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Activation of protein kinase R by hepatitis C virus RNA-dependent RNA polymerase

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ABSTRACT

Hepatitis C virus (HCV) was shown to activate protein kinase R (PKR), which inhibits expression of interferon (IFN) and IFN-stimulated genes by controlling the translation of newly transcribed mRNAs. However, it is unknown exactly how HCV activates PKR. To address the molecular mechanism(s) of PKR activation mediated by HCV infection, we examined the effects of viral proteins on PKR activation. Here, we show that expression of HCV NS5B strongly induced PKR and eIF2α phosphorylation, and attenuated MHC class I expression. In contrast, expression of Japanese encephalitis virus RNA-dependent RNA polymerase did not induce phosphorylation of PKR. Co-immunoprecipitation analyses showed that HCV NS5B interacted with PKR. Furthermore, expression of NS5B with polymerase activity-deficient mutation failed to phosphorylate PKR, suggesting that RNA polymerase activity is required for PKR activation. These results suggest that HCV activates PKR by association with NS5B, resulting in translational suppression of MHC class I to establish chronic infection.

1. Introduction

Over 70 million people worldwide are chronically infected with hepatitis C virus (HCV) and are at risk of developing chronic hepatitis, cirrhosis, and hepatocellular carcinoma. HCV infection has been successfully treated with direct-acting antivirals, but the molecular mechanisms that have been associated with persistent or chronic HCV infection are not fully understood. Host cells exhibit innate immunity in response to invasion by viral pathogens. Interferons (IFNs) are major cytokines that are responsible for the induction of an antiviral state, and are produced by infected cells and dendritic cells (Takeuchi and Akira, 2009), which subsequently induce a variety of IFN-stimulated genes (ISGs). As a result, many viruses have evolved the ability to suppress IFN signaling and/or the function of ISGs in infected cells.

Protein kinase R (PKR), a double-stranded RNA-dependent serine/threonine protein kinase, is an antiviral protein transcriptionally induced by IFNs in response to stress and viral infection (Garcia et al., 2006). Activated PKR phosphorylates the α subunit of the eukaryotic translation initiation factor 2 (eIF2α), which results in the shutdown of cellular and viral protein synthesis (Williams, 1999). Therefore, PKR was proposed to act as an antiviral agent through its ability to be activated by dsRNA and to trigger the inhibition of viral translation. It had been reported that HCV E2 and NS5A interact with PKR and repress its function (Gale et al., 1997; Taylor et al., 1999). More recent studies revealed that HCV also activates PKR, which leads to inhibition of IFN, ISGs, and MHC class I expression by controlling the translation of newly transcribed mRNAs, to negatively control the antiviral action of host immunity (Kang et al., 2014; Arnaud et al., 2010; Garaigorta and Chisari, 2009), although this mechanism is not well defined.

In vitro binding experiments using purified PKR protein showed that PKR can be activated upon binding to HCV internal ribosome entry site (IRES), (Toroney et al., 2010; Shimoike et al., 2009). However, it is unknown exactly how HCV infection activates PKR in HCV-infected cells, in which viral RNA is replicated in specific compartments surrounded by two layers of membranes (Paul et al., 2013). To address the molecular mechanism(s) of PKR activation mediated by HCV infection, we examined the effects of viral proteins on PKR activation using cells that do not support viral genome replication to exclude the effects of viral RNA intermediates. In the present study, we showed that NS5B expression activated PKR and induced eIF2α phosphorylation following

