

mice harboring the core gene, increased ROS production has been observed.⁷⁻⁹ A recent study found, by the proteomic profiling of biopsy specimens, that an impairment in key mitochondrial processes, including fatty acid oxidation and oxidative phosphorylation, and in the response to oxidative stress occurs in HCV-infected human liver with advanced fibrosis.¹⁰ Therefore, it is probable that the HCV core protein affects mitochondrial functions because such pathogenesis is observed in both HCV core-transgenic mice and HCV-infected patients.¹¹⁻¹³

The recent progress in proteomics has opened new avenues for disease-related biomarker discovery. Among proteomics approaches, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a technique for the separation and identification of proteins in a sample by displacement in two dimensions oriented at right angles to one another. This method is generally used as a component of proteomics and is the step used for the isolation of proteins for further characterization by mass spectrometry. 2D-PAGE is particularly useful when comparing two related samples such as healthy and diseased tissue. For example, proteins that are more abundant in diseased tissue may represent novel drug targets or diagnostic markers. In fact, several candidate biomarkers for many human cancers have been identified by this approach.¹⁴ There are, however, tens of thousands of proteins in a cell, differing in abundance over six orders of magnitude. 2D-PAGE is not sensitive enough to detect rare proteins, and hence many proteins are not resolved. Therefore, splitting a sample into different fractions is often necessary to reduce the complexity of protein mixtures prior to 2D-PAGE. For this advantage, Lescuyer et al.¹⁵ performed a 2D-PAGE of human mitochondrial proteins derived from the placenta and identified proteins mainly by peptide mass fingerprinting.

In this study, we performed a 2D-PAGE of mitochondria isolated from HepG2 cells stably expressing the HCV core protein and identified several proteins of different expressions when compared with control HepG2 cells. Among up-regulated proteins in the core-expressing cells, we focused on prohibitin, which functions as a mitochondrial protein chaperon, and found that the core protein interacts with prohibitin and represses the interaction between prohibitin and subunit proteins of cytochrome c oxidase (COX), which may lead to decreases in the expression level of the proteins and in COX activity. These results may explain the pathogenesis of liver disease in HCV infection including ROS induction.

Materials and Methods

Cells and Purification of Mitochondria. Hep39 cells,¹⁶ which stably express the HCV core protein, and

control HepG2 cells (Hepswx) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1 mg/mL G418. Mitochondria were purified using Nycodenz (Nycomed Pharma, Zürich, Switzerland) according to the protocols reported by Okado-Matsumoto et al.¹⁷ For transient transfection experiments, HepG2 cells were transfected with a core-expression plasmid using TransIT-LT1 (Mirus Bio, Madison, WI). Huh7 cells harboring HCV genotype 1b full-genomic (RCYM1)¹⁸ or subgenomic replicon (5-15), and livers of 3-month-old core-gene transgenic mice² were also used for the analysis.

2D-PAGE. Gel electrophoresis in the first dimension was performed using an immobilized pH gradient gel (Immobiline Dry Strip gel, pH 4-7 linear, 13 cm; GE Healthcare, Uppsala, Sweden). The two-dimensional separation was performed on 12.5%, 14 × 16 cm², SDS polyacrylamide gels. After the electrophoresis, gels were silver-stained using a silver staining kit (GE Healthcare) according to the manufacturer's protocols. The stained gels were scanned and electronic images of the gels were analyzed using ImageMaster 2D Elite software (GE Healthcare).

In-Gel Digestion and Matrix-Assisted Laser Desorption Ionization, Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). Protein spots on the gels were excised and a "control" piece was cut from a blank region of the gel and processed in parallel with the sample. In-gel digestion with trypsin was performed as reported.¹⁹ The resulting peptides were concentrated using Zip-Tip C18 (Millipore, Bedford, MA). The peptide mixtures were eluted from Zip-Tip with 75% acetonitrile in 0.1% trifluoroacetic acid (TFA). The matrix (α -cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% TFA) was deposited on a dried sample target. Then 0.5- μ L aliquots of the analyte solution were deposited onto matrix surfaces and the solvent was allowed to evaporate at ambient temperature. The digests were analyzed with a TOF mass spectrometer, PE Biosystems Voyager DE STR MALDI (Foster City, CA).

Database Analysis. For protein identification the measured monoisotopic masses of the peptides were analyzed using MS-Fit provided by UCSF (<http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm>).

