Heat Shock Protein 72 Is Associated with the Hepatitis C Virus Replicase Complex and Enhances Viral RNA Replication*

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The NS5A protein of the hepatitis C virus (HCV) is an integral component of the viral replicase. It also modulates cellular signaling and perturbs host interferon responses. The multifunctional characteristics of NS5A are mostly attributed to its ability to interact with various cellular proteins. This study aimed to identify the novel cellular factors that interact with NS5A and decipher the significance of this interaction in viral replication. The NS5A-interacting proteins were purified by the tandem affinity purification (TAP) procedure from cells expressing NS5A and identified by mass spectrometry. The chaperone protein Hsp72 was identified herein. In vivo protein-protein interaction was verified by co-immunoprecipitation and an in situ proximity ligation assay. In addition to NS5A, Hsp72 was also associated with other members of the replicase complex, NS3 and NS5B, suggesting that it might be directly involved in the HCV replication complex. Hsp72 plays a positive regulatory role in HCV RNA replication by increasing levels of the replicase complex, which was attributed either to the increased stability of the viral proteins in the replicase complex or to the enhanced translational activity of the internal ribosome entry site of HCV. The fact that the host chaperone protein Hsp72 is involved in HCV RNA replication may represent a therapeutic target for controlling virus production.

The hepatitis C virus (HCV), a hepativirus in the Flaviviridae family, is the leading cause of acute and chronic hepatitis worldwide (1, 2). Currently, around 170 million individuals are chronically infected with HCV. HCV infection often leads to liver cirrhosis and hepatocellular carcinoma (1–3). The replication of HCV is entirely cytoplasmic. The HCV lifecycle starts when enveloped virus particles attach to the cell membrane and interact with specific surface receptor(s). After internalization and membrane fusion in an endosome, the HCV genome is released into the cytoplasm. This genome serves not only as the messenger RNA for the translation of viral proteins but also as the template for viral RNA replication. All viral proteins are directly or indirectly associated with the endoplasmic reticulum (ER) membrane, where replication and assembly take place. The HCV non-structural proteins, NS3/NS4A, NS4B, NS5A, and NS5B, and likely several host-derived factors function as a replicase complex, which executes the replication process. Once the newly synthesized nascent RNA is packaged in the particle, the virion forms by budding into the ER and leaves the cell through the secretory pathway (4–7).

Among the HCV viral proteins, NS5A has been demonstrated to be multi-functional, which supports the replication and survival of HCV. The requirement for NS5A in HCV RNA replication has been supported by numerous lines of evidence (8–17). The other important functions of NS5A lie in its potential to perturb the interferon responses and modulate the signaling pathways of host cells; those are mostly dependent on its interaction with cellular molecules (11, 18–26).

The heat shock protein 70 (Hsp70) family consists of a number of homologous chaperone proteins: Hsp70–1t, -2, -5, -6, -9, Hsp72, and Hsc70, which differ from each other in amino acid sequence, expression levels and subcellular localization (27). All of the Hsp70 proteins have two conserved domains, the ATPase domain and the substrate-binding domain, and function involves a tightly controlled conformational shift between these two domains. ATPase activity is critical for the functions of all Hsp70s, and the residue essential for ATPase catalytic activity has been mapped to Lys-71 (28). The EEVD motif located at the C terminus of Hsp70 is responsible for interactions with other chaperones or co-chaperones (27, 29).

Hsp70 proteins possess a wide range of housekeeping functions, including folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of secretory proteins, and control of the activity of regulatory proteins (29). In this study, we aimed to find novel cellular molecules that interact with NS5A and influence viral RNA replication. Hsp72 was identified as a target by the tandem affinity purification (TAP) procedure. The interaction of Hsp72 with various viruses has been previously reported to play a role in many aspects of the viral lifecycle, including cell entry, genome replication, viral gene expression, viral protein folding, virion assembly, and even virus-induced cell transformation (30). Here we demonstrate that Hsp72 positively regulates HCV replication by increasing levels of the HCV replicase complex.

