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Complex formation of the nonstructural protein 3 of hepatitis C virus with the p53 tumor suppressor

Satoshi Ishido, Hak Hotta*

Department of Microbiology, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan

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Abstract By co-immunoprecipitation analysis, we demonstrated that wt-p53 formed a complex with non-structural protein (NS) 3 of hepatitis C virus, both in the absence and the presence of NS4A, a viral cofactor that strongly associates with NS3. Deletional analysis revealed that a portion near the N-terminus of NS3 (amino acids (aa) 1055 and 1200), which is different from the NS4A binding site, was necessary for the complex formation with wt-p53. On the other hand, a portion near the C-terminus of wt-p53 (aa 301–360), which has been reported to contain the oligomerization domain, was important for the complex formation with NS3.

Key words: Hepatitis C virus; Non-structural protein 3; p53 tumor suppressor

1. Introduction

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an enveloped virus, whose genome is single-strand, positive-sense RNA of approximately 9.5 kb. The HCV genome encodes a polyprotein consisting of about 3010–3033 amino acid (aa) residues. This polyprotein has been shown to be cleaved co- and post-translationally into mature viral proteins, which are arranged in the order of NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH [1–3]. The function of certain HCV proteins has been determined. For example, a polyprotein encompassing NS2 and an N-terminal portion of NS3 functions as metalloproteinase to autocatalytically cleave off NS3 [1]. The processed NS3 then possesses serine proteinase activity at its N-terminal portion [2] and RNA helicase activity at its C-terminal portion [3,4]. NS4A is known to be a cofactor for NS3, which augments the serine proteinase activity [5–8]. Also, NS5B is thought to be an RNA-dependent RNA polymerase [9].

HCV causes either acute or chronic persistent infection, with the latter potentially leading to liver cirrhosis and hepatocellular carcinoma [10,11]. The viral genome and antigens have been detected in affected liver cells [12,13]. Recently, it was reported that NS3 [14] or core protein [15] might be involved in malignant transformation of NIH3T3 cells. We previously reported that a C-terminally truncated form of NS3 inhibited actinomycin D-induced apoptosis possibly through an inhibition of p53 accumulation [16] and that NS3 was co-localized with p53 upon co-expression [17]. These results suggest that NS3 might interact with the wild-type (wt)-p53 tumor suppressor to inhibit apoptosis. In this study, we examined the possible association between NS3 and wt-p53 by using vaccinia virus-T7 hybrid expression system. Immunoprecipitation analysis revealed that NS3 formed a complex with wt-p53 through an N-terminal portion of NS3 and a C-terminal portion of wt-p53. It was also demonstrated that the complex formation between NS3 and wt-p53 took place even in the presence of NS4A. The functional implication of their association in carcinogenesis is discussed.

2. Materials and methods

2.1. Construction of expression plasmids

Construction of NS3 and NS4A expression plasmids, pBSns3/1027–1657, pBSns3/1027–1459, pBSns3/1201–1459, and pBSns4A/1658–1711 (see Fig. 1A), was described previously [17,18]. To obtain pBSns3/1027–1245 FLAG, pBSns3/1027–1459 was digested with *Nde*I and *Bam*HI and ligated to an oligonucleotide encoding the peptide FLAG (DYKDDDDK). To obtain pBSns3/1055–1459 and pBSns3/1055–1200FLAG, the corresponding portions were amplified from pBSns3/1027–1459 by using two sets of primers NS3-S1/NS3-R1 and NS3-1/NS3-R2, respectively (Table 1). The amplicons were subcloned into the unique *Eco*RI site, or *Eco*RI and *Bam*HI sites, respectively, of pBlueScript II SK$^-$ (Stratagene). A series of deletion mutants of wt-p53 were obtained as described below. First, the *Xho*I fragment of pCDM8VAarg/neo, which encodes the full-size wt-p53 (p53F), was ligated to *Xho*I-treated pBlueScript II SK$^-$ (Stratagene) to generate pBS53/1–393 (Fig. 1B). To obtain pBS53/40–393, the corresponding portion was amplified from pBS53/1–393 by using primer 53-S1 and 53-R1 (Table 1). To obtain pBS53/1–360, pBS53/1–318 and pBS53/1–300, corresponding portions were each amplified from pBS53/1–393 by using sets of primers 53-S2/53-R2, 53-S2/53-R3, and 53-S2/53-R4, respectively. These PCR products were digested with *Eco*RI and *Xho*I and inserted into the *Eco*RI and *Xho*I sites of pBlueScript II SK$^-$.