Immunoblotting and Immunoprecipitation. Purified mitochondria were lysed and sonicated in RIPA buffer, then centrifuged at 16,000 rpm for 10 minutes. Protein concentration was determined using a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). The samples were separated by sodium dodecyl sulfate (SDS)-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Japan), then blocked with BlockAce (Snow Brand, To-

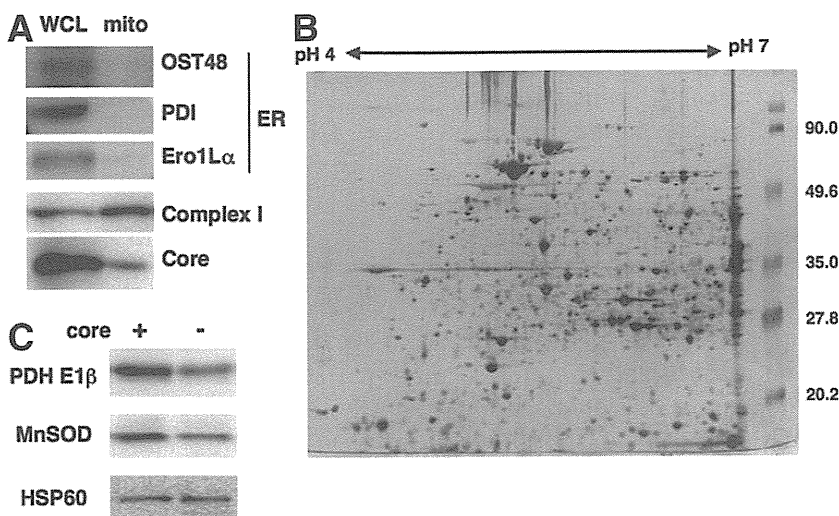


Fig. 1. 2D-PAGE of mitochondria purified from core-expressing cells. (A) Whole-cell lysates (WCL) and purified mitochondria (mito) derived from core-expressing cells were subjected to SDS-PAGE and immunoblotted with anti-core, anti-subunit of complex I (mitochondrial protein), or anti-OST48, PDI, Ero1L α (ER proteins) antibodies. (B) Purified mitochondria of core-expressing cells were subjected to 2D-PAGE and the gel was stained with silver. The numbers shown on the right are molecular weights. (C) Purified mitochondria of core-expressing and control cells were subjected to SDS-PAGE and blotted with an anti-E1 β subunit of PDH (PDH E1 β), anti-MnSOD, or anti-HSP60 antibody.

kyo, Japan). The membrane was subsequently incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Antibodies against the core protein (Anogen, Mississauga, Canada), manganese superoxide dismutase (MnSOD) (BD Biosciences, San Jose, CA), prohibitin (Neomarkers, Fremont, CA), oligosaccharyl-transferase-48 (OST48), heat shock protein (HSP) 60 (Santa-Cruz Biotechnology, Santa Cruz, CA), pyruvate dehydrogenase (PDH), ubiquinol-cytochrome c oxidoreductase, COX (Molecular Probes, Eugene, OR), protein disulfide isomerase (PDI), ER protein endoplasmic oxidoreduction-1 (Ero1)-L α , and I κ B α (Cell Signaling Technology, Danvers, MA), were used as primary antibodies. For immunoprecipitation experiments, cells were lysed in NET-N buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and the lysates were incubated with anti-prohibitin overnight followed by the addition of protein Sepharose 4B (GE Healthcare), then washed with the same buffer five times. Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with specific antibodies.

Determination of COX Activity. COX activity was determined with a MitoProfile Rapid Microplate Assay Kit (MitoSciences, Eugene, OR) using 10 μ g of purified mitochondria. The assay was performed three times independently.

Statistical Analysis. Results are expressed as means \pm SE. The significance of the difference in means was determined by Student's *t* test or Mann-Whitney's *U* test.

Results

Presence of HCV Core Protein in Purified Mitochondria. Increasing evidence suggests that the HCV

core protein is localized to mitochondria as well as to ER and the nucleus. Therefore, we first investigated whether the core protein is expressed in the mitochondria of core-expressing (Hep39) cells used in this study. We used Ny-codenz discontinuous gradients to extract mitochondria as described.¹⁷ In the mitochondria derived from core-expressing HepG2 cells, the core protein was detected by immunoblotting, whereas ER resident proteins such as an ER-specific type I transmembrane protein OST48, ER-resident molecular chaperon PDI, and ER membrane-associated N-glycoprotein Ero1-L α , were not (Fig. 1A). In this fraction, reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase, complex I of mitochondrial oxidative phosphorylation system, was more strongly expressed than that in the whole cell. These results indicate that the purified mitochondria fraction was free of ER, and that a portion of the core protein was localized to the mitochondria in core-expressing cells.

Proteomics Analysis of Mitochondria by 2D-PAGE. For proteomics analysis, purified mitochondrial proteins derived from core-expressing cells were subjected to 2D-PAGE followed by silver-staining of the gel. In this study we analyzed only acidic proteins using IPG strips covering pH 4 to pH 7 because the analysis of acidic proteins by 2D-PAGE is relatively easy. The mitochondrial fraction was also extracted from Heps wx, a control cell line resistant to G418 but does not express the core protein, then similarly subjected to 2D-PAGE and used for comparing the expression pattern. We repeated the above procedure (purification of mitochondria, 2D-PAGE, and silver-staining) five times, and confirmed a similar expression pattern in core-expressing cells. The representative gel image is shown in Fig. 1B. ImageMaster 2D Elite software detected about 1100 spots on the silver-stained acidic gel, i.e., at pH 4-7 and Mrs of 20-100 kDa. The number of

Table 1. Proteins of Differential Expression in Mitochondria of Core-Expressing Cells

