Hepatitis C Virus Core Protein Inhibits Tumor Suppressor Protein Promyelocytic Leukemia Function in Human Hepatoma Cells

Kerstin Herzer, Sandra Weyer, Peter H. Krammer, Peter R. Galle, and Thomas G. Hofmann

Abstract
Tumor suppressor protein promyelocytic leukemia (PML) is implicated in apoptosis regulation and antiviral response. PML localizes predominantly to PML-nuclear bodies (PML-NB), nuclear macromolecular complexes regulating tumor suppressor protein p53 activity. Consistent with the function of PML in the cellular antiviral response, PML-NBs represent preferential targets in viral infections. In the case of hepatitis C virus (HCV) infection, important characteristics are nonresponsiveness to IFN therapy and development of hepatocellular carcinoma. However, the mechanisms which lead to the development of hepatocellular carcinoma are largely unknown. Here, we show that HCV core protein localizes to the cell nucleus in PML-NBs, where it colocalizes with p53. The HCV core interacts with endogenously expressed PML isoform IV (PML-IV), a key regulator of p53 activity. Importantly, we show that HCV core protein inhibits PML-IV–induced apoptosis and interferes with the coactivator function of PML-IV for proapoptotic p53 target genes including CD95 (Fas/APO-1). In particular, we found that the HCV core inhibits p53-mediated target gene expression by predominantly targeting the coactivator function of PML-IV because HCV core–mediated p53 target gene repression was absent in PML-ablated cells. HCV core expression abrogated both p53 serine 15 phosphorylation and lysine 382 acetylation, two p53-activating posttranslational modifications which were previously linked to an increased PML-NB formation. Taken together, our results suggest a potential mechanism for HCV-associated development of hepatocellular carcinoma through HCV core–mediated inactivation of the PML tumor suppressor pathway. (Cancer Res 2005; 65(23): 10830-7)

Introduction
About 170 million people worldwide are infected with the hepatotropic hepatitis C virus (HCV; ref. 1). HCV infection is among the leading causes of viral hepatitis, and chronic HCV infection is associated with liver cirrhosis and formation of hepatocellular carcinoma (2). HCV is a small RNA virus coding for a limited number of four structural and six nonstructural polypeptides which regulate HCV replication and encapsidation of the viral genome (3). One of the structural components, the HCV core protein, is essential for the packaging of the HCV genome. Besides its structural role in particle formation, the HCV core protein also influences important biological programs of its host cell. For example, HCV core protein has been described to facilitate cellular transformation (4), and its expression in transgenic mice was linked to the development of hepatocellular carcinoma (5). Consistently, HCV core interacts with a number of cellular host factors, including tumor suppressor protein p53 (6–10), a key regulator of the cellular response to genotoxic stress (11) and antiviral response (12). Persistence of infection is a prerequisite for chronic viral infection, which is often found in HCV patients. To persist in its host cells, HCV developed mechanisms to evade the immune system and to circumvent its elimination by immune cells (13). Despite our steadily increasing knowledge about HCV–host cell interaction, the molecular mechanisms which contribute to HCV core–mediated transformation and carcinogenesis are currently incompletely understood.

Tumor suppressor protein promyelocytic leukemia (PML) localizes predominantly to distinct nuclear domains termed PML-nuclear bodies (PML-NB), multiprotein complexes implicated in apoptosis regulation, cellular senescence, and antiviral response (14, 15). PML-NBs are present in almost every human cell type analyzed thus far, and appear as discrete nuclear dots in immunofluorescence. In leukemic blasts of patients suffering from acute promyelocytic leukemia, the PML gene is reciprocally fused with the retinoic acid receptor-α (RARα) gene, thus, resulting in expression of an oncogenic PML-RARα fusion protein (16). PML-RARα protein leads to disruption of PML-NBs in numerous tiny microspeckles, and its expression is sufficient for cellular transformation and induction of leukemia (16). Treatment of acute promyelocytic leukemia patient with all-trans retinoic acid or arsenic trioxide (As2O3) leads to the reformation of PML-NBs and triggers either terminal differentiation or apoptosis of the blasts, which finally results in disease remission (16).