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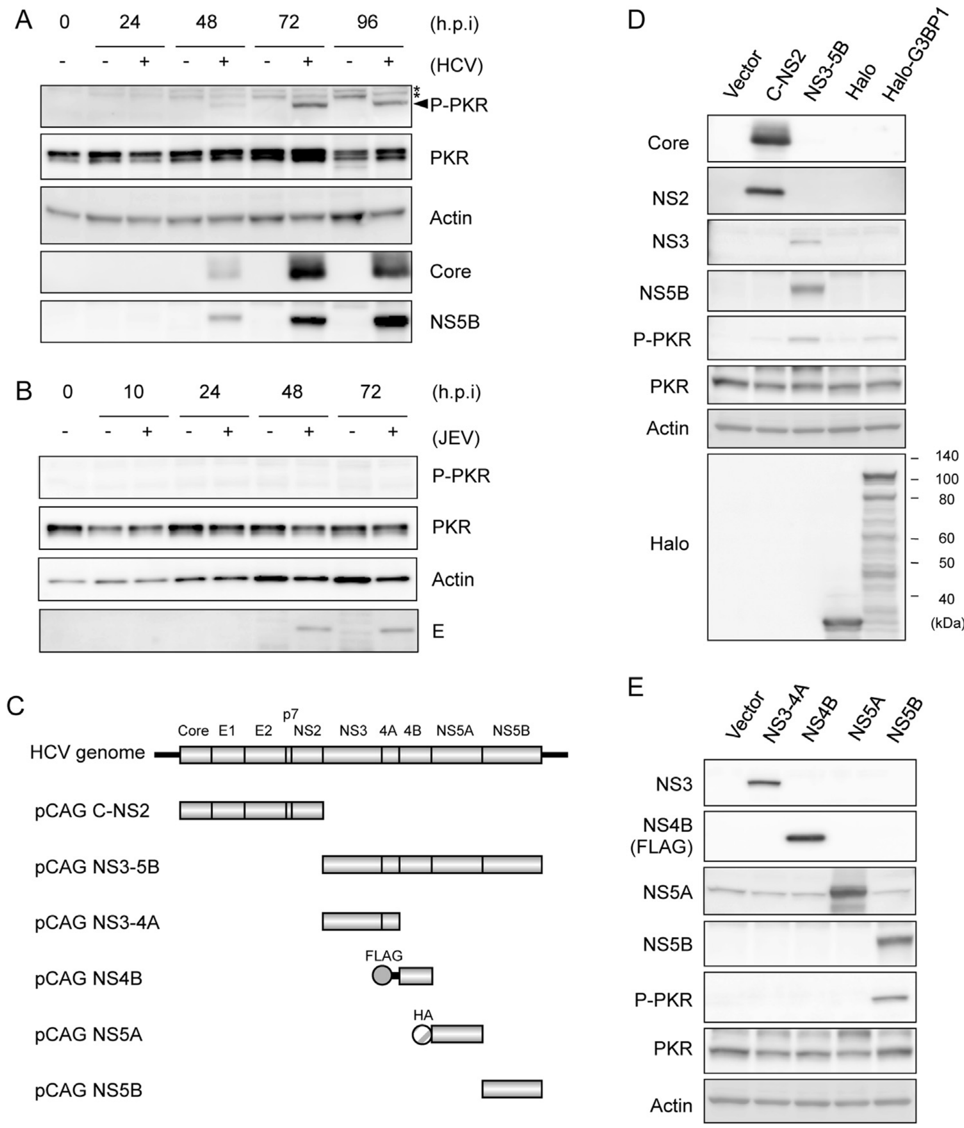

Fig. 1. PKR activation by HCV infection and NS5B expression. Huh7.5.1 cells were infected with HCVcc (A) or JEV (B). Cells were lysed at the indicated time points, followed by immunoblotting using antibodies against the indicated proteins. Arrowhead indicates P-PKR. Non-specific bands are marked with an asterisk. (C) Schematic diagram of the plasmid used in this study. (D and E) 293 T cells were transfected with the indicated expression plasmids, harvested 48 h post-transfection, and the indicated proteins were detected by immunoblotting.

translational shutoff and suppression of MHC class I expression.

2. Results

2.1. PKR activation by HCV infection or NS5B expression

To test whether HCV infection activates PKR, Huh7.5.1 cells were infected with cell culture-produced HCV (HCVcc). Viral proteins, core and NS5B, were detected in cells at 48 h post-infection. In accordance with the expression of viral proteins, phosphorylated PKR was detected in HCV-infected cells (Fig. 1A), whereas the total amount of PKR was not affected by HCV infection. In contrast, phosphorylated PKR was not detected in cells infected with Japanese encephalitis virus (JEV), as shown in Fig. 1B. These results indicate that HCV infection, but not JEV infection, triggers cells to activate PKR.

Next, we examined the effects of viral protein expression on PKR activation in 293 T cells transfected with HCV protein-encoding plasmids (Fig. 1C). As shown in Fig. 1D, PKR phosphorylation was detected in cells expressing NS3–5B or Ras GTPase-activating protein-binding protein 1 (G3BP1), which is known to recruit PKR to stress granules (SGs) for activation (Reineke and Lloyd, 2015). Therefore, we further examined the effects of each nonstructural protein on PKR activation. Expression of each nonstructural protein revealed PKR phosphorylation in cells expressing NS5B (Fig. 1E).

2.2. RNA-dependent RNA polymerase (RdRp) activity of NS5B is required for PKR activation and modulation of cap-dependent translation

PKR is known to be activated by dsRNA (Garcia et al., 2006), and it has also been shown that stable stem-loop structures essential for replication of viral RNA genome are located in the coding region of C-terminal NS5B (You et al., 2004; Lee et al., 2004). Therefore, to determine whether PKR is activated by expressed NS5B protein or mRNA molecules coding NS5B, pCAG NS5B-fs, which contains a frameshift mutation in the 5′ portion of the NS5B coding sequence, was constructed (Fig. 2A). In addition, we constructed pCAG NS5B-GND, which contains a point mutation at the GDD motif in NS5B to abolish RdRp activity (Suzuki et al., 2012). Whereas NS5B expression phosphorylated PKR and eIF2α, PKR and eIF2α activation was not observed in cells expressing NS5B-GND or cells transfected with plasmid pCAG NS5B-fs.

