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Distinct role of 4E-BP1 and S6K1 in regulating autophagy and hepatitis B virus (HBV) replication

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ABSTRACT

Aims: To investigate the role and underlying mechanism of 4E-BP1 and S6K1 in regulating autophagy and hepatitis B virus (HBV) replication.
Main methods: The mRNA relative expression of HBx and its DNA level were detected by real-time PCR. The relative levels of hepatitis B surface antigen (HBsAg) were measured by enzyme-linked immunosorbent assay (ELISA). HBx DNA level of HepG2 cells transfected with pcDNA3.1(+)-HBV1.3 plasmids were detected by Southern blot. Moreover, we determined autophagy through electron microscopy, confocal microscopy and Western blot.
Key findings: Rapamycin promoted autophagy and the X protein synthesis concomitantly with elevation in Akt phosphorylation and Beclin1 expression. Either Beclin1 or Akt depletion suppresses the Rapa-enhanced HBV replication, whereas mTOR silencing inhibited HBV replication concurring with a decreased in both S6K1 and 4E-BP1 phosphorylation. Unexpectedly, Akt inhibitor suppressed Rapa-dependent autophagic flux and increased the level of p62/SQSTM1. While S6K1 ablation impaired autophagy and decreased X protein expression, 4E-BP1 silencing slightly influenced autophagy and increased X protein level.
Significance: The underlying mechanism of 4E-BP1 and S6K1, two main downstream effectors of mTOR, in mediating HBV replication and HBV-induced autophagy remains largely unknown. Here, we propose that Akt is required for both HBV replication and Rapa-induced autophagy, and 4E-BP1 and S6K1 play a distinct role in the virus replication and autophagic process.

1. Introduction

Hepatitis B virus (HBV) belongs to the *Hepadnavirus* family and its infection is a significant public health problem in worldwide [1]. HBV is a small-DNA virus that contains a relaxed circular and partially double-stranded DNA genome coding for the viral envelop, core proteins, viral polymerase, and HBx protein (X). The X protein transcriptional activity is regulated through a protein-protein interaction, and was reported to be necessary for viral replication [2,3]. Recently, numbers of studies have revealed that macroautophagy (hereafter referred to as autophagy) is involved in HBV DNA replication [4,5]. In addition, Akt has been found to mediate the transcription of viral RNA and the replication of DNA [6].

Akt, also known as protein kinase B, is a serine/threonine kinase and a downstream effector of phosphoinositide 3-kinase (PI3K). In mammals, triple Akt isoforms exist and are encoded by three separate genes [7]. PI3K/Akt signaling is commonly activated in various types of cancers and represents a major cell survival pathway. One well-known downstream effector of Akt is the mammalian target of rapamycin (mTOR) signaling pathway. mTOR, also known as FRAP, is a 289-kDa serine/threonine protein kinase and a member of the PI3K-related kinase family [8]. It is found in two distinct complexes, mTORC1 and mTORC2, and regulates many aspects of cellular functions [8,9]. mTORC1, which is primarily activated by the PI3K/Akt, is rapamycin-sensitive, whereas the mTORC2 is rapamycin-insensitive and can activate Akt. In general, mTORC1 is a negative regulator of autophagy [7,10]. S6K1 (p70 S6 kinase) and eIF4E-binding protein (4E-BP1) are two main downstream substrates of mTOR [11]. The activity of S6K1, which is required for cell growth and G1 cell cycle progression, is controlled by multiple phosphorylation events, such as the activation of mTOR [12,13]. Phosphorylation of Thr389, however, most closely correlates with S6K1 activity, could be blocked by Rapa [14].

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Translation repressor protein 4E-BP1 inhibits cap-dependent translation by binding to the translation initiation factor eIF4E (Eukaryotic initiation factor 4E) and the phosphorylation of 4E-BP1 at threonine 45 disrupts this interaction and results in activation of cap-dependent translation [15,16].

Rapa (also known as sirolimus) is an acute specific inhibitor of mTORC1 and a commonly used autophagy inducer [17]. Rapa treatment can activate PI3K/Akt and extracellular signal-regulated, mitogen-activated protein kinase (ERK/MAPK) signaling pathways through the feedback loops [18,19]. In addition, Rapa was found to stimulate viral protein synthesis in picornavirus infection, and enhanced HBV production in HepG2.2.15 cells [20,21].

In the present study, we have investigated the internal links among the autophagy, Akt signaling and the induced HBV replication. Our results reveal that Akt has a positively regulatory role in Rapa-dependent autophagy via S6K1 and promotes HBV replication through 4E-BP1 inhibition.

2. Materials and methods

2.1. Chemicals and antibodies

API-2 (Triciribine hydrate), Akti-1/2 (Akt1/2 kinase inhibitor), chloroquine (CQ), rapamycin (Rapa), polyclonal antibodies of LC3 were purchased from Sigma-Aldrich. Total Erk1/2, Phospho(T389)- and total-S6K, Phospho(*S*473)- and total-Akt, total-4E-BP1, Phospho (T2448)- and total-mTOR antibodies were obtained from Cell Signaling Technology. Antibodies of p62, Beclin1, and actin were acquired from Santa Cruz. Phospho (T45)-4E-BP1 antibody was purchased from Abcam.

2.2. Plasmids and siRNAs

GFP-LC3 and pcDNA3.1(+)-HBV1.3 plasmids were kind gifts of Dr. Tamotsu Yoshimori and Dr. Meng respectively. The siRNA specific for Akt1/2, Beclin1, LC3, mTOR, S6K1 and 4E-BP1 were purchased from Santa Cruz Biotechnology along with the control siRNA.

