Hepatitis B virus polymerase inhibits RIG-I- and Toll-like receptor 3-mediated beta interferon induction in human hepatocytes through interference with interferon regulatory factor 3 activation and dampening of the interaction between TBK1/IKKe and DDX3

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Hepatitis B virus (HBV) infection remains one of the most serious health problems worldwide. Whilst studies have shown that HBV impairs interferon (IFN) production from dendritic cells in chronic hepatitis B patients, it remains unknown whether HBV inhibits IFN production in human hepatocytes. Using transient transfection assays in a primary human hepatocyte cell line (PH5CH8), this study demonstrated that HBV polymerase inhibits IFN-β promoter activity induced by Newcastle disease virus, Sendai virus or poly(I:C) in a dose-dependent manner, whilst ectopic expression of the HBV core and X proteins had no effect on IFN-β promoter activity. In addition, HBV polymerase blocked cellular IFN-β expression and consequent antiviral immunity revealed by an infection protection assay. Furthermore, overexpression of key molecules on the IFN-β induction axis, together with HBV polymerase, resulted in a block of IFN-β promoter activity triggered by RIG-I, IPS-1, TRIF, TBK1 and IKKe, but not by an IFN regulatory factor 3 dominant-positive mutant (IRF3-5D), suggesting that HBV polymerase prevents IFN-β expression at the TBK1/IKKe level. Further studies showed that HBV polymerase inhibited phosphorylation, dimerization and nuclear translocation of IRF3, in response to Sendai virus infection. Finally, it was shown that HBV polymerase-mediated dampening of the interaction between TBK1/IKKe and DDX3 may be involved in the inhibitory effect on IFN-β induction. Taken together, these findings reveal a novel role of HBV polymerase in HBV counteraction of IFN-β production in human hepatocytes.

INTRODUCTION

Hepatitis B virus (HBV), one of many pathogens seriously jeopardizing global health, can establish persistent infection in the human liver and is closely associated with chronic hepatitis, cirrhosis and hepatocellular carcinoma (Ganem & Prince, 2004; Liaw & Chu, 2009). However, the underlying mechanism resulting in chronic hepatitis B infection has remained elusive. Human hepatocytes, the major target of HBV infection, are the only confirmed site of replication for the virus. It has been reported that hepatocytes have an innate immune response (Crispe, 2009; Lucifora et al., 2010). Nevertheless, HBV has developed some sophisticated mechanisms to evade or subvert key aspects of the antiviral activity of liver cells (Foster et al., 1991; Wu et al., 2009).

The type I interferon (IFN) system, an indispensable part of the host innate immune response, exerts an immediate antiviral response through induction of numerous functional proteins against the virus life cycle and activates the adaptive immune response. Type I IFNs can be induced by a Toll-like receptor (TLR)- or RIG-I-like helicase (RLH)-
mediated signalling pathway. The upstream pathways are distinct for these two different signals: TRIF is essential for a TLR3-induced IFN response, whilst IPS-1 is required for a RIG-I-induced IFN response. These two pathways converge at the level of TBK1/IKKε, which can phosphorylate IFN regulatory factor 3 (IRF3) and IRF7, two important transcriptional factors required for induction of type I IFNs (Takeuchi & Akira, 2009). In addition, it has been reported that the DEAD-box helicase DDX3 is also a crucial component in induction of type I IFNs that can contribute to activation of IRFs and ultimately the IFN-β promoter (Schröder et al., 2008; Soulat et al., 2008).

A growing body of evidence also suggests that HBV can eliminate type I IFNs. In chronic hepatitis B infection, there is a functional impairment of plasmacytoid dendritic cells, which are the predominant producers of IFN-α during virus infection (van der Molen et al., 2004). In addition, it has been reported that hepatitis B virus surface antigen (HBsAg), hepatitis B virus e antigen (HBeAg) and HBV virions almost completely suppress TLR-mediated antiviral activity and cytokine induction in murine liver parenchymal cells, indicating that HBV can counteract the TLR-mediated innate immune response in the microenvironment surrounding the infection foci (Wu et al., 2009). Although it is known that the RLH-mediated signalling pathway plays a critical role in the detection of invading pathogens and induction of IFN-β, little is known about whether and how HBV disturbs cytoplasmic RLH-mediated IFN-β induction in human hepatocytes.

In this paper, we identified HBV polymerase as a potent inhibitor of IFN-β induction in human hepatocytes. Expression of HBV polymerase led to inhibition of promoter activity and transcription of IFN-β, IFN-β expression and antiviral immunity in a primary hepatocytic cell line, PH5CH8. In addition, we demonstrated that HBV polymerase interfered with IFN-β induction at the TBK1/IKKε level. Furthermore, expression of HBV polymerase inhibited Sendai virus (SeV)-induced endogenous IRF3 phosphorylation, dimerization and nuclear translocation. Finally, our results showed that DDX3 may be involved in the inhibition of IFN-β induction by HBV polymerase.