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‡ The abbreviations used are: HCV, hepatitis C virus; HSC, heat shock cognate protein; Hsp, heat shock protein; IP, immunoprecipitation; PLA, proximity ligation assay; TAP, tandem affinity purification.
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**EXPERIMENTAL PROCEDURES**

*Cell Lines—* Human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mmol/liter L-glutamine, 1 IU/ml penicillin G, and 1 μg/ml streptomycin. Huh7.5.1 and its derivative P1, which stably carries the HCV subgenomic replicon pSGR-JFH1, were maintained in the above culture medium with the addition of non-essential amino acids.

*Constructs—* A DNA fragment encoding NS3/NS4A, NS4B, NS5A, or NS5B of HCV genotype 1b (NCBI Accession No. gi:329763) was cloned into the pFlag-CMV2 vector with the Flag tag sequence fused in-frame at the 5’-end of the inserted gene. The NS5A deletion clones were constructed as previously described (31). To construct the N-TAP-Flag:NS5A- and the N-TAP-Flag:NS4B-expressing plasmids, the TAP tag fragment was first amplified by polymerase chain reaction (PCR) from plasmid pZome1-N (EUROSCARF, Frankfurt, Germany) and then fused in-frame to the 5’-end of the Flag-NS5A or Flag-NS4B sequences. The sequence frame and the expression of TAP-fused in-frame to the 5’/H11032pZome1-N (EUROSCARF, Frankfurt, Germany) and then amplified by polymerase chain reaction (PCR) from plasmid (31). To construct the N-TAP-Flag-NS5A- and the N-TAP-Flag-NS4B protein were confirmed by DNA sequencing and Western blot analysis, respectively. The wild-type Hsp72 gene, obtained by reverse transcription (RT)-PCR from the total RNA of 293T cells, was cloned into the pcDNA3 vector with a Myc tag at the C terminus. The ATPase catalytic site mutant (K71A) was constructed by the site-directed mutagenesis method described previously (32). The mutant clone was confirmed using DNA sequencing.

*Purification of NS5A-interacting Cellular Proteins—* 293T cells (3 × 10⁵) were transfected with 100 μg of the TAP-Flag-NS5A DNA, TAP-Flag-NS4B DNA, or the TAP vector DNA. At 48 h after transfection, cells were harvested with TAP lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.2% Nonidet P-40, 1 mM DTT), IPP300 buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM DTT), and Tobacco Etch Virus (TEV) protease cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM EDTA), IPP300 buffer (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.1% Nonidet P-40 and 1 mM DTT), and Tobacco Etch Virus (TEV) protease cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, and 1 mM DTT). The cell lysate was incubated with IgG Sepharose (GE Healthcare) at 4 °C for 2 h. The Sepharose was washed sequentially with IPP150 buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM DTT), IPP300 buffer (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.1% Nonidet P-40 and 1 mM DTT), and Tobacco Etch Virus (TEV) protease cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, and 1 mM DTT). The Sepharose was mixed with AcTEV protease (Invitrogen) in TEV protease cleavage buffer at 4 °C for 18 h. The supernatant was collected and then incubated with calmodulin Sepharose (GE Healthcare) in calmodulin-binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM MgOAc, 1 mM imidazole, 2 mM CaCl₂, and 1 mM β-mercaptoethanol) at 4 °C for 4 h. The calmodulin Sepharose was washed three times with binding buffer, and 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 0.02% (w/v) bromphenol blue) was then added to the Sepharose beads. After boiling, the supernatant was subjected to SDS-PAGE analysis. The protein bands were visualized by silver staining following the manufacturer’s instructions (PlusOne Silver Staining Kit, GE Healthcare) and cut into slices for further protein identification by LC-MS/MS.

*Antibodies—* The antibodies used in this study were as follows: mouse anti-NS5A monoclonal antibody (Austral Biologicals), rabbit anti-NS3 polyclonal antibody (self-prepared), rabbit anti-NS5B polyclonal antibody (Santa Cruz Biotechnology), mouse anti-Flag M2 monoclonal antibody (Sigma), mouse anti-Myc monoclonal antibody (LTK BioLaboratories, Tao-yuan, Taiwan), rabbit anti-Hsp72 polyclonal antibody (Stressgen), mouse anti-β-actin monoclonal antibody (Sigma), TrueBlot Ultra horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (eBioscience), HRP-conjugated sheep anti-mouse IgG secondary antibody (GE Healthcare), and goat anti-rabbit HRP secondary antibody (DAKO, Denmark).

