NS3 protein of *Hepatitis C virus* associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner

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The N-terminal 198 residues of NS3 (NS3-N) of *Hepatitis C virus* (HCV) subtype 1b obtained from 29 patients, as well as full-length NS3 (NS3-Full), were analysed for their subcellular localization, interaction with the tumour suppressor p53 and serine protease activity in the presence and absence of the viral cofactor NS4A. Based on the subcellular-localization patterns in the absence of NS4A, NS3-N sequences were classified into three groups, with each group exhibiting either dot-like, diffuse or a mixed type of localization. Chimeric NS3-Full sequences, each consisting of an individual NS3-N and a shared C-terminal sequence, showed the same localization patterns as those of the respective NS3-N. Site-directed mutagenesis experiments revealed that a single or a few amino acid substitutions at a particular position(s) of NS3-N altered the localization pattern. Interestingly, NS3 of the dot-like type, either NS3-N or NS3-Full, interacted with p53 more strongly than that of the diffuse type, in both the presence and the absence of NS4A. Moreover, NS3-N of the dot-like type suppressed *trans*-activating activity of p53 more strongly than that of the diffuse type. Serine protease activity did not differ significantly between the two types of NS3. In HCV RNA replicon-harbouring cells, physical interaction between NS3 and p53 was observed consistently and p53-mediated transcriptional activation was suppressed significantly compared with HCV RNA-negative control cells. Our results collectively suggest the possibility that NS3 plays an important role in the hepatocarcinogenesis of HCV by interacting differentially with p53 in an NS3 sequence-dependent manner.

**INTRODUCTION**

Chronic, persistent infection with *Hepatitis C virus* (HCV) often leads to liver cirrhosis and hepatocellular carcinoma (HCC) (Saito *et al.*, 1990). However, the exact mechanisms of HCV-associated pathogenesis and carcinogenesis are largely unknown.

HCV possesses a single-stranded, positive-sense RNA genome of 9-6 kb, which encodes a polyprotein of approximately 3000 aa. The polyprotein is processed into at least 10 structural and non-structural (NS) viral proteins by cellular and viral proteases (Reed & Rice, 2000). One of the viral proteases, the NS3 serine protease, has become a research focus, as it is indispensable for virus replication and, therefore, would be a good target for antiviral drugs. The serine protease is encoded in the N-terminal portion of NS3 and is responsible for cleavage at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions. NS4A, a cofactor for NS3, stabilizes it to augment its serine protease activity, being virtually essential for complete cleavage of the HCV polyprotein (Reed & Rice, 2000). The C-terminal portion of NS3 possesses the NTPase/helicase activity (Kim *et al.*, 1995), which is essential for viral RNA replication.

In addition to its key role in the life cycle of HCV, possible involvement of NS3 in viral persistence and hepatocarcinogenesis has been studied. For example, NS3 was reported to transform NIH3T3 (Sakamuro *et al.*, 1995) and rat fibroblast (Zemel *et al.*, 2001) cells. We also demonstrated that NIH3T3 cells constitutively expressing C-terminally truncated NS3 (aa 1–433) were more resistant to actinomycin D-induced apoptosis than control cells (Fujita *et al.*, 1996). It was also reported that NS3 could block transforming growth factor-β/Smad3-mediated apoptosis (Cheng *et al.*, suppl.)
Moreover, the NS3–4A complex was shown to suppress beta interferon (IFN-β) induction by inhibiting retinoic acid-inducible gene I-mediated activation of IFN regulatory factor 3, counteracting innate immune responses to help establish persistent HCV infection (Foy et al., 2003, 2005; Breiman et al., 2005).

