The NS5A protein of hepatitis C virus partially inhibits the antiviral activity of interferon

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The non-structural protein 5A (NS5A) of some hepatitis C virus (HCV) isolates has been implicated in the inhibition of the antiviral activity of interferon (IFN). In the present study, the possible inhibitory effects of NS5A from two isolates of HCV subtype 1b, HCV-1bJk and M094AJk, and their chimeric form on the antiviral activity of IFN were examined. HCV-1bJk and M094AJk are categorized as IFN resistant and IFN sensitive, respectively, based on the sequences of the IFN-sensitivity determining region (ISDR). When encephalomyocarditis virus was used as a challenge virus, NS5A was shown to eliminate the antiviral activity of IFN, with inhibition being more prominent with HCV-1bJk NS5A than with M094AJk NS5A. Moreover, the inhibition was significantly weaker in cells expressing a chimeric NS5A that had a short stretch of 49 amino acids (aa 2209–2257), including the ISDR sequence, from M094AJk in the backbone of the HCV-1bJk sequence than in cells expressing the original NS5A from HCV-1bJk. These results suggest an important role for the 49 aa sequence, including the ISDR, in the inhibition of IFN-mediated antiviral activity. On the other hand, only a slight reduction of IFN antiviral activity by HCV-1bJk NS5A was observed when vesicular stomatitis virus was used as a challenge virus, and barely any reduction was observed when Japanese encephalitis virus was used. These results may reflect differential importance of each of the IFN-mediated signalling pathways in conferring resistance against different viruses.

Introduction

Hepatitis C virus (HCV) easily establishes a persistent infection, leading to chronic liver disease that includes chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Houghton, 1996). The HCV genome exhibits a considerable degree of sequence variation, on the basis of which HCV can be classified into at least six major genotypes and a total of more than 60 subtypes (Doi et al., 1996; Mellor et al., 1995). It has been reported that the prevalence of each subtype varies in different geographical areas (Apichartpiyakul et al., 1994; Doi et al., 1996; Mellor et al., 1995; Soetjipto et al., 1996) and that virus pathogenicity and sensitivity to interferon (IFN) treatment appear to vary with different subtypes (Kanai et al., 1992; Pozzato et al., 1991; Soetjipto et al., 1996). The viral genome encodes a polyprotein precursor, consisting of 3010–3033 amino acid (aa) residues (Kato et al., 1990), which is cleaved by a combination of the host signal peptidase and two virus-encoded proteases to generate at least 10 viral proteins: C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Houghton, 1996).

It has been reported that non-structural protein 5A (NS5A) of HCV undergoes differing degrees of phosphorylation to give two phosphorylated forms, p56 and p58, with the latter being a hyperphosphorylated form of the former (Asabe et al., 1997; Kaneko et al., 1994). Phosphorylation occurs predominantly on serine, with a minor fraction of threonine residues being phosphorylated (Reed et al., 1997). NS5A was shown to possess a nuclear localization signal and to be localized on the perinuclear membrane (Ide et al., 1996; Tanji et al., 1995). It was also demonstrated that a form of NS5A truncated at the amino terminus functioned as a potent transcriptional activator (Kato et al., 1997; Tanimoto et al., 1997). Enomoto et al. (1996) reported a clinical observation that mutations in the amino acid sequence of NS5A, especially in a region between aa 2209 and 2248, were associated with
improved responsiveness to IFN in Japanese patients infected with HCV subtype 1b, and the region has therefore been designated the IFN-sensitivity determining region (ISDR). In support of this finding, it was shown that NS5A bound through the ISDR and an adjacent region to double-stranded RNA-dependent protein kinase (PKR), a key molecule for the antiviral activity of IFN (Sen & Ransohoff, 1993; Vilcek & Sen, 1996), to interfere with its kinase activity (Gale et al., 1997, 1998). However, controversial clinical observations were reported that suggested that mutations in the ISDR of European HCV isolates were not associated with IFN sensitivity (Squadrito et al., 1997; Zeuzem et al., 1997). Moreover, direct evidence for the inhibitory effects of NS5A on IFN activity has yet to be demonstrated. In the present paper, we report that NS5A partially inhibits the antiviral activity of IFN, with the reduction varying with different NS5A sequences. We also demonstrate that a short stretch of NS5A sequence, including the ISDR, is involved in the inhibition of IFN-mediated antiviral activity.