2.3. Cell culture and Western blot analysis

HepG2 and HepG2.2.15 cell lines were grown in DMEM medium containing 10% fetal bovine serum, and antibiotics. Cells were grown to 70–80% confluency before indicated chemical treatment. The transfections were carried out using Lipofectamine 2000 or Attractene according to the manufacturer's protocol. For siRNA interference, cells were grown to 30% confluence and transfected using DharmaFECT. Cells were split and cultured overnight before exposure to stimulations after 48 h transfection. Whole cell lysates were prepared with lysis using Triton X-100/glycerol buffer [22]. Western blot assay was performed using appropriate primary antibodies and horseradish peroxidase-conjugated suitable secondary antibodies, followed by detection with enhanced chemiluminescence (Pierce Chemical). Several X-ray films were analyzed to verify the linear range of the chemiluminescence signals and the quantifications were carried out using densitometry.

2.4. Confocal microscopy

HepG2.2.15 cells were transiently transfected with GFP-LC3 for 24 h, and split and grown on coverslips overnight before indicated treatment. Cells were fixed with freshly prepared 4% paraformaldehyde and observed with confocal microscopy.

2.5. Electron microscopy

Electron microscopy was performed as described [22]. The morphometric analysis of the area fraction between autophagosomes and total cell was calculated by using Photoshop CS4 software. The autophagosomes areas were copied and pasted to a new layer, then the total pixels of autophagosomes part and whole cell part, were obtained by using the histogram tool. The data of the area ratio were non-normally distributed and are presented as the mean of at least 20 cells counted for each group.

2.6. Real-time PCR assay

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, 15596-018) according to the manufacturer's protocol. 1 μg of total RNA was reversely transcribed using PrimeScript™ RT reagent Kit (TaKaRa). Total cellular DNA was extracted from the HepG2.2.15 cells after indicated treatment as the templates, then HBx level detected by real-time PCR were used as to represent the HBV DNA level. Real-time PCR was carried out using a SYBR real-time PCR kit (Sigma-Aldrich) in a Eppendorf Mastercycle EP (Eppendorf AG 22331, Hamburg). The following primers were for HBx: upstream primer 5′-AAGACTGGGAGGA GTTGGGG-3′, downstream primer 5′-GGCAGAGGGGAAAAAGTTGC-3′; the primers of β-actin are as following: upstream primer 5′-GCCTGAC GGCCAGGTCATCAC-3′, downstream primer 5′-CGGATGTCCACGTCA CAC TTC-3′.

2.7. ELISA detection

HBsAg levels in the supernatant of cell culture were detected using HBsAg diagnostic kits according to the manufacturer's instructions using TECAN plate reading at 450 nM.

2.8. DNA extraction and Southern blot

Total cellular DNA was extracted from transfected cells (6-well) at 48 h post-transfection and digested with EcoR I restricted enzyme. DNA samples were separated on 1% agarose gels and transferred to a nitrocellulose membrane (Amersham). After ultraviolet crosslinking and prehybridization, the membranes were hybridized with equal quantity of alkaline phosphatase-labeled HBx-specific probe from a random-primed labeling kit (GE Healthcare). The signal was detected by exposure to an X-ray film.

2.9. Statistical analysis

The quantifications of the chemiluminescence signals were carried out using densitometry, and normally distributed data are shown as mean ± SD and were analyzed using one-way analysis of variance and the Student-Newman-Keuls post-hoc test. Data are shown as mean ± SD in Graphs.

3. Results

3.1. Rapa promotes HBV replication and increases Beclin1 expression

It has been demonstrated that prolonged treatment with PI3K-AKT-mTOR pathway inhibitors markedly promoted HBV replication in HBV replicating and natural infection models. The PI3K-AKT-mTOR pathway was therefore identified to be a negative regulator of HBV replication. However, in our present study, we knocked down mTOR in HepG2.2.15 cells and observed that the deprivation of mTOR significantly increased HBx mRNA expression (Fig. 1A and Fig. 5B). To confirm the role of mTOR in HBV replication, we employed Rapa, an acute specific inhibitor of mTORC1 and commonly used autophagic inducer [17], in the following tests. Used electron microscopy, we observed that the treatment of Rapa increased vacuoles context (Fig. 1B, indicated by black arrow). Immunoblotting assay revealed that Rapa upregulated the levels of both LC3-II and Beclin1 (Fig. 1C). As has been suggested, here we utilized total Erk1/2 (extracellular signal-regulated protein kinase