The tumour-suppressor protein p53 functions principally to control cell-cycle arrest and apoptosis upon various cellular stresses, ensuring completion of DNA repair and the integrity of the genome (Levine, 1997). It has been documented that oncogenic viral proteins, such as papillomavirus E6 (Münger & Howley, 2002; Longworth & Laimins, 2004), adenovirus E1B 55K (Martin & Berk, 1998), simian virus 40 large T antigen (Sheppard et al., 1999) and hepatitis B virus X protein (Truant et al., 1995), inhibit p53-mediated apoptosis via interacting with p53. In the case of HCV, NS5A (Lan et al., 2002) and core protein (Kao et al., 2004) were reported to suppress p53-dependent apoptosis. Our previous studies showed that NS3 colocalized with p53 in the nucleus (Ishido et al., 1997; Muramatsu et al., 1997) and that they formed a complex through an N-terminal portion of NS3 (aa 29–174) and a C-terminal portion of p53 (Ishido & Hotta, 1998). In a clinical setting, we found a strong correlation between NS3 sequence diversity and p53 interactions at the tumour suppressor protein p53 functions. In the case of HCV, oncogenic viral proteins, such as papillomavirus E6 (Münger & Howley, 2002; Longworth & Laimins, 2004), adenovirus E1B 55K (Martin & Berk, 1998), simian virus 40 large T antigen (Sheppard et al., 1999) and hepatitis B virus X protein (Truant et al., 1995), inhibit p53-mediated apoptosis via interacting with p53. In the case of HCV, NS5A (Lan et al., 2002) and core protein (Kao et al., 2004) were reported to suppress p53-dependent apoptosis. Our previous studies showed that NS3 colocalized with p53 in the nucleus (Ishido et al., 1997; Muramatsu et al., 1997) and that they formed a complex through an N-terminal portion of NS3 (aa 29–174) and a C-terminal portion of p53 (Ishido & Hotta, 1998). In a clinical setting, we found a strong correlation between HCC and predicted secondary structure of an N-terminal portion of NS3 (Ogata et al., 2003). These observations prompted us to investigate the possible correlation between NS3 sequence diversity and p53 interaction. We report here that subcellular localization of NS3 and its interaction with p53 vary with different NS3 sequences.

**METHODS**

**Plasmid construction.** cDNA fragments encoding the N-terminal 198 residues of NS3 (NS3-N; aa 1–198) of HCV subtype 1b (HCV-1b) isolates were described previously (Ogata et al., 2002, 2003). *Bam*HI and *Hind*III recognition sites were introduced by PCR into the 5′ and 3′ ends of the cDNAs, respectively. The cDNAs were digested with *Bam*HI and *Hind*III and subcloned into pcDNA3.1/Myc-His(−)C (Invitrogen). A single point mutation(s) was introduced into some plasmids by using a QuickChange site-directed mutagenesis kit (Stratagene). Expression plasmids for Myc-tagged full-length NS3 (NS3-Full) of different HCV isolates, MKC1a, M-H05-5, M-45, M-H17-2 and M-42, were reported elsewhere (Hidajat et al., 2005). To express NS3–4A in cis, the corresponding region was amplified from pTMns2-5B/810-2721 (Muramatsu et al., 1997) and subcloned into pcDNA3.1/Myc-His(−)C to generate pcDNA3.1/MKC1a/4A. Expression plasmids for chimeric NS3-Full flanked with residues were derived from MKC1a/4A. They were designated pcDNA3.1/M-H05-5/4A and pcDNA3.1/M-H17-2/4A. The NS3 sequences were subcloned also into pSG5 (Stratagene).

An EcoRI fragment encoding full-length NS4A was obtained from pBSns4A (Muramatsu et al., 1997) and subcloned into pcDNA3.1/Myc-His(−)C and pSG5. Myc-tagged NS4A was amplified from pFK5B/2884Gly (a kind gift from Dr R. Bartenschlager, University of Heidelberg, Heidelberg, Germany) and subcloned into pEF1/Myc-His (Invitrogen). An expression plasmid for Myc-tagged NS4B was reported elsewhere (Tanaka et al., 2006). To express a polypeptide consisting of full-length NS5A and C-terminally truncated NS5B (NS5A/5BAC; aa 1973–2720 of the entire HCV polyprotein), the corresponding region was amplified from pTMns2-5B/810-2721 (Muramatsu et al., 1997) and subcloned into pcTM1 (Moss et al., 1990).

An Xhol fragment encoding full-length wild-type p53 was obtained from pBlSp53-1-393 (Ishido & Hotta, 1998) and subcloned into pcDNA3.1/Myc-His(−)C. pSG5/p53 (Flores et al., 2002) was also used.

