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Single-point mutations of hepatitis C virus NS3 that impair p53 interaction and anti-apoptotic activity of NS3

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Abbreviations: HCV, hepatitis C virus; aa, amino acid; GST, glutathione S transferase; PBS, phosphate-buffered saline; wt, wild-type; mut, mutant-type.

Abstract

The N-terminal domain of NS3 of hepatitis C virus (HCV) possesses serine protease activity, which is essential for virus replication. This portion is also implicated in malignant transformation of hepatocytes. We previously demonstrated that an N-terminal portion of NS3 formed a complex with the tumor suppressor p53 and suppressed actinomycin D-induced apoptosis. We report here that single-point mutations of NS3 at position 106 from Leu to Ala (L106A), and position 43 from Phe to Ala (F43A) to a lesser extent, significantly impaired complex formation with p53. Moreover, the L106A mutation impaired an otherwise more distinct anti-apoptotic activity of NS3. F43A and L106A mutations also inhibited serine protease activity of NS3. These results collectively suggest the possibility that Leu^{106} and Phe^{43} are involved in p53 interaction and serine protease activity, and therefore, can be a good target for certain low-molecular-weight compound(s) to inhibit both oncogenic and replicative abilities of HCV.

Key words: hepatitis C virus; NS3; p53; tumor suppressor; protein-protein interaction; point-mutation; apoptosis; serine protease

Introduction

Hepatitis C virus (HCV) was identified as the major etiological agent of most transfusion-associated non-A, non-B hepatitis in 1989 [1,2]. It has been estimated that more than 170 million people are infected with HCV worldwide [3]. HCV easily establishes persistent infection, which may be mild, or sometimes even asymptomatic, in early phases of the disease, but after a decade or two, may cause liver cirrhosis and eventually hepatocellular carcinoma [4].

HCV, an enveloped RNA virus, belongs to genus *Hepacivirus*, family *Flaviviridae* [5]. The viral genome, single-stranded, positive-sense RNA of about 9.6 kb, consists of 5'- and 3'-untranslated regions, and a large open reading frame that encodes a polyprotein precursor of 3,010-3,030 amino acids (aa) [5,6]. The polyprotein is cleaved by the host signal peptidase and virus-encoded two proteases to generate mature viral proteins; four structural proteins, such as Core, E1, E2, p7, and six nonstructural proteins, such as NS2, NS3, NS4A, NS4B, NS5A and NS5B [5,7,8].

HCV NS3 is a multifunctional protein essential for virus replication. Its N-terminal one-third is responsible for chymotrypsin-like serine protease activity that cleaves at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions [9,10]. A minimal portion of the NS3 serine protease activity has been mapped to a region between aa 33 and 178 [11]. Its C-terminal half possesses RNA helicase activity [9,10]. In addition to its important roles in virus replication, NS3 is implicated in malignant transformation. For example, an N-terminal portion of NS3 was reported to transform NIH3T3 cells [12] and rat fibroblast cells [13]. We also observed that an N-terminal portion of NS3 inhibited apoptosis of NIH3T3 cells [14], which is a prerequisite for malignant transformation.

The tumor suppressor p53 is a key molecule in the signaling pathway of apoptosis

induced by DNA damage or deregulated cell cycle [15]. Some DNA tumor viruses are known to interact with p53 to inactivate its function. For example, human papillomavirus E6 forms a ternary complex with p53 and E6AP, an E3 ubiquitin ligase, and this complex formation results in the ubiquitination and subsequent degradation of p53 [16,17]. It has also been demonstrated that simian virus 40 T antigen binds to and inactivate the function of p53 [18]. Similarly, adenovirus E1B [19] and hepatitis B virus X protein [20] were reported to suppress p53 function although their direct binding to p53 appears to be weak.

As for the possible interaction between HCV NS3 and p53, it was reported that NS3 repressed p53-dependent transcriptional activity of $p21^{waf1}$ [21]. Also, we have demonstrated that p53 enhances nuclear accumulation of NS3 [22, 23] and that NS3, through its N-terminal portion, forms a complex with p53 [24]. In this study, we defined a minimum p53-binding region(s) of NS3 and identified single-point mutations that impaired the interaction with p53. We also examined possible biological significance of those mutations.

Materials and Methods

Expression plasmids. cDNA encoding an N-terminal region of NS3 (aa 1 to 198) of the HCV isolate no. 43 [25] (GenBank accession no. **AB072085**) was amplified using primers NS3/M/B and NS3/AS/H (Table 1), and subcloned into pcDNA3.1/Myc-His(-)C (pcDNA) (Invitrogen) to generate pcDns3/1-198. Various deletion mutants of pcDns3/1-198 (see Fig. 1A) were constructed using appropriate sets of primers, NS3-del-S and NS3-del-AS. To express the full-length NS3 (NS3-Full), the entire NS3 sequence was amplified from pBSns3/1-631 [23] (GenBank accession no. **D45172**) using primers NS3/M/B and NS3F/HindIII, and subcloned into pcDNA. The resultant plasmid was designated pcDns3-Full. All the NS3 fragments expressed from these plasmids could be detected by

anti-Myc antibody. Expression plasmids for NS3 fragments tagged with glutathione S transferase (GST) and Myc-His were also constructed. In brief, *Hinc*II-*Bam*HI fragment of pGEX-4T-1 (Amersham Biosciences) containing GST sequence was subcloned into pBlueScript II SK- to generate pBS-GST. An *EcoR*I–*BamH*I fragment of pBS-GST was introduced to pcDNA to generate pcDgst. Various deletion mutants of NS3 were amplified from pcDns3/1-198 and subcloned into pcDgst.

