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Interaction of hepatitis C virus core protein with Hsp60 triggers the production of reactive oxygen species and enhances TNF-α-mediated apoptosis

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ABSTRACT

The hepatitis C virus (HCV) core protein is the primary protein component of the nucleocapsid that encapsidates the viral RNA genome. Besides its role as a viral structural protein, the core protein is implicated in HCV chronic infection-associated liver diseases by induction of reactive oxygen species (ROS) production and modulation of apoptosis. Here, we show that interaction of the core protein, through its N-terminal domain (amino acids 1–75), with heat shock protein (Hsp60) is critical for the induction of ROS production, leading to sensitization of core protein-expressing cells to apoptosis induced by tumor necrosis factor-α (TNF-α). Moreover, overexpression of Hsp60 rescued the core protein-expressing cells from cell death by reducing ROS production. Collectively, our results suggest that impairment of Hsp60 function through binding of HCV core protein contributes to HCV viral pathogenesis by ROS generation and amplification of the apoptotic effect of TNF-α.

1. Introduction

Hepatitis C virus (HCV) infection often leads to chronic viral hepatitis, which over time evolves into liver cirrhosis with a high risk of developing hepatocellular carcinoma (HCC) [1–3]. Several studies have suggested that oxidative stress, which is induced either by viral proteins or host immune responses, is one of major determinants of the HCC development [4]. Since chronic HCV infection is associated with an enhancement of TNF-α production by macrophages and cytotoxic T lymphocytes [5,6], TNF-α further induces oxidative stress by stimulating the release of ROS, which may in turn induce massive liver steatosis, and plays a central role in liver injury during elimination of virus-infected cells *via* apoptosis [7–10].

HCV core protein is the viral nucleocapsid protein that binds and packages the viral RNA genome. Besides its role as a viral structural protein, the core protein is known to induce reactive oxygen species (ROS) production in tissue cultures and animal models [11–13]. However the mechanism(s) by which the core protein induces ROS production are largely unknown. In an attempt to gain a better understanding of how HCV core protein is involved in HCV viral pathogenesis by interacting with cellular proteins, we previously used a proteomic approach and found that heat shock protein 60 (Hsp60) is one of the cellular protein that binds to the core protein [14]. Hsp60, a stress response molecular chaperon [15], is primarily but not exclusively compartmentalized in mitochondria matrix [16] and has a crucial function in folding/refolding of mitochondria-imported pre-proteins [17]. In addition to its function in folding of macromolecules in mitochondria, Hsp60 has been shown to have potent anti-apoptotic role in both the cytosol and mitochondria [18,19].

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These observations prompted us to test whether the interaction between Hsp60 and the HCV core protein is linked to ROS production and the regulation of apoptosis. We report here that the core protein directly interacts with Hsp60 and induces ROS production, which potentiates TNF-α-induced apoptotic cell death. Thus, our data reveal a novel mechanism of Hsp60 regulation through an interaction with the HCV core protein, which may contribute to liver diseases and carcinogenesis.

2. Materials and methods

2.1. Cell lines and culture

The human hepatoma cell line, Huh7, was grown in RPMI-1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin under standard culture conditions (5% CO_2, 37 °C). The human hepatoma stable cell line Huh7TR-4 expressing the tetracycline repressor and the Huh7TR-core expressing HCV core protein in a tetracycline-inducible manner [14] were maintained in the presence of blasticidin S (10 μg/ml) and Zeocine (100 μg/ml). Core protein expression in Huh7TR-core was induced by addition of 1 μg/ml tetracycline for 48 h, unless otherwise specified.

2.2. Plasmids

The pcDNA3.1-Flag-core expressing Flag-epitope-tagged full-length HCV core protein was described previously [14]. For expression of the enhanced green fluorescence (EGFP)-fused HCV core protein and its deletion derivatives, pEGFP-C(1–191), pEGFP-C(1–75), pEGFP-C(1–121), pEGFP-C(1–173), pEGFP-C(76–191) and pEGFP-C(99–191) were constructed by cloning the cDNAs encoding the amino acid sequence indicated within parenthesis. Partial HCV core gene fragments were generated by conventional PCR using Vent DNA polymerase (NEB) with pCV-J4L6S [20] as a template. The amplified PCR products were digested with EcoRI and BamHI and inserted into pEGFP-C1 (Clontech). The HA-tagged Hsp60-expression vector, pcDNA3-Hsp60-HA, was provided by Yasuo Tanaka (University of Tokyo, Japan).

