Hepatitis C virus genotype-3a core protein enhances sterol regulatory element-binding protein-1 activity through the phosphoinositide 3-kinase–Akt-2 pathway

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INTRODUCTION

Infection with hepatitis C virus (HCV) results in the development of hepatitis, steatosis, fibrosis, cirrhosis, hepatocellular carcinoma and the eventual need for liver transplantation. Different HCV genotypes cause different clinical pathologies (Amoroso et al., 1998). Clinical evidence has shown that genotype 3a (HCV-3a) may have a direct cytopathic effect on hepatocytes, with approximately 70 % of patients developing steatosis, or fatty liver, in the absence of other contributing factors such as obesity, diabetes and alcoholism (Kumar et al., 2002; Poynard et al., 2003a; Rubbia-Brandt et al., 2000). Furthermore, clearance of HCV-3a virus decreases or abolishes the presence of steatosis (Castera et al., 2004). The presence of steatosis can hamper successful clearance of the virus during therapy as well as increase the progression of the liver to cirrhosis (Patton et al., 2004).

Sterol regulatory element binding protein-1 (SREBP-1) is a key transcription factor that activates genes involved in lipid and cholesterol metabolism such as fatty acid synthase through binding of the sterol regulatory element (SRE) in the gene promoters (Horton et al., 2002; Latasa et al., 2000). SREBP-1 localizes on the endoplasmic reticulum (ER) in a complex with SREBP cleavage activating protein (SCAP) until low cholesterol and/or lipid levels or increased insulin induces the complex to move into the Golgi, where SREBP-1 is cleaved and the amino-terminal end is released into the cytoplasm to localize in the nucleus and regulate transcription of lipid metabolism genes (Bengoechea-Alonso & Ericsson, 2007; Raghow et al., 2008). Once in the nucleus, SREBP-1 can be modified by phosphorylation at multiple serine residues by a number of protein kinases, including mitogen-activated protein kinase (MAPK), protein kinase A (PKA), and glycogen synthase kinase-3β (GSK-3β) (Bengoechea-Alonso & Ericsson, 2009; Kotzka et al., 1998; Lu & Shyy, 2006; Punga et al., 2006; Roth et al., 2000). Phosphorylation of SREBP-1 can either enhance or decrease its transcriptional activity (Bengoechea-Alonso & Ericsson, 2007). The influence of HCV core on this transcription factor could play an important role in the development of steatosis during HCV infection.
Akt, an important kinase downstream of phosphoinositide 3-kinase (PI3K) (Engelman et al., 2006; Taniguchi et al., 2006), is known to influence the regulation of SREBP. Activation of Akt results in increased synthesis and nuclear accumulation of SREBPs, as well as increased expression of FAS and overall increased levels of cellular fatty acids (Porstmann et al., 2005). There are three isoforms of Akt. Akt-2 is primarily expressed in insulin-sensitive tissues. Knockout of Akt-2 in mice caused insulin resistance and a diabetes mellitus-like syndrome, while knockout of Akt-1 or Akt-3 did not affect metabolism. This suggests that Akt-2 may play an important role in regulating metabolism pathways (Cho et al., 2001). However, the role of Akt-2 in modulating HCV-associated metabolic abnormalities has not been studied.

Previous research has shown that the core protein of HCV-3a may have a differential mechanism in regulating lipid metabolism during infection in comparison to other genotypes, although the exact molecular mechanisms are not well understood (Abid et al., 2005; Hourioux et al., 2007; Pazienza et al., 2009; Piodi et al., 2008). Our previous study has shown that HCV-3a core protein upregulates fatty acid synthase gene transcription in an SREBP-1-dependent manner (Jackel-Cram et al., 2007). In this study, we further investigated whether HCV-3a core protein enhances SREBP-1 activity through PI3K and Akt-2. Our results showed that HCV-3a core protein could increase SREBP-1 activity and the PI3K–Akt-2 pathway played an important role in this process. Our results identified an important mechanism of HCV-associated steatosis.