**Methods**

**Plasmid construction.** The entire NSSA region of HCV-1bJ cDNA (Tanji et al., 1995; Kaneko et al., 1994) was amplified by PCR with primers 5' SacGAGCTCTAAAGGATGTT 3': underline, SacI recognition site; boldface letters, translation initiation codon) and 5' SacGAGCTCTAAAGGATGTT 3': underline, SacI recognition site; boldface letters, complementary sequence of a stop codon). The primers were designed so that the amplified fragments could mediate expression of full-length NS5A immediately after the methionine residue when placed downstream of an appropriate promoter. Similarly, the entire NSSA region of another strain of HCV (M094AJk) (Takehara et al., 1995) was amplified by PCR with primers 5' TATATAAGCTCTAGTCCGGATCCTGGCTCAGGGATGTT 3': underline, SacI recognition site; boldface letters, translation initiation codon) and 5' TATATAAGCTCTAGTCCGGATCCTGGCTCAGGGATGTT 3': underline, SacI recognition site; boldface letters, complementary sequence of a stop codon). After treatment with SacI, the amplified fragments were each subcloned in the correct orientation into the unique SacI site of pSG5 expression vector, which mediates expression of the cloned gene under the regulation of the simian virus 40 early promoter (Green et al., 1988). The resultant plasmids were named pSGns5a-Jk and the control were treated with murine IFN-α (kindly provided by T. Imaqawa, Osaka University) that had been raised against NS5A antigen expressed in Escherichia coli (Manabe et al., 1994). To analyse the phosphorylation status of NS5A, cell lysates were treated with E. coli C75 alkaline phosphatase (Takara Shuzo) at 37 °C for 60 min and subjected to immunoblot analysis, as described above.

**Effects of NS5A on antiviral activity of IFN.** NS5A-expressing cells and the control were treated with murine IFN-α/β (Hotta et al., 1984) (kindly provided by M. Kohase, National Institute of Infectious Diseases, Tokyo) or mock-treated for 18 h and challenged with encephalomyocarditis virus (EMCV) [strain DK-27 (Dan et al., 1995); kindly provided by Y. Seto, Keio University, Tokyo], vesicular stomatitis virus (VSV, New Jersey strain) or Japanese encephalitis virus (JEV, Nakayama strain). Culture fluids were taken every day after infection and virus titres were measured by plaque assay on either L929 (EMCV and VSV) or Vero cells (JEV).

**Statistical analysis.** The data obtained were analysed statistically by one-way analysis of variance (Duncan’s multiple-range test) using a computer program (SPSS/PC+ Statistics 4.0).

**Results**

**Establishment of NS5A-expressing cells and the control**

The NS5A sequences that were amplified from HCV-1bJ and M094AJ cDNAs and subcloned into the pSG5 plasmid...
Inhibition of IFN by HCV NS5A

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Fig. 2. Immunofluorescence analysis of NS5A-expressing cells. L929 (a–c) and NIH3T3 (d–f) transfectants harbouring pSG5 (a, d), pSGns5A-Jk (b, e) and pSGns5a-94AJk (c, f) were subjected to indirect immunofluorescence analysis using anti-NS5A monoclonal antibody. Transfectants shown are LpSG-1 (a), Lns5a-J-1 (b), Lns5a-94AJ-1 (c), 3T3pSG (d), 3T3ns5a-J (e) and 3T3ns5a-94AJ (f).

differed from their respective parental sequences by three and one amino acids, respectively, and are referred to as HCV-1bJk and M094AJk (k stands for Kobe). All of the changes were found in other HCV strains at the corresponding positions and are therefore unlikely to represent artefactual sequences. The NS5A sequence of HCV-1bJk differed from that of M094AJk by 42 aa, including four amino acids in the ISDR (Fig. 1). The ISDR sequence of HCV-1bJk is the prototype for IFN-resistant HCV strains (Enomoto et al., 1996). On the other hand, M094AJk, which was isolated from a partially responding patient who showed rebound viraemia after responding once to IFN treatment, can be categorized as an IFN-sensitive strain on the basis of the number of mutations in the ISDR sequence (Enomoto et al., 1996).