Single-point mutations were introduced using QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instruction. For plasmid-based expression, the Myc-tagged NS3 fragments described above were subcloned into pSG5 vector (Stratagene) downstream of simian virus 40 early promoter.

An expression plasmid for Myc-tagged NS4B, designated pEFns4B, was constructed by amplifying the corresponding sequence from pM094AJ (HCV-1b) [26] using primers NS4B-1-S and NS4B-Xba-AS, and subcloned into pEF1/Myc-His (Invitrogen). A coding region for NS5A/5BΔC polyprotein (aa 1973 to 2721 in the entire HCV polyprotein) was amplified from pTMns2-5b(810-2721) [23] using primers 5A5BncoI and 5A5BpstI, and subcloned into pTM1 vector to generate pTM-NS5A/5BΔC. This plasmid was used to express a substrate for the NS3 serine protease.

To express p53, *Xho*I fragments of pcDM8VAarg/neo and pcDM8A431/neo [22,23,27], which harbor the entire sequences of wild-type (wt) and mutant-type (mut) p53, respectively, were subcloned into pcDNA and pSG5 vectors. Resultant plasmids were designated pcDwt-p53, pcDmut-p53 and pSGwt-p53. pSGwt-p53 was used for plasmid-based expression.

Transient protein expression. HeLa and Huh7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum at

37°C in 5% CO_2. For protein expression, vaccinia virus-T7 hybrid expression and plasmid-based expression systems were used, as reported previously [22–24,28]. After overnight cultivation, the protein expression was examined by the following analyses.

Immunoprecipitation and immunoblotting. Cells were lysed in a buffer consisting of 10 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA. After being clarified by centrifugation, cell lysates were incubated with anti-p53 rabbit antiserum (Santa Cruz) for 1 h at 4°C and then further incubated with protein A sepharose beads (Amersham) on a rotator for 30 min at 4°C. Normal rabbit serum served as a control. After being washed with the buffer, the immunoprecipitates were subjected to SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore). After being blocked with 5% skim milk, the membrane was incubated with mouse monoclonal antibody against either the Myc peptide (Santa Cruz Biotechnol.) or p53 (Ab-1; Calbiochem), and subsequently with peroxidase-labeled goat anti-mouse IgG (MBL, Nagoya, Japan). The protein bands were visualized by using an enhanced chemiluminescence (Amersham) and their intensity measured by LAS-1000 (Fuji Film).

Double-staining immunofluorescence. Cells were fixed with 3.7% paraformaldehyde for 15 min at 37°C and cold methanol for 10 min. After being washed with phosphate-buffered saline (PBS), the cells were incubated with anti-Myc mouse monoclonal antibody (Santa Cruz) and anti-p53 rabbit antiserum (Santa Cruz) for 1 h. The cells were washed with PBS and then incubated with FITC-conjugated goat anti-mouse IgG (MBL) and Cy3-conjugated donkey anti-rabbit IgG (Chemicon) for another 1 h. The cells were observed with a laser scanning confocal microscope (Carl Zeiss).

NS3-expressing cell lines. NIH3T3 cells were maintained in Dulbecco's modification of Eagle's medium containing 10% heat-inactivated fetal calf serum at 37°C in a CO_2 incubator, as described previously [14]. The cells were transfected with pSGns3/1-180, pSGns3/1-180/L106A, or the pSG5 empty vector, together with pSV2-neo as a selection marker, and cultivated in the presence of a neomycin derivative (G418, 600 μg/ml, GIBCO). After 2 weeks, stable transformants were cloned using cloning cylinders and tested for NS3 expression by immunoblotting. Five clones each strongly expressing NS3/1-180 or NS3/1-180/L106A, and the non-expressing control, were selected and mixed to avoid clonal variation.

Quantitative apoptosis assays. Cells were seeded into 6-mm well of a 96-well plate at a concentration of 5×10^3 cells/well and incubated overnight. The cells were then treated with actinomycin D (10 ng/ml) for 3 days. The number of the living cells was monitored every day by using cell proliferation reagent WST-1 (Roche), according to the manufacturer's protocol. In brief, 100 μl of culture medium containing 10% WST-1 was added to each well. After 1 h, the absorbance was measured at 450 nm with a microplate reader.

Measurement of serine protease activity of NS3. Various forms of NS3 were co-expressed with NS5A/5BΔC polyprotein, a substrate for the NS3 serine protease. The cells were lysed and probed with subjected to immunoblot analysis using anti-NS5A mouse monoclonal antibody (kindly provided by I. Yoshida, Research Institute for Microbial Diseases, Kan-Onji Branch, Kagawa, Japan) and peroxidase-conjugated goat anti-mouse IgG (MBL). Intensity of the bands corresponding to the cleaved NS5A and the uncleaved NS5A/5BΔC was measured. Arbitrary units of serine protease activity of each NS3 were calculated by the following formula.

Protease activity (arbitrary unit) = NS5A ÷ (NS5A/5BΔC + NS5A)

Results

Minimum regions of NS3 responsible for complex formation with p53. We previously demonstrated that an N-terminal portion of NS3 (aa 29 to 174) was involved in complex formation with wt-p53 [24]. In this study we aimed to narrow down the p53-binding region(s) and to determine a residue(s) responsible for the interaction. We expressed a number of deletion mutants of NS3 (Fig. 1A). Initial immunoprecipitation experiments revealed that two non-overlapping regions of NS3, one spanning from 1 to 90 and the other from 91 to 198, each formed a complex with p53 (Fig. 1B, lanes 6 and 8). The specificity of the interaction between NS3 and p53 was secured by control experiments (lanes 1 and 2), and also confirmed by the lack of interaction between p53 and a control protein, HCV NS4B (Fig. 1C). These results suggested the possible presence of at least two separate regions responsible for p53 binding. We then tried to express further deleted NS3 sequences. However, those shorter fragments were quite unstable and only poorly detectable, which made the analysis practically impossible. Therefore, we expressed NS3 fragments tagged with GST and Myc-His to overcome this problem.