2.2. Transient expression by vaccinia virus-T7 hybrid expression system

HeLa cells were used in this study unless otherwise stated. Cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) [19]. After 1 h, the cells were transfected with the expression plasmids using Lipofectin reagent (Life Technologies) according to the manufacturer's protocol. After cultivation for 16 h, the cells were analyzed for the complex formation of the expressed proteins, as described below.

2.3. Immunoprecipitation analysis

Cells transfected with the plasmids were labeled at 12 h postinfection with 25 μCi of ^{35}S-translabel (Amersham) per ml in serum-free medium. Cell lysates were prepared in RIPA buffer consisting of 150 mM NaCl, 0.5% Triton X-100, and 10 mM Tris-HCl (pH 7.5). After being clarified by centrifugation, the cell lysates were incubated for 1 h at 4°C with either one of the anti-NS3, anti-p53 (clone 421; Oncogene Science) or anti-FLAG M2 monoclonal antibodies (Kodak) and 10 μl of protein G/protein A Sepharose (Oncogene Science). After being washed six times with RIPA buffer, the immunoprecipitates were subjected to SDS-PAGE and visualized by using BAS2000 system.

*Corresponding author. Fax: (81) (78) 351-6347.
E-mail: hotta@kobe-u.ac.jp

Abbreviations: HCV, hepatitis C virus; NS, non-structural protein; aa, amino acid; ΔC, carboxy-terminally truncated; ΔNΔC, amino- and carboxy-terminally truncated; F, full-size; ΔN, amino-terminally truncated; wt-p53, wild-type p53; mt-p53, mutant-type p53