All of the plasmid constructs were verified for the correct sequence by DNA sequencing.

**Cell culture and protein expression.** Huh-7 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For protein expression, cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (VT7-3) (Fuerst et al., 1986). After 1 h, the cells were transfected with the expression plasmids by using Lipofectin reagent (Invitrogen). After cultivation overnight, the proteins expressed in the cells were analysed by co-immunoprecipitation, immunoblot and immunofluorescence techniques, as described below. For the luciferase reporter assay, Huh-7 cells were transfected with plasmids by using Fugene 6 transfection reagent (Roche) and cultivated for 24 h before analysis.

Huh-7 cells stably harbouring an HCV subgenomic RNA replicon were prepared as described previously (Taguchi et al., 2004; Hidajat et al., 2005), using pFK5B/2884Gly (Lohmann et al., 2001). Cured Huh-7 cells were prepared by treating the HCV replicon-harbouring cells with IFN-α (1000 IU ml<sup>−1</sup>) for 1 month (Hidajat et al., 2005). Full-length HCV RNA-harbouring Huh-7 cells, designated O, and IFN-cured cells, designated Oc, were described previously (Ikeda et al., 2005).

**Indirect immunofluorescence.** Cells expressing Myc-tagged NS3 were fixed with methanol at −20 °C for 20 min and incubated with an anti-Myc mouse mAb (9E10; Santa Cruz Biotech) for 1 h at room temperature. In some experiments, an anti-NS3 mouse mAb (AA-3; a kind gift from Dr I. Fuke, Research Foundation for Microbial Diseases, Osaka University, Kaga, Japan) was used to detect NS3-Full. An anti-haemagglutinin (HA) mouse mAb (HA11; Covance Inc.) served as a control IgG. After being washed with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (MBL) and observed under a laser-scanning confocal microscope (LSM510 version 3.0; Carl Zeiss).

**Immunoprecipitation and immunoblotting.** Cells expressing NS3 (Myc-tagged or untagged) and p53 were lysed in a stringent RIPA buffer containing 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.1% sodium deoxycholate and protease inhibitor cocktail (Roche) for 30 min on ice. The cell lysates were centrifuged and the supernatants were cleared by mixing with 0.25 µg normal rabbit IgG (Santa Cruz Biotech) and 15 µl protein A–Sepharose beads (Amersham Biosciences) at 4 °C for 30 min on a rotator to reduce non-specific precipitation. The cleared lysates were incubated with anti-p53 rabbit polyclonal antibody (FL-393; Santa Cruz Biotech) at 4 °C for 1 h and subsequently with 15 µl protein A–Sepharose beads for another 1 h. The beads were washed six times with RIPA buffer and the immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting (see below). To analyse the interaction of NS3 expressed in the context of HCV RNA replication with p53, the HCV subgenomic or full-length RNA replicon-harbouring cells were lysed in a mild RIPA buffer without 0.1% SDS and 0.1% sodium deoxycholate. The lysates were subjected to immunoprecipitation analysis in the same way as described above, except that the beads were washed with PBS instead of RIPA buffer. Anti-FLAG rabbit polyclonal antibody (Sigma) served as a control.
Sequence-dependent interaction between HCV NS3 and p53

Immunoblot analysis was performed as described previously (Hidajat et al., 2005). Mouse mAbs against Myc (9E10), NS3, NS4A (S4-13; a kind gift from Dr I. Fuke) and p53 (Ab-1; Calbiochem) were used as primary antibodies and peroxidase-labelled goat anti-mouse IgG (MBL) as a secondary antibody. The protein bands were visualized by an enhanced chemiluminescence method (ECL; Amersham Biosciences) and the intensity of the bands was quantified by using NIH Image 1.61.