*Co-immunoprecipitation—* 293T cells (4.5 × 10⁶) were co-transfected with Hsp72-myc DNA and the genes coding for the Flag-tagged viral antigens. At 48 h after transfection, cells were harvested using RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, 50 mM leupeptin, and 50 μg/ml aprotinin). The cell lysate was first treated with RNase A (50 μg/ml) or vehicle at 4 °C for 30 min, followed by incubation with the indicated antibody for 16 h. Protein G Sepharose beads (Sigma) were added and then incubated for another 1 h. The Sepharose was then sequentially washed with high-salt buffer (1 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.2% Nonidet P-40), low-salt buffer (0.1 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.2% Nonidet P-40, and 1 mM EDTA), RIP A buffer, and wash buffer (50 mM NaCl, 25 mM Tris-HCl, pH 8.0, and 0.2% Nonidet P-40). The Sepharose pellet was resuspended in 2× SDS sample buffer. After boiling, the supernatant was subjected to SDS-PAGE analysis.

*Transfection of HCV Replicon RNA and Replication Analysis—* The HCV subgenomic replicon pSGR-JFH1 was transcribed in vitro using the T7 MEGAscript kit (Ambion) following the manufacturer’s instructions. A total of 3 μg of the in vitro transcribed HCV replicon RNA were transfected into Huh7.5.1 cells using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, the culture medium was replaced with fresh medium containing G418 (0.8 μg/ml). One of the selected clones was named P1. Quantification of HCV RNA in the replicon cells was performed by quantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a normalization control.

*Lentivirus-mediated Hsp72 Knockdown or Overexpression—* The lentiviral clone expressing short hairpin RNA (shRNA) targeting human Hsp72 was purchased from the National RNAi Core Facility, Academia Sinica, Taiwan. The lentiviral particles carrying the shRNA or the wild-type or mutant form of Hsp72 cDNA were produced by transfecting the constructs along with the packaging plasmid pCMV-ΔR8.91 and the envelope plasmid pMD.G into 293T cells using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, the medium was replaced with DMEM containing 5% FCS. The viral particles in the culture medium were harvested 48 h later and stored at −80 °C until use. The knockdown or overexpression experiments were performed as follows. P1 cells or Huh7.5.1 cells (4 × 10⁵/well) were seeded in a 12-well plate. After 24 h, 50% of the medium in each well was removed and polybrene was added.
to a final concentration of 8 μg/ml. The shRNA- or cDNA-expressing lentiviruses (MOI = 1) were added to the cells. After an overnight incubation at 37 °C, the medium was replaced with fresh medium and incubated for another 72 h. The cells were then used to evaluate the expression of Hsp72 or for further experiments.

In Situ Proximity Ligation Assay—Cells on coverslips were fixed with 1% formaldehyde for 5 min followed by permeabilization with 0.5% Triton X-100 in PBS (PBST) for 3 min. Cells were then incubated in blocking solution for 30 min at 37 °C. After blocking, two different primary antibodies from different hosts (mouse anti-NS5A antibody (1:400 dilution) and rabbit anti-NS3 (1:1,200 dilution) or rabbit anti-Hsp72 antibody (1:100 dilution)) were incubated with the cells overnight at 4 °C, and the cells were then washed three times with PBST. To detect primary antibodies with the in situ proximity ligation assay (PLA), the PLA probes mouse PLUS and rabbit MINUS (Olink Bioscience, Sweden) were added at a 1:5 dilution in antibody dilution buffer (Olink Bioscience, Sweden) for 60 min at 37 °C. After washing the coverslips with PBST three times, the probe was detected using in situ PLA detection kit 613 (Olink Bioscience) according to the manufacturer’s instructions. Images of cells were acquired with an Olympus LSM Fluoview FV1000 confocal laser scanning microscope (Olympus) and then analyzed by the image analysis software Blob Finder V3.2, which automatically counts the number of spots per cell.

Internal Ribosome Entry Site (IRES) Reporter Assays—293T cells were infected with lentiviral vector expressing wild-type Hsp72-myc or shRNA targeting Hsp72 at an MOI of 5. Three days later, cells were co-transfected with control vector DNA or pFlag-NS5A DNA along with a bicistronic reporter construct which contains a Renilla luciferase (RLuc) at the first cistron and a Firefly luciferase (FLuc) at the second cistron. The translation of RLuc is cap-dependent, whereas the translation of FLuc is HCV IRES-dependent. Two days after transfection, cell lysates were harvested and analyzed for activity levels of both luciferases using the Dual-Glo Luciferase Assay System (Promega).

