Hepatitis C virus NS5A protein interacts with 2′,5′-oligoadenylate synthetase and inhibits antiviral activity of IFN in an IFN sensitivity-determining region-independent manner

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INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus, which readily persists in infected patients and is the most common pathogen of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma in industrialized countries (Lauer & Walker, 2001). HCV-infected patients are treated with IFN-α, alone or in combination with ribavirin, but the IFN treatment is effective in only half or fewer of the treated patients (McHutchison et al., 1998). The HCV genome exhibits a considerable degree of sequence variation and HCV is now classified into at least six genotypes and more than 60 subtypes (Mellor et al., 1995; Doi et al., 1996; Robertson et al., 1998). Clinico-pathological features, such as the severity of liver injury, development of hepatocellular carcinoma, viraemia titres and responsiveness to IFN treatment, are likely to vary with different subtypes (Bruno et al., 1997; Nousbaum et al., 1995) and even with different strains of the same subtype (Enomoto et al., 1996; Song et al., 1999; Lusida et al., 2001; Ogata et al., 2002, 2003). The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues, which is processed by the signal peptidase of the host cell and virally encoded proteases to generate at least 10 viral proteins (Reed & Rice, 2000).

The non-structural protein 5A (NS5A) of HCV has been implicated in inhibition of antiviral activity of IFN. While previous studies have suggested an interaction between NS5A and the double-stranded RNA-dependent protein kinase (PKR), the possibility still remains that interaction with another molecule(s) is involved in the NS5A-mediated inhibition of IFN. In the present study, we investigated a possible interaction between NS5A and 2′,5′-oligoadenylate synthetase (2-5AS), another key molecule in antiviral activity. We observed that NS5A physically interacted with 2-5AS in cultured cells, with an N-terminal portion of NS5A [aa 1–148; NS5A(1–148)] and two separate portions of 2-5AS (aa 52–104 and 184–275) being involved in the interaction. Single point mutations at residue 37 of NS5A affected the degree of the interaction with 2-5AS, with a Phe-to-Leu mutation (F37L) augmenting and a Phe-to-Asn mutation (F37N) diminishing it. Virus rescue assay revealed that the full-length NS5A (NS5A-F) and NS5A(1–148), the latter of which contains neither the IFN sensitivity-determining region (ISDR) nor the PKR-binding domain, significantly counteracted the antiviral activity of IFN. Introduction of a F37N mutation into NS5A(1–148) impaired the otherwise more significant IFN-inhibitory activity of NS5A(1–148). It was also found that the F37N mutation was highly disadvantageous for the replication of an HCV RNA replicon. Taken together, our results suggest the possibility that NS5A interacts with 2-5AS and inhibits the antiviral activity of IFN in an ISDR-independent manner.
mutations in the ISDR inversely correlated with HCV RNA titres in patients infected with HCV-1b, HCV-1c and HCV-2a (Lusida et al., 2001). In experimental settings, NS5A has been demonstrated to rescue encephalomyocarditis virus (EMCV) replication in IFN-treated cell cultures (Polyak et al., 1999; Song et al., 1999). It was also reported that NS5A inhibited antiviral activity of IFN by binding to the double-stranded RNA-dependent protein kinase (PKR) through the ISDR and its adjacent region, called the PKR-binding region (aa 237–302) (Gale et al., 1997, 1998). However, apparently controversial observations were reported in that the ISDR sequence variation did not account for different IFN resistance in patients (Duverlie et al., 1998) and also in an HCV subgenomic RNA replicon system (Guo et al., 2001). Moreover, expression of NS5A or the entire HCV polyprotein was reported to counteract the antiviral effect of IFN in a PKR-independent, ISDR-independent manner (Francois et al., 2000; Podevin et al., 2001). The possibility therefore still remains that a molecule(s) other than PKR is involved in the NS5A-mediated inhibition of IFN.

In addition to PKR, the antiviral effects of IFN are executed through the functions of various proteins including 2’,5’-oligoadenylate synthetase (2-5AS), RNase L and Mx (Staeheli & Pavlovic, 1991; Hassel et al., 1993; Sen & Ransohoff, 1993; Li et al., 1998). The role of the human MxA protein, in regulating HCV infection might be marginal (Frese et al., 2001). In the present study we investigated a possible interaction between NS5A and 2-5AS. We report here that the N-terminal one-third of NS5A [NS5A(1–148)], which does not contain the ISDR or PKR-binding domain, physically interacted with 2-5AS and counteracted the antiviral activity of IFN. Introduction of a point mutation (Phe to Asn) to residue 37 of NS5A(1–148) significantly reduced 2-5AS-binding activity and negated the otherwise more significant IFN-inhibitory activity of NS5A(1–148). The same mutation introduced to an HCV subgenomic RNA replicon abolished its replication competence. These results collectively suggest that NS5A interacts with 2-5AS and inhibits the antiviral activity of IFN in an ISDR-independent manner.