**Luciferase reporter assay.** p53-Luc (Stratagene), which contains the *Photinus pyralis* (firefly) luciferase reporter gene driven by a basic promoter element plus an inducible cis-enhancer element, containing 14 repeats of the p53-binding sequence (TTCCTGGAACCTTGCCTGT), was used as a reporter plasmid. pRL-SV40 (Promega), which expresses *Renilla* luciferase, was used as a control plasmid to check transfection efficiency. Huh-7 cells prepared in a 24-well tissue-culture plate were transfected transiently with p53-Luc (10 ng), pRL-SV40 (1 ng), pSG5/p53 (5 ng) and pSG5/NS3-N or pSG5/NS3-Full (250 ng) in the absence or presence of pSG5/NS4A (75 ng). After 24 h, the cells were harvested and a luciferase assay was performed by using the Dual-Luciferase Reporter Assay system (Promega), as described previously (Kadoya et al., 2005). Firefly and *Renilla* luciferase activities were measured by using a Luminescencer-JNR AB-2100 (Atto). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample.

**NS3 serine protease activity.** HeLa cells transiently coexpressing N5SA/5BAC and Myc-tagged NS3 were lysed in gel-loading buffer containing 50 mM Tris/HCl (pH 6.8), 5 % 2-mercaptoethanol, 2 % SDS, 0-1 % bromophenol blue and 10 % glycerol. The lysates were separated by SDS-PAGE and analysed by immunoblotting using anti-N5SA (B926; a kind gift from Dr I. Fuke) and anti-Myc antibodies (9E10). Intensity of the bands corresponding to the cleaved N5SA and the uncleaved N5SA/5BAC was measured. Arbitrary units of serine protease activity of each NS3 were calculated by the following formula: protease activity (arbitrary units) = N5SA/(NS5A/5BAC + N5SA).

**RESULTS**

**NS3-N sequences of different HCV-1b isolates exhibit distinct subcellular-localization patterns in a sequence-dependent manner**

We first examined the subcellular localization of NS3-N in HeLa cells. As shown in Fig. 1(a), we noticed three distinct patterns of NS3-N localization: (i) dot-like staining in both the cytoplasm and the nucleus, (ii) diffuse staining predominantly in the cytoplasm and (iii) a mixed pattern of the former two. Of the 29 HCV-1b isolates tested, 15 (52 %) exhibited exclusively the dot-like staining, nine (31 %) the diffuse staining and the remaining five (17 %) the mixed pattern. The subcellular-localization patterns of four NS3-N sequences each from the dot-like, diffuse and mixed staining groups are also shown in Supplementary Fig. S1 (available in JGV Online). Similar results were obtained when NS3-N sequences were expressed in Huh-7 cells (data not shown), suggesting that the distinct localization patterns among different NS3 sequences are not restricted to a particular cell line.

In order to see which amino acid residue(s) affected the subcellular localization of NS3, we determined the sequences of all 29 isolates. Some of the sequences showing the typical localization patterns, along with a standard sequence, are shown in Fig. 1(b). For more information, the sequences of all 29 isolates are shown in Supplementary Fig. S2 (available in JGV Online). We did not find any common amino acid residue(s) that was/were associated with a particular localization pattern. We noticed, however, that a substitution at position 17 or 18 (Ile to Val) was observed with some NS3-N of the dot-like pattern, but not with any NS3 sequences of the other localization patterns. Also, a substitution(s) at positions 150–153 (Val to Ala, Ile to Val) appeared to be more frequent in NS3 sequences of the dot-like pattern. To examine the possible importance of those substitutions, we introduced a point mutation(s) to some NS3-N of the dot-like pattern (Fig. 1c). Introduction of two point mutations at positions 18 and 153 into NS3-N of isolate H05-5 did not alter the localization pattern. However, introduction of an additional two point mutations at positions 80 and 122 altered the localization pattern significantly, with the majority of the cells exhibiting the typical diffuse staining. Similarly, introduction of two mutations at positions 17 and 86, but not of either one alone, into NS3-N of isolate 45 altered the localization pattern from dot-like to diffuse staining. As for isolate 63, a single point mutation at position 150 alone was enough to change the localization pattern of NS3-N. These results suggest that residues at positions 17 or 18, 80–86 and/or 150–153 play an important role in determining the localization pattern of some, but not all, NS3 sequences.

