Virus Research 141 (2009) 55–62

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Identification of the nonstructural protein 4B of hepatitis C virus as a factor that inhibits the antiviral activity of interferon-alpha

Jing Xu[a,b], Shufeng Liu[b], Yihui Xu[b], Po Tien[a,**], Guangxia Gao[b,*]

[a] Wuhan University, Wu Han, Hubei Province, China
[b] Institute of Biophysics, Chinese Academy of Sciences, No. 15 Da-Tun Road, Chao-Yang District, Beijing 100101, China

ARTICLE INFO

Article history:
Received 30 August 2008
Received in revised form
25 December 2008
Accepted 8 January 2009
Available online 29 January 2009

Keywords:
IFN-alpha resistance
Hepatitis C virus
NS4B

ABSTRACT

Interferon-alpha (IFN-alpha) is the most commonly used therapeutics for the treatment of chronic viral infection. However, many viruses are resistant to IFN-alpha treatment to some degrees through encoding inhibitors of the IFN-alpha producing or signaling pathway. Multiple HCV viral proteins have been reported to be involved in IFN-alpha resistance. To develop a method to screen for factors that inhibit the antiviral activity of IFN-alpha, a mini-library of HCV genome was transduced into the Huh7 cells containing the HCV subgenomic replicon (CON1 HCV S2204I) and screened for the factor that rendered the cells more resistant to IFN-alpha treatment. A fragment of nonstructural protein 4B (NS4B), named tNS4B, was isolated. Expression of tNS4B or the full-length NS4B in CON1 HCV S2204I or naïve Huh7 cells inhibited the protection of the cells by IFN-alpha treatment from vesicular stomatitis virus (VSV) infection. In Huh7 cells expressing NS4B or tNS4B, IFN-alpha-induced phosphorylation levels of signal transducer and activator of transcription 1 (STAT1) were reduced. Furthermore, expression of NS4B reduced IFN-alpha-induced expression levels of type I interferon receptor and a reporter driven by the ISRE promoter. In conclusion, we have developed a method to screen for IFN-alpha resistance factors and identified HCV NS4B as such a factor.

1. Introduction

Interferon-alpha (IFN-alpha) is the most commonly used therapeutics for the treatment of hepatitis C. However, most HCV isolates are resistant to IFN-alpha to some degrees (Fried et al., 2002; Saracco et al., 2001). The efficacy of IFN-alpha treatment differs depending on many factors, including age, sex, pre-treatment viral load, fibrosis stage and HCV genotype (Poynard et al., 2000). As far as genotype is concerned, the response rate to IFN-alpha is lower in genotype 1b than in other genotypes (Fried et al., 2002; Liang et al., 2000; Zeuzem et al., 2004).

Type I interferons, including IFN-alpha and IFN-beta, directly target human hepatocytes in both autocrine and paracrine manners, and initiate interferon signaling pathways by activation of the Janus kinase (JAK) – signal transducers and activators of transcription factor (STAT) (Darnell et al., 1994; Doly et al., 1998). Upon type I interferon binding to the heterodimeric type I interferon receptor (IFNAR), the receptor-associated tyrosine kinases JAK1 and tyrosine kinase 2 (Tyk2) are activated. Tyk2 then phosphorylates IFNAR1 on tyrosine-466, creating a docking site to accept STAT2 transferred from IFNAR2, resulting in kinase-mediated phosphorylation of STAT2 and STAT1. The activated STAT1 and STAT2 heterodimerize and translocate into the nucleus, where they bind to interferon regulatory factor-9 (IRF-9) to form a transcription factor complex, interferon-stimulated gene factor 3 (ISGF3). This complex binds to interferon-stimulated response elements (ISREs) present in promoters of a large set of target genes important in antiviral responses and initiates the transcription of these genes. Type I interferons also activate other STATs in human hepatocytes, but the roles of these STATs in IFN-mediated antiviral response are less clear (Darnell et al., 1994; van Boxel-Dezaire et al., 2006).

Type I interferons induce interferon-responsive antiviral effector mechanisms, including 2′,5′-oligoadenylate synthetase (2′,5′-OAS), double-stranded RNA activated protein kinase (PKR) and MxA, a GTPase that blocks transport of viral ribonucleoproteins to the nucleus (Samuel, 2001). Such antiviral activities are part of the early or innate host immune response to limit viral infection.

The mechanism by which HCV evades IFN-alpha treatment has been extensively studied. Several HCV proteins have been reported to be involved in interfering with the antiviral effect of IFN-alpha, including the structural proteins Core and E2, and nonstructural proteins NS3/4A and NS5A (Foy et al., 2003; Gale et al., 1997; Lin et al., 2006; Taylor et al., 1999).

NS5A was the first reported to be involved in determining the response rates of HCV subtypes to IFN-alpha through binding to PKR and thereby preventing its activation (Gale et al., 1997). Enomoto et al. described a correlation between the number of mutations

* Corresponding authors. Tel.: +86 10 64888545; fax: +86 10 64877837.
** Co-corresponding authors. Tel.: +86 27 68752897; fax: +86 27 68752897.
E-mail addresses: tienpo@sun.im.ac.cn (P. Tien), gaogx@moon.ibp.ac.cn (G. Gao).

0168-1702/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2009.01.001

within a 40 amino acid sequence of NS5A and the response rate to IFN-alpha therapy in genotype 1b-infected patients (Enomoto et al., 1995). These results were later confirmed by some Japanese clinical studies, but were not in accordance with most Western European and American studies (Arase et al., 1999; Enomoto et al., 1996; Sarrazin et al., 2000).

A stretch of 12-amino acids in E2, named PKR-eIF2α phosphorylation homology domain (PePHD), was reported to be involved in PKR inhibition (Taylor et al., 1999). Taylor et al. provided evidence implying that the E2 protein acts as a competitive inhibitor of PKR and suggested that E2 determines the response rates of HCV of different subtypes to IFN-alpha (Taylor et al., 1999). However, studies in a small number of patients infected with HCV genotypes 1 and 3 revealed that in some patients responding to IFN-alpha treatment, the virus had a PePHD sequence identical to that of the HCV 1b subtype, calling Taylor's hypothesis into question (Abid et al., 2000). The involvement of E2 in determining the IFN-alpha response rates was further challenged by the clinical observations on HCV2a/b isolates (Le Guillou-Guillemette et al., 2007; Saito et al., 2003).

The Core protein has also been reported to be involved in IFN-alpha resistance by a few groups. The Core protein seems to inhibit IFN-alpha response through inhibiting STAT1 phosphorylation, although different groups reported different mechanisms by which Core inhibits STAT1 activation (de Lucas et al., 2005; Lin et al., 2005; Melen et al., 2004). The correlation between the sequence of Core and clinical IFN-alpha response has not been documented.

