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

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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

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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