2.3. Protein digestion, mass spectrometric analysis and database searching

Protein spots were excised from a stained two-dimensional gel, processed, and digested with a bovine trypsin solution (10 μg/ml) (Roche), as described previously [14]. Digested peptides were analyzed by a MALDI-TOF/TOF tandem mass spectrometer (ABI 4700 Proteomics Analyzer, Applied Biosystems). The MS/MS spectra were then used to search for a matching protein using the MASCOT search program (http://www.matrixscience.com), as described [14].

2.4. Immunoprecipitation and Western blot analysis

Cell lysates were prepared in lysis buffer (1% Triton X-100, 100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4 and 17.5 mM β-glycerophosphate) supplemented with a protease inhibitor cocktail (Roche). Cleared cell lysates were incubated with mouse monoclonal anti-Hsp60 antibody (LK-1 clone, Stressgen) for 1 h with gentle agitation at 4 °C. Immune complexes were then recovered by adsorption to Protein G-Sepharose (Qiagen) for 1 h at 4 °C. For immunoprecipitation of the Flag-tagged HCV core protein, Huh7 cells were transfected with the pcDNA3.1-Flag-core expression vector using fuGENE6 (Roche) and harvested at 48 h post-transfection. Cell lysates were incubated with an anti-Flag peptide antibody M2-conjugated agarose beads (Sigma–Aldrich) for 2 h at 4 °C. For Western blot analysis, immunoprecipitates or lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and blotted onto nitrocellulose membranes. Membranes were analyzed by immunoblotting, as described previously [14], using various primary antibodies [rabbit anti-Flag antibodies (Sigma–Aldrich), HCV patient serum (kindly provided by Dr. K. H. Han at Yonsei University School of Medicine), rabbit anti-GFP antibody (Santa Cruz Biotechnology), rabbit anti-poly (ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology) detecting full-length (116 kDa) and the cleaved fragment (89 kDa) of PARP, anti-caspase 3 antibody (Cell Signaling Technology) detecting full-length (35 kDa) and the cleaved fragment (17 kDa), and anti-vimentin antibody (V9 clone, Sigma–Aldrich)] known to detect intact vimentin (58 kDa) and its cleavage products (47, 41, 28 and 22 kDa) [21]], and appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoblots were developed with the ECL detection kit (GE Healthcare Life Sciences) according to the manufacturer's recommendations.

2.5. Confocal microscopy

Flag-tagged core protein in cells permeabilized with 0.2% Triton X-100 was visualized by immunostaining with anti-Flag and FITC-conjugated anti-rabbit IgG antibodies (Sigma–Aldrich), as described previously [14]. For the staining of mitochondria, cells were incubated in medium containing 100 nM Mitotracker Red CMXRos (Molecular Probes) for 45 min. Hsp60 was detected using a mouse monoclonal anti-Hsp60 antibody and a Texas-red conjugated anti-mouse IgG antibody (Vector Laboratories). Nuclei were visualized by staining with 1 μM 4′,6′-diamidino-2-phenylindole (DAPI) in PBS for 10 min. Confocal images were obtained using a BIO-RAD Radiance 2000 multi-photon laser scanning confocal microscope.

2.6. Subcellular fractionation and protease accessibility assay

Subcellular organelles were fractionated by differential centrifugation as described previously [22]. Mitochondrial fraction was treated with proteinase K (0–10 μg/ml) in the absence or presence of Triton X-100 (1%) for 30 min on ice. The reaction was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride. Samples were then analyzed by immunoblotting.

2.7. Intracellular ROS analysis

Huh7TR-core cells were pre-incubated with 10 mM of the antioxidant N-acetylcysteine (NAC; Sigma–Aldrich) for 12 h, washed, and induced with tetracycline (1 μg/ml), unless otherwise specified, for 24 h. In control experiments, Huh7TR-4 cells were challenged with H_2O_2 (800 μM) for 30 min at 37 °C. Cells were incubated with 5 μM fluorescent probe, 5′,6′-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (CM-H_2DCFDA, Molecular Probes) in the dark for 60 min. Intracellular ROS, resulting in the oxidative production of dichlorofluorescin, was detected under a fluorescence microscope. Alternatively, cells were pelleted and resuspended in 5 μM CM-H_2DCFDA in PBS. Cells were then incubated in the dark for 15 min and washed three times in PBS. Fluorescence intensity representing ROS levels was recorded on the FL-1 channel of FACScan (Becton-Dickinson), and data were analyzed using the CellQuest software.

2.8. Flow cytometric analysis of apoptosis

Transiently transfected Huh7 cells and stable Huh7 cell lines expressing the core protein were treated with TNF-α (Peprotech: 20 ng/ml) and cyclohexamide (CHX, Sigma–Aldrich: 10 μg/ml) for the indicated time (0, 3, 6 and 12 h). Apoptosis was determined by Annexin V membrane staining according to manufacturer's instruction (Annexin V-FITC detection kit, Pharmingen). After TNF-α and CHX treatment, the cells were detached with trypsin, mixed with cells floating in the culture medium, and collected by centrifugation. Cells were washed twice with PBS and resuspended in 100 μl of 1× binding buffer (19 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml and stained with annexin V-FITC. The level of apoptosis was determined by flow cytometric analysis using a FACScan flow cytometer.