PML expression exerts potent growth-suppressive (17) and apoptosis-inducing activities (18, 19), and PML-deficient mice and cells exhibit defects in multiple apoptosis pathways (20). Furthermore, PML deficiency has been linked to increased susceptibility to viral pathogens (21, 22). It is now established that PML-NBs act as platforms which are involved in the control of p53 activity (23). Consistent with this model, PML isoform IV (PML-IV; ref. 24) serves as a p53 coactivator and colocalizes with numerous factors regulating p53 posttranslational modification, in particular, its phosphorylation and acetylation in PML-NBs, thereby regulating p53 target gene expression and effector function (18, 19, 25–27). Although previous studies showed that HCV core protein interacts with p53 and affects its function (6–10), the reported effects of HCV core on p53 activity are not consistent.
The aim of our study was to elucidate molecular mechanisms which may contribute to HCV-associated pathogenesis, in particular, the development of hepatocellular carcinoma. By focusing on the cellular function of the HCV core protein, we uncovered a previously unidentified link between HCV core and PML-NBs. We found that HCV core protein targets PML-NBs and inactivates the PML tumor suppressor pathway by interfering with the apoptosis-inducing function of PML-IV. Our findings indicate a potential mechanism for HCV infection–associated carcinogenesis, and suggest the HCV core protein as a potential therapeutic target in the treatment of HCV-associated hepatocellular carcinoma.

Materials and Methods

Cell lines and culture conditions. HepG2 and Hep3B cells obtained from the American Type Culture Collection (Rockville, MD) were maintained in DMEM (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated FCS (Invitrogen), 10 mmol/L HEPES (Invitrogen), 5 mmol/L L-glutamine (Invitrogen) and 100 μg/mL gentamicin (Invitrogen) at 37°C and 5% CO2.

Transient transfection and luciferase assays. Transient transfections of Hep3B cells were done using LipofectAMINE (Life Technologies, Gaithersburg, MD), and of HepG2 cells using FuGene6 (Roche, Applied Science, Mannheim, Germany) following the manufacturer's instructions. For the immunoprecipitation experiments, cells were transfected in 10 cm dishes, on the next day, cells were trypsinized, pooled, and split on 6 cm dishes and treated as indicated.

For luciferase assays, cells were plated on six-well plates. The luciferase reporter constructs were cotransfected with control vector pcDNA3, or the expression plasmid for HCV core and wild-type p53 as indicated. One hundred nanograms of Renilla luciferase vector (Promega, Madison, WI) were cotransfected to determine transfection efficiencies. Total DNA amounts were kept equal in all transfections. Cells were harvested after 36 hours and the assay was done according to the manufacturer's instructions. Luciferase activity was measured using the DualLumat LB9507 (Berthold, Wildbad, Germany). Duplicate measurements were done for all experiments. Protein concentrations were determined using the bicinchoninic acid assay (Bio-Rad GmbH, Munich, Germany).

Western blotting and antibodies. Western blot analysis was done as published previously (13). The HCV core antigen was detected with the monoclonal antibody MAI-080 obtained from ABR (Golden, CO). The mouse monoclonal p53-phospho-Ser15 (16G8) and the rabbit polyclonal p53-phospho-Ser 20 and p53-acetylated lysine 382 (Lys382) antibodies were purchased from New England Biolabs, Santa Cruz Biotech, Santa Cruz, CA.

Expression constructs and reagents. The HCV core expression vectors (pcDNA3-HCV core and adenoviral HCV-core vector; ref. 13) as well as the CD95 receptor promoter–driven luciferase reporter gene (28) have been described previously. HCV core sequence was PCR-amplified from HCV strain H77 1a, as described previously (13). The PML-IV expression vector was a kind gift from Giannino Del Sal (Area Science Park, Trieste, Italy; ref. 18), the PIG3 reporter was kindly provided by Matthias Dobbelstein (University of Southern Denmark, Odense, Denmark; ref. 29) and the p53 expression constructs was a gift from Giannino Del Sal (Area Science Park, Trieste, Italy; ref. 18), the PIG3 reporter was kindly provided by Matthias Dobbelstein (University of Southern Denmark, Odense, Denmark; ref. 29) and the p53 expression constructs was a gift from Giannino Del Sal (Area Science Park, Trieste, Italy; ref. 18). Arsenic trioxide was purchased from Sigma-Aldrich (Seelze, Germany).