RESULTS

HSC70 and Hsp72 Are NS5A-interacting Cellular Proteins—To identify novel cellular factors interacting with HCV NS5A, we used the TAP method to purify proteins that interact with NS5A in vivo (33). The construct used for TAP purification contained three tags in tandem at the N terminus: protein A, the TEV protease cleavage site, and the calmodulin-binding protein. The NS5A coding sequences were fused to the C termini of the tags. After first purifying with IgG Sepharose, cleaving with TEV protease, and then further purifying with calmodulin Sepharose, the NS5A-interacting proteins were separated from other lysate proteins. The purified proteins were analyzed by SDS-PAGE and visualized using silver staining. The empty vector and DNA expressing TAP-Flag-NS4B were used as purification controls. Results from several independent experiments all indicated that in addition to the highly abundant Flag-NS5A protein, indicated by the arrowhead and asterisks in Fig. 1A (representing the full-length and the degraded forms of Flag-NS5A, respectively, as proven by the Western blot analysis shown in Fig. 1B), two cellular proteins with molecular masses of about 70 kDa (bands a and b) were reproducibly co-purified at much higher levels than the others (Fig. 1A, lane 2). LC-MS/MS analysis consistently indicated that the upper band had a high hit score matching heat shock cognate protein 70 (HSC70) and that the lower band matched heat shock protein 72 (Hsp72). The TAP-Flag-NS4B protein also had a similar but much weaker association (lane 3), whereas the ∼19 kDa protein encoded from the empty vector did not have any association with HSC70 or Hsp72 (lane 1), although it also contained the TAP sequences. These results suggested that HSC70 and Hsp72 may specifically interact with HCV viral proteins.

Both HSC70 and Hsp72 are Hsp70 chaperone proteins. They share 85% homology in terms of amino acid sequences. HSC70 is a constitutively expressed chaperon protein whereas Hsp72 is a stress-inducible protein and can be induced by various stresses, such as heat shock, UV radiation and osmotic stress. HSC70 has previously been demonstrated to be part of the HCV viral particles and plays roles in the budding and entry processes of the HCV lifecycle, but has no effects on intracellular HCV RNA replication (34). Hsp72 (or Hsp70–1) is a general name for Hsp70–1a and Hsp70–1b which are encoded by two cross-linked genes, HSPA1A and HSPA1B, respectively (35, 36). These two proteins share all but 2 of 641 amino acids; therefore, they are more than 99% identical. In eukaryotic cells, Hsp72 is found in virtually all cellular compartments under normal conditions. This study elected to further investigate the function of Hsp72 in HCV RNA replication.


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**A**

| Hsp72-myc: | Flag-NS5A: |
|------------|------------|
| +          | +          |
| -          | +          |
| -          | -          |

IP: anti-Flag

WB: anti-Myc

**B**

Lysate WB: anti-Myc

Lysate WB: anti-Flag

**C**

Lysate WB: anti-Myc

Lysate WB: anti-β-actin

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**FIGURE 2. In vivo interaction between NS5A and Hsp72.** 293T cells were co-transfected with pFlag-NS5A DNA and total cell lysates were harvested 48 h after transfection for co-IP assays. A, IP was performed using an anti-Flag antibody, and the precipitates were analyzed by Western blotting using an anti-Myc antibody. B, IP was performed using an anti-Myc antibody, and the precipitates were analyzed by Western blotting using an anti-Flag antibody. C, interaction of NS5A with endogenous Hsp72. 293T cells were transfected with vector or pFlag-NS5A DNA. Proteins immunoprecipitated with an anti-Flag antibody were subjected to Western blotting using an anti-Hsp72 antibody. The expression of Hsp72-myc and Flag-NS5A in the input lysates is shown.

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**The Middle Region of NS5A Is Required for Protein-Protein Interaction**—To verify the interaction between NS5A and Hsp72, co-immunoprecipitation (co-IP) experiments were performed on 293T cells co-expressing Flag-NS5A and Hsp72-myc. As shown in Fig. 2A, the anti-Flag immunoprecipitates pulled down Hsp72-myc. Reciprocal co-IP experiments also showed that the anti-Myc antibody precipitated Flag-NS5A (Fig. 2B). Consistent with these results, endogenous Hsp72 was co-immunoprecipitated from 293T cells that only expressed Flag-NS5A using the anti-Flag antibody (Fig. 2C).