3. Results

3.1. HCV core protein binds to Hsp60

Previously we identified Hsp60 as one of the cellular proteins that interacts with the HCV core protein [14]. In the present work, we were able to further confirm the identity of this protein by tandem mass spectrometry. Collision-induced dissociation spectra for seven selected parental ions from MALDI-TOF mass spectrometry identified the following peptide sequences that match the Hsp60 protein sequence: TVIIEQSWGSPK (aa 61–62), GYISPYFINTSK (aa 212–223), CEFQDATVLLSEK (aa 227–239), DRVTDALNATR (aa 409–419), CEFQDAYVLLSEK (aa 227–239), CIPALDSLTPANEDQK (aa 437–452) and NAGVEGSLIVEK (aa 472–483). Coimmunoprecipitation assays, using cell extracts from the Huh7TR-core cells expressing the core protein under the control of a tetracycline-inducible promoter, confirmed the interaction between the core protein and Hsp60, by demonstrating the presence of the core protein in the immunocomplexes precipitated with the anti-Hsp60 antibody (Fig. 1A, lane 5). In control experiments, no core protein was detected when Hsp60 was immunoprecipitated with normal rabbit serum (lane 3). The interaction between the core protein and Hsp60 observed in Huh7TR-core cells was also detected in Huh7 cell by overexpressing a Flag-tagged core protein (Fig. 1B, lane 5).

To map the Hsp60-interacting domain, we expressed various GFP-tagged core protein truncations (Fig. 1C) in Huh7 cells and examined Hsp60-immunoprecipitates for the presence of core protein by immunoblot blot analysis. The result showed that the interacting domain of the core protein is its N-terminal domain (aa 1–75), since the core proteins lacking this domain, core (76–191) and core (99–191), did not bind to the Hsp60 protein, while the full-length core protein and its truncations including this domain [core (1–75), core (1–121) and core (1–173)] were co-immunoprecipitated with Hsp60 (Fig. 1D).

3.2. Cellular localization of the core protein and Hsp60

Previously it was shown that Hsp60 resides primarily in the mitochondria matrix [16], whereas there are several different pools of the core protein, which localize to the surface of the ER [23], lipid droplets [24,25], and to mitochondria [12,26–28]. To determine if the interaction between Hsp60 and the core protein occurs in the mitochondria, Huh7 cells were transfected with a Flag-tagged core protein expression vector. At 48 h post-transfection, the cells were fixed, permeabilized, and dual labeled with an anti-Flag antibody and the mitochondria dye MitoTracker (Fig. 2A) or anti-Hsp60 antibody (Fig. 2B). Core protein was predominantly in the cytosol with intense perinuclear staining. A fraction of the core protein colocalized with mitochondria, exhibiting a punctate distribution (Fig. 2A). Dual immunofluorescence staining of Hsp60 and core protein also demonstrated their partial colocalization in the cytosol (Fig. 2B).

To determine if the core protein associates with Hsp60 in the mitochondria, we next determined the distribution of core protein and Hsp60 in subcellular fractions of HCV core protein-expressing cells. As expected, the majority of Hsp60 was present in the mitochondrial fraction (Fig. 2C, lanes 2 and 6), while the core protein was detected in both mitochondrial and microsomal fractions (lanes 6 and 7). To further characterize the core protein association with mitochondria, we performed a proteinase K accessibility assay using the mitochondrial fractions. The results revealed that the core protein in the mitochondrial fraction was resistant to proteinase K treatment as much as Hsp60 (Fig. 2D), which is known to locate primarily in the inner membrane of mitochondria [16]. Core protein, like Hsp60, was however completely digested when the treatment was carried out in the presence of 1% Triton X-100, suggesting that it appears to be not associated with the detergent resistant membranes on the mitochondria. Altogether, these data indicate that at least a fraction of the core protein resides in the mitochondria.