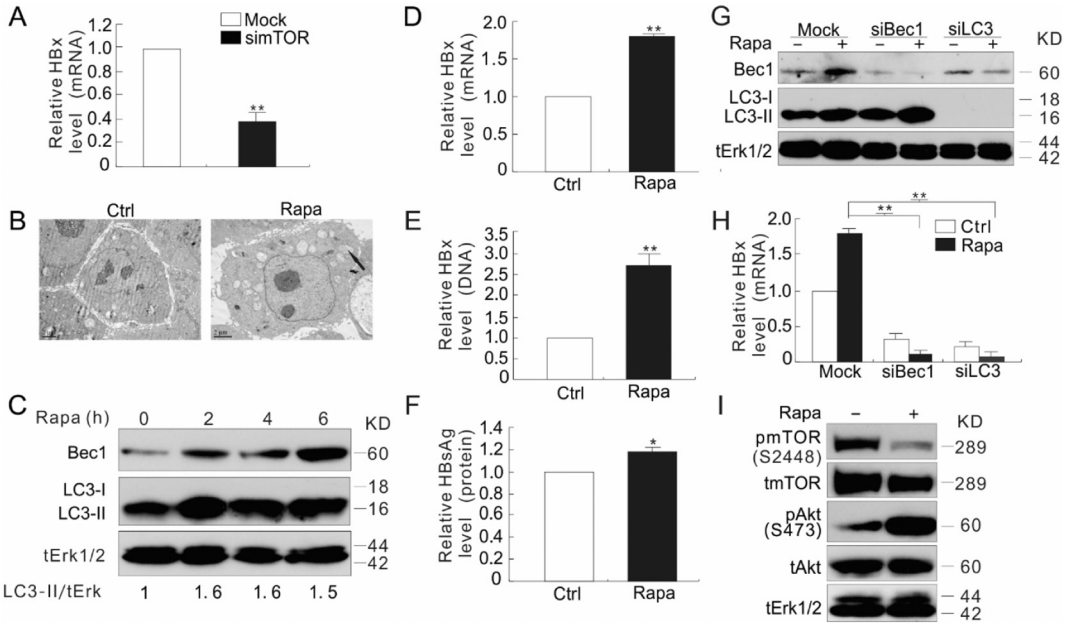

Fig. 1. Rapa promotes HBV replication and increases Akt activity. (A) The expression of HBx was detected by real-time PCR following deprivation of mTOR. (B) Electron microscopy was performed on HepG2.2.15 cells following Rapa (50 nM) treatment for 4 h. (C) HepG2.2.15 cells were treated with Rapa for up to 6 h and the lysates were detected by Western blot. (D and E) After Rapa treated for 4 h, the total RNA (D) and DNA (E) were extracted, and then performed real-time PCR to detect the HBx at mRNA and DNA levels. (F) HepG2.2.15 cells were treated with Rapa for 6 h, and the cell culture mediums were gathered and measured using ELISA. (G) HepG2.2.15 cells were transfected with siRNA of Beclin1 (siBec1) or LC3 for 48 h. The cells were subjected to Rapa for 4 h before analysis by Western blot using the indicated antibodies. (H) The expression of HBx for samples in (G) was detected by real-time PCR. (I) HepG2.2.15 cells were treated with Rapa for 4 h and the lysates were detected by Western blot. The data are presented as mean ± SD from three independent experiments. The single asterisk denotes the group is statistically different from the control groups ($p < 0.05$), whereas double asterisk means $p < 0.01$.

1/2) as the loading control [23]. Unexpectedly, unlike the knockdown of mTOR, Rapa challenge increased HBV replication by measuring the expression of HBx mRNA (Fig. 1D). To further exam the influence of Rapa on the virus replication, we extracted total DNA and HBV DNA level was detected by monitoring HBx DNA amount using real-time PCR. It demonstrated that Rapa increased the expression of HBx at DNA level (Fig. 1E). In ELISA, we found that HBsAg (HBV surface antigen) was increased upon Rapa challenge (Fig. 1F) [24]. Consistent with the conception that autophagy is required for HBV replication [4,21], the knockdown either Beclin1 or LC3 reduced HBV replication, which failed to be enhanced in response to Rapa treatment (Fig. 1G and H). Similar to former reports [25], we observed that Rapa increased the phosphorylation of Akt, suggesting that Akt may be required for Rapa to affect HBV replication (Fig. 1I).

3.2. Akt inhibition attenuates autophagic flux in HepG2.2.15 cells

API-2, which is a cell-permeable tricyclic nucleoside that selectively inhibits the cellular phosphorylation/activation of Akt [26], and we found that its presentation reduced the amount of autophagosomes in HepG2.2.15 cells (Fig. 2A, indicated by black arrow). Using confocal microscope, we observed that API-2 decreased the dots number of GFP-LC3 (Fig. 2B, indicated by white arrow). Immunoblotting analysis revealed that API-2 challenge obviously attenuated the phosphorylated Akt level and decreased the ratio of LC3-II to total Erk1/2 in a concentration-dependent manner (Fig. 2C). In addition, API-2 increased the expression of p62 (Fig. 2C), a well-known substrate of autophagy [27]. These results indicated that API-2 might inhibit autophagic flux in the cells. To confirm aforementioned results, we employed another Akt inhibitor, Akti-1/2, in the experiment. It demonstrated that Akti-1/2 completely inhibited basal autophagy at both 0.5 and 1 h time points, as CQ failed to further accumulate LC3-II in the treated cells (Fig. 2D). Together, we propose that Akt activity is likely required for early autophagic process at least in HepG2.2.15 cell.

3.3. Rapa fails to stimulate HBV replication in Akt-depleted cells

Since autophagy was reported to play an essential role in HBV replication, we next detected the virus propagation when Akt signaling was suppressed. Notably, API-2 decreased the expression of HBx at both mRNA and DNA levels (Fig. 3A and B). While Rapa challenge alone increased the levels of X protein mRNA by > 40%, API-2 showed to inhibit Rapa-dependent X protein expression (Fig. 3C). ELISA assay revealed that API-2 also decreased Rapa-induced HbsAg secretion (Fig. 3D). Similarly, Akti-1/2 inhibited both basal and the induced mRNA expression of X protein (Fig. 3E). In order to confirm aforementioned results, Southern blot and real-time PCR were carried out in HepG2 cells transiently transfected with HBV1.3 plasmids. Accordingly, Rapa treatment increased the amount of the HBx DNA, whereas API-2 decreased the levels of both basal and Rapa-induced HBx DNA (Fig. 3F and G), indicating that Akt is indeed required for HBV replication.

3.4. The deprivation of Akt attenuates autophagic flux and reduces HBV replication

To further investigate the regulatory role of Akt in autophagy and HBV replication, small interference RNA of Akt was introducing into HepG2.2.15 cells. The deprivation of Akt attenuated Rapa-dependent accumulation of LC3-II, whereas combination of Rapa and CQ was unable to increase LC3-II in Akt1/2-depleted cells (Fig. 4A and B). While Akt1/2 loss led to a decrease in HBx DNA levels (Fig. 4C), X protein mRNA expression of both basal and Rapa-induced were significantly suppressed in Akt1/2-deprived cells compared to the Mock control (Fig. 4D). Together with above obtained results, we reasonably believed that Akt was actually required for autophagic process and HBV replication in this cell line.