Protein Name	Fold Change (Mean \pm SD)
Increased	
Succinyl-CoA:ketoacid CoA transferase	10.43 \pm 1.29
NADH-specific isocitrate dehydrogenase a subunit precursor	9.64 \pm 4.66
Unknown	8.65 \pm 2.40
GrpE-like protein co-chaperon	5.71 \pm 0.49
Leucine aminopeptidase	4.26 \pm 1.14
Pyruvate dehydrogenase E1 component b subunit	3.79 \pm 1.34
CG015alt2	3.18 \pm 0.80
HSP70	3.11 \pm 1.39
Prohibitin	2.60 \pm 0.24
3-Hydroxyisobutyrate dehydrogenase	2.47 \pm 0.77
HSPC108	2.46 \pm 0.69
MnSOD	2.35 \pm 0.65
Ubiquinol-cytochrome c oxidoreductase core I protein	2.00 \pm 0.23
Decreased	
Aldehyde dehydrogenase 2	0.12 \pm 0.02
Aldehyde dehydrogenase 5 precursor	0.25 \pm 0.03
ATP synthase a subunit isoform 1	0.50 \pm 0.09
Reference protein	
HSP60	1.02 \pm 0.02

protein spots was smaller than those reported in a recent study investigating the human placental mitochondrial proteome.¹⁵

We then compared the intensity of the spots between core-expressing and control cells. Analysis of repeated experiments by Student's *t* test revealed 13 increased and three decreased spots in intensity in core-expressing cells. These spots were excised and digested with trypsin, then proteins were identified by mass spectrometry. The names of the identified proteins are listed in Table 1. Among them were proteins related to mitochondrial respiratory chain, protein chaperons, and lipid metabolism. Because antibodies to some of these proteins are commercially available, expression levels of the proteins were examined by immunoblotting. The expression levels of the PDH-E1 β subunit and MnSOD, which were identified as increased proteins, were higher in core-expressing cells than in control cells (Fig. 1C), whereas that of HSP60, which was identified as having a similar expression, was unchanged.

Up-regulation of Prohibitin by the Core Protein. Among the identified proteins, we focused on prohibitin, an up-regulated protein in mitochondria of core-expressing cells (Fig. 2A). Prohibitin is a mitochondrial protein associated with cell proliferation.²⁰ It also works as a chaperon of mitochondrial proteins.^{21,22} We confirmed an increased prohibitin expression level in core-expressing cells

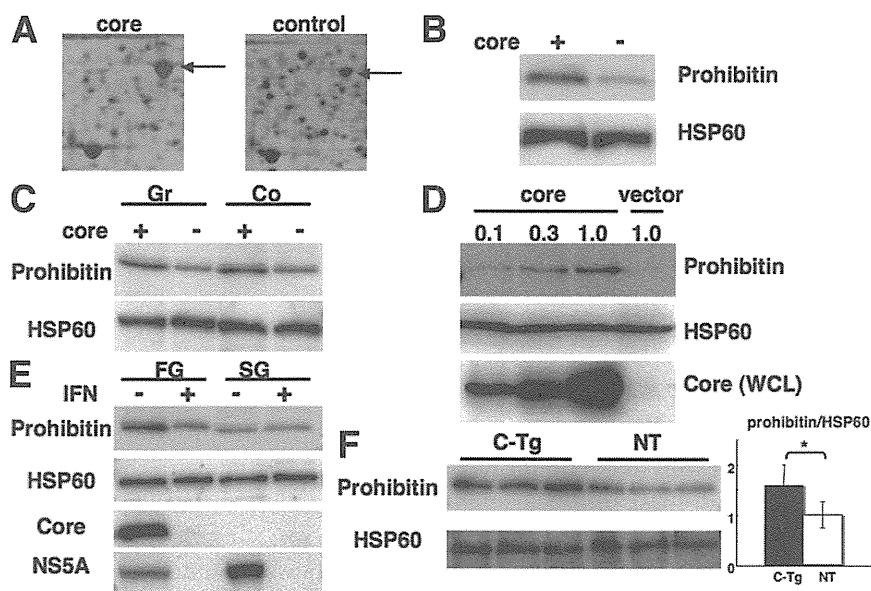


Fig. 2. Up-regulation of prohibitin in core-expressing cells. (A) Protein spot corresponding to prohibitin (arrow) in 2D-PAGE. (B) Purified mitochondria from core-expressing or control cells were subjected to SDS-PAGE and immunoblotted with anti-prohibitin or anti-HSP60 antibody. (C) Mitochondria were purified from growing (Gr) or confluent (Co) cells in 100-mm dishes and subjected to SDS-PAGE, then immunoblotted with an anti-prohibitin or anti-HSP60 antibody. (D) HepG2 cells in six-well plates were transfected with different amounts (μ g) of core-expressing plasmid and mitochondrial proteins were analyzed by immunoblotting with anti-prohibitin or anti-HSP60 antibody. The expression levels of the core protein in whole-cell lysates (WCL) were also determined. (E) Cells harboring HCV replicon were untreated or treated with IFN and expression levels of prohibitin in mitochondria were determined. Expression of HCV core and NS5A proteins was also examined. FG, full-genomic replicon cells; SG, subgenomic replicon cells. (F) Expression levels of prohibitin in mitochondria were determined in liver tissues HCV core-gene transgenic and nontransgenic mice. Prohibitin/HSP60 expression levels were determined by densitometry. C-Tg, core-gene transgenic mouse; NT, nontransgenic littermate ($n = 3$) * $P < 0.05$.

