

Research Article

CK1 α upregulates the IFNAR1 expression to prompt the anti-HBV effect of type I IFN in hepatoma carcinoma cellsJing Xiong¹, Yanjun Jiang¹, Jinru Zhang, Yanmeng Chen, Yuan Hu*

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ABSTRACT

Casein kinase 1 α (CK1 α) mediates the phosphorylation and degradation of interferon- α/β receptor 1 (IFNAR1) in response to viral infection. However, how CK1 α regulates hepatitis B virus (HBV) replication and the anti-HBV effects of IFN- α are less reported. Here we show that CK1 α can interact with IFNAR1 in hepatoma carcinoma cells and increased the abundance of IFNAR1 by reducing the ubiquitination levels in the presence of HBV. Furthermore, CK1 α promotes the IFN- α triggered JAK-STAT signaling pathway and consequently enhances the antiviral effects of IFN- α against HBV replication. Our results collectively provide evidence that CK1 α positively regulates the anti-HBV activity of IFN- α in hepatoma carcinoma cells, which would be a promising therapeutic target to improve the effectiveness of IFN- α therapy to cure CHB.

1. Introduction

Hepatitis B virus (HBV) infection remains a severe global public health problem (Nguyen et al., 2020). Despite the availability of preventive vaccines, more than 240 million people worldwide still suffer from chronic hepatitis B infection (CHB), which is a high-risk factor for developing liver cirrhosis and hepatocellular carcinoma (HCC) (Poh et al., 2015).

Currently, there are two major antiviral therapies approved for the clinical CHB treatment, including nucleos(t)ide analogs and interferon-alpha (IFN- α) (Pietro Lampertico et al., 2017). The advantages of IFN- α therapy include a definite course of treatment, a relatively higher rate of HBV e antigen (HBeAg) seroconversion and a low recurrence rate after drug withdrawal (Konerman and Lok, 2016). However, IFN- α therapy responds in a limited number of CHB patients (Van Zonneveld et al., 2004). Therefore, it is important to investigate the cellular host factors that affect the antiviral activity of IFN- α .

IFN- α belongs to type I IFNs. In the type I IFN signaling pathways, IFN- α binds to its receptors (IFNAR1 and IFNAR2) and triggers intracellular Janus kinase/signal transducers and activators of transcription pathway (JAK/STAT) (Schreiber, 2017). The phosphorylated STAT1, STAT2 (pSTAT1/pSTAT2) and interferon regulatory factor 9 (IRF9) form IFN-stimulated gene factor 3 (ISGF3), which then activates the promotion

of interferon-stimulated response element (ISRE) and upregulates the expression of hundreds of interferon-stimulated genes (ISGs), such as APOBEC3G, SAMHD1, and MX1. Finally, these ISGs exert multiple antiviral functions against HBV replication in the HBV life cycle (Janahi and McGarvey, 2013; Sommer et al., 2016; Wang et al., 2020). Therefore, the levels of IFNAR1 are important to evaluate the effectiveness of IFN- α to control viral replication (Xia et al., 2018), and epigenetic modifying factor and enzyme were reported to mediate the phosphorylation or ubiquitination of IFNAR1, thus is crucial to regulate the antiviral function of IFN- α (Kumar et al., 2003; Zheng et al., 2011).

Casein kinase 1 α (CK1 α), a member of the CK1 protein family, is widely distributed on the cell membrane and nucleus. CK1 α has a serine/threonine-protein kinase activity, which plays an important role in maintaining the autophagy, cell cycle, cell metabolism and host defense response (Jiang et al., 2018). Recently, CK1 α has reported to phosphorylate IFNAR1 at Ser535 *in vitro* and mediate the phosphorylation-dependent degradation of IFNAR1 in response to endoplasmic reticulum (ER) stress and viral infection (Liu et al., 2009). Meanwhile, CK1 α promotes influenza A virus (IAV) replication by regulating the degradation of IFNAR1 and IFNAR2 caused by hemagglutinin of IAV (Xia et al., 2018), strengthening the importance of CK1 α to contribute to viral replication. However, it is unclear if CK1 α controls HBV replication or whether CK1 α regulates the antiviral function of IFN- α against HBV replication.

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Our study identifies that CK1 α interacts with IFNAR1 in HepG2-NTCP cells that supported HBV infection by LC-MS analysis. However, different from that in IAV infection, CK1 α increased the abundance of IFNAR1, leading to an enhancement of the IFN- α signaling pathway. As a result, CK1 α greatly improves the efficacy of IFN- α against HBV replication, while it does not directly regulate the HBV replication in hepatoma carcinoma cells, which could provide a new understanding of the molecular mechanism of CK1 α regulate the anti-HBV effect of IFN- α , and suggest CK1 α as an attractive therapeutic target for improving the effectiveness IFN- α therapy to cure CHB.

2. Materials and methods

2.1. Cell culture and transfection

Human embryo kidney 293T cells, human hepatoma Huh7 cells, and HepG2-NTCP cells were cultured in Dulbecco's modified Eagle's medium DMEM (Gibco, Beijing, China) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37 °C in a 5% CO₂ incubator. According to the manufacturer's instructions, cells were transfected with indicated plasmids or specific siRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

2.2. Chemical inhibitors, plasmids and siRNA

CK1 α inhibitor D4476 (S7642, Houston, USA) was purchased from Selleckchem, cycloheximide (CHX, M4879, Houston, USA) and proteasome inhibitor MG132 (M1902, Houston, USA) from AbMole, and recombinant human interferon- α 2a were purchased from MCE (HY-P7022, New Jersey, USA). The concentration of D4476, CHX or MG132 was 5 μ mol/L, 100 μ g/mL and 20 μ mol/L. IFN- α was used at the concentration of 1000 IU/mL. The human expression plasmids encoding HA-IFNAR1 (HG13222-CY) and Flag-CK1 α (HG30031-CF) were constructed by Sino Biological Company (Beijing, China). The mutant plasmid (HA-IFNAR1Ser535A) was constructed by PCR-based mutagenesis. The ISRE luciferase reporter plasmid was a kind gift from professor Yong Lin. Small interfering RNAs (siRNAs) targeting individual CK1 α were synthesized by the Shanghai Jima Company. All specific siRNA sequences are listed in [Supplementary Table S1](#).

2.3. Virus concentration and HBV infection

Virus production and HBV infection were conducted as described previously ([Chen et al., 2018](#)). Briefly, HBV inoculum was concentrated from filtered the supernatants of HepAD38 cells by 30% PEG8000 precipitation overnight, centrifuged at 3059 \times g for 30 min at 4 °C and dissolved by opti-MEM (Gibco, New York, USA). For HBV infection, HepG2-NTCP cells were infected with HBV particles at 1000 genome equivalents (GE) per cell in the presence of 4% PEG8000 for 24 h.

