CLASS II PHOSPHATIDYLINOSITOL 3-KINASE 2B IS A NOVEL TARGET FOR THE POTENTIAL DEVELOPMENT OF ANTIVIRAL DRUGS AGAINST THE HEPATITIS B VIRUS

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Abstract

Phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases that regulate crucial cellular processes, including cell survival, proliferation and intracellular vesicular trafficking. PI3Ksphosphorylate the 3-hydroxyl position of phosphatidylinositol (P1), P(4)P and P(4,5)P. Three PI3Ks classes have been described, according to their structure, substrate preference, and function, of which class II PI3Ks are least investigated. Class II PI3K comprises the PI3K-C2α, PI3K-C2β, and PI3K-C2γ isoforms with different cellular localisations involved in vesicular trafficking. PI3K-C2β, in particular, regulates endocytosis and endosomal signalling and, therefore, could interfere with the life-cycle of viruses that depend on the endosomal pathway for trafficking and morphogenesis, such as the Hepatitis B virus (HBV). HBV is an important human pathogen causing severe liver disease and hepatocellular carcinoma development, which results in the death of more than 500,000 chronically-infected patients every year. In this study, we modulated PI3K-C2β expression in hepatoma cells by using the CRISPR/Cas9 genome editing technology and investigated the consequences on HBV production. Our results showed significant inhibition of HBV particles release from cells depleted of PI3K-C2β, adding to the repertoire of host cell factors regulating HBV production, a novel target for the potential development of antiviral inhibitors.

Keywords: antiviral targets, HBV, PI3K2β

Introduction

Hepatitis B virus (HBV) remains a major health issue, despite the availability of a safe and effective vaccine. WHO estimates that approximately 250 million people are chronic HBV carriers at high risk of developing hepatocellular carcinoma (HCC) [33]. Current treatment is based on the so-called Direct Acting Antivirals (DAAs) and on immune response modulators that can block viral replication. DAAs are nucleoside or nucleotide analogues that inhibit the HBV polymerase, effectively suppressing HBV replication. However, these drugs need long-term administration and often induce resistance mutations. In addition, many HBV chronic carriers do not respond to current therapies. Therefore, novel antiviral targets need to be identified and used for drug development [37]. HBV is a small DNA virus of the Hepadnaviridae family with a unique life cycle [18, 25]. The infectious virion (also called the Dane particle) has a lipid membrane displaying three viral surface antigens, S (small), M (middle), and L (large), around a nucleocapsid containing the hepatitis B core protein (Hbc) and the 3.2 kb relaxed circular (RC) partially double-stranded DNA genome, covalently attached to the viral polymerase (Pol) [5]. Studies have shown that following virus attachment to heparin sulfate receptors that promote virus particle internalisation
by endocytosis [3, 35]. Depending on the cell type, HBV entry may occur by caveolin [20] or clathrin-mediated endocytosis [11], both pathways depending on dynamin-2 activity. Following the internalisation of the HBV particles, the envelope is removed within the endosomal network, and the released nucleocapsid is transferred to the nuclear pore complex by a directed transport [28]. In the nucleus, the RC-DNA is converted to a covalently closed circular (ccc) DNA that acts as a template for the transcription of viral pre-genomic (pg) RNA and the subgenomic RNAs [14]. Following the release into the cytosol, a single molecule of pgRNA is incorporated into newly forming capsids together with Pol, which uses it as a template for genomic DNA synthesis [2]. The final particle maturation step consists of the nucleocapsid envelopment occurring at late endosomal membranes forming multi-vesicular bodies (MVB) [16, 32]. This process is followed by the extracellular release of virus particles using the exosome pathway [4, 23]. Interestingly, the envelope proteins can also self-assemble at the endoplasmic reticulum (ER) membrane, independent of the nucleocapsid presence, to form large amounts of non-infectious subviral particles (SVPs) that are secreted via the constitutive secretory pathway [26].

Phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases that coordinate many cellular processes, including cell survival, proliferation, and intracellular vesicular trafficking, through different signalling pathways. PI3Ks phosphorylate membrane phosphoinositides at the 3’ position of their inositol rings resulting in phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) [21]. Accumulated evidence indicates that many viruses activate PI3Ks pathways to inhibit apoptosis and promote viral replication [6].