3.3. Core protein–Hsp60 interaction triggers induction of ROS production

Overexpression of the core protein in cell culture or in transgenic mice is known to induce ROS production [11–13]. We first confirmed that core protein expression in Huh7TR-core cells induced ROS production. Huh7TR-core cells were pretreated with the antioxidant NAC for 12 h and core protein expression was induced with 1 μg/ml of tetracycline for 24 h, then ROS were fluorescently stained with the ROS-sensitive fluorophore DCF-DA. ROS staining revealed that expression of the core protein induced ROS production, as did H_2O_2 treatment of the parental Huh7TR-4 cells, which do not express the core protein (Fig. 3A). ROS production levels were also determined by flow cytometry, and showed that intracellular ROS level increased with an increase of core protein expression level (Fig. 3B, left panel). We also noticed that, even in uninduced Huh7TR-core cells, the endogenous ROS levels were slightly higher compared to the parental Huh7TR-4 cells, which was likely due to low-level leaky expression of the core protein in the Huh7TR-core cells (Fig. 3B, right panel and inset therein). The ROS level in induced Huh7TR-core cells was comparable to the ROS level in Huh7TR-4 cells treated with H_2O_2 (800 μM) for 30 min. More importantly, we found that expression of core protein truncations that lack the Hsp60 interaction domain (core 99–191) failed to induce ROS production (Fig. 3C), suggesting that a physical interaction is crucial for ROS generation, possibly by interfering with normal Hsp60 functions. This hypothesis was confirmed by demonstrating that overexpression of the HA-tagged Hsp60, suppressed ROS production in the Huh7TR-core cells (Fig. 3D).

3.4. ROS production induced by core protein–Hsp60 interaction sensitizes cells to TNF-α-induced apoptosis

Double staining of core protein and Hsp60 revealed partial overlapping signals on the mitochondria (Fig. 2A and B), and the proteinase K sensitivity assay with the mitochondrial fraction (Fig. 2C and D) suggested the presence of a fraction of core protein in the mitochondria. These find-