PII: S0014-5793(98)01312-X

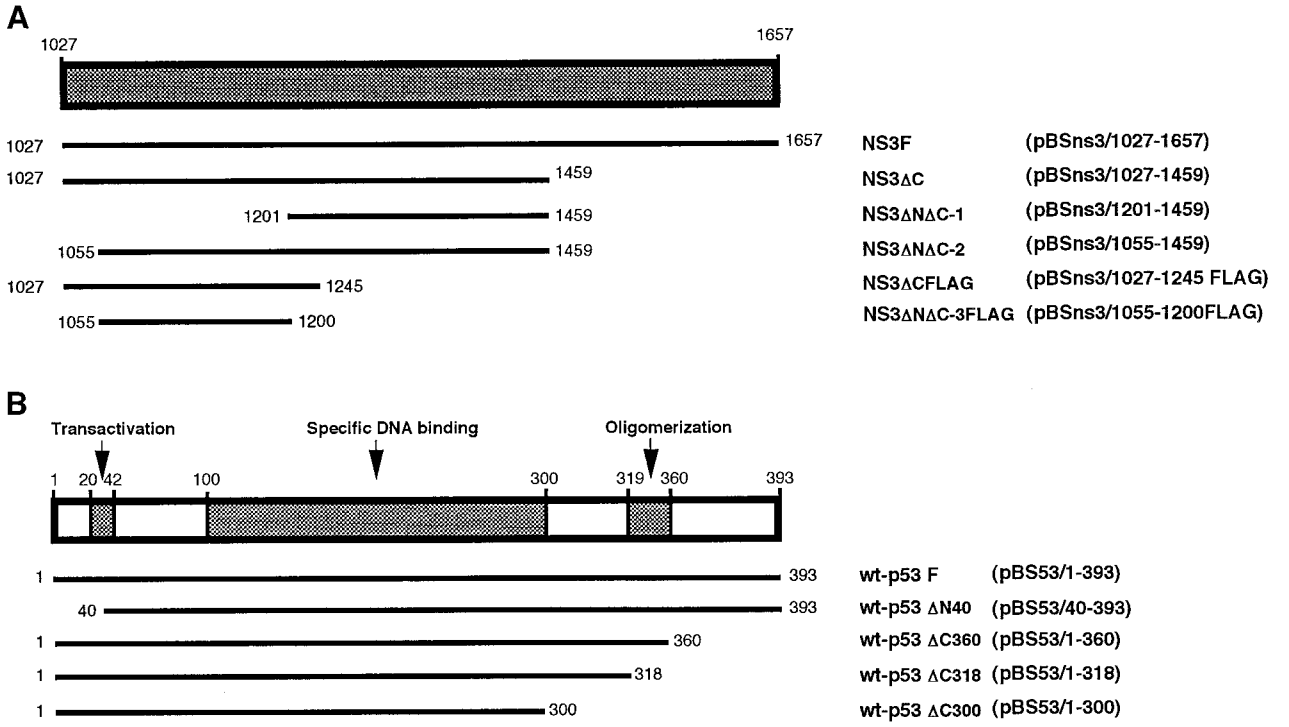

Fig. 1. Schematic representation of the open reading frame encoding full-size and N- and/or C-terminally deleted forms of NS3 and wt-p53. A: NS3. B: wt-p53. Various functional domains are shown on the top. The numbers indicate amino acid positions.

3. Results and discussion

3.1. NS3 forms a complex with wt-p53

By immunofluorescence analysis in vaccinia virus-T7 hybrid expression system, we previously demonstrated that NS3F and NS3ΔC, but not NS3ΔNΔC, were each co-localized with wt-p53 [17]. In the present study, we examined the possible complex formation between NS3 and p53 by immunoprecipitation analysis. In the initial experiment, we used NS3F, NS3ΔC and NS3ΔNΔC-1 (the same as NS3ΔNΔC in the previous study) (Fig. 1). Each form of NS3 was co-expressed with the full-size wt-p53 (p53F) in HeLa cells. Immunoprecipitation analysis was performed using anti-NS3 monoclonal antibody, anti-p53 monoclonal antibody or control IgG. Anti-NS3 monoclonal antibody co-precipitated wt-p53 with NS3F and NS3ΔC, but not with NS3ΔNΔC-1 (Fig. 2A–C, lane 2). Likewise, anti-p53 monoclonal antibody co-precipitated NS3F and NS3ΔC, but not NS3ΔNΔC-1, with wt-p53 (Fig. 2A–C, lane 3). Thus, NS3 was likely to form a complex with wt-p53 through an N-terminal portion of NS3 (aa 1027–1200). This portion has been shown to contain interaction domains for NS4A [6–8,11] and NS5B [20].

HeLa cells have been reported to express the E6 and E7 oncoproteins of human papilloma virus [21], the former of which can interact with wt-p53 [22]. To exclude the possibility that the complex formation between NS3 and wt-p53 was influenced by the E6 protein expressed in HeLa cells, we performed the experiments using BHK-21 cells. Practically the same results as those in Fig. 2 were obtained with BHK-21 cells (data not shown), suggesting that the E6 protein has nothing to do with the observed complex formation between NS3 and wt-p53.

We previously reported that a mutant-type (mt)-p53, which was localized exclusively in the cytoplasm, inhibited the nuclear localization of NS3F and NS3ΔC [17,18]. We then examined their possible interaction by immunoprecipitation

Table 1
Nucleotide sequences of primers used in this study

Designation	Nucleotide sequence[a]	Enzyme
NS3-S1	5′-CCGGGAATTCAAC**ATG**GTCGAGGGAGAGGTTCAGG-3′	*Eco*RI
NS3-R1	5′-GTCGAATTC**CTA**GGTGACACATGTGTTACA-3′	*Eco*RI
NS3-R2	5′-TGTCGGATCC**TCA**CTTGTCATCGTCGTCCTTGTAGTCGGACTCTACGGGCACAAAGTCCACCGCCTT-3′	*Bam*HI
53-S1	5′-GCCGCTCGAGGCA**ATG**GATGATTTGATGCTGTCCC-3′	*Xho*I
53-R1	5′-GTGGGAATTC**TCA**GTCTGAGTCAGGCCCTTCTGT-3′	*Eco*RI
53-S2	5′-AGCCCTCGAGCCTTCCGGGTCACTGCC**ATG**GAGG-3′	*Xho*I
53-R2	5′-GAGCGAATTC**CTA**CCCTGGCTCCTTCCCAGCCTG-3′	*Eco*RI
53-R3	5′-CCAGGAATTC**CTA**CTTTGGCTGGGGAGAGGAGCTGGT-3′	*Eco*RI
53-R4	5′-TAGTGAATTC**TCA**GGGCAGCTCGTGGTGAGGCTC-3′	*Eco*RI

[a]The enzyme recognition sites are underlined. The translation initiation codons and complementary sequences of stop codons are shown in boldface letters.