Transient overexpression of NS3/4A, but not E2 or NS5A, blocks the activation of interferon regulatory factor-3 (IRF-3), the major transcription factor inducing expression of a variety of cellular interferon-stimulated genes and thereby inhibits interferon production induced by virus infection (Foy et al., 2003). The HCV NS3/4A protease functions through cleaving the Toll-like receptor 3 adaptor protein (TRIF) (also known as CARDIF, MAVS, IPS-1 or VISA) and blocking retinoic acid-inducible gene-1 (RIG-I) signaling and thereby restoring virus-induced IRF-3 phosphorylation (Foy et al., 2005; Ferreon et al., 2005; Li et al., 2005). The N-terminal domain of NS3 (amino acids 1–180) with a serine protease located in is required for this cleavage and the NS4A functions as an cofactor for the protease (Ferreon et al., 2005).

Despite some controversies, the above studies suggest that HCV encode multiple mechanisms to block the IFN-alpha pathway.

HCV NS4B is a highly hydrophobic nonstructural protein, with 244 amino acids for genotype 1b. NS4B is localized to the endoplasmic reticulum (ER) and induces a pattern of cytoplasmic foci positive for markers of the ER through four transmembrane segments. By introducing glycosylation sites at various positions in NS4B, it has been demonstrated that the C-terminus is cytoplasmic and N-terminal portion is luminal (Lundin et al., 2003). NS4B induces intracellular membrane alterations (Egger et al., 2002). The altered membranous web is thought to be the viral replication site (Egger et al., 2002; Gosert et al., 2003). Mutagenesis studies of the nucleotide binding motif of NS4B suggest that NS4B is also a helper factor for the HCV RNA dependent RNA polymerase (Piccininni et al., 2002; Einav et al., 2004).

In the present study, using the HCV subgenomic replicon system, we developed a method to screen for viral factors that inhibit the antiviral activity of IFN-alpha and identified HCV NS4B as a factor that may contribute to IFN-alpha resistance.

2. Materials and methods

2.1. Constructs

Retroviral vector pBabe-HAZ-SrfI was modified from pBabe-HAZ, which contains a hemagglutinin (HA) epitope tag at the 5′-end, a Zeocin resistance gene at the 3′-end and cloning sites in between (Gao et al., 2002). To generate pBabe-HAZ-SrfI, a SrfI restriction site was inserted by cloning a stretch of synthetic double-stranded oligonucleotides (5′-GAATTCGGGCCCGGGCG-GGGCGGCCGC-3′) between the EcoRI and NotI sites in pBabe-HAZ.

pBabe-tNS4B and pBabe-NS4B are retroviral vectors expressing HA-tagged HCV tNS4B and NS4B of subtype 1b, respectively. The cDNA isolated from the C4 cells, which encodes HA-tagged tNS4B, was cloned into retroviral vector pBabe-puro (Morgenstern and Land, 1990) using BamHI and SalI sites to generate pBabe-C4. pBabe-tNS4B, pBabe-NS4B and pBabe-NS5A were generated by cloning the PCR-derived fragments of tNS4B, NS4B or NS5A into pBabe-C4 to replace the original tNS4B sequence using EcoRI and SalI sites. The sequences of forward primers (FPs) and reverse primers (RPs) are listed below, with the built-in restriction sites underlined.

tNS4B-FP: 5′-CGGGATCC GCCACCATGGCTTATCCATAT-3′;
tNS4B-RP: 5′-ACGCGTCGACTCAACTTGGCGCGGCCGCTCCAGC-GATG-3′;
NS4B-FP: 5′-GGAATTCGCCTCACACCTCCCTTAC-3′;
NS4B-RP: 5′-ACGCGTCGACTCAGCATGGCGTGGAGCAGTC-3′;
NS5A-FP: 5′-GGAATTCTCCGGCTCGTGGCTA
NS5A-RP: 5′-ACGCGTCGACTCAGCAGCAGACGACGTCCTC

The ISRE-Luc plasmid was kindly provided by Dr. Chen Wang of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, China.

2.2. Construction of the HCV genomic library and selection of interferon-resistant clones

An infectious clone of subtype 1b HCV genome (HC-J4) (Yanagi et al., 1998) was fragmented using a nebulizer following a previously described procedure (Evans et al., 2004). Briefly, the DNA was sheared by hydrodynamic forces, and the fragments were "polished" with polymerases and phosphorylated with T4 polynucleotide kinase (New England Biolabs), followed by size fractionation using agarose gel electrophoresis. The fragments of 0.2–1.0 kb were cloned into retroviral vector pBabe-HAZ-SrfI. The library was co-transfected into 293T cells with pGag-Pol, a plasmid-expressing murine leukemia virus Gag-Pol, and pVSV-G, a plasmid-expressing vesicular stomatitis virus (VSV) glycolprotein, to produce library-transducing viruses to infect CON1 HCV S2204I cells. The library-transduced cells were treated with IFN-alpha at a final concentration of 100 IU/ml for three times and cultured in media-containing G418 (Invitrogen) at a final concentration of 1 mg/ml until single colonies appeared.

2.3. Cell culture and assays

All the cells were maintained in DMEM supplemented with 10% fetal calf serum. CON1 HCV S2204I cells, a Huh-7 cell line expressing HCV subgenomic replicon, were kindly provided by Dr. Charles Rice of Rockefeller University, USA (Blight et al., 2000). To generate the cell lines expressing tNS4B, NS4B or NS5A in CON1 HCV S2204I or Huh7 cells, pBabe-tNS4B, pBabe-NS4B or pBabe-NS5A was cotransfected into 293T cells with pGag-Pol and pVSV-G to produce pseudotyped virus to transduce CON1 HCV S2204I or Huh7 cells. The transduced cells were selected with puromycin (Sigma) at a final concentration of 4 μg/ml.

The IFN-alpha antagonizing activity of tNS4B, NS4B or NS5A was assessed on the basis of their protective effect on VSV infection against IFN-alpha, as described by Familletti et al. and Johnston et al. with some modifications (Familletti et al., 1981; Johnston et al., 2005). Briefly, cells were seeded at full confluency, treated with IFN-alpha at the concentration of 100 IU/ml for 16 h, washed twice

with PBS, and infected with 1000 pfu of VSV for 2 h. The infection media were replaced with fresh media and the cells were incubated for additional 24 h. The titers of the viruses collected from the supernatants of the infected cells were measured on HeLa cells by counting the plaques visualized by Gimesa staining.