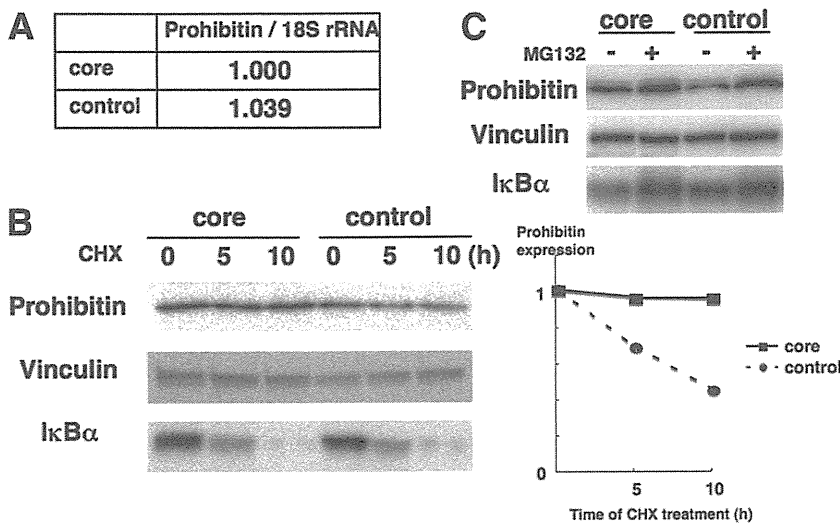


Fig. 3. Increased protein stability of prohibitin in core-expressing cells. (A) RNA was extracted from core-expressing and control cells, and the amount of specific mRNA was determined by real-time PCR with specific primers/probe against prohibitin. The amount of prohibitin mRNA was standardized by that of 18S ribosomal RNA (18S rRNA). (B) Cells were incubated with 100 ng/mL cycloheximide and harvested at the timepoints indicated above the lanes (numbers are hours of cycloheximide treatment). Whole-cell lysates were subjected to SDS-PAGE and immunoblotted with anti-prohibitin, anti-IκBα, or anti-vinculin (as an internal standard) antibody. The intensity of each band was measured by densitometry, and expression levels (prohibitin/vinculin) are shown in the right panel. (C) Cells were harvested after incubation with 20 μM MG132 for 8 hours and subjected to immunoblotting with anti-prohibitin, anti-IκBα, or anti-vinculin antibody.

by immunoblotting (Fig. 2B). Because prohibitin is associated with cell proliferation, it is possible that prohibitin expression changed according to the cell proliferative status. As shown in Fig. 2C, core-expressing cells had high prohibitin expression levels in the cells in both confluent growth and growing statuses compared with control cells. We also determined the expression levels in cells synchronized with aphidicolin followed by l-mimosine treatment and found an increased expression level in core-expressing cells (data not shown). To exclude the possibility that the increased prohibitin expression level is due to the expansion of limited cell clones, not specific to the core protein expression, we examined prohibitin expression in cells transiently expressing the core protein and found that prohibitin expression level increased dose-dependently in core-expressing cells (Fig. 2D). We also examined the prohibitin expression levels in Huh7 cells harboring full- or subgenomic HCV replicon. For this purpose, we used interferon (IFN)-treated replicon cells (cured cells) as a control. Core and nonstructural (NS)5A proteins were not detected after treatment of full-genomic replicon cells with IFN, suggesting a successful elimination of replicon. Prohibitin expression levels in cells with full-genomic replicon were increased compared with those in IFN-treated cured cells, whereas levels of prohibitin expression were low in subgenomic replicon cells regardless of IFN-treatment (Fig. 2E). In addition, prohibitin expression levels were also increased in livers of 3-month-old transgenic mice expressing the core protein compared with those in nontransgenic littermates (Fig. 2F).

We next sought to determine the mechanism of the increased steady-state level of prohibitin in core-expressing cells. To determine prohibitin messenger RNA (mRNA) expression, we performed a real-time polymerase chain reaction (PCR) using specific primers/probe.

No difference in prohibitin mRNA was observed between core-expressing and control cells (Fig. 3A). We next determined the stability of prohibitin in these cells. By treating the cells with cycloheximide, the expression levels of prohibitin gradually decreased in control cells (Fig. 3B). On the other hand, in core-expressing cells prohibitin was hardly degraded by cycloheximide treatment for 10 hours, whereas IκBα was equally degraded in both cells. This result suggests that prohibitin was stabilized in the presence of the core protein. Because prohibitin has been shown to be degraded by proteasome,²³ we examined expression levels of prohibitin in the presence of proteasome inhibitor MG132. By treatment with MG132, prohibitin expression was increased to the similar level in core-expressing and control cells. These results suggest that the core protein may inhibit proteasomal degradation of prohibitin by some mechanism, including the prevention of degradation by interaction with the core protein. Then, core-expressing cells were lysed and subjected to immunoprecipitation with an anti-prohibitin antibody. As shown in Fig. 4, the core protein was coimmunoprecipitated with an anti-prohibitin antibody. To exclude a non-specific interaction with the antibody or Sepharose beads, cells expressing a small amount of prohibitin by transfection with small interfering RNA (siRNA) against prohibitin were also examined. In these cells the amount of the coimmunoprecipitated core protein decreased. In addition, the core protein was not coimmunoprecipitated by control immunoglobulin G (IgG), indicating a specific interaction of prohibitin with the core protein. These results suggest that prohibitin expression increased in core-expressing cells owing to the increased stability presumably by interaction with the core protein.

