Activation of Pattern Recognition Receptor-mediated Innate Immunity Inhibits the Replication of Hepatitis B Virus in Human Hepatocyte-derived Cells

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Running Title: Innate immune response inhibits HBV replication

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Recognition of virus infections by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), activates signaling pathways leading to the induction of inflammatory cytokines that limit viral replication. To determine the effects of PRR-mediated innate immune response on HBV replication, a 1.3mer HBV genome was cotransfected into HepG2 or Huh7 cells with plasmid expressing TLR adaptors, myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF), or RIG-I/MDA5 adaptor, interferon promoter stimulator 1 (IPS-1). The results showed that expressing each of the three adaptors dramatically reduced the levels of HBV mRNA and DNA in both HepG2 and Huh7 cells. However, HBV replication was not significantly affected by treatment of HBV genome-transfected cells with culture media harvested from cells transfected with each of the three adaptors, indicating that the adaptors-induced antiviral response was predominantly mediated by intracellular factors, rather than secreted cytokines. Analyses of involved signaling pathways revealed that activation of NFκB is required for all three adaptors to elicit antiviral response in both HepG2 and Huh7 cells. However, activation of IRF3 is only essential for induction of antiviral response by IPS-1 in Huh7 cells, but not in HepG2 cells. Furthermore, our results suggest that besides NFκB, additional signaling pathway(s) are required for TRIF to induce a maximum antiviral response against HBV. Knowing the molecular mechanisms by which PRR-mediated innate defense responses control HBV infections could potentially lead to the development of novel therapeutics that evoke the host cellular innate antiviral response to control HBV infections.
Heaptitis B virus (HBV) is a noncytopathic, hepatotropic virus belonging to the \textit{hepadnaviridae} family. Infection of HBV can be either transient or chronic in nature (13, 30). Transient infection frequently leads to acute hepatitis and, in rare cases, results in fatal, fulminant hepatitis(30). Chronic infection represents a major public health burden affecting an estimated 400 million individuals worldwide and carries a high risk for the development of chronic active hepatitis, cirrhosis and primary hepatocellular carcinoma(43). As for many other viruses, the outcomes of HBV infections and pathogenesis of the associated diseases are determined by virus-host interactions, largely mediated by innate and adaptive immune responses (8, 73).

Virus infection elicits a rapid and potent innate immune response in mammalian cells to produce pro-inflammatory cytokines and chemokines that limit virus replication and coordinate adaptive antiviral immunity(50). Central to this cellular response is the virus-induced production of alpha/beta interferons (IFN-α/β), which play an indispensable role in host defense against virus infections(22, 45, 61). In the last decade, tremendous progresses have been made in uncovering the cellular pattern recognition receptors (PRRs) that sense diverse pathogen-associated molecular patterns (PAMPs) and deciphering the molecular pathways that couple pathogen recognition to the induction of IFNs and other cytokines(50, 66, 71). Notably, mammalian cells sense virus infections predominantly \textit{via} either endosomal Toll-like receptors 3, 7/8 and 9 (TLR3, TLR7/8, and TLR9) or cytoplasmic caspase activation and recruitment domain (CARD)-containing DEx(D/H) box RNA helicases, including retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5)(1, 79). While the four TLRs are activated by selective binding of viral double stranded RNA (TLR3), single-stranded poly-U RNA (TLR7/8) or unmethylated CpG DNA motifs present in viral genomes (TLR9) in the endosomes(2, 24, 39), RIG-I and MDA5 recognize cytoplasmic viral RNAs bearing
distinguishable structural features from cellular RNA, such as the presence of triphosphates at the 5’ terminus (31, 51). As illustrated in Fig. 1, recognition of such virus-associated molecular patterns by each of the cellular PRRs recruits their distinct adaptor proteins which activate signaling cascades to induce cytokine production in virally infected cells. For examples, binding of viral RNA to RIG-I/MDA5 induces conformation changes that lead to the exposure of their CARD domains (65). Through homotypic CARD interaction with the interferon promoter stimulator 1 (IPS-1, also known as CARDIF, MAVS and VISA) adaptor protein, the RIG-I/MDA5 is recruited onto the outer membrane of the mitochondria to form a macromolecular signaling complex that serves to activate downstream interferon regulatory factor 3 (IRF3), nuclear factor κ B (NFκB), c-Jun/ATF2 and other transcription factors that stimulate the transcription of IFNs and certain IFN-stimulated genes (ISGs) (38, 44, 59, 77). Similarly, engagement of the TLRs with viral nucleic acids recruits distinct adaptor proteins TRIF (for TLR3) or MyD88 (for TLR7/8 and TLR9), which initiates signaling pathways to activate critical transcription factors, such as NFκB, IRF3, IRF7 or IRF5 among others, to induce expression of pro-inflammatory cytokines and other cellular genes (37).

Microarray analyses of host cellular gene expression profiles in response to infections of the variety of viruses in cultured cells and tissue samples obtained from infected people and animals in the last decade reveal that induction of ISG expression, a indicator of the activation of PRR-mediated host cellular antiviral responses, is observed in almost all the virus infections examined, with rare exceptions (34, 72). One of such exceptions is HBV infection of chimpanzees (73). While it had been shown that induction of ISGs in the livers of chimpanzees and humans was a hallmark of hepatitis C virus (HCV) infections (5, 6, 27, 40, 60, 62), HBV infection of chimpanzees did not induce the expression of ISGs as the virus spreads throughout
the liver (73). This surprising and unprecedented observation implies that HBV either fails activate or has evolved ability to inhibit the activation of the PRR-mediated innate immune responses.