RESULTS

Generation and characterization of HCV-3a core/1b chimeric replicon

It is important to study the function of a viral protein in the context of viral replication. However, efforts to establish an HCV-3a genomic replicon have not been successful so far. Therefore, we established a human hepatoma (Huh-7) cell line harbouring a genomic replicon containing HCV-3a core coding sequence in a full-length 1b genomic replicon backbone. The expression of core and NS5B proteins was demonstrated by Western blotting in the chimeric replicon cells (Fig. 1). The lysates of the HCV-1b replicon were used as controls (Fig. 1). These results demonstrated that we successfully generated a chimeric HCV-3a core/1b genomic replicon. Although the levels of NS5B in these two replicon cells were comparable, the level of HCV-3a core was lower than that of HCV-1b core, consistent with the findings when the core proteins of HCV-1b and -3a were expressed after transient transfection (Jackel-Cram et al., 2007). The reason for the different expression levels of core proteins of different HCV genotypes is not clear and is currently under investigation in our laboratory.

To determine the replication of HCV-1b and HCV-3a core/1b replicons, we performed a transient replication assay after electroporating luciferase-containing replicon RNAs into Huh-7 cells followed by luciferase assay 4 and 24 h after electroporation. As shown in Fig. 1(c), luciferase activities increased more than twofold at 24 h in comparison to baseline levels at 4 h for both HCV-1b and HCV-3a core/1b replicons, suggesting replication of both replicons. Interestingly, substitution of the coding sequence of the core protein with genotype HCV-3a in an HCV-1b backbone did not affect genomic RNA replication, since both replicons had similar luciferase levels at 24 h.

HCV-3a core is associated with increased level of mature SREBP-1 dependent upon PI3K

SREBP-1 is regulated post-translationally by cleavage into a mature and active transcription factor with a molecular mass of 68 kDa (Brown et al., 2000). To investigate whether HCV-3a core can modulate SREBP-1 activity, we examined the level of mature SREBP-1 (mSREBP-1) in the presence of HCV-3a core protein. Our experiments demonstrated that, in comparison to controls, the level of mSREBP-1 protein was higher in the presence of HCV-3a core.
core after transient expression, while no change was detected after transient expression of HCV-1b core (Fig. 2a). The increase of mSREBP-1 was more pronounced in the chimeric HCV-3a core/1b replicon cells (Fig. 2b). Once again, the level of mSREBP-1 was not increased in HCV-1b replicon cells in comparison to controls (Fig. 2b). These results demonstrated that HCV-3a core could increase mSREBP-1 level.

Since the activity of SREBP-1 can also be regulated by phosphorylation, we investigated whether the phosphorylation of SREBP-1 was modulated in HCV replicon cells using a method described previously (Waris et al., 2007). For this purpose, Huh-7, HCV-1b, and chimeric HCV-3a core/1b replicon cells were transfected with a plasmid expressing Flag-tagged mature SREBP-1a. SREBP-1a was then immunoprecipitated with a Flag-tag-specific antibody and blotted with a pool of phospho-Ser antibodies or the Flag antibody. Consistent with the endogenous mSREBP-1 levels as demonstrated in Fig. 2(b), the level of mSREBP-1a was higher in the chimeric HCV-3a core/1b replicon cells than those in control Huh-7 and HCV-1b replicon cells (Fig. 2c). Western blotting with phospho-Ser antibodies demonstrated that the levels of phosphorylated SREBP-1a were increased in both HCV-1b and chimeric HCV-3a core/1b replicon cells in comparison to control Huh-7 cells. The degree of SREBP-1a phosphorylation in chimeric HCV-3a core/1b replicon cells was higher than in HCV-1b replicon cells. However, the difference between these two replicons in phospho-SREBP-1a was much smaller than the total mSREBP-1a levels (Fig. 2c). These results demonstrated that both HCV-1b and chimeric HCV-3a core/1b replicons can stimulate SREBP-1a phosphorylation.