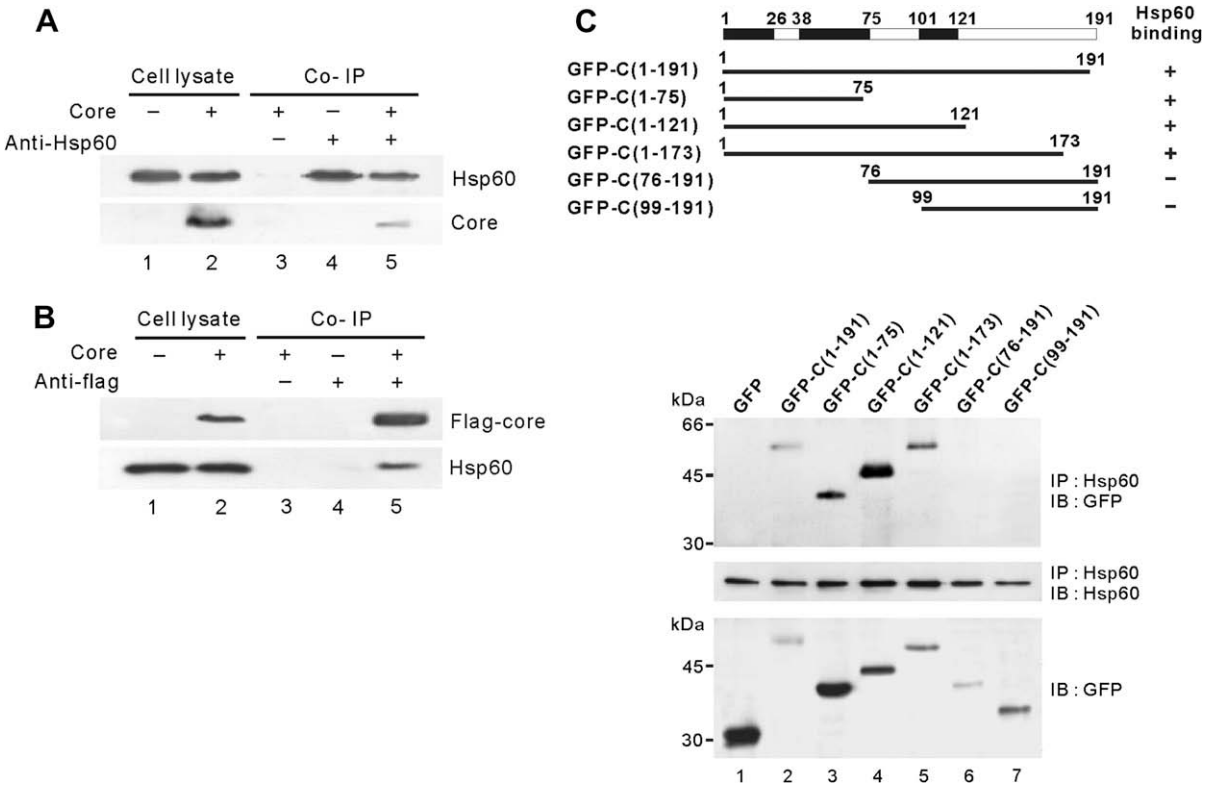

Fig. 1. Hsp60 binds to HCV core protein. (A) Cellular lysates of Huh7-TR cell that do not expressing HCV core protein (−) or Huh7-TR-core cells that express the core protein (+) were used to immunoprecipitate (IP) protein complexes using an anti-Hsp60 antibody (+) or an irrelevant antibody (−). Cell lysates and the immunoprecipitated complexes were analyzed by immunoblotting for Hsp60 and the HCV core protein. (B) Cellular lysates of Huh7 cells transfected with a vector expressing a Flag-tagged core protein (+) or the vector alone (−) were used to immunoprecipitate protein complexes using an anti-Flag M2 agarose beads (+) or with an irrelevant antibody (−). Cell lysates and immunoprecipitated complexes were analyzed for Flag-tagged core protein and Hsp60. (C) Mapping of the regions in the core protein responsible for its interaction with Hsp60. (Top panel) Schematic representation of the full-length HCV core protein and the five truncations fused to GFP at the N-terminus. Amino acid numbers of each domain are indicated in parentheses. (Bottom panel) Cell lysates from Huh7 cells transfected with the indicated plasmids shown above the blot were used to immunoprecipitate protein complexes using an anti-Hsp60 antibody and analyzed by immunoblot (IB) for the GFP-fusion core proteins using a GFP-specific antibody (first panel) and an anti-Hsp60 antibody (second panel). The expression levels of the GFP-core fusion proteins in total lysates (20 μg) of Huh7 cells at 48 h post-transfection were determined by immunoblotting with a GFP-specific antibody (third panel).

ings prompted us to investigate whether the interaction between the core protein and Hsp60 is involved in regulation of apoptosis, since previous studies demonstrated that Hsp60 has anti-apoptotic functions in both the mitochondria and cytosol [18,19]. Huh7TR-4 and Huh7TR-core cells, which were induced with tetracycline for 24 h, were treated with TNF-α (20 ng/ml) and CHX (10 μg/ml) for 0, 3, 6 and 12 h. CHX prevents *de novo* protein synthesis and was included in the treatment to enhance apoptosis, since Huh7 cells were relatively resistant to TNF-induced apoptosis. Immunoblot analyses revealed that PARP and procaspase-3 cleavage occurred more rapidly in core protein-expressing cells (Fig. 4A). Vimentin, which is known to be cleaved at multiple sites at an early stage of apoptosis by caspases 3, 6 and 7 [29,30], also underwent rapid degradation in the core protein-expressing cells. Flow cytometric analysis of an early marker of apoptosis, using FITC-conjugated annexin V, confirmed that core protein expression sensitizes cells to apoptosis induced by TNF-α (Fig. 4B).

We next tested whether the direct interaction of the core protein with Hsp60, which led to onset of oxidative stress, was responsible for the sensitization of core protein-expressing cells to TNF-α. Huh7 cells transiently expressing either GFP-C(1–191) or GFP-C(99–191) were treated with TNF-α and CHX for 12 h before FACS analysis for annexin V-stained cells. We found that GFP-C(1–191) but not GFP-C(99–191) potentiated cells to undergo apoptosis, indicating that direct interaction between the core protein and Hsp60 was responsible for the sensitization of cells to TNF-α-induced apoptosis (Fig. 4C). Furthermore, co-expression of Hsp60 in Huh7 cells expressing the GFP-C(1–191) protected cells from the enhanced TNF-α-induced apoptosis (Fig. 4D). These results suggest that binding of core protein to Hsp60 may interfere with its chaperone function and/or negatively regulate its anti-apoptotic functions, rendering the core protein-expressing cells more susceptible to TNF-α-induced apoptosis.

4. Discussion

In this study, we addressed several important questions about role of Hsp60 in HCV core protein-mediated induction of oxidative stress. Is the interaction between the core protein and Hsp60 crucial for ROS production? Is there a pool of the core protein that localizes to the mitochondria matrix where it would be able to interfere with the chaperone functions of Hsp60? Dose ROS production induced by the interaction between the core protein and Hsp60 sensitize cells to TNF-α? We demonstrate that induction of ROS production in HCV core protein-expressing cells is triggered by the binding of the core protein to Hsp60, which could inactivate the Hsp60 chaperone function in the mitochondria and sensitize core protein-expressing cells to undergo TNF-α-induced apoptosis.

