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Hepatitis E Virus Methyltransferase Inhibits Type I Interferon Induction by Targeting RIG-I

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The type I interferons (IFNs) play a vital role in activation of innate immunity in response to viral infection. Accordingly, viruses have evolved to employ various survival strategies to evade innate immune responses induced by type I IFNs. For example, hepatitis E virus (HEV) encoded papain-like cysteine protease (PCP) has been shown to inhibit IFN activation signaling by suppressing K63-linked de-ubiquitination of retinoic acid-inducible gene I (RIG-I) and TANK-binding kinase 1 (TBK1), thus effectively inhibiting down-stream activation of IFN signaling. In the present study, we demonstrated that HEV inhibits polyinosinic-polycytidylic acid (poly(I:C))-induced IFN-β transcriptional induction. Moreover, by using reporter assay with individual HEV-encoded gene, we showed that HEV methyltransferase (MeT), a non-structural protein, significantly decreases RIG-I-induced IFN-β induction and NF-κB signaling activities in a dose-dependent manner. Taken together, we report here that MeT, along with PCP, is responsible for the inhibition of RIG-I-induced activation of type I IFNs, expanding the list of HEV-encoded antagonists of the host innate immunity.

Keywords: Hepatitis E virus, methyltransferase, RIG-I

Introduction

Hepatitis E virus (HEV) is one of hepatitis viruses that cause liver disease [1]. Annually, roughly 20 million people are infected with HEV worldwide according to The World Health Organization (WHO). Hepatitis E virus transmission is known to be prevalent in developing countries [2–4], but in recent years, it has also been detected in industrialized countries such as UK, France, and Germany [5]. HEV infection is mostly asymptomatic or a self-limiting acute illness, rarely progressing to persistent infection. However, its infection in pregnant women can cause high mortality rate up to 20~30% [6, 7]. The HEV variants are classified into 7 genotypes (GT1-7), among which four major genotypes are associated with human infection. GT1 and GT2 HEV are restricted to human infection, whereas GT3 and GT4 HEV are zoonotic, infecting both human and animals such as domestic pig, deer, and wild boar [2, 8, 9].

HEV is a non-enveloped virus with a single-stranded, positive-sense RNA genome, which is approximately 7.2 kb in length. Its genome consists of the 5′ untranslated region (UTR), three overlapping open reading frames (ORF1, ORF2, ORF3) and the 3′ UTR followed by poly A [10–12]. The ORF1 encodes 7 non-structural replication proteins: methyltransferase, Y-domain, papain-like cysteine protease (PCP), hypervariable region (HVR), X-domain, RNA helicase, and RNA-dependent RNA polymerase (RdRp), all of which are involved in the viral genome replication and

polyprotein processing [13–15]. ORF2 encodes the viral capsid protein that plays important functions in virus infection and virion assembly [16–18]. ORF3 encodes a small phosphoprotein of 113 or 114 amino acids that is essential for virus egress from infected cells [19–21]. Recently, Nair *et al*. showed that ORF4 is expressed only under endoplasmic reticulum (ER) stress and interacts with multiple viral proteins, stimulating activity of viral RdRp [22].

Type I Interferon (IFN) is the first line defense against viral infection, stimulating a series of subsequent anti-viral immune responses [23–26]. Recent studies suggest that type I IFNs are also involved in the pathogenesis of chronic inflammatory disease as well as in anti-cancer immunity [25, 27]. Upon virus infection into the cells, pathogen recognition receptors (PRRs), such as retinoic-acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and Toll-like receptors (TLRs), recognize the viral genome [28–30] and rapidly activate type I IFN production through a cascade of downstream signaling molecules, such as mitochondrial antiviral-signaling protein (MAVS) [31–33], TIR-domain-containing adapter-inducing interferon-β (TRIF) [34, 35], I kappa B kinase epsilon (IKKε)/TANK-binding kinase 1 (TBK1) [36–40], interferon regulatory factor 3 (IRF3) [41–43], IRF7 [44], and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [33, 45, 46]. Activation of those factors leads to expression of IFN-α/β and interferon-stimulated genes (ISGs) that induce efficient antiviral immune responses in an endocrine and paracrine manner [47, 48]. Interestingly enough, pathogenic viruses have evolved a slew of immune escape strategies to avoid and/or disrupt the host immune system [49, 50]. For example, hepatitis C virus (HCV) encodes viral proteins, such as core, E2, NS3/4A, and NS5A, which have been shown to be involved in innate immune evasion by directly or indirectly regulating the type I IFN production signaling cascade [51], thus contributing to the establishment of chronic viral infection [52–58].