Impaired Chaperon Function of Prohibitin in Core-Expressing Cells. We next examined the effect of

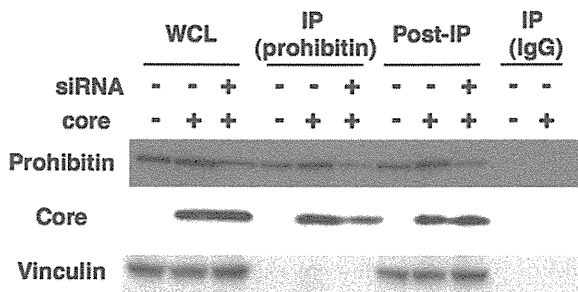


Fig. 4. Interaction of the core protein with prohibitin. Core-expressing and control cells were transfected with or without siRNA against the prohibitin gene, then harvested and lysed in NET-N buffer 3 days after transfection. Whole-cell lysates (WCL) were immunoprecipitated (IP) with an anti-prohibitin antibody or control IgG and immunoblotted with anti-prohibitin or anti-core antibody. Supernatants after the immunoprecipitation were harvested and similarly immunoblotted (Post-IP).

the interaction of prohibitin with the core protein on the function of prohibitin. Prohibitin works as a chaperon of mitochondrial proteins. Nijtmans et al.²¹ demonstrated that prohibitin exerts a chaperon function particularly for the stabilization of mitochondrial DNA-encoded proteins. COX is a mitochondrial respiratory complex IV formed by 14 subunits, 10 of which are encoded by nuclear DNA and the rest by mitochondrial DNA.²⁴ We examined the interaction of prohibitin with subunit II of COX encoded by mitochondrial DNA. As shown in Fig. 5A, the level of COX II coimmunoprecipitated with an anti-prohibitin antibody was decreased in core-expressing cells, although the amount of immunoprecipitated prohibitin was higher than that in control cells. On the other hand, the subunit IV of COX encoded by nuclear DNA was similarly coimmunoprecipitated between core-expressing and control cells. When prohibitin expression was decreased by siRNA transfection, coimmunoprecipitation of COX subunits was similarly decreased with the amount of immunoprecipitation of prohibitin itself being low. We next determined expression levels of COX subunits in the mitochondria in these cells. Expression levels of mitochondrial DNA-encoded subunits I and II in core-expressing cells were decreased, whereas the levels of nuclear DNA-encoded subunits IV and VIb were similar to those in control cells. When transfected with prohibitin-siRNA, expression levels of all of the COX subunits examined were decreased in both core-expressing and control cells, suggesting that protein levels of these subunits are dependent on prohibitin (Fig. 5B, see Supporting Fig. 1 for densitometry). Similar data were observed when blots for COX II and IV were developed together in the same membrane (Supporting Fig. 2). We also determined COX activity in these cells and found that core-expressing cells had a significantly decreased COX activity (about 70% of that in control cells, Fig. 5C). These results

suggest that interaction of prohibitin with the core protein is associated with an impaired function of prohibitin as a mitochondrial chaperon, which may trigger disordered assembly and function of mitochondrial respiratory complexes.

Discussion

In the present study we analyzed expression levels of mitochondrial proteins in HepG2 cells expressing the HCV core protein and identified a set of proteins with different expressions. Some of those proteins were related to the mitochondrial respiratory chain (Table 1). Because the core protein was shown to be associated with the induction of oxidative stress,⁷⁻⁹ the core protein may modulate the expression and function of proteins forming mitochondrial respiratory complexes, which naturally