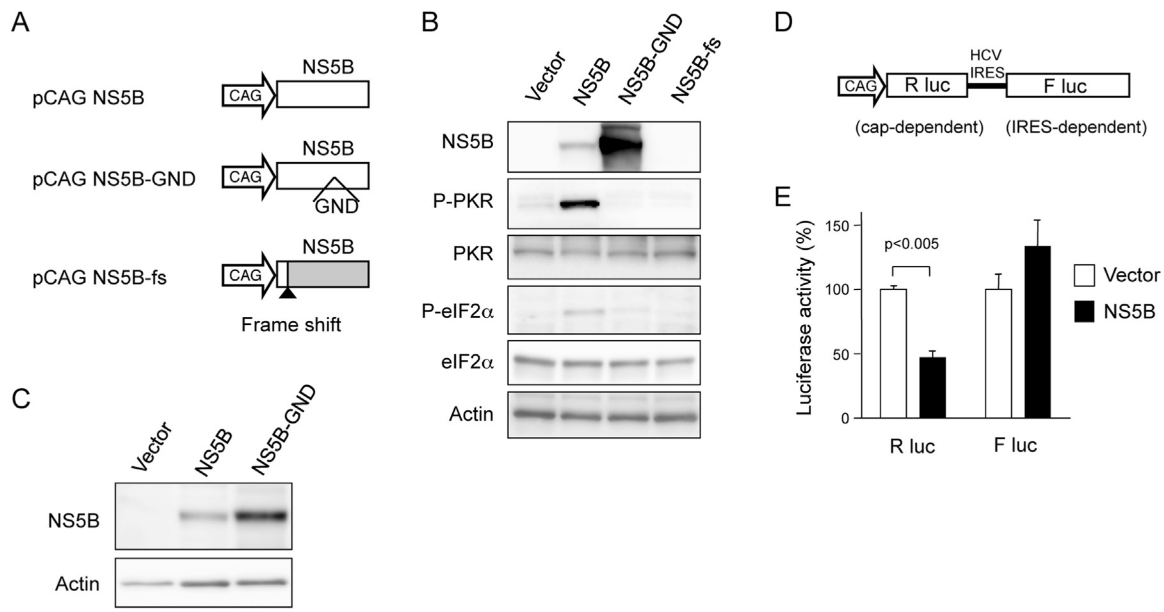

Fig. 2. NS5B RdRp activity is required for PKR activation and modulation of cap-dependent translation. (A) Schematic diagram of HCV NS5B plasmids used in this study. 293T cells (B) or Huh7.5.1 cells (C) were transfected with the indicated expression plasmids, harvested 48 h post-transfection, and the indicated proteins were detected by immunoblotting. (D) Schematic diagram of the bicistronic expression plasmid used in this study. (E) 293T cells were transfected with the bicistronic reporter plasmid with or without NS5B-expression plasmid. The firefly and *Renilla* luciferase activities in the cells were measured 48 h posttransfection.

It should be noted that NS5B expression levels were lower when compared to those of NS5B-GND in 293T cells (Fig. 2B). A similar observation was reproduced in human hepatoma Huh7.5.1 cells (Fig. 2C), which is in accordance with the proposed model that translation is suppressed by eIF2α phosphorylation in NS5B-expressing cells.

To investigate whether NS5B-induced translational shutoff affects HCV IRES-dependent translation, we examined cap-dependent and HCV IRES-dependent translation with or without NS5B expression using a bicistronic reporter plasmid, as shown in Fig. 2D. HCV NS5B expression reduced cap-dependent translation, but not HCV IRES-dependent translation (Fig. 2E). These results suggest that PKR and eIF2α phosphorylation by NS5B expression has little effect on HCV IRES-dependent translation of HCV proteins, whereas cap-dependent translation is suppressed.

2.3. Transcriptional induction of ISGs by expression of viral RdRp

As HCV NS5B was shown to activate innate immune signaling in the absence of HCV RNA replication components by synthesis of small dsRNA (Yu et al., 2012), we tested whether NS5B expression triggers innate immune responses and leads to transcriptional induction of ISGs. Expression of NS5B, but not NS5B-GND, induced mRNA of OAS1, MX2, IFI44, and IFIT1, but did not affect PKR mRNA levels (Fig. 3A). Furthermore, transcriptional induction of ISGs was observed in cells expressing JEV NS5 or cells transfected with poly(I:C) (Fig. 3B), suggesting that viral RdRp expression induces synthesis of ISG mRNAs, possibly via dsRNA synthesized by RdRp, which triggers innate immune responses. Interestingly, PKR was specifically activated by HCV NS5B expression, but not JEV NS5 expression (Fig. 3C), whereas poly(I:C) transfection induced moderate PKR activation. Therefore, PKR is considered to be specifically phosphorylated by HCV NS5B, but not by general synthesized RNA by viral RdRp.

2.4. NS5B expression induces SG formation

As global reduction of protein synthesis by stress-induced eIF2α phosphorylation is linked to the assembly of SGs, we examined whether NS5B expression induces SG formation. An SG marker protein, G3BP1, was accumulated and localized close to the NS5B-enriched region in cells expressing FLAG-tagged NS5B, but not in cells expressing FLAG-NS5B GND or FLAG-tagged JEV NS5 (Fig. 4). These results suggest that HCV NS5B expression, but not that of the polymerase-deficient mutant or JEV NS5, induced SG formation.