On the contrary, little is known on the HEV-encoded mechanisms of type I IFN evasion [59]. Treatment of recombinant IFN-α on HEV replicon-harboring cells revealed that HEV replication was partially inhibited. These results imply for HEV-encoded antagonist(s) of IFN-α-induced antiviral immune response [60, 61]. For example, HEV PCP protein seems to suppress K63-linked de-ubiquitination of RIG-I and TBK1, which is essential for their activation and IFN-β expression [62–64]. However, detailed mechanisms of PCP-mediated evasion strategy against type I IFN response have not been delineated yet. Moreover, it is still likely that HEV-encoded proteins, other than PCP, may also be involved in antagonizing the type I IFN response. Therefore, we aimed to screen each and every HEV protein for their inhibitory activities on type I IFN signaling molecules. Here, we demonstrate that besides PCP, HEV methyltransferase (MeT) is a strong antagonist of the type I IFN by inhibiting RIG-I-mediated activation. Furthermore, RIG-I-mediated activation of NF-κB is also inhibited by HEV MeT. Taken together, these results establish a novel function of HEV MeT in the inhibition of and thus the immune evasion of type I IFNs.

Materials and Methods

Annotation of HEV Genomes

A set of 3 different viral genome sequences were obtained from Genbank. The genes for each viral protein were annotated with significant similarity in amino acid sequences based on a genotype 3 HEV (47832c; Genbank: KC618403), compared with genotype 1 HEV L08816 and AF082843, which were isolated from swine.

Cells and Reagents

HEK293T cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% streptomycin (Gibco, USA) in a humidifying incubator at 37°C with 5% CO_2 [65–69]. A549/D3 and A549/HEV cells [70] were maintained in DMEM supplemented with 5% calf bovine serum and 1% streptomycin in a humidifying incubator at 37°C with 5% CO_2. Detailed procedures for cell culture were described elsewhere [71–77]. For transfection, polyethylenimmine (PEI) solution was prepared (Sigma-Aldrich) and DNA:PEI ratio was 1:2 to form DNA-transfection reagent complex [78–81]. High molecular weight polyinosinic-polycytidylic acid (poly(I:C)) was purchased from Invivogen [82].

Plasmid Construction and Western Blotting

cDNA corresponding to the individual gene of HEV (GT3, 47832c) was generated by reverse transcriptase (MMLV, Thermo Fischer Scientific) followed by PCR using specific primers. Resulting cDNA was cloned into p3xFLAG-CMV-10 (Sigma-Aldrich). Plasmid encoding each HEV gene was transfected into HEK293T cells and assessed for its expression by anti-FLAG antibody (M2, Sigma-Aldrich). Detailed procedures were described elsewhere [83–87].

Luciferase Reporter Assay

HEK293T cells were seeded in a 6-well plate the day before transfection and then transfected for 24 h with various plasmids, as indicated in the figures. The cells were lysed using reporter lysis 5× buffer (Promega, USA). Luciferase activity in the cell

lysates was determined using luciferase assay system (Promega, USA), and the luminescence was measured by the Glomax (Promega, USA) according to the manufacturer's instructions. Luminescence of firefly luciferase was normalized by beta-galactosidase activity.

Statistical Analysis

Data are shown as one representative example from at least two independent experiments. The statistical significance was determined by the Student's t test. $P < 0.05$ was considered statistically significant.