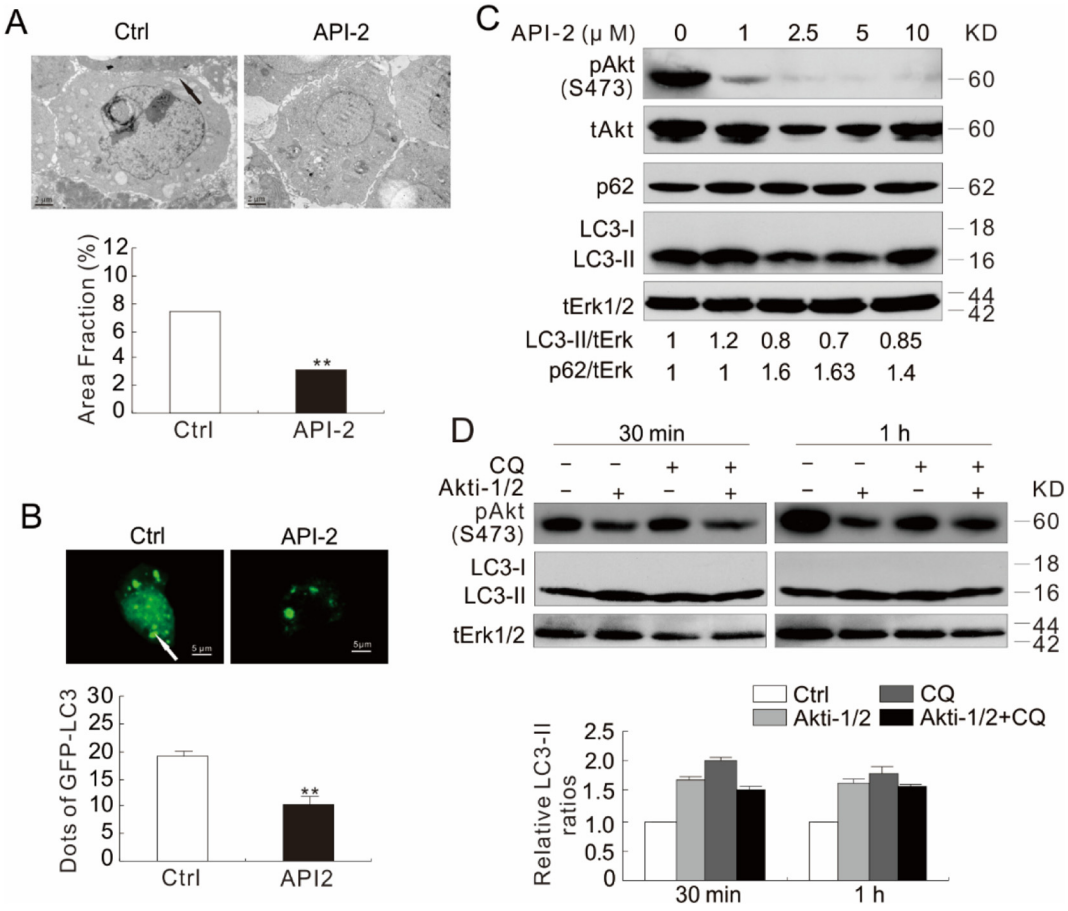

Fig. 2. Akt inhibition blunts autophagy. (A) Electron microscopy was performed on HepG2.2.15 cells following API-2 (2.5 μM) treatment for 4 h. The data are presented as the mean of at least 20 cells counted for each group. (B) HepG2.2.15 cells were transfected with GFP-LC3 for 24 h. After treatment with API-2 for 1 h, punctate GFP-LC3 in each cell was counted, and at least 50 cells were included for each group. Data represent the mean ± SD was shown in graph. The data are presented as mean ± SD from three independent experiments. The double asterisk denotes the group is significantly different from the control groups ($p < 0.01$). (C) HepG2.2.15 cells were treated with API-2 (1–10 μM) for 6 h. The lysates were analyzed by Western blot with the antibodies indicated. The adjusted ratio of LC3-II was presented below the blots. (D) HepG2.2.15 cells were treated with Akti-1/2 (1 μM) for 30 min, or 1 h in the presence or absence of CQ before analysis with Western blot.

3.5. Rapa and mTOR deprivation display a differentially regulatory role in the phosphorylation of 4E-BP1 and S6K1

Although always used as a specific inhibitor of mTOR, Rapa treatment and mTOR depletion indeed functioned differentially on HBV replication. Thus, we speculated that the downstream molecules of mTOR might play a role in HBV replication. To prove the hypothesis, we examined the phosphorylation of S6K1 and 4E-BP1, two main downstream substrates of mTOR [11]. Unexpectedly, Rapa failed to suppress 4E-BP1 phosphorylation even at high concentration, whereas it completely inhibited S6K1 phosphorylation (Fig. 5A), indicating that Rapa-insensitive events are involved in the regulation of 4E-BP1 in the HepG2.2.15 cells. In contrast, the deprivation of mTOR led to an inhibition of both S6K1 and 4E-BP1 phosphorylation (Fig. 5B). Additionally, API-2 markedly upregulated 4E-BP1 phosphorylation and influenced slightly the phosphorylation of S6K1 (Fig. 5C). Notably, we observed that API-2 remarkably increased 4E-BP1 protein level (Fig. 5C). Since viruses are absolutely dependent on the translational machinery residing in their hosts [28], the loss of 4E-BP1 phosphorylation possibly activated the translational repressor, prevented the dissociation of 4E-BP1 from eIF4E, and limited ribosome recruitment [15,16]. Thus, the increased expression of 4E-BP1 may be one of the reasons for API-2 to attenuate the HBV replication, and loss of HBV replication in the mTOR-depleted cells is probably caused by 4E-BP1 dephosphorylation.

3.6. 4E-BP1 and S6K1 function differentially in HBV replication and autophagy

To confirm aforementioned hypothesis, we silenced 4E-BP1 and S6K1 separately in HepG2.2.15 cells. The deprivation of 4E-BP1 resulted in no inhibition on basal autophagy as CQ further accumulated LC3-II (Fig. 6A). On the contrary, CQ failed to promote LC3-II accumulation in S6K1-depleted cells, suggesting an inhibited autophagic flux (Fig. 6B). Former study suggested that S6K1 loss inhibited autophagy through upregulation of Akt signaling [29], we then double depletion Akt with either S6K1 or 4EBP1. Unexpectedly, combined depletion of Akt1/2 with either 4E-BP1 or S6K1 completely impaired the autophagic flux (Fig. 6C and D). Similar to the increased in 4E-BP1 phosphorylation, its loss could release eIF4E to promote cap-dependent translation. As a result, we observed that 4E-BP1 depletion increased the X protein mRNA level. In contrast, knockdown of S6K1 inhibited X protein mRNA expression (Fig. 6E). Moreover, we observed that API-2 did not reduce the mRNA expression of X protein in 4E-BP1-deficient cells (Fig. 6F), in which Rapa failed to augment X protein mRNA levels (Fig. 6F). Consequently, it is likely that Akt regulates HBV replication via 4E-BP1 and mediates autophagy through S6K1.