2.4. Co-IP and Western blot

The Co-IP was conducted as described previously ([Chen et al., 2020](#)). The cells were co-transfected with HA-tagged IFNAR1 and Flag-tagged CK1 α . After 48 h of transfection, the cells were lysed in RIPA lysis buffer, incubated with protein G agarose beads, then immunoprecipitated with anti-HA antibody or anti-Flag antibody at 4 °C overnight. The immunoprecipitated proteins were analyzed by Western blot with indicated antibodies. The following antibodies were used: CK1 α (1:500, 55192-1-AP, rabbit, Proteintech, Rosemont, IL, USA), IFNAR1 (1:500, ab45172, rabbit, Abcam, Cambridge, MA, USA), STAT1 (1:1000, 14994, rabbit, CST, Danvers, MA, USA), pSTAT1 (1: 1000, 7649, rabbit, CST), STAT2 (1:1000, 72604, rabbit, CST), p-STAT2 (1:1000, 88410, rabbit, CST), Flag-tag (1:1000, MA-1-91878, mouse, Thermo, San Jose, CA, USA), HA-tag (1:1000, 26183, mouse, Thermo), β -actin (1:3000, sc-47778, mouse, Santa Cruz, Dallas, TX, USA), and ubiquitin (1:500, AF0306,

rabbit, Beyotime, Shanghai, China). The mouse monoclonal antibody against HBV core protein (HBc) was a kind gift from Prof. Xuefei Cai in our lab. The density of the immunoblot band was analyzed by Image J (National Institutes of Health, Bethesda, MD, USA).

2.5. Mass spectrometry (MS) analysis

HepG2-NTCP cells were infected with the HBV particles, then HA-IFNAR1 or control vector was transfected in HepG2-NTCP cells, followed by IFN- α treatment for 24 h. After 48 h of transfection, cells were harvested and immunoprecipitated with an anti-HA monoclonal antibody as described above. SDS-PAGE separated the immunoprecipitated proteins, and the bands were excised and digested with trypsin for further LC-MS analysis by the Applied Protein Technology company (Shanghai, China).

2.6. CHX chase assay and ubiquitination assays

The half-life of IFNAR1 was determined by a CHX chase assay. Huh7 cells were transfected with Flag-CK1 α or empty plasmid, respectively. The cells were treated with CHX after transfection at the different times indicated in the figure, and then Western blot was conducted. To detect the ubiquitination levels of IFNAR1, Flag-CK1 α , HA-IFNAR1WT or HA-IFNAR1 Ser535A, and Myc-ubiquitin plasmids were co-transfected into HEK293T cells. After treatment with proteasome inhibitor MG132 with or without HBV, the cells were harvested and analyzed by Co-IP and Western blot.

2.7. Luciferase assay for ISRE promoter activity

Huh7 cells inoculated in 24-well plates were co-transfected with CK1 α expression plasmid along with ISRE luciferase reporter plasmid and Renilla luciferase reporter plasmid. At 24 h post-transfection, the cells were treated with IFN- α (1000 IU/mL) for 16 h and then harvested. According to the manufacturer's instruction, the luciferase activities were measured with the Dual-Luciferase Reporter Assay kit (E1910, Promega, Madison, USA).

2.8. RNA extraction and quantitative RT-PCR (qRT-PCR)

According to the manufacturer's instructions, total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the RNA was reverse transcribed into cDNA with Primescript RT reagent Kit with gDNA Eraser (RR047A, Takara, Dalian, China). cDNA was amplified by real-time quantitative PCR (qPCR) to analyze the transcriptional levels of target gene. The GAPDH was referred to as a normalization control. The relative level of the target gene was performed and calculated by the 2^{- $\Delta\Delta$ Ct} method. All qPCR primers are shown in [Supplementary Table S1](#).

2.9. Enzyme-linked immunosorbent assay

The cultured mediums were collected and examined for HBV surface antigen (HBsAg) or HBeAg following the instructions of ELISA assay (Kehua, Shang Hai, China).

2.10. HBV DNA extraction, Southern blot and qPCR

HBV core-associated DNA was extracted and detected as described previously ([Chen et al., 2018](#)). Briefly, HBV DNA from core particles in the cytoplasm was collected through a sucrose density gradient and treated with proteinase K digestion. After phenol/chloroform was extracted, the isolated viral DNA was detected by Southern blot or qPCR. The primers used to quantify HBV DNA are listed in [Supplementary Table S1](#). The extracted viral DNA was detected using a DIG high prime

