequence (siRNA3) for the next experiments. After cells were transfected with siFKBP38 and treated with staurosporine, there were an increasing number of apoptotic cells in NS5A stable cells compared to the cells treated with control siRNA. More importantly, almost no differences could be detected in vector cells treated with siFKBP38 or control siRNA (Fig. 6B). DAPI staining also showed that more condensed or fragmented nuclei could be observed in NS5A stable cells treated with siFKBP38 (Fig. 6C). To further confirm the above results, we also detected the cleavage of PARP products which could be cleaved by activated Caspase-3 after cells undergoing apoptosis. Results showed that more cleaved PARP products (85 kDa) could be detected after treatment of siFKBP38 in NS5A stable cells, while no differences could be seen in vector cells (Fig. 6D). Taken together, these results suggested NS5A could specifically inhibit apoptosis by interaction with FKBP38.

4. Discussion

HCV infection often leads to chronic hepatitis, liver cirrhosis and HCC. The mechanism for HCV persistent infection is still unclear. Among different antiviral defense systems developed by the cell, the programmed cell death, or apoptosis, significantly contributes to clear the virus infected cells. To survive, viruses elaborate multiple protective strategies to interfere apoptosis at different levels. The anti-apoptotic property of NS5A has been widely investigated. NS5A can protect against the tumor necrosis factor alpha-mediated apoptotic cell death [17,18], interact with Bin1 and inhibit apoptosis [22]. It can also directly interact with p53 and inhibit p53-mediated apoptosis by sequestering p53 in cytoplasm [19]. NS5A was also demonstrated to form a complex with phosphatidylinositol 3-kinase in EGF signaling pathway, enhance the PI3K-AKT pathway and contribute to cell survival in virus-infected cells [21]. Recently, it was also reported that NS5A contained three