4. Discussion

A new finding in the present study is that Akt signaling is required

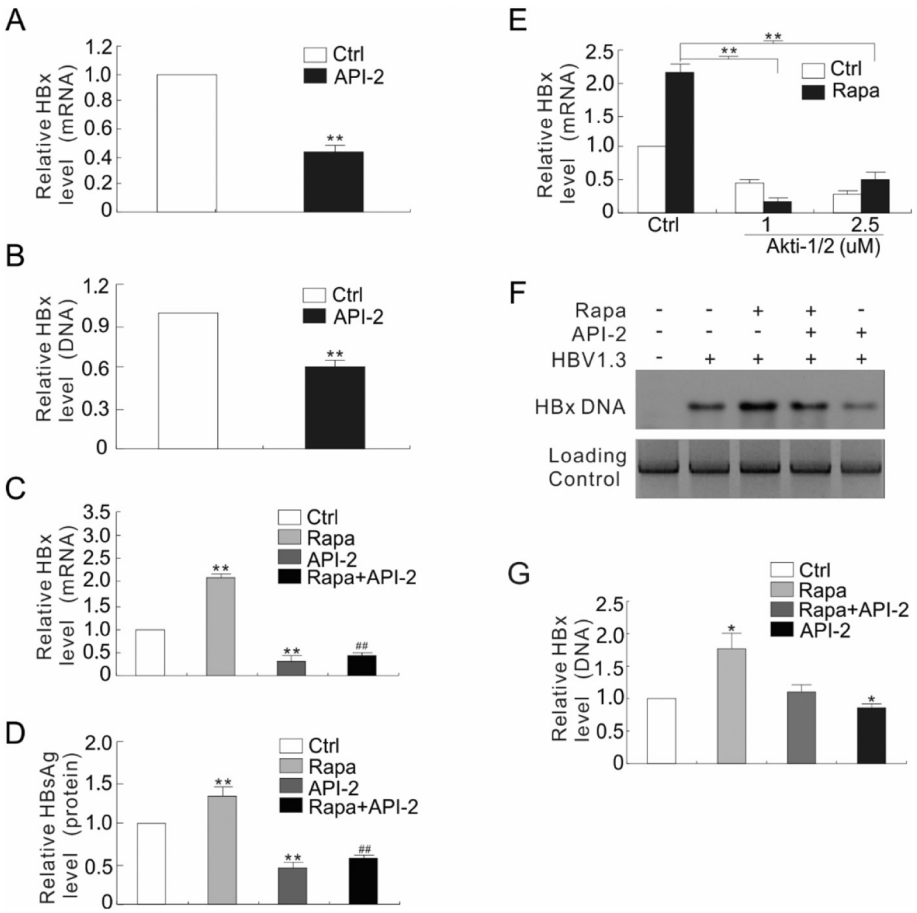

Fig. 3. Akt suppression impairs HBV replication stimulated by Rapa. (A and B) HepG2.2.15 cells were treated with API-2 (2.5 μM) for 4 h, then the RNA and DNA were extracted, respectively. (C) RNA was obtained from HepG2.2.15 cells following treatment with API-2 in the presence or absence of Rapa (50 nM) for 4 h. Then HBx was detected by real-time PCR at either mRNA or DNA level. (D) HepG2.2.15 cells were treated with Rapa in the presence or absence of API-2 for 6 h, and the cell culture mediums were gathered and measured using ELISA. (E) HepG2.2.15 cells were treated with 1 or 2.5 μM Akti-1/2 in the presence or absence of Rapa for 4 h. The expression of HBx was detected by real-time PCR. The data are presented as mean ± SD from three independent experiments. The double asterisk denotes the group is significantly different from the control groups ($p < 0.01$), and double octothorpe indicates significantly different from API-2 group. (F–G) After transfection with HBV1.3 plasmids for 24 h, HepG2 cells were exposed indicated treatments. Then the HBx DNA levels were measured by Southern blot using HBx-specific probe (F) and real-time PCR (G). The asterisk denotes the group is significantly different from the control group ($p < 0.05$).