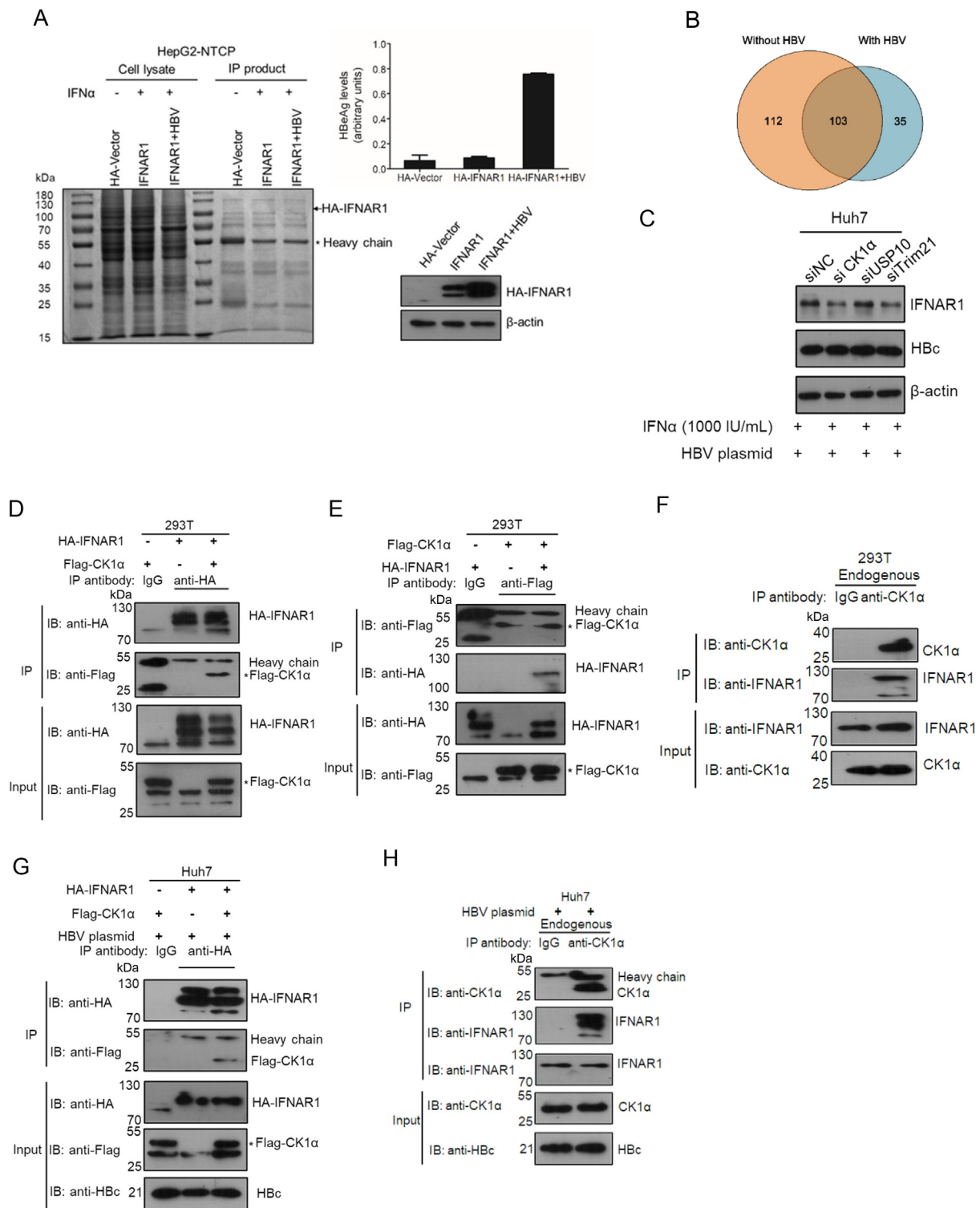


Fig. 1. Identification of interaction between CK1 α and IFNAR1 by MS and co-IP. **A** HepG2-NTCP cells were infected with or without HBV at 1000 genome equivalents (GE) per cell and then transfected with 6 μ g HA-IFNAR1 expression plasmid or 6 μ g empty vector as a control. After being stimulated with IFN- α (1000 IU/mL) for 24 h, the HBeAg levels in the supernatant were measured by ELISA. The cell lysates were immunoprecipitated by an anti-HA antibody and analyzed by SDS-PAGE. **B** Diagrams describe the number of proteins interacting with IFNAR1 identified in the MS data in the absence or presence of HBV. **C** Effects of the siCK1 α , siUSP10 or siTrim21 on IFNAR1 expression. Huh7 cells were transfected with 50 nmol/L siCK1 α , siUSP10 or siTrim21, after being stimulated with IFN- α (1000 IU/mL) for 24 h, the protein level of IFNAR1 was measured by Western blot. **D** CK1 α interacts with IFNAR1. HEK293T cells were co-transfected with the 6 μ g HA-IFNAR1 expression plasmid and 6 μ g Flag-CK1 α expression plasmid. At 48 h post-transfection, the cell lysates were immunoprecipitated with IgG or anti-HA antibodies and detected by Western blot. **E** Reverse Co-IP analysis was detected for the interaction between exogenous Flag-CK1 α and HA-IFNAR1 by immunoprecipitation with anti-Flag antibody in HEK293T cells. **F** The interaction of endogenous CK1 α and IFNAR1 was investigated by Co-IP analysis. HEK293T cells lysates were immunoprecipitated by IgG or anti-CK1 α antibodies and detected by Western blot. **G**, **H** Co-IP analysis for interaction between CK1 α and IFNAR1 in the presence of HBV in Huh7 cells. Huh7 cells were co-transfected with the 4 μ g HA-IFNAR1 expression plasmid, 4 μ g Flag-CK1 α expression plasmid and 4 μ g HBV expression plasmid. After 48 h post-transfection, the cell lysates were immunoprecipitated with IgG, anti-HA antibody or anti-CK1 α as indicated and detected by Western blot.

DNA labeling and detection starter kit II (11585614910, Roche Diagnostics GmbH, Germany) for Southern blot.

2.11. MTS assay

The cytotoxicity of D4476 in Huh7 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays (Promega). Briefly, Huh7 cells in a 96-well plate were treated with D4476 at the concentration of 0, 5, 10 $\mu\text{mol/L}$ for 3 days at 37 °C, then stimulated by IFN- α (1000 IU/mL) for 24 h. Finally, the MTS assays were conducted according to the manufacturer's instructions.

2.12. Statistical analyses

All experiments were performed at least three times. Data are expressed as the mean \pm standard deviation. The statistical analyses were performed by GraphPad Prism 5. Statistical analysis was used a *t*-test or one-way ANOVA with a Tukey post-hoc test. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. MS analysis confirms CK1 α interacts with IFNAR1

As IFNAR1 is a key factor in regulating the antiviral function of IFN- α , thus we sought to identify the possible cellular proteins that can interact with IFNAR1 and potentially regulate the anti-HBV function of IFN- α by MS analysis. HepG2-NTCP cells were infected with HBV at 1000 GE per cell, then HBeAg in supernatants was measured by ELISA assay to confirm the successful HBV infection. Next, the cells were transfected with HA-tagged IFNAR1 or control plasmid, followed by IFN- α treatment for 24 h. While the HA-IFNAR1 was efficiently expressed, the cell lysates were immunoprecipitated using an antibody against HA, and the Co-IP products were subjected to LC-MS analysis (Fig. 1A). We analyzed all the proteins precipitated with HA-IFNAR1 with or without HBV by LC-MS analysis. The identified proteins for which more than five unique peptides detected were selected for further analysis. Totally, 215 proteins were identified to interact with IFNAR1 in the absence of HBV, while 35 proteins exclusively interact with IFNAR1 in the presence of HBV (Fig. 1B, Supplement Table S2). Of the identified proteins for IFNAR1-interacting proteins, the F-box and WD repeat domain containing 11 (Fbxw11/ β -TrCP2) was previously reported to interact with IFNAR1 (Kumar et al., 2004) (Table 1). The epigenetic modifying enzymes, such as USP10, TRIM21, CK1 α , were selected for further study. As the levels of IFNAR1 were remarkably changed by silencing the endogenous expression of CK1 α (Fig. 1C), then we continue to investigate whether CK1 α could regulate the anti-HBV function of IFN- α by interacting with IFNAR1.

First, since CK1 α was physiologically associated with IFNAR1 in hepatoma carcinoma cells revealed by MS data, we conducted the Co-IP experiment to confirm this interaction. It was shown that Flag-CK1 α interacted with HA-IFNAR1 both in Co-IP or reverse Co-IP experiments in physiological condition using HEK293T cells (Fig. 1D and E). We further

Table 1

A list of IFNAR1 interacting proteins identified by LC-MS analysis.

Identified proteins	Unique peptides of MS spectra		
	HA-vector	HA-IFNAR1	HA-IFNAR1+HBV
Interferon alpha and beta receptor subunit 1 (IFNAR1)	0	15	15
Casein kinase 1 alpha (CSNK1A1/CK1 α)	0	8	6
F-box and WD repeat domain containing 11 (β -TrCP2)	0	5	6
Ubiquitin carboxyl-terminal hydrolase 10 (USP10)	0	12	8
E3 ubiquitin-protein ligase TRIM21	0	15	14

The data indicate the unique peptides of proteins interacting HA-IFNAR1.

tested whether the endogenous CK1 α could interact with IFNAR1 and observed that IFNAR1 was detected in CK1 α immunoprecipitates (Fig. 1F). In addition, in the presence of HBV, CK1 α is still bound with IFNAR1 in Huh7 cells as demonstrated with Co-IP assay (Fig. 1G and H).