RESULTS

Effects of HBV proteins on IFN-β promoter activity in human hepatocytes

In the HBV life cycle, three viral proteins (HBx, core and polymerase) can localize in the cellular cytoplasm (Seeger & Mason, 2000). To determine whether these viral proteins interfere with IFN-β induction, we performed a functional screen assay using an IFN-β promoter activity reporter system. Primary hepatocytic PH5CH8 cells capable of IFN-β induction in response to Newcastle disease virus (NDV), SeV or poly(I: C) were transfected with a construct for HBV polymerase, core or HBx. In parallel, hepatitis C virus (HCV) NS3/4A, a well-known inhibitor of IFN-β induction (Ferreon et al., 2005; Li et al., 2005c), was used as a positive control. Similar to HCV NS3/4A, HBV polymerase significantly repressed IFN-β promoter activity induced by infection of NDV, SeV and direct addition of poly(I: C) into the culture medium compared with empty vector control (Fig. 1a, left panel). In contrast, expression of the HBV core or HBx did not significantly reduce IFN-β promoter activity although their expression levels were slightly higher than HBV polymerase (Fig. 1a, right panel). These results indicated that HBV polymerase can be involved in manipulation of IFN-β induction by RIG-I or TLR3 in HBV infection.

To investigate further the effect of HBV polymerase on IFN-β promoter activity on a whole HBV genome scale, we compared the response of HepG2 cells harbouring wild-type HBV or polymerase-null HBV (HBV-ΔPol). Given that HepG2 cells are deficient in their response to extra-cellular poly(I: C) and have a diminished response to SeV infection (Li et al., 2005a and our unpublished data), we co-transfected the cells with constructs expressing RIG-I, IPS-1 or TRIF, together with wild-type HBV or HBV-ΔPol. As expected, in the cells transfected with the wild-type HBV, IFN-β promoter activity stimulated by RIG-I, IPS-1 or TRIF was reduced when compared with that in cells lacking HBV polymerase expression (Fig. 1b–d).

To exclude the possibility of non-specific effects on IFN-β promoter activity, we examined whether HBV polymerase had an effect on the unrelated p53 promoter activity. No inhibitory effect of HBV polymerase on the p53 promoter was detected (Fig. 1e). Overall, these results strongly indicated that HBV polymerase interferes with IFN-β induction by both TLR3 and RIG-I signals.

HBV polymerase inhibits promoter activity and expression of the IFN-β gene in human hepatocytes in a dose-dependent manner

To characterize further the inhibitory effect of HBV polymerase on IFN-β production in human hepatocytes, increasing amounts of HBV polymerase were transfected into PH5CH8 cells followed by NDV infection. IFN-β promoter activity induced by NDV was significantly decreased with increasing expression of HBV polymerase (Fig. 2a), indicating that HBV polymerase can inhibit IFN-β promoter activity in a dose-dependent manner. Similar results were also obtained with SeV infection and poly(I: C) treatment (data not shown).

In addition, we determined the effect of HBV polymerase on endogenous IFN-β transcription. Different amounts of HBV polymerase were transfected into PH5CH8 cells followed by NDV infection or direct addition of poly(I: C) into the culture medium, and the level of IFN-β mRNA was quantified using real-time PCR. As expected, the cells produced less IFN-β mRNA with increasing expression of HBV polymerase, indicating that HBV polymerase also
inhibits endogenous IFN-β gene transcription in a dose-dependent manner (Fig. 2b).

Furthermore, we examined the amount of IFN-β produced by PH5CH8 cells in the presence of HBV polymerase. PH5CH8 cells were transfected with the indicated amounts of HBV polymerase and then challenged by NDV infection. The results showed that IFN-β levels in the supernatant induced by NDV were inversely proportional to HBV polymerase expression levels (Fig. 2c), thus indicating that HBV polymerase inhibits IFN-β expression in PH5CH8 cells in a dose-dependent manner.

Taken together, these results indicated that HBV polymerase is a vital regulator that negatively modulates the production of IFN-β in human hepatocytes.

HBV polymerase impairs IFN-β-mediated protective antiviral immunity

Blocking the spread of virus infection is one of the most important functions of IFN-β produced by infected cells. To address the effect of HBV polymerase on this function, we analysed the induced antiviral activity of PH5CH8 cells transfected with HBV polymerase through a virus infection protection assay (Fig. 3a). The antiviral activities of supernatants harvested from transfected PH5CH8 cells challenged with NDV were compared based on the extent of green fluorescent protein-tagged NDV (NDV–GFP) replication in Vero cells. Viral GFP expression was decreased in Vero cells pre-treated with the supernatant of NDV-treated PH5CH8 cells and IFN-α (Fig. 3b, lanes 2 and 7), whereas GFP expression levels in Vero cells pre-treated with supernatant from the NS3/4A group was almost identical to the mock group (Fig. 3b, lanes 1 and 6), suggesting that NS3/4A could abolish IFN-β expression in PH5CH8 cells challenged by NDV. In the HBV polymerase groups, the supernatants gradually lost the ability to block virus replication with increasing HBV polymerase expression (Fig. 3b, lanes 3–5). These data indicated that the innate antiviral response in PH5CH8 cells was substantially decreased in the presence of HBV polymerase.