The regions of NS5A required for the interaction with Hsp72 were further investigated. Eight deletion mutants of NS5A were previously constructed, as illustrated in Fig. 3A (31). Each of them was co-transfected with Hsp72-myc DNA into 293T cells, and the interaction of Hsp72 and NS5As was determined using a co-IP assay. In cells containing mutants D3, D4, and D5, the interaction was significantly reduced (Fig. 3B), indicating that the middle region of NS5A, from amino acids 221 to 302, was required for the interaction with Hsp72.

**Hsp72 Has A Positive Regulatory Role in HCV Replication**—To understand whether the NS5A-Hsp72 interaction is involved in HCV replication, the impact of Hsp72 overexpression or knockdown on HCV replication was studied in an HCV subgenomic replicon cell line (P1) by measuring HCV RNA (Fig. 4A) and protein levels (Fig. 4B). When Hsp72 was overexpressed, levels of HCV RNA and proteins were all significantly increased compared with levels observed in the control replicon cells (lane 4 versus lane 2). Conversely, Hsp72 knockdown markedly reduced HCV replication (lane 3 versus lane 2).

expression of the K71A mutant, which had a mutation at the catalytic site, Lys-71, also significantly reduced HCV replication (lane 5), suggesting a potential dominant negative property of the mutant protein. Collectively, these results demonstrate that Hsp72 may function as a positive regulator of HCV replication and that the ATPase activity of Hsp72 is essential for this regulation.

**Hsp72 Interacts with Multiple Components of the HCV Replicase Complex**—HCV RNA replication is carried out by the replicase complex, which is mainly composed of HCV non-structural proteins and some host proteins. Given that Hsp72 interacts with NS5A and up-regulates HCV replication, we proposed that Hsp72 might participate in the replicase complex. Therefore, we investigated whether Hsp72 also interacted with other replicase proteins. First, Flag-tagged NS3/NS4A, NS4B, or NS5B was co-expressed with Hsp72-myc in 293T cells. Anti-Flag and anti-Myc antibodies were used to perform co-IP and reciprocal co-IP experiments, respectively. In addition to NS5A, both NS3 and NS5B were also found to form complexes with Hsp72-myc (Fig. 5A). The interaction between Hsp72 and NS4B was too weak to be seen, which seemed contradictory to results obtained using the TAP purification procedure (Fig. 1A, lane 3). We reasoned that the conditions necessary for successful use of the RIPA buffer in co-IP assays could be more stringent than those required for use of the TAP buffer. Therefore, the RIPA buffer, but not the TAP buffer, was able to dissociate the weak interaction between Flag-NS4B and Hsp72. The interaction between Hsp72 and HCV viral proteins was not mediated by RNA, as RNase A treatment prior to the co-IP assays did not diminish the interaction (Fig. 5A). The interaction between Hsp72 and NS3/NS4A, NS5A, or NS5B was further examined in HCV replicon cells that over-expressed Hsp72-myc. NS3, NS5A, and NS5B were detected in the anti-Myc precipitates, and the interaction was also independent of the presence of RNA (Fig. 5B, lane 3). These data strongly suggest that Hsp72 is directly associated with the HCV replicase complex in replicon cells.
**Hsp72 Enhances HCV Replication by Increasing Levels of the Replicase Complex**—We speculated that Hsp72 might enhance HCV replication by stabilizing and concurrently increasing the amount of replicase complex. To prove this notion, anti-NS5A was used to pull down the NS5A-containing replicase complex from P1 replicon cells in which Hsp72 was either knocked down or overexpressed. As shown in Fig. 6A, overexpression of Hsp72 significantly increased the amounts of NS3 and NS5B complexed with NS5A (lane 4 versus lane 2), whereas Hsp72 knockdown diminished the amounts of replicase complex (lane 3).