To resolve these issues and determine whether activation of PRR-mediated innate immunity in human hepatoma cells could inhibit HBV replication, we investigated the induction and antiviral effects of TLRs- and RIG-I/MDA5-mediated host cellular innate immune responses against HBV in HepG2 and Huh7 cells via overexpression of the three PRR adaptors, IPS-1, TRIF and MyD88. Our results demonstrated that expressing each of the three adaptors potently inhibited HBV replication, predominantly through intracellular antiviral pathway(s), rather than secreted cytokines. Most strikingly, we found that unlike IFN-α/β inhibition of HBV replication in mouse hepatocytes, where the cytokines prevented pregenomic (pg) RNA-containing nucleocapsid formation(74), the innate immune response-elicited by expression of adaptor proteins in human hepatoma cells limited HBV replication by reducing the steady-state levels of viral mRNAs. Analysis of involved signaling pathways revealed that activation of NFκB is essential for all three adaptors to elicit antiviral response in both HepG2 and Huh7 cells, but activation of IRF3 is only essential for induction of antiviral response by IPS-1 in Huh7 cells, but not HepG2 cells. Our results thus suggest that although HBV fails to activate host cellular innate defense pathways, it is extremely sensitive to the antiviral effects elicited by PRR-mediated host cellular antiviral response in human hepatocyte-derived cells. Further analysis of the intracellular antiviral pathway could potentially lead to the identification of molecular targets for the development of antivirals that evoke the intricate cellular antiviral pathway(s) and eliminate HBV infections.
MATERIALS AND METHODS

Cell culture and kinase inhibitors. HepG2 cells were obtained from ATCC and maintained in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Huh7 cells were maintained as previously described(20). MAPK inhibitors SB202190, U0126 and SP600125 were purchased from Calbiochem.

Plasmid construction. Plasmids pHBV1.3 and pCMV-HBV were described previously(19). Plasmid pS that expresses HBV small (S) envelope proteins (HBsAg) was described previously and kindly provided by Volker Bruss (University of Gottingen, Germany)(55). Plasmid pcHBs-V5, which expressed C-terminally V5 tagged HBsAg, was generated by insertion of a 0.8kb PCR fragment spanning the HBsAg coding region into pcDNA3.1/V5-His-TOPO vector. Plasmids expressing C-terminally HA-tagged MyD88, TRIF with deletion of carboxyl terminal 68 amino acids (saTRIF), and constitutively active (or super-active) interferon regulatory factor 3 (saIRF3) proteins were purchased from Invivogen (San Diego, California). Plasmids expressing wild-type IκB-alpha (IκBα) and dominant negative IκB-alpha (DN-IκBα) were obtained from Christoph Seeger (Fox Chase Cancer Center, Philadelphia). Plasmids expressing other adaptors or cellular signaling proteins used in this study were constructed via similar approaches described previously(35). Briefly, to obtain cDNA clones of human RIG-I, MDA5, CARD domains of RIG-1 and MDA5, IPS-1 and IRF3, first strand cDNA was made from Huh7 cell-derived total RNA with oligo-(dT)12-18 primer and Superscript III DNA polymerase (Invitrogen) by following the manufacturer’s direction. Full-
length cDNA for each of the fore-mentioned genes with N-terminal FLAG tag was amplified by PCR (primer sequences are available upon request). The purified PCR fragments were digested with restriction enzymes Alf II and Not I and cloned into pCDNA5/FRT/ΔCAT vector that was derived from pCDNA5/FRT/CAT (Invitrogen) by removing CAT-coding sequence with Apa I and Xho I digestion and self-ligation(35). The plasmid expressing the dominant-negative IRF3 (DN-IRF3) was generated by amplification of the coding region for the amino acid 55 to 452 of IRF3 (42) and cloned into pCDNA5/FRT/ΔCAT as described above. The identity of each cDNA clone was verified by nucleotide sequence analysis.

**Cell transfection.** HepG2 and Huh7 cells were seeded into 35mm diameter collagen coated dishes at a density of 1.2 x 10^6 cells per dish in antibiotics-free complete DMEM/F12 medium. After 6 hours, each well was transfected with a total of 4 µg plasmid with Lipofectamine 2000 (Invitrogen) by following the manufacturer’s directions. Transfected cells were harvested at the indicated times. The levels of HBV mRNAs and core associated DNA were determined by Northern and Southern blot hybridization, respectively.

**Nucleic acid analysis.** Intracellular viral core DNA was extracted as described previously(18). Briefly, cells from one 35mm diameter dish were lysed with 0.5 ml of lysis buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% NP40 (0.1% for Huh7 cells) and 2% sucrose at 37°C for 10 minutes. Cell debris and nuclei were removed by centrifugation and the supernatant was mixed with 130 µl of 35% PEG-8000 containing 1.5 M NaCl. After 1 hour incubation in ice, viral nucleocapsids were pelleted by centrifugation at 12,000 g for 10 min at 4°C, followed by 1 hour digestion at 37°C in 400 µl of digestion buffer containing 0.5 mg/ml pronase (Calbiochem), 0.5% SDS, 150 mM NaCl, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA. The digestion mixture was extracted twice with phenol and DNA was precipitated with ethanol,
dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). One third of the DNA sample from each plate was resolved by electrophoresis into a 1.5% agarose gel. Gel was then subjected to denaturalization in a solution containing 0.5 M NaOH and 1.5 M NaCl, followed by neutralization in a buffer containing 1 M Tris-HCl (pH7.4) and 1.5M NaCl. DNA was then blotted onto Hybond-XL membrane (GE Healthcare) in 20X SSC buffer.

Total cellular RNA was extracted with TRIzol reagents (Invitrogen), by following the instructions of the manufacturer. Ten microgram of total RNA was resolved in 1.5% agarose gel containing 2.2 M formdelhyde and transferred onto Hybond-XL membrane in 20X SSC buffer.

For the detection of HBV DNA and RNA, membranes were probed with either an α-32P-UTP (800 Ci/mmol, Perkin Elmer) labeled minus or plus strand specific full-length HBV riboprobe. Hybridization was carried out in 5 ml EKONO hybridization buffer (Genotech) with 1 hour pre-hybridization at 65°C and overnight hybridization at 65°C followed by a 1 hour wash with 0.1X SSC and 0.1% SDS at 65°C. The membrane was exposed to a phosphoimager screen and hybridization signals were quantified with QuantityOne software (Bio-Rad).

**Western blot analysis.** Transfected cells in a 35-mm diameter dish were washed once with PBS buffer and lysed in 200 µl of 1X Lamini buffer. Twenty microliters of the cell lysate was resolved on a sodium dodecyl sulfate-10% polyacrylamide gel and transferred onto PVDF membrane (Bio-Rad). The membrane was blocked with PBS containing 0.1% Tween 20 and 5% nonfat dry milk and probed with antibodies against Flag tag (Sigma), HA tag (Clontech), TRIF (Invivogen) and β-actin (Chemicon International). Bound antibodies were revealed by HPR-labeled secondary antibodies and visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) according to the protocol of the manufacturer.
RT PCR. For semi-quantitative detection of IFN-β, IFN-λ1, ISG56, MxA and β-actin mRNA, total cellular RNA was extracted with TRIzol and cDNA was synthesized with oligo-(dT)\textsubscript{12-18} primer and Superscript III DNA polymerase (Invitrogen) by following the manufacturer's direction. The PCRs were carried out in a 25-µl reaction mixture with the Advantage cDNA PCR kit (Clontech). The PCR annealing temperatures selected varied depending on the primers selected for amplification. The prime sequences are available upon request.