2.5. Interaction of HCV NS5B with PKR in mammalian cells

As PKR is specifically activated by HCV NS5B, we examined whether HCV NS5B interacts with PKR in mammalian cells. FLAG-tagged NS5B with polymerase-deficient mutation (F-NS5B GND) was co-expressed in 293T cells with myc-tagged PKR K296R (myc-PKR K296R), as expression of NS5B with polymerase activity or expression of PKR-WT decreased exogenous protein expression levels due to translational shutoff possibly through PKR activation (Fig. 2B and unpublished data). Myc-PKR K296R co-immunoprecipitated with FLAG-NS5B GND (Fig. 5A). Furthermore, immunohistochemical staining revealed that NS5B is colocalized with endogenous PKR (Fig. 5B).

2.6. NS5B expression attenuates MHC class I expression

A number of viruses have evolved mechanisms to decrease MHC class I expression in order to escape CD8 + T cell responses. As we confirmed that HCV NS5B expression resulted in PKR and eIF2α phosphorylation, we examined whether MHC class I expression on the cell surface is regulated by expression of HCV NS5B. MHC class I expression was reduced in HCV NS5B-expressing cells when compared with cells expressing NS5B-GND or JEV NS5 (Fig. 6A). HLA-a, HLA-b, and HLA-c mRNA expression levels were not affected by HCV NS5B expression (Fig. 6B), supporting the notion that NS5B regulates MHC class I expression at the translational level.

3. Discussion

Host cells exhibit innate immunity in response to invasion by viral pathogens. HCV can suppress these innate immune responses, resulting in chronic infection. One reason for this is viral NS3–4A protease, which cleaves MAVS, an essential component of the RIG-I pathway (Meylan et al., 2005), and TRIF, an adaptor molecule in the TLR3 pathway (Li et al., 2005), to prevent signaling of pathogen recognition receptors

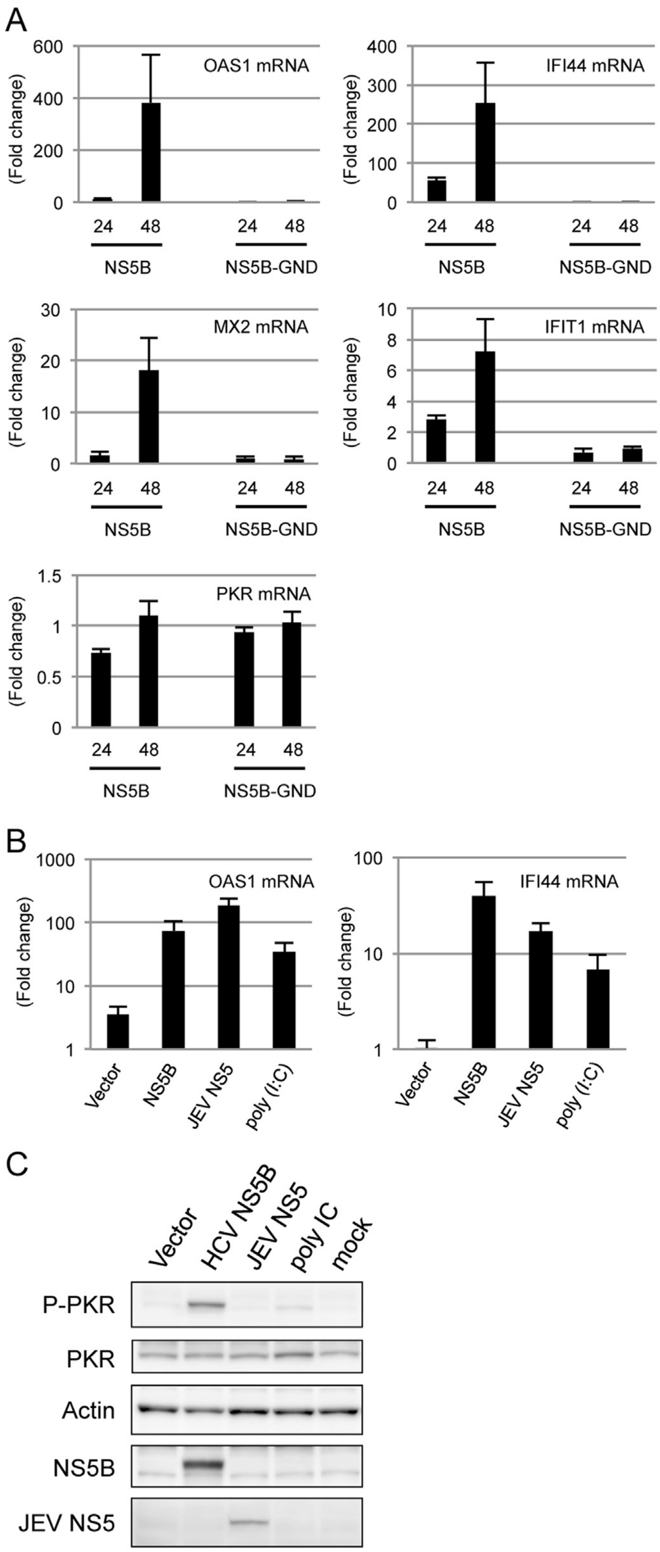

Fig. 3. Induction of synthesis of ISG mRNAs by expression of viral RdRp. (A) 293T cells were transfected with the indicated plasmids and harvested 24 h and 48 h post-transfection. ISG mRNA levels were determined by RT-qPCR and expressed as fold induction. 18S rRNA quantification from the same samples was used for normalization. The value for empty vector was set at 1. (B and C) 293T cells were transfected with the indicated plasmids or poly(I:C) and harvested 48 h post-transfection. (B) OAS1 and IFI44 mRNA levels were determined by qRT-PCR and shown as fold induction. 18S rRNA quantification from the same samples was used for normalization. The value for empty vector was set at 1. (C) The indicated proteins were detected by immunoblotting.