3.2. CK1 α increases the abundance of IFNAR1 in the presence of HBV and exogenous IFN- α treatment

IFN- α , binding to the IFNAR1, triggers the JAK-STAT signaling pathway, including STAT1 and STAT2 phosphorylation, forming the complex with activated STAT1/2 heterodimers and IRF9. Then, the complex binds to the ISRE in the promoter region of hundreds of ISGs to interfere with virus replication (Schreiber, 2017). As CK1 α participates in regulating the abundance of IFNAR1 in the presence of HBV, we investigated the effect of CK1 α modulating the IFN- α triggered JAK-STAT signaling pathway in the presence of HBV. First, the toxicity of CK1 α specific inhibitor-D4476 combined with IFN- α treatment at Huh7 cells was determined by MTS assays. Huh7 cells were pretreated with D4476 (5 $\mu\text{mol/L}$), followed with or without IFN- α treatment (1000 IU/mL) for 24 h. As shown in Supplementary Fig. S1, D4476 combined with IFN- α treatment has no potential effect on cell apoptosis. In this physiological condition, D4476 combined with IFN- α treatment increased by 34% of the abundance of endogenous IFNAR1, and a minor increase of phosphorylation of STAT1 and STAT2 (Fig. 2A, lane 3 vs 4), indicating CK1 α caused IFNAR1 a minor degradation in physiological condition. On the contrary, in the presence of HBV and exogenous IFN- α treatment, the expression of endogenous IFNAR1 was reduced by 44% by D4476 treatment (Fig. 2B, lane 3 vs lane 4), while its mRNA levels did not be altered determined by qRT-PCR analysis (Fig. 2B, right panel). To confirm the positive effect of CK1 α modulating the IFN- α triggered JAK-STAT signaling pathway in the presence of HBV, we further analyzed the phosphorylation levels of STAT1 and STAT2. As shown in Fig. 2B, in the presence of HBV, the D4476 treatment remarkably inhibited the phosphorylation of STAT1 and STAT2 induced by IFN- α treatment. Conversely, ectopic expression of CK1 α increased the abundance of IFNAR1 by 65% at HBV transfection-replication models (Fig. 2C). Again, HepG2-NTCP cells were infected with HBV, treated with CK1 α inhibitor or ectopic expression of Flag-CK1 α , and then the expression of IFNAR1 was measured by Western blot. Similarly, inhibition of CK1 α decreased the level of IFNAR1 while overexpression of CK1 α increased the level of IFNAR1 in HepG2-NTCP cells infected with HBV particles (Fig. 2D and E). These data demonstrated that CK1 α down-regulated the abundance of IFNAR1 in physiological conditions, while it upregulated the abundance of IFNAR1 both in the presence of HBV and exogenous IFN- α treatment. This suggested CK1 α positively regulated the IFN- α triggered JAK-STAT signaling pathway in the presence of HBV, which is quite different from results of IAV infection (Xia et al., 2018).

3.3. CK1 α promotes the IFN- α antiviral signaling pathway in the presence of HBV

Next, to investigate the potential effect of CK1 α on ISRE activity, we analyzed the ISRE promoter activation in the presence of HBV. The Huh7 cells were either treated with D4476 or transfected with specific siRNA

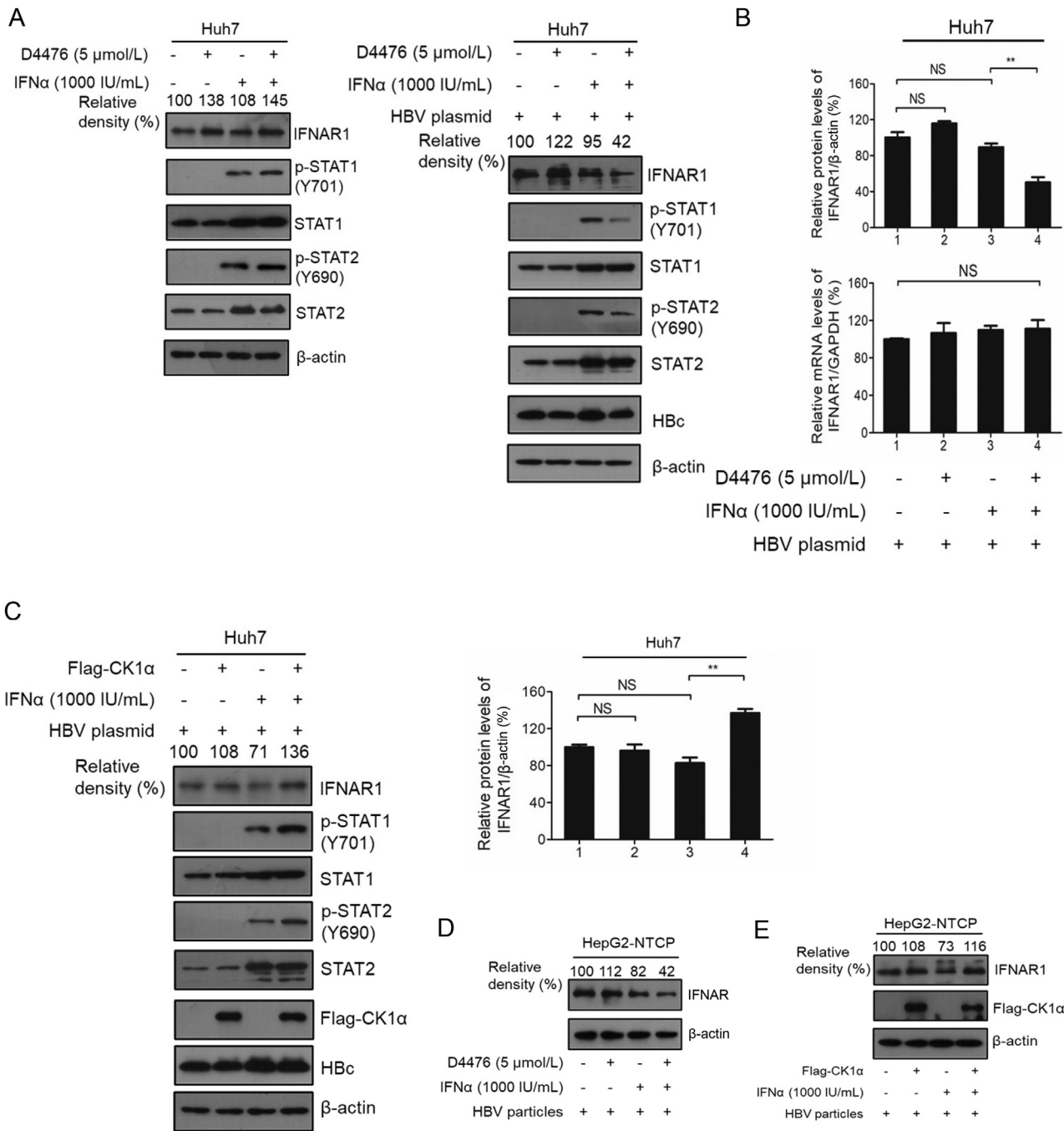


Fig. 2. Effects of CK1α on the abundance of IFNAR1 and type I IFN signaling pathway. **A** Huh7 cells were pretreated by D4476 (5 μmol/L) or DMSO for 24 h and then stimulated by IFN-α (1000 IU/mL). The levels of IFNAR1, STAT1, STAT2 were analyzed by Western blot. **B** Huh7 cells were treated with 5 μmol/L D4476, then transfected with 1 μg HBV expression plasmids, followed by stimulation of IFN-α (1000 IU/mL). Cell lysates were analyzed by Western blot as indicated (left panel). The expression of IFNAR1 was detected by Western blot or qPCR (right panel). **C** The effect of overexpression of Flag-CK1α on the expression of IFNAR1 at transfection-replication model. Huh7 cells were co-transfected with 1 μg Flag-CK1α and 1 μg HBV expression plasmids, followed by stimulation of IFN-α (1000 IU/mL) for 24 h. Cell lysates were analyzed by Western blot as indicated. **D, E** HepG2-NTCP cells were treated with 5 μmol/L D4476 or overexpression of Flag-CK1α, and infected with HBV particles (1000 GE/cell) and stimulated by IFN-α (1000 IU/mL). The levels of IFNAR1 were measured by Western blot. The relative level of IFNAR1/β-actin was calculated for the intensity measured by Image J software. Data represent the mean and standard deviation of three experiments. Statistical significance was determined by one-way ANOVA with Tukey post-hoc test (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, NS, not significant).