RNA interference. For RNA interference (RNAi), the following oligonucleotide-targeting sequence flanked by the recommended sequences (30), was inserted into the pSUPER vector to knock-down PML. PML sense, 5'-GAGCTCAAGTGGCACATCA-3'; PML antisense, 5'-TGATGTCGCACTT-3'; this target region is present in all PML isoforms. In the control experiments, empty pSUPER vector was used. The short interfering RNA sequences were verified by BLAST searches to confirm their specificity.

Determination of cell death. As a measurement of apoptotic cell death, DNA fragmentation was quantified by subdiploid DNA content analysis as described previously (13, 31). Analysis was done with a BD Biosciences (Erembodegem, Belgium) FACS flow cytometer and CellQuest software. Specific apoptosis was calculated as 100% × (experimental apoptosis – spontaneous apoptosis in control (%)) / (100% – spontaneous apoptosis in control (%)).

Cell survival assays. Cells were transfected with the expression vectors indicated and subsequently seeded into two culture dishes per sample. For 2 days, one culture dish was harvested and analyzed by immunoblotting and the second dish was fixed and stained with crystal violet as described previously (26). In brief, cells were rinsed twice with PBS and incubated with fixation buffer containing 10% (v/v) methanol and 10% (v/v) acetic acid. After 1 hour, cells were stained with crystal violet [5% (w/v) in fixation buffer] for 30 minutes. Subsequently, cells were rinsed twice with water and air-dried.

Immunofluorescence stainings. Cells were grown in 12-well plates on coverslips and transfected with 1 μg expression vector, washed once with PBS, and fixed for 5 minutes at –20°C with methanol/acetic (1:1). After being dried, cells were blocked for 30 minutes at room temperature in PBS containing 5% (v/v) goat serum. Cells were then incubated for 60 minutes with the primary antibodies at room temperature, subsequently washed five times (5 minutes each) in PBS before incubation for 45 minutes with the appropriate fluorochrome-conjugated secondary antibodies. The mouse monoclonal HCV core antibody, MAI-080 (ABR), and the rabbit polyclonal PML antibody, H-238 (Santa Cruz Biotech), were used as primary antibodies. Alexa-488-coupled goat anti-mouse (Molecular Probes, Eugene, OR) and Texas red–coupled goat anti-rabbit antibodies (The Jackson Laboratory, Bar Harbor, ME) were used as secondary antibodies. Chromosomal DNA was stained with 4′,6-diamidino-2-phenylindole. Stained cells were mounted on glass slides and examined with an epifluorescence microscope (Axioplan-2; Zeiss, Jena, Germany). Images were processed using the Adobe Photoshop 6.0 software.

Commmunonprecipitations. Cells were grown on 10 cm dishes and transiently transfected with 5 μg of the indicated constructs. After 10 hours, cells were lysed and further processed. Coimmunoprecipitation was done after the lysis of (2-3) × 106 cells in high-salt lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 10% (v/v) glycerol, 1% (v/v) NP40, 300 mmol/L NaCl, 150 mmol/L KCl, 5 mmol/L EDTA, 1 mmol/L DTT, 10 mmol/L NaF, 0.5 mmol/L sodium vanadate, 10 μg/mL leupeptin, and 10 μg/mL aprotinin]. After centrifugation, equal amounts of protein contained in the supernatant were diluted with lysis buffer lacking NaCl and KCl to a final concentration of 100 mmol/L NaCl and 50 mmol/L KCl. After the lysates had been precleared with 5 μg control antibody and Protein-A/G-Sepharose beads, 5 μg rabbit anti-PML antibodies (H-238; Santa Cruz Biotech) or 5 μg anti-HCV core antibodies and Protein-A/G-Sepharose beads were added and incubated overnight at 4°C on a rotating wheel. The beads were washed thrice in ice-cold PBS containing 10 mmol/L NaF and 0.5 mmol/L sodium vanadate. Proteins bound to the control antibodies and to the anti-PML antibodies were eluted by boiling for 3 minutes in SDS sample buffer and further analyzed by SDS-PAGE and Western blotting.