**NFκB DNA binding activity assay.** Cell nuclear extracts were obtained by using Nuclear Extract kit (Active Motif), the protein concentration was determined by BCA assay (Pierce). Two and half micrograms of nuclear extract were subjected to NFκB p65 DNA binding ELISA assay (Active Motif) according to the manufacturer’s protocol.

**RESULTS**

**Activation of the host cellular innate immune response inhibits HBV replication in HepG2 and Huh7 cells.** Study of HBV and host cell interactions is hampered by the lack of cell culture systems for efficient viral infection. As an alternative, HBV genome replication can be initiated by transfection of HBV DNA that is able to transcribe pg RNA into a handful of human hepatocyte-derived tumor cell lines, such as HepG2 and Huh7 cells. However, as for many tumor-derived cell lines, the two cell lines are not completely competent in innate immune response. For example, neither HepG2 nor Huh7 cells express TLR3 receptors and thus, do not respond to the treatment with double stranded (ds) RNA (41). Moreover, activation of RIG-I and/or MDA5-mediated cellular innate immunity in cultured cells is usually achieved by
transfection of their cognate ligands, such as 5’-triphosphate RNA and dsRNA, respectively. However, cytoplasm-delivered dsRNA could potentially interact with other cellular dsRNA binding proteins, such as double stranded RNA dependent protein kinase (PKR) and adenosine deaminase act on RNA 1 (ADAR 1), and might directly affect viral replication (14, 54). To overcome these limitations and avoid the confounding effects of RNA transfection, the viral PRR-mediated host cellular innate immune responses in hepatocyte-derived cells were activated by over-expression of the three major adaptor proteins for TLRs (TRIF and MyD88) and RIG-I/MDA5 (IPS-1) (Fig. 1), which efficiently activate cellular responses similar to that initiated by binding of nucleic acid ligands to their cognate PRRs(12, 59). Because overexpression of full-length TRIF induces apoptosis, we utilized in this study a mutant or super-activated (sa) TRIF that lacks the C-terminal 68aa, but activates promoters containing NFκB and ISRE motifs at levels comparable to wild-type TRIF, without induction of apoptosis (36). In addition, to independently evaluate the antiviral effects of RIG-I and MDA5-pathways, the innate immune responses mediated by the two PRRs were activated by over-expression of either the full-length RIG-I and MDA5 protein or the CARD domains derived from the two receptors(78).

Accordingly, HepG2 cells were cotransfected with a 1.3mer HBV genome and control vector or plasmids expressing full-length RIG-I or MDA5, the CARD domains of RIG-I and MDA5 (designated as RN230 and MN300, respectively), and the adaptor proteins MyD88, saTRIF and IPS-1. As a control, one set of HBV genome transfected cells were treated with 500 IU/ml IFN-α that displays almost a maximum antiviral effect of the cytokine against HBV (data not shown). Cells were harvested four days after transfection. Intracellular HBV mRNA and core-associated HBV DNA replicative intermediates were analyzed by Northern and Southern blot hybridization, respectively. Expression of desired proteins in transfected cells was
confirmed by Western blot analyses (Fig. 2A, lower panel). The results demonstrated that except for cells co-transfected with plasmid expressing full-length RIG-I, the steady-state levels of HBV mRNAs were reduced in all other cells that were treated with IFN-α or that over-expressed full-length MDA5, RN230, MN300, or each of the three adaptors (Fig. 2A, upper panel, also see the left panel of Fig. 2C for quantitative comparison). Interestingly, the levels of core-associated HBV DNA were decreased in proportion of HBV mRNA reductions in the cells under the corresponding treatment conditions (Fig. 2A, middle panel, and comparing the left two panels of Fig. 2C). These results suggested that the primary effect of the antiviral responses either induced by IFN-α treatment or elicited by overexpression of PRR adaptors and CARDs of RIG-I and MDA5 was to down-regulate viral RNAs. However, the possibility that the innate immune responses also inhibited additional steps in HBV nucleocapsid assembly and DNA replication could not be ruled out. Similarly, a strong inhibition on HBV replication, as demonstrated by the reduced levels of both HBV mRNA and DNA, were observed in Huh7 cells cotransfected with plasmids expressing each of the three PRR adaptors (Fig. 2B and right two panels of Fig. 2C). Furthermore, no evidence of cytotoxicity was observed for any experimental set, as determined by microscopic observation and quantitatively measured by cotransfection of cells with plasmids expressing green fluorescent protein (GFP) and individual adaptors or control vector. Results from the later experiment demonstrated that the percentage of GFP-positive cells in cultures cotransfected with plasmids expressing GFP and each of the three adaptor proteins did not differ from that in cells cotransfected with control vector and GFP-expressing plasmid (data not shown).

Taking together, the above results clearly demonstrated that over-expression of saTRIF and IPS-1 induced a strong antiviral response that non-cytopathically inhibited HBV replication.
in both HepG2 and Huh7 cells. In agreement with a previous report by Xiong and colleagues, overexpression of MyD88 also inhibited HBV replication (76), albeit to a lesser extent, compared with the other two adaptors (Fig. 2C). Moreover, activation of either RIG-I or MDA5 pathway by overexpression of their respective CARDs in HepG2 cells induced a robust antiviral response against HBV (Fig. 2A, Lanes 4 and 6). In corroboration with their ability to activate innate immune response upon overexpression (53), only full-length MDA5, but not RIG-I, was able to inhibit HBV replication in HepG2 cells (Fig. 2, Lanes 3 and 5). This is most possibly due to the distinct structural features of the two PRRs. RIG-I contains a C-terminal repressor domain which intra-molecularly interacts with the CARDs to prevent its auto-activation. Binding of its agonists induces RIG-I multimerization and conformation change that relieves auto-repression and exposes the CARDs. On the contrary, MDA5 lacks such an internal repressor domain and, consequently, induces innate immune response upon over-expression (53, 78).