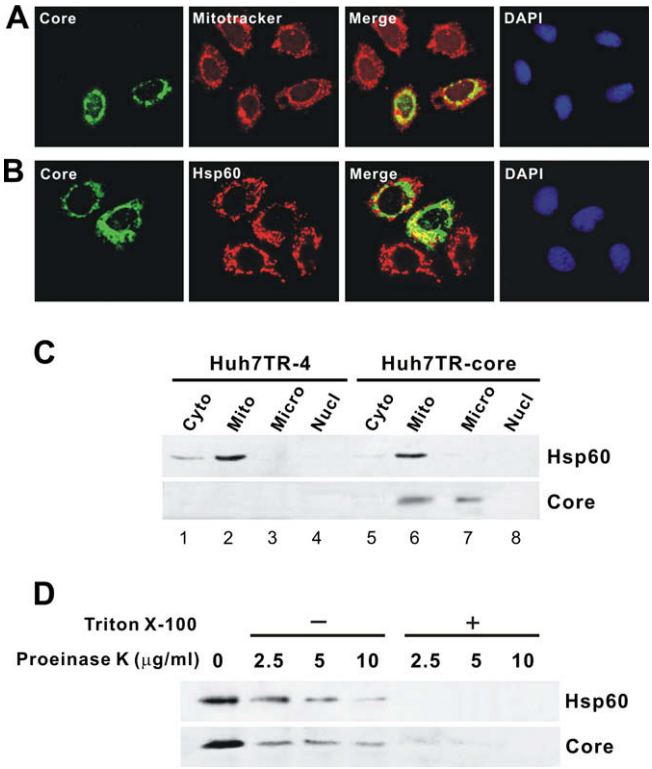

Fig. 2. Subcellular localization of HCV core protein. (A) Huh7 cells were transfected with a plasmid expressing the Flag-tagged HCV core protein. At 48 h post-transfection, cells were examined by indirect double immunofluorescence staining. Ectopic expression of the Flag-tagged core protein was monitored by using anti-Flag antibodies and a FITC-conjugated anti-rabbit IgG antibody (green). Mitochondria were stained with Mitotracker Red CMXRos (red). (B) The Huh7 cells transfected as in (A) were used for immunostaining of the Flag-tagged core protein (green) or for detection of Hsp60 (red) with an anti-Hsp60 antibody and Texas-red conjugated anti-mouse IgG antibody. Merged images are shown in the third panel. Nuclei were visualized by DAPI staining. (C) Co-fractionation of the core protein with mitochondria and its presence in the matrix of the mitochondria. Cytosolic (Cyto), mitochondrial (Mito), microsomal (Micro) and nuclear (Nucl) fractions were prepared by differential centrifugation of cell lysates from the Huh7TR-4 or Huh7TR-core. Each subcellular fraction (20 μg) was used for immunoblot analysis for the indicated proteins. (D) Proteinase K accessibility assay. The mitochondrial fractions of Huh7TR-core were incubated with increasing concentrations of proteinase K in the absence (−) or presence (+) of 1% Triton X-100 and analyzed by immunoblotting with anti-Hsp60 and anti-HCV core antibodies.

Previous studies showed that the core protein localizes to the surface of the endoplasmic reticulum [23] and lipid droplets [24,25] in the cytosol, as well as to the mitochondria [12,26–28]. We were able to confirm the presence of the core protein in both mitochondrial and microsomal fractions of core protein-expressing cells. Immunofluorescence studies confirmed the association of the core protein with mitochondria. More importantly, we found, by a proteinase K accessibility assay, that a portion of core protein that is associated with the mitochondria was resistant to the protease treatment unless the mitochondrial membrane integrity is disturbed by the non-ionic detergent Triton X-100. These results support our conclusion that a fraction of the HCV core protein is localized in mitochondria. Consistent with our findings, immunoelectron microscopy analysis of core protein-expressing cells suggested a portion of the core protein localized in the matrix of mitochondria [26].

Mitochondria are the primary site of ROS generation, caused by an imbalance of the redox milieu [31]. A previous study demonstrated that HCV core protein impairs the mitochondria electron transport system in transgenic mice [32]. These data supports our notion that core protein binding to Hsp60 may interfere with the chaperone function of Hsp60 in mitochondria, leading to mitochondrial dysfunction and ROS production. Mitochondrial dysfunction might be induced by misfolding of mitochondrial proteins, because the majority of these proteins are encoded by nuclear genetic material and need to be refolded in the mitochondria by the help of chaperone complexes composed of Hsp60 and Hsp10 [15].