Results

Hepatitis C virus core protein localizes to promyelocytic leukemia-nuclear bodies and interacts with promyelocytic leukemia protein. To assess the subcellular localization of the HCV core protein in human hepatoma cells, indirect immunofluorescence stainings of HepG2 cells transfected with an HCV expression construct were done. HCV core protein was predominantly detected in the cytoplasmic compartment (Fig. 1A). However, a small fraction of HCV core protein also localized to the cell nucleus in distinct nuclear bodies (Fig. 1A). This observation was highly reproducible in numerous experiments (data not shown).

The number and the size of the HCV-positive nuclear domains observed was reminiscent of nuclear domains called PML-NBs. To find out whether HCV core protein in fact may localize to PML-NBs, we co-stained HCV core and endogenous PML, which serves as a marker protein for these domains, in human hepatoma cells.
treated with arsenic trioxide, a drug increasing PML-NB formation. Consistent with our initial observation, immunofluorescence stainings showed that a fraction of HCV core protein colocalizes with endogenous PML inside the PML-NBs (Fig. 1B and C). Furthermore, staining of HCV core and endogenous p53 in these cells showed colocalization of both proteins inside PML-NBs (Fig. 1D), indicating that HCV core expression does not interfere with PML-NB recruitment of p53. Of note, for currently unknown reasons, treatment with arsenic trioxide led to a redistribution of the cytoplasmic HCV core protein pool into the nucleus (compare Fig. 1A and Fig. 1B).

Next, we investigated whether the HCV core could form a protein complex with PML in human hepatoma cells either with ectopically expressed or with endogenously expressed PML. To this end, HCV core protein was immunoprecipitated from lysates of HCV core expressing HepG2 cells, which were either left untreated or treated with arsenic trioxide to increase PML-NB formation. The immune-complexes yielded were analyzed by Western blotting.
Importantly, endogenous PML readily coprecipitated with HCV core protein (Fig. 1E, lane 2), and coprecipitation was increased after the induction of PML-NB formation by arsenic trioxide (Fig. 1E, lane 4). Immunoprecipitation analyses of PML-IV and HCV core–expressing cells indicated that the HCV core, in fact, forms a complex with PML-IV, as the endogenously expressed PML isoform coprecipitated with HCV core exactly comigrated with ectopically expressed PML-IV (Fig. 1F, lane 6). Control immunoprecipitations using HepG2 cells transfected with empty vector (Fig. 1E, left) showed the specificity of the observed HCV core–PML interaction. Reciprocal immunoprecipitation analyses also revealed specific coimmunoprecipitation of HCV core protein both with endogenously (Fig. 1F, lanes 2 and 4) and exogenously expressed PML-IV protein (Fig. 1F, lane 6). Immunoblot analysis of the cell lysates used for the immunoprecipitation analysis revealed similar amounts of HCV core and PML (Fig. 1G). Taken together, these findings indicate that HCV core protein forms a protein complex with PML-IV.