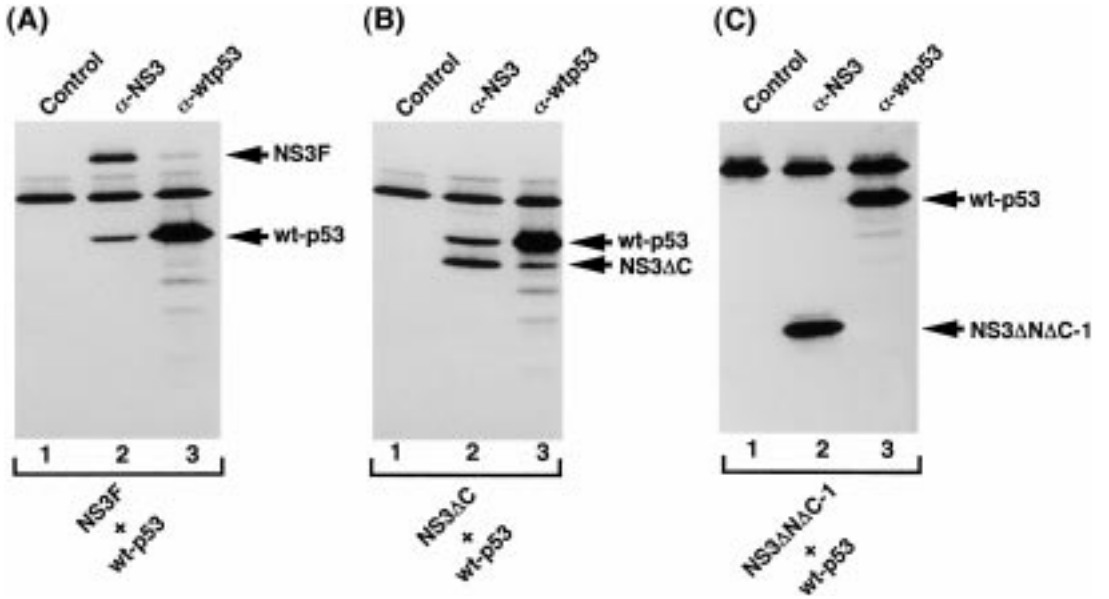

Fig. 2. Complex formation of NS3 with wt-p53. Lysates of cells expressing wt-p53 and NS3F (A), NS3ΔC (B) or NS3ΔNΔC-1 (C) were immunoprecipitated with normal mouse IgG (lane 1), anti-NS3 (lane 2) or anti-p53 monoclonal antibody (lane 3). Immunocomplex was separated by SDS-PAGE and analyzed by autoradiography.

analysis. The result revealed that NS3ΔC, but not NS3ΔNΔC-1, formed a complex with mt-p53 (data not shown).

3.2. *Even in the presence of NS4A, NS3 forms a complex with wt-p53*

NS3 has been known to form a stable complex with NS4A. Therefore, we examined the effect of NS4A on NS3-p53 complex formation. NS3ΔC, NS4A and wt-p53 were co-expressed, and immunoprecipitation analysis was performed. Anti-NS3 monoclonal antibody co-precipitated both wt-p53 and NS4A with NS3ΔC, although the same monoclonal antibody did not co-precipitate wt-p53 or NS4A in the absence of NS3ΔC (Fig. 3). This result confirmed our previous immunofluorescence observation that NS3 was co-localized with wt-p53 in the presence of NS4A, though in fewer occasions than in the absence of NS4A [17]. Taken together, these results indicate that, even in the presence of NS4A, NS3 can form a complex with wt-p53.