PI3Ks are divided into three classes according to their structure, localisation and functional role. Class II PI3Ks are the least studied members of the PI3K family and include three isoforms with broad tissue expression, PI3K-C2α, PI3K-C2β and PI3K-C2γ [36]. Unlike class I and III PI3Ks, class II PI3Ks produce PI(3)P and PI(3,4)P2, but not PI(3,4,5)P3, possibly indicating modulation of different downstream signalling pathways [1, 8].

Of the class II PI3Ks, PI3K-C2β was recently found to localise in lysosomes and late endosomes [22]. As the endosomal pathway plays a critical role in the HBV life-cycle [11, 13, 19, 31], we hypothesised that PI3K-C2β might be involved in the HBV production. To test our hypothesis, we used the CRISPR/Cas9 genome editing technology to deplete hepatoma cells of PI3K-C2β expression and investigated the functional consequences on the HBV life-cycle. Our study demonstrates that the production of both viral particles and SVPs is significantly inhibited in these cells suggesting that PI3K-C2β is a key cellular factor regulating HBV production and a potential target for developing antiviral drugs.

Materials and Methods

Cells cultures
Huh7 cells were maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 100 units/mL penicillin-streptomycin at 37°C in a 5% CO2 atmosphere.

Generation of PI3K-C2β knockout clones by CRISPR/Cas9 genome editing
The first exon of the PI3K-C2β gene was analysed to design 20 base pairs (bp) sequences targeting this region. The resulting sequences:

5'-TTTAGAGCTTACTATTCCAGAGG-3'(S1) and 5'-AGAGCTGTTGCTAAAGAAAAAAGA-3'(S2).

(Invitrogen, USA) were each cloned into pSpCas9n(BB)-2A-Puro (PX462) V2.0 plasmid, also called pSpCas9n for short (Addgene, #62987), following BbsI digestion (NEB). The resulting plasmids called pSpCas9n(S1) and pSpCas9n(S2) were used to transform E. coli cells and further amplified from selected colonies. The identity of all constructs was confirmed by DNA sequencing.

Huh7 cells were seeded in 6-well plates (Corning) at 70% confluence and transfected the next day with 5 μg of empty pSpCas9n vector (control) or 5 μg of an equimolar mixture of pSpCas9n(S1) and pSpCas9n(S2), using Lipofectamine 3000 (Invitrogen), according to the manufacturer's protocol. The following day, fresh DMEM medium supplemented with 3 μg/mL puromycin dihydrochloride (Santa Cruz, sc-108071) was added to cells and changed every two days for the next two weeks. The resulting cell populations (bulk) were subjected to limited dilution cloning in 96-well plates (Corning). Selected Huh-7 knockout clones, denoted b1, b3, b5 and b6, were transferred to larger cell culture flasks and further amplified. To confirm genomic editing, nuclear DNA was extracted from selected clones using Trizol Reagent (Thermo Fisher, USA) and amplified by PCR using the 5-TTTAGA CCTACTATTCCAGAG-3 and 5'-AGAGCTGTTGCG TGAAAAAGAAA-3' primers flanking the region of interest.

Quantification of HBV production by quantitative (q)PCR
Huh7 cells were transfected with plasmid pTRiExHBV1.1 containing 1.1 units of the HBV genome by using Lipofectamine 3000 [17]. The following day cells were washed ten times with DMEM and further cultured for 9 days, with fresh DMEM medium, added every day. On day 9, supernatants were collected, and cells were harvested following a short trypsin treatment. Cells were pelleted, washed three times with ice-cold PBS and lysed with 1% Triton in PBS supplemented with protease inhibitors for 30 min on ice. The Viral HBV
DNA was extracted from both cells and supernatants and quantified by qPCR using the Rotor-Gene 6000 instrument (Qiagen) and a standard curve generated by ten-fold dilutions of known amounts of HBV DNA. Specific HBV primers used in this assay have been described elsewhere [17].

Quantification of HBV-SVPs secretion by ELISA
The amount of HBV-SVPs released from transfected Huh7 cells was quantified using the MonolisaHBsAg Ultra Kit (Bio-Rad), according to the protocol recommended by the manufacturer. The results were obtained as ratios of signal to cut-off value and were converted to folds of HBsAg secretion relative to control.