**METHODS**

**Plasmid construction.** The FLAG peptide-coding sequence was introduced to the pcDNA3.1(−)/myc-His expression plasmid (Invitrogen) to generate the pcDNA-FLAG vector. The full-length NS5A of HCV-1bJk strain (Song et al., 1999, 2000) was subcloned into pcDNA-FLAG in frame with the FLAG peptide and the N-terminal 232 residues of NS5A was ligated to NheI fragment of pcDNA-FLAG-NS5A-F encoding the FLAG-tagged N-terminal 232 residues of NS5A was ligated to NheI-treated pcDNA3.1(−)/myc-His in the proper orientation to generate pcDNA-FLAG-NS5A-FLAG-F (A). The coding sequence for glutathione S-transferase (GST) in pGEX-4T-1 (Pharmacia Biotech) was cloned into the unique SmaI site of pBS-GST. This vector plasmid was designated pBS-GST. pBS-GST was digested with BamHI, the ends filled in by treatment with the Klenow fragment of *Escherichia coli* DNA polymerase I and then self-ligated to create an in-frame stop codon after the GST sequence. This plasmid, designated pBS-GST, was used to express control GST. The full-length coding sequences for NS5A, NS5A(1–148) and NS5A(1–148)F37N were each subcloned into the pCAGGS expression vector (Niwa et al., 1991) to generate pCAGGS-NS5A-F, pCAGGS-NS5A(1–148) and pCAGGS-NS5A(1–148)F37N, respectively, and used to establish stable transformants, as described below.

The plasmid pMA25 containing the entire coding sequence except the first three amino acid residues for murine 2-5AS (DDBJ/EMBL/GenBank accession no. X04958) was a kind gift from Y. Sokawa, Kyoto Institute of Technology, Kyoto, Japan. The mouse 2-5AS belongs to the isoform 1a since its sequence is 99.5% identical to a standard sequence of the isoform 1a (accession no. BC013715). The sequence was fused in frame to the influenza virus HA epitope and cloned into the pSG5 expression vector (Stratagene). The resultant plasmid was designated pSG-HA-2-5AS-F.

The coding sequence for glutathione S-transferase (GST) in pGEX-4T-1 (Pharmacia Biotech) was cloned into the unique Smal site of pBlueScript II SK− (Stratagene). This vector plasmid was designated pBS-GSTBan. The GST of this plasmid was digested with BamHI, the ends filled in by treatment with the Klenow fragment of *Escherichia coli* DNA polymerase I and then self-ligated to create an in-frame stop codon after the GST sequence. This plasmid, designated pBS-GST, was used to express control GST. The full-length coding sequences for NS5A

**Fig. 1.** Schematic representation of the full-length and various deletion mutants of NS5A (A) and 2-5AS (B). (A) The ISDR spans aa 237–276 and the PKR-binding domain (PKR-BD) aa 237–302. The FLAG peptide or GST was fused at the N terminus of NS5A. The numbers indicate amino acid positions. Results of the binding experiments are shown on the right. (B) The positions of the P-loop motif and KR-rich region (Yamamoto et al., 2000) are shown. GST or the HA peptide was fused at the N terminus of 2-5AS. Results of the binding experiments are shown on the right.
Table 1. Oligonucleotide primers used in this study