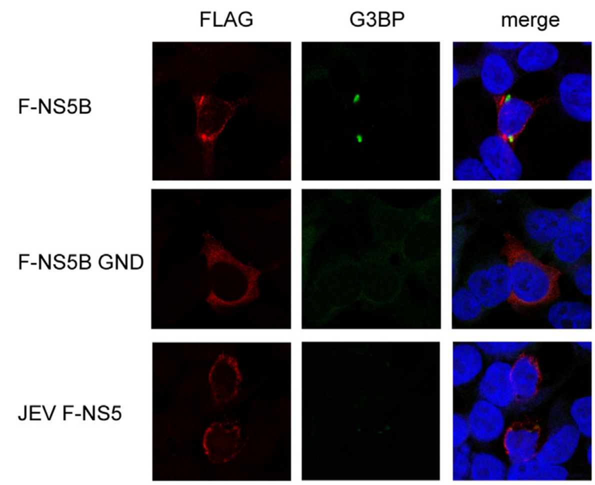

Fig. 4. NS5B expression induces SG formation. 293T cells were transfected with indicated plasmids. Two days post-transfection, cells were fixed, permeabilized with Triton X-100, and then subjected to immunofluorescence staining using anti-FLAG and G3BP antibodies.

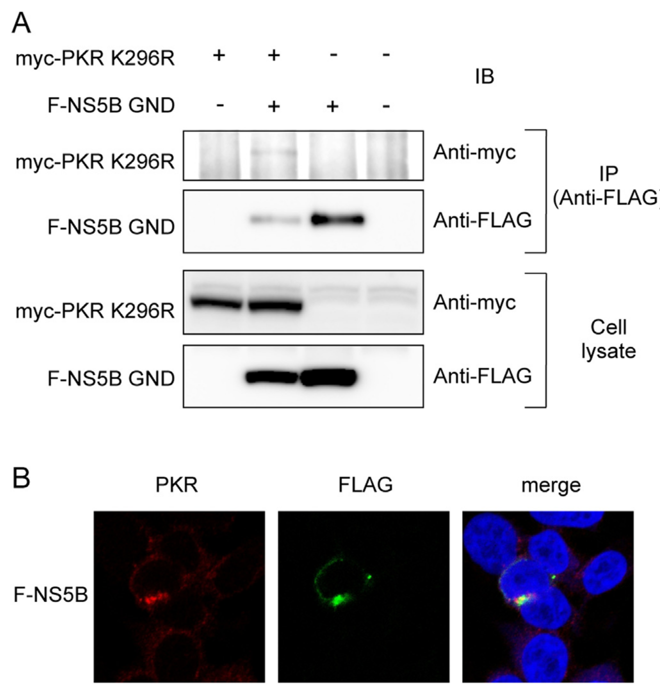

Fig. 5. Interaction of HCV NS5B protein with PKR in mammalian cells. (A) 293T cells were co-transfected with a FLAG-tagged NS5B GND expression plasmid in the presence of a myc-PKR expression plasmid. Cell lysates of transfected cells were immunoprecipitated with anti-FLAG antibody. The resulting precipitates and whole cell lysates used in immunoprecipitation (IP) were examined by immunoblotting using anti-FLAG or anti-myc antibody. An empty plasmid was used as a negative control. (B) 293T cells were transfected with a FLAG-tagged NS5B (WT) expression plasmid. Two days post-transfection, cells were fixed, permeabilized with Triton X-100, and then subjected to immunofluorescence staining using anti-FLAG and PKR antibodies.

(PRRs). HCV has also been shown to activate PKR, which leads to inhibition of antiviral protein expression by controlling translation (Garcia et al., 2006; Arnaud et al., 2010). In this study, we demonstrated that HCV NS5B expression strongly induced PKR and eIF2α phosphorylation. PKR could be activated due to interaction with NS5B and possibly by synthesized RNA derived from cellular RNA by NS5B in the absence of viral RNA, resulting in translational suppression of genes related to host immune responses.