Results

HEV Inhibits IFN-β Transcriptional Activation Induced by Poly(I:C)

A549/D3 cell line was subcloned from A549 cells for its high susceptibility to HEV [88], which was also shown to support HEV replication and virus production at high levels. Interestingly, HEV infection was stably maintained over time, resulting in the generation of an HEV stable cell line, A549/HEV [88], which was utilized in this study for investigating the mechanism(s) of HEV-mediated disruption

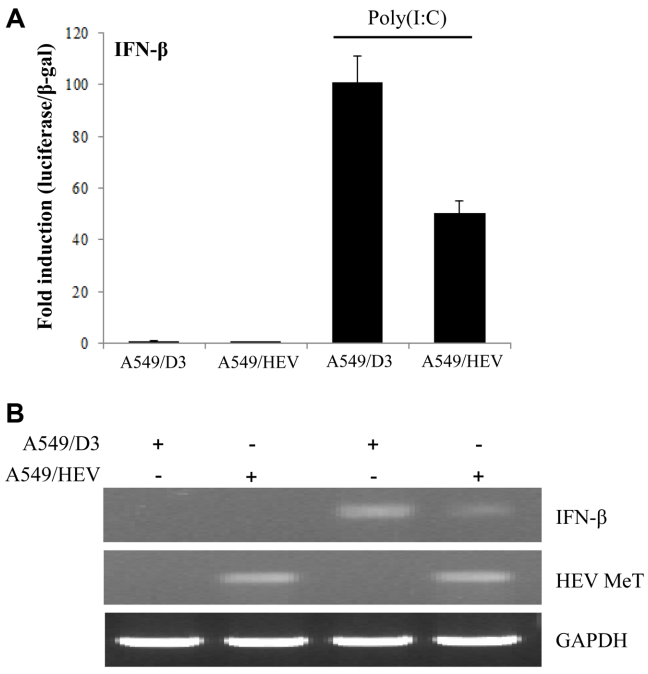

Fig. 1. Poly(I:C)-induced IFN-β reporter activities decreased in HEV infected cells.

A549/D3 and A549/HEV cells were seeded at 1×10^6 in a 6-well plate. After 24 h, cells were treated with 1 μg/ml of poly(I:C) for 12 h. Cells were subsequently subjected to either IFN-β reporter assay (**A**) or RT-PCR (**B**).

of type I IFN signaling. Previously, poly(I:C)-treated A549 cells expressed type I IFN proteins at increasing levels in a time-dependent manner (12–72 h) [89]. As IFN-β is a key factor for innate immune response and is a major target of viral evasion [46, 90], we sought to test whether HEV modulates poly(I:C)-mediated induction of type I IFN expression in A549/D3 and A549/HEV cells. Two cell lines were co-transfected with IFN-β-luciferase (IFN-β-luc) reporter and beta-galactosidase (β-gal) plasmid, and treated with 1 μg/ml poly(I:C) for 12 h. Poly(I:C)-mediated induction of IFN-β reporter activity (Fig. 1A) and expression of IFN-β mRNA (Fig. 1B) were significantly inhibited in the context of HEV infection. These results strongly suggest for the presence of HEV-encoded antagonistic mechanisms against type I IFN signaling.

HEV MeT and PCP Protein Inhibits Poly(I:C)-Induced IFN-β Production

With an aim to screen out HEV protein(s) that inhibit type I IFN signaling, we first annotated each HEV gene encoded in the genome of HEV 47832c strain (GT3) as shown in Fig. 2A. ORF1 is translated into a polypeptide, which is proteolytically cleaved to 7 nonstructural replication proteins. Each nonstructural gene as well as ORF2 and ORF3 was cloned into the plasmid, p3xFLAG-CMV10, and its protein expression was analyzed by Western blotting (Fig. 2B). To examine which viral protein(s) are responsible for the inhibition of IFN signaling, HEK293T cells were co-transfected with IFN-β-luc reporter and β-gal plasmids, and each viral gene-expressing plasmid. At 24 h post-transfection, cells were stimulated with 5 μg/ml poly(I:C) for 8 h to activate RIG-I-like receptor (RLR)-mediated IFN signaling. Poly(I:C)-induced IFN-β reporter activity was roughly 30% suppressed by the expression of MeT (Fig. 3), suggesting that MeT plays a role in antagonizing RLR-mediated activation of innate immune responses. As expected, HEV-encoded PCP significantly reduced poly(I:C)-mediated induction of IFN-β promoter activation (Fig. 3). Therefore, it seems that our result recapitulated previous findings while providing new insights as well.