To determine the role of PI3K in this process, cells were treated with Ly294002, a PI3K inhibitor, prior to Western blotting. Ly294002 has been used to inhibit PI3K activity in Huh-7 cells previously (Mannova & Beretta, 2005; Waris et al., 2007). As shown in Fig. 2, application of Ly294002 decreased mSREBP-1 protein levels after HCV-3a core protein transient expression and in HCV-3a core/1b replicon cells. These results indicated that HCV-3a core could increase the level of mature SREBP-1 protein which was dependent on PI3K activity.

Akt phosphorylation is increased in the presence of HCV-3a core protein

Akt is a downstream kinase of PI3K and previous studies have shown that Akt is involved in modulating SREBP-1 activity and Akt-2 in particular plays a significant role in regulating metabolism (Cho et al., 2001). We therefore wanted to examine the role of Akt-2 in SREBP-1 activation by HCV-3a core. To this end, it was necessary to demonstrate whether HCV-3a core protein expression could modulate Akt phosphorylation, indicative of its activity. We used Huh-7 cells transfected with HCV-3a core-expressing plasmid or the HCV-3a core/1b replicon cells to determine Akt phosphorylation in Western blotting. The level of serine phosphorylation of Akt was increased after HCV-3a core transient expression (Fig. 3a) and in HCV-3a core/1b replicon cells (Fig. 3b) in comparison to controls. To determine whether Akt phosphorylation induced by HCV-3a core was regulated by PI3K, cells were treated with Ly294002, a PI3K inhibitor. As shown in Fig. 3, application of the PI3K inhibitor Ly294002 decreased Akt phosphorylation.
indicating PI3K dependence. These results indicated that HCV-3a core could increase the level of Akt phosphorylation which was dependent on PI3K activity.

**Inhibition of PI3K causes decreased SRE-driven transcription activation by HCV-3a core**

Our results thus far demonstrated that PI3K is involved in modulating mSREBP-1 levels by HCV-3a core protein (Fig. 2). We wished to confirm these findings by another reliable reporter for SREBP-1 activity. We chose to use an SRE-luciferase construct in which the luciferase expression is directly controlled by three copies of SRE sequences (ATCACCCCAC, pSRE-Luc) (Amemiya-Kudo et al., 2002). The rationale is that if SREBP-1 activity is increased, more active SREBP-1 will bind to the SRE sequence, which will in turn result in increased luciferase activity driven by SRE (Amemiya-Kudo et al., 2002; Hawkins et al., 2008). For this purpose, plasmid pSRE-Luc was transfected either together with an HCV-3a core-expressing plasmid into Huh-7 cells or alone into HCV-3a core/1b replicon cells. Cells were treated with DMSO or Ly294002 prior to luciferase assay. Our results showed that treatment with Ly294002 was associated with significantly lower SRE-driven transcription in comparison to control when HCV-3a core was expressed alone (Fig. 4a) and in HCV-3a core/1b replicon cells (Fig. 4b). It is noteworthy that the luciferase readings were much lower in replicon cells than in transiently transfected cells (compare Fig. 4b to 4a), even though the same amounts of reporter plasmids were used. The reason for this difference is not clear and requires further investigation.

These results indicate that activation of SREBP-1 and hence SRE-driven transcription by HCV-3a core is indeed dependent on PI3K.

**Inhibition of Akt-2 activity causes decreased SREBP-1 activation by HCV-3a core protein**

Activity of Akt has been linked to the processing of SREBP-1 and Akt-2 in particular plays a critical role in modulating metabolism (Porstmann et al., 2005). As such, we wanted to examine the role of Akt-2 in SREBP-1 activation by HCV-3a core. We once again used pSRE-Luc as a read-out for SREBP-1 activity. To specifically inhibit Akt-2 activity, we transfected cells with a dominant-negative (DN) form of Akt-2 together with SREBP-1 reporter pSRE-Luc. As shown in Fig. 5, expression of a DN form of Akt-2 resulted in significantly lower SRE-driven transcription when HCV-3a core protein was transiently
expressed (Fig. 5a) and in HCV-3a core/1b replicon cells (Fig. 5b).