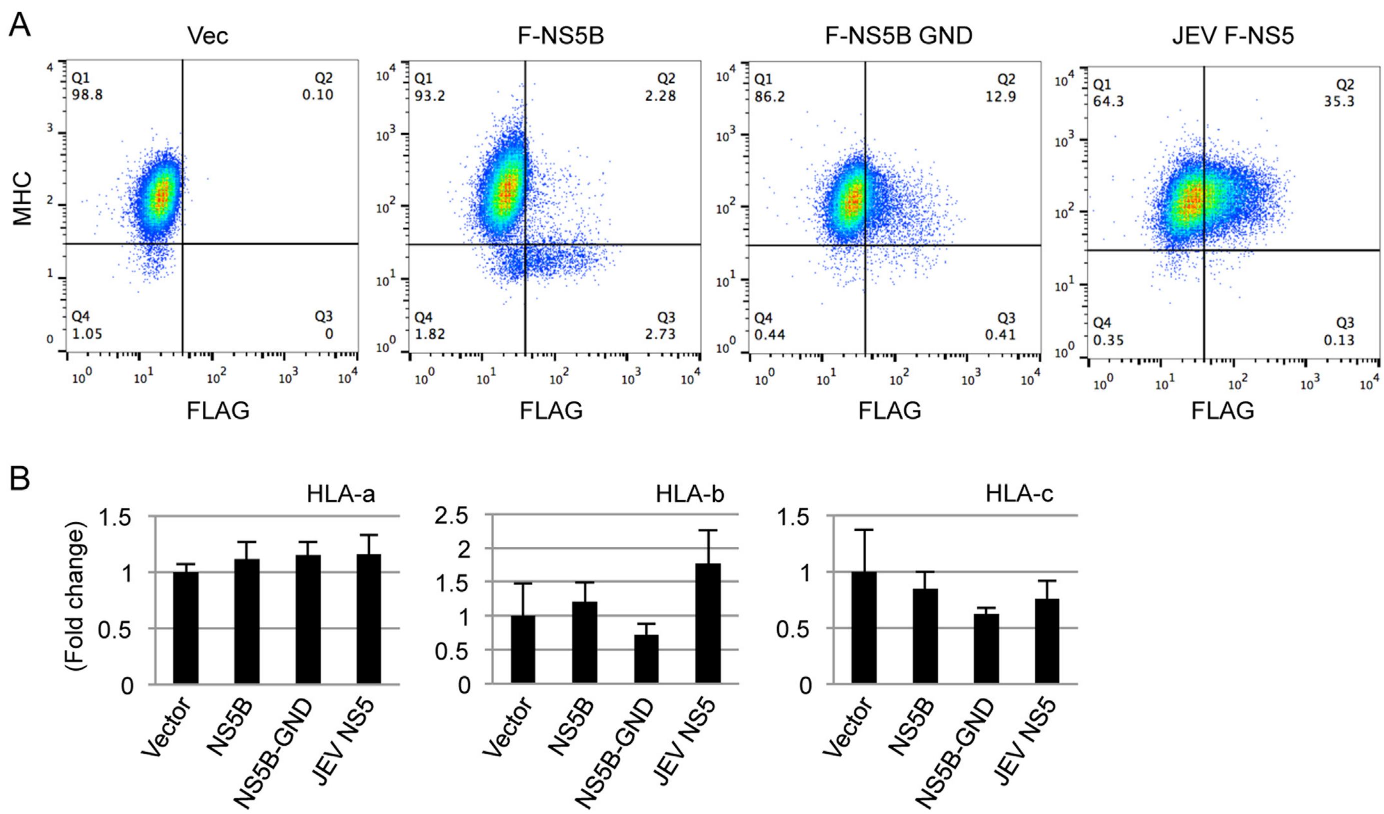

Fig. 6. NS5B expression attenuates MHC class I expression. (A) 293T cells were transfected with the indicated plasmids. Two days post-transfection, the cell surface was stained using Alexa Fluor 488-labeled anti-human HLA-ABC and then fixed, permeabilized with Triton X-100, and subjected to immunofluorescence staining using anti-FLAG antibodies. (B) 293T cells were transfected with the indicated plasmids and harvested 48 h post-transfection. HLA-A, HLA-B, and HLA-C mRNA expression was determined by qRT-PCR and expressed as fold induction. 18S rRNA quantification from the same samples was used for normalization. The value for empty vector was set at 1.

PKR has been shown to be activated by HCV infection in vitro and in vivo (Arnaud et al., 2010, 2011; Garaigorta and Chisari, 2009; Kang et al., 2009; Mitchell et al., 2017) in accordance with our data (Fig. 1). It has also been reported that PKR can be activated upon binding to HCV IRES, which is highly structured and conserved among genotypes, through in vitro binding assays (Toroney et al., 2010; Shimoike et al., 2009). Although HCV RNA is considered to replicate in membranous replication factories associated with double-membrane vesicles (DMVs) (Romero-Brey et al., 2012), it is unknown whether HCV RNA, including IRES, interacts with PKR inside or outside of the DMVs. Alternatively, the possibility that HCV proteins activate PKR has not been completely excluded because dsRNA is not the only substrate for PKR activation. In fact, a limited number of proteins have been shown to activate PKR in the absence of RNA (Burugu et al., 2014). Therefore, in this study, we explored whether HCV proteins are able to activate PKR. To this end, we took advantage of cells that do not support viral genome replication to exclude the effects of viral RNA intermediates on PKR activation. Our data clearly showed that expression of NS5B protein activated PKR without the viral genome.