Activation of the ISRE-Luc reporter expression by IFN-alpha treatment was measured with the luciferase reporter assay. Cells were seeded in 35 mm plates and transfected on the following day using Fugene 6 (Roche Diagnostics) with the reporter plasmid expressing firefly luciferase under the control of ISRE promoter. To normalize transfection efficiency, 0.1 μg of pTK-RL (Promega), which expresses Renilla luciferase under the control of a constitutive promoter, was included in each transfection. Approximately 48 h posttransfection, the luciferase activities were measured using the Dual-luciferase assay kit (Promega) following the manufacturer's instructions.

2.4. Isolation of the cDNA from the IFN-alpha-resistant cells

To recover the cDNA from the IFN-alpha-resistant cells, total RNA was isolated using the RNAeasy Mini Kit (Qiagen). First-strand cDNA was prepared using MLV-RT and amplified in a 50 μl PCR reaction with the Expand High Fidelity PCR kit (Roche Diagnostics) under the following conditions: 10 cycles of 94 °C for 15 s, 50 °C for 30 s, 72 °C for 60 s each cycle, followed by 20 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 60 + 5 s each cycle. The forward primer was 5′-GCTTATCCATATGATGTTCCAGATT-3′ and the reverse primer was 5′-GCACCGGAACGGCACTGGTCAACTT-3′.

2.5. Antibodies

The rabbit polyclonal antibody against NS4B was kindly provided by Dr. Charles Rice of Rockefeller University, USA. The rest antibodies are commercially available, including antibodies against HA (Santa Cruz, USA, rabbit, catalog number sc-805), IFNAR (Abcam, UK, goat, catalog number ab10739), P-STAT1 (Cell Signal Technology, USA, rabbit, catalog number 9171), STAT1 (Cell Signal Technology, USA, rabbit, catalog number 9172), P-STAT3 (Cell Signal Technology, USA, rabbit, catalog number 9131), STAT3 (Cell Signal Technology, USA, rabbit, catalog number 9132), P-Jak1 (Cell Signal Technology, USA, rabbit, catalog number 3331), Jak1 (Cell Signal Technology, USA, rabbit, catalog number 3332), P-Tyk2 (Cell Signal Technology, USA, rabbit, catalog number 9321) and Tyk2 (Cell Signal Technology, USA, rabbit, catalog number 9312).

2.6. Quantification of relative protein expression levels

The results of Western blotting were analyzed by the NIH Image J software (http://rsb.info.nih.gov/ij). The relative intensity of the band of interest was normalized by that of the loading control (β-actin in most cases).

3. Results

3.1. Isolation of cell lines resistant to IFN-alpha treatment

To establish a strategy to isolate IFN-alpha resistance genes, we set out to develop a screening method using the HCV subgenomic replicon system to identify the viral factors that protect the replicon from IFN-alpha treatment (Fig. 1). The HCV subgenomic replicon can stably self-replicate in Huh-7 cells, rendering the cells capable of growing in G418-containing media. When the cells are treated with IFN-alpha at the concentration of 100 IU/ml, the replication is inhibited and the cells become sensitive to G418 (Blight et al., 2000; Lohmann et al., 1999). Although the HCV replicon already expresses some nonstructural proteins, we speculated that expression of an HCV protein that is capable of rendering the cells resistant to IFN-alpha but not included in the replicon should help to increase the threshold for IFN-alpha treatment to inhibit the replication of the replicon. Even if the protein that has IFN-alpha antagonizing activity was included in the replicon, additional expression of this protein might also improve the cells' resistance to IFN-alpha treatment.

The genome of a subtype 1b HCV infectious clone was fragmented and cloned into a retroviral vector, with an HA tag fused at the 5′-end to provide a start codon and an epitope for future detection of the protein by Western blotting (see Section 2 for details). The Huh7 cells harboring HCV subgenomic replicon (CON1 HCV S2204I cells) (Blight et al., 2000) were transduced with the HCV library at low multiplicity of infection (m.o.i. = 0.01) such that one CON1 HCV S2204I cell received only one copy of a HCV fragment. The transduced cells were repeatedly treated with IFN-alpha at the concentration of 100 IU/ml and selected in G418-containing media. The surviving cells were retested for their resistance to IFN-alpha

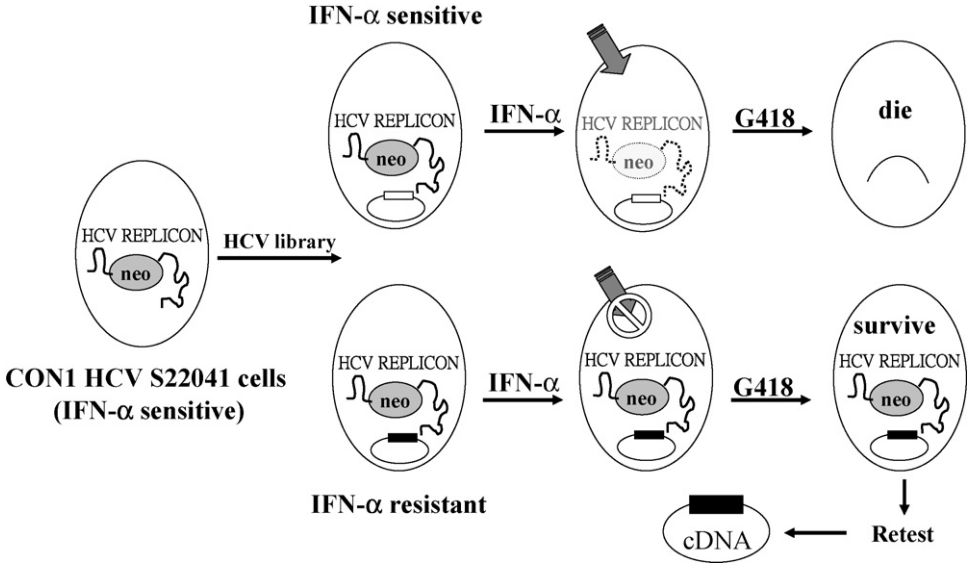

Fig. 1. Strategy for isolating HCV viral factor (s) that renders CON1 HCV S2204I cells resistant to IFN-alpha treatment.