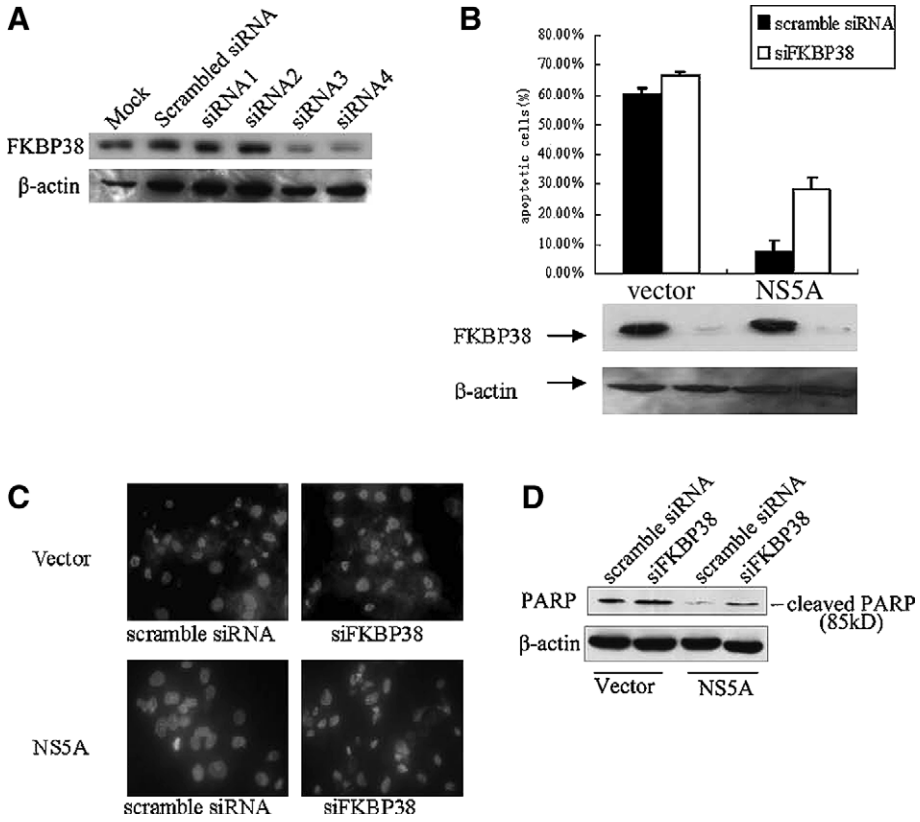

Fig. 6. Inhibition of FKBP38 specifically restores apoptosis in NS5A stably expressing Huh7 hepatoma cells. (A) The expression of endogenous FKBP38 was suppressed by RNA interference. Cells were transfected with water (Mock), scramble control siRNA or four candidate siRNA$_s$ targeting FKBP38. The content of FKBP38 was determined by immunoblotting. The expression of β-actin was determined as an internal control. The inhibition efficiency was determined by three independent experiments. (B) After NS5A stably expressing Huh7 cells and vector cells were transfected with scramble siRNA or siFKBP38 for 48 h, cells were incubated with staurosporine for 8 h and the percentage of apoptotic cells was counted using FACS by propidium iodide staining (upper), the inhibitory efficiency of FKBP38 was determined by immunoblotting, β-actin as internal control (lower). (C and D) Cells described above were also stained with DAPI to observe the condensation of nucleus (C) or cell lysates were immunoblotted to determine the cleavage products of PARP (D).

BH domains and the BH2 domain of NS5A were required to bind Bax and inhibit apoptosis by sequestering it in the nucleus [20]. Although these evidences give some clues for the role of NS5A in mediating apoptosis, the overall anti-apoptosis mechanism developed by NS5A is still unknown. It is presumed that different pathways are involved.

FKBP38 belongs to a family of immunosuppressant FK506-binding proteins (FKBPs), which is a subfamily of a super family of peptidylprolyl *cis*/*trans* isomerases (PPIase). PPIase is known to be involved in many cellular processes such as cell signaling, protein trafficking and transcription [28,29]. So far little has been known about its function in HCV infection until very recently another subfamily member cyclophilin B has been shown to interact with NS5B and regulate viral replication [30]. To date, seven mammalian FKBPs have been found in human. However, little has been known about their role in mediating apoptosis except FKBP38. In our work, we also tested the interaction between NS5A and other FKBP member such as FKBP51. However, no interaction was found, suggesting the association between FKBP38 and NS5A maybe specific.

It is interesting that NS5A also contains three Bcl-2 homologies, whereas FKBP38 has been demonstrated to associate with Bcl-2 and exert its anti-apoptosis function by targeting Bcl-2 on the outer-membrane of mitochondria. The NS5A interacting domain was also located on the region of aa 148–236 containing a BH1 domain. There have been a great number of evidences showing that many viruses such as herpesvirus saimiri (HVS), Epstein Barr virus, etc. have been found to employ BH domains to evade apoptosis during infection. By using BH domains these viral proteins could interact with pro-apoptotic Bcl-2 members such as Bax to inhibit apoptosis [26,27]. Our results imply HCV may also adopt similar strategy to evade apoptosis and establish persistent viral infection.

In addition to BH domains within NS5A, the subcellular compartment staining also defines that both NS5A and FKBP38 localize in the mitochondria and ER. The result of ER localization of FKBP38 is consistent with the very recent reports [31,32]. More importantly, it was shown that, besides ER, NS5A could also reside in the mitochondria. It should be noted that most other viral proteins could localize in the mitochondria and exert their functions. For example, the X protein of hepatitis B virus (HBx) localizes in mitochondria and leads to the alteration of transmembrane potential [33]. Moreover, other viral proteins such as vMIA protein of cytomegalovirus, Kaposi's sarcoma-associated herpesvirus mitochondrial K7 protein, Vpr protein from human immunodeficiency virus-1, all localize in the mitochondria and exert their pro- or anti-apoptotic functions [34]. Considering that Bcl-2 expression is deficient in liver tumor cells and suppression of FKBP38 in NS5A stable Huh7 hepatoma cells could result in the increasing number of apoptotic cells while vector cells could not, we speculate that NS5A may mimic the function of Bcl-2 and thus inhibit apoptosis.

In summary, we have found a novel interaction between NS5A and FKBP38 and their mitochondrial and ER localization. More importantly, such interaction implies a new mechanism for NS5A to evade apoptosis. However, the exact mechanism downstream the interaction need to be further elucidated.

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