HCV E2 and NS5A have been shown to interact with PKR and repress its function (Gale et al., 1997; Taylor et al., 1999). However, the effect of PKR on HCV replication remains controversial. It has been reported that HCV infection is not affected by reduction of PKR (Garaigorta and Chisari, 2009). In contrast, antiviral activity of PKR directed against HCV replication was also reported (Kang et al., 2009). A more recent study supported the notion that HCV activates PKR to escape from antiviral host immunity. Garaigorta et al. proposed that HCV activates PKR, which is advantageous for the virus because of ISG translation suppression (Garaigorta and Chisari, 2009). Kang et al. also reported that IFN-induced MHC class I expression is attenuated in HCV-infected cells by PKR activation (Kang et al., 2014). Although PKR activation induces eIF2α phosphorylation, resulting in suppression of cap-dependent protein translation in cells, HCV IRES-dependent translation has been shown to be resistant to eIF2α phosphorylation (Shimoike et al., 2009; Terenin et al., 2008) in accordance with our data (Fig. 2E). This could be explained by the observation that eIF2α phosphorylation did not compromise translation from HCV genomic RNA (Terenin et al., 2008; Dabo and Meurs, 2012).

Several studies have already reported that expression of a viral RdRp can activate innate immune signaling in cells without the requirement of viral RNA replication (Yu et al., 2012; Naka et al., 2006; Moriyama et al., 2007; Painter et al., 2015; Zhang et al., 2016). Viral RdRp uses host RNAs as a template to produce dsRNAs in cells, even in the absence of viral genomic RNA, and these dsRNAs are recognized by PRRs, which trigger IFN production, albeit by different mechanisms. We also showed that expression of HCV NS5B and JEV NS5 induced synthesis of ISG mRNAs without the requirement of viral RNA replication, and ISG induction was abrogated by the introduction of polymerase-defective mutation (Fig. 3). Therefore, activation of the innate immune response by the replicase of a positive-strand RNA virus may be a general property of RNA virus infection. Nevertheless, expression of HCV NS5B, but not JEV NS5, specifically activated PKR. Therefore, PKR activation might be a unique strategy to modulate host immunity for persistent HCV infection. It is not clear whether dsRNA generated from NS5B in the replication complex, or NS5B protein that is not located in the replication complex, activated PKR in HCV-infected cells, as it has been shown that not only RdRp alone, but also the viral replication complex can generate non-specific dsRNA from host RNA (Nikonov et al., 2013). Further study with HCV-infected hepatic cells is needed to clarify the mechanism of PKR activation in viral genome-

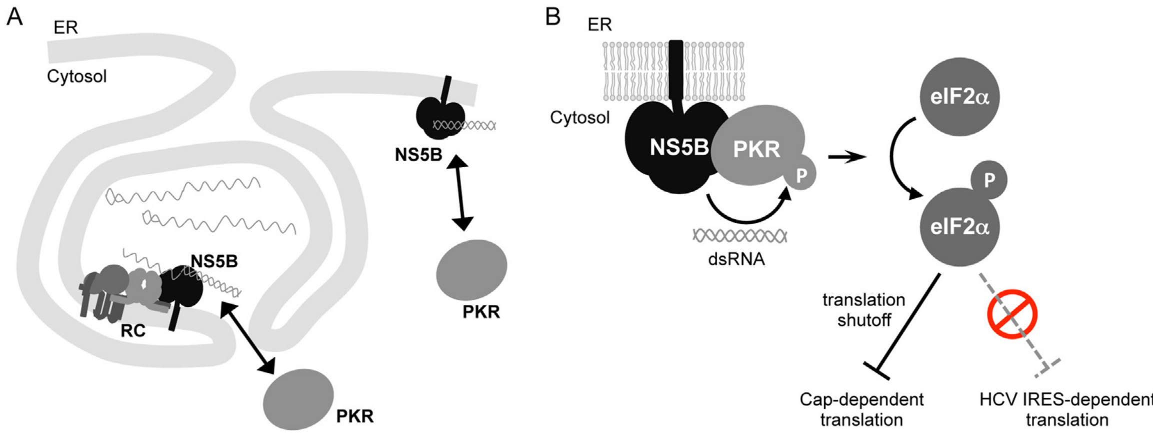

Fig. 7. Proposed model of PKR activation by HCV NS5B. (A) PKR binds to HCV NS5B located in double-membrane vesicles as the replication complex (RC), or NS5B located outside of the DMVs. (B) PKR interacts with NS5B, recognizes synthesized dsRNA from cellular RNAs as templates by NS5B, and is phosphorylated. eIF2α phosphorylation subsequently occurs, leading to the inhibition of general translation including ISGs. However, HCV IRES-dependent translation is not affected.

replicating cells. As the RdRp activity of NS5B is a prerequisite for PKR phosphorylation, we proposed a model of PKR activation by HCV NS5B, as shown in Fig. 7. PKR is activated by synthetic RNA from HCV NS5B through proximity or binding to PKR. Then, PKR phosphorylates eIF2α, following suppression of cap-dependent translation. In fact, we observed reduced expression levels of HLA in NS5B-expressing cells (Fig. 6A), whereas we could not completely exclude the possibility that degradation of HLA is promoted, or that its translocation to the cell surface is suppressed. Under these conditions, HCV IRES-dependent translation is not regulated (Fig. 2E).

