



# Impairment of interferon regulatory factor-3 activation by hepatitis C virus core protein basic amino acid region 1

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## ABSTRACT

Interferon regulatory factor-3 (IRF-3), a key transcriptional factor in the type I interferon system, is frequently impaired by hepatitis C virus (HCV), in order to establish persistent infection. However, the exact mechanism by which the virus establishes persistent infection has not been fully understood yet. The present study aimed to investigate the effects of various HCV proteins on IRF-3 activation, and elucidate the underlying mechanisms. To achieve this, full-length HCV and HCV subgenomic constructs corresponding to structural and each of the nonstructural proteins were transiently transfected into HepG2 cells. IFN- $\beta$  induction, plaque formation, and IRF-3 dimerization were elicited by Newcastle disease virus (NDV) infection. The expressions of IRF-3 homodimer and its monomer, Ser386-phosphorylated IRF-3, and HCV core protein were detected by immunofluorescence and western blotting. IFN- $\beta$  mRNA expression was quantified by real-time PCR (RT-PCR), and IRF-3 activity was measured by the levels of IRF-3 dimerization and phosphorylation, induced by NDV infection or polyriboinosinic:polyribocytidylic acid [poly(I:C)]. Switching of the expression of the complete HCV genome as well as the core proteins, E1, E2, and NS2, suppressed IFN- $\beta$  mRNA levels and IRF-3 dimerization, induced by NDV infection. Our study revealed a crucial region of the HCV core protein, basic amino acid region 1 (BR1), to inhibit IRF-3 dimerization as well as its phosphorylation induced by NDV infection and poly (I:C), thus interfering with IRF-3 activation. Therefore, our study suggests that rescue of the IRF-3 pathway impairment may be an effective treatment for HCV infection.

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## 1. Introduction

Hepatitis C virus (HCV), a flavivirus comprising a positive-sense, single-stranded RNA (ssRNA) of approximately 9.6 kb [1], causes persistent disease in infected individuals, possibly leading to chronic liver injury [2]. Despite the approximately 170 million individuals worldwide suffering from HCV infection that ranges from chronic hepatitis to hepatocellular carcinoma (HCC) [3,4], the exact mechanism by which the virus establishes persistent infection is not fully resolved.

The innate immune system is activated immediately upon infection as the first line of host defense against invading pathogens, with type I interferon (IFN) signaling being the crucial step

in the antiviral response [5]. The IFN system is, therefore, a prime target of HCV and other viruses in order to establish persistent infections [6], wherein the disruption of the type I IFN-activation pathway forms the most efficient strategy for HCV. Studies on HCV IFN-interference mechanisms have revealed that the HCV proteins NS5A and E2 selectively inhibit the double-stranded RNA-activated protein kinase (PKR) [7,8], an IFN-inducible antiviral molecule that controls transcription and translation [6]. IFN- $\beta$ , a crucial molecule in type I IFN signaling, is regulated by several cellular factors associated with the activation of interferon regulatory factor-3 (IRF-3), leading to its rapid induction following viral infection [9,10]. However, IFN- $\beta$  induction is impaired in HCV-infected cells, thus resulting in the disruption of IFN downstream signaling cascade [11].

IRF-3, a key constitutively expressed transcriptional factor localized in the cytoplasm in its inactive form [9], is activated upon

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phosphorylation, whereby it translocates to the nucleus to act as a transcriptional factor for positive regulatory domain (PRD) 1 of the IFN- $\beta$  promoter. IRF-3 activation also induces phosphorylation of Ser385 and Ser386 or the serine/threonine (Ser/Thr) cluster between amino acids (aa) 396 and 405 (located at the C-terminus of IRF-3), a step that is essential for dimerization and nuclear translocation.

A previous study by Foy et al. showed that the NS3/4A serine protease derived from a subgenomic replicon participates in the suppression of the cellular pathway that activates IRF-3 [12]. The NS3/4A protein of HCV disrupts signaling of the double-stranded RNA (dsRNA) receptors, retinoic acid-inducible gene-I (RIG-I), and Toll-like receptor 3 (TLR3) by inducing proteolysis of interferon promoter stimulator-1 (IPS-1) [13–15] and Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein inducing IFN- $\beta$  (TRIF) [16], as well as by suppressing the downstream activation of IFN- $\beta$  [13].