<table>
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<tr>
<th>Primer</th>
<th>Nucleotide sequence (5'→3')*</th>
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<tr>
<td>NSSA-1</td>
<td>TATAGAATTCTTCCGGATGCTGGTAAAG</td>
</tr>
<tr>
<td>NSSA-27</td>
<td>TATAGGATCCTTCCGGGAAATTGCCG</td>
</tr>
<tr>
<td>NSSA-109-R</td>
<td>TATAGAATTCCTACGCCCTGGAATATGT</td>
</tr>
<tr>
<td>NSSA-148-R</td>
<td>TATAGAATTCCTTTGCGGGCCGGAAC</td>
</tr>
<tr>
<td>NSSA-F37L</td>
<td>AATTGCCGGGAGTCCCTTTCtacTCATGCCAACGCGGGTACAAGG</td>
</tr>
<tr>
<td>NSSA-F37L-R</td>
<td>CTTGTATACCCTGCGTGGCATGAAaaaGAGGAATCCCGGCAATTT</td>
</tr>
<tr>
<td>NSSA-F37N</td>
<td>AATTGCCGGGAGTCCCTTTCcaccTCAATCCGACGATGGTACAAGG</td>
</tr>
<tr>
<td>NSSA-F37N-R</td>
<td>CTTGTATACCCTGCGTGGCATGAggtAAAAGGGACCTCCCGGCAATT</td>
</tr>
<tr>
<td>NSSA-F37S</td>
<td>AATTGCCGGGAGTCCCTTTCaaccTCATGCCAACGCGGGTACAAGG</td>
</tr>
<tr>
<td>NSSA-F37S-R</td>
<td>CTTGTATACCCTGCGTGGCATGAggaGAAAGGGACTCCCGGCAATT</td>
</tr>
<tr>
<td>NSSA-F37Y</td>
<td>AATTGCCGGGAGTCCCTTTCaatcTCAATCCGACGATGGTACAAGG</td>
</tr>
<tr>
<td>NSSA-F37Y-R</td>
<td>CTTGTATACCCTGCGTGGCATGAgtaGAAAGGGACTCCCGGCAATT</td>
</tr>
<tr>
<td>2-5AS-1</td>
<td>TATAGGATCCTGAGCAGCAGGACCCAGG</td>
</tr>
<tr>
<td>2-5AS-52</td>
<td>TATAGGATCTACACCCATGAGGGTCCTCC</td>
</tr>
<tr>
<td>2-5AS-60-R</td>
<td>TATAGGATCTACACCCATGAGGGTCCTCC</td>
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<td>2-5AS-104-R</td>
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<tr>
<td>2-5AS-144-R</td>
<td>TATAGGATCCTAATTCTTCTTGTAT</td>
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<td>2-5AS-184</td>
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<tr>
<td>2-5AS-235-R</td>
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<tr>
<td>2-5AS-275-R</td>
<td>TATAGGATCCTAATTCTTCTTGTAT</td>
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*Enzyme recognition sites are underlined. Sequences complementary to a stop codon are shown in bold and those for site-directed mutagenesis in lower-case letters.

and 2-5AS were each subcloned in frame to the GST sequence into the unique BamHI site of pBS-GST-Bam to generate pBS-GST-NSSA-F and pBS-GST-2-5AS-F, respectively. Plasmids for various deletion mutants of GST-tagged 2-5AS (see Fig. 1B) were constructed by cloning the PCR products, which had been amplified using appropriate sets of primers (Table 1), into the unique BamHI site of pBS-GST-Bam.

We also used the entire coding sequence for human 2-5AS isoform 1 (DDBJ/EMBL/GenBank accession no. D00068) in pHE25AS (a kind gift from Y. Sokawa). The human 2-5AS sequence was fused in frame with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) for 1 h and then transfected with the expression plasmids, as described above. To verify that comparable amounts of the proteins were being analysed, the cell lysates were directly (without pull-down) subjected to immunoblotting using appropriate antibodies, as described below.

Transient and stable expression. Transient expression was performed as described previously (Muramatsu et al., 1997). In brief, HeLa cells, maintained in Dulbecco’s modified Eagle’s medium supplemented with 10 % fetal bovine serum, were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) for 1 h and then transfected with the expression plasmids using Lipofectin reagents (Life Technologies). After cultivation for 12–16 h, the cells were subjected to immunoblotting using appropriate antibodies, as described below. To establish cell clones stably expressing NSSA, mouse fibroblast L929 and human hepatoma Huh-7 cell lines were used. The cells were co-transfected with a selection plasmid, pSV2neo, and either pCAGGS-FLAG-NSSA-F, pCAGGS-FLAG-NSSA(1-148), pCAGGS-FLAG-NSSA(1-148)F37N or the pCAGGS vector using FuGene 6 transfection reagents (Roche). After cultivation in the presence of G418 (1 mg ml⁻¹) for 2–3 weeks, resultant colonies were cloned using cloning cylinders.

Huh-7 human hepatoma cells harbouring the HCV subgenomic RNA replicon were also generated by transfecting RNA that had been transcribed in vitro from pFKS5B2884Gly (Lohmann et al., 2001), followed by G418 selection.

GST pull-down assay. HeLa or Huh-7 cells were transiently transfected with the expression plasmids for GST-tagged NSSA and HA-tagged 2-5AS or GST-tagged 2-5AS and FLAG-tagged NSSA, as described above. After 12–16 h, the cells were washed once with PBS and lysed in NETN buffer consisting of 150 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl (pH 7.4) and 0.5 % NP40. The lysates were centrifuged at 14 000 r.p.m. for 5 min and the supernatants were mixed with 20 µl glutathione-conjugated Sepharose beads at 4 °C for 90 min. The beads were washed five times with NETN buffer, and possible association between NSSA and 2-5AS was analysed by immunoblotting using appropriate antibodies, as described below.

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Immunoblot analysis. Samples dissolved in a solution consisting of 50 mM Tris/HCl (pH 6.8), 100 mM dithiothreitol, 2 % SDS, 0.1 % bromphenol blue and 10 % glycerol were resolved by SDS-PAGE and electrophoretically blotted on to a PVDF filter (Bio-Rad). After blocking in PBS containing 3 % non-fat dried milk, the filters were incubated with mouse monoclonal antibodies against NSSA (a kind gift from I. Fuke, Research Institute for Microbial Diseases,
Kan-Onji Branch, Osaka University, Kan-Onji, Kagawa, Japan), the FLAG (F-3165; Sigma) or HA peptide (16B12; BabCO). After five washes with PBS containing 0-5% Tween 20, the filters were incubated with peroxidase-labelled goat anti-mouse IgG (MBL). After a further five washes, the protein bands were visualized by an enhanced chemiluminescence method (Amersham Pharmacia). The intensity of the signals was quantified by using NIH image 1.62 software.