In chronic HCV infection, enhanced hepatocyte apoptosis occurs through the intrinsic apoptosis pathway *via* mitochondria, because external activation by death signaling is insufficient to induce apoptosis in hepatocytes [33–35]. ROS overproduction is known to stimulate many forms of apoptosis [36]. The results presented here showed ROS generation induced by the core protein–Hsp60 interaction potentiates apoptosis induced by TNF-α in core protein-expressing Huh7 cells. Hsp60 has been shown in several systems used to study apoptosis to be released into the cytoplasm during apoptosis [37]. Therefore, binding of core protein to the Hsp60 in the cytosol might further precipitate the apoptotic process by further hampering the

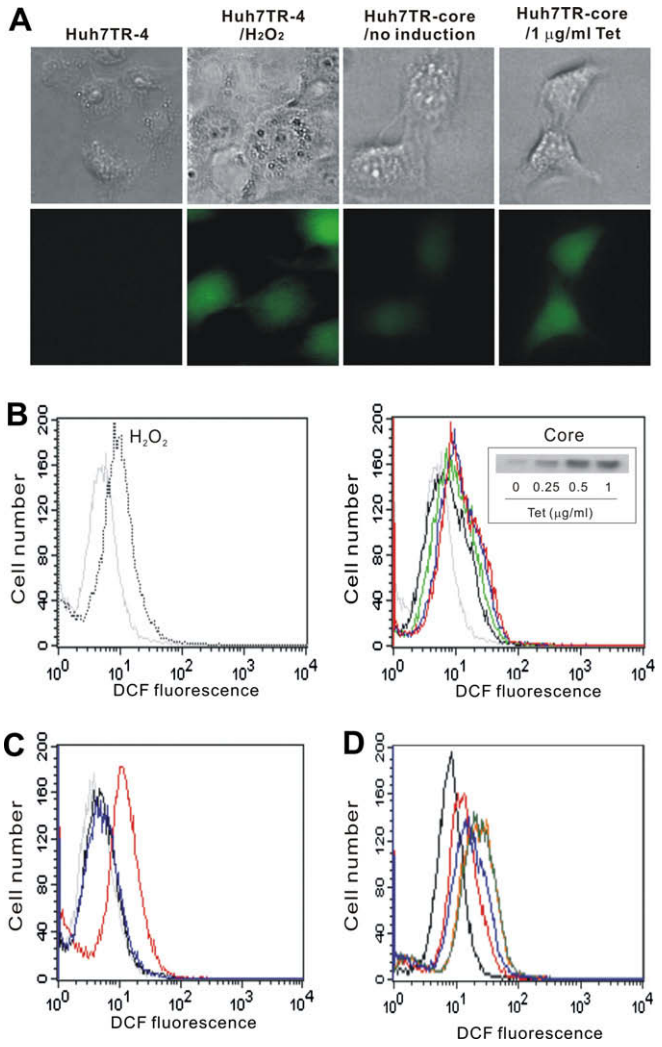

Fig. 3. HCV core protein induces ROS production through its direct interaction with Hsp60. (A) Huh7TR-core cells were pre-incubated with NAC (10 mM) for 12 h, washed, and then induced with tetracycline (Tet, 1 μg/ml) for 24 h. Huh7TR-4 cells were similarly pretreated with NAC and challenged with H_2O_2 (800 μM) for 30 min. The DCF-DA probe (5 μM) was then added to the cells and imaged at 10 min after the fluorescence probe addition. (B) ROS production monitored by flow cytometry. (Left panel) ROS production in Huh7TR-4 cells stimulated with H_2O_2 as in (A). The grey and black patterns represented the histogram obtained with no treatment or H_2O_2 treatment, respectively. (Right panel) ROS production in Huh7TR-core cells induced with increasing concentrations of tetracycline (no induction, black; 0.25 μg/ml, green, 0.5 μg/ml, blue, 1 μg/ml, red). The grey pattern represents the histogram obtained from non-treated Huh7TR-4 cells. Inset shows the expression pattern of the core protein, analyzed by immunoblotting of the cell lysates of induced Huh7TR-4 cells. (C) Induction of ROS production by HCV core protein–Hsp60 interaction. Huh7 cells were grown in 6-well plates, pre-incubated with NAC (10 mM) for 12 h, washed, and left untransfected (grey) or transfected with 3 μg of pEGFP (black), pEGFP-C(1–191) (red), or pEGFP-C(99–191) (blue). ROS production was measured at 48 h post-transfection as in (B). (D) Reduction of HCV core protein-induced ROS production by Hsp60 overexpression. Huh7TR-core cells, pretreated with NAC as in (A), were left untreated (red) or induced with tetracycline (1 μg/ml, orange) for 24 h. Induced cells were transfected with pcDNA3.1 (green) or the Hsp60-expression vector pcDNA3-Hsp60-HA (blue). After 24 h, intracellular ROS levels were measured by flow cytometry as in (B). The black pattern represents the histogram obtained from Huh7TR-4 cells.

anti-apoptotic activities of Hsp60. This possibility is supported by a previous study showing that Hsp60 binds to Bax in the cytosol and negatively regulates its pro-apoptotic function by preventing translocation to the mitochondria [18]. Another anti-apoptotic role of Hsp60 has recently been elucidated [19]. Survivin located in the mitochondria to inhibit apoptosis [38], is stabilized by forming a complex with Hsp60, which refolds survivin upon internalization into the mitochondria. Thus, binding of the HCV core protein to Hsp60 may interfere with the formation of the Hsp60-survivin complex and inhibit the anti-apoptotic function of the survivin in the mitochondria. Furthermore, Hsp60 release from the mitochondria during apoptosis also would further contribute to the promotion of apoptosis by concomitant destabilization of survivin in the mitochondria.