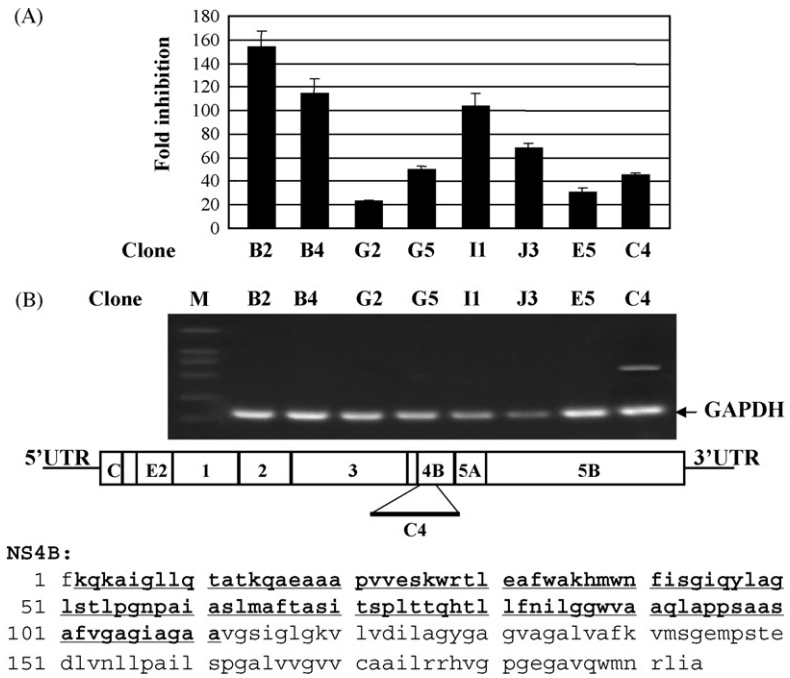

Fig. 2. (A) Confirmation of the cell lines that were resistant to IFN-alpha treatment. The cells were treated with IFN-alpha (1000 IU/ml) for 16 h and then infected with VSV. Twenty-four hours postinfection the supernatants were collected and the virus in the supernatant was titered by the VSV plaque assay. Fold inhibition is calculated as the titer of the virus produced by the resistant cells divided by that of the virus produced by the control CON1 HCV S2204I cells. The data are means ± S.D. of three independent experiments. (B) Isolation of the cDNA from the IFN-alpha-resistant cell lines. Total RNA was isolated from the resistant clones and reverse transcribed. The transduced cDNA was recovered by PCR using vector specific primers (upper panel). GAPDH was used as a control. The recovered cDNA was sequenced and aligned with the HCV genome (lower panel). The amino acid sequence encoded by the recovered cDNA is underlined. B2, B4, G2, G5, I1, J3, E5 and C4 are cell lines isolated from the screening that were resistant to IFN-alpha treatment.

treatment as judged by an increase in the susceptibility to VSV infection after IFN-alpha treatment.

In one selection, 198 clones survived. These clones were retested for the resistance to IFN-alpha treatment by the VSV propagation assay (see Section 2 for details). Compared with the empty vector-transduced control cells, 8 clones displayed more than 20-fold resistance to IFN-alpha treatment (Fig. S1 for the original data and Fig. 2A for fold inhibition). These cells were further analyzed.

3.2. Isolation of a truncated NS4B from the IFN-alpha-resistant cells

To recover the cDNAs from the IFN-alpha-resistant cells, total RNA was isolated and the transduced cDNA was recovered by RT-PCR. Out of the eight resistant clones, only in the clone C4 was a cDNA recovered (Fig. 2B, upper panel). Sequence analysis of the recovered cDNA from clone C4 revealed that it is a fragment of HCV genome corresponding to part of the NS4B coding sequence (Fig. 2B, lower panel). This fragment was thus named truncated NS4B (tNS4B).

3.3. Expression of NS4B was sufficient to confer IFN-alpha resistance

To confirm that tNS4B was the causal agent of the IFN-alpha resistance, the cDNA encoding HA-tagged tNS4B was cloned into retroviral vector pBabe-puro and introduced into CON1 HCV S2204I cells. The transduced cells were selected in puromycin. Bulk-tansduced cells and three individual clones were used for further analyses. Compared with original C4 cell line, the bulk-transduced tNS4B cells displayed similar magnitude of inhibition to the antiviral activity of IFN-alpha (Fig. S2A for the original data and Fig. 3A for fold inhibition). Three individual clones expressing different levels of tNS4B were also used for analysis for their resistance to IFN-alpha treatment. Expression of tNS4B indeed reduced the inhibitory effect of IFN-alpha on VSV propagation and the magnitude of reduction was roughly correlated with the expression levels of tNS4B (Fig. S2B for the original data and Fig. 3B for fold inhibition).

To test whether the full-length NS4B also had the IFN-alpha antagonizing activity, NS4B was introduced into naïve Huh7 cells. The better studied NS5A was used as a positive control. Three cell lines each expressing different levels of tNS4B, NS4B or NS5A were analyzed by the VSV propagation assays. NS4B and tNS4B displayed similar IFN-alpha antagonizing activities as NS5A (Fig. S3 for the original data and Fig. 4A for fold inhibition). The expression level of NS4B in the Huh7 cells expressing the highest level of NS4B (Huh7-NS4B clone 1 in Fig. 4A) was compared with the expression level of NS4B in the CON1 HCV S2204I cells (Fig. 4B). The expression levels were relatively comparable. Taken together, these results established that expression of NS4B or tNS4B reduced the antiviral activity of IFN-alpha.

3.4. NS4B inhibited IFN-alpha-induced activation of STAT1

STAT1 is an important player in type I interferon signaling pathway and it has been reported that viruses could antagonize the antiviral activity of interferons by inhibiting the activation of STAT1 (Rodriguez et al., 2004; Takeuchi et al., 2001). In an attempt to understand the mechanisms by which NS4B inhibited the activity of IFN-alpha, we analyzed whether NS4B affected the IFN-alpha-induced activation of STAT1. The Huh7 cells that expressed the highest level of NS4B (Huh7-NS4B clone 1 in Fig. 4A) were treated with IFN-alpha following serum starvation and the levels of IFN-alpha-induced STAT1 phosphorylation were detected by Western blotting. Compared with Huh7 control cells, the levels of phosphorylated STAT1 were significantly reduced in Huh7-NS4B cells