**Hepatitis C virus core protein colocalizes and interacts with promyelocytic leukemia protein in the absence of p53.** Both the HCV core protein (6, 7) and PML-IV (18, 19) are known interaction partners of tumor suppressor protein p53. Therefore, we asked the question whether PML-NB recruitment of the HCV core is mediated by interaction with p53, which could corecruit the core to PML-NBs by binding PML-IV. To this end, we analyzed the subcellular localization of HCV core protein in p53-deficient Hep3B hepatoma cells treated with arsenic trioxide. Notably, HCV core protein readily colocalized with PML in PML-NBs in p53-deficient Hep3B cells (Fig. 2A), clearly demonstrating that the HCV core is not recruited to PML-NBs by its interaction with p53. In addition, immunoprecipitation analyses of lysates from Hep3B cells expressing HCV core protein and endogenous PML revealed strong complex formation of both proteins in the absence of p53 (Fig. 2B, lane 2). Interestingly, complex formation was not further increased after arsenic trioxide treatment, suggesting that the PML-HCV core protein complexes already reached a level of saturation (Fig. 2B, lane 4). Coimmunoprecipitation analyses of ectopically expressed PML-IV and HCV core protein confirmed that the PML isoform coprecipitating with HCV core in the absence of p53 was also PML-IV. In addition, reciprocal immunoprecipitation analyses showed specific coimmunoprecipitation of HCV core protein with endogenously (Fig. 2C, lanes 2 and 4) and exogenously expressed PML-IV (Fig. 2C, lane 6). Immunoblot analysis of the cell lysates used for immunoprecipitation analysis confirmed similar levels of HCV core and PML expression (Fig. 2D). In summary, these findings show that PML-NB recruitment of HCV core and its interaction with PML-IV occur independent of its established interaction with p53.

**The hepatitis C virus core protein inhibits protein promyelocytic leukemia–mediated apoptosis.** To define the functional relevance of this protein-protein interaction, we next examined whether the interaction between HCV core and PML has any consequences for PML-dependent biological functions, including cell growth inhibition and induction of apoptosis.

As expected, cell survival assays done on HepG2 cells revealed that PML-IV expression strongly reduced cell growth (Fig. 3A, top). Interestingly, coexpression of HCV core efficiently rescued PML-mediated reduction of cell survival (Fig. 3A, top), indicating that the HCV core counteracts PML function. Immunoblot analysis of cell lysates from HepG2 cells transfected with empty vector (Fig. 3C, lane 2) and HCV core (Fig. 3C, lane 5) showed the specificity of the observed HCV core–PML interaction. Reciprocal immunoprecipitation analyses also revealed specific coimmunoprecipitation of HCV core protein both with endogenously (Fig. 3C, lanes 2 and 4) and exogenously expressed PML-IV protein (Fig. 3C, lane 6). Immunoblot analysis of the cell lysates used for the immunoprecipitation analysis revealed similar amounts of HCV core and PML (Fig. 3G). Taken together, these findings indicate that HCV core protein forms a protein complex with PML-IV.
lysates prepared from aliquots of the cells confirmed similar expression levels of HCV core and PML-IV in the different transfections (Fig. 3A, bottom).

Because PML was also reported to exert p53-independent growth-suppressive effects, we did a similar experiment in p53-deficient Hep3B cells (Fig. 3B). PML-IV expression only slightly reduced cell growth, indicating that the growth-suppressive effect of PML in these cells strongly depends on p53. Of note, the growth-suppressive effect of PML-IV was not rescued by core expression, strongly suggesting that HCV core specifically targets the p53-dependent effector arm of PML.

Next, we studied whether PML expression might reduce cell growth by induction of apoptosis. After expression of PML-IV in HepG2 cells, analysis of the subdiploid DNA content, a hallmark of apoptosis, by FACS analysis revealed up to 25% apoptosis after 24 hours (Fig. 3C). Notably, PML-IV-induced apoptosis was strongly impaired by HCV core expression (Fig. 3C). In contrast, expression of PML-IV in Hep3B cells showed only minor effects on apoptosis induction, and this effect was not significantly affected by HCV core coexpression (Fig. 3D). Thus, these findings indicate that HCV core protein specifically inhibits PML-induced apoptosis by eliminating the p53-dependent PML effector pathway.

**Hepatitis C virus core protein inhibits promyelocytic leukemia-isofrom IV–mediated p53 activation.** PML-NBs are implicated in regulating p53 activity, and PML-IV induces apoptosis by activating the p33 signaling pathway (18, 19). Thus, we investigated the role of HCV core in PML-IV-mediated p53 activation. To measure the influence of HCV core on PML-induced gene expression of genes involved in p53-dependent apoptosis, PML-IV was coexpressed in HepG2 cells along with luciferase reporter gene constructs controlled by promoters of p53 target genes involved in apoptosis. Transcription from the CD95 receptor–promoter was readily induced by PML-IV expression (Fig. 4A). Importantly, promoter induction was significantly inhibited in the presence of HCV core (Fig. 4A). Similar effects were observed on PIG3- and bax promotor–driven luciferase reporter constructs (data not shown). Consistently, regulation of the CD95 receptor was also reflected by increased protein levels as revealed by immunoblotting (Fig. 4B).