Thus, the HCV core protein appears to lower the threshold necessary for a cell to undergo apoptosis through a diverse set of mechanisms, primarily due to the amplification of the death signaling pathway by the accumulation of

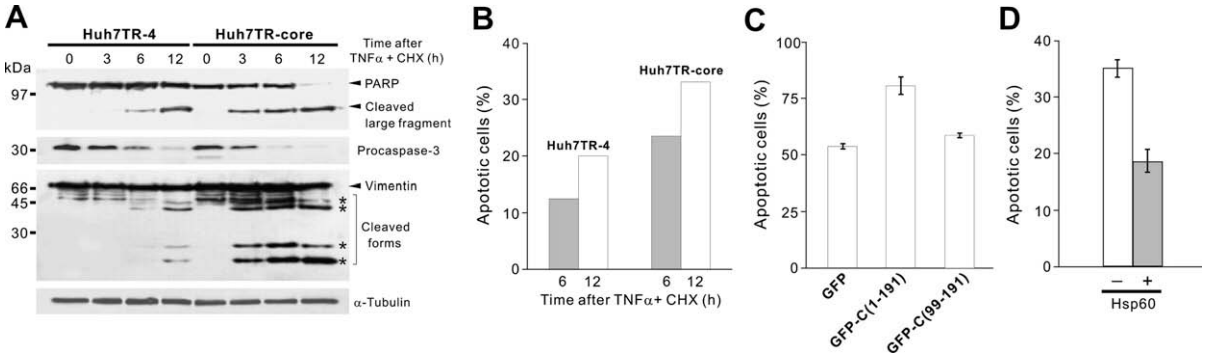

Fig. 4. Rescue of HCV core protein-expressing cells from TNF-α-induced apoptosis by overexpression of Hsp60. (A) Analysis of PARP, procaspase-3 and vimentin cleavages in tetracycline-induced Huh7TR-4 and Huh7TR-core cells by immunoblot analysis after treatment with TNF-α (20 ng/ml) and CHX (10 μg/ml) for 0, 3, 6 and 12 h. Levels of intact PARP (116 kDa), the large fragment of cleaved PARP (89 kDa), procaspase-3 (32 kDa), intact vimentin (58 kDa) and cleaved forms of vimentin (47, 41, 28 and 22 kDa; indicated by asterisks) were assessed by immunoblot analysis of whole cell lysates with various specific antibodies for the indicated proteins. Immunoblotting with anti-α-tubulin was used as an internal control for equal loading. (B) Huh7TR-4 and induced Huh7TR-core cells were exposed to TNF-α and CHX for 6 and 12 h, and the percentage of annexin V positive cells was determined by flow cytometry for early apoptosis. (C) Direct binding of the core protein to Hsp60 is responsible for the sensitization to cell death induced by TNF-α. Huh7 cells were transfected with the vectors expressing indicated proteins GFP- or GFP-core fusion proteins. At 48 h post-transfection, cells were treated with TNF-α and CHX for 12 h, and annexin V positive cells in the transfected cells were determined as in (B). The results represent the mean ± standard deviation from triplicate samples. (D) Hsp60 overexpression rescued the core protein-expression cells from TNF-α-induced apoptosis. The tetracycline-induced Huh7TR-core cells were either left untransfected (white bar) or transfected (grey bar) with the HA-tagged Hsp60-expression vector. Apoptotic cells were measured as in (C).

ROS [35,36]. Indeed, enhanced hepatocyte apoptosis during chronic HCV infection is known to be associated with liver diseases including fulminant hepatitis and fibrogenesis [39]. In addition, increased ROS production is likely to lead to liver steatosis, by exacerbating oxidative mechanisms, and liver carcinogenesis, by DNA oxidative damage, during chronic HCV infection [4].

In summary, we have provided evidence that the HCV core protein–Hsp60 interaction is linked to the induction of ROS production and sensitization of cells to TNF-α-induced apoptosis. Our results raise the possibility that an interaction with core protein might affect chaperone activity and/or anti-apoptotic function of Hsp60 in the mitochondria or cytosol. Since overexpression of Hsp60 could compensate for such Hsp60 dysfunctions, it may be a potential target for a therapy overcoming oxidative stresses in liver injury during chronic infection of HCV.

Conflicts of interest statement

None declared.

Acknowledgments

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