To test the possibility of other cytokines in the supernatant contributing to the antiviral effect, we further characterized the role of IFN-β in protective antiviral immunity in PH5CH8 cells using an IFN-β neutralizing antibody. When co-incubated with neutralizing antibody, the supernatant of PH5CH8 cells treated with NDV was deprived of its ability to block NDV–GFP replication (Fig. 3c, lane 2). Once the neutralizing antibody had been inactivated by three cycles of freezing and thawing, the neutralization effect dissipated accordingly, as shown by the inhibition of viral GFP expression in Vero cells (Fig. 3c, lane 3). In conclusion, our results revealed that it is IFN-β induced by NDV that exerts the main antiviral effect in the protective antiviral response.
HBV polymerase inhibits induction of IFN-β at the TBK1/IKKε level

The RLH and TLR signalling cascades that elicit IFN-β gene induction involve sensors, adaptors, kinases and transcriptional factors. To uncover the level at which HBV polymerase inhibits induction of IFN-β at the TBK1/IKKε level.

Fig. 2. HBV polymerase inhibits RIG-I- and TLR3-induced IFN-β promoter activity, transcription and expression in a dose-dependent manner. (a) PH5CH8 cells in 48-well plate were co-transfected with pIFN-β-Luc, pRL-TK and the indicated amounts of HBV polymerase (Pol) or NS3/4A. After 36 h, all of the cells were infected with 100 HAU NDV ml⁻¹ for 12 h and then harvested for luciferase activity (upper panel). The expression level of HBV polymerase was determined by immunoblotting (IB) using anti-Flag antibody (lower panel). (b) PH5CH8 cells in 24-well plates were transfected with increasing amounts of HBV polymerase. After 36 h, the cells were infected with 100 HAU NDV ml⁻¹ or treated with 50 μg poly(I : C) ml⁻¹ for an additional 6 h before measurement of IFN-β mRNA by real-time RT-PCR. (c) The amount of IFN-β produced by PH5CH8 cells in 12-well plates transfected with the indicated plasmids was determined by ELISA. *, P<0.05.

Fig. 3. HBV polymerase impairs cellular IFN-β-dependent antiviral immunity. (a) Scheme outlining the infection protection assay. The detailed protocol is described in Methods. (b) Vero cells were pre-treated with supernatant from NDV-treated PH5CH8 cells transfected with empty vector, Flag–Pol (Pol) or NS3/4A or with IFN-α (100 IU ml⁻¹) for 12 h, and then infected with NDV–GFP overnight. (c) Active or inactive IFN-β neutralizing antibody (10 μg ml⁻¹) was mixed with the supernatant from NDV-treated PH5CH8 cells and added to Vero cells for 12 h. The cells were then infected with NDV–GFP overnight. The GFP expression levels in (b) and (c) were examined by immunoblotting (IB) and microscopy.
polymerase interferes with IFN-β induction, 293T cells were co-transfected with constructs of key regulatory molecules on the IFN-β induction axis and HBV polymerase. Expression of RIG-I, ARIG-I, TRIF, IPS-1, TBK1, IKKe and an IRF3 dominant-positive mutant (IRF3-5D) in 293T cells enhanced IFN-β promoter activity (Fig. 4a–g). As a positive control, expression of HCV NS3/4A, which can cleave IPS-1 and TRIF (Li et al., 2005b, c; Meylan et al., 2005), blocked IFN-β promoter activity triggered by RIG-I, ARIG-I, TRIF and IPS-1 (Fig. 4a–d), but not TBK1, IKKe or IRF3-5D (Fig. 4e–g). However, in the presence of HBV polymerase, inhibition of IFN-β promoter activity was observed for all effectors tested except IRF3-5D (Fig. 4a–g). To confirm this observation, HEK293 cells were co-transfected with TBK1, IKKe or IRF3-5D and the indicated amounts of HBV polymerase. As expected, HBV polymerase inhibited IFN-β promoter activity triggered by TBK1 and IKKe in a dose-dependent manner (Fig. 4h), whilst it had no effect on activation by IRF3-5D, even at the highest HBV polymerase expression level (Fig. 4i). These results suggested that HBV polymerase inhibits IFN-β induction at the level of TBK1/IKKc.