**Co-immunoprecipitation analysis.** HeLa cells transiently transfected with the expression plasmids were lysed with NETN buffer and the lysates were clarified by centrifugation. The resultant supernatants were incubated at 4°C for 3 h with 0.5 µg anti-HA rabbit polyclonal antibody (Y-11; Santa Cruz Biotechnology) to immunoprecipitate HA-tagged 2-5AS. Normal rabbit IgG served as a control. The mixtures were then incubated with 10 µl of protein G-coupled Sepharose (Amersham Pharmacia). After six washes with NETN buffer, the immunoprecipitates were subjected to immunoblot analysis using anti-FLAG antibody to detect NS5A.

**Immunofluorescence analysis.** HeLa cells transiently transfected with the expression plasmids were fixed with 95% ethanol and double-stained with anti-FLAG mouse monoclonal antibody and anti-HA rabbit polyclonal antibody. After five washes with PBS, the cells were incubated with Texas-Red-conjugated anti-mouse IgG (Amersham Life Science) and FITC-conjugated anti-rabbit IgG (MBL). After a further five washes with PBS, the cells were analysed by confocal laser-scanning microscopy (MRC-1024; Bio-Rad).

**IFN antiviral activity assay.** Cells were seeded in six-well tissue culture plates at a density of 2 x 10⁵ cells per well and cultivated for 24 h. The cells were treated with recombinant mouse IFN-α2A (PBL Biomedical Laboratory) or human IFN-α2a (Roche) at concentrations of 5 and 25 U ml⁻¹ or left untreated for another 24 h, then inoculated with 50–60 p.f.u. EMCV (strain DK-27) per well (Dan et al., 1995; Song et al., 1999). After 1 h with intermittent rocking, fresh medium containing 1% methylcellulose was added to each well of the plates. After cultivation for 2 days, the plates were stained with crystal violet and the number of plaques in each well was counted. The percentage of plaques on IFN-treated cells compared with untreated cells was calculated.

**RESULTS**

**NS5A-F co-localizes and physically interacts with 2-5AS in mammalian cells**

We first investigated the subcellular localization of NS5A and 2-5AS. FLAG-tagged NS5A-F and HA-tagged 2-5AS-F were transiently expressed in HeLa cells using a vaccinia virus–T7 hybrid expression system. Consistent with our previous observations and those of others (Ghosh et al., 2000; Song et al., 1999, 2000), both NS5A-F and 2-5AS-F were found to localize in the cytoplasmic perinuclear region. Confocal laser-scanning microscopy analysis revealed that NS5A partially co-localized with 2-5AS (Fig. 2A).

We then examined possible complex formation between NS5A and 2-5AS in mammalian cells using a GST pull-down assay and co-immunoprecipitation analysis. In the GST pull-down assay, GST-tagged 2-5AS-F or the control GST was expressed with or without NS5A-F in HeLa cells and pulled down by glutathione-conjugated Sepharose beads, which were then subjected to immunoblotting using anti-NS5A monoclonal antibody. The result clearly demonstrated that GST-tagged 2-5AS-F, but not the control GST, pulled down NS5A-F (Fig. 2B, left panel). Similarly, when GST-tagged NS5A-F or the control GST was co-expressed with HA-tagged 2-5AS or the control HA, pulled down by glutathione–Sepharose beads and probed with anti-HA monoclonal antibody to detect 2-5AS, the result showed that GST-tagged NS5A-F, but not the control GST, pulled down HA-tagged 2-5AS-F (Fig. 2B, right panel).

**The N-terminal 148 residues of NS5A are involved in the interaction with 2-5AS**

Various deletion mutants of FLAG-tagged NS5A (see Fig. 1A) were co-expressed with GST-tagged 2-5AS-F to determine the region responsible for the interaction with anti-NS5A monoclonal antibody. The result clearly demonstrated that GST-tagged 2-5AS-F, but not the control GST, pulled down NS5A-F (Fig. 2B, left panel). Similarly, when GST-tagged NS5A-F or the control GST was co-expressed with HA-tagged 2-5AS or the control HA, pulled down by glutathione–Sepharose beads and probed with anti-HA monoclonal antibody to detect 2-5AS, the result showed that GST-tagged NS5A-F, but not the control GST, pulled down HA-tagged 2-5AS-F (Fig. 2B, right panel).