targeting CK1α, then co-transfected with the luciferase reporter plasmid and HBV expression plasmid, followed by IFN-α treatment. As shown in Fig. 3A and B, IFN-α stimulated a high ISRE promoter activity, but D4476 treatment or knockdown the endogenous CK1α suppressed the IFN-α activated ISRE promoter activity remarkably. Conversely, exogenous expression of CK1α enhanced the ISRE promoter activity significantly (Fig. 3C). As the activation of the ISRE promoter stimulates ISG expression,

we examined whether CK1α has the same positive effects on ISG expression upon IFN-α treatment. As shown in Fig. 3D, the D4476 treatment significantly reduced the mRNA levels of ISG15, ISG56, and A3G induced by IFN-α using the qPCR analysis. Conversely, exogenous expression of CK1α increased the ISG15, ISG56, and A3G transcripts induced by IFN-α (Fig. 3E). These data confirmed that CK1α executed a positive effect on the IFN-α triggered signaling pathway in the presence of HBV.

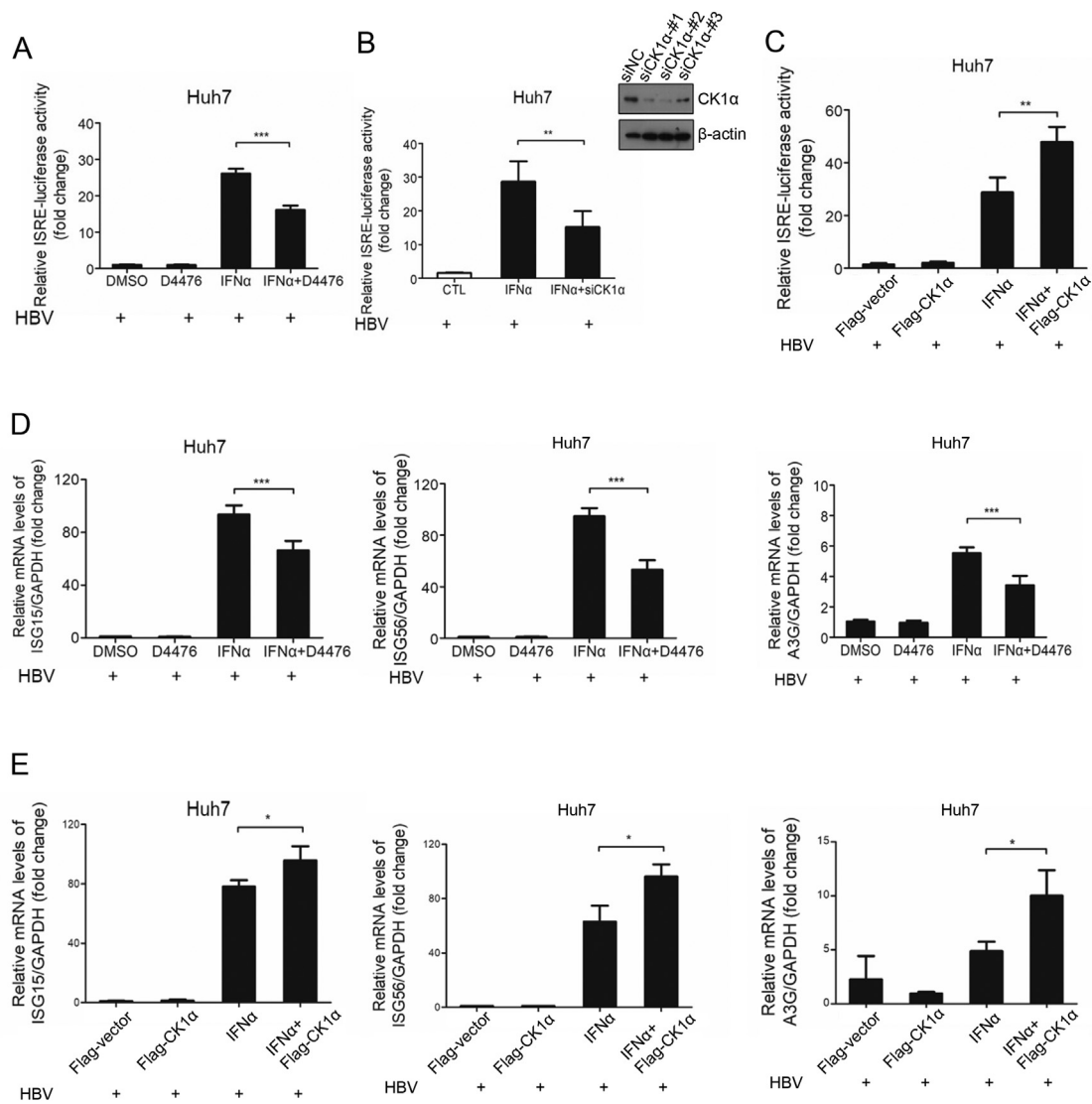


Fig. 3. CK1 α prompts IFN- α mediated ISRE activity and ISG induction. **A–C** Huh7 cells were co-transfected with 100 ng/well ISRE luciferase reporter plasmid and 10 ng/well Renilla luciferase reporter plasmid along with D4476 (5 μ mol/L) treatment (**A**) or siRNA targeting CK1 α (**B**) or 1 μ g Flag-CK1 α (**C**) expression plasmid. The luciferase activity was measured after IFN- α (1000 IU/mL) stimulation for 16 h. **D, E** Huh7 cells were treated as above, then the ISG15, ISG56 and APOBEC3G mRNA levels were detected by real-time PCR. The relative mRNA level of target gene/GAPDH was analyzed by the $2^{-\Delta\Delta C_t}$ method. Data represent the mean and standard deviation of three experiments. Statistical significance was determined by one-way ANOVA with Tukey post-hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.4. CK1 α contributes to the anti-HBV function of IFN- α

Next, we determined whether CK1 α would regulate the anti-HBV function of IFN- α . Firstly, CK1 α was knocked down by transfection of specific siRNAs into Huh7 cells, followed by exogenous IFN- α treatment (1000 IU/mL). Four days later, the HBsAg levels in the supernatants and the core-associated viral DNA were measured by ELISA or Southern blot. IFN- α treatment suppressed viral HBsAg or DNA levels. Although CK1 α silencing did not affect the viral replication (Fig. 4A, group A), this led to a decrease of antiviral effects of exogenous IFN- α against HBV replication (Fig. 4A, group B). Similar results were observed by D4476 treatment at Huh7 cells (Fig. 4B). Again, we evaluated the effects of D4476 treatment toward the antiviral effects of exogenous IFN- α in the HBV infection system. HepG2-NTCP cells were treated with D4476 and were then infected with viral particles. We observed that the anti-HBV effects of IFN- α were suppressed by D4476 treatment (Fig. 4C). Finally, Huh7 cells were co-transfected with HBV expression plasmid and Flag-CK1 α , followed by exogenous IFN- α treatment. The HBsAg levels and viral DNA levels were remarkably reduced in the overexpression group (Fig. 4D,

group B) but not in the mock group (Fig. 4D, group A). Similar results were observed in the HepG2-NTCP cells (Fig. 4E). Therefore, we concluded that CK1 α could prompt the anti-HBV effect of exogenous IFN- α .