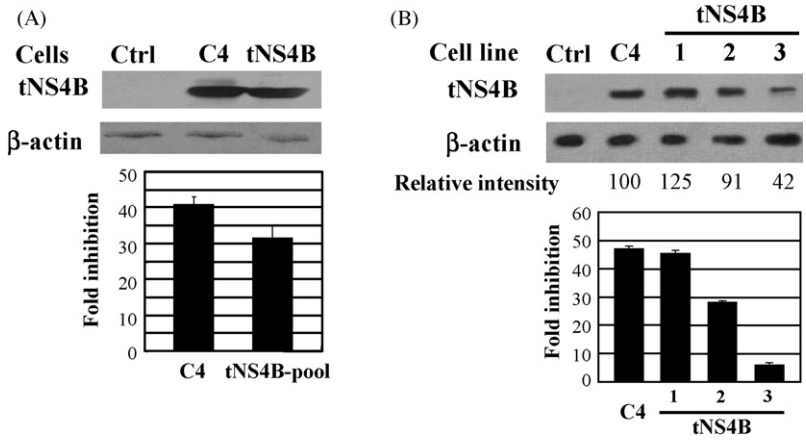

Fig. 3. Expression of tNS4B was sufficient to render CON1 HCV S22041 cells resistant to IFN-alpha treatment. CON1 HCV S22041 cells were transduced with tNS4B and selected in puromycin (4 μg/ml). (A) The expression levels of tNS4B in a pool of the transduced cells and in the C4 cells were analyzed by Western blotting (upper panel). The resistance of these cells to IFN-alpha treatment was assayed as described in the legend to Fig. 2A (lower panel). Ctrl, CON1 HCV S22041 control cells; C4, C4 cells; tNS4B, tNS4B-transduced cells. (B) Individual clones of the tNS4B-transduced CON1 HCV S22041 cells were picked, expanded and analyzed for the expression levels of tNS4B, as measured by Western blotting (upper panel). The cell lines expressing different levels of tNS4B were assayed for their resistance to IFN-alpha treatment as described in the legend to Fig. 2A (lower panel). The relative protein expression levels of tNS4B are indicated. The data are means ± S.D. of three independent experiments.

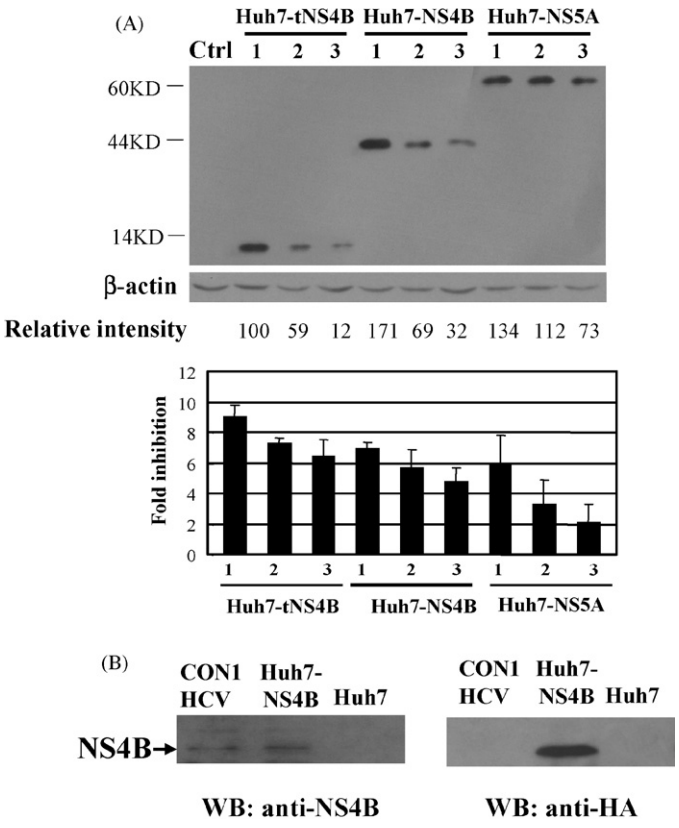

Fig. 4. (A) Comparison of the IFN-alpha antagonizing activities of tNS4B, NS4B and NS5A in Huh7 cells. Huh7 cells were transduced with retroviral vectors expressing tNS4B, NS4B or NS5A and selected in puromycin (4 μg/ml). Individual clones were picked, expanded and analyzed for protein expression levels by Western blotting (upper panel). The relative protein expression levels are indicated. The inhibitory effects of these proteins on IFN-alpha response were assayed as described in the legend to Fig. 2A. Ctrl: empty vector transduced Huh7 cells. The data are means ± S.D. of three independent experiments. (B) Comparison of the protein levels of NS4B expressed in CON1 HCV S22041 cells and in Huh7-NS4B cells by Western blotting using the indicated antibodies. CON1 HCV, CON1 HCV S22041 cells; Huh7-NS4B, Huh7-NS4B clone 1 cells in Fig. 4A; Huh7, naïve Huh7 cells.

(Fig. 5A). Similar experiments were also performed without serum starvation and similar results were observed (Fig. S4).

To test whether NS4B specifically inhibits the signaling of IFN-alpha or generally inhibits STAT1 activation, the cells were treated with IFN-gamma under the same condition. No obvious difference was detected between control cells and NS4B-expressing cells in IFN-gamma-induced STAT1 phosphorylation (Fig. 5B), suggesting that the inhibitory effect of NS4B is specific to the IFN-alpha signaling pathway.

In an attempt to further understand the mechanisms underlying the inhibitory effect of NS4B on the IFN-alpha signaling pathway, the IFN-alpha-induced phosphorylation levels of JAK1, TYK2 and STAT3 were compared and no obvious difference was detected between the control cells and the NS4B-expressing cells (Fig. 6). It is worth noting that the IFN-alpha induction condition was optimized such that the phosphorylation level of each signal transducer was not saturated (data not shown).

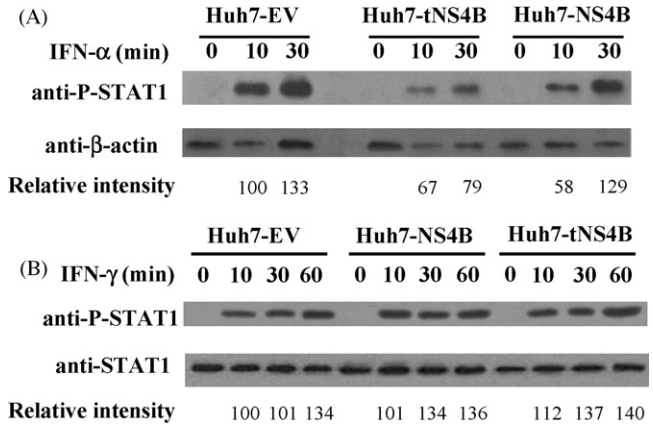

Fig. 5. Inhibition of IFN-alpha-induced STAT1 activation by NS4B. The indicated cell lines were serum-starved for 12 h and treated with the indicated interferon for the indicated time. The Huh7-NS4B cell lines are the clones that expressed the highest level of the protein (clone 1 in Fig. 4). The cells were lysed, and the proteins were resolved by electrophoresis and subject to Western blotting with the indicated antibodies. The relative protein levels of phosphorylated STAT1 (P-STAT1) are indicated. The data are representative of three independent experiments. (A) Tyrosine phosphorylation of STAT1 upon stimulation with IFN-alpha (1000 IU/ml). (B) Tyrosine phosphorylation of STAT1 upon stimulation with IFN-gamma (1000 IU/ml).