To investigate whether MeT-mediated inhibition was RIG-I-mediated, HEK293T cells were co-transfected with IFN-β-luc, β-gal and RIG-I-expressing plasmids together with an increasing amount of HEV MeT-expressing plasmid. Interestingly, MeT inhibited RIG-I-mediated activation of IFN-β signaling in a dose-dependent manner (Fig. 4A, top panel). Of note, RIG-I protein levels were not changed even when increasing levels of MeT protein were present (Fig. 4A, bottom panel).

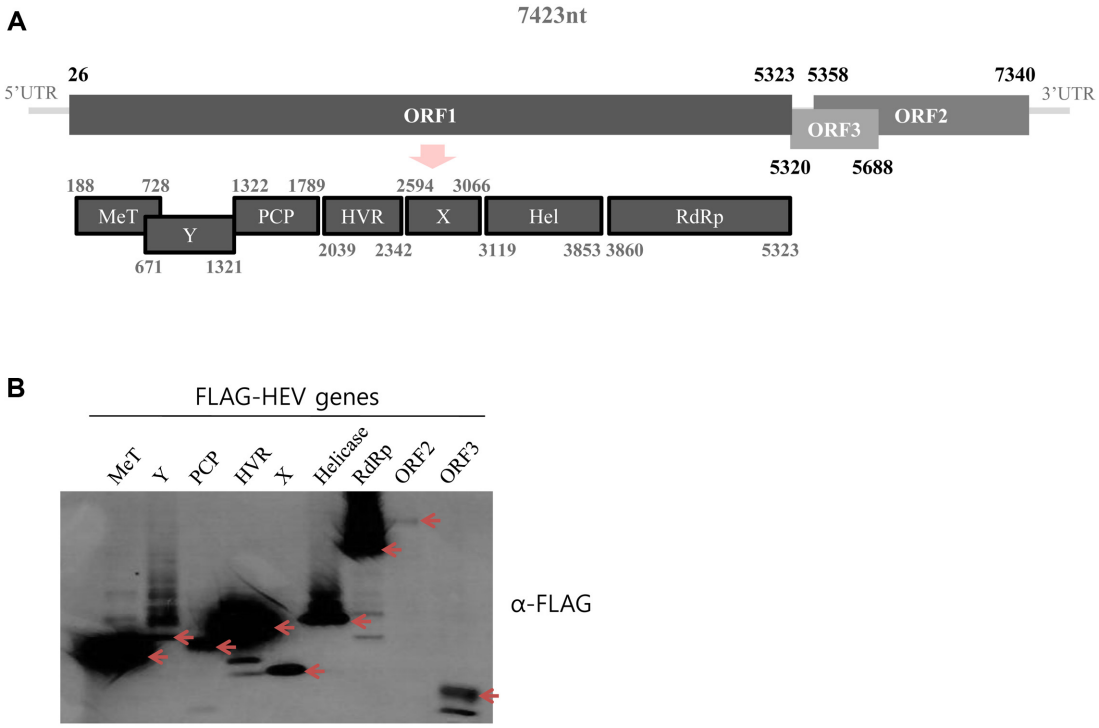

Fig. 2. Annotation of HEV-encoded genes and expression of them in fusion to FLAG tag at the N-terminus.
(**A**) Schematic diagram of viral proteins encoded in the HEV genome. ORF1 encodes seven nonstructural proteins, methyltransferase, Y, papain-like cysteine protease, hypervariable region (HVR), X, RNA helicase, and RNA-dependent RNA polymerase. (**B**) Each of viral genes was cloned into a Flag-tag vector and transfected into HEK293T cells. Cell extracts (20 μg) were loaded onto a SDS-PAGE gel and analyzed by immunoblot with anti-FLAG antibody (M2).