3.5. CK1 α does not regulate the HBV replication in hepatoma carcinoma cells lacking a strong innate immune response

Our above data revealed that CK1 α prompted the anti-HBV effect of exogenous IFN- α , while it had no potential effect on HBV replication both in HBV transfection-replication and HepG2-NTCP infection models (Figs. 4 and 5A). The possible reason for this may be due to the relative lower efficiency of the innate immune response and not sensing viral DNA to promote the production of type I IFN in HepG2-NTCP infected with HBV particles (Lauterbach-Rivière et al., 2020). To address this question, HepG2-NTCP were infected with different amounts of viral particles, and then the mRNA levels of ISGs such as ISG15, ISG54, or interferon- β were measured by qPCR. As shown in Fig. 5B and C, although the viral DNAs or RNAs accumulated and could be measured

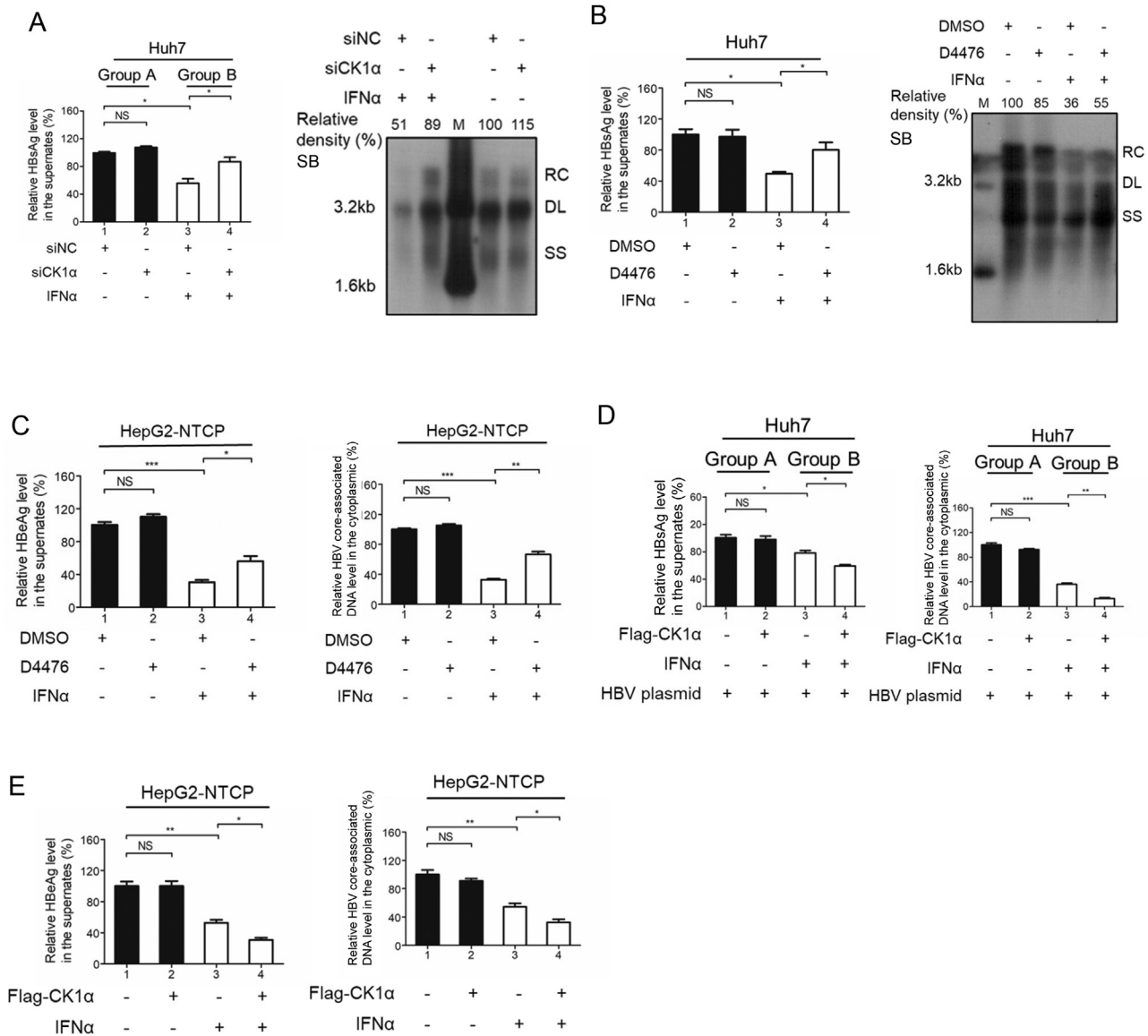


Fig. 4. Effect of knockdown or overexpression of CK1α on the anti-HBV activity of IFN-α. **A, B** Silencing the endogenous expression or inhibition of CK1α reduced the antiviral function of IFN-α against HBV. Huh7 cells were transfected with 50 nmol/L siCK1α (**A**) or treated with 5 μmol/L D4476 for 24 h (**B**) and then transfected with 2 μg HBV expression plasmids. After stimulated by 1000 IU/mL IFN-α for consecutive three days, the HBsAg levels in the supernatant were measured by ELISA, and the HBV core-association DNA was analyzed by Southern blot (SB). The relative level of viral DNA was calculated for the intensity measured by Image J software. RC, relaxed circular; DL, double-stranded; SS, single-stranded. **C** HepG2-NTCP cells were pretreated by D4476 (5 μmol/L) for 24 h, then infected with HBV particles (1000 GE/cell) and followed by IFN-α treatment (1000 IU/mL). The HBeAg levels in the supernatant were measured by ELISA, and the HBV core-association DNA was analyzed by qPCR. **D, E** Exogenous expression of CK1α prompted the antiviral function of IFN-α against HBV. **D** Huh7 cells were co-transfected with 1 μg HBV expression plasmids and 1 μg Flag-CK1α expression plasmids, or HepG2-NTCP cells were infected with HBV particles (1000 GE/cell). After being stimulated by IFN-α (1000 IU/mL) for consecutive three days, the HBsAg levels in the supernatant were measured by ELISA, and the HBV core-association DNA was analyzed by qPCR. Data represent the mean and standard deviation of three experiments. Statistical significance was determined by one-way ANOVA with Tukey post-hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS, not significant).

after 6 days of infection, no remarkable increasing mRNA levels of ISG15, ISG 54, and interferon-β were detected in HBV infected group even in higher virus titer (2000 GE/cell) compared with poly(I:C) stimulation group, confirming that DNA innate immune responses are poorly active in HepG2-NTCP cells, consistent with a previous report (Cheng et al., 2017; Lauterbach-Rivière et al., 2020).