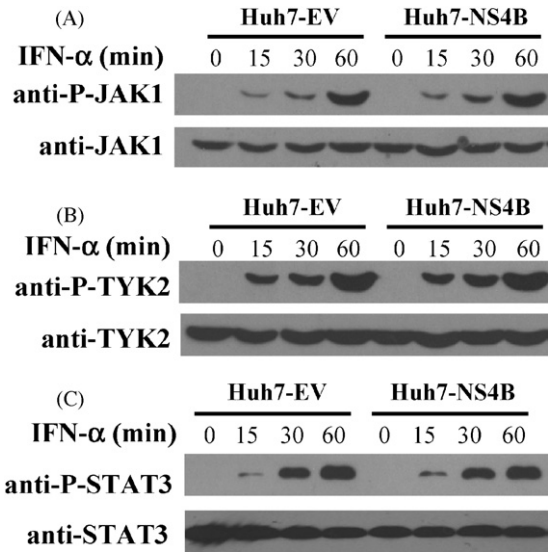

Fig. 6. Effects of NS4B on the activation of other IFN-alpha signal transducers. The indicated cell lines were serum-starved for 12 h and treated with the indicated interferon for the indicated time. The cells were lysed, and the proteins were resolved by electrophoresis and subject to Western blotting with the indicated antibodies. The data are representative of three independent experiments. (A) Tyrosine phosphorylation of JAK1 upon stimulation with IFN-alpha (1000 IU/ml). (B) Tyrosine phosphorylation of TYK2 upon stimulation with IFN-alpha (1000 IU/ml). (C) Tyrosine phosphorylation of STAT3 upon stimulation with IFN-alpha (1000 IU/ml).

3.5. NS4B inhibited IFN-alpha-induced activation of ISRE promoter and expression of type I interferon receptor

IFN-alpha treatment of cells results in the upregulation of a repertoire of downstream genes. To further demonstrate the inhibitory effect of NS4B on IFN-alpha signaling, we compared the IFN-alpha-induced expression of ISRE-Luc reporter in control cells and NS4B-expressing cells. Huh7 cells expressing different levels of NS4B or tNS4B were transfected with a luciferase reporter under the control of ISRE promoter and assayed for the IFN-alpha-induced luciferase expression. Expression of the reporter was reduced by about 50% (Fig. 7A).

We next analyzed the effect of NS4B in Huh7 cells on IFN-alpha-induced expression of type I interferon receptor. In the control Huh7 cells, treatment of the cells with IFN-alpha resulted in increased expression of the receptor. In comparison, in NS4B or tNS4B-expressing cells, the increase in the receptor expression level was reduced (Fig. 7B).

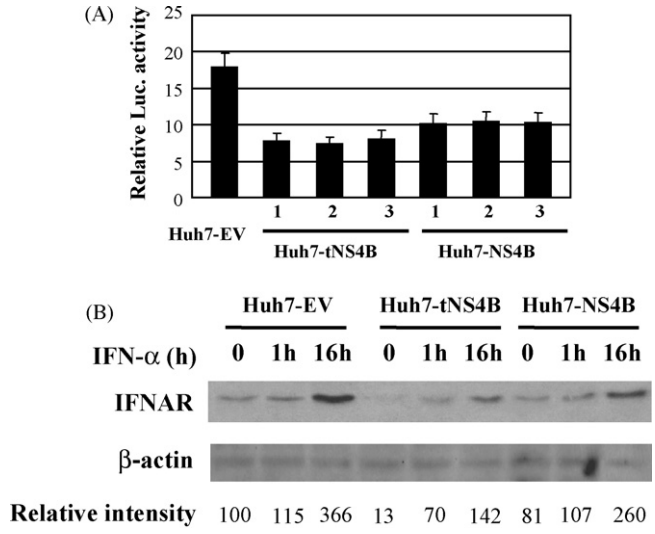

Fig. 7. Inhibition of IFN-alpha-induced downstream gene expression by NS4B. (A) The ISRE-Luc reporter plasmid was transfected into the indicated cells. Twenty-four hours post transfection, the cells were treated with IFN-alpha (1000 IU/ml) for 30 min and cultured for an additional 24 h. Then the cells were lysed and the luciferase activities were measured. The data are means ± S.D. of three independent experiments ($p \leq 0.03$). (B) Inhibition of IFN-alpha-induced expression of IFNAR by tNS4B and NS4B. The indicated cell lines were treated with IFN-alpha at a final concentration of 1000 IU/ml for the indicated time. The expression levels of IFNAR were detected by Western blotting using the indicated antibodies. The data are representative of two independent experiments. The relative protein expression levels of IFNAR are indicated.

4. Discussion

Type I interferons play important roles for the host to prevent virus infection. To establish efficient infection, viruses have evolved multiple mechanisms to antagonize interferons. Identification and characterization of these mechanisms should help better understand the virus–host interplay during the process of viral infection and may eventually lead to development of efficient antiviral therapeutics. In this report, we developed a screening method to isolate factors that can inhibit the antiviral activity of IFN-alpha and identified HCV NS4B as such a factor. This method should be also useful for isolating IFN-alpha resistance factors of other viruses, especially those with large genomes. Furthermore, this method could be modified to isolate host factors involved in IFN-alpha resistance.

Out of the eight IFN-alpha-resistant clones only from one clone was cDNA recovered. The lack of cDNAs in the other seven clones could be accounted for by a few possibilities. First, the INF-alpha resistance phenotype is caused by spontaneous mutations of the cells. Cultured cells are well known for their genetic instability. Our experience with somatic cell screenings is that the background is usually relatively high due to spontaneous mutations. Secondly, the RT-PCR method was not sensitive enough to recover some cDNAs. However, the same RT-PCR method, including primers and PCR conditions, has been successfully used to isolate much longer inserts. Thus, although the possibility exists that the RT-PCR rescue is not sensitive enough, we tend to believe this is unlikely the reason. Thirdly, the IFN-alpha resistance phenotype could be caused by some adaptive mutations in the subgenomic replicon. To investigate this possibility, the subgenomic replicons were recovered from the cDNA-negative IFN-alpha-resistant clones and sequenced, and no adaptive mutations are found (data not shown). In summary, we tend to believe that the cDNA-negative-resistant clones arose from spontaneous mutations.

Besides NS4B, multiple HCV viral factors, such as E2, core, NS3/4A and NS5A, have been reported to be involved in IFN-alpha resistance. In the screening reported here, only a fragment of NS4B was recovered. The failure to isolate other factors is likely due to the fact that the screening we report here is a relatively small-scale screening. In addition, the library was made from small fragments of HCV genome, which may not include all the IFN-alpha resistance factors. To recover more IFN-alpha resistance factors a large-scale screening using different libraries should be needed.