PRR-mediated innate immune response reduces the steady-state levels of viral RNAs via post-transcriptional mechanisms. To determine if the observed reduction of HBV RNAs is due to transcriptional inhibition or post-transcriptionally accelerated decay of viral RNA, we intended to distinguish if the adaptor-induced reduction of HBV mRNAs was a HBV promoter-dependent or HBV RNA sequence-dependent phenomenon. To this end, plasmids expressing each of the three PRR adaptors or control vector were cotransfected into HepG2 cells with either pCMVHBV in which the HBV pgRNA transcription is under the control of a cytomegalovirus immediate early (CMV-IE) promoter or, plasmid pS in which the 2.1kb HBV subgenomic mRNA is transcribed under the direction of a SV40 early promoter (illustrated in Fig. 3A). Cells were harvested 2 days post transfection and the levels of HBV mRNA were
determined by Northern blot assay. As shown in Fig. 3B and C, the levels of both HBV pgRNA in pCMVHBV transfected cells and 2.1kb mRNA in pS transfected cells were dramatically reduced upon the overexpression of saTRIF and IPS-1, but to a lesser extent in MyD88 overexpressing cells. These results essentially corroborated with the observations obtained in pHBV1.3 transfected cells as shown in Fig. 2A and thus suggested that the innate immunity elicited by the PRR adaptors either inhibited the transcriptional activity of the CMV and SV40 promoters or post-transcriptionally reduced the levels of HBV RNAs in a HBV RNA sequence-dependent manner.

To investigate these two possibilities, plasmid pcHBs-V5 that transcribed a 1.0 kb truncated HBV mRNA spanning only the coding region of HBsAg under the control of a CMV-IE promoter and 0.8 kb neomycin phosphotransferase II (NTP-II) mRNA under the direction of a SV40 early promoter (illustrated in Fig. 3A) were cotransfected into HepG2 cells with the control vector or plasmids expressing each of the three adaptors. As shown in Fig. 3D, the levels of both HBsAg and NTP-II mRNAs in HepG2 cells were not significantly affected by any of the three adaptors. These results thus suggest that the reduction of HBV mRNAs by the three adaptors in HepG2 cells is most likely due to post-transcriptional decay of viral RNAs. Interestingly, because HBV pgRNA and 2.1kb mRNA (Fig. 3B and C), but not the 1.0kb truncated HBsAg mRNA (Fig. 3D), are efficiently reduced in HepG2 cells that over-express the PRR adaptors, it is reasonable to conclude that RNA sequence element(s) located in the 3’ overlapping region of the three HBV mRNA species may be critical for the selective reduction of viral RNAs.
Analysis of cellular pathways activated by overexpression of the adaptors. As illustrated in Fig. 1, recognition of PAMPs by TLRs stimulates the recruitment of a set of intracellular TIR-domain-containing adaptors which include MyD88 and TRIF. MyD88 is a universal adaptor for all TLRs with exception of TLR3 and activates MAP kinases (MAPKs) (ERK, JNK and p38) and transcription factor NFκB to control the expression of inflammatory cytokine genes in many types of cells. TRIF is recruited by TLR3 and TLR4 to activate MAPKs, NFκB and transcription factor IRF3, all of which are required for activation of IFN-β and IFN-λ1 genes(47). Similarly, IPS-1 mediates RIG-I and MDA5 signaling to activate multiple intracellular signaling pathways including MAPKs, NFκB and IRF3, which culminate to induction of IFNs and other cellular genes(59). To determine if the proper cellular responses are activated by the expression of the adaptors in the hepatoma cell lines under the condition of HBV replication, the DNA binding activity of NFκB and induction of IFN-β, IFN-λ1, ISG56 and MxA mRNAs were determined in the HBV genome-transfected HepG2 and Huh7 cells. As shown in Fig.4, the results reveal the following observations. First, as expected, all three adaptors activate NFκB, as measured by p65 DNA binding activity with a commercial ELISA kit (Active Motif). Moreover, the activation can be efficiently inhibited by a dominant negative IκBα, which bears N-terminal 39aa deletion and thus, cannot be phosphorylated by IKK-α/β (Fig. 4A) (67). Second, based on the induction of ISG56 (Fig. 4B), which depends on activation of IRF3, but not NFκB (11, 15), it can be deduced that only IPS-1 and saTRIF, but not MyD88, efficiently activated IRF3. Third, mRNAs for both IFN-β and IFN-λ1 were induced by IPS-1 and saTRIF, but not MyD88. Consistently, MxA gene expression, which relies on JAK-STAT signaling pathway, was only induced in cells that IFNs were induced. Finally, as expected, the constitutively active form of IRF3 (saIRF3), generated by the substitution of the Ser-Thr
residues in the carboxyl terminus with the phosphomimetic Asp(42), potently induced ISG56 and IFNs, and as a consequence, the MxA gene is also induced(28). In summary, these results suggest that overexpression of the three PRR adaptors elicited the authentic innate immune responses in human hepatocyte-derived cells, essentially as observed in other cell types (78).

**Inhibition of HBV replication by PRR-mediated innate immune response is an intracellular event.** It has been shown that, besides the secretion of IFNs, chemokines and other pro-inflammatory cytokines, the PRRs-activated cellular innate immune response could also induce the expression of several ISGs and other cellular genes that might directly inhibit viral infections(57, 58). Moreover, although both IFN-α and IFN-λ inhibited HBV replication in Huh7 and HepG2 cells, the antiviral potency of the cytokines was not as great as that of the PRR adaptor-elicited innate immunity (Fig. 2A). It is, therefore, interesting to know whether the observed antiviral effects elicited by the adaptors depend on the secreted cytokines or intracellular proteins.

To investigate these two possibilities, cultured media were harvested between 24 to 96 h post transfection from HepG2 or Huh7 cells transfected with plasmids expressing each of the three adaptors or control plasmid and applied onto pHBV1.3 transfected HepG2 and Huh7 cells, respectively. As controls, the pHBV1.3 transfected cells were left untreated or treated with 500IU/ml IFN-α or 400ng/ml IFN-λ1. As shown in Fig. 5, in agreement with the results shown above, IFN-α and IFN-λ1 modestly reduced the levels of HBV RNAs in both HepG2 and Huh7 cells (Fig. 5, lanes 2 and 3). However, it is surprising that the viral RNA levels were not affected or reduced less than 20% in HepG2 and Huh7 cells treated with conditioned medium harvested from the cells transfected with plasmid expressing TRIF, MyD88 or saIRF3 (Fig. 5, lanes 6 to 8).
On the contrary, treatment of cells with the conditioned medium harvested from the IPS-1-overexpressed cells resulted in a reduction of HBV mRNA levels in Huh7 cells by approximately 33%, which is similar with that observed in Huh7 cells treated with either IFN-α or IFN-λ (Fig. 5, lane 5), but apparently at a lesser extent in comparison with that observed in cells transfected with IPS-1-expressing plasmid, which reduced the level of viral RNA by 80% (Fig. 2B, lane 3 and Fig. 2C).