HBV polymerase inhibits IRF3 phosphorylation, dimerization and nuclear translocation

IRF3, a key transcriptional factor in IFN-β induction, is regulated through phosphorylation by the TBK1 and IKKe kinases. Upon phosphorylation, IRF3 dimerizes and translocates into the nucleus, ultimately driving IFN-β transcription (Hiscott, 2007). In light of our results indicating that HBV polymerase inhibited IFN-β induction at the level of TBK1/IKKc, we further studied the phosphorylation, dimerization and nuclear translocation of IRF3 in the presence of HBV polymerase.

To examine the effect of HBV polymerase on IRF3 phosphorylation, PH5CH8 cells transfected with the indicated amounts of HBV polymerase were challenged with SeV infection. The results showed that IRF3 phosphorylation was inhibited with increasing HBV polymerase expression (Fig. 5a). Likewise, the effect of HBV polymerase on IRF3 dimerization was studied by native gel electrophoresis. As expected, SeV infection induced dimerization of IRF3 in the absence of HBV polymerase (Fig. 5b, lanes 2 and 4). In contrast, a dose-dependent reduction in dimerized IRF3 was observed in the presence of the polymerase (Fig. 5b, lanes 5–7). Next, we examined the subcellular localization of endogenous IRF3 with or without HBV polymerase expression. Immunofluorescence observations showed that, compared with empty vector control, IRF3 nuclear translocation was significantly reduced in response to SeV stimulation in PH5CH8 cells expressing HBV polymerase (Fig. 5c, and Supplementary Fig. S1a, available in JGV Online). This indicated that HBV polymerase inhibits IRF3 nuclear translocation. Similar results were also obtained in SeV-infected HepG2 cells co-transfected with trace amounts of RIG-I as well as wild-type HBV or HBV-ΔPol (Fig. 5d).

Fig. 4. HBV polymerase inhibits IFN-β induction at the TBK1/IKKc level. 293T cells in 48-well plates were co-transfected with pIFN-β-Luc, pRL-TK and Flag–Pol (Pol) (200 ng) or NS3/4A (200 ng) along with 50 ng of expression plasmids encoding RIG-I (a), ΔRIG-I (b), IPS-1 (c), TRIF (d), TBK1 (e), IKKe (f) or IRF3-5D (g). After 36 h, the cells were harvested for luciferase activity. (h) HEK293 cells in 24-well plates were co-transfected with TBK1/IKKc together with the indicated amounts of Flag–Pol. After 36 h, the cells were harvested for luciferase activity (left panel). Expression of the indicated proteins was determined by immunoblotting (IB; right panel). (i) HEK293 cells in 48-well plate were co-transfected with IRF3-5D together with the indicated amounts of Flag–Pol. After 36 h, the cells were harvested for luciferase activity.
However, no co-localization between HBV polymerase and IRF3 was observed (Fig. 5c). To confirm this result, Flag-tagged HBV polymerase was pulled down to analyse its binding proteins using immunoprecipitation. HBV polymerase and DDX3, but not IRF3, were detected in the immunoprecipitation complex (see Supplementary Fig. S1b). Thus, these results indicated that HBV polymerase interferes with IRF3 activation and that this inhibitory effect may be indirect.

**DDX3 is involved in the inhibition of IFN-β induction by HBV polymerase**

DDX3 has been reported to be involved in TBK1/IKKε-mediated IRF activation and type I IFN induction (Schröder et al., 2008; Soulat et al., 2008). Moreover, DDX3 has been isolated from a Flag-tagged HBV polymerase immunoprecipitation complex using an anti-Flag pull-down assay (Wang et al., 2009). Based on these studies, we speculated that DDX3 may also be involved in the inhibition of IFN-β induction by HBV polymerase. We first confirmed the involvement of endogenous DDX3 in IFN-β induction in our system using a DDX3 antisense construct (DDX3-AS) (Fig. 6a), and detected the co-localization of HBV polymerase and DDX3 in the cytoplasm (see Supplementary Fig. S2a, available in JGV Online). Next, we analysed the interaction between TBK1/IKKε and DDX3 in the presence of HBV polymerase. The amount of TBK1/IKKε was compared in the absence or presence of HBV polymerase. Consistent with previous reports (Schröder et al., 2008; Soulat et al., 2008; Wang et al., 2009), TBK1/IKKε and HBV polymerase were detected in the DDX3 immunoprecipitation complex. However, less TBK1/IKKε was detected in the absence of HBV polymerase when compared with the absence of HBV polymerase (Fig. 6b and Supplementary Fig. S2b), suggesting that HBV polymerase can dampen the interaction between TBK1/IKKε and DDX3 through competitive binding of DDX3. Furthermore, HBV polymerase also inhibited the phosphorylation of DDX3 by TBK1/IKKε (Fig. 6c and Supplementary Fig. S2c). Finally, we examined whether overexpression of DDX3 could restore the inhibitory effect of HBV polymerase. As
expected, HBV polymerase-mediated inhibition of TBK1- and SeV-induced IFN-β promoter activation was rescued by DDX3 in a dose-dependent manner (Fig. 6d, e). However, HCV NS3/4A inhibition of IFN-β promoter was not reversed by overexpression of DDX3 (Fig. 6e), which suggests that DDX3 interaction is at the heart of HBV polymerase inhibitory effects. All these results indicated that DDX3 is involved in the inhibition of IFN-β induction by HBV polymerase.