GST- and/or Myc-tagged NS3 fragments used in this study are shown in Fig. 1A. Using these fragments, we identified three separate regions; NS3/41-90, /61-110 and /121-160 formed a complex with p53 (Fig. 1D, lanes 3, 9 and 15). On the other hand, NS3/46-90, /61-105 and /121-155 fail to interact with p53 (lanes 4, 10 and 16), suggesting that residues at positions from 41 to 45, from 106 to 110, and from 156 to 160 were likely to involved in the interaction.

To identify the responsible residues, we introduced a single-point mutation and tested

the ability of each NS3 mutant to interact with p53. The results clearly demonstrated that a single-point mutation of NS3 at position 43 from Phe to Ala (F43A) abolished the interaction with p53 (Fig. 2, lane 5). Likewise, single-point mutations at position 106 from Leu to Ala (L106A) and position 158 from Val to Ala (V158A) abolished the interaction with p53 (lanes 9 and 15). These results obtained with GST- and Myc-tagged short fragments of NS3 suggested the possible importance for Phe, Leu and Val at positions 43, 106 and 158, respectively, in complex formation with p53.

Residues of NS3 responsible for complex formation with p53. We then introduced the F43A and L106A mutations into Myc-tagged NS3/1-180 and NS3-Full, and examined their possible interaction with p53. The results obtained demonstrated that the introduction of L106A mutation into NS3/1-180 markedly inhibited interaction with p53 while F43A mutation only weakly impaired it (Fig. 3A). On the other hand, V158A mutation did not affect the interaction (data not shown). When introduced to NS3-Full, L106A mutation, again, markedly inhibited interaction with p53 while F43A mutation only weakly (Fig. 3B). These results suggested an important role for Leu at position 106, and Phe at position 43 to a lesser extent, in the interaction between NS3 and p53.

We also examined the possible effect of those single-point mutations on the interaction with mut-p53, which has a mutation at position 273 from Arg to Gln and, unlike wt-p53, localizes exclusively in the cytoplasm [22]. The result showed that, when introduced to NS3/1-180, L106A mutation markedly impaired the interaction with mut-p53 (Fig. 3C). F43A mutation also impaired the interaction with mut-p53, though to a lesser extent than that observed with the L106A mutation. These results are consistent with those obtained with wt-p53 (see Fig. 3A).

Effects of single-point mutations on subcellular localization of NS3 in the presence and absence of wt-p53. We have reported that NS3-Full and its C-terminally truncated form (NS3/1-433) were each co-localized with wt-p53 almost exclusively in the nucleus when transiently expressed in HeLa cells [22,23]. In this study, we investigated the subcellular localization of NS3/1-180, NS3-Full, and their point-mutants both in the presence and absence of wt-p53 in Huh7 cells using plasmid-based expression system. NS3/1-180 was localized both in the cytoplasm and the nucleus when expressed alone (Fig. 4, panel *a*), and was localized almost exclusively in the nucleus when co-expressed with wt-p53 (panel *b*), with the results being consistent with our previous observations [22,23]. NS3/1-180/F43A exhibited practically the same localization patterns as those of the parental NS3/1-180 both in the absence and presence of wt-p53 co-expression (panels *e* and *f*). However, NS3/1-180/L106A was localized exclusively in the cytoplasm, exhibiting a dot-like pattern, irrespective of p53 co-expression (panels *i* and *j*). Similar results were obtained when L106A mutation was introduced into NS3-Full; the L106A mutant showed exclusive cytoplasmic localization, irrespective of wt-p53 co-expression (panels *q* and *r*). Similar localization patterns of NS3/1-180, NS3-Full and their point-mutants in the presence and absence of wt-p53 were consistently observed using vaccinia virus-T7 hybrid expression system in HeLa cells (data not shown).

Impairment of anti-apoptotic activity of NS3 by the L106A mutation. Cells were treated with actinomycin D for 3 days and examined for apoptosis. Consistent with our previous observations with a longer NS3 sequence encoding aa 1 to 433 [14], cells stably expressing NS3/1-180 showed a higher degree of resistance against actinomycin D-induced apoptosis than the non-expressing control. The otherwise distinct anti-apoptotic activity of NS3 was significantly impaired by the L106A mutation (Fig. 5). Significant impairment of NS3

anti-apoptotic activity by the L106A mutation was also evident 2 days after actinomycin D treatment (data not shown). Similar results were reproducibly obtained in separate experiments.

Effects of single-point mutations on serine protease activity of NS3. The NS3 serine protease cleaves NS5A/5BΔC polyprotein into two fragments, NS5A and NS5BΔC. By using this system, we compared serine protease activities of various single-point mutants of NS3. As shown in Fig. 6A, introduction of either F43A or L106A mutation into NS3/1-180 markedly impaired the otherwise strong serine protease activity whereas the inhibitory effect of V158A mutation was marginal. Similarly, when introduced into NS3-Full, L106A mutation markedly impaired the serine protease activity while F43A mutation impaired it moderately (Fig. 6B).