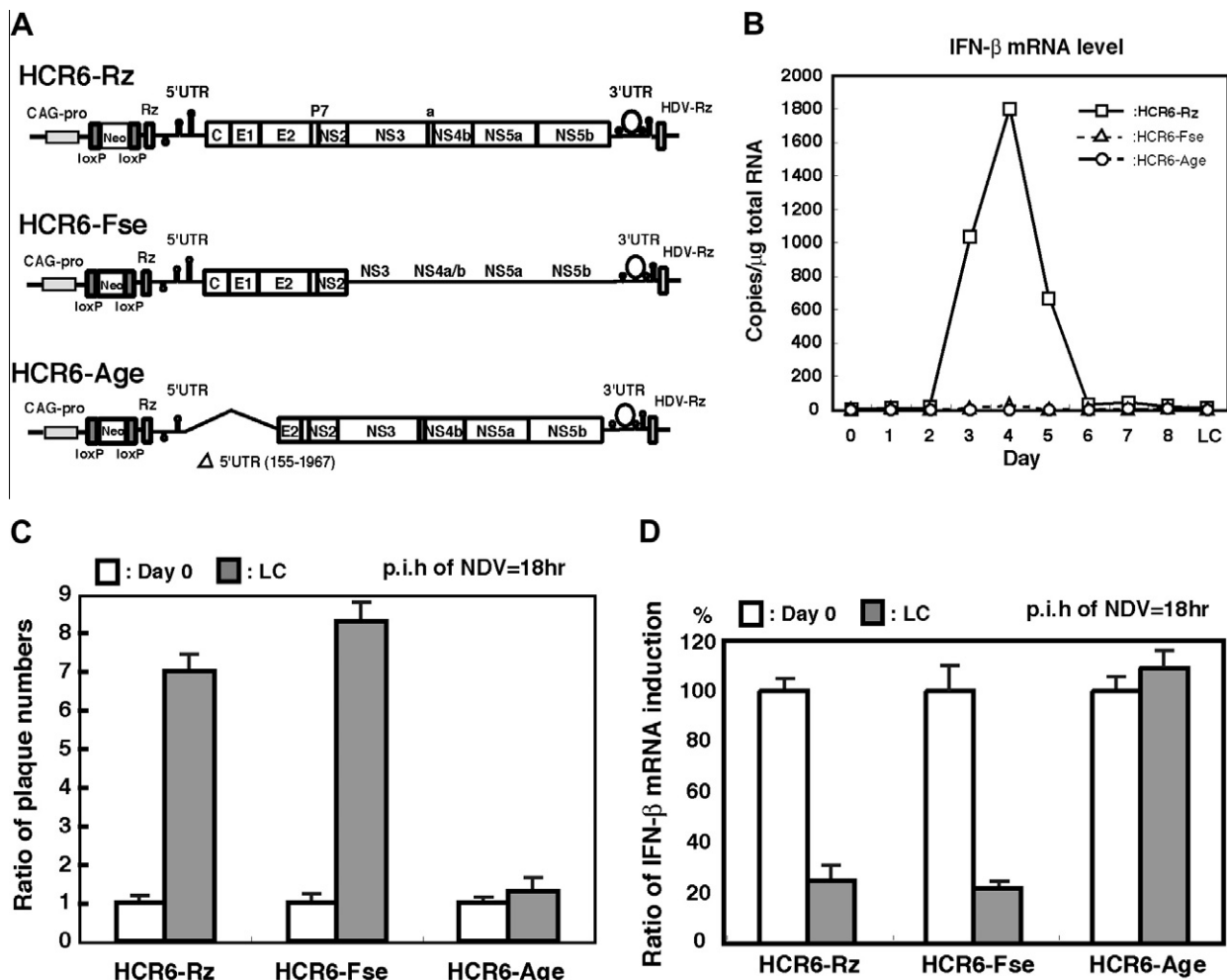
In view of the above observations and the emerging data on the role of HCV in regulating the IRF-3 pathway by additional

mechanisms, we aimed to investigate the effects of various HCV proteins on IRF-3 activation, and further elucidate the underlying novel mechanisms.

## 2. Material and methods

### 2.1. Transient expression of the HCV core proteins E1, E2, and NS3–4A

HepG2 cells were transfected to express E1, E2, or NS3–4A HCV core protein under the control of EF promoter (Invitrogen). The HCV core expression vectors were derived from HCR6 (genotype 1b), HCR24–12K (genotype 2a), or HCR24–12Q (genotype 2a). The E1, E2, and NS3 clones derived from HCR6 contained either the full-length cDNAs encoding the core protein or 1 of the 3 different deletions (deletion mutants), each of which lacked 1 of the 3 basic amino acid regions (BR), BR1 deletion (aa 4–14), BR2 deletion (aa 37–44), and BR3 deletion (aa 57–72) [17]. HepG2 cells were transfected with 4  $\mu$ g of the core cDNA (amino acids 1–191), E1 (amino



**Fig. 1.** (A) Structures of the conditional expression vectors for HCV RNAs and proteins. The cDNA clones that displayed highest level of homology to the consensus sequences among the 3 clones were used to construct HCR6-Rz (nt 1–9611). HCR6-Fse clone harbored a termination codon introduced at nucleotide 3606 and truncated HCR6-Age clone lacked nucleotides 155–1967. They were flanked with ribozyme (Rz) and hepatitis D virus ribozyme (HDV-Rz) sequences under the control of the CAG promoter in the Cre/loxP switching expression cassette, which consisted of the neomycin resistance gene, as a stuffer region flanked by the loxP sequence. (B) IFN- $\beta$  mRNA levels in the cell lines HCR6-Rz, HCR6-Fse, and HCR6-Age before and after the expression of the HCV protein. The results are expressed as copy numbers per microgram of total RNA, as quantified by RT-PCR. (C) Efficiency of NDV plaque formation. Plaque assays were performed on Vero cells for NDV infectivity in HCR6-Rz, HCR6-Fse, or HCR6-Age before and after the expression of the HCV genome. The plaque numbers were counted 3 days after NDV inoculation. The ratio indicates the plaque numbers after the expression of the HCV genome divided by the plaque numbers before the expression of the HCV genome. Day 0, before the expression of the HCV genome; Day 48, after the expression of the HCV genome. (D) Suppression of IFN- $\beta$  mRNA induction by HCV expression, 18 days after NDV inoculation. Day 0, before the expression of the HCV genome; Day 48, after the expression of the HCV genome. The results are expressed relative to the levels on Day 0 (100%) in each of the 3 HCV-expressing systems. p.i.h., post-inoculation hour.

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