Hence, these results suggest that the observed strong inhibition of HBV replication by the three PRR adaptors is predominantly mediated by inducible intracellular factor(s), rather than secreted cytokines. It will be interesting to determine the intracellular signaling pathways that mediated the antiviral responses elicited by the PRR adaptors, which will ultimately lead to the identification of cellular factors that decrease the levels of HBV RNAs.

**Role of MAP kinase-mediated signaling in the inhibition of HBV replication.** As mentioned in a previous section, MAP kinases, including ERK, JNK and p38, are activated essentially in all the PRR-mediated innate immune responses and play pivotal roles in the induction of pro-inflammatory cytokines (7, 80). To examine if any of the three major MAP kinase-mediated signal transduction pathways was required for the inhibition of HBV replication by the three adaptors, HepG2 cells were cotransfected with pHBV1.3 and plasmids expressing each of the three adaptors and control plasmid. Twelve hours after the transfection, cells were left untreated or treated with 25 μM SB202190, 10 μM U0126, or 25 μM SP600125, selective inhibitors of p38 MAPK, ERK or JNK, respectively. The concentrations of the kinase inhibitors used in this study were adopted from previous reports demonstrating that the desired kinases were inhibited in HepG2 cells under these conditions (23, 75, 81). The cells were harvested at
day 4 after transfection and the levels of HBV RNAs were measured by Northern blot hybridization. Consistent with results presented in Fig. 2, cotransfection of pHBV1.3 with plasmid expressing IPS-1, saTRIF or MyD88 reduced the levels of HBV RNA (Fig. 6A, lanes 1 to 4). Interestingly, treatment of HBV genome-transfected cells with p38, ERK or JNK inhibitors in the absence of PRR adaptor overexpression reduced the level of HBV pgRNA by approximately 30, 70 and 60%, respectively (Fig. 6, comparing lane 1 to lanes 5, 9 and 13, respectively). This result implied that the basal level activities of the three MAPKs might be required for the maximum transcription of HBV mRNAs in HepG2 cells. However, as shown in Fig. 6B, overexpression of the three adaptors resulted in similar extents of HBV RNA reductions in the absence or presence of the MAPK inhibitors and thus suggested that none of the inhibitors is able to attenuate the inhibitory effects of the three adaptors on HBV replication.

**Role of NFκB and IRF3-mediated signaling in the inhibition of HBV replication.** In addition to the MAPKs, NFκB and IRF3 are two key transcription factors for PRR-mediated induction of pro-inflammatory cytokines, IFNs and many other cellular genes(29). To determine their roles in the antiviral responses elicited by the three PRR adaptors, the activation of NFκB and the function of activated IRF3 can be selectively inhibited by the well-characterized dominant-negative (DN)-IκB-α and IRF3, respectively(28). To provide a basis for further pathway analysis, we first examined the effects of disrupting the NFκB and IRF3-mediated signaling pathways on HBV replication without overexpression of PRR adaptors. As shown in Fig. 7, the results demonstrated that expression of either wild-type or dominant-negative IκB and IRF3 did not affect the levels of HBV RNAs in the two cell lines. However, it is interesting that over-expression of saIRF3 activated a similar innate immune response (Fig. 4B, comparing lanes
7 and 14) and induced ISG56 expression (Fig. 7, lane 3) in both HepG2 and Huh7 cells, but only dramatically reduced levels of HBV RNA and DNA in Huh7 cells (Fig. 7A and B, lane 3, and data not shown). The reason for the cell line specific antiviral response induced by saIRF3 is currently not known.

We next investigated the role of the two transcription factor-mediated signaling pathways in the PRR adaptor-elicited innate immunity against HBV. HepG2 and Huh7 cells were either left untreated or transfected with control plasmid or cotransfected with pHBV1.3 and plasmids expressing each of the three adaptors and plasmids expressing the dominant negative IRF3 and/or IκB-α. Cells were harvested at 4 days after transfection and the levels of viral RNAs were determined by Northern blot hybridization analysis. Induction of ISG56 mRNA (Fig. 8A and B, middle panel) served as an indicator for IRF3 activation in the transfected cells. The results from these experiments (Fig. 8A and B) revealed the following observations. First, as shown in Fig. 2, TRIF and IPS-1 induced a strong reduction of viral mRNAs (Fig. 8A and B, top panel) in both HepG2 and Huh7 cells, but the antiviral effects of MyD88 are less potent (Fig. 8A and B, comparing lane 3 with lanes 4 to 6). Second, it was DN-IκBα, but not DN-IRF3, that restored the levels of HBV RNA in the two cell lines transfected with MyD88 (Fig. 8A and B, comparing lanes 6, 9 and 12). Third, although ISG56 mRNA was induced by IPS-1 and TRIF in both HepG2 and Huh7 cells, and as expected, the induction was inhibited by expression of DN-IRF3, the levels of HBV mRNAs were only restored by DN-IRF3 in Huh7, but not HepG2 cells that overexpressed IPS-1(Fig. 8A and B, comparing lanes 4 and 7). On the contrary, cotransfection of plasmid expressing DN-IκBα efficiently attenuated the antiviral effects of IPS-1 in both HepG2 and Huh7 cells (Fig. 8A and B, comparing lanes 4 and 10). Finally, TRIF induced antiviral response can only be partially attenuated by expression of DN-IκBα in the two cell lines, but
expression of DN-IRF3 had no effect (Fig. 8A and B, comparing lanes 5, 8 and 11), indicating that additional unknown signaling pathway(s) are required to mediate TRIF-induced antiviral response against HBV in human hepatoma cells.

Taken together, the results presented in this section suggest that NFκB is required for all three adaptors to elicit antiviral response in both HepG2 and Huh7 cells. However, despite it was efficiently activated by TRIF or IPS-1 in both HepG2 and Huh7 cell, as indicated by ISG56 mRNA induction and inhibition by DN-IRF3, the IRF3 is only essential for induction of an antiviral response by IPS-1 in Huh7 cells.