Discussion

We demonstrated in the present study that single-point mutations of NS3, either NS3/1-180 or NS3-Full, at position 106 from Leu to Ala (L106A), and position 43 from Phe to Ala (F43A) to a lesser extent, inhibited complex formation with p53 (Fig. 3). We previously reported that a 146-residue fragment near the N-terminus of NS3 (aa 29 to 174) was involved in the interaction with p53 [22–24]. We also noticed that secondary structure of the N-terminal 120 residues of NS3 was well correlated with the risk for development of hepatocellular carcinoma in HCV-infected patients [25]. These results suggest an important role for the N-terminal portion of NS3, especially Phe and Leu at positions 43 and 106, respectively, in the pathogenesis of HCV, such as anti-apoptotic status [14], a mutation-prone phenotype [29, 30], malignant transformation of cultured cells [12,13] and eventually

development of hepatocellular carcinoma in patients [25]. We previously reported that N-terminal two-thirds of NS3 (aa 1 to 433) inhibited actinomycin D-induced apoptosis [14]. In the present study, we observed that even a shorter NS3 fragment, NS3/1-180, inhibited actinomycin D-induced apoptosis and that the L106A mutation significantly impaired otherwise more distinct anti-apoptotic activity of NS3/1-180 (Fig. 5). Our results collectively suggest the possibility that the L106A mutation impairs NS3 anti-apoptotic activity through decreased p53 binding.

Inhibition of apoptosis is a prerequisite for malignant transformation of the cell. However, another cellular event(s) is required for malignant transformation. In fact, we observed that, despite distinct inhibition of apoptosis, NIH3T3 cells stably expressing the N-terminal two-thirds of NS3 [14] or NS3/1-180 (data not shown) did not exhibit typical malignant phenotypes, such as higher population density in anchorage-dependent culture, colony formation in anchorage-independent culture and tumor formation in nude mice. Our results appear to be inconsistent with a previous report by other researchers that an N-terminal portion of NS3 flanked with a short stretch of a C-terminal portion of NS2 mediated malignant transformation of NIH3T3 cells [12]. This discrepancy might be explained by the differences in aa sequences and expression levels of NS3, and/or cellular characteristics, etc. Further studies are needed to fully understand the mechanism of malignant transformation by HCV.

L106A and F43A mutations also inhibited serine protease activity of NS3 (Fig. 6). Due to the impairment of the enzymatic activity essential for virus replication [5], HCV strains with such a mutation(s) would not replicate efficiently and, therefore, they would become less prevalent in a patients population. Indeed, we have not found the F43A or L106A mutations among ~200 HCV clinical isolates so far tested [25].

Crystal structure analysis of the serine protease domain of NS3 reported previously

[31,32] suggest that the residues at positions 43 and 106 are localized spatially in the close proximity to Ser at position 139, one of the catalytic triad of the NS3 serine protease activity. This may explain why a single-point mutation, such as L106A, impairs two unrelated NS3 properties, p53-binding and serine protease activity. These results collectively imply the possibility that certain low-molecular-weight compound(s) that specifically interacts with the residues at position 106 or 43 interferes with both oncogenic and replicative abilities of HCV.

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References

[1] G. Kuo, Q.-L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, G. E. Tegtmeier, F. Bonino, M. Colombo, W.-S. Lee, C. Kuo, K. Berger, J.R. Shuster, L.R. Overby, D. W. Bradley, M. Houghton, An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis, Science 244 (1989) 362-364.

[2] Q.-L. Choo, G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, M. Houghton, Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome, Science 244 (1989) 359-362.

[3] World Health Organization, Weekly Epidemiol. Rec. 74 (1999) 425-427.

[4] I. Saito, T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Q.-L. Choo, M. Houghton, G. Kuo, Hepatitis C virus infection is associated with the development of hepatocellular carcinoma, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 6547-6549.

[5] K. E. Reed, C. M. Rice, Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties, Curr. Top. Microbiol. Immunol. 242 (2000) 55-84.

[6] N. Kato, M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, K. Shimotohno, Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 9524-9528.

[7] Q.-L. Choo, K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, M. Houghton, Genetic organization and diversity of the hepatitis C virus, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 2451-2455.

[8] M. Hijikata, N. Kato, Y. Ootsuyama, M. Nakagawa, K. Shimotohno, Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 5547-5551.

[9] Y. Tanji, M. Hijikata, S. Satoh, T. Kaneko, K. Shimotohno, Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing, J. Virol. 69 (1995) 1575-1581.

[10] R. Bartenschlager, V. Lohmann, T. Wilkinson, J. O. Koch, Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation, J. Virol. 69 (1995) 7519-7528.

[11] K. Yamada, A. Mori, M. Seki, J. Kimura, S. Yuasa, Y. Matsuura, T. Miyamura, Critical point mutations for hepatitis C virus NS3 proteinase, Virology 246 (1998) 104-112.

[12] D. Sakamuro, T. Furukawa, T. Takegami, Hepatitis C virus nonstructural protein NS3 transform NIH3T3 cells, J. Virol. 69 (1995) 3893-3896.

[13] R. Zemel, S. Gerechet, H. Greif, L. Bachmatove, Y. Birk, A. Golan-Goldhirsh, M. Kunin, Y. Berdichevsky, I. Benhar, R. Tur-Kaspa, Cell transformation induced by hepatitis C virus NS3 serine protease, J. Viral Hepat. 8 (2001) 96-102.

[14] T. Fujita, S. Ishido, S. Muramatsu, M. Itoh, H. Hotta, Suppression of actinomycin D-induced apoptosis by the NS3 protein of hepatitis C virus, Biochem. Biophys. Res. Commun. 229 (1996) 825-831.

[15] A. J. Levine, p53, the cellular gatekeeper for growth and division, Cell 88 (1997) 323-331.