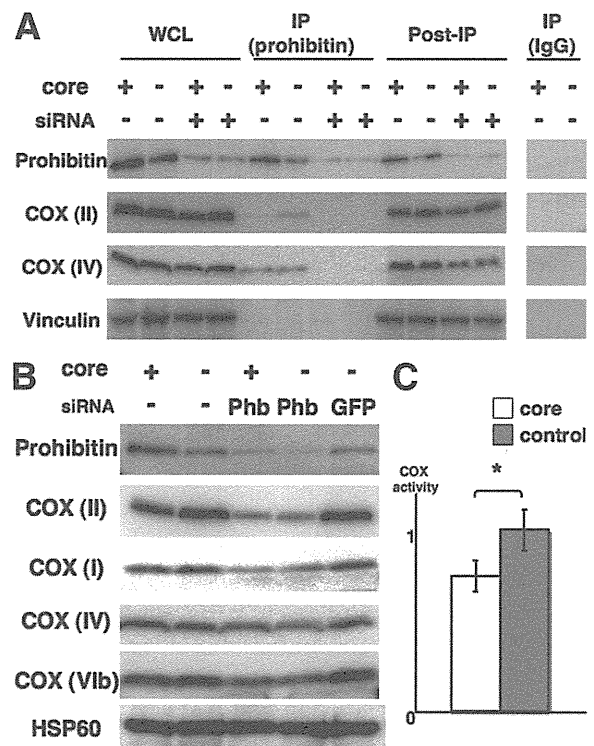


Fig. 5. Effects of core-prohibitin interaction on interaction/expression of COX subunit proteins and COX activity. (A) Whole-cell lysates (WCL) of core-expressing and control cells were subjected to immunoprecipitation with an anti-prohibitin antibody or control IgG, and the interaction of prohibitin with COX subunits was determined by immunoblotting of immunoprecipitated proteins (IP). Supernatants after the immunoprecipitation were harvested and similarly immunoblotted (Post-IP). (B) Cells were transfected with or without siRNA against the prohibitin (Phb) or GFP gene and harvested 3 days after transfection for purification of mitochondria. Purified mitochondria were subjected to SDS-PAGE and immunoblotted with several anti-COX subunits antibodies. The expression levels of HSP60 were also examined as an internal control. (C) COX activity was determined by measuring cytochrome c oxidation. The activity was normalized by taking the average rate of control cells as 1. Data shown are means \pm SE ($n = 5$). * $P < 0.05$.

leads to ROS accumulation. In addition, MnSOD, which plays a key role in protecting cells from oxidative damage, was up-regulated in core-expressing cells, reflecting ROS increase in the cells. Several protein chaperons such as HSP70 and GrpE-like protein co-chaperon were also identified as up-regulated proteins. Because these proteins are known to be important in the mitochondrial protein-import mechanisms, the modulated expression of these proteins may be associated with the different expressions of the identified mitochondrial proteins.

Prohibitin, a mitochondrial protein chaperon, was identified as an up-regulated protein in core-expressing cells. Prohibitin is a ubiquitously expressed and highly conserved protein that was originally determined to play a predominant role in inhibiting cell-cycle progression and cellular proliferation by attenuating DNA synthesis.^{20,25} Prohibitin is present in the nucleus and interacts with transcription factors that are important in cell cycle progression. In core-expressing cells used in this study, prohibitin was also detected in the nucleus and its expression level was also higher than that in control Hepswx cells or HepG2 cells (data not shown). The growth rate of core-expressing cells, however, was similar to that of control cells (data not shown). The physiological significance of the high expression level of prohibitin in the nucleus remains to be determined, but it may be related to enhanced apoptosis by Fas ligand, as shown by Ruggieri et al.,¹⁶ because prohibitin interacts with E2F, Rb, and p53 and modulates the transcription activity of these factors and induces apoptosis.^{26,27}

Mitochondrial prohibitin acts as a protein chaperon by stabilizing newly synthesized mitochondrial translation products through direct interaction.²¹ We examined the interaction between prohibitin and mitochondrially encoded subunit II of COX and found a suppressed interaction between these proteins in core-expressing cells. In addition, there are several studies that showed the association of prohibitin with the assembly of mitochondrial respiratory complex I as well as complex IV (COX).^{21,28} Complex I also consists of both nuclear- and mitochondrial-DNA-encoded subunits; therefore, it is probable that the assembly and function of complex I are impaired by the core protein. We attempted to examine the interaction of prohibitin with the mitochondrial DNA-encoded subunit of complex I, but commercially available antibodies against this subunit could not detect the protein itself by immunoblotting (data not shown). With respect to the complex I function, we found a decreased complex I activity in core-expressing cells (H. Miyoshi et al., manuscript in preparation). Other groups have also shown that complex I activity is decreased in the liver of transgenic mice harboring HCV core and envelope genes⁹

as well as in cultured cells.²⁹ From these findings, the interaction between prohibitin and the core protein may impair the function of complex I as well as complex IV, leading to an increase in ROS production. In fact, the suppression of the prohibitin function is shown to result in an increased production of ROS,³⁰ a phenomenon observed in core-expressing cells used in this study (Miyoshi et al., in prep.) as well as in the liver of core-gene transgenic mice.^{7,8} Interestingly, Berger and Yaffe³¹ showed that loss of function of prohibitin leads to an altered mitochondrial morphology, that is, the loss of the normal reticular morphology and organized mitochondrial distribution. In hepatocytes from the core-gene transgenic mice, we observed a change in morphology of mitochondria, a disappearance of the double structure of mitochondrial membranes.² These changes in mitochondrial morphology are somewhat different, but the dysfunction of prohibitin may be responsible for the morphological abnormality of mitochondria observed in the core-gene transgenic mice.