SDS-PAGE and Western blotting
Cell lysates were clarified by centrifugation for 10 min at 10,000x g, and the total amount of protein was quantified using the bicinichonic acid (BCA) assay (Pierce). Equal amounts of proteins were heat-denatured in the presence of 5 mM dithiothreitol (DTT) on sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose membranes using a wet blotter (Bio-Rad) then incubated with mouse anti-PI4Kδ (Santa Cruz, sc-377064) at 1/300 dilution in PBS with 1% BSA or rabbit anti-tubulin antibodies (Abcam, ab15246) at 1/10,000 dilution in PBS with 5% skimmed milk, followed by incubation with either mouse IgGk light chain binding protein (Santa Cruz, sc-516102) or mouse anti-rabbit antibodies (Santa Cruz, sc-2357) conjugated to horseradish peroxidase (HRP), diluted 1/10,000 in PBS. The proteins were visualised using the ECL detection system (Thermo Scientific, USA) followed by autoradiography.

Rescue of PI3K-C2β expression in PI3K-C2β knockout clones
Control (Ctrl) Huh7 cells and the PI3K-C2β knockout clone b3 were seeded in 24-well plates and transfected with either 2 μg of pcDNA3.1 or increasing concentrations of pMyc-DDK-PI3KC2β fusion plasmid (Origene, RC218354), as indicated in Figure 3 and Figure 4. The total DNA was adjusted to 2 μg for all samples by adding the corresponding amounts of pcDNA3.1. The following day cells were transfected with 0.3 μg of pTriEx-HBV1.1 for five days, then collected and transfected again with pMyc-DDK-PI3KC2β for 3 more days, following the same protocol as on day one. Cells and supernatants were harvested, and the amount of HBV and SVPs were determined by either qPCR or ELISA, as described above.

Results and Discussion
In this study, we applied a CRISPR/CAS9 genome editing protocol to modulate PI3K-C2β expression in Huh7 cells, which are permissive for HBV replication, assembly and secretion of fully infected virions and SVPs. Further, we investigated the effects on the viral cycle. Several Huh7 PI3K-C2β knockout clones denoted b1, b3, b5 and b6, with similar growth rates as the control cells, were randomly picked and subjected to protein analysis by SDS-PAGE and western blot. Both PI3K-C2β and tubulin (as loading control) were evidenced on western blots with specific antibodies (Figure 1). Interestingly, while PI3K-C2β was still detected in clone b1, most likely reflecting the successful inactivation of only one allele encoding for the PI3K-C2β, the protein was virtually undetectable in the remaining b3, b5 and b6 clones, confirming a complete knockout (Figure 1). Of these, clones b3 and b5 were retained to further investigate the HBV and SVPs production. A bulk cell population resulting after the genome editing, before the clonal selection, was also included in this analysis. Cells were transfected with the HBV genome, and secretion of viral particles and SVPs were monitored by qPCR and ELISA, respectively. As shown in Figure 2, the amount of SVPs released from clones b3 and b5 was significantly lower than from control cells. Similarly, the production of viral particles was inhibited by 60 - 90% in PI3K-C2β knockout cells.

A phenotype rescue experiment was designed to validate this result and ascertain that the observed inhibition is indeed due to the absence of PI3K-C2β in these cells, whereby PI3K-C2β expression was restored in the b3 clone by transient transfection with increasing concentrations of the pMyc-DDK-PI3KC2β plasmid. Importantly, production of both SVPs and viral particles was restored in b3 cells to levels similar to those in control cells, already at the lowest concentration of the expression plasmid used (Figure 3). These results confirm that PI3K-C2β is required for HBV and SVPs production in hepatoma cells.