[16] K. Münger, P. M. Howley, Human papillomavirus immortalization and transformation functions, Virus Res. 89 (2002) 213-228.

[17] M. S. Longworth, L. A. Laimins, Pathogenesis of human papillomaviruses in differentiating epithelia, Microbiol. Mol. Biol. Rev. 68 (2004) 362-372.

[18] C. S. Sullivan, J. M. Pipas, T antigens of simian virus 40: molecular chaperones for viral replication and tumorigenesis, Microbiol. Mol. Biol. Rev. 66 (2002) 179-202.

[19] C. Endter, T. Dobner, Cell transformation by human adenoviruses, Curr. Top. Microbiol. Immunol. 273 (2004) 163-214.

[20] S. Murakami, Hepatitis B virus X protein: a multifunctional viral regulator, J. Gastroenterol. 36 (2001) 651-660.

[21] H. J. Kwun, E. Y. Jung, J. Y. Ahn, M. N. Lee, K. L. Jang, p53-dependent transcriptional repression of $p21^{waf1}$ by hepatitis C virus NS3, J. Gen. Virol. 82 (2001) 2235-2241.

[22] S. Ishido, S. Muramatsu, T. Fujita, Y. Iwanaga, W.-Y. Tong, Y. Katayama, M. Itoh, H. Hotta, Wild type, but not mutant-type, p53 enhances nuclear accumulation of the NS3 protein of hepatitis C virus, Biochem. Biophys. Res. Commun. 230 (1997) 431-436.

[23] S. Muramatsu, S. Ishido, T. Fujita, M. Itoh, H. Hotta, Nuclear localization of the NS3 protein of hepatitis C virus and factors affecting the localization, J. Virol. 71 (1997) 4954-4961.

[24] S. Ishido, H. Hotta, Complex formation of the nonstructural protein 3 of hepatitis C virus with the p53 tumor suppressor, FEBS Lett. 438 (1998) 258-262.

[25] S. Ogata, R. H. Florese, M. Nagano-Fujii, R. Hidajat, L. Deng, Y. Ku, S. Yoon, T. Saito, S. Kawata, H. Hotta, Identification of hepatitis C virus (HCV) subtype 1b strains that are highly, or only weakly, associated with hepatocellular carcinoma on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein, J. Clin. Microbiol. 41 (2003) 2835-2841.

[26] T. Takehara, N. Hayashi, Y. Miyamoto, M. Yamamoto, E. Mita, H. Fusamoto, T. Kamada, Expression of the hepatitis C virus genome in rat liver after cationic liposome-mediated in vivo gene transfer, Hepatology 21 (1994) 746-751.

[27] T. Tamura, N. Aoyama, H. Saya, H. Haga, S. Futami, M. Miyamoto, T. Koh, T. Ariyasu,

M. Tachi, M. Kasuga, R. Takahashi, Induction of Fas-mediated apoptosis in p53-transfected human colon carcinoma cells, Oncogene 11 (1995) 1939-1946.

[28] R. H. Florese, M. Nagano-Fujii, Y. Iwanaga, R. Hidajat, H. Hotta, Inhibition of protein synthesis by the nonstructural proteins NS4A and NS4B of hepatitis C virus, Virus Res. 90 (2002) 119-131.

[29] K. Machida, K. T.-N. Cheng, V. M.-H. Sung, S. Shimodaira, K. L. Lindsay, A.M. Levine, M.-Y. Lai, M. M. C. Lai, Hepatitis C virus induces a mutator phenotype: enhanced mutations of immunoglobulin and protooncogenes, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 4262-4267.

[30] K. Machida, K. T.-H. Cheng, V. M.-H. Sung, K. J. Lee, A. M. Levine, M. M. C. Lai, Hepatitis C virus infection activates the immunologic (type II) isoform of nitric oxide synthase and thereby enhances DNA damage and mutations of cellular genes, J. Virol. 78 (2004) 8835-8843.

[31] R. A. Love, H. E. Parge, J. A. Wickersham, Z. Hostomsky, N. Habuka, E. W. Moomaw, T. Adachi, Z. Hostomska, The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site, Cell 87 (1996) 331-342.

[32] J. L. Kim, K. A. Morgenstern, C. Lin, T. Fox, M. D. Dwyer, J. A. Landro, S. P. Chambers, W. Markland, C. A. Lepre, E. T. O'Malley, S. L. Harbeson, C. M. Rice, M. A. Murcko, P. R. Caron, J. A. Thomson, Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide, Cell 87 (1996) 343-355.

Figure legends

Fig. 1. Minimum regions of NS3 responsible for complex formation with wt-p53. (A) Schematic diagram of the HCV genome and NS3. NS3-Full, NS3/1-198 and various deletion mutants are depicted. (B) Various deletion mutants of Myc-tagged NS3 were expressed without (lane 2) or with wt-p53 (lanes 1 and 3 to 8) in HeLa cells by using vaccinia-T7 hybrid expression method. Cell lysates were immunoprecipitated using anti-p53 rabbit antiserum (lanes 2 to 8) or with normal rabbit IgG (lane 1), and the immunoprecipitates were probed with anti-Myc antibody to detect NS3 (upper panel). The lysates were directly (without being immunoprecipitated with anti-p53 antibody) probed with anti-Myc antibody to verify comparable degrees of expression of the NS3 mutants (middle panel). Efficient immunoprecipitation of wt-p53 with anti-p53 antibody was also verified (lower panel). (C) Myc-tagged NS3/1-198 and full-length NS4B were each co-expressed with wt-p53. Cells expressing NS3 alone (lane 1) were also prepared. Cell lysates were immunoprecipitated with anti-p53 antibody, and the immunoprecipitates were probed with anti-Myc antibody (upper panel). The lysates were directly probed with anti-Myc antibody to verify the expression of NS3/1-198 and NS4B (middle panel). Efficient immunoprecipitation of wt-p53 was also verified (lower panel). (D) NS3 mutants tagged with GST and Myc were expressed without (lanes 1, 7 and 12) or with wt-p53 (lanes 2 to 6, 8 to 11, and 13 to 16). Cell lysates were immunoprecipitated using anti-p53 antibody, and then probed with anti-Myc antibody (upper panel). The lysates were directly probed with anti-Myc antibody (middle panel). Efficient immunoprecipitation of wt-p53 was also verified (lower panel).