We also used a newly developed technique, in situ proximity ligation assay (PLA), to detect the amounts of the replicase complex in vivo. The protein-protein interaction was sensitively and specifically demonstrated with pairs of proximity probes, which can be detected by rolling circular amplification in situ (37). First, the interaction of Hsp72 and NS5A, shown as the red fluorescent dots in Fig. 6B, was confirmed by PLA with a pair of rabbit anti-Hsp72 and mouse anti-NS5A antibodies.

Next, the interaction between NS3 and NS5A, representing the formation of the replicase complex, was investigated under conditions of Hsp72 overexpression or knockdown. P1 replicon cells were first treated with RNase A (50 μg/ml) before the co-IP experiments. Degradation of cellular RNA was confirmed by RNA gel analysis (data not shown). The expression of the Flag-tagged non-structural genes and Hsp72-myc in the input lysates is shown.

**Hsp72 Increases the Stability of Viral Proteins and Enhances HCV IRES Activity**—The Hsp70 family of proteins modulates the maturation and functions of their substrates through their interactions with other cellular factors. In this study, we found that Hsp72 enhances HCV replication by increasing the stability of viral proteins and improving the activity of the IRES.

![Figure 4](image-url) **Hsp72 enhances HCV replication.** The effects of Hsp72 on HCV RNA replication were determined using either quantitative RT-PCR (A) or Western blotting (B). P1 cells, which stably carry the HCV subgenomic replicon, were infected with control lentiviral vector, the lentiviral vector expressing wild-type or K71A mutant Hsp72, or the lentiviral vector expressing shRNA targeting Hsp72, at an MOI of 1. Cellular RNA and lysates were harvested 72 h after lentiviral infection and analyzed by qRT-PCR or Western blotting, respectively. Interferon-α (500 IU/ml) treatment was used as a control that completely abolished HCV RNA replication.

![Figure 5](image-url) **Interaction of Hsp72 with other members of the HCV replicase complex.** A, interaction of Hsp72 with HCV non-structural proteins. 293T cells were co-transfected with pHsp72-myc DNA and vector or Flag-tagged NS3/NS4A, NS4B, NS5A, or NS5B DNA. Cell lysates were harvested 48 h after transfection for co-IP experiments with anti-Flag and anti-Myc antibodies, respectively. In one set of experiment, cell lysates were first treated with RNase A (50 μg/ml) before the co-IP experiments. Degradation of cellular RNA was confirmed by RNA gel analysis (data not shown). The expression of the Flag-tagged non-structural genes and Hsp72-myc in the input lysates is shown. B, interaction of Hsp72 with HCV non-structural proteins in HCV replicon cells. P1 cells were infected with the lentiviral vector expressing wild-type pHsp72-myc at an MOI of 1. Cell lysates were harvested 72 h after lentiviral infection for co-IP experiments with an anti-Myc antibody. The precipitates were subjected to Western blotting with an anti-NS3, anti-NS5A, or anti-Hsp72 antibody. RNase A means that the lysates were treated with RNase A prior to the co-IP experiments.
chaperone activities. Therefore, suppression of Hsp expression may decrease the stability of their substrates, leading to proteasomal degradation. To determine whether this is how Hsp72 acts on HCV viral proteins, we added the proteasomal inhibitor, MG132, to the P1 replicon cells in which Hsp72 was knocked down. Discernible recovery of the NS3, NS5A, and NS5B proteins was indeed observed (Fig. 7A). These data suggested that Hsp72 could increase levels of replicase complex via chaperone activity, thereby stabilizing the complex.

Because Hsp72 strongly interacts with NS5A, and NS5A has been reported to stimulate HCV IRES activity (38), we sought to determine whether Hsp72 could increase levels of the replicase complex by enhancing HCV IRES activity. Bicistronic reporter DNA, in which RLuc was translated by the cap-dependent mechanism and FLuc was co-transfected with or without pFlag-NS5A DNA into the P1 replicon cells under conditions of Hsp72 overexpression or knockdown. As shown in Fig. 7B, in the absence of NS5A (white bars), knockdown of Hsp72 reduced HCV IRES activity to 0.63-fold relative to the control lentivirus-transduced cells (p < 0.01), whereas overexpression of Hsp72 increased this factor 1.29-fold (p < 0.05). The results indicated that Hsp72 per se could enhance HCV IRES activity. Moreover, in the control lentivirus-transduced cells HCV NS5A also stimulated HCV IRES activity by 1.3-fold in comparison to cells without NS5A. Addition of NS5A to the cells overexpressing Hsp72 further enhanced IRES activity to 1.8-fold of control levels, but the stimulatory effects of NS5A were completely abolished when Hsp72 was knocked down. The results suggest that the stimulatory effect of NS5A on HCV IRES activity was Hsp72-dependent.