Two clones each of the L929 transfectants expressing NS5A from HCV-1bJk (Lns5a-J-1 and Lns5a-J-2) and M094AJk (Lns5a-94AJ-1 and Lns5a-94AJ-2), as well as two control clones (LpSG-1 and LpSG-2), were obtained. Indirect immunofluorescence analysis revealed NS5A expression in > 80% of the total population of Lns5a-J-1 and Lns5a-94AJ-1 (Fig. 2 b, c). Practically identical results were obtained with Lns5a-J-2 and Lns5a-94AJ-2 (data not shown). Also, uncloned NIH3T3 transfectants expressing NS5A from HCV-1bJk (3T3ns5a-J) and M094AJk (3T3ns5a-94AJ), and a non-expressing control (3T3pSG), were obtained. NS5A expression was confirmed in > 80% of the 3T3ns5a-J and 3T3ns5a-94AJ cells (Fig. 2 e, f). NS5A-expressing cells, either L929 or NIH3T3 transfectants, exhibited a diffuse staining pattern in the cytoplasm with the nuclei being unstained. The intensity of NS5A expression did not appear to differ between HCV-1bJk and M094AJk.

Immunoblot analysis revealed that HCV-1bJk NS5A formed two bands in both L929 and NIH3T3 transfectants, with the upper band of 58 kDa (p58) being significantly less prominent than the lower band of 56 kDa (p56) (Fig. 3 a). In the case of M094AJk, on the other hand, p58 showed nearly the same intensity as p56. The total intensity of p56 and p58 for each cell line was virtually the same among the NS5A-expressing cells tested, suggesting an almost equal level of NS5A expression in each cell. It should also be noted that p58 disappeared after alkaline phosphatase treatment, with p56 being detected as a single band (Fig. 3 b). This result is consistent with previous observations (Kaneko et al., 1994; Reed et al., 1997).

NS5A partially inhibits the antiviral activity of IFN against EMCV

We tested possible inhibitory effects of NS5A on IFN-mediated antiviral activity using EMCV, VSV and JEV as
Fig. 3. Immunoblot analysis of NS5A-expressing cells. (a) L929 and NIH3T3 transfectants harbouring pSG5, pSGns5A-Jk, pSGns5a-94AJk or pSGns5a-J/AJ/Jk were subjected to immunoblot analysis using anti-NS5A monoclonal antibody. Lanes 1 and 2, two independent control clones (LpSG-1 and LpSG-2); 3 and 4, two independent clones (Lns5a-J-1 and Lns5a-J-2) expressing HCV-1bJk NS5A; 5 and 6, two independent clones (Lns5a-94AJk-1 and Lns5a-94AJk-2) expressing M094AJk NS5A; 7, 3T3pSG; 8, 3T3ns5a-J; 9, 3T3ns5a-94AJ; 10 and 11, two independent clones (Lns5a-J/AJ/Jk-1 and Lns5a-J/AJ/Jk-2) expressing the chimeric NS5A. The positions of p56 and p58 are indicated. (b) Cell lysates, either mock-treated (−, lanes 1, 3 and 5) or treated with E. coli alkaline phosphatase (BAP) (+, lanes 2, 4 and 6), were analysed as in (a). Lanes 1 and 2, Lns5a-J-1; 3 and 4, Lns5a-94AJ-1; 5 and 6, Lns5a-J/AJ/Jk-1.