![Fig. 2. NS5A-F and 2-5AS-F co-localize and physically interact with each other in HeLa cells. (A) Confocal laser-scanning immunofluorescence microscopy analysis. Cells transiently expressing FLAG-tagged NS5A-F and HA-tagged 2-5AS-F were stained for NS5A-F (left panel) and 2-5AS-F (middle panel). The two images were merged to show their co-localization (right panel). (B) Physical interaction between NS5A-F and 2-5AS-F in HeLa cells. Left upper panel: lysates of cells expressing control GST (lanes 1 and 2) or GST-tagged 2-5AS-F (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of NS5A-F were pulled down and probed with anti-NS5A monoclonal antibody. Left lower panel: verification of comparable amounts of NS5A-F in the lysates. Right upper panel: lysates of cells expressing control GST (lanes 5 and 6) or GST-tagged NS5A-F (lanes 7 and 8) in the absence (lanes 5 and 7) or presence of HA-tagged 2-5AS-F (lanes 6 and 8) were pulled down and probed with anti-HA monoclonal antibody. Right lower panel: verification of comparable amounts of 2-5AS-F in the lysates.](image-url)
2-5AS. All the NS5A deletion mutants tested were localized in the cytoplasm (data not shown). Immunoblot analysis using anti-FLAG antibody revealed that FLAG-tagged NS5A(1–232) and NS5A(1–148), but not NS5A(1–109) or NS5A(27–148), were efficiently pulled down by GST-tagged 2-5AS-F (Fig. 3A, upper panel). It should be noted that NS5A(1–232) and NS5A(1–148) bound to 2-5AS-F more efficiently than did NS5A-F. Comparable degrees of expression of NS5A-F and the deletion mutants (Fig. 3A, lower panel) and GST-tagged 2-5AS-F (data not shown) in each transfected cell culture were verified. Neither NS5A(1–67) nor NS5A(1–87) were pulled down by GST-tagged 2-5AS-F (data not shown).

We also performed co-immunoprecipitation analysis to confirm the complex formation between NS5A and 2-5AS. HA-tagged 2-5AS-F was expressed in HeLa cells with or without FLAG-tagged NS5A(1–148). The cell lysates were immunoprecipitated using anti-HA or control antibody and probed with anti-FLAG antibody. As shown in Fig. 3(B), anti-HA antibody (directed against HA-tagged 2-5AS-F), but not the control antibody, co-immunoprecipitated FLAG-tagged NS5A(1–148) from the lysates of cells expressing both NS5A(1–148) and 2-5AS-F (lane 9); the same anti-HA antibody did not co-immunoprecipitate FLAG-tagged NS5A(1–148) from the lysates of cells expressing NS5A(1–148) alone or 2-5AS-F alone (lanes 3 and 6). NS5A-F was also co-immunoprecipitated with HA-tagged 2-5AS-F (data not shown). These results thus confirmed that NS5A forms a complex with 2-5AS in mammalian cells.

Two separate portions of 2-5AS are independently involved in the interaction with NS5A

To determine the region(s) of 2-5AS responsible for the interaction with NS5A, various deletion mutants of GST-tagged 2-5AS (see Fig. 1B) were co-expressed with FLAG-tagged NS5A(1–148), pulled down by glutathione-conjugated Sepharose beads and subjected to immunoblot analysis using an anti-FLAG antibody. GST-tagged 2-5AS(1–104), 2-5AS(52–144) and 2-5AS(184–275), but not 2-5AS(1–60), 2-5AS(184–235) or the control GST, pulled down NS5A(1–148) (Fig. 3C, upper panel). Comparable degrees of expression of FLAG-tagged NS5A(1–148) (Fig. 3C, lower panel), GST-tagged 2-5AS-F and the deletion mutants (data not shown) in each transfected cell

Fig. 3. An N-terminal region of NS5A (aa 1–148) and two separate regions of 2-5AS (aa 52–104 and aa 184–275) are involved in the interaction between NS5A and 2-5AS. (A) Upper panel: lysates of HeLa cells expressing control GST (lanes 1–5) or GST-tagged 2-5AS-F (lanes 6–10) together with FLAG-tagged NS5A-F (lanes 1 and 6) and its deletion mutants (lanes 2–5 and 7–10) were pulled down and probed with anti-FLAG monoclonal antibody. Lower panel: verification of comparable amounts of NS5A in the lysates. (B) Lysates of cells expressing FLAG-tagged NS5A(1–148) alone (lanes 1–3), HA-tagged 2-5AS-F alone (lanes 4–6) or both (lanes 7–9) were either left untreated (input 5% of total; lanes 1, 4 and 7) or immunoprecipitated with control rabbit IgG (lanes 2, 5 and 8) or anti-HA polyclonal rabbit antiserum (lanes 3, 6 and 9). The immunoprecipitates were probed with an anti-FLAG monoclonal antibody. The arrowhead indicates NS5A(1–148). (C) Upper panel: lysates of cells expressing control GST (lane 1), GST-tagged 2-5AS-F (lane 2) or its deletion mutants (lanes 3–7) together with FLAG-tagged NS5A(1–148) were pulled down and probed with anti-FLAG monoclonal antibody. Lower panel: verification of comparable amounts of NS5A(1–148) in the lysates.
culture were verified. Collectively, these results suggested that two separate regions of 2-5AS (aa 52–104 and aa 184–275) are independently involved in physical interaction with NS5A(1–148).