To further confirm these findings, Akt-2 knockdown cells were created by stably transducing Huh-7 cells with a lentivirus construct containing Akt-2-specific small hairpin RNA (shRNA). The level of Akt-2 protein was reduced in Akt-2 shRNA cells in comparison to control shRNA (Fig. 6a). When these cells were transfected with pSRE-Luc and HCV-3a core protein-expressing plasmid, knockdown of Akt-2 had significantly decreased transcriptional activity from SRE, while control shRNA did not have such an effect (Fig. 6b). These results indicate that Akt-2 is involved in SREBP-1 activation by HCV-3a core.

**DISCUSSION**

It becomes increasingly clear that HCV infection is affecting every aspect of host metabolism and therefore hepatitis C has been classified as a metabolic syndrome (Negro, 2006; Sheikh et al., 2008). Interferon combined with ribavirin, the only approved therapy, is effective for only about 50% of the patients (Poynard et al., 2003b). Novel therapeutics are urgently needed but the progress is very slow because of a lack of knowledge on the mechanisms of how HCV causes liver diseases, including steatosis.

Our previous results have shown that HCV-3a core may contribute to steatosis by upregulating transcription of fatty acid synthase in an SREBP-1-dependent manner (Jackel-Cram et al., 2007). However, whether and how HCV-3a core protein modulates SREBP-1 activity is not known. In this study, we sought to investigate this question. We used two different models by either transiently expressing core protein after transfection or establishing a chimeric genomic replicon containing the HCV-3a core sequence in an HCV-1b backbone (Fig. 1). HCV replicons have been widely used for HCV research as they express all of the HCV proteins as a result of HCV RNA replication (Pietschmann et al., 2002) and the chimeric replicon established in this study is a valuable system for investigating the functional roles of HCV-3a core protein.

SREBP-1 is translated as a precursor protein that is cleaved into its mature, nuclear localized and active form. Our results show that HCV-3a protein, when expressed alone or in HCV-3a/1b replicon cells, can increase the level of mature SREBP-1 (Fig. 2), whereas the level of precursor...
SREBP-1 is largely unchanged (data not shown). It is noteworthy that the level of mSREBP-1 in HCV-3a core/1b replicon cells is higher than in cells expressing HCV-3a core after transient transfection (Fig. 2). The reason for this difference is not clear but it may be due to the fact that all replicon cells express HCV-3a core protein, while only a certain percentage of cells express core after transfection. In addition, other HCV proteins, such as NS2 and NS4B, which are only present in replicon cells, may contribute to higher mSREBP-1 levels as previous studies have shown these proteins can increase mSREBP-1 (Oem et al., 2008; Park et al., 2009).

Phosphorylation of SREBP-1 is an important mechanism of regulating its activities through affecting protein stability and/or DNA-binding capability (Bengoechea-Alonso & Ericsson, 2007). We showed that phosphorylation of SREBP-1a was stimulated in both HCV-1b and chimeric HCV-3a core/1b replicon cells (Fig. 2c). Our results are in agreement with a previous publication showing enhanced SREBP-1 phosphorylation after HCV infection (Waris et al., 2007). Since the impact of SREBP-1 phosphorylation on its activity is complex, future studies are needed to investigate the role of SREBP-1 phosphorylation in HCV pathogenesis.

Several studies have shown that HCV affects the PI3K–Akt signalling pathway. For example, it has been shown that HCV promotes cell survival through PI3K (Mannova & Beretta, 2005; Street et al., 2004). In addition, activation of SREBP-1 and SREBP-2 processing by HCV-2a JFH-1 infection is also through PI3K (Waris et al., 2007). Adding to the functions of PI3K in the context of HCV, we showed that PI3K is required for SREBP-1 activation by HCV-3a core protein (Figs 2 and 4). As a downstream kinase of PI3K, Akt is an important node in modulating a wide range of signal transduction pathways (Brazil et al., 2004). Due to its proven role in metabolism, we are particularly interested in investigating the role of Akt-2. We showed that inhibition of Akt-2 kinase activity by overexpressing a DN mutant or knocking down Akt-2 expression by shRNA abrogated SREBP-1 activation by HCV-3a core (Figs 5 and 6), indicating that Akt-2 is indeed involved in SREBP-1 activation by HCV-3a core.