To test for the p53-dependence of the HCV core–mediated effect, p53-deficient Hep3B hepatoma cells were transfected with a luciferase reporter gene controlled by the CD95 receptor–promoter, together with various combinations of vectors encoding p53, PML-IV, or HCV core (Fig. 4C). Whereas PML-IV alone failed to induce CD95 receptor–dependent transcription in the absence of p53, it triggered gene expression in the presence of p53, indicating that PML activates CD95 receptor transcription via p53. Interestingly, induction of CD95 receptor–transcription was significantly impaired in the presence of HCV core protein (Fig. 4C). These data show that HCV core interferes with the coactivator function of PML-IV on p53.

Next, we addressed the question of whether the observed inhibition of p53 activity by the HCV core in the absence of PML-IV (Fig. 4C) was because of the interaction of HCV core with endogenous PML. Therefore, we measured p53-mediated transcription of the CD95 receptor reporter gene in HepG2 cells, where PML expression was reduced by RNAi by the expression of a short hairpin (sh) RNA (pSUPER-PML), which specifically targets PML. In contrast to the control pSUPER vector, cotransfection of the shPML construct strongly reduced expression of overexpressed PML-IV in HepG2 cells (Fig. 4D, right) and expression of endogenous PML as judged by immunofluorescence staining (data not shown). Notably, down-regulation of PML by RNAi strongly interfered with p53-mediated reporter gene transcription (Fig. 4D, left), indicating that endogenous PML is critical for this process. To find out whether HCV core–mediated suppression of p53-dependent transcription depends on the presence of PML, we ablated endogenous PML and measured the effect of HCV core on p53-dependent transcription. Strikingly, after PML down-regulation, the inhibitory effect of HCV core on p53-mediated transcription...
was abolished. Taken together, these results indicate that the HCV core predominantly inhibits p53-regulated transcription by eliminating the p53 coactivator function of PML.

Hepatitis C virus core inhibits p53 serine 15 phosphorylation and lysine 382 acetylation. Previously, increased PML-NB formation after overexpression of oncogenic Ras or PML-IV could be linked to increased site-specific phosphorylation of p53 at serine 15 (Ser15; ref. 27), a phosphorylation known to be critical for p53 activation. Because arsenic trioxide treatment, similar to PML overexpression, leads to increased PML-NB formation, we determined the phosphorylation status of p53 after this treatment using p53 phosphospecific antibodies. Treatment with arsenic trioxide resulted in a pronounced increase in p53 Ser15 phosphorylation (Fig. 5A). In contrast, no increase in p53 Ser20 phosphorylation was detected after arsenic trioxide treatment (Fig. 5A). Importantly, in the presence of HCV core protein, p53 Ser15 phosphorylation was strongly reduced (Fig. 5A). Because the acetylation of p53 at Lys382, an event which has been previously linked to PML-NB recruitment of p53 (25, 26), was not found to be increased after arsenic trioxide treatment (Fig. 5A), we used the DNA-damaging chemotherapeutic drug doxorubicin (Adriamycin) to test the effect of HCV core expression on p53 Lys382 acetylation. Interestingly, doxorubicin-triggered p53 acetylation was strongly reduced upon HCV core expression (Fig. 5B). These findings indicate that the HCV core protein targets PML-mediated p53 activation by inhibiting its site-specific phosphorylation and acetylation, and thus, provides a molecular mechanism for its transforming and antiapoptotic function.

Discussion

Chronic HCV infection is often associated with the development of hepatocellular carcinoma. Although HCV core protein expression has been previously linked to the development of hepatocellular carcinoma in a transgenic mouse model (5), the molecular mechanisms leading to HCV-associated hepatocellular carcinoma remain largely unclear.