MeT Inhibits RIG-I-Mediated NF-κB Activation

For a full activation of IFN-β promoter activity, adequate NF-κB activation is prerequisite. To investigate if HEV MeT downregulates RIG-I-mediated activation of NF-κB, HEK293T cells were co-transfected with NF-κB-luc, β-gal, and RIG-I expressing plasmids together with an increasing amount of HEV MeT-expressing plasmid. When 1 μg of FLAG-tagged MeT-expressing plasmid was transfected, NF-κB activity was around 40% inhibited. And the inhibitory activity was left unchanged in the presence of higher levels of MeT expression, suggesting that RIG-I-mediated signaling-induced activation of NF-κB is inhibited by MeT in a dose-dependent manner. However, as seen in IFN-β reporter assay (Fig. 4A, bottom panel), RIG-I protein levels were not perturbed (Fig. 4B, bottom panel).

Discussion

Upon viral infection, the first line defense activated in the host cell is type I IFNs. MDA5 and RIG-I are sensors of viral mRNA. Both molecules recognize the RNA genome of invading pathogens, previous reports have shown that RIG-I and MDA5 have similar functions. However, the type and magnitude of their activation depends on the nature of their cognate ligands and stimuli [91, 92]. The nature and structure of the RNA molecules recognized by RLR's is known to vary. RIG-I binds to relatively short 5′ triphosphate RNA, and the RNA should be blunted. However, MDA5 does not have those restrictions and can bind to relatively long RNA's. Upon recognition, these molecules induce and activate MAVS on the mitochondrial outer membrane. MAVS, in turn, activates TBK1 and IKKε, phosphorylating IRF3. Phosphorylated IRF3 dimerizes with itself or phosphorylates IRF7, translocating into the nucleus and consequently transcribing IFN-β mRNA. Therefore, it is of paramount importance to analyze which molecule of the two (MDA5 or RIG-I) recognizes HEV genome and how HEV counteracts the IFN-mediated innate immunity.

When HEV-infected or naïve A549/D3 cells were transfected with poly(I:C), which activates IFN-β expression mainly through RIG-I-Like receptors, poly(I:C)-mediated activation

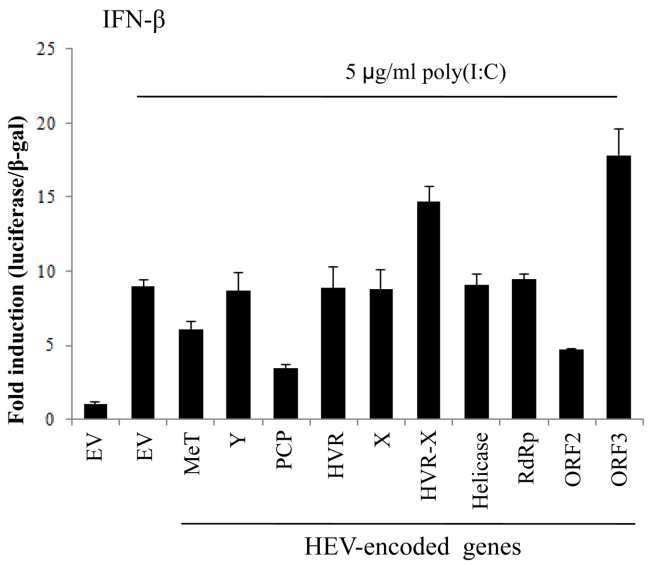

Fig. 3. HEV MeT, PCP, and ORF2 inhibits poly(I:C)-induced IFN-β induction.

Plasmids encoding HEV genes were transfected with IFN-β-luc and β-gal plasmids into HEK293T cells. At 24 h post-transfection, cells were treated with poly(I:C) at 5 μg/ml for 8 h and the luciferase activity was then measured. EV; empty vector, MeT; methyltransferase, PCP; papain-like cysteine protease, HVR; hypervariable region, RdRp; RNA-dependent RNA polymerase.