During the course of this work, the involvement of HCV NS4B in IFN-alpha resistance was reported by other groups (Keskinen et al., 2002; Munoz-Jordan et al., 2005). Keskinen et al. analyzed the antiviral effect of various HCV proteins against VSV infection in human osteosarcoma cells and reported that expression of NS4B impaired the IFN-alpha-induced antiviral activity (Keskinen et al., 2002). The underlying mechanism was not further studied. Munoz-Jordan et al. analyzed the activity of HCV NS4B to inhibit the activation of ISRE reporter and reported negatively results (Munoz-Jordan et al., 2005). Consistent with these findings, in the present study, expression of HCV NS4B in Huh7 cells inhibited IFN-alpha-induced antiviral activity by about sevenfold in the VSV production

assay (Fig. 4A), but reduced IFN-alpha-induced ISRE reporter expression by less than 50% (Fig. 7A). The VSV production assay, which measures the activity of HCV NS4B to inhibit the protection of cells by IFN-alpha from VSV infection, should better reflect the antiviral activity of NS4B. The involvement of NS4B of other Flaviviruses has also been reported (Liu et al., 2005; Munoz-Jordan et al., 2003), suggesting that the involvement of NS4B in IFN-alpha resistance might be a conserved mechanism in Flaviviruses.

The involvement of NS4B in IFN-alpha resistance was also implicated in clinical studies. Welker et al. analyzed the correlation between IFN-alpha response rates and NS4B sequences, and reported that a rapid initial HCV RNA decline could be associated with a higher frequency of nonconservative amino acid exchanges within NS4B compared with patients with a nonrapid HCV RNA decline (Welker et al., 2007). It would be interesting to systematically analyze the correlation between the NS4B sequences and their IFN-antagonizing activities in cultured cells.

The truncated NS4B displayed the same IFN-alpha antagonizing activity as the full-length NS4B (Fig. 4A). HCV NS4B is reported to be localized to the ER through four transmembrane domains (TMDs), with the C-terminal domain in the cytoplasm and the N-terminal domain in the lumen (Lundin et al., 2003). tNS4B contains the N-terminal domain, TMD1 and most of TMD2 of NS4B, which should position the protein in ER in a similar manner as the counterpart in NS4B. It is possible that the IFN-alpha antagonizing activity requires only the portion corresponding to tNS4B. Database search revealed that Phe102 and Ile124 of NS4B are predicted as proteasomal cleavage sites with very high scores at netchop (www.cbs.dtu.dk/Services/NetChop). It would be interesting to find out whether such a truncated form of NS4B also exists *in vivo*.

NS4B displayed higher inhibitory effect on IFN-alpha in CON1 HCV S2204I cells than in naïve Huh7 cells (Figs. 3 and Fig. 4A), suggesting that other IFN-resistance genes expressed from the replicon synergize with the transduced NS4B for IFN-alpha resistance. Our preliminary results revealed that coexpression of NS4B and NS5A displayed higher IFN-alpha antagonizing activity than NS4B or NS5A alone (data not shown). How these factors interplay in IFN-alpha resistance and the underlying mechanisms await further investigation.

In NS4B-expressing cells the IFN-alpha-induced activation of STAT1 was reduced while IFN-gamma-induced STAT1 activation was not affected (Fig. 5), suggesting that the inhibitory effect of NS4B on STAT1 activation is specific to IFN-alpha receptors. Direct interaction between NS4B and IFN-alpha receptors could not be detected (data not shown). This could be accounted for by a weak interaction, if any, between NS4B and IFN-alpha receptors or by the possibility that the interaction was indirect. Further investigation is needed to elucidate the mechanism by which HCV NS4B inhibits IFN-alpha-induced activation of STAT1.

Acknowledgements

We thank Dr. Charles Rice of Rockefeller University, USA, for providing CON1 HCV S2204I cells, Dr. Chen Wang of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, China, for providing ISRE-Luc reporter plasmid.

Grant Support: This work was partially supported by grants to Guangxia Gao from the National Science Foundation (30770099) and the Ministry of Science and Technology (973 Program 2006CB504302) of China.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.virusres.2009.01.001.

References

Abid, K., Quadri, R., Negro, F., 2000. Hepatitis C virus, the E2 envelope protein, and alpha-interferon resistance. Science 287 (5458), 1555.

Arase, Y., Ikeda, K., Chayama, K., Murashima, N., Tsubota, A., Suzuki, Y., Saitoh, S., Kobayashi, M., Kumada, H., 1999. Efficacy and changes of the nonstructural 5A GENE by prolonged interferon therapy for patients with hepatitis C virus genotype 1b and a high level of serum HCV-RNA. Intern. Med. 38 (6), 461–466.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290 (5498), 1972–1974.

Darnell Jr., J.E., Kerr, I.M., Stark, G.R., 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264 (5164), 1415–1421.

de Lucas, S., Bartolome, J., Carreno, V., 2005. Hepatitis C virus core protein downregulates transcription of interferon-induced antiviral genes. J. Infect. Dis. 191 (1), 93–99.

Doly, J., Civas, A., Navarro, S., Uze, G., 1998. Type I interferons: expression and signalisation. Cell Mol. Life Sci. 54 (10), 1109–1121.

Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H.E., Moradpour, D., Bienz, K., 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virol. 76, 5974–5984.

Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Izumi, N., Marumo, F., Sato, C., 1995. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J. Clin. Invest. 96 (1), 224–230.

Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Ogura, Y., Izumi, N., Marumo, F., Sato, C., 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N. Engl. J. Med. 334 (2), 77–81.

Evans, M.J., Bacharach, E., Goff, S.P., 2004. RNA sequences in the Moloney murine leukemia virus genome bound by the gag precursor protein in the yeast three-hybrid system. J. Virol. 78, 7677–7684.

Einav, S., Elazar, M., Danieli, T., Glenn, J.S., 2004. A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. J. Virol. 78, 11288–11295.

Familletti, P.C., Rubinstein, S., Pestka, S., 1981. A convenient and rapid cytopathic effect inhibition assay for interferon. Methods Enzymol. 78 (Pt A), 387–394.

Ferreon, J.C., Ferreon, A.C., Li, K., Lemon, S.M., 2005. Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3/4A protease. J. Biol. Chem. 280, 20483–20492.

Foy, E., Li, K., Wang, C., Sumpter Jr., R., Ikeda, M., Lemon, S.M., Gale Jr., M., 2003. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science 300 (5622), 1145–1148.

Foy, E., Li, K., Sumpter, R., Loo, Y.M., Johnson, C.L., Wang, C., et al., 2005. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid- inducible gene-I signaling. Proc. Natl. Acad. Sci. U.S.A. 102, 2986–2991.

Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales Jr., F.L., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J., Yu, J., 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347 (13), 975–982.