Together, these results demonstrated that Hsp72 could enhance HCV RNA replication by increasing levels of the replicase complex, which could be mediated by the increased stability of viral proteins in the complex or by the enhanced translational efficiency of HCV IRES, leading to the production of more viral proteins.

DISCUSSION

The formation of a membrane-associated replicase complex composed of replicating viral RNA, viral proteins, altered cellular membranes, and some cellular proteins is a hallmark of all positive-stranded RNA viruses. In the case of HCV, replication likely takes place in an ER-derived membrane alteration referred to as the “membranous web” (4–7). The NS3/NS4A, NS4B, NS5A, and NS5B proteins of HCV constitute the viral portion of the replicase complex and are necessary and sufficient for HCV RNA replication in the cell, whereas the identities of the cellular proteins in the HCV replicase complex remained to be determined. With the improvement in proteomic exploration, increasing numbers of cellular pro-
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In this study, we used the TAP method to purify NS5-interacting proteins. The method has been described as an efficient protocol to purify intracellular protein complexes (33). The tandem tags present in the fusion protein allow for stepwise purification, which improves specificity greatly in comparison to the results obtained using the traditional single tag method. Using this method, HSC70 and Hsp72 were reproducibly identified as targets interacting with NS5A (and weakly with NS4B). Previous study has demonstrated that HSC70 is present in HCV viral particles. HSC70 modulates HCV infectivity and virus release but does not affect HCV RNA replication (34). Thus, it remains to be determined whether the interaction of HCV non-structural proteins with HSC70 has anything to do with the formation of viral particles. On the other hand, this study demonstrated that Hsp72 significantly increased HCV RNA replication. The results were consistent with a recent study by Gonzalez et al. (47) who demonstrated that knockdown of Hsp72 expression by siRNA or treating replicon cells with a heat shock protein inhibitor, Quercetin, reduced HCV RNA replication. All these results indicated that the Hsp70 family of proteins could participate in multiple steps of the HCV lifecycle.

Chaperone proteins are highly abundant in the cell and control cellular protein quality. These proteins are involved in protein translation, folding, trafficking, and degradation (48). However, they are also utilized by various viruses to enhance viral replication. In the course of viral proliferation, a large number of viral proteins are synthesized in a relatively short period of time, during which protein folding can become a limiting step. Therefore, most viruses may need cellular chaperones to circumvent this limitation. Among chaperone proteins, Hsp90 has been implicated in HCV replication. Hsp90 is thought to enhance the maturation of the NS2/NS3 (40, 49) and to form a complex with FKBP8 and NS5A to participate in HCV replication (44). This study demonstrated that Hsp72 chaperone activity could also increase levels of the replicase complex, leading to enhanced viral RNA replication. Hsp72 could stabilize the individual components of the replicase complex via its chaperon activity, or it could enhance the translational efficiency of HCV IRES, which could in turn augment the stimulatory effect of NS5A on HCV IRES activity. Both mechanisms are possible and not mutually exclusive. The latter mechanism has been demonstrated previously by Gonzalez et al. (47).

The effect of Hsp72 in increasing HCV replicase levels was also confirmed by the in situ PLA technique. The advantages of this technique are the high specificity and the great sensitivity, due to the use of two specific primary antibodies and the amplification procedure, respectively, which allow for detection of the interactions between two proteins in low abundance. Using this assay, we were able to observe the in situ interaction between Hsp72 and NS5A as well as the association between NS3 and NS5A, representing the replicase complex, in the replicon cells. The protein complexes observed were dispersed around the cell nucleus. The speckled pattern of the complexes is similar to previous reports in which the HCV replication complexes were observed using immunofluorescence staining (50). Thus, we believe that this assay should provide a reliable tool to faithfully represent in situ protein interactions.