Fig. 4. Partial inhibition by NS5A of IFN-mediated antiviral activity against EMCV in L929 transfectants. L929 transfectants, either untreated (open symbols) or treated with 100 U/ml IFN (filled symbols), were inoculated with EMCV and virus titres in the culture fluids were determined. (○, ●) Mean titres of the control (LpSG-1 and LpSG-2); (△, ■) mean titres of clones (Lns5a-J-1 and Lns5a-J-2) expressing HCV-1bJk NS5A; (□) mean titres of clones (Lns5a-94AJ-1 and Lns5a-94AJ-2) expressing M094AJk NS5A; (◇) titre of the inoculum. Geometric means and standard deviations are shown. *, P < 0.05, compared with 3T3pSG (control).

Challenge viruses. There are a number of key molecules required for IFN to induce antiviral activity, such as PKR, 2–5A synthetase/RNase L and Mx protein (Sen & Ransohoff, 1993; Vilcek & Sen, 1996). VSV is a standard virus for IFN assay and virus replication has been reported to be inhibited through the PKR and Mx protein pathways (Vilcek & Sen, 1996) but not through the 2–5A synthetase/RNase L pathway (Chebath et al., 1987; Coccia et al., 1990; Hassel et al., 1993). On the other hand, EMCV has been shown to be inhibited translationally through the 2–5A synthetase/RNase L pathway (Vilcek & Sen, 1996; Li et al., 1998). JEV is a member of the family Flaviviridae, which also includes HCV.

A representative result obtained with EMCV is shown in Fig. 4. When infected with EMCV without IFN treatment, L929 transfectants produced progeny virus at $10^{8.1} – 10^{8.2}$ p.f.u./ml, irrespective of NS5A expression. IFN treatment (100 U/ml) prior to EMCV infection suppressed virus production drastically by 4–4 orders of magnitude in the NS5A-negative control cells. On the other hand, the IFN-mediated suppression of virus yield was inhibited to some extent in cells expressing HCV-1bJk NS5A, with the reduction being only 2–9 orders of magnitude. Partial inhibition of IFN-mediated antiviral activity was also observed in cells expressing M094AJk NS5A, but it was weaker than that observed in cells expressing HCV-1bJk NS5A.

NIH3T3 transfectants were less sensitive to IFN treatment; only a slight reduction in virus production was achieved after treatment with 100 U/ml IFN prior to EMCV infection (data not shown). Therefore, the cells were treated with 5000 U/ml IFN and infected with EMCV. This dose of IFN suppressed
A short stretch of NS5A sequence that includes the ISDR (aa 2209–2257) plays an important role in the inhibition of IFN-mediated antiviral activity

NS5A from HCV-1bJk differed from that of M094Ajk by 42 aa, including four amino acids of the ISDR (Fig. 1). It has recently been reported that a short stretch of 66 aa of NS5A (aa 2209–2274), which includes the ISDR, binds to PKR and interferes with its function (Gale et al., 1998). To determine whether or not this region is involved in the inhibition of IFN-mediated antiviral activity, we generated stable L929 transformants that expressed a chimeric NS5A with a short stretch of 49 aa, including the ISDR, of M094Ajk in the backbone of the HCV-1bJk sequence (Lns5a-J/AJ/J-1 and Lns5a-J/AJ/J-2). Immunoblot analysis revealed that the chimeric NS5A, like M094Ajk NS5A, formed two bands (p56 and p58) of practically the same intensity (Fig. 3).

As shown in Fig. 6, the chimeric NS5A clearly inhibited IFN-mediated antiviral activity to some extent. However, the reduction was significantly less than that observed with HCV-1bJk NS5A.