Fig. 2. Single-point mutations of NS3 responsible for complex formation with wt-p53. GST- and Myc-tagged NS3/31-90, NS3/61-130, NS3/121-198 and their single-point-mutants

were expressed in HeLa cells without (lanes 1, 7 and 13) or with wt-p53 (lanes 2 to 6, 8 to 12, and 14 to 17). Cell lysates were immunoprecipitated using anti-p53 antibody, and probed with anti-Myc antibody to detect NS3 (upper panel). The lysates were directly probed with anti-Myc antibody (middle panel). Efficient immunoprecipitation of wt-p53 was also verified (lower panel). WT, wild-type (without point-mutation).

Fig. 3. F43A and L106A mutations in NS3/1-180 and NS3-Full impair complex formation with wt-p53. (A) Myc-tagged NS3/1-180 and its point mutants were expressed in HeLa cells without (lane 1) or with wt-p53 (lanes 2 to 5). Cell lysates were immunoprecipitated using anti-p53 antibody, and probed with anti-Myc antibody to detect NS3 (upper panel). The lysates were directly probed with anti-Myc antibody (middle panel). Efficient immunoprecipitation of wt-p53 was also verified (lower panel). Amounts of NS3 co-immunoprecipitated with p53 were normalized to the total amounts of NS3 in the lysates. The normalized values, considered as p53-binding capacity of NS3, are depicted in the graph (bottom). (B) Similar analysis was performed using NS3-Full. (C) Similar analysis was performed using mut-p53 and NS3/1-180. WT, wild-type (without point-mutation).

Fig. 4. L106A, but not F43A, mutation alters subcellular localization of NS3/1-180 and NS3-Full in the presence of wt-p53. NS3/1-180, NS3-Full and their single-point mutants were each expressed without or with wt-p53 in Huh7 cells by plasmid-based expression system. The cells were stained with anti-Myc mouse monoclonal antibody and anti-p53 rabbit antiserum, followed by FITC-conjugated anti-mouse IgG (green) and Cy3-conjugated anti-rabbit IgG (red). Merged pictures are shown on the right. WT, wild-type (without point-mutation).

Fig. 5. L106A mutation impairs anti-apoptotic activity of NS3/1-180. Percent cell survival after actinomycin D treatment (10 ng/ml, 3 days) is shown. Data represent the mean ± SD obtained from triplicate cultures of two independent experiments. Expression levels of NS3/1-180 and its L106A mutant in mixed cultures of five clones each are shown at the bottom.

Fig. 6. F43A and L106A mutations in NS3/1-180 and NS3-Full impair the serine protease activity. (A) NS5A/5BΔC, a substrate for NS3 serine protease, was expressed in HeLa cells without (lane 1) or with Myc-tagged NS3/1-180 (lane 2) and its point mutants (lanes 3 to 6). Cell lysates were probed with anti-NS5A antibody to detect uncleaved and cleaved NS5A fragments (upper panel). The lysates were also probed with anti-Myc antibody to verify a comparable degree of NS3 expression in each lane (lower panel). Serine protease activities were measured, as described in the Materials and methods, and depicted in the graph (bottom). (B) Similar analysis was performed using NS3-Full.

Table 1. Oligonucleotide primers used for PCR amplification.

Primer	**Sequence** [a]	**Amplified direction**
NS3/M/B	5'- GCAAGGATCCGCC**ATG**GCGCCTATCACGGCCTA -3'	Forward
NS3/AS/H	5'- CTCCAAGCTTGGAATGTTTGCGGTA -3'	Reverse
NS3FHindIII	5'- CTCCAAGCTTGAGTGACCTCTAGGT -3'	Reverse
NS3-del-S [b]	5'- GCAAGAATCCGCC**ATG** $(N)_{15\sim18}$–3'	Forward
NS3-del-AS [b]	5'- $(n)_4$ AAGCTTC $(N)_{13\sim18}$ -3'	Reverse
NS4B-1-S	5'- CCGCGAATTCAAC**ATG**GCGTGGAGCAGTC -3'	Forward
NS4B-Xba-AS	5'- TATATCTAGACATGGCGTGGAGCAG -3'	Reverse
5A5BNcoI	5'- CCACC**AT**GGCTCCGGCTCGTGGCTCAG -3'	Forward
5A5BpstI	5'- CAGGCTGCAGAGGCCTTCAAGTAACATGTG -3'	Reverse

[a] Restriction enzyme recognition sites are underlined. The translation initiation codon is shown in boldface letters.

[b] A group of primers used to amplify various deletion mutants of NS3 (see Fig. 1A). N, nucleotide residues either identical or complementary to those of the isolate no. 43 [25] (GenBank accession no. **AB072085**); n, arbitrarily added residues that help ensure the recognition of the following sequences by the respective restriction enzymes.