We concluded that prohibitin overexpression is due to increased stability induced by the interaction with the core protein. In this study we showed that prohibitin might be degraded by proteasome, although we could not detect ubiquitinated forms of prohibitin. If the degradation is mediated by ubiquitin as reported,²³ it is possible that the interaction with the core protein interferes with ubiquitin-binding and protects prohibitin from degradation by proteasome. Some posttranslational protein modifications such as phosphorylation are other possible factors for the stabilization, because prohibitin can be serine-phosphorylated³²; however, in our examination no serine/threonine/tyrosine phosphorylation of prohibitin was detected in core-expressing cells (data not shown). Thus far, there are no studies showing that prohibitin stabilization leads to a suppressed function as a mitochondrial chaperon. Therefore, this finding is novel and noteworthy because the prohibitin expression level has been considered to be proportional to the chaperon function. Prohibitin is highly expressed in several human tumors.^{33,34} In addition, a 2D-PAGE of the hepatoma cell line HCC-M identified prohibitin as a positively regulated protein.³⁵ In these studies, the mechanism of prohibitin overexpression was not elucidated, but considering that prohibitin is associated with the inhibition of cell proliferation, the function of prohibitin is suppressed by stabilization by some molecules in the tumor, similar to the mechanism we suggest in the current study.

In addition to HepG2 cells constitutively expressing the core protein, increased prohibitin expression levels were also found in livers of core-gene transgenic mice.

The difference in expression levels between the transgenic mice and nontransgenic littermates, however, was a little bit smaller than that in the studies of HepG2 cells. This may be due to the low expression level of the core protein in the transgenic mice compared with that in core-expressing HepG2 cells because the expression level of prohibitin was proportionally increased to that of the core protein as shown in this study (Fig. 2D). Otherwise, there might be some *in vivo* mechanism for suppressing prohibitin expression in mice.

In this study, COX subunit IV as well as II were found to interact with prohibitin (Fig. 5A). Although there are no studies demonstrating that prohibitin also works as chaperon for nuclear DNA-encoded mitochondrial proteins as far as we investigated, knockdown of prohibitin expression by siRNA led to decreases in expression levels of both nuclear (COX IV, VIb) and mitochondrial (COX I, II) DNA-encoded subunits in mitochondria (Fig. 5B and Supporting Figs. 1 and 2). We showed that COX IV interacts with prohibitin (Fig. 4), suggesting that prohibitin also works for stable expression of nuclear DNA-encoded COX IV. Degrees of decrease in COX IV and VIb expression, however, were smaller than those in I and II. Prohibitin might contribute to stabilization of COX IV and VIb by mechanism(s) other than chaperon function. Steglich et al.³⁶ showed that prohibitin regulates protein degradation by the m-AAA protease in mitochondria. Recently, Da Cruz et al.³⁷ showed that SLP-2, a member of the stomatin gene family, interacts with prohibitin and regulates the expression of mitochondrial proteins such as COX IV and ND6 of complex I encoded by nuclear DNA by AAA proteases. In view of these findings, COX IV and VIb expression in mitochondria is dependent on prohibitin but other factors may also be involved in the attainment of stable expression of these subunits. The expression levels of COX II and IV in the whole-cell lysates were not so drastic among cell samples (Fig. 5A) compared to those in the mitochondria (Fig. 5B). The reason is not clear, but it is possible that redundant proteins such as improperly folded proteins by lack of chaperons were included in the whole-cell lysates.

In summary, we analyzed mitochondrial proteins in core-expressing HepG2 cells by proteomics analysis and identified prohibitin as an up-regulated protein. The dysfunction of prohibitin induced by the core protein may lead to ROS overproduction in the mitochondrion, which plays a key role in the pathogenesis of chronic hepatitis C. The restoration of prohibitin function might be a therapeutic option for correcting the dysregulated assembly and dysfunction of mitochondrial respiratory chain complexes.

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References

1. Suzuki R, Suzuki T, Ishii K, Matsuura Y, Miyamura T. Processing and functions of Hepatitis C virus proteins. *Intervirology* 1999;42:145-152.
2. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998;4:1065-1067.
3. Naas T, Ghorbani M, Alvarez-Maya I, Lapner M, Kothary R, De Repentigny Y, et al. Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. *J Gen Virol* 2005;86:2185-2196.
4. Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. *J Virol* 2006;80:7199-7207.
5. Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, et al. Molecular determinants for subcellular localization of hepatitis C virus core protein. *J Virol* 2005;79:1271-1281.
6. Schwer B, Ren S, Pietschmann T, Kartenbeck J, Kaehlcke K, Bartenschlager R, et al. Targeting of hepatitis C virus core protein to mitochondria through a novel C-terminal localization motif. *J Virol* 2004;78:7958-7968.
7. Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, et al. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001;61:4365-4370.
8. Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, et al. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002;122:366-375.
9. Korenaga M, Wang T, Li Y, Showalter LA, Chan T, Sun J, et al. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 2005;280:37481-37488.
10. Diamond DL, Jacobs JM, Paeper B, Proll SC, Gritsenko MA, Carithers RL Jr, et al. Proteomic profiling of human liver biopsies: hepatitis C virus-induced fibrosis and mitochondrial dysfunction. *HEPATOLOGY* 2007;46:649-657.
11. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997;78(Pt 7):1527-1531.
12. Moriya K, Todoroki T, Tsutsumi T, Fujie H, Shintani Y, Miyoshi H, et al. Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. *Biochem Biophys Res Commun* 2001;281:1207-1212.
13. Fujie H, Yotsuyanagi H, Moriya K, Shintani Y, Tsutsumi T, Takayama T, et al. Steatosis and intrahepatic hepatitis C virus in chronic hepatitis. *J Med Virol* 1999;59:141-145.
14. Cho WC. Contribution of oncoproteomics to cancer biomarker discovery. *Mol Cancer* 2007;6:25.
15. Lescuyer P, Strub JM, Luche S, Diemer H, Martinez P, Van Dorsselaer A, et al. Progress in the definition of a reference human mitochondrial proteome. *Proteomics* 2003;3:157-167.
16. Ruggieri A, Harada T, Matsuura Y, Miyamura T. Sensitization to Fas-mediated apoptosis by hepatitis C virus core protein. *Virology* 1997;229:68-76.
17. Okado-Matsumoto A, Fridovich I. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. *J Biol Chem* 2001;276:38388-38393.
18. Murakami K, Ishii K, Ishihara Y, Yoshizaki S, Tanaka K, Gotoh Y, et al. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. *Virology* 2006;351:381-392.

19. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996;68:850-858.
20. Mishra S, Murphy LC, Murphy LJ. The prohibitins: emerging roles in diverse functions. *J Cell Mol Med* 2006;10:353-363.
21. Nijtmans LG, de Jong L, Artal Sanz M, Coates PJ, Berden JA, Back JW, et al. Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. *EMBO J* 2000;19:2444-2451.
22. Back JW, Sanz MA, De Jong L, De Koning LJ, Nijtmans LG, De Koster CG, et al. A structure for the yeast prohibitin complex: structure prediction and evidence from chemical crosslinking and mass spectrometry. *Protein Sci* 2002;11:2471-2478.
23. Thompson WE, Ramalho-Santos J, Sutovsky P. Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. *Biol Reprod* 2003;69:254-260.
24. Fontanesi F, Soto IC, Horn D, Barrientos A. Assembly of mitochondrial cytochrome c-oxidase, a complicated and highly regulated cellular process. *Am J Physiol Cell Physiol* 2006;291:C1129-C1147.
25. Mishra S, Murphy LC, Nyomba BL, Murphy LJ. Prohibitin: a potential target for new therapeutics. *Trends Mol Med* 2005;11:192-197.
26. Fusaro G, Dasgupta P, Rastogi S, Joshi B, Chellappan S. Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. *J Biol Chem* 2003;278:47853-47861.
27. Joshi B, Ko D, Ordonez-Ercan D, Chellappan SP. A putative coiled-coil domain of prohibitin is sufficient to repress E2F1-mediated transcription and induce apoptosis. *Biochem Biophys Res Commun* 2003;312:459-466.
28. Bourges I, Ramus C, Mousson de Camaret B, Beugnot R, Remacle C, Cardol P, et al. Structural organization of mitochondrial human complex I: role of the ND4 and ND5 mitochondria-encoded subunits and interaction with prohibitin. *Biochem J* 2004;383:491-499.
29. Piccoli C, Scrima R, Quarato G, D'Aprile A, Ripoli M, Lecce L, et al. Hepatitis C virus protein expression causes calcium-mediated mitochondrial bioenergetic dysfunction and nitro-oxidative stress. *HEPATOLOGY* 2007;46:58-65.
30. Theiss AL, Idell RD, Srinivasan S, Klapproth JM, Jones DP, Merlin D, et al. Prohibitin protects against oxidative stress in intestinal epithelial cells. *FASEB J* 2007;21:197-206.
31. Berger KH, Yaffe MP. Prohibitin family members interact genetically with mitochondrial inheritance components in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1998;18:4043-4052.
32. Ross JA, Nagy ZS, Kirken RA. The PHB1/2 phosphocomplex is required for mitochondrial homeostasis and survival of human T cells. *J Biol Chem* 2008;283:4699-4713.
33. Coates PJ, Nenutil R, McGregor A, Picksley SM, Crouch DH, Hall PA, et al. Mammalian prohibitin proteins respond to mitochondrial stress and decrease during cellular senescence. *Exp Cell Res* 2001;265:262-273.
34. Asamoto M, Cohen SM. Prohibitin gene is overexpressed but not mutated in rat bladder carcinomas and cell lines. *Cancer Lett* 1994;83:201-207.
35. Seow TK, Ong SE, Liang RC, Ren EC, Chan L, Ou K, et al. Two-dimensional electrophoresis map of the human hepatocellular carcinoma cell line, HCC-M, and identification of the separated proteins by mass spectrometry. *Electrophoresis* 2000;21:1787-1813.
36. Steglich G, Neupert W, Langer T. Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. *Mol Cell Biol* 1999;19:3435-3442.
37. Da Cruz S, Parone PA, Gonzalo P, Bienvenut WV, Tondera D, Jourdain A, et al. SLP-2 interacts with prohibitins in the mitochondrial inner membrane and contributes to their stability. *Biochim Biophys Acta* 2008;1783:904-911.