Inhibition of IFN-mediated antiviral activity by NS5A was only slight against VSV and barely observed against JEV

When VSV was used as a challenge virus, no inhibition of IFN (100 U/ml)-mediated antiviral activity by HCV-1bJk NS5A was observed 1 day post-infection (Fig. 7a). IFN effects are generally assessed by measuring virus titres 1 day post-infection. However, we continued to measure virus titres until 2 days post-infection, in the hope that we could detect a slight, otherwise undetectable difference. Indeed, we could observe significant reduction of IFN-mediated antiviral activity by

![Fig. 6. Involvement of a 49 aa sequence (aa 2209–2257), including the ISDR, of NS5A in the partial inhibition of IFN-mediated antiviral activity against EMCV. L929 transfectants, either untreated (open symbols) or treated with 100 U/ml IFN (filled symbols), were inoculated with EMCV and virus titres in the culture fluids were determined. (○, ●) Mean titres of the control (LpSG-1 and LpSG-2); (△, ▲) mean titres of clones (Lns5a-J-1 and Lns5a-J-2) expressing HCV-1bJk NS5A; (□, ■) mean titres of clones (Lns5a-J/AJ/J-1 and Lns5a-J/AJ/J-2) expressing a chimeric NS5A with a short stretch of 49 aa, including the ISDR, of M094Ajk in the backbone of the HCV-1bJk sequence; (◇) titre of the inoculum. Geometric means and standard deviations are shown. *, P < 0.05, compared with the control. †, P < 0.05, compared with Lns5a-J/AJ/J-1 and Lns5a-J/AJ/J-2.]

![Fig. 7. Effects of NS5A on IFN-mediated antiviral activity against VSV and JEV. Cells, either untreated (open symbols) or treated with IFN (filled symbols) at concentrations of 100 (a, b) or 10 000 U/ml (c), were inoculated with VSV (a) or JEV (b, c) and virus titres in the culture fluids were determined. (○, ●) Mean titres of the control (LpSG-1 and LpSG-2); (△, ▲) mean values of clones (Lns5a-J-1 and Lns5a-J-2) expressing HCV-1bJk NS5A; (◇) titre of the inoculum. Geometric means and standard deviations are shown. *, P < 0.05, compared with the control.]

![Virus titre (log10 p.f.u./ml)]

![Time post-infection (h)]

![Virus titre (log10 p.f.u./ml)]

![Time post-infection (h)]

![Virus titre (log10 p.f.u./ml)]

![Time post-infection (h)]
HCV-1bJk NS5A 2 days post-infection. This same tendency was observed reproducibly under these experimental conditions. On the other hand, significant inhibition was not observed in cells expressing M094AJk NS5A (data not shown).

JEV was more resistant to IFN treatment than EMCV and VSV; treatment of L929 transfectants with 100 U/ml IFN reduced JEV production by only 3–0 orders of magnitude, whereas the same IFN treatment reduced production of EMCV and VSV by 4–4 and 5–8 orders of magnitude, respectively (Figs 4 and 7a). Moreover, no significant difference was observed between NS5A-expressing cells and the control in terms of IFN-mediated reduction of JEV production (Fig. 7b). IFN treatment at a higher dose (10,000 U/ml) resulted in the reduction of JEV production to practically the same extent as that observed with EMCV or VSV after treatment with 100 U/ml IFN. Even under these experimental conditions, no significant difference was observed between NS5A-expressing cells and the control (Fig. 7c). Practically identical results were obtained with M094AJk NS5A (data not shown). Thus, HCV NS5A did not appear to inhibit IFN-mediated antiviral activity further in JEV-infected cells.

Discussion

In the present study, we demonstrated that NS5A partially inhibited the antiviral activity of IFN against EMCV and that the degree of reduction of IFN-mediated antiviral activity varied among NS5A from different strains, principally according to the different amino acid sequences of a short stretch of 49 aa (aa 2209–2257), including the ISDR (Figs 4 and 5). The inhibition of IFN activity was observed reproducibly in the cells used in this study, as well as in other cell clones prepared in separate transfection experiments (data not shown), suggesting that the inhibition was specific to NS5A expression. Also, a trend was noticed that the lower the level of NS5A expression, the less prominent the reduction of IFN-mediated antiviral activity (data not shown). Thus, our present results support the hypothesis that HCV strains with the prototype ISDR sequence are more resistant to IFN than those with mutated ISDR sequences (Enomoto et al., 1996). In this context, it was recently reported that a 66 aa sequence of NS5A (aa 2209–2274), including the ISDR, was responsible for the formation of complexes with, and the functional inhibition of, PKR (Gale et al., 1998).