The exact mechanism by which HCV-3a core protein activates SREBP-1 activity through the PI3K–Akt-2 pathway is not clear. SREBP-1 can be regulated by transcription and/or by translocation from ER to Golgi followed by proteolytic processing (Brown et al., 2000; Horton et al., 2002). Our results did not show significant effects of either core or Akt-2 on SREBP-1 transcription (C. Jackel-Cram, L. Qiao & Q. Liu, unpublished data). Therefore, it is tempting to speculate that HCV-3a core protein might enhance the ER to Golgi translocation of SREBP-1 protein, which results in increased mature SREBP-1 level through Akt-2 activity. In supporting this hypothesis, Akt has been shown to be able to increase ER to Golgi transport of SREBP and mature SREBP levels (Du et al., 2006; Porstmann et al., 2005).

In this study, we investigated the effect of HCV-3a core protein on SREBP-1 activity by transient transfection and chimeric genomic replicon. It would be interesting to extend this investigation by using infectious HCV viruses containing HCV-3a core. Until now, infectious HCV-3a viruses have not been generated from cell culture. Intergenotypic 3a/JFH1 chimeric viruses containing core to NS2 of HCV-3a could be used (Gottwein et al., 2007; Pietschmann et al., 2006).

How HCV causes steatosis is not well understood. Previous studies and the results presented here indicate a critical role of HCV core protein in modulating lipid metabolism (Abid et al., 2005; Hourioux et al., 2007; Jackel-Cram et al., 2007). Other HCV proteins, such as NS2, NS4B, and NS5A have also been shown to be able to modulate lipogenic gene expression (Oem et al., 2008; Park et al., 2009; Waris et al., 2007). Future studies should determine whether different HCV proteins modulate lipid metabolism through different mechanisms.

In summary, our results further elucidate the molecular mechanisms of steatosis associated with HCV infection by demonstrating an important role of PI3K–Akt-2 pathway in mediating the effects of the HCV-3a core protein on the activity of SREBP-1, a master transcription factor in lipid metabolism. This study may provide rationale for developing effective therapeutics against hepatitis C by targeting PI3K–Akt-2 pathway especially in light with promising progress being made in the field of developing small inhibitors for PI3K and Akt (Engelman, 2009; Yap et al., 2008).

**METHODS**

**Plasmids, antibodies and inhibitors.** Plasmid pSRE-Luc containing three copies of SRE sequences was kindly provided by Dr Shimano (Amemiya-Kudo et al., 2002). To generate HCV-3a core/1b replicon, HCV-3a core coding sequence (Jackel-Cram et al., 2007) was cloned in place of the 1b core in the 1b replicon plasmid pHCV N Neo C-5B (Ikeda et al., 2002) through PCR-mediated recombination (Fang et al., 1999) (Fig. 1a). To perform HCV transient replication assays, the neomycin phosphotransferase (Neo) coding sequence in HCV-1b and HCV-3a core/1b replicon constructs was replaced by the firefly luciferase gene through molecular cloning. To generate a plasmid expressing mature SREBP-1a with a Flag-tag at the amino terminus, the coding sequence of aa 1–517 of human SREBP-1a (Shimano et al., 2008; Park et al., 2009; Hourioux et al., 2007; Waris et al., 2007; Jackel-Cram et al., 2007) was cloned into the pCMV2-Flag vector (Sigma). All plasmids were confirmed by DNA sequencing.