**Mutation of residue 37 of NS5A affects its interaction with 2-5AS**

We previously noticed that a single point mutation of NS5A at residue 37 might be correlated with serum HCV RNA titres (data not shown). Therefore, we were interested in testing the possible effects of NS5A mutations at residue 37 (F37L, F37N, F37S and F37Y) on its interaction with 2-5AS. FLAG-tagged mutants of NS5A(1–148) were co-expressed with GST-tagged 2-5AS-F or the control GST in HeLa cells. The cell lysates were subjected to a GST pull-down assay using glutathione-conjugated Sepharose beads, followed by immunoblot analysis using an anti-FLAG antibody. The F37L mutation of NS5A significantly augmented complex formation with 2-5AS, whereas the F37N mutation significantly decreased it (Fig. 4A, upper panel). F37S or F37Y mutations did not significantly affect the complex formation. A comparable degree of expression of NS5A(1–148) and the single point mutants (Fig. 4A, lower panel) in each transfected cell culture was verified. Mean values of relative degrees of complex formation between each NS5A mutant and 2-5AS obtained from four independent experiments are shown in Fig. 4(B).

**NS5A-F and NS5A(1–148) counteract the antiviral activity of IFN**

To test possible inhibitory effects of NS5A on the antiviral activity of IFN, we established L929 cell clones stably expressing NS5A-F, NS5A(1–148) or NS5A(1–148)F37N as well as a non-expressing control. Cells were treated with IFN or left untreated, and challenged with 50 p.f.u. EMCV. Consistent with our previous observations (Song et al., 1999), the numbers of plaques formed on cells that had not been treated with IFN were almost the same among the cell clones tested, irrespective of NS5A expression and NS5A mutation (data not shown). IFN at concentrations of 5 and 25 U ml⁻¹ suppressed EMCV replication in the non-expressing control cells by 70 and 95 % (as judged by the relative virus titres of 30 and 5 %), respectively (Fig. 5A). The antiviral activity of IFN against EMCV was significantly reduced in cells expressing NS5A-F or NS5A(1–148).

NS5A(1–148)F37N, which was shown to interact with 2-5AS only weakly (see Fig. 4), was less effective in counteracting the antiviral activity of IFN compared with the wild-type (WT) NS5A(1–148) (Fig. 5B). This result suggested that the F37N mutation negated an otherwise more evident inhibitory effect of NS5A(1–148) on IFN antiviral activity.

The possible inhibitory effects of NS5A on antiviral activity of IFN were also assessed using Huh-7 human hepatoma cells. We first confirmed the physical interaction between NS5A and human 2-5AS in this cell type. As shown in Fig. 6(A), NS5A(1–148), NS5A(1–148)F37L and NS5A(1–148)F37N physically interacted with GST-tagged wild-type (WT) NS5A(1–148) (lanes 1 and 6) and its single point mutants (lanes 2–5 and 7–10) were pulled down and probed with an anti-FLAG monoclonal antibody. Lower panel: verification of comparable amounts of NS5A in the lysates. (B) The intensities of the bands for NS5A that had been pulled down were measured and the ratios compared with total input were calculated. Means±SD of four independent experiments are shown. *P<0.05, compared with WT.

**Fig. 4.** Single point mutations at residue 37 of NS5A influence its interaction with 2-5AS. (A) Upper panel: lysates of HeLa cells expressing control GST (lanes 1–5) or GST-tagged 2-5AS-F (lanes 6–10) together with FLAG-tagged wild-type (WT) NS5A(1–148) (lanes 1 and 6) and its single point mutants (lanes 2–5 and 7–10) were pulled down and probed with an anti-FLAG monoclonal antibody. Lower panel: verification of comparable amounts of NS5A in the lysates. (B) The intensities of the bands for NS5A that had been pulled down were measured and the ratios compared with total input were calculated. Means±SD of four independent experiments are shown. *, P<0.05, compared with WT.
another mutant replicon possessing the F37N mutation did not generate any colonies after selection in G418-containing medium, although the wild-type HCV replicon and the F37L mutant generated substantial numbers of G418-resistant colonies (Fig. 7B). Similar results were reproducibly obtained, suggesting that the F37N mutation is highly disadvantageous for replication of the HCV genome.