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![Figure 1](image)

Figure 1.
Modulation of PI3K-C2β expression in Huh7 cell lines by using the CRISPR/Cas9 genome editing technology. Expression of PI3K-C2β in selected Huh7 knockout clones (b1, b3, b5 and b6), following the CRISPR/Cas9 gene editing and control (Ctrl) cells, were analysed by western blot (upper panel). Tubulin expression in the same cells was used as a sample loading control (lower panel).
Production of HBsAg and HBV particles is inhibited in PI3K-C2β knockout Huh7 cells

The mixed Huh7 cell populations resulted after the CRISPR/Cas9 genome editing (bulk), the PI3K-C2β knockout (clones b3 and b5) and control (Ctrl) cell lines were transfected with pTriEx-HBV1.1 and further cultured for 9 days. The release of the HBsAg (A) and virus particles (B) in cell supernatants were determined by ELISA (A) and qPCR (B), respectively. p values for replicate group comparisons were calculated using Student’s unpaired t-test, *, p < 0.05; **, p < 0.01.

Rescue of HBsAg and HBV particles production in PI3K-C2β knockout Huh7 cells after exogenous expression of PI3K-C2β

Control (Ctrl) cells and the PI3K-C2β knockout Huh7 clone b3 were transfected with either pcDNA3.1 or increasing concentrations of pMyc-DDK-PI3KC2β, as indicated. The following day cells were transfected with pTriEx-HBV1.1 for 5 days, then collected and transfected again with pMyc-DDK-PI3KC2β for 3 more days. The release of the HBsAg (A) and virus particles (B) in cell supernatants were determined by ELISA (A) and qPCR (B), respectively, p values for replicate group comparisons were calculated using Student’s unpaired t-test, *, p < 0.05; **, p < 0.01.

PI3K-C2β regulates HBV replication in Huh7 cells

Control (Ctrl) Huh7 cells and the PI3K-C2β knockout clone b3 were transfected with either pcDNA3.1 or increasing concentrations of pMyc-DDK-PI3KC2β, as indicated. The following day cells were transfected with pTriEx-HBV1.1 for 5 days, then collected and transfected again with pMyc-DDK-PI3KC2β for 3 more days. The amount of HBV nucleocapsids was determined in cell lysates by qPCR (B). p values for replicate group comparisons were calculated using Student’s unpaired t-test, *, p < 0.05; **, p < 0.01.

Inhibition of HBV virions and SVPs release from PI3K-C2β knockout cells could be a consequence of perturbed particle trafficking or viral replication. To discriminate between these possibilities, the amount of HBV DNA-containing nucleocapsids was quantified in Huh7 cells with either knockout or rescued PI3K-C2β expression (Figure 4). Interestingly, the amount of HBV nucleocapsids detected in the PI3K-C2β knockout b3 cell line was significantly decreased, by 80%, as compared with control. This result strongly suggests that HBV replication, rather than particle trafficking, is severely impaired in cells lacking PI3K-C2β and points to this enzyme as a novel cell factor involved in the HBV life-cycle. Recovery of HBV replication in the b3 cell line overexpressing PI3K-C2β confirms once again that the observed phenotype is modulated by this protein.

It is interesting to note that PI3K-C2β localisation in late endosomes and lysosomes was shown to inhibit the activity of the mammalian target of rapamycin (mTORC1) [9, 22], a highly conserved Ser/Thr kinase.
involved in important cellular processes, such as cell proliferation, metabolism and survival [15]. The mTOR pathway is also a negative regulator of autophagy [29], which in turn, is an essential process for HBV replication [27]. Not surprisingly, Akt/mTOR signalling inhibitors significantly enhanced HBV transcription and replication [34]. Therefore, it is tempting to speculate that the proviral role played by PI3K-C2β on HBV replication observed in this study could be mediated by the inhibition of the mTOR signalling. Future studies will address in more depth the molecular mechanism of the HBV regulation by PI3K-C2β and whether early autophagy and mTOR pathways are implicated in this process.

Conclusions
In this study, we have shown for the first time, to the best of our knowledge, that PI3K-C2β plays an important role in the HBV life-cycle. Using CRISPR/Cas9 genome editing to modulate PI3K-C2β expression in Hu7 cells, we demonstrated that the production of both HBV particles and SVPs is significantly inhibited in the absence of this enzyme. Exogenous PI3K-C2β expression restored HBV production, indicating a specific effect. Identification of PI3K-C2β as a novel host cell factor regulating HBV replication adds to the repertoire of antiviral targets to develop much needed, improved HBV therapies.

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Conflict of interest
The authors declare no conflict of interest.

References