The hyperphosphorylation of HCV-1bJk NS5A was reported to occur only in the presence of NS4A, which could form a complex leading to a conformational change of NS5A so that additional serine residues in the central portion of NS5A were exposed and able to be phosphorylated (Asabe et al., 1997; Lin et al., 1997). We have observed that, when expressed alone, HCV-1bJk NS5A was present mostly in the less phosphorylated form, as demonstrated by the predominance of p56 (Fig. 3a), this result being basically consistent with previous observations (Asabe et al., 1997). On the other hand, evident hyperphosphorylation of M094AJk NS5A, as indicated by the presence of p58, was observed even in the absence of NS4A. It was confirmed that p58 disappeared after alkaline phosphatase treatment, with p56 being detected as a single band (Fig. 3b). These results suggest that the conformation of NS5A varies among different HCV isolates, resulting in a different degree of exposure of the serine/threonine residues to be phosphorylated. It should also be noted that the chimeric NS5A formed two bands, p56 and p58, of practically the same intensity, as did M094AJk NS5A (Fig. 3a). Therefore, it is likely that a difference in a limited portion of NS5A (aa 2209–2257) affects the conformation and/or phosphorylation status of NS5A, which would modulate its ability to inhibit the antiviral activity of IFN.

We used L929 and NIH3T3 mouse fibroblast cell lines as parental cells because the relative importance for each of the IFN-induced antiviral pathways, such as 2–5A synthetase/RNase L and PKR, in various virus infections has been well characterized using these cells (Chebath et al., 1987; Coccia et al., 1990; Hassel et al., 1993; Li et al., 1998). Our present results clearly demonstrated that the antiviral activity of IFN against EMCV was partially inhibited, by one to two orders of magnitude, in NS5A-expressing cells (Figs 4 and 5). On the other hand, inhibition of IFN-mediated antiviral activity was much less evident when VSV was used as a challenge virus and was barely observed when JEV was used (Fig. 7). These results might reflect a differential importance for each of the IFN-mediated signalling pathways in conferring resistance against different viruses. It has been documented that the replication of EMCV, but not VSV, is inhibited through the 2–5A synthetase/RNase L pathway (Chebath et al., 1987; Coccia et al., 1990; Hassel et al., 1993; Sen & Ransohoff, 1993; Vileck & Sen, 1996). We assume that, besides interacting with PKR (Gale et al., 1997, 1998), NS5A might interact with a molecule(s) that is involved in the 2–5A synthetase/RNase L pathway. This hypothesis needs to be verified experimentally.

It is unlikely that the observed inhibition of IFN-mediated antiviral activity was a mere reflection of a cytotoxic effect due to overexpression of a protein. The rationale for this statement is as follows: (i) the inhibition was observed rather specifically with EMCV among the three viruses tested; (ii) in the absence of IFN treatment, the NS5A-expressing cells produced virtually the same amount of progeny virus as the control cells; and (iii) the NS5A-expressing cells grew normally when compared to the control cells (data not shown).

Inhibition of IFN-mediated antiviral activity by NS5A was not observed when JEV was used as a challenge virus. There are two possible explanations for this phenomenon: (i) IFN-inducible antiviral signalling pathways that can be inhibited by NS5A do not play an important role in establishing an antiviral state against JEV; or (ii) a JEV protein(s), presumably NS5, interferes with the antiviral activity of IFN through the same mechanism as HCV NS5A and, as a result, an otherwise detectable effect of NS5A is masked. The observation that the
antiviral activity of IFN was much weaker in JEV-infected cells than in cells infected with EMCV or VSV (Figs 4 and 7) favours the latter explanation.

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