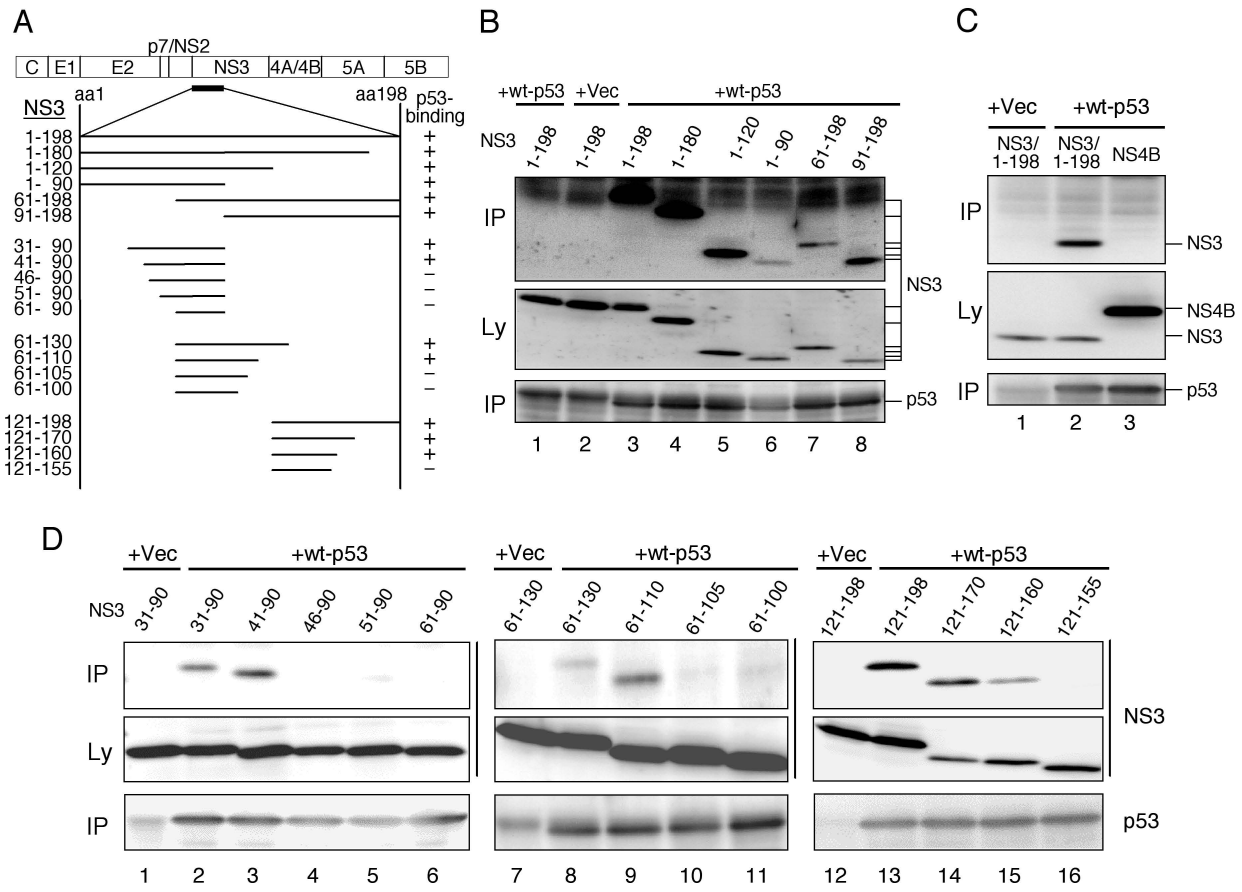

Fig. 1. Tanaka et al.

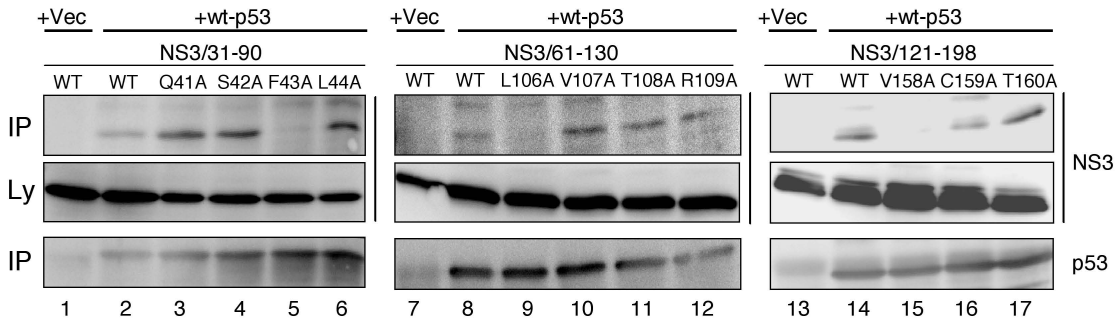

Fig. 2. Tanaka et al.