**DISCUSSION**

IFN-β induction is one of the first-phase characteristics of activation of the type I IFN system. TLRs and RLHs, as molecular sensors to viral products, can trigger downstream adaptor aggregation and subsequent activation of TBK1/IKKe, which ultimately phosphorylate IRF3. IRF3, together with other related transcription factors, can drive IFN-β production in a concerted manner (Taniguchi & Takaoka, 2002). To evade recognition and the immune response, viruses have sophisticatedly generated viral partners to target the critical molecules in the IFN-β induction pathway and have them hijacked or degraded, resulting in a relatively favourable environment for viral proliferation (Roy & Mocarski, 2007). For example, HCV NS3/4A can cleave TRIF and IPS-1 and consequently lead to acquired deficiency of IFN-β induction in infected cells (Li et al., 2005b, c; Meylan et al., 2005). Ebola virus VP35, a component of the viral RNA polymerase complex, can also subvert host type I IFN induction via suppression of IRF3 activation (Basler et al., 2003; Cardenas et al., 2006). Here, we found that HBV polymerase could disturb RIG-I- and TLR3-mediated IFN-β induction (Figs 1–3). Unlike HCV NS3/4A, the action target of HBV polymerase was defined at the TBK1/IKKe level (Fig. 4). Although HBV polymerase inhibited endogenous IRF3 activation (Fig. 5), a physical interaction was not detected (Supplementary Fig. S1b), which suggests that other molecular mechanisms underlie the inhibitory effect on IFN-β induction.

![Fig. 6.](https://www.microbiologyresearch.org) DDX3 is involved in the inhibition of IFN-β induction by HBV polymerase. (a) PH5CH8 cells in 24-well plates were transfected with pIFN-β-Luc, pRL-TK and the indicated amounts of DDX3-AS. After 48 h, cells were treated with 100 HAU SeV ml⁻¹ for 12 h before the luciferase activity assay (upper panel) and evaluation of DDX3-AS-mediated silencing of DDX3 by immunoblotting (IB; lower panel). (b) 293T cells in a 10 cm diameter dish were co-transfected with 5 µg TBK1, DDX3, Flag–Pol or empty vector. Cells were harvested for immunoprecipitation and immunoblotting at 48 h post-transfection. (c) HEK293 cells in 12-well plates were co-transfected with the indicated expression constructs. Cells were harvested for immunoblotting using the indicated antibodies at 48 h post-transfection. (d) 293T cells in 24-well plates were co-transfected with pIFN-β-Luc, pRL-TK, TBK1 and Flag–Pol as well as the indicated amounts of DDX3. After 36 h, the cells were harvested for luciferase activity (left panel). The expression of Myc–DDX3 and Flag–Pol were confirmed by immunoblotting (right panel). (e) PH5CH8 cells in 24-well plates were co-transfected with pIFN-β-Luc, pRL-TK and Flag–Pol or NS3/4A as well as the indicated amounts of DDX3. After 36 h, the cells were treated with 100 HAU SeV ml⁻¹ for 12 h and subjected to a luciferase assay.
Recent studies have identified DDX3 as another crucial molecule in TBK1/IKKe-mediated activation of IRFs and IFN-β synthesis (Schröder et al., 2008; Soulat et al., 2008). In addition, DDX3 also interacts with HBV polymerase and impedes viral reverse transcription by incorporation into nucleocapsids (Wang et al., 2009). In this study, as no physical interaction was detected between IRF3 and HBV polymerase (Supplementary Fig. S1b), we speculated that DDX3 might be a candidate target for HBV inhibition of IFN-β induction at the TBK1/IKKe level. We found that HBV polymerase could dampen the interplay between TBK1/IKKe and DDX3 via competitive interaction (Fig. 6b and Supplementary Fig. S2b) and consequently impede DDX3 phosphorylation (Fig. 6c and Supplementary Fig. S2c). Furthermore, overexpression of DDX3 could rescue IFN-β promoter activity suppressed by HBV polymerase (Fig. 6d, e). This suggests that HBV polymerase can hijack endogenous DDX3 and subsequently dampen the interaction between TBK1/IKKe and DDX3 and ultimately inhibit IFN-β induction. This mechanism is similar to that of the vaccinia virus K7 protein (Kalverda et al., 2009; Schröder et al., 2008). Moreover, as the effect of HBV polymerase on TBK1 was more significant than on IKKe (Fig. 4h), we speculated that the difference in the effect of HBV polymerase on the two isozymes (TBK1 and IKKe) could be associated with the different affinity of the two kinases for DDX3. Interestingly, DDX3 ATPase activity is required for the inhibition of HBV replication but not for IRF activation and type I IFN induction (Schröder et al., 2008; Soulat et al., 2008; Wang et al., 2009). Comprehensive analysis of the two sides of the interaction between DDX3 and HBV polymerase may shed light on the role of DDX3 in host cells against HBV infection. On the one hand, HBV might eliminate IFN-β induction via HBV polymerase hijacking of DDX3. On the other hand, host cells could still impede viral genome replication through impregnation of DDX3 into viral nucleocapsids even if IFN-β induction is blocked, which would partially explain the sophisticated strategy of host cells to suppress HBV replication. In addition, according to previous studies, no direct interaction has been observed between DDX3 and IRF3 (Schröder, 2010; Soulat et al., 2008), and it is thus interesting to study whether and how DDX3 can impact on TBK1/IKKe activity and consequently affect the activation of IRF3. One hypothesis is that DDX3, as well as being one of the substrates of TBK1/IKKe, may also function as a scaffold protein like TANK protein to facilitate TBK1/IKKe activation.