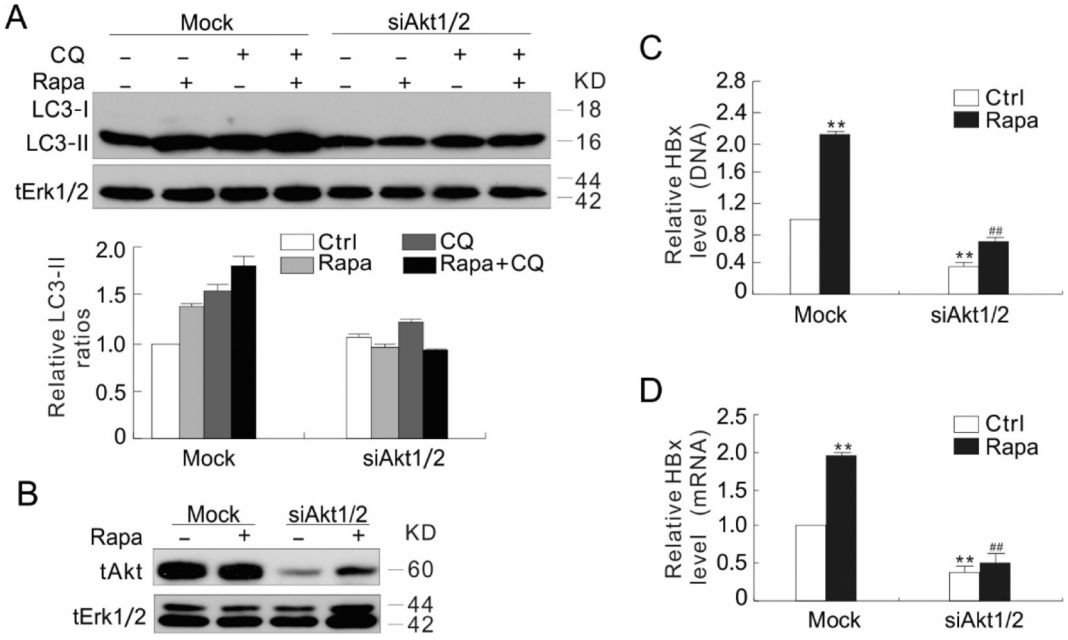

Fig. 4. Akt deprivation inhibits Rapa-promoted autophagy. HepG2.2.15 were transfected with the indicated siRNAs for 48 h. (A and B) Cell lysates were analyzed by Western blot following Rapa treatment for 1 h in the presence or absence of CQ. Relative levels of LC3-II were calculated and presented in graphs. (C and D) After Rapa treated for 4 h, the total DNA (C) and RNA (D) were extracted, and then performed real-time PCR to detect the HBx at mRNA and DNA levels. The data are presented as mean ± SD from three independent experiments. The double asterisk denotes the group is significantly different from the Mock control groups ($p < 0.01$), and double octothorpe indicates significantly different from Mock Rapa group.

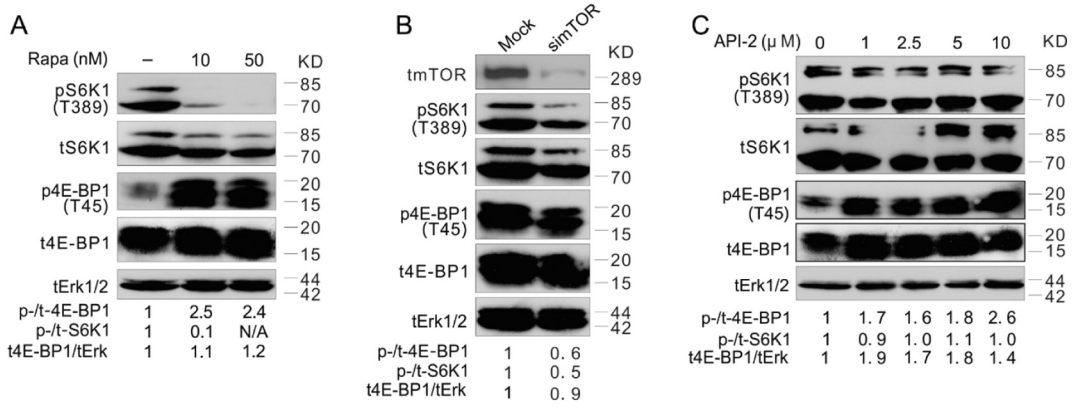

Fig. 5. 4E-BP1 and S6K1 behaves differentially under certain stimulus. (A) HepG2.2.15 cells were treated with Rapa (10 and 50 nM) for up to 4 h. (B) Cells were transfected with siRNA of mTOR for 48 h. (C) Cells were treated with API-2 (0–10 μM) for 6 h. The lysates of all above treatments were analyzed by Western blotting using indicated antibodies. Similar experiments repeated twice.

for Rapa-dependent autophagy, and S6K1 and 4E-BP1 have a distinct regulatory role in both autophagy and HBV replication. Numbers of viruses were able to induce either partial or complete autophagic process, which may affect virus replication either positively or negatively [30,31]. Consistent with former reports [4,5], here we also found that autophagy played a positively role in HBV replication.

As the upstream regulator of mTOR, Akt is usually considered as a suppressor of autophagy [17,32]. In malignant glioma cell lines, Rapa-induced autophagy was synergistically augmented by adding either PI3K or Akt inhibitor [17]. One study has revealed that mTORC1 positively regulated autophagy by increasing Akt activity [29]. Nevertheless, it demonstrated that the oxidative stress induced autophagy accompanied by Akt activation [33]. Moreover, HCV was found to induce autophagy concurring with the activation of mTOR [34]. Given that Akt can either be an upstream or downstream regulator of mTOR [7], therefore, it is highly possible that Akt may also play positive role in autophagy regulation. Except for inhibiting autophagy when mTORC1 is suppressed, Akt appeared to be required for the activation of autophagy induced by either Rapa or 4E-BP1 silencing. The inhibition of Akt not only blocked Rapa-induced autophagy, but also suppressed HBV replication. Besides, it is well known that Rapa treatment usually induced an elevated activation of Akt through feedback mechanism [18]. Since autophagy was inhibited in Akt/S6K1- or Akt/4E-BP1-depleted cells, therefore, the cooperation between mTOR and Akt signaling in regulation of autophagy may be existed. We speculate whether autophagy is induced or blocked by the Akt inhibition depend on the cell type, or treatment conditions such as concentration and stimulation time, and the crosstalk between Akt and mTOR signaling pathway. One study has demonstrated that prolonged starvation and persistent autophagy induction can trigger negative feedback that result in reactivation of mTOR, which is necessary for the reformation of lysosomes [35]. Alternatively, HBV replication probably requires the participation of autophagy and an adequate quantity of Akt signaling.