**NS3-N binds to p53 and inhibits its trans-activating activity in an NS3 sequence-dependent manner**

We previously reported that a region near the N terminus of NS3 (aa 29–174) was involved in complex formation with p53 (Ishido & Hotta, 1998). In this study, we examined whether interaction between NS3-N and p53 differs with different NS3-N sequences. We selected two NS3-N sequences each from the dot-like (H05-5 and 45) and diffuse (H17-2 and 42) staining groups. Co-immunoprecipitation analysis demonstrated that NS3-N of isolate H05-5 interacted with p53 most strongly, followed by that of isolate 45, both in the absence (Fig. 2a) and the presence (Fig. 2b) of NS4A. On the other hand, NS3-N of the diffuse-staining group interacted only weakly with p53. The specificity of the interaction between NS3-N and p53 was confirmed by a control experiment, in which neither NS4A nor NS4B bound to p53 under the same experimental conditions (Fig. 2c, left and centre panels). The specificity of the NS3–p53 interaction was also secured by another control experiment using an irrelevant (anti-FLAG) antibody (Fig. 2c, right panel).

Next, we examined the possible effect of NS3-N on p53 function. The plasmid p53-Luc harbours 14 copies of p53-responsive elements and a minimum promoter upstream of a luciferase gene, and is used to monitor p53-dependent transcriptional activity. Interestingly, NS3-N of H05-5 and that of isolate 45 inhibited p53-dependent transcription of the luciferase gene strongly and moderately, respectively.
(Fig. 2d). On the other hand, no inhibition was observed with NS3-N of isolate 42 and even a slight increase in p53-dependent transcription was observed with NS3-N of H17-2.

NS3 forms a stable complex with its cofactor NS4A, which may counteract the NS3-mediated inhibitory action of p53-dependent transcription. In fact, we observed that inhibition of the p53-dependent transcription by NS3-N of the H05-5 isolate was alleviated to some extent, but not completely, by coexpression of NS4A (Fig. 2e).

To further test the possibility that the alteration in the localization pattern of NS3-N affects its interaction with p53, we compared NS3-N of H05-5 with its point mutant H05-5/mut (Fig. 1c) in terms of their p53-binding abilities and inhibitory effects on p53-dependent transcription. The result obtained demonstrated that NS3-N of H05-5/mut, which showed diffuse localization, had weaker p53-binding capacity (Fig. 3a) and exerted weaker inhibition on p53-dependent transcription (Fig. 3b) compared with NS3-N of the parental H05-5, showing the dot-like localization. Similar results were obtained with isolates 45 and 63 and their point mutants (data not shown). Our results thus suggest that NS3-N of the dot-like localization pattern interacts with p53 more strongly and inhibits p53-mediated transcriptional activation more efficiently than that of the diffuse localization.

**NS3-Full sequences exhibit the same subcellular-localization patterns as those of NS3-N sequences derived from the same isolates and interact differentially with NS4A and p53 in an NS3 sequence-dependent manner**