Gale Jr., M.J., Korth, M.J., Tang, N.M., Tan, S.L., Hopkins, D.A., Dever, T.E., Polyak, S.J., Gretch, D.R., Katze, M.G., 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 230 (2), 217–227.

Gao, G., Guo, X., Goff, S.P., 2002. Inhibition of retroviral RNA production by ZAP, a CCCH-type zinc finger protein. Science 297 (5587), 1703–1706.

Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H.E., Bienz, K., Moradpour, D., 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. 77 (9), 5487–5492.

Johnston, J.B., Nazarian, S.H., Natale, R., McFadden, G., 2005. Myxoma virus infection of primary human fibroblasts varies with cellular age and is regulated by host interferon responses. Virology 332 (1), 235–248.

Keskinen, P., Melen, K., Julkunen, I., 2002. Expression of HCV structural proteins impairs IFN-mediated antiviral response. Virology 299, 164–171.

Le Guillou-Guillemette, H., Vallet, S., Gaudy-Graffin, C., Payan, C., Pivert, A., Goudeau, A., Lunel-Fabiani, F., 2007. Genetic diversity of the hepatitis C virus: impact and issues in the antiviral therapy. World J. Gastroenterol. 13 (17), 2416–2426.

Li, K., Foy, E., Ferreon, J.C., Nakamura, M., Ferreon, A.C., Ikeda, M., Ray, S.C., Gale Jr., M., Lemon, S.M., 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc. Natl. Acad. Sci. U.S.A. 102, 2992–2997.

Liang, T.J., Rehermann, B., Seeff, L.B., Hoofnagle, J.H., 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann. Intern. Med. 132 (4), 296–305.

Lin, W., Choe, W.H., Hiasa, Y., Kamegaya, Y., Blackard, J.T., Schmidt, E.V., Chung, R.T., 2005. Hepatitis C virus expression suppresses interferon signaling by degrading STAT1. Gastroenterology 128 (4), 1034–1041.

Lin, W., Kim, S.S., Yeung, E., Kamegaya, Y., Blackard, J.T., Kim, K.A., Holtzman, M.J., Chung, R.T., 2006. Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. J. Virol. 80 (18), 9226–9235.

Liu, W.J., Wang, X.J., Mokhonov, V.V., Shi, P.Y., Randall, R., Khromykh, A.A., 2005. Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. J. Virol. 79 (3), 1934–1942.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285 (5424), 110–113.

Lundin, M., Monne, M., Widell, A., Von Heijne, G., Persson, M.A., 2003. Topology of the membrane-associated hepatitis C virus protein NS4B. J. Virol. 77 (9), 5428–5438.
Melen, K., Fagerlund, R., Nyqvist, M., Keskinen, P., Julkunen, I., 2004. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. J. Med. Virol. 73 (4), 536–547.
Morgenstern, J.P., Land, H., 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic. Acids. Res. 18 (12), 3587–3596.
Munoz-Jordan, J.L., Laurent-Rolle, M., Ashour, J., Martinez-Sobrido, L., Ashok, M., Lipkin, W.I., Garcia-Sastre, A., 2005. Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J. Virol. 79 (13), 8004–8013.
Munoz-Jordan, J.L., Sanchez-Burgos, G.G., Laurent-Rolle, M., Garcia-Sastre, A., 2003. Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. U.S.A. 100 (24), 14333–14338.
Piccininni, S., Varaklioti, A., Nardelli, M., Dave, B., Raney, K.D., McCarthy, J.E., 2002. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. J. Biol. Chem. 277, 45670–45679.
Poynard, T., McHutchison, J., Goodman, Z., Ling, M.H., Albrecht, J., 2000. Is an "a la carte" combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? The ALGOVIRC Project Group. Hepatology 31 (1), 211–218.
Rodriguez, J.J., Cruz, C.D., Horvath, C.M., 2004. Identification of the nuclear export signal and STAT-binding domains of the Nipah virus V protein reveals mechanisms underlying interferon evasion. J. Virol. 78 (10), 5358–5367.
Saito, T., Ito, T., Ishiko, H., Yonaha, M., Morikawa, K., Miyokawa, A., Mitamura, K., 2003. Sequence analysis of PePHD within HCV E2 region and correlation with resistance of interferon therapy in Japanese patients infected with HCV genotypes 2a and 2b. Am. J. Gastroenterol. 98 (6), 1377–1383.
Samuel, C.E., 2001. Antiviral actions of interferons. Clin. Microbiol. Rev. 14 (4), 778–809, table of contents.
Saracco, G., Ciancio, A., Olivero, A., Smedile, A., Roffi, L., Croce, G., Colletta, C., Cariti, G., Andreoni, M., Biglino, A., Calleri, G., Maggi, G., Tappero, G.F., Orsi, P.G., Terreni, N., Macor, A., Di Napoli, A., Rinaldi, E., Ciccone, G., Rizzetto, M., 2001. A randomized 4-arm multicenter study of interferon alfa-2b plus ribavirin in the treatment of patients with chronic hepatitis C not responding to interferon alone. Hepatology 34 (1), 133–138.
Sarrazin, C., Berg, T., Lee, J.H., Ruster, B., Kronenberger, B., Roth, W.K., Zeuzem, S., 2000. Mutations in the protein kinase-binding domain of the NS5A protein in patients infected with hepatitis C virus type 1a are associated with treatment response. J. Infect. Dis. 181 (2), 432–441.
Takeuchi, K., Komatsu, T., Yokoo, J., Kato, A., Shioda, T., Nagai, Y., Gotoh, B., 2001. Sendai virus C protein physically associates with Stat1. Genes Cells 6 (6), 545–557.
Taylor, D.R., Shi, S.T., Romano, P.R., Barber, G.N., Lai, M.M., 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 285 (5424), 107–110.
van Boxel-Dezaire, A.H., Rani, M.R., Stark, G.R., 2006. Complex modulation of cell type-specific signaling in response to type I interferons. Immunity 25 (3), 361–372.
Welker, M.W., Hofmann, W.P., Welsch, C., von Wagner, M., Herrmann, E., Lengauer, T., Zeuzem, S., Sarrazin, C., 2007. Correlation of amino acid variations within nonstructural 4B protein with initial viral kinetics during interferon-alpha-based therapy in HCV-1b-infected patients. J. Viral. Hepat. 14 (5), 338–349.
Yanagi, M., St Claire, M., Shapiro, M., Emerson, S.U., Purcell, R.H., Bukh, J., 1998. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. Virology 244 (1), 161–172.
Zeuzem, S., Hultcrantz, R., Bourliere, M., Goeser, T., Marcellin, P., Sanchez-Tapias, J., Sarrazin, C., Harvey, J., Brass, C., Albrecht, J., 2004. Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. J. Hepatol. 40 (6), 993–999.