Interestingly, Rapa was found to suppress the activation of S6K1, but failed to inhibit the phosphorylation of 4E-BP1, indicating that the phosphorylation of 4E-BP1 and S6K1 is distinctly mediated in the HepG2.2.15 cells. Similar to our observation, the activation of S6K1 were completely Rapa-sensitive in RSV (Rous sarcoma virus) transformed fibroblasts; the dephosphorylation of 4E-BP1 was only partially responsive to Rapa challenge [36]. In encephalomyocarditis virus and poliovirus infected cells, Rapa was demonstrated to stimulate viral protein synthesis and augment the shutoff of host protein synthesis with the dephosphorylation of 4E-BP1. Thus, Rapa mediated viral protein synthesis could be the phosphorylation of 4E-BP1 dependent and independent. In fact, similarly to phosphorylation upregulation, deprivation of 4E-BP1 would release eIF4E to promote cap-dependent translation, and here we observed that 4E-BP1 silencing increased the X protein mRNA expression level, which was not inhibited by API-2. Rapa

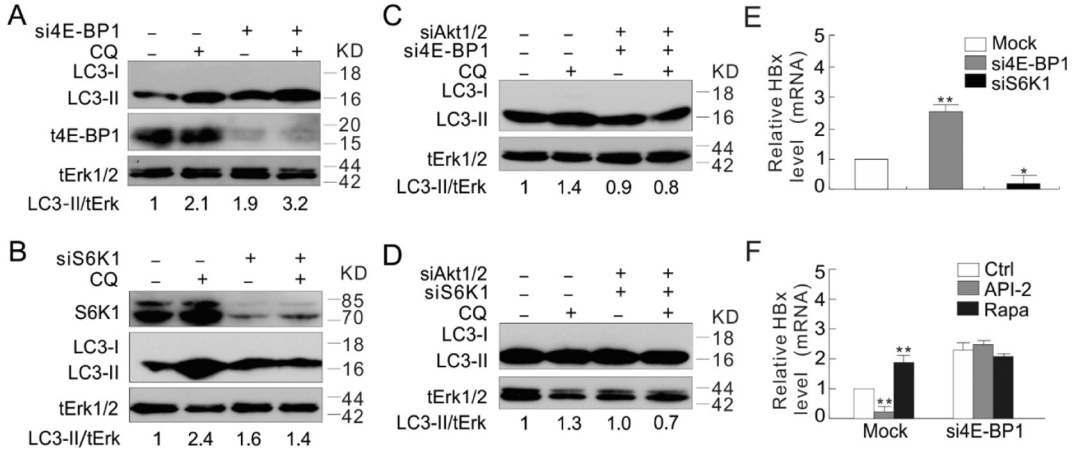

Fig. 6. 4E-BP1 and S6K1 differentially regulates HBV replication and autophagy. HepG2.2.15 cells were transfected with the indicated siRNAs for 48 h. (A, B, C and D) The lysates were analyzed by Western blot following CQ treatment for 2 h. Relative LC3-II level was calculated and presented in graphs or below the bolts. (E and F) HBx expressions were detected by real-time PCR with no treatment (C), with or without API-2 (2.5 μM) and Rapa (50 nM) treatment for 4 h (F). The data are presented as mean ± SD from three independent experiments. The single asterisk denotes the group is statistically different from the control groups ($p < 0.05$), whereas double asterisk means $p < 0.01$.

alone promoted the HBV replication that failed to further increase in 4E-BP1-silenced cells. Therefore, we assumed that 4E-BP1 may be the key regulator for the Akt regulated HBV replication. Unlike S6K1, 4E-BP1 deprivation had no inhibitory effect on autophagy, thus, Akt is likely to positively regulate autophagy via S6K1. The phosphorylation level of S6K1 was decreased in response to Rapa treatment, however, silence of S6K1 was found to decrease the HBV replication. That maybe due to that: on the one hand, deprivation of S6K1 inhibited viability of the host cells and resulted in downregulated protein synthesis; on the other hand, S6K1 may have a kinase-independent activity during this process.

5. Conclusions

In summary, the data presented here clearly showed that autophagy blockage attenuated the HBV replication. Akt positively regulated autophagy through S6K1 and mediated HBV via 4E-BP1. Future work in this direction will enable us to better understand the regulatory mechanism of autophagy and offer help in the management of HBV infection.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