As shown above, NS3-N exhibited a distinct subcellular-localization pattern when expressed alone (see Fig. 1). Moreover, we have reported that NS3, either NS3-N or NS3-Full, enters the nucleus when
**Fig. 2.** Physical and functional interactions between NS3-N and p53 in an NS3 sequence-dependent manner. NS3-N and p53 were coexpressed in the absence (a) and presence (b) of NS4A. Cells that did not express NS3-N served as a control. Cell lysates were immunoprecipitated by using an anti-p53 antibody and probed by immunoblotting using an anti-Myc antibody to detect NS3-N (top row). Efficient immunoprecipitation of p53 was verified (second row). Lysates were probed directly (without being immunoprecipitated with anti-p53 antibody) with anti-Myc and anti-p53 antibodies, respectively, to verify comparable expression levels of NS3-N (third row) and p53 (bottom row). The intensity of the bands for NS3-N co-immunoprecipitated with p53 was quantified and normalized to the expression levels of NS3-N in the lysates. Filled columns and bars represent mean ± SD obtained from three independent experiments. The p53-binding intensity of NS3-N of the isolate H05-5 was expressed as 1 - 0. *P < 0.01; †P < 0.05, compared with isolate 42. (c) Cells expressing Myc-tagged H05-5, NS4A or NS4B together with p53 were analysed by immunoprecipitation using an anti-p53 antibody (left). Lysates were probed directly with anti-Myc and anti-p53 antibodies, respectively (middle). Cells expressing Myc-tagged H05-5 with or without p53 were analysed by immunoprecipitation using an irrelevant (anti-FLAG) antibody (right). (d) Inhibition of p53-dependent transcription by NS3-N in an NS3 sequence-dependent manner. pSG5-based NS3-N expression plasmids were each co-transfected with pSG5/p53, p53-Luc and pRL-SV40 in Huh-7 cells and cultivated for 24 h. Firefly luciferase activity was measured and normalized to Renilla luciferase activity. The luciferase activity in the control cells without NS3-N expression was expressed arbitrarily as 1 - 0. Results are shown as mean ± SD from three independent experiments. *P < 0.01; †P < 0.05, compared with the control. Expression levels of NS3-N in the cells are shown at the bottom. (e) Inhibition of p53-dependent transcription by NS3-N of H05-5 in the absence (filled bars) and presence (open bars) of NS4A. Results are shown as mean ± SD from three independent experiments. *P < 0.01; †P < 0.05, compared with the control.
coexpressed with p53 and that the p53-mediated nuclear localization of NS3 is inhibited by NS4A in an NS3 sequence-dependent manner (Muramatsu et al., 1997). Therefore, we examined the subcellular-localization patterns of NS3-Full of different sequences, both when expressed alone and when coexpressed with p53 and/or NS4A. The NS3-Full sequences tested differ from each other only in the N-terminal 180 residues that are derived from the clinical isolates, with the C-terminal 451 residues being shared among all the strains tested (Fig. 4a; Hidajat et al., 2005). When expressed alone, NS3-Full of all four strains exhibited the same subcellular-localization patterns as those of NS3-N of the same strains (Fig. 4b; data not shown for M-45 and M-42). When coexpressed with NS4A and/or NS4A, NS3-Full was localized in the cytoplasm, especially in perinuclear regions, regardless of the strain tested. Interestingly, when p53 was additionally coexpressed with NS4A, NS3-Full of the dot-like type (M-H05-5 and M-45) showed an increased tendency to accumulate in the nucleus together with p53 (Fig. 4b), with nearly 25% of the cells exhibiting nuclear localization of NS3 (Fig. 4c). Concomitant expression of NS4A in the cytoplasm of the same cells was confirmed by double-staining immunofluorescence analysis (data not shown), the result being consistent with our previous observation (Ishido et al., 1997). On the other hand, NS3-Full of the diffuse type (M-H17-2, M-42 and MKC1a) was localized almost exclusively in the cytoplasm together with NS4A. Similar results were obtained when NS3-N sequences of different isolates were coexpressed with p53 and/or NS4A (data not shown).

We then tested complex formation between NS3-Full of the five different sequences and p53. The results demonstrated clearly that NS3-Full of the dot-like type (M-H05-5 and
M-45) interacted with p53 more strongly than that of the diffuse type (M-H17-2, M-42 and MKC1a), both in the absence and presence of NS4A (Fig. 5a, b). In this connection, it should be noted that the interaction between NS3-Full and p53 was weaker in the presence of NS4A than in its absence. We also examined the interaction of NS3 with p53 when full-length NS3–4A was expressed in cis, where NS3–4A complex formation occurs more efficiently than in trans. The results demonstrated that M-H05-5/4A interacted with p53 more strongly than did M-H17-2/4A (Fig. 5c), again suggesting NS3 sequence-dependent interaction with p53.

NS3 binds to p53 and inhibits its trans-activating activity in HCV RNA replicon-harbouring cells

In order to determine whether NS3 expressed in the context of HCV replication interacted with p53, we used Huh-7 cells harbouring an HCV subgenomic RNA replicon and examined physical and functional interactions between NS3 and p53. Co-immunoprecipitation analysis revealed that NS3 interacted physically with p53 in HCV subgenomic RNA replicon-harbouring cells, albeit with much lower efficiency than in the plasmid-based expression system (Fig. 6a). The specificity of the interaction between NS3 and p53 was confirmed by the lack of interaction between NS4A and p53 in HCV subgenomic RNA-harbouring cells (Fig. 6c). Next, we compared trans-activating activity of p53 between HCV RNA replicon-harbouring cells and the HCV-negative controls (parental and cured Huh-7 cells). We observed that p53-dependent transcription was suppressed significantly in cells harbouring an HCV RNA replicon, either subgenomic or full-length, compared with the parental and cured Huh-7 cells (Fig. 6d, e). These results suggest collectively that NS3 expressed in the context of HCV replication inhibits p53 function.