In the present study, we uncover a novel link between HCV core protein and PML-NBs, nuclear domains involved in apoptosis regulation. We provide evidence that a fraction of HCV core protein colocalizes with PML and p53 in PML-NBs of human hepatoma cells. In addition, we show that the HCV core forms a protein complex with PML-IV, the PML isoform acting as a cofactor for p53 function. As PML-NB recruitment of HCV core protein was also found in p53-deficient hepatoma cells, we could clearly exclude a role for p53, which can bind both HCV core (6, 7) and PML-IV (18, 19) in PML-NB recruitment of the HCV core. Moreover, our experiments revealed that the HCV core abrogates the apoptosis-inducing function of PML-IV by blocking its coactivator function on p53. Consistently, induction of various proapoptotic p53 target genes, namely CD95 (Fas/APO-1), PIG3, and bax (data not shown), was significantly reduced by HCV core expression.

The crucial finding of our study, that HCV core–mediated transcriptional inactivation of p53 is strictly dependent on PML expression, came from reporter gene assays where endogenous PML expression was reduced by RNAi. In addition, reporter gene activation mediated by p53 expression largely relied on PML expression. These findings strongly suggest that in HepG2 hepatoma cells, HCV core protein predominantly interferes with p53 function by targeting PML and not only p53, as suggested by previous reports (6–10). In this context, it is also important to mention that our findings could provide an explanation for the contradictory reports about the effect of HCV core expression on p53 function, because they show a prominent role for PML in this process.

Figure 4. HCV core impairs PML-mediated p53 activation. A, HepG2 cells were transfected with the indicated combinations of PML-IV (1 μg) and HCV core (1 μg) expression vector along with a luciferase reporter gene driven by the CD95 receptor promoter. Transfected DNA amounts were equalized by the addition of empty vector. Columns, mean; bars, SD from at least three independent experiments. B, HepG2 cells were transfected with empty control vector or with combinations of expression plasmids for p53, HCV core, and PML-IV along with a CD95 receptor reporter gene as indicated. Columns, mean; bars, SD from at least three independent experiments. C, Hep3B cells were transfected with empty control vector or with combinations of expression plasmids for p53 and HCV core along with a CD95 receptor luciferase reporter gene and pSUPER or pSUPER-PML as indicated (left). Cells were harvested and luciferase activity was measured. Columns, mean; bars, SD from at least three independent experiments. D, Hep3B cells were transfected with a PML-IV expression vector along with pSUPER or pSUPER-PML as indicated (right). Total cell lysates were analyzed by immunoblotting with the antibodies indicated.
Intriguingly, HCV core expression blocked arsenic trioxide–described a crucial role for PML-NBs in regulating p53 activity by phosphorylation and acetylation. Numerous previous studies mainly controlled through its subcellular localization and post-function of PML-IV. mechanism for how HCV core protein inactivates the effector induced p53 Lys382 acetylation, thus providing a potential molecular with the antibodies indicated. doxorubicin for 24 hours and total cell lysates were analyzed by immunoblotting PML-NBs. vector or a HCV core expression construct were treated with 50 Cancer Res 2005; 65: (23). December 1, 2005 10836 www.aacrjournals.org p53 Ser 15 phosphorylation after increased PML-NB formation, also increased p53 Ser 15 phosphorylation. Cells lysates were analyzed by Western blotting for the phosphorylation of p53 Ser15 and Ser20 using phosphospecific antibodies, and for p53 and HCV core protein expression. A representative experiment is shown. B, HepG2 cell transfected with empty vector or a HCV core expression construct were treated with 50 μg/mL doxorubicin for 24 hours and total cell lysates were analyzed by immunoblotting with the antibodies indicated. C, a proposed model of HCV core function in PML-NBs.

scenario, which could be differentially expressed in the particular cell system used in these studies.