3.3. *The wt-p53-binding site of NS3 is different from the NS4A-binding site*

To look into the relationship between NS3-p53 complex and NS4A, we examined the binding sites of NS3 against wt-p53 and NS4A. A stretch of N-terminal 28 aa of NS3 has been shown to be important for binding to NS4A [5,8]. Therefore, we constructed a plasmid to express NS3ΔNΔC-2, in which the N-terminal 28 aa were deleted (Fig. 1). NS3ΔNΔC-2 and wt-p53 were co-expressed, and immunoprecipitation analysis was performed. Each of anti-NS3 and anti-p53 monoclonal antibodies precipitated both NS3ΔNΔC-2 and wt-p53 (Fig. 4A), clearly demonstrating complex formation between the two proteins. On the other hand, no complex formation was observed between NS3ΔNΔC-2 and NS4A (Fig. 4B, lanes 2 and 3), while NS3ΔC formed a complex with NS4A under the same conditions (Fig. 4B, lanes 5 and 6). These results strongly suggest that the wt-p53-binding site is different from the NS4A-binding site, and are in good agreement with the observation that, even in the presence of NS4A, NS3 formed a complex with wt-p53 (Fig. 3). Our results, taken together, also suggest that wt-p53-binding site is located in a portion near the N-terminus of NS3 between aa 1055–1200.

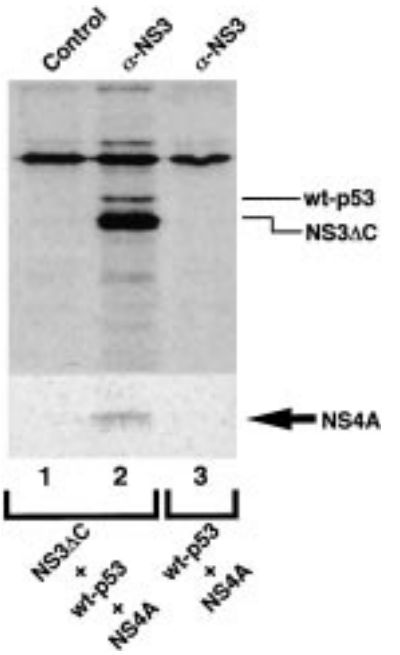

Fig. 3. The effect of NS4A on the complex formation between NS3 and wt-p53. Lysates of cells expressing NS3ΔC, wt-p53 and NS4A (lanes 1 and 2) or lysates of cells expressing wt-p53 and NS4A (lane 3) were immunoprecipitated with normal mouse IgG (lane 1) or anti-NS3 monoclonal antibody (lanes 2 and 3). Immunocomplex was separated by SDS-PAGE and analyzed by autoradiography.