Fig. 5. Physical interaction between NS3-Full and p53 in an NS3 sequence-dependent manner. NS3-Full of different strains in the absence (a) and presence of NS4A coexpression in trans (b) were analysed as described in the legend to Fig. 2(a). Filled columns and bars represent mean ± SD obtained from three independent experiments. The p53-binding intensity of M-H05-5 was expressed as 1.0. *P < 0.01 compared with M-42. (c) M-H05-5/4A and M-H17-2/4A (NS3–4A coexpression in cis) were analysed as described in the legend to Fig. 2(a), except that anti-NS3 antibody was used instead of anti-Myc antibody. Filled columns and bars represent mean ± SD obtained from three independent experiments. The p53-binding intensity of M-H05-5/4A was expressed as 1.0. †P < 0.05 compared with M-H17-2/4A.

Serine protease activity of NS3-Full in the absence and presence of NS4A

The N-terminal portion of NS3 possesses a serine protease activity that can cleave the NS5A/5B junction even in the absence of NS4A (Lin et al., 1994). By using NS5A/5BΔC as a substrate, we compared the serine protease activities of NS3-Full of different subcellular-localization patterns. A tendency was noted that, in the absence of NS4A, NS3-Full of the dot-like type showed slightly weaker protease activity than that of the diffuse type (Fig. 7). This difference might be attributable, at least partly, to the fact that NS5A/5BΔC was localized diffusely in the cytoplasm (Kim et al., 1999; Mottola et al., 2002; data not shown) and, therefore, could be recognized more easily by NS3 of the same localization pattern than by NS3 of the other type. In the presence of
NS4A, on the other hand, all of the NS3-Full sequences, which accumulated at a perinuclear region of the cytoplasm (see Fig. 4b), exhibited an enhanced and comparable degree of serine protease activity among the five strains (Fig. 7). Similar results were obtained when Huh-7 cells were used instead of HeLa cells (data not shown).

DISCUSSION

In the present study we demonstrated that, when expressed alone, NS3 of HCV-1b isolates, either NS3-N or NS3-Full, exhibited distinct subcellular-localization patterns, i.e. (i) dot-like staining both in the cytoplasm and the nucleus, (ii) diffuse staining predominantly in the cytoplasm and (iii) a mixed type, in a sequence-dependent manner (Figs 1 and 4). Although no significant correlation has been observed so far between the localization patterns of NS3 and the HCC status of the patients, it was interesting to find that NS3-N and NS3-Full of the dot-like staining pattern interacted with p53 more strongly than that of the diffuse-staining pattern (Figs 2a, 3a and 5a). Similar results were obtained when NS3 was coexpressed with NS4A (Figs 2b, 5b and 5c). We also observed that both NS3-N and NS3-Full of the dot-like staining pattern, but not those of the diffuse pattern, were more prone to colocalize with p53 in the nucleus even in the presence of NS4A (Fig. 4). Luciferase reporter analysis demonstrated that NS3-N of the dot-like type, but not that of the diffuse type, suppressed p53-dependent transcriptional activation significantly (Figs 2d and 3b).

When cells are exposed to a variety of stresses, p53 is induced to accumulate in the nucleus, where it functions as a transcription factor for cell-cycle regulators such as p21 (Levine, 1997). Our present results demonstrated that NS3-N of isolate H05-5 inhibited p53-dependent transcription of a reporter gene strongly (Figs 2d and 3b). We need to assess two possible mechanisms for the NS3-N-mediated p53 inhibition: NS3 might inhibit either p53 expression or p53 function itself. Our results showed that p53 expression levels were not altered significantly by NS3-N, irrespective of the localization patterns (Fig. 2a, b, bottom). Similar results that neither p53 mRNA nor protein levels were downregulated by NS3 were reported by Kwun et al. (2001). Overexpression of p53 was even observed in hepatocytes of some, if not all, HCV-infected patients (Loguerico et al., 2003). It is likely, therefore, that NS3-N inhibits p53 function by interacting with it physically.