[1] H.B. El-Serag, K.L. Rudolph, Hepatocellular carcinoma: epidemiology and molecular carcinogenesis, Gastroenterology 132 (2007) 2557–2576, https://doi.org/10.1053/j.gastro.2007.04.061.
[2] M.J. Bouchard, L.H. Wang, R.J. Schneider, Calcium signaling by HBx protein in hepatitis B virus DNA replication, Science 294 (2001) 2376–2378, https://doi.org/10.1126/science.294.5550.2376.
[3] Z.M. Xu, et al., Enhancement of hepatitis B virus replication by its X protein in transgenic mice, J. Virol. 76 (2002) 2579–2584, https://doi.org/10.1128/Jvi.76.5.2579-2584.2002.
[4] D. Sir, et al., The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 4383–4388, https://doi.org/10.1073/pnas.0911373107.
[5] Y.J. Tian, D. Sir, C.F. Kuo, D.K. Ann, J.H.J. Ou, Autophagy required for hepatitis B virus replication in transgenic mice, J. Virol. 85 (2011) 13453–13456, https://doi.org/10.1128/Jvi.06064-11.
[6] H.T. Guo, et al., Regulation of hepatitis B virus replication by the phosphatidylinositol 3-kinase-Akt signal transduction pathway, J. Virol. 81 (2007) 10072–10080, https://doi.org/10.1128/Jvi.00541-07.
[7] P.T. Bhaskar, N. Hay, The two TORCs and Akt, Dev. Cell 12 (2007) 487–502, https://doi.org/10.1016/j.devcel.2007.03.020.
[8] M. Laplante, D.M. Sabatini, mTOR signaling in growth control and disease, Cell 149 (2012) 274–293, https://doi.org/10.1016/j.cell.2012.03.017.
[9] S. Wullschleger, R. Loewith, M.N. Hall, TOR signaling in growth and metabolism, Cell 124 (2006) 471–484, https://doi.org/10.1016/j.cell.2006.01.016.
[10] Z.F. Yang, D.J. Klionsky, Eaten alive: a history of macroautophagy, Nat. Cell Biol. 12 (2010) 814–822, https://doi.org/10.1038/ncb0910-814.
[11] N. Hay, N. Sonenberg, Upstream and downstream of mTOR, Genes Dev. 18 (2004) 1926–1945, https://doi.org/10.1101/gad.1212704.
[12] N. Pullen, G. Thomas, The modular phosphorylation and activation of p70(s6k), FEBS Lett. 410 (1997) 78–82, https://doi.org/10.1016/S0014-5793(97)00323-2.
[13] M. Saitoh, et al., Regulation of an activated S6 kinase 1 variant reveals a novel mammalian target of rapamycin phosphorylation site, J. Biol. Chem. 277 (2002) 20104–20112, https://doi.org/10.1074/jbc.M201745200.
[14] A. Dufner, G. Thomas, Ribosomal S6 kinase signaling and the control of translation, Exp. Cell Res. 253 (1999) 100–109, https://doi.org/10.1006/excr.1999.4683.
[15] M.I. Ayuso, M. Hernandez-Jimenez, M.E. Martin, M. Salinas, A. Alcazar, New hierarchical phosphorylation pathway of the translational repressor eIF4E-binding protein 1 (4E-BP1) in ischemia-reperfusion stress, J. Biol. Chem. 285 (2010) 34355–34363, https://doi.org/10.1074/jbc.M110.135103.
[16] A. Pause, et al., Insulin-dependent stimulation of protein-synthesis by phosphorylation of a regulator of 5'-cap function, Nature 371 (1994) 762–767, https://doi.org/10.1038/371762a0.
[17] H. Takeuchi, et al., Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors, Cancer Res. 65 (2005) 3336–3346, https://doi.org/10.1158/0008-5472.Can-04-3640.
[18] P.P. Hsu, et al., The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling, Science 332 (2011) 1317–1322, https://doi.org/10.1126/science.1199498.
[19] Y.H. Yu, et al., Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling, Science 332 (2011) 1322–1326, https://doi.org/10.1126/science.1199484.
[20] L. Beretta, Y.V. Svitkin, N. Sonenberg, Rapamycin stimulates viral protein synthesis and augments the shutoff of host protein synthesis upon picornavirus infection, J. Virol. 70 (1996) 8993–8996.
[21] W.J. Huang, et al., Rapamycin enhances HBV production by inducing cellular autophagy, Hepat. Mon. 14 (2014), https://doi.org/10.5812/hepatmon.20719.
[22] Q. Lu, et al., Akt inhibition attenuates rasfonin-induced autophagy and apoptosis through the glycolytic pathway in renal cancer cells, Cell Death Dis. 6 (2015), https://doi.org/10.1038/cddis.2015.344.
[23] S. Mizrachy-Schwartz, N. Kravchenko-Balasha, H. Ben-Bassat, S. Klein, A. Levitzki, Optimization of energy-consuming pathways towards rapid growth in HPV-transformed cells, PLoS One 2 (2007), https://doi.org/10.1371/journal.pone.0000628.
[24] L. Galluzzi, et al., Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on cell death 2012, Cell Death Differ. 19 (2012) 107–120, https://doi.org/10.1038/cdd.2011.96.
[25] X. Wan, B. Harkavy, N. Shen, P. Grohar, L.J. Helman, Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism, Oncogene 26 (2007) 1932–1940, https://doi.org/10.1038/sj.onc.1209990.
[26] L. Yang, et al., Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt, Cancer Res. 64 (2004) 4394–4399, https://doi.org/10.1158/0008-5472.Can-04-0343.
[27] D. Kliosnky, Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition) (vol 12, pg 1, 2015), Autophagy 12 (2016) 443, https://doi.org/10.1080/15548627.2016.1147886.
[28] C. Arias, D. Walsh, J. Harbell, A.C. Wilson, I. Mohr, Activation of host translational control pathways by a viral developmental switch, PLoS Pathog. 5 (2009), https://doi.org/10.1371/journal.ppat.1000334.
[29] X.H. Zeng, T.J. Kinsella, Mammalian target of rapamycin and S6 kinase 1 positively regulate 6-thioguanine-induced autophagy, Cancer Res. 68 (2008) 2384–2390, https://doi.org/10.1158/0008-5472.Can-07-6163.
[30] N.J. Lennemann, C.B. Coyne, Catch me if you can: the link between autophagy and viruses, PLoS Pathog. 11 (2015), https://doi.org/10.1371/journal.ppat.1004685.
[31] L.T. Lin, P.W.H. Dawson, C.D. Richardson, Viral interactions with macroautophagy: a double-edged sword, Virology 402 (2010) 1–10, https://doi.org/10.1016/j.virol.2010.03.026.
[32] J. Zhao, et al., FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells, Cell Metab. 6 (2007) 472–483, https://doi.org/10.1016/j.cmet.2007.11.004.
[33] R. Castino, I. Fiorentino, M. Cagnin, A. Giovia, C. Isidoro, Chelation of lysosomal Iron protects dopaminergic SH-SY5Y neuroblastoma cells from hydrogen peroxide toxicity by precluding autophagy and Akt Dephosphorylation, Toxicol. Sci. 123 (2011) 523–541, https://doi.org/10.1093/toxsci/kfr179.
[34] S. Shrivastava, J.B. Chowdhury, R. Steele, R. Ray, R.B. Ray, Hepatitis C virus upregulates Beclin1 for induction of autophagy and activates mTOR signaling, J. Virol. 86 (2012) 8705–8712, https://doi.org/10.1128/Jvi.00616-12.
[35] L. Yu, et al., Termination of autophagy and reformation of lysosomes regulated by mTOR, Nature 465 (2010) 942–U911, https://doi.org/10.1038/nature09076.
[36] Z. Tuhackova, V. Sovova, E. Sloncova, C.G. Proud, Rapamycin-resistant phosphorylation of the initiation factor-4E-binding protein (4e-BPI) in v-src-transformed hamster fibroblasts, Int. J. Cancer 81 (1999) 963–969, https://doi.org/10.1002/(Sici)1097-0215(19990611)81:6<963::Aid-Ijc20>3.0.Co;2-C.