3.6. CK1α increases the abundance of IFNAR1 and stability in the presence of HBV and IFN-α treatment

The above data indicated CK1α prompted the classical IFNα-induced JAK-STAT signaling pathway, and consequently contributed to the anti-

HBV effect of IFN-α both in transfection-replication and infection models. Then we explored the underlying mechanism by which CK1α prompted the anti-HBV effects of IFN-α by increasing the IFNAR1 protein levels as CK1α did not affect the transcripts of IFNAR1. First, to address how CK1α influences the stability of IFNAR1, we examined the protein stability of IFNAR1 by treating Huh7 with protein synthesis inhibitor CHX with or without CK1α expression. Again, in the physiological condition, endogenous IFNAR1 protein levels after treatment with CHX decreased more rapidly in the presence of CK1α (Fig. 6A), while in the presence of HBV and IFN-α, overexpression of CK1α increased the IFNAR1 protein levels (Fig. 6B). These data indicated CK1α upregulated the IFNAR1 protein by increasing its stability in the presence of HBV compared with

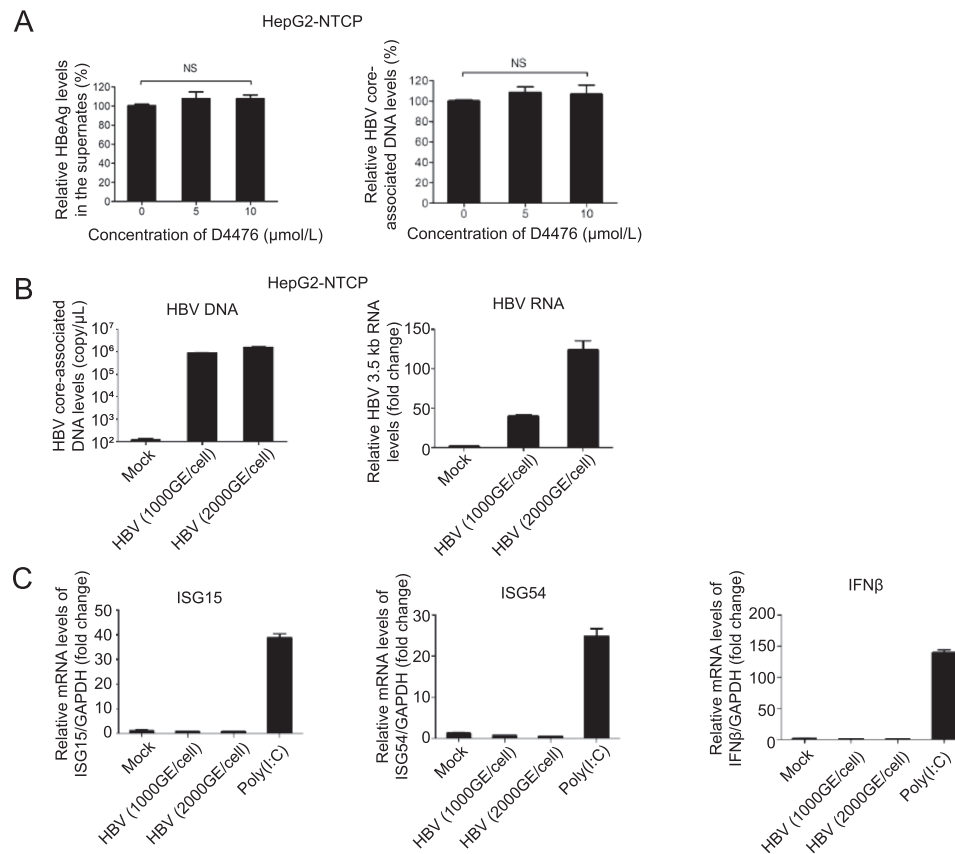


Fig. 5. CK1 α does not regulate HBV replication in hepatoma carcinoma cells. **A** HepG2-NTCP cells were infected with HBV particles (1000 GE/cell) and treated with D4476 (5 μ mol/L). After six days, the HBeAg levels in the supernatant were measured by ELISA, and HBV core-associated DNA was analyzed by qPCR. **B** The relative levels of HBV DNA (left) or 3.5 kb RNA (right) in the cytoplasm in the HepG2-NTCP cells infected with different doses of HBV particles. **C** Relative RNA expression of ISG15, ISG54 and interferon β in the HBV particle infection. The relative mRNA level of target gene/GAPDH was analyzed the $2^{-\Delta\Delta Ct}$ method. Poly(I:C) (1 μ g/mL) were served as positive control. Data represent mean and standard deviation of three experiments. Statistical significance was determined by one-way ANOVA with Tukey post-hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS, not significant).

physiological conditions. Finally, we examined whether CK1 α increased the protein levels of IFNAR1 via the ubiquitin-proteasome pathway. As shown in Fig. 6C, in the physiological condition, overexpression of CK1 α increased the ubiquitination levels of IFNAR1 as measured by ubiquitination assays in 293T cells. In line with previous report (Liu et al., 2009), phosphorylation of IFNAR1 Ser535 was required for CK1 α mediated phosphorylation and ubiquitination of IFNAR1. On the contrary, the ubiquitination levels of IFNAR1 were decreased by overexpression of CK1 α combined with the HBV and IFN- α treatment (Fig. 6D). This regulation was still dependent on the residue Ser535 of IFNAR1, stressing the activity of CK1 α was required in mediating the phosphorylation and ubiquitination of IFNAR1 in the presence of HBV.

4. Discussion

IFN- α , including pegylated IFN- α (PEG-IFN- α), has been widely used as first-line antiviral therapy for curing CHB (Pietro Lampertico et al., 2017; Terrault et al., 2018). Several clinical researches reports that the virological parameters such as HBeAg, HBV DNA and HBV genotype were associated with the response to IFN- α therapy for CHB (Zhao et al., 2007; Buster et al., 2008). However, the host factors that affect the anti-HBV effectiveness of IFN- α were less reported.

IFNAR1, binding with type I IFN, is triggered the type I IFN induced antiviral responses (Fuchs, 2013). Therefore, the levels of IFNAR1 are important to control viral replication. CK1 α has been suggested to modulate the phosphorylation and subsequent degradation of IFNAR1

and IFNGR1 response to IAV infection (Xia et al., 2018). However, whether CK1 α is crucial to HBV replication or the anti-HBV function of IFN- α is less reported. We identified that CK1 α interacted with IFNAR1 by mass spectrometry in HepG2-NTCP cells that supported HBV infection (Fig. 1), confirming that CK1 α would be involved with IFN- α triggered antiviral function during HBV infection. However, the function of CK1 α in mediating the effectiveness of IFN- α against HBV replication is quite different from that in IAV infection for the following phenomenon. CK1 α increased the abundance of IFNAR1 and IFN- α triggering classical JAK-STAT pathways (Figs. 2 and 3) in the presence of HBV, consequently contributing to the anti-HBV function of IFN- α in HBV-transfection and infection cells (Fig. 4). Furthermore, we revealed that CK1 α increased the abundance of IFNAR1 by inhibiting its proteasomal pathways (Fig. 6). Therefore, CK1 α may indirectly mediate the IFNAR1 degradation process by modification of an unknown protein involved in the IFNAR1 degradation process in the presence of HBV. The activity of CK1 α is required in this process as the CK1 α inhibitor-D4476 reduced the abundance of IFNAR1 in the presence of HBV. Further research would be conducted to seek this potential factor involved in the process. These results provide a new understanding of the molecular mechanism of CK1 α mediating the degradation of IFNAR1 in response to viral infection.