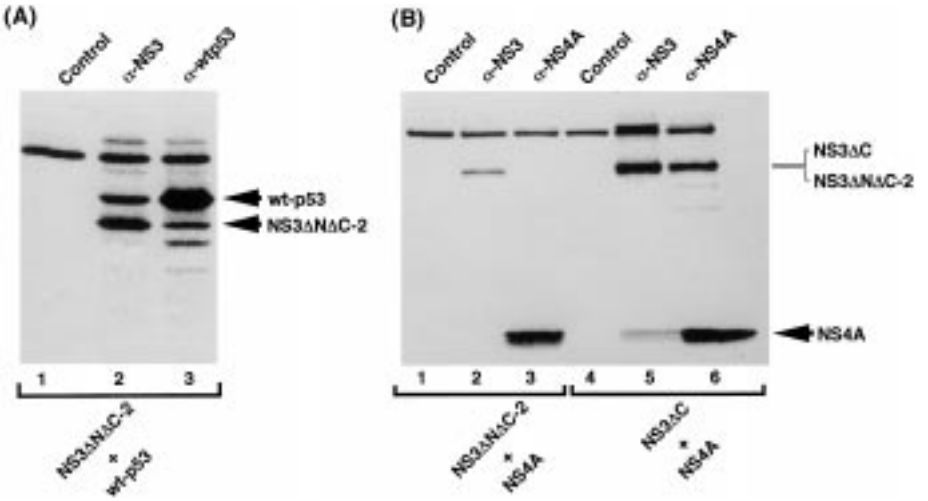

Fig. 4. Comparison between the wt-p53-binding site and NS4A-binding site of NS3. A: Lysates of cells expressing NS3ΔNΔC-2 and wt-p53 were immunoprecipitated with normal mouse IgG (lane 1), anti-NS3 (lane 2) or anti-wt-p53 monoclonal antibody (lane 3). Immunocomplex was separated by SDS-PAGE and analyzed by autoradiography. B: Lysates of cells expressing NS4A and NS3ΔNΔC-2 (lanes 1–3) or NS3ΔC (lanes 4–6) were immunoprecipitated with normal mouse IgG (lanes 1 and 4), anti-NS3 (lanes 2 and 5) or anti-NS4A monoclonal antibody (lanes 3 and 6).

3.4. The portion near the N-terminus of NS3 (aa 1055–1200) forms a complex with wt-p53

To directly assess the possibility that the portion near the N-terminus of NS3 (aa 1055–1200) is important for the association between NS3 and p53, NS3ΔNΔC-3FLAG (Fig. 1A) was co-expressed with wt-p53. As shown in Fig. 5, NS3ΔNΔC-3FLAG and wt-p53 were co-precipitated with each of the respective monoclonal antibodies, suggesting that the portion of NS3 between aa 1055 and 1200 is involved in the complex formation with wt-p53.

3.5. A portion near the C-terminus of wt-p53 is involved in the complex formation with NS3

To determine the portion of wt-p53 involved in the complex formation with NS3, we performed deletion mutational analysis of wt-p53. wt-p53 has been shown to have various functional domains, such as transactivation [23], DNA binding [24] and oligomerization domains [25] (Fig. 1B). We focused on transactivation and oligomerization domains of wt-p53. We first examined wt-p53ΔN40 and wt-p53ΔC300, which lack the transactivation and oligomerization domains, respectively (Fig. 1B). When co-expressed with NS3ΔCFLAG, wt-p53ΔN40 was co-precipitated with anti-FLAG monoclonal antibody, and similarly, NS3ΔCFLAG was co-precipitated with anti-p53 monoclonal antibody (Fig. 6A, lanes 2 and 3). When wt-p53ΔC300 was expressed with NS3ΔCFLAG, however, co-precipitation of the two proteins was not observed (Fig. 6A, lanes 5 and 6). To determine the important portion of wt-p53 in more detail, expression plasmids for wt-p53ΔC318 and wt-p53ΔC360 were constructed (Fig. 1B). When wt-p53ΔC360 was expressed with NS3ΔCFLAG, co-precipitation of the two proteins was still clearly observed (Fig. 6B, lanes 1 and 2). However, when wt-p53ΔC318 was expressed with NS3ΔCFLAG, co-precipitation of the two molecules became very faint (Fig. 6B, lanes 3 and 4). These results suggest that a portion near the C-terminus of wt-p53 (aa 301–360) is important for complex formation between NS3 and wt-p53. Since the region between aa 301 and 360 has been shown to include oligomerization domain of wt-p53 [25], NS3 might interfere with wt-p53 oligomerization.

In conclusion, our present data demonstrate that NS3 can form a complex with p53. The complex formation is likely to result from direct interaction between the two molecules, because no specific band other than NS3s and wt-p53s was detected in the co-immunoprecipitation analysis. In the present study, NS3s and wt-p53s were expressed abundantly by using a strong expression system [19]. We do not know, at present, whether or not the same interaction takes place in HCV-infected cells. In this connection, Errington et al. [26]