The final outcome of virus infection depends on the balance of host response and virus countermeasures. However, the host immune response, including innate immunity and adaptive immunity, is contingent on the host recognition of invading viruses. HCV, a hepatotropic RNA virus, has been reported to trigger a hepatic innate immune response through the RIG-I signalling pathway (Saito et al., 2008). Unlike HCV, it remains controversial whether HBV, a hepatotropic DNA virus, can be recognized by host cells in HBV infection (Lucifora et al., 2010; Wieland & Chisari, 2005). Our study here demonstrated that HBV polymerase can inhibit IFN-β induction by RIG-I or TLR3 signal. Similarly, 2TGH cells stably expressing the terminal protein domain of HBV polymerase have no response to dsRNA (Foster et al., 1991). Moreover, HBsAg, HBeAg and even virion particles can also suppress TLR-mediated innate immunity and cytokine induction in primary hepatocytes (Wu et al., 2009). All these findings provide evidence that HBV has developed strategies to subvert the host innate immune system; however, they also imply that HBV has the possibility of being recognized by host cells. There may be some leakage of viral DNA or RNA into the cytoplasm, either during entry of HBV into the cells or during exportation of viral RNA to the cytoplasm, which is then recognized by potential DNA or RNA sensors. It is worth noting that DNA viruses can be recognized through cellular RNA polymerase III coupled with the RIG-I pathway (Ablasser et al., 2009; Chiu et al., 2009). Therefore, to comprehensively elucidate the interaction between HBV and host innate immunity, both the recognition of HBV by the host immune system and how viral nucleic acids and proteins including HBV polymerase participate in the active evasion of recognition are worthy of further investigation.

In conclusion, our findings suggest a novel role of HBV polymerase in the inhibition of IFN-β induction in human hepatocytes. Further investigations will be conducted to confirm this novel function in stable cell lines and clinical samples, which will open up an exciting future for improving IFN treatment by triggering the pathway or molecules targeted by HBV.

METHODS

Cell culture. PH5CH8 (a gift from Nobuyuki Kato, Okayama University Graduate School of Medicine and Dentistry, Japan), a simian virus 40 large T antigen-immortalized non-neoplastic human hepatocyte cell line with intact capacity for type I IFN induction (Li et al., 2005a), was maintained as described previously (Noguchi & Hirohashi, 1996). HEK293, 293T, HepG2 and Vero cells (obtained from the Cell Bank of the Chinese Academy of Science) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU ml⁻¹; Gibco) and streptomycin (100 μg ml⁻¹; Gibco), in a 5% CO₂ atmosphere at 37 °C.