HCV core antibody was purchased from Anogen and NS5B antibody was obtained from Dr Wilson (Saskatoon, Canada). SREBP-1 antibody was obtained from Santa Cruz Biotechnology. Antibodies for Akt, Akt-2, phospho-Ser Akt and β-actin were purchased from Cell Signaling Technology. Anti-Flag monoclonal antibody was from Sigma. Phospho-Serine antibodies were purchased from Enzo Life Sciences. Inhibitor Ly294002 was purchased from EMD Biosciences. Calyculin A, a serine/threonine phosphatase inhibitor, was from Cell Signaling Technology.

**Cell culture, transfections and generation of the HCV-3a core/1b chimeric replicon.** Huh-7 cells were transfected by using the

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calcium phosphate precipitation method as described previously (Graham & van der Eb, 1973).

Huh-7 cells having an HCV-1b genomic replicon were kindly provided by Dr Lemon (Ikeda et al., 2002). To generate an HCV-3a core/1b chimeric replicon, RNA produced by in vitro transcription from linearized plasmid DNA was electroporated into Huh-7 cells. Cells were selected using G418 and the resultant colonies were examined for viral protein expression by Western blotting. An interferon-cured Huh-7 cell line was used as a control cell line for replicon experiments.

**Western blotting and immunoprecipitation analysis.** Huh-7 cells were harvested 48 h after transfection with Cell Lysis Buffer (Cell Signaling Technology) containing 10 μM PMSF according to the manufacturer’s protocol. For immunoprecipitation of Flag-tagged SREBP-1a protein, cells were treated with 100 nM Calyculin A for 1 h prior to lysis as per the manufacturer’s instructions. The cells were then lysed with RIPA buffer consisting of 150 mM NaCl, 10 mM Tris, pH 7.8, 1% Triton-X, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and 100 mM Calyculin A. Cell lysates were incubated with an anti-Flag antibody or normal IgG (as a negative control) followed by addition of protein G–Sepharose (GE Healthcare). After washing with RIPA buffer for four times, the immunoprecipitates were collected by centrifugation and dissolved in an SDS protein loading buffer. Proteins were subjected to SDS-PAGE, blotted onto PVDF membranes, blocked in 5% skimmed milk in PBS or 3% BSA plus 1% PEG-8000, and placed in primary antibody overnight at 4°C. Blots were washed and incubated with a secondary antibody (Li-Cor) for 1 h and then washed again. Blots were then scanned using a Li-Cor Odyssey scanner at appropriate wavelengths and fluorescent intensity of the protein bands was quantified by using the Odyssey software. For Western blotting with phospho-Ser antibodies, the membrane was incubated with a pool of six phospho-Ser antibodies (Phosphoserine Detection Set; Enzo Life Sciences) followed by biotin-conjugated goat anti-mouse IgM (Caltag Laboratories). After a further incubation with peroxidase-conjugated Streptavidin (Jackson ImmunoResearch Laboratories), the membrane was developed with the ECL Advance Detection reagents (GE Healthcare). Band intensities were determined by the Quantity One software (Bio-Rad).

**Luciferase assays.** Cells were plated at 4 × 10^4 per well of 24-well plates and cultured overnight. Cells were transfected with a maximum of 1.05 μg plasmid, including a Renilla luciferase internal control plasmid. PI3K inhibitor LY294002 was added when indicated. Cells were harvested in Passive Cell Lysis Buffer and luciferase activity was assayed with the Dual Luciferase Assay system (Promega). Luciferase levels were normalized to the level of Renilla luciferase activity and statistical significance was determined by a Student’s t-test.

Replication of HCV genomic replicons was determined by luciferase assay after electroporation of luciferase-containing replicon RNAs into Huh-7 cells as described previously (Lohmann et al., 2003).

**Generation of Akt-2 knockdown cells.** Akt-2-specific shRNA lentiviral construct with a puromycin resistance gene (Open Biosystems) was co-transfected with lentivirus packaging plasmids into HEK293T cells (Brownlie et al., 2009). The resulting lentiviral particles were used to infect Huh-7 cells. At 24 h post-infection, 2.5 μg puromycin ml⁻¹ was added to select for stably transduced cells. Akt-2 knockdown was confirmed by Western blotting. A control shRNA against GAPDH was used to generate control cells.

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