Fig. 6. Physical and functional interactions between NS3 and p53 in HCV RNA replicon-harbouring cells. p53 was expressed ectopically in Huh-7 cells harbouring either HCV subgenomic (a) or full-length (b) RNA replicon and the parental Huh-7 cells by using a vaccinia virus–T7 hybrid expression method. Cell lysates were analysed as described in Methods. (c) The lack of interaction between NS4A and p53 in HCV subgenomic RNA replicon-harbouring Huh-7 cells was confirmed. Inhibition of p53-dependent transcription in Huh-7 cells harbouring either HCV subgenomic (d) or full-length (e) RNA replicon. Cells were co-transfected with p53-Luc (10 and 50 ng, filled and open bars, respectively), pSG5/p53 (10 ng) and pRL-SV40 (1 ng) as an internal control. After 24 h, firefly luciferase activity was measured and normalized to Renilla luciferase activity. Luciferase activities in the parental Huh-7 cells were expressed arbitrarily as 1-0. Cured cells also served as a control. Results are shown as mean ± SD from three independent experiments. *P < 0.01; †P < 0.05, compared with the parental and cured Huh-7 cells.
We previously reported that a region of p53 near the C terminus (aa 301–360) was involved in complex formation with NS3 (Ishido & Hotta, 1998). This region includes the p53 oligomerization domain (aa 324–355) (Levine, 1997). It is known that the p53 tetramer binds to the p53-response element on promoter sequences most efficiently and, therefore, is most effective in trans-activation of its target genes (McLure & Lee, 1998; Weinberg et al., 2004). Recently, it was reported that proteins of the S100 family disrupted p53 tetramerization via binding to its tetramerization domain (Fernandez-Fernandez et al., 2005). Therefore, it is reasonable to assume that interaction of NS3-N with p53 interferes with its tetramer formation and DNA binding, thereby inhibiting p53-dependent transcriptional activation. It was also reported that a C-terminal portion of p53 (aa 364–393) negatively regulated its DNA-binding capacity (Müller-Tiemann et al., 1998) and that the 14-3-3 proteins could associate with this region to counteract the negative regulation, which resulted in increased DNA binding of p53 (Waterman et al., 1998). It is tempting to speculate that, by binding to a nearby region of p53, NS3-N may impair the association of 14-3-3 proteins with p53, which results in comparably decreased DNA binding of p53. Moreover, p53 is subject to post-translational modifications, including phosphorylation and acetylation, that affect p53 function (Appella & Anderson, 2001). Further study is needed to determine whether such p53 modification status is affected, either directly or indirectly, by NS3-N.

Consistent with the results obtained from transient-expression experiments, physical interaction between NS3 and p53 was also observed in Huh-7 cells harbouring either an HCV subgenomic or full-length RNA replicon, albeit to a smaller extent than in the transient-expression system (Fig. 6). It should be noted that NS3 expressed by the full-length RNA replicon is detected more strongly by the anti-NS3 antibody used in this study than that of the subgenomic RNA replicon. In HCV RNA replicon-harbouring cells, the HCV non-structural proteins are incorporated into the HCV RNA replication complex and, therefore, it is conceivable that only a minor fraction of NS3 is available for the interaction with p53. Nevertheless, p53-mediated transcriptional activation was suppressed significantly in HCV RNA replicon-harbouring cells compared with the controls (Fig. 6d, e). We must consider the possibility that not only NS3, but also other HCV proteins, are involved in the observed p53 inhibition. In fact, interaction between NS5A and p53 has been reported (Lan et al., 2002; Qadri et al., 2002).

In conclusion, our present results have demonstrated that NS3 of HCV-1b can be divided into three groups based on the subcellular-localization patterns and that NS3 of the dot-like localization pattern interacts with, and inhibits the function of, the tumour suppressor p53 more strongly than that of the diffuse type. The observed difference may account, at least partly, for a different degree of the oncogenic capacity of different HCV-1b isolates.

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