HCV utilizes many host factors that are required for viral propagation. Several cellular proteins have been reported as NS5B interacting proteins, such as hPLIC1 (Gao et al., 2003), VAP-A and B (Gao et al., 2004; Hamamoto et al., 2005), CyPB (Watashi et al., 2005), ESRα (Watashi et al., 2007), ATG5 (Guevin et al., 2010), and FASN (Huang et al., 2013), which are important for the formation of replication complexes. In contrast, PKR, a newly identified NS5B binding protein, does not appear to be required for replication, as HCV replicates in PKR-knockout cells (Chang et al., 2006). Our study is the first, to our knowledge, to reveal a novel NS5B-binding host factor that is not required for viral RNA replication, but has an important role in regulation of translation.

In conclusion, we confirmed that HCV NS5B expression phosphorylates PKR and eIF2α. PKR could be activated by interaction with NS5B and possibly by synthesized RNA by NS5B, resulting in translational suppression of genes related to host immune responses, although HCV IRES-dependent translation is not affected. These findings provide insight into understanding the molecular mechanisms of HCV invasion against host immune responses.

4. Materials and methods

4.1. Cell culture

Human embryonic kidney 293T cells and human hepatoma Huh7.5.1 cells were maintained in Dulbecco's modified Eagle medium supplemented with nonessential amino acids, 100 U of penicillin/ml, 100 mg of streptomycin/ml, and 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator.

4.2. Plasmids

Plasmid pCAGC-NS2 was previously described as pCAGC-NS2/JFH1 (Masaki et al., 2010). Plasmid expressing N-terminally hemagglutinin-tagged NS5A was also previously described (Masaki et al., 2008). To generate NS3–5B, NS3–4A, Flag-tagged NS4B, and NS5B expression plasmids, each respective cDNA was amplified from full-length JFH-1 cDNA by PCR. The resultant fragments were cloned into pCAGGS.

pCAG NS5B-GND, which contains a point mutation at the GDD motif in NS5B to abolish RdRp activity, was constructed by oligonucleotide-directed mutagenesis. pCAG NS5B-fs, which contains a frameshift mutation through a 4-nt insertion at the BsrGI site, was constructed from pCAG NS5B.

To generate the JEV NS5 expression plasmid pCAG JE NS5, cDNA encoding JEV NS5 was amplified from JEV subgenomic replicon plasmid (Suzuki et al., 2014). The resultant fragments were cloned into pCAGGS.

To generate the bicistronic reporter plasmid, HCV IRES (1–407 nt of H77c strain) (Yanagi et al., 1997) and firefly *luc* gene from pGL3-Basic were inserted into the *Xba*I site of pRL-CMV (Promega).

pCAG myc-PKR K296R, a cDNA clone of human PKR with catalytically inactive mutation possessing an N-terminal myc tag, was constructed by PCR from the plasmid previously described (Shimoike et al., 2009). The resultant linear fragment was inserted under the control of a CAG promoter of pCAGGS.

HaloTag Control Vector and pFN21AE3935 (Promega) were used for expression of HaloTag and Halo-human G3BP1, respectively.

4.3. Antibodies

Mouse monoclonal antibodies against actin (AC-15), FLAG (M2), and rabbit polyclonal antibodies against FLAG (F7425) were obtained from Sigma-Aldrich. Rabbit monoclonal antibodies against phosphorylated PKR on threonine 446 (ab32036) and rabbit polyclonal antibodies against phosphorylated eIF2α on serine 51 (ab4837) were obtained from Abcam. The phosphorylation of threonine 446 is directly related to PKR activation and coupled with dimerization of kinase domain (Dey et al., 2005). Rabbit polyclonal antibodies against PKR (K-17; sc-707), eIF2α (sc11386), and Myc (A-14; sc-7) were obtained from Santa Cruz Biotechnology. Mouse monoclonal antibodies against human G3BP (BD611127) and Alexa Fluor 488-Mouse anti-Human HLA-ABC (DX17) were obtained from BD Pharmingen. Mouse monoclonal antibodies against HCV core (2H9) and rabbit polyclonal antibodies against NS2, NS3, NS5A, NS5B, and JEV E were previously described (Suzuki et al., 2013; Saga et al., 2016).

4.4. Reagents

Poly(I:C) was obtained from IMGENEX. FuGENE 6 transfection reagent (Promega) was used for plasmid transfection in accordance with the manufacturer's instructions.

4.5. Viruses

HCVcc derived from JFH-1 containing adaptive mutations in E2 (N417S), p7 (N765D), and NS2 (Q1012R) was generated as described previously (Suzuki et al., 2012). The JEV Nakayama strain was described previously (Suzuki et al., 2014).

4.6. RNA extraction and qRT-PCR

Total cellular RNAs isolated by Isogen (Nippon Gene) were transcribed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time PCR was performed using an ABI StepOnePlus Real-Time PCR system and TaqMan chemistry. Each target was run in triplicate with TaqMan Gene Expression Master Mix. Primer and probe sets for relative quantification were selected from the Assays-on-Demand product list (Applied Biosystems). The results were normalized against 18S rRNA levels. Primers and probes used for analysis were as follows: human gene eukaryotic 18S rRNA (4319413E), MX2 (Hs01550811_m1), OAS1 (Hs00973637_m1), IFI44 (Hs00951349_m1), IFIT1 (Hs03027069_s1), PKR (Hs00169345_m1), HLA-a (Hs01058806_g1), HLA-b (Hs00818803_g1), and HLA-c (Hs00740298_g1).

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