**DISCUSSION**

Antiviral effects of IFN are executed by a variety of antiviral proteins, including PKR, 2-5AS, RNase L and Mx proteins (Staeheli & Pavlovic, 1991; Hassel et al., 1993; Sen & Ransohoff, 1993; Li et al., 1998). HCV NS5A has been reported to bind to and inhibit the function of PKR through the ISDR of NS5A (Gale et al., 1997, 1998). However, PKR-independent, ISDR-independent IFN inhibition by NS5A has also been suggested (Duverlie et al., 1998; Francois et al., 2000; Guo et al., 2001; Podevin et al., 2001). Moreover, NS5A was reported to up-regulate interleukin 8 expression, which counteracted IFN activity possibly through inhibiting 2-5AS (Polyak et al., 2001; Girard et al., 2002). With respect to the role for 2-5AS in HCV infection, controversial observations in clinical settings have been reported indicating that 2-5AS activity in peripheral blood mononuclear cells or liver cells correlated well with IFN responsiveness (Grander et al., 1996; Podevin et al., 1997), whereas serum 2-5AS activity did not (Murashima et al., 2000). On the other hand, 2-5AS and, in turn, RNase L have been shown to be activated by HCV RNA (Han & Barton, 2002). It has also been reported that the 2-5AS gene was transcriptionally activated by HCV core protein (Naganuma et al., 2000). Thus, the significance of possible inhibition and activation of 2-5AS in HCV infection is yet to be clarified and, to our knowledge, direct interaction between 2-5AS and HCV proteins has not been documented so far.

In the present study we have demonstrated that HCV NS5A physically interacts with 2-5AS, with the N-terminal region of NS5A (aa 1–148) and two separate regions of 2-5AS (aa 52–104 and aa 184–275) being involved in the interaction (Figs 2 and 3). We used two different 2-5AS molecules: the isoform 1a (p42) of mouse 2-5AS and the isoform 1 (p40) of human 2-5AS. While the overall sequence similarity between them is 68 %, the NS5A-binding portions show even higher sequence similarities (83 and 85 %, respectively). Our results also demonstrated that NS5A(1–148) inhibited the antiviral activity of IFN against Fig. 5. NS5A-F and NS5A(1–148) inhibit the antiviral activity of IFN in L929 mouse cells. (A) Two clones each of L929 cells stably expressing NS5A-F (F cl.-1 and -2) or NS5A(1–148) (148 cl.-1 and -2) and non-expressing controls (Cont cl.-1 and -2) were treated with IFN or left untreated for 24 h. Cells were then infected with EMCV (50 p.f.u.) and plaques formed on the monolayer cells were counted. The percentage of plaques on IFN-treated cells compared with untreated controls was calculated. Means ± SD of four independent experiments are shown. * P<0.01, compared with the non-expressing control. Expression levels of NS5A are shown below. (B) Effect of the F37N mutation on the IFN-inhibitory activity of NS5A(1–148). Two clones each of L929 cells stably expressing wild-type (WT) NS5A(1–148) or its single point mutant NS5A(1–148)F37N and non-expressing control were treated with IFN (5 U ml⁻¹) or left untreated, and then infected with EMCV (50 p.f.u.). The percentage of plaques on IFN-treated cells compared with the untreated controls was calculated. Means ± SD of four independent experiments are shown. † P<0.01, compared with WT. Expression levels of NS5A are shown below.
EMCV, as did the full-length NS5A-F, in both mouse and human cell culture systems (Figs 5 and 6). Since NS5A(1–148) does not harbour the ISDR or PKR-binding domain, IFN inhibition by NS5A(1–148) is likely independent of ISDR and PKR. The major phosphorylation sites of NS5A (Reed & Rice, 1999; Katze et al., 2000) and residues undergoing adaptive mutation in NS5A of HCV replicons (Blicht et al., 2000; Krieger et al., 2001; Lohmann et al., 2001), which are located in the central and C-terminal portions of NS5A, are unlikely to be involved in the interaction with 2-5AS or IFN inhibition, since NS5A(1–148) does not contain these residues. We could not confirm, however, the inhibition of 2-5AS enzymic activity in NS5A-expressing cells (data not shown). We assume that the

Fig. 6. NS5A physically interacts with human 2-5AS and inhibits the antiviral activity of IFN in Huh-7 human hepatoma cells. (A) Upper panel: lysates of cells transiently expressing control GST (lanes 1–3) or GST-tagged Hu-2-5AS-F (lanes 4–6) in the presence of FLAG-tagged wild-type (WT) NS5A(1–148) (lanes 1 and 4) and its single point mutants (lanes 2, 3, 5 and 6) were pulled down and probed with an anti-FLAG monoclonal antibody. Lower panel: verification of comparable amounts of NS5A in the lysates. (B) Huh-7 cells stably expressing NS5A-F or NS5A(1–148) or the non-expressing control were treated with IFN or left untreated for 24 h. Cells were then infected with EMCV (50 p.f.u.) and plaques formed on the monolayer of cells were counted. The percentage of plaques on IFN-treated cells compared with the untreated controls was calculated. Filled and open columns represent the results obtained with cells treated with 5 and 25 U IFN ml⁻¹, respectively. The mean ± SD of four independent experiments is shown. *, P<0·01, compared with the control Huh-7 cells. Expression levels of NS5A are shown below.