of IFN-β promoter activity was over 60% suppressed in the context of HEV infection (Fig. 1A). Reduction in luciferase activity was well-correlated with significant decrease in mRNA levels of IFN-β (Fig. 1B). These data strongly suggest that HEV may encode viral mechanism(s) that inhibit RLR-mediated activation of IFN-β. It turns out that HEV-encoded MeT, PCP and ORF2 are responsible for the inhibition of HEV (Fig. 3). As PCP has been shown to inhibit RIG-I-mediated IFN signaling, subsequent studies focused on the role of MeT in the RIG-I-mediated activation of IFN signaling. Of note, MeT inhibited RIG-I-mediated IFN-β induction in a dose-dependent manner (Fig. 4A, top panel), suggesting that MeT may directly or indirectly target RIG-I. Further studies are warranted to investigate molecular mechanisms that underlie MeT-mediated inhibition of RIG-I. It is interesting to note that protein levels of RIG-I are not perturbed even in the presence of increasing amounts of MeT (Fig. 4A, bottom panel), suggesting that MeT may regulate RIG-I-mediated signaling at the post-translational level. Currently, studies are underway to fathom its molecular mechanisms. It is well established that the activation of NF-κB is required for full activation of IFN-β signaling. Therefore, it is important to analyze if RIG-I-mediated activation of NF-κB is also regulated by the expression of MeT. Interestingly, MeT significantly inhibited RIG-I-

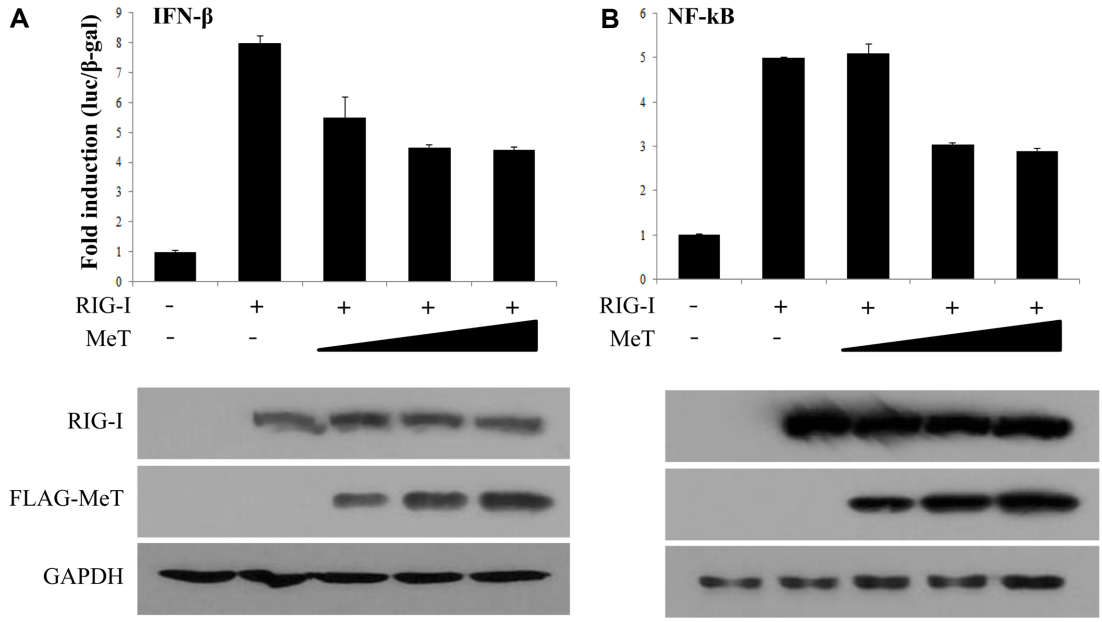

Fig. 4. HEV MeT inhibits RIG-I-induced IFN-β and NF-kB induction.

HEK293T cells were transfected for 24 h with IFN-β (**A**) or NF-κB reporter (**B**) construct, β-gal, and RIG-I expression plasmids as well as with increasing amounts (0.5, 1, and 2 μg) of FLAG-tagged MeT expression plasmid before subjected to luciferase assay. Shown above are the data from three independent experiments.

mediated activation of NF-κB activities in a dose-independent manner (Fig. 4B). Taken together, these data suggest that MeT inhibits both RIG-I-mediated signaling and NF-κB activity, leading to attenuation of IFN-β production. Therefore, MeT can be added to the list of HEV-encoded antagonists of IFN-β signaling that can help effectively counteract and thus develop strong therapeutic strategies for HEV infection.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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