FIGURE 7. Hsp72 increases the stability of viral proteins and enhances HCV IRES activity. A, effect of Hsp72 knockdown on viral protein stability. P1 cells were infected with lentiviral vector expressing control shRNA or shRNA targeting Hsp72. Cell lysate proteins were analyzed 72 h after lentiviral infection by Western blotting with anti-NS3, anti-NS5A, anti-NS5B, or anti-Hsp72 antibody. In one set of experiment, MG132 (100 nM) was added during the last 24 h of culture to inhibit proteasomal degradation. **, p < 0.01 and *, p < 0.05, as compared with the cells infected with control lentiviral vector and transfected with control vector DNA.

In this study, we used the TAP method to purify NS5-interacting proteins. The method has been described as an efficient protocol to purify intracellular protein complexes (33). The tandem tags present in the fusion protein allow for stepwise purification, which improves specificity greatly in comparison to the results obtained using the traditional single tag method. Using this method, HSC70 and Hsp72 were reproducibly identified as targets interacting with NS5A (and weakly with NS4B). Previous study has demonstrated that HSC70 is present in HCV viral particles. HSC70 modulates HCV infectivity and virus release but does not affect HCV RNA replication (34). Thus, it remains to be determined whether the interaction of HCV non-structural proteins with HSC70 has anything to do with the formation of viral particles. On the other hand, this study demonstrated that Hsp72 significantly increased HCV RNA replication. The results were consistent with a recent study by Gonzalez et al. (47) who demonstrated that knockdown of Hsp72 expression by siRNA or treating replicon cells with a heat shock protein inhibitor, Quercetin, reduced HCV RNA replication. All these results indicated that the Hsp70 family of proteins could participate in multiple steps of the HCV lifecycle.

Chaperone proteins are highly abundant in the cell and control cellular protein quality. These proteins are involved in protein translation, folding, trafficking, and degradation (48). However, they are also utilized by various viruses to enhance viral replication. In the course of viral proliferation, a large number of viral proteins are synthesized in a relatively short period of time, during which protein folding can become a limiting step. Therefore, most viruses may need cellular chaperones to circumvent this limitation. Among chaperone proteins, Hsp90 has been implicated in HCV replication. Hsp90 is thought to enhance the maturation of the NS2/NS3 (40, 49) and to form a complex with FKBP8 and NS5A to participate in HCV replication (44). This study demonstrated that Hsp72 chaperone activity could also increase levels of the replicase complex, leading to enhanced viral RNA replication. Hsp72 could stabilize the individual components of the replicase complex via its chaperon activity, or it could enhance the translational efficiency of HCV IRES, which could in turn augment the stimulatory effect of NS5A on HCV IRES activity. Both mechanisms are possible and not mutually exclusive. The latter mechanism has been demonstrated previously by Gonzalez et al. (47).

The effect of Hsp72 in increasing HCV replicase levels was also confirmed by the in situ PLA technique. The advantages of this technique are the high specificity and the great sensitivity, due to the use of two specific primary antibodies and the amplification procedure, respectively, which allow for detection of the interactions between two proteins in low abundance. Using this assay, we were able to observe the in situ interaction between Hsp72 and NS5A as well as the association between NS3 and NS5A, representing the replicase complex, in the replicon cells. The protein complexes observed were dispersed around the cell nucleus. The speckled pattern of the complexes is similar to previous reports in which the HCV replication complexes were observed using immunofluorescence staining (50). Thus, we believe that this assay should provide a reliable tool to faithfully represent in situ protein interactions.
Hsp72 Enhances HCV RNA Replication

The domain in NS5A required for Hsp72 interaction was mapped to the middle region of NS5A between amino acids 221 and 302. This region overlaps with the previously defined low complexity sequence (LCS) I and domain II of NS5A and has been established as indispensable for viral RNA replication (51). Many mutations that increase RNA replication of HCV replicons have been mapped to this region (8, 9, 51). This region also contains the protein kinase R (PKR)-binding domain/interferon sensitivity-determining region of NS5A, which was used to interrupt PKR function (52, 53). The finding that NS5A associates with Hsp72 in this region provides a novel function for this domain, which may be related to the replication efficiency of HCV replicons.

In summary, we have demonstrated that the Hsp70 chaperone can interact with multiple members of the HCV replicase complex, stabilize them, and lead to their accumulation in the cell, thus enhancing HCV RNA replication. The importance of Hsp chaperones to HCV replication represents a target for the development of anti-HCV therapies. Indeed, several Hsp inhibitors have been shown to effectively attenuate HCV production (44, 47).

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