DISCUSSION

Our work presented in this report shows that either IFN treatment or over-expression of the adaptors that mediate the signaling of the major viral PRRs non-cytopathically inhibited HBV replication in human hepatocyte-derived cell lines (Fig. 2). Moreover, three lines of evidence support the notion that the PRR adaptor-elicited antiviral response was mediated by intracellular factors, rather than secreted cytokines. First, the adaptor-induced antiviral response was far more potent than that induced by high concentrations of IFN-α or IFN-λ (Fig. 2 and Fig. 5). Second, treatment of HBV genome-transfected HepG2 and Huh7 cells with culture medium harvested from the adaptor-expressing plasmid-transfected HepG2 and Huh7, respectively, did not significantly inhibit HBV replication (Fig. 5). Finally, analyses of the involved signal transduction pathways in the adaptor-induced antiviral response suggested that activation of IRF3 is only essential for induction of antiviral response by IPS-1 in Huh7 cells, but not essential for all three adaptors to induce antiviral response in HepG2 cells (Fig. 8). It is well-known that
IRF3 is essential for the induction of type I and type III IFNs by both IPS-I and TRIF. Hence, IFNs should not be the mediators of adaptor-induced antiviral response in HepG2 cells (Fig. 8). In addition, it is worth noting that the primary effect of either IFNs or host cellular innate immunity elicited by the PRR adaptors on HBV replication in HepG2 and Huh7 cells is to reduce the steady-state levels of viral RNAs, but not to inhibit viral DNA replication (Figs. 2 and 3).

Previous studies with HBV transgenic mice or duck hepatitis B virus (DHBV) infected primary duck hepatocytes indicated that there are several distinct intracellular mechanisms that can be activated by innate and/or adaptive immune responses to limit hepadnavirus replication. First, it was convincingly demonstrated that IFN-α/β treatment of HBV replicating mouse hepatocytes or DHBV-replicating primary duck hepatocytes and chicken hepatoma cells does not affect the level of viral RNA, but selectively inhibits the formation of pgRNA containing nucleocapsids. As a consequence, viral DNA replication is inhibited in the cells treated with the cytokines (21, 56, 74). Second, it was shown that adoptive transfer of HBV surface antigen (HBsAg)-specific cytotoxic T lymphocytes (CTLs) or injection of IL-2 resulted in a post-transcriptional down-regulation of viral mRNA in IFN-γ and tumor necrosis factor alpha (TNF-α)-dependent mechanisms (17, 68). Third, acute infection of LCMV or repeated injection of IFN-α/β inducer polyI:C into HBV transgenic mice inhibited HBV RNA transcription (70). Finally, a single intravenous injection of ligands specific for TLR3, TLR4, TLR5, TLR7 and TLR9 into HBV transgenic mice noncytolytically inhibited HBV replication within 24 h in an IFN-α/β-dependent manner (32). Thus far, despite several reports suggesting that IFN treatment of human hepatocytes-derived cells inhibited HBV RNA transcription or post-transcriptionally reduced viral mRNA levels, the inhibition of pgRNA-containing capsid formation by IFNs or other cytokines had not yet been convincingly demonstrated in any of human cell lines (52, 69).
Hence, it is possible that the molecular mechanism by which the innate immunity controls HBV replication is species-specific.

Analysis of signaling pathways required for each of adaptors to elicit antiviral responses against HBV revealed that activation of NFκB is essential for all three adaptors to elicit antiviral response in both HepG2 and Huh7 cells. However, activation of IRF3 is only essential for induction of antiviral response by IPS-1 in Huh7 cells, but not HepG2 cells. It is paradoxical that despite the fact that expression of ISG56, IFN-β and IFN-λ1 was as efficiently induced in HepG2 cells as in Huh7 cells (Fig. 4B, comparing lanes 7 and 14), salRF3 only elicited robust antiviral response in Huh7 cells (Fig. 7A and B, lane 3). Consistent with this observation, the IRF3 is only essential for induction of an antiviral response by IPS-1 in Huh7 cells, but not in HepG2 cells. These findings suggest that IPS-1 might down-regulate the levels of HBV mRNA via distinct mechanisms in HepG2 and Huh7 cells (Figs. 7 and 8). Moreover, our results also suggest that additional signaling pathway(s) other than MAPKs, NFκB and IRF3 are required for TRIF to induce a maximum antiviral effect against HBV.

In our effort to determine if the observed reduction of HBV RNAs upon overexpression of the three adaptors is via the transcriptional and/or post-transcriptional mechanisms, we found that both HBV pgRNA and 2.1kb subgenomic RNA can be efficiently reduced by the three adaptors in a HBV promoter-independent manner. However, the level of a 1.0kb truncated HBV mRNA that spans only the coding region of HBV small envelope protein (HBsAg) was not affected by the three adaptors in HepG2 cells (Fig. 3). These results suggest that the reduction of HBV mRNAs by all three adaptors in HepG2 cells is most likely due to post-transcriptional decay of viral RNAs and RNA sequence element(s) located in the 3’ overlapping region of HBV mRNAs is critical for the selective reduction of viral RNA. Although it is not yet known whether
the same or distinct effector molecules are induced by the different PRR adaptors in HepG2 cells to down-regulate HBV RNA, microRNAs (miRNAs) and/or lupus associated antigen (La) are two cellular factors that might possibly accelerate the post-transcriptional decay of viral RNAs and thus, deserve a serious consideration in future studies.

MicroRNAs are small non-coding RNAs of approximately 22 nucleotides in length. They are derived from cellular transcripts and sequence-specifically bind to their targeting mRNAs to result in either mRNA cleavage or translational repression (3, 4, 33). MiRNAs have been demonstrated to play an important role in defending the hosts against virus infection in plants and invertebrates (10), but it has been argued that in mammals, this sequence-specific innate antiviral immunity has been replaced by a sequence-nonspecific, double-stranded RNA-triggered, interferon-mediated innate host defense mechanism (9, 49). However, recent studies suggest that cellular miRNAs can be induced by TLR signaling and IFN treatment to regulate innate immune signal transduction or even directly inhibit the replication of HCV (48) (46, 64) (63). It is, therefore, entirely possible that certain cellular miRNAs could be induced by the innate immune response and directly catalyze viral RNA decay or indirectly modulate cellular RNA decay enzyme expression.