Plasmids and transfection. The following expression plasmids were generously provided: pIFN-β-Luc (Dr Rongtuan Lin, McGill University, Canada); TBK1 and IKKe (Dr Katherine A. Fitzgerald, University of Massachusetts Medical School, MA, USA); TRIF, IRF3–GFP and IRF3-5D (Professor John Hiscott, McGill University); haemagglutinin (HA)–DDX3 and Myc–DDX3 (Professor Andrew G. Bowie, Trinity College Dublin, Ireland); RIG-1 and IPS-1 (Takashi Hirohashi, Kyoto University, Japan); AGR1-1 was amplified from RIG-1 and cloned into a modified pcDNA3.1 vector containing three tandem Flag epitopes in frame at the 5’ end of the cloning site; HCV NS3/4A was amplified from cDNA of HCV replicon cells and cloned into the pcDNA3.1 vector by Dr Zhigang Yi (Fudan University, PR China); DDX3-AS (Kuan-Teh Jeang, National Institutes of Allergy and Infectious Diseases, MD, USA); HBV-Apol (Professor Jianming...
Hu, The Pennsylvania State University, PA, USA) is a vector containing whole HBV genome with a point mutation changing the polymerase AUG to ACG making it deficient for polymerase synthesis while keeping the core protein unaltered; myc-p53 (Professor Jianxin Gu, Fudan University, PR China); pp53-Luc (Professor Lan Ma, Fudan University), Flag-Pol (pcDNA3.1-3 Flag-Pol, and pQXIP-3 Flag-Pol), pHBV1.3 (Professor Yumei Wen, Fudan University). HBV-Core and HBV-X were amplified from EGFP-Core and EGFP-X (Wu et al., 2007), respectively, and cloned into pcDNA3.1-3 Flag to make pcDNA3.1-3 Flag-Care and pcDNA3.1-3 Flag-HBx. All of the plasmids were sequenced and the indicated protein expression was confirmed by immunoblotting. PH5CH8, HEK293, 293T and HepG2 cells were transfected with the indicated plasmids using Fugene HD (Roche) or Lipofectamine 2000 (Invitrogen), according to the manufacturers’ instructions.

Poly(I:C), NDV, NDV–GFP and SeV. Poly(I:C) was purchased from Sigma. NDV, NDV–GFP (a gift from Dr Yan Yuan, University of Pennsylvania, PA, USA) and SeV were propagated and purified from specific-pathogen-free-maintained chicken eggs. PH5CH8 cells were treated with 50 μg poly(I:C) ml−1 added into the culture medium or infected with 100 haemagglutinin units (HAU) ml−1 of the indicated virus and harvested 6 h later for RNA extraction or 12 h later for the luciferase reporter assay.

Dual-luciferase reporter assay. Cells (3 × 10⁴ per well) were seeded in a 48-well plate for culture overnight. The cells were then transfected with 20 ng pIFN-β-Luc, 5 ng pRL-TK (expressing Renilla luciferase; Promega) and the indicated amounts of expression plasmids. After 36 h, cells were mock treated or treated with poly(I:C), NDV or SeV for an additional 12 h. All cells were lysed with passive lysis buffer and assayed for luciferase activity with a Dual-Luciferase Assay Kit (Promega). Firefly luciferase activities were normalized based on Renilla luciferase activities. The fold induction of promoter activity was calculated by dividing the normalized luciferase activity of stimulated cells by that of mock-treated cells. All reporter assays were repeated at least three times. The data shown are mean values ± s.d. from one representative experiment.

Real-time RT-PCR. Total cellular RNA was extracted with TRizol reagent (Invitrogen), treated with DNase I (Takara) to remove genomic DNA contamination and reverse-transcribed using Toyobo Ace reverse transcriptase. The cDNA samples were subjected to real-time PCR using primers specific for IFN-β and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): IFN-β forward, 5′-GATTCATCTAGCA-CGTGCCCTGG-3′, and reverse, 5′-CTTCAGGTAATGCGAATCCC-3′; GAPDH forward, 5′-GGTAATGTTGAAAGACCTCATGA-3′, and reverse, 5′-ATGCCAGTGCTCCGCTCAG-3′. For comparisons, transcription of IFN-β was normalized to that of GAPDH.

IFN-β ELISA. The level of IFN-β in the culture medium was measured using an ELISA kit for human IFN-β (USCN Life Science) according to the manufacturer’s instructions.

Immunofluorescence. PH5CH8 cells or HepG2 cells transfected with the indicated plasmids were seeded into a chambered coverglass system (Lab-Tek) for 24 h, then fixed with 35% paraformaldehyde, permeabilized by the addition of 0.1% Triton X-100, and blocked with 10% FBS for 2 h. IRF3 was detected by staining with rabbit anti-human IRF3 (1:300 dilution; Santa Cruz Biotechnology) followed by Alexa 488-coupled goat anti-rabbit IgG (1:1000; Jackson Immunologicals). Flag-tagged HBV polymerase was detected by staining with anti-Flag antibody (1:2000; Sigma) followed by Cy3-coupled goat anti-mouse IgG (1:1000; Jackson Immunologicals), and the nuclei were counterstained with the DNA-specific stain TO-PRO-3 (1:1000; Invitrogen) or 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Invitrogen). The subcellular localization of IRF3 (green) and HBV polymerase (red) was observed with an Olympus Fluoview FV1000 confocal microscope or Zeiss Axiovert 200 fluorescent microscope.