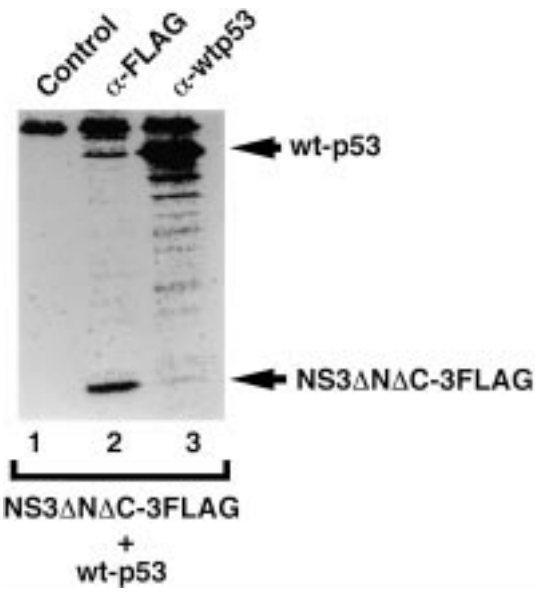

Fig. 5. Complex formation between wt-p53 and a portion near the N-terminus of NS3 (aa 1055–1200). Lysates of cells expressing NS3ΔNΔC-3FLAG and wt-p53 were immunoprecipitated with normal mouse IgG (lane 1), anti-FLAG (lane 2) or anti-p53 monoclonal antibody (lane 3). Immunocomplex was separated by SDS-PAGE and analyzed by autoradiography.

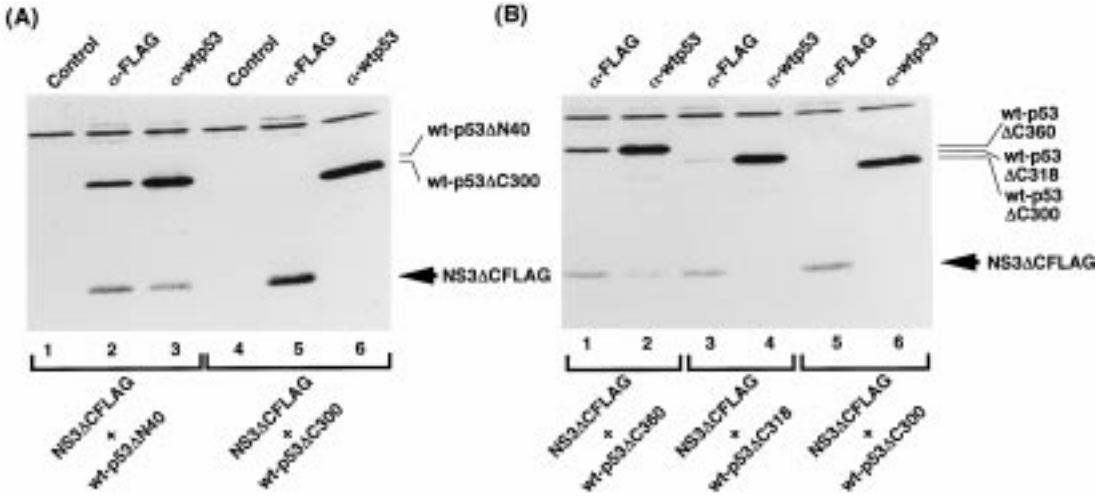

Fig. 6. Complex formation between NS3ΔCFLAG and various forms of wt-p53. A: Lysates of cells expressing NS3ΔCFLAG and wt-p53ΔN40 (lanes 1–3) or wt-p53ΔC300 (lanes 4–6) were immunoprecipitated with normal mouse IgG (lanes 1 and 4), anti-FLAG (lanes 2 and 5) or anti-p53 monoclonal antibody (lanes 3 and 6). Immunocomplex was separated by SDS-PAGE and analyzed by autoradiography. B: Lysates of cells expressing NS3ΔCFLAG and wt-p53ΔC360 (lanes 1 and 2), wt-p53ΔC318 (lanes 3 and 4) or wt-p53ΔC300 (lanes 5 and 6) were immunoprecipitated with anti-FLAG (lanes 1, 3 and 5) or anti-p53 monoclonal antibody (lanes 2, 4 and 6).

reported prominent nuclear accumulation of NS3 in a minor proportion of HCV-infected hepatocytes in patients. The authors did not test the expression of p53 in these hepatocytes. It would be worthwhile to examine for the possible interaction between NS3 and p53 in such HCV-infected hepatocytes.

Recently, NS3 was reported to transform NIH3T3 cells [14] and inhibit actinomycin D-induced apoptosis [16]. These results imply the possibility that NS3 interferes with the function of p53, which would eventually lead to malignant transformation of the cells.

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