In agreement with a previous report demonstrating increased p53 Ser15 phosphorylation after increased PML-NB formation following Ras- and PML-IV-induced cellular senescence (32), we found that induced PML-NB formation in PML-IV-expressing cells by treatment with arsenic trioxide, a potent inducer of PML-NB formation, also increased p53 Ser15 phosphorylation. Intriguingly, HCV core expression blocked arsenic trioxide–mediated p53 Ser15 phosphorylation as well as doxorubicin-induced p53 Lys382 acetylation, thus providing a potential molecular mechanism for how HCV core protein inactivates the effector function of PML-IV.

p53 is a highly regulated unstable protein whose activity is mainly controlled through its subcellular localization and post-translational modifications, in particular, through site-specific phosphorylation and acetylation. Numerous previous studies described a crucial role for PML-NBs in regulating p53 activity by regulating p53 posttranslational modification, and showed that besides p53, multiple p53 regulators, including acetyltransferase CBP (19, 25, 26), p53 kinases hCdks1/Chk2 (33), and HIPK2 (26, 34) localize to PML-NBs. Because PML-NBs are important centers controlling growth control and apoptosis, this might explain why PML-NBs are preferential targets in viral infection.

Similar to HCV core protein, other viral polypeptides are also capable of interacting with PML, and of disabling its biological function in apoptosis regulation, growth suppression, and cellular senescence. For example, adenoviral E1A protein abrogates oncogenic Ras- and PML-IV-induced cellular senescence by overriding PML function (32). In addition, high-risk human papilloma virus-18 E6 protein targets PML-NBs and abrogates PML-IV-induced cellular senescence by mediating PML degradation (35). These reports, along with our data presented here, strongly suggest that transforming and oncogenic viruses developed strategies to neutralize PML tumor suppressor function, thus suggesting PML as a key obstacle counteracting cellular transformation and carcinogenesis (Fig. 5B). This assumption is consistent with a previous report demonstrating compromised PML expression in numerous human cancers of multiple histologic origins (36). Unfortunately, the role of PML in HCV-associated hepatocellular carcinoma was not addressed in this study.

Because induction of apoptosis is a central mechanism to counteract tumorigenesis, our finding that HCV core expression inactivates the apoptosis-inducing effect of tumor suppressor PML provides a possible explanation for the previously reported transforming activity and carcinogenic function of HCV core (4, 5). Given that PML and its associated PML-NBs regulate p53 activity (23), and, in addition, PML is a crucial mediator of p53-induced cell death (37), the functional inactivation of PML through the HCV core presumably compromises p53 effector function in HCV-infected cells, thereby contributing to chromosomal instability, which is a hallmark of human cancers.

IFNs are powerful antiviral cytokines, and IFN-α is widely used to treat HCV-infected patients (38). Interestingly, a recent report identified p53 as a key regulator of IFN-α/β-mediated antiviral response (12). Because IFNs are potent inducers of PML expression and strongly increase PML-NB number and size (39), this suggests that the up-regulated PML levels and increased PML-NB formation contributes to the therapeutic outcome by reactivating the PML tumor suppressor pathway, which is targeted by the HCV core protein. In HCV patients who do not respond to IFN-treatment, the PML levels reached may not be sufficient to efficiently reanimate the PML tumor suppressor pathways. Future studies will warrant important insight into how HCV-associated hepatocellular carcinoma is linked to PML and its associated nuclear bodies, and whether HCV core protein may be usable as a therapeutic target to treat HCV-associated hepatocellular carcinoma.

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Figure 5. HCV core abrogates p53 Ser15 phosphorylation and Lys382 acetylation. A, either empty control vector or HCV core expression vector was transfected in HepG2 cells as indicated. After 24 hours, cells were treated with 1 μmol/L As2O3 for 12 hours or left untreated as indicated. Cell lysates were analyzed by Western blotting for the phosphorylation of p53 Ser15 and Ser20 using phosphospecific antibodies, and for p53 and HCV core protein expression. A representative experiment is shown. B, HepG2 cell transfected with empty vector or a HCV core expression construct were treated with 50 μg/mL doxorubicin for 24 hours and total cell lysates were analyzed by immunoblotting with the antibodies indicated. C, a proposed model of HCV core function in PML-NBs.
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