Immunoprecipitation. 293T or HepG2 cells were seeded into 10 cm dishes (2 × 10⁶ cells) and cultured overnight to 80–90% confluency before co-transfection with the indicated construct. Cells were harvested after 48 h in 1 ml lysis buffer [25 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% NP-40 containing 1× Roche protease inhibitors]. DDx3 polyclonal IgG was pre-coupled to Protein A/G PLUS–Agarose (Santa Cruz Biotechnology) for 1 h at 4°C before incubation with the cell lysates for 4 h at 4°C. The immune complexes were precipitated, washed and analysed by SDS-PAGE and immunoblotting.

Immunoblotting. Cellular extracts were subjected to immunoblotting analysis as described previously (Wu et al., 2007). The antibodies used were as follows: mouse anti-Flag, mouse anti-Myc, mouse anti-β-actin (Sigma), rabbit anti-DDx3, rabbit anti-IRF3 (Santa Cruz Biotechnology), mouse anti-HA, rabbit anti-phospho-IRF3, rabbit anti-TBK1, rabbit anti-IKKz (Cell Signalling), and peroxidase-conjugated secondary goat anti-mouse and anti-rabbit antibodies (Amersham Biosciences). Protein bands were visualized using an ECL Plus Western blotting system (Perkin-Elmer) followed by exposure to Kodak Bio-Max film.

Native PAGE. The IRF3 dimerization assay was performed as described previously with a modification (Iwamura et al., 2001). Briefly, 2 × 10⁵ cells were seeded into a 12-well plate and cultured overnight. The PH5CH8 cells were transfected with the indicated amounts of HBV polymerase (empty vector was used to balance the total amount of DNA) using Fugene HD. After 24 h, cells were selected with puromycin (3 μg ml⁻¹) for 24 h and then infected with SeV or mock infected for an additional 6 h. The cells were harvested with 30 μl ice-cold lysis buffer [50 mM Tris/HCl, (pH 7.5), 150 mM NaCl and 0.5% NP-40 containing 1× Roche protease inhibitors]. After centrifugation at 13 000 g for 10 min, supernatants were quantified using a BCA assay (Thermo Scientific) and diluted with 2× native PAGE sample buffer [125 mM Tris/HCl (pH 6.8), 30% glycerol and 0.1% bromophenol blue], and then 20 μg of total protein was applied to a pre-run 7.5% native gel for separation. After electrophoresis, the gel was soaked in SDS electrophoresis buffer [25 mM Tris/HCl (pH 8.3), 250 mM glycine and 0.1% SDS] for 10 min at room temperature before the proteins were transferred onto a nitrocellulose membrane for immunoblotting.

Infection protection assay. PH5CH8 cells seeded in a 12-well plate and transfected with the indicated plasmids were challenged with NDV (100 HAU ml⁻¹) for 3 h and then rinsed five times with PBS and cultured for an additional 12 h. The culture medium was centrifuged for collection of the supernatants. Vero cells were pretreated with these supernatants for 12 h and then infected with NDV–GFP (40 HAU ml⁻¹) overnight, followed by observation under a Zeiss Axiovert 200 fluorescent microscope and analysis by immunoblotting. The neutralization assay was performed by the addition of IFN-β neutralizing antibody (10 μg ml⁻¹; R&D Systems) into the supernatant.

Statistical analysis. All results were confirmed in at least three independent experiments in triplicate within each experiment. Data were analysed using Student’s t-test and expressed as the mean ± s.d. A value of P<0.05 was considered to be statistically significant.
NOTE ADDED IN PROOF

Similar results to ours concerning the interaction with DDX3 were presented by other authors at the meeting on the Molecular Biology of Hepatitis B Viruses held in Tours, France, in September 2009 (Wang & Ryu, 2009), and have recently been accepted for publication (Wang & Ryu, 2010).

ACKNOWLEDGEMENTS

We thank Nobuyuki Kato (Okayama University, Japan) for kindly providing PH5CH8 cells and Rongtuan Lin, John Hiscott (McGill University, Canada), Katherine Fitzgerald (University of Massachusetts Medical School, MA, USA), Takashi Fujita (Kyoto University, Japan), Kuan-Teh Jeang (National Institutes of Allergy and Infectious Diseases, MD, USA), Jianming Hu (The Pennsylvania State University, PA, USA), Yumei Wen, Jianxin Gu and Lan Ma (Fudan University, PR China) for their plasmids. We are grateful to Nathalie Grandvaux (University of Montreal, Canada) for immunoblotting advice. We are indebted to Professor Andrew Bowie and Dr Martina Schroeder (Trinity College Dublin, Ireland) for M yc–DDX3 and helpful discussions. This work was financially supported by a Chinese State Basic Research Foundation Grant (2005CB522902), National High Tech Project (2006AA 02A411), National Megaprojects for Infectious Diseases (2008ZX10203) and the Program for Outstanding Medical Academic Leader of Shanghai to Z. Y. and a grant from the National Youth Foundation of China (2003).

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