Fig. 7. Analysis using HCV subgenomic RNA replicons. (A) Antiviral activity of IFN is counteracted by HCV replicons. Huh-7 cells harbouring an HCV replicon (WT) or its F37L mutant and the parental control cells without the HCV replicon (Cont) were treated with IFN or left untreated for 24 h. Cells were infected with EMCV (50 p.f.u.) and the number of plaques formed on the monolayer was counted. The percentage of plaques on IFN-treated cells compared with the untreated controls was calculated. Filled and open columns represent the results obtained with cells treated with 5 and 25 U IFN ml⁻¹, respectively. The mean ± SD of four independent experiments is shown. *, P<0·01, compared with the control Huh-7 cells. Expression levels of NS5A are shown below. (B) F37N mutation of NS5A impairs replication competence of HCV replicon. An HCV subgenomic RNA replicon transcribed from pFK5B2884Gly (WT) and mutant HCV replicons possessing the F37L or F37N mutations were transfected into Huh-7 cells and G418-resistant colonies were obtained. A few colonies were formed but had Phe (WT) at the 37th residue of NS5A.
possible inhibition might have been masked due to a technical limitation, since 2-5AS in lysates of the cells, either NS5A-expressing cells or the control, is unavoidably activated to some extent during the experimental procedures.

In our previous study on NS5A sequence diversity among HCV isolates in Indonesia (Lusida et al., 2001), we noticed that a mutation at residue 37 of NS5A might be correlated with HCV viraemia titres (data not shown). In an attempt to find a correlation between the mutation and IFN inhibition, we introduced various point mutations: F37L, F37N, F37S and F37Y. Interestingly, our results revealed that NS5A(1–148)F37L interacted with 2-5AS twice as strongly as the wild-type NS5A(1–148) (Fig. 4). However, the interaction between another mutant, NS5A(1–148)F37N, and 2-5AS was much weaker than that between the wild-type NS5A(1–148) and 2-5AS. This weaker interaction may account for the weaker inhibitory effect of NS5A(1–148)F37N on the antiviral activity of IFN compared with the wild-type NS5A(1–148). Moreover, our result suggests that the F37N mutation is highly disadvantageous for the replication of the HCV subgenomic RNA replicon (Fig. 7B). It should be noted that, while Phe, Leu and Tyr are hydrophobic and found at this position in clinical isolates of HCV, Asn is hydrophilic and has not been found so far at this position in clinical isolates.

There are a number of functional domains in the N-terminal half of NS5A. The N-terminal ~30 residues of NS5A have been reported to include a membrane-anchor domain that determines the cytoplasmic localization of NS5A (Satoh et al., 2000; Song et al., 2000; Brass et al., 2002). It has also been demonstrated that the N-terminal half of NS5A (aa 1–224) binds to apolipoprotein A1 and co-localizes with the HCV core protein on lipid droplets (Shi et al., 2002). Moreover, a region of NS5A spanning from aa 105 to 162 forms a complex with NS5B and modulates its RNA-dependent RNA polymerase activity (Shirotu et al., 2002). The 2-5AS-binding region of NS5A determined in the present study (aa 1–148) overlaps those domains. For 2-5AS, we identified two independent regions that are responsible for the interaction with NS5A: one spanning from aa 52 to 104 and the other from aa 184 to 275. The former region contains an ATP-binding motif (P-loop) followed by an Asp76-Ala-Asp78 sequence (D-box), while the latter contains a region with a high Lys and Arg content (KR-rich region). The P-loop, D-box and KR-rich region are important for the enzymic activity of 2-5AS, and mutations in these motifs impair the enzymic activity (Yamamoto et al., 2000). It is likely, therefore, that NS5A interferes with 2-5AS functions by binding to the active sites.

Whether NS5A(1–148) or its equivalent(s) is actually generated in the cell would be an interesting issue to address. We have observed that NS5A was cleaved to generate a cleavage product of 19 kDa in FL cells undergoing apoptosis and that the cleavage was inhibited by the caspase inhibitor Z-VAD (data not shown). An NS5A cleavage product of ~19 kDa in apoptotic cells was previously reported by Satoh et al. (2000), with the estimated cleavage site being residue 154. Goh et al. (2001) also reported NS5A cleavage by a caspase-like protease(s) that was activated by co-expressed HCV core protein. Collectively, these results suggest the possibility that NS5A(1–148) or its equivalent(s) is generated in the cell under certain conditions and interferes with 2-5AS functions more strongly than does full-length NS5A.

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