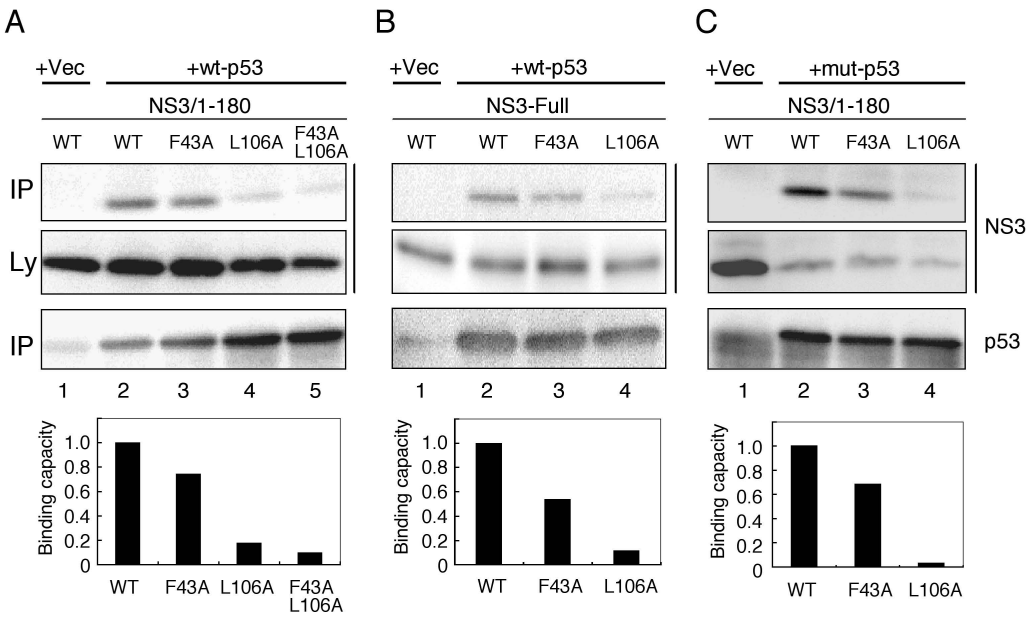

Fig. 3. Tanaka et al.

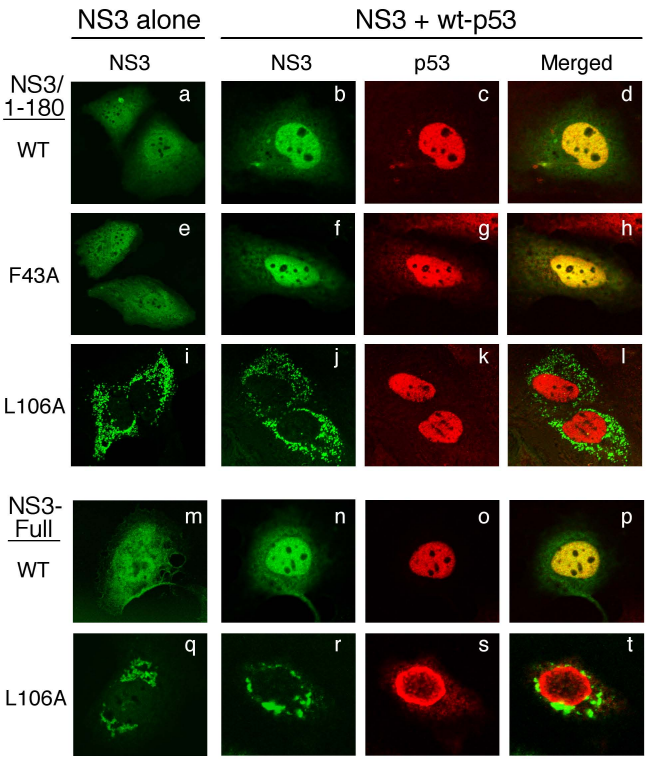

Fig. 4. Tanaka et al.

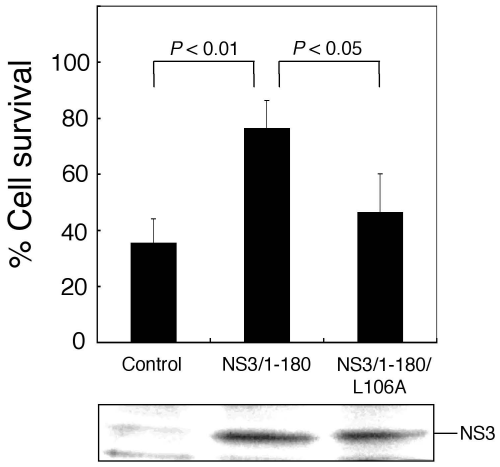

Fig. 5. Tanaka et al.

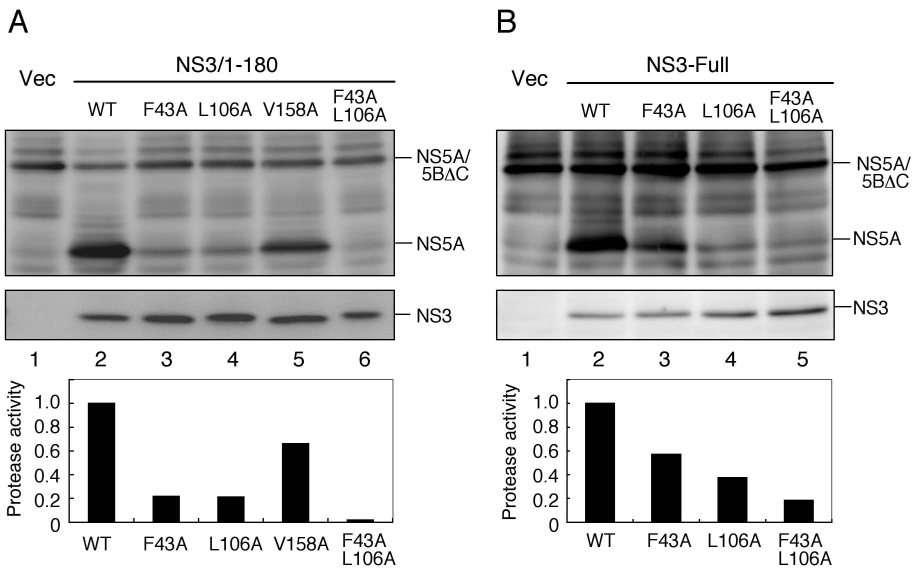

Fig. 6. Tanaka et al.