Innate immunity is the first line of defense against virus infection. After viral infections, the viral genetic information is recognized by a variety of pattern recognition receptors including Toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MAD5), then triggers innate immune responses to

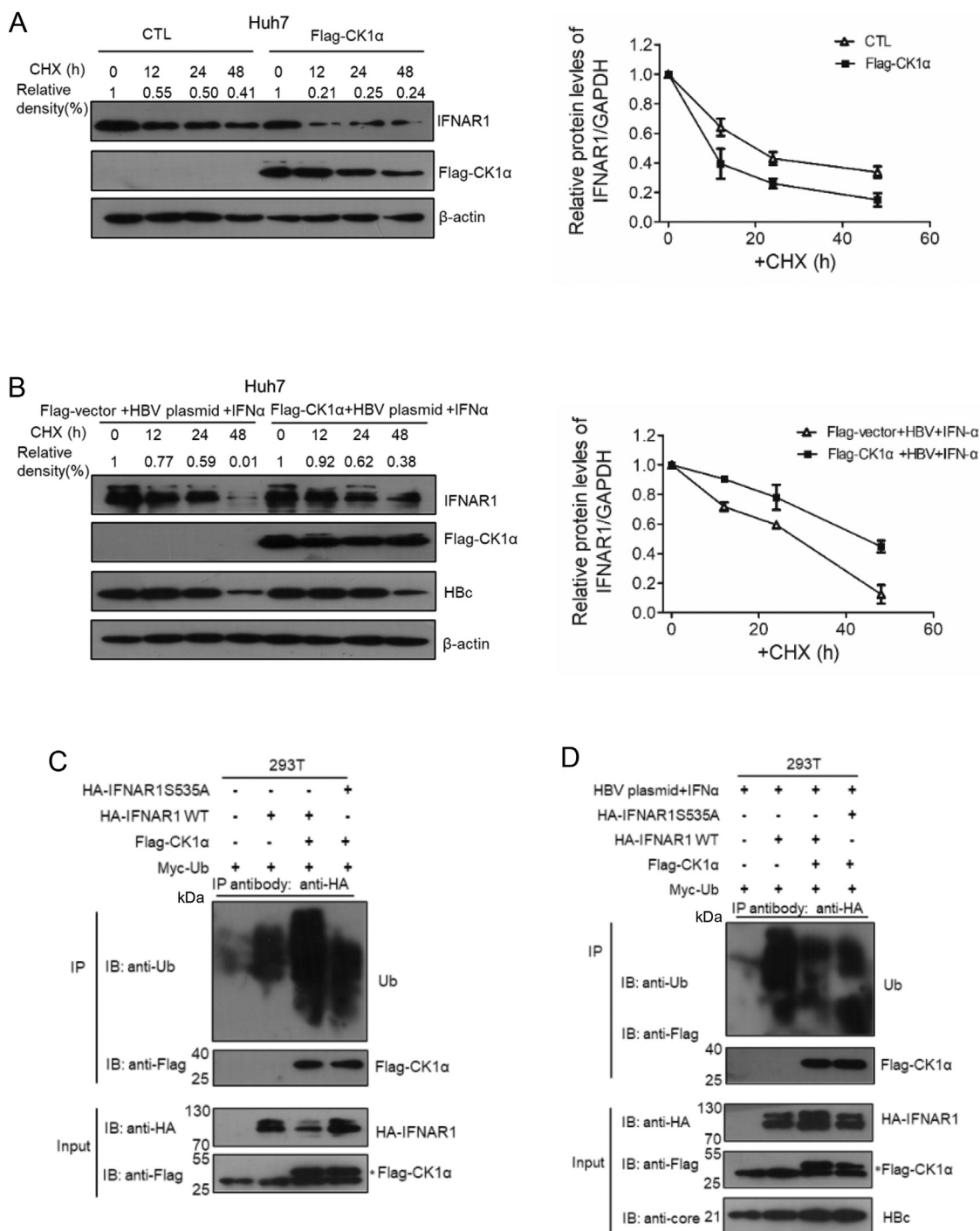


Fig. 6. CK1 α increases the stability and reduces the ubiquitination of IFNAR1 protein in the presence of HBV and exogenous IFN- α treatment. **A, B** Overexpression of CK1 α decreased the stability of IFNAR1 protein levels in physiological condition (A), while increased the stability of IFNAR1 protein levels in the presence of HBV and exogenous IFN- α treatment (B). **A** Huh7 cells were first transfected with Flag-CK1 α expression plasmid (1 μ g), followed by 100 μ g/mL CHX treatment for the indicated time points. **B** Huh7 cells were co-transfected with Flag-CK1 α (0.5 μ g) and HBV expression plasmid (0.5 μ g), then stimulated by IFN- α (1000 IU/mL), followed by 100 μ g/mL CHX treatment for the indicated time points. The steady-state levels of endogenous IFNAR1 protein were measured by Western blot, and the levels of IFNAR1/ β -actin were measured by Image J. Data represent mean and standard deviation of three experiments. **C, D** The IFNAR1 ubiquitination levels were modulated by CK1 α in HEK293T cells with or without HBV. HEK293T cells were transfected with the Flag-CK1 α expression plasmid (2 μ g), Myc-Ub expression plasmid (2 μ g), HA-IFNAR1 expression plasmid (2 μ g) or HA-IFNAR1 Ser535A expression plasmid (2 μ g), followed by treatment with MG132 (20 μ mol/L) for 6 h. The cell lysates were immunoprecipitated with anti-HA antibodies and detected by Western blot with indicated antibodies.

induce interferon to exert antiviral functions (Brubaker et al., 2015; Roers et al., 2016). However, the induction of an innate response to HBV infection is still a matter of debate (Sato et al., 2015; Cheng et al., 2017). MAD5 had been suggested to be sensing HBV in HBV-transfected cells (Lu, 2013), yet another report indicated that hepatocytes did not respond

to productive HBV infection (Lauterbach-Rivière et al., 2020). Our study finds that HBV infection does not induce the interferon response in HepG2-NTCP cells (Fig. 5), in accordance with other's report (Cheng et al., 2017). Consequently, although CK1 α promoted the anti-HBV function of IFN- α by increasing the stability of IFNAR1 (Figs. 4 and 6),

it has no potential function to regulate viral replication directly in HBV plasmid-transfected Huh7 cells or HBV-infected HepG2-NTCP cells due to lack of effective inducing interferon production. Further research would test whether CK1 α could regulate HBV DNA replication by targeting IFNAR1 at immune-competent hepatocytes such as primary human hepatocyte (PHH).

5. Conclusions

In summary, our work demonstrates that CK1 α increases the stability of IFNAR1, enhancing the efficacy of IFN- α against HBV replication. This work expands our knowledge of the molecular mechanism of CK1 α in the antiviral function of IFN- α , and CK1 α may be an attractive therapeutic target for improving the effectiveness of IFN- α therapy to cure CHB clinically.

Data availability

All the data generated during the current study are included in the manuscript.

Ethics statement

This article does not contain any studies with human or animal subjects performed by any of the authors.

Author contributions

Jing Xiong: methodology, investigation, formal analysis, writing-original draft preparation. Yanjun Jiang: methodology, investigation, formal analysis, validation. Jinru Zhang: investigation, formal analysis. Yanmeng Chen: investigation, funding acquisition. Yuan Hu: funding acquisition, conceptualization, supervision, writing-reviewing and editing.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2022.08.004>.

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