Alternatively, as mentioned above, selective decay of HBV mRNA has been previously observed in the livers of HBV transgenic mice injected with HBsAg-specific CTLs or cytokines such as IL-2. It was shown that the process is mediated by TNF-α and IFN-γ (17, 68). In an attempt to identify host factors involved in degradation of the viral RNA, the mouse La protein (mLa) was identified as an HBV RNA-specific binding protein, which presumably binds and stabilizes HBV RNA in the nuclei of hepatocytes (25). It was found that the CTLs or IL-2 treatment resulted in the processing of full-length mLa, which render viral RNA vulnerable for
degradation by cellular nucleases. Moreover, the mLα binding site was mapped to a predicted stem-loop structure within an element 91 nucleotides long located at the 5’-end of the post-transcriptional regulatory element of HBV(16, 26). Interestingly, our results presented in Fig. 3 demonstrated that the nucleotide sequence in the non-translational region of HBV 2.1kb mRNA, where the mLα binding site is located, is critical for HBV RNA decay induced by the three PRR adaptors. Hence, it will be interesting to determine if the adaptors induce the processing of human La antigen in HepG2 and Huh7 cells and examine the role of human La in HBV RNA decay in the innate immune response elicited by each of the three adaptors.

In summary, although it is not yet known how the PRR adaptors-elicited cellular innate immunity down-regulates the levels of HBV RNAs, the results presented clearly indicate the existence of an intricate antiviral mechanism in human hepatocytes that could be activated under certain conditions and potently inhibit HBV replication in a non-cytopathic fashion. Noncytopathic inhibition of viral gene expression is an attractive therapeutic means of controlling infections, especially in the case of HBV where the 3.5-kb RNA serves as the template for reverse transcription of the encapsidated viral DNA genome. Further understanding the nature of these antiviral mechanisms should provide valuable insights into novel strategies for the development of antivirals that evoke the antiviral response to eliminate HBV infection.
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REFERENCES


FIGURE LEGENDS

Fig. 1. Schematic representation of the major viral pattern recognition receptor-mediated signaling pathways. The three PRR adaptors and their relationship with the two downstream transcription factors (NFκB and IRF3) and the activated target genes examined in this study are illustrated. See text for the detailed discussion.

Fig. 2. Over-expression of the CARD domain of RIG-I or MDA5, and three adaptor proteins IPS-1, TRIF and MyD88 potently inhibits HBV replication in human hepatocyte-derived tumor cell lines. (A) A plasmid that encodes pgRNA of wild-type HBV (pHBV1.3) was co-transfected with control plasmid (lane 2) or plasmids that express RIG-I (lane 4), CARD of RIG-I (RN230, lane 5), MDA5 (lane 6), CARD of MDA5 (MN300, lane 7), IPS-1 (lane 8), TRIF (lane 9) or MyD88 (lane 10) into HepG2 cells. In these transfection experiments, 2 µg of pHBV1.3 was cotransfected with 2 µg of control plasmid or 1 µg of control plasmid plus 1 µg of plasmid expressing proteins under investigation into a 35mm dish of cells. Lane 1 was loaded with sample derived from pHBV1.3 and control plasmid cotransfected cells and lane 2 is loaded with samples derived from pHBV1.3 and control plasmid cotransfected cells treated with 500 IU/ml IFN-α which is added at 12 hrs after transfection. Cells were harvested at 4 day after transfection and the levels of viral RNAs and core-associated DNA were determined by Northern (upper panel) and Southern (middle panel) blot hybridization analyses, respectively. For RNA analysis, each lane was loaded with 10 µg of total RNA. Ribosomal RNAs (28S and 18S) were presented as loading controls. The positions of HBV 3.5 kb, 2.4 kb, and 2.1 kb RNAs are indicated. For DNA analysis, HBV core DNA was probed with a genome-length, minus strand
specific HBV riboprobe. The positions of relaxed circular (RC), single-stranded (SS) DNAs are indicated. Proper expression of intended proteins by the transfected plasmids was confirmed by Western blot analysis with antibodies described in Materials and Methods and the levels of β-actin serve as loading controls (Lower panel). (B) Huh7 cells were cotransfected with 2 µg of pHBV1.3 and 2 µg of control plasmid (lane 1 and 2) or 1 µg of control plasmid plus 1 µg of plasmid expressing IPS-1 (lane 3), TRIF (lane 4) and MyD88 (lane 5). One set of the control plasmid transfected cells (lane 2) was treated with 500 IU/ml IFN-α at 12 hrs after transfection. Cells were harvested at 4 day after transfection and the levels of viral RNAs and core-associated DNA were determined as described above. (C) Levels of HBV mRNAs and DNA in (A) and (B) were determined by a phosphoimager with QuantityOne software (Bio-Rad) and plotted as the percentage of the RNA or DNA levels in control cells that were cotransfected with pHBV1.3 and vector plasmids (lane 1).

Fig. 3. Reduction of HBV RNA levels induced by the innate immune responses does not depend on the nature of the promoters. (A) Schematic representation of the RNA species and the corresponding promoter that directs the RNA transcription in the plasmids used in this study. The numbers indicate the HBV DNA sequence with 1 at the unique EcoRI site of HBV genome. (B) HepG2 cells were cotransfected with 2 µg of pCMV-HBV and 2 µg of control plasmid (lanes 1 to 3) or 1 µg of control plasmid plus 1 µg of plasmid expressing IPS-1 (lane 4), TRIF (lane 5) and MyD88 (lane 6). The control plasmid cotransfected cells were left untreated (lane 1) or treated with 500 IU/ml IFN-α (lane 2) and 400 ng/ml IFN-λ1 (lane 3), respectively. Cells were harvested at 4 day after transfection and the levels of viral RNAs were determined by Northern blot hybridization. Ribosomal RNAs (28S and 18S) were presented as loading controls. The
positions of HBV 3.5kb, 2.4kb, and 2.1kb RNAs are indicated. (C) HepG2 cells were cotransfected with 2 µg of pS and 2 µg of control plasmid (lane 1) or 1 µg of control plasmid plus 1 µg of plasmid expressing IPS-1 (lane 2), TRIF (lane 3) and MyD88 (lane 4), respectively. Cells were harvested at 2 day after transfection and the levels of HBV 2.1 kb mRNA were determined by Northern blot hybridization. Ribosomal RNAs (28S and 18S) were presented as loading controls. (D) HepG2 cells were cotransfected with 2 µg of pcHBs-V5 and 2 µg of control plasmid (lane 1) or 1 µg of control plasmid plus 1 µg of plasmid expressing IPS-1 (lane 2), TRIF (lane 3) and MyD88 (lane 4), respectively. Cells were harvested at 2 day after transfection and the levels of 1 kb HBV mRNA and 0.8 kb neomycin phosphotransferase II (NTP-II) mRNA were determined by Northern blot hybridization. Ribosomal RNAs (28S and 18S) were presented as loading controls. Relative RNA level in each sample is expressed as the percentage of RNA level in control cells cotransfected with vector plasmid (lane 1 of Fig. 3B, C and D), and is presented underneath each of the blots.

**Fig. 4. Expression of the three adaptor proteins activates desired intracellular signaling pathways.** (A) NFκB DNA binding assay. Huh7 cells were untransfected or transfected with indicated plasmids. 2 days later, 2.5µg of the nuclear extract were subjected to the p65 DNA binding ELISA assay following the manufacturer’s direction. (B) HepG2 (lanes 1 to 7) or Huh7 (lanes 8 to 14) cells were cotransfected with 2 µg of pHBV1.3 and 2 µg of control plasmid (lanes 1 to 3 and 8 to 10) or 1 µg of control plasmid plus 1 µg of plasmid expressing IPS-1 (lanes 4 and 11), TRIF (lanes 5 and 12), MyD88 (lanes 6 and 13) and constitutively active IRF3 (saIRF3, lanes 7 and 14), respectively. Cells were harvested at 2 day after transfection and the levels of ISG56, IFN-β, IFN-λ1, MxA and β-actin mRNAs were determined by RT-PCR assay.
**Fig. 5. Antiviral effects induced by the innate immune response are intracellular events, but not mediated by secreted cytokines.** HepG2 (upper panel) and Huh7 (lower panel) cells were transfected with 1.6 µg of pHBV1.3 per well in 12-well plates. 12 hours after the transfection, culture media were replaced with fresh DMEM/F12 (lane 1), fresh DMEM/F12 containing 500 IU/ml IFN-α (lane 2) or 400 ng/ml IFN-λ1 (lane 3), or conditioned media prepared by combination of equal volumes of fresh DMEM/F12 with medium harvested from HepG2 and Huh7 cells transfected with control plasmid (lane 4) or plasmid expressing IPS-1 (lane 5), TRIF (lane 6), MyD88 (lane 7) or saIRF3 (lane 8). The transfected cells were cultured for additional 48 hours with same medium change at 24 hour under the condition described above and then harvested for total RNA extraction. Levels of HBV RNA were determined by Northern blot hybridization. Ribosomal RNAs (28S and 18S) were presented as loading controls. Relative RNA level in each sample is expressed as the percentage of RNA level in control cells cotransfected with pHBV1.3 and vector plasmid, but left untreated (lane 1), and is presented underneath each of the blots.

**Fig. 6. The three major MAPKs are not involved in the inhibition of HBV replication by the innate immune response.** (A) HepG2 cells were cotransfected with 2 µg of pHBV1.3 and 2 µg of control plasmid (lanes 1, 5, 9 and 13) or 1 µg of control plasmid plus 1 µg of plasmid expressing IPS-1 (lanes 2, 6, 10 and 14), TRIF (lanes 3, 7, 11 and 15) and MyD88 (lanes 4, 8, 12 and 16). Twelve hours after transfection, cells were left untreated (lane 1 to 4, group A) or treated with 25 µM MAPK p38 inhibitor SB202190 (lanes 5 to 8, Group B), 10 µM ERK inhibitor U0126 (lanes 9 to 12, Group C) or 25 µM JNK inhibitor SP600125 (lanes 13 to 16, ...
Group D). DMSO concentration in all experiment groups was normalized at 0.1%. Culture media and the inhibitor were replaced every other day and cells were harvested at day 4 after transfection. Total RNA was extracted and analyzed by Northern blot hybridization. Ribosomal RNAs served as loading control. RNA level in each sample is expressed as the percentage of RNA level in control cells cotransfected with pHBV1.3 and vector plasmid, but left untreated (lane 1). (B) RNA levels in the adaptor-expressing plasmids transfected cells were plotted as the percentage of that in cells cotransfected with pHBV1.3 and vector plasmid, and left untreated (lane 1) or treated with the indicated kinase inhibitors in the absence of adaptor protein overexpression (lanes 5, 9 or 13), respectively.

Fig. 7. Antiviral effects of IRF3 and NFKB-mediated signaling pathways in HepG2 and Huh7 cells. HepG2 (upper panel) or Huh7 (Lower panel) cells were cotransfected with 2 µg of pHBV1.3 and 2 µg of control plasmid (lane 1) or plasmid expressing wild-type IRF3 (lane 2), saIRF3 (lane 3), DN-IRF3 (lane 4), wild-type IκBα (lane 5), DN-IκBα (lane 6) or DN-IRF3 and DN-IκB-α in combination (lane 7). Cells were harvested at 4 day after transfection and the levels of viral RNAs were determined by Northern blot hybridization analysis. Induction of ISG56 mRNA serves as an indicator of IRF3 activation in transfected cells. Ribosomal RNAs (28S and 18S) were presented as loading controls. The positions of HBV 3.5kb, 2.4kb, and 2.1kb RNAs are indicated. Relative RNA level in each sample is expressed as the percentage of RNA level in control cells cotransfected with pHBV1.3 and vector plasmid (lane 1), and presented underneath each of the blots.

Fig. 8. Role of IRF3 and NFKB in mediating the antiviral response elicited by host cellular
innate immunity. HepG2 (A) or Huh7 (B) cells in a 35mm-diameter dish were either left untreated (lane 1), transfected with 4 μg of control plasmid (lane 2) or cotransfected with 4 μg of plasmids containing 2 μg pHBV1.3, 1 μg plasmid expressing each of the three adaptors and 1 μg plasmid expressing DN-IRF3 and/or DN-IκBα, plus 1 μg of control plasmid (omitted when DN-IRF3 and DN-IκBα were in combination). Cells were harvested at 4 day after transfection and the levels of viral RNAs were determined by Northern blot hybridization. Induction of ISG56 mRNA serves as an indicator of IRF3 activation in transfected cells. Ribosomal RNAs (28S and 18S) were presented as loading controls. The positions of HBV 3.5kb, 2.4kb, and 2.1kb RNAs are indicated. RNA level in each sample is expressed as the percentage of that in control cells cotransfected with pHBV1.3 and vector plasmid (lane 3), and is presented underneath the blots.
Fig. 2
Fig. 3
Fig. 5
Fig. 6

A

B
Fig. 7
**Fig. 8**

(A) pHBV1.3 +

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% of control:

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(B) pHBV1.3 +

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lane:

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