Activation of sterol regulatory element-binding protein 1c and fatty acid synthase transcription by hepatitis C virus non-structural protein 2

Jae-Ku Oem,† Candice Jackel-Cram,† Yi-Ping Li,† Yan Zhou,† Jin Zhong, Hitoshi Shimano, Lorne A. Babiuk, and Qiang Liu

1Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E3, Canada
2Institut Pasteur of Shanghai, Shanghai, PR China
3University of Tsukuba, Tsukuba City, Ibaraki, Japan
4University of Alberta, Edmonton, Alberta, Canada

Received 2 October 2007
Accepted 9 January 2008

Sterol regulatory element-binding protein 1c (SREBP-1c) is a member of the basic helix–loop–helix leucine zipper family of transcription factors (Horton et al. 2002; Eberle et al., 2004). SREBP-1c expression is regulated at the transcription level (Desvergne et al., 2006). For instance, liver X receptor (LXR) activates SREBP-1c transcription by binding to the LXR element (LXRE) sequences in the SREBP-1c promoter (Yoshikawa et al., 2001; Tarling et al., 2004; Dif et al., 2006). SREBP-1c can also regulate its own transcription in a positive feed-back loop through binding to the sterol regulatory elements (SREs) in the SREBP-1c promoter (Amemiya-Kudo et al., 2000). The newly synthesized, precursor SREBP-1c protein is bound to the endoplasmic reticulum (ER). Following proteolytic digestions, the amino-terminal domain is released and transported to the nucleus as an active transcriptional factor (Brown et al., 2000). The processed, mature SREBP-1 proteins are modified by phosphorylation by protein kinases, such as mitogen-activated protein kinase (MAPK), protein kinase A (PKA) and glycogen synthase kinase-3 β (Kotzka et al., 1998; Roth et al., 2000; Lu & Shyy, 2006; Punga et al., 2006). The impact of phosphorylation on the transcriptional activity of SREBP-1 is less clear. For instance, phosphorylation by MAPK appears to enhance the transcriptional activity of SREBP-1, whereas PKA phosphorylation suppresses the function of SREBP-1 (Kotzka et al., 1998, 2000; Lu & Shyy, 2006). A major function of SREBP-1c is to activate genes involved in the synthesis of fatty acid and their incorporation into triglycerides and phospholipids (Horton et al., 2002; Eberle et al., 2004). As such, abnormal higher levels of SREBP-1c will result in lipid accumulation in the liver and cause steatosis (Ferre & Foufelle, 2007). Fatty acid synthase (FAS) is a well established target gene of SREBP-1c (Latasa et al., 2000; Amemiya-Kudo et al., 2002).

Steatosis is an important clinical manifestation associated with hepatitis C virus (HCV) infection (Asselah et al., 2006). The development of steatosis in HCV infections is a complex process that likely involves both host and viral factors. As a major transcription factor for lipogenic gene

†These authors contributed equally to this work.

†Present address: National Veterinary Institute, Technical University of Denmark, Høje Taastrup, Denmark.

expression, SREBP-1 may play a major role in this process. For instance, HCV infection enhances the proteolytic processing of SREBP-1 (Waris et al., 2007). The expression of HCV core and non-structural protein 4B (NS4B) proteins also enhances SREBP processing and lipid accumulation (Yamaguchi et al., 2005; Waris et al., 2007; Kim et al., 2007). Our previous research showed that HCV core protein can activate the FAS promoter in an SREBP-1-dependent manner (Jackel-Cram et al., 2007). Given the key role of SREBP-1c in hepatic steatosis, we investigated whether HCV NS2 protein may regulate SREBP-1c expression and thus be involved in causing steatosis. In this study, we showed that HCV NS2 protein increases SREBP-1c transcription, protein expression and proteolytic processing. We further showed that FAS transcription is also upregulated by HCV NS2, as a consequence of SREBP-1c activation. Our results suggest that HCV NS2 is a contributing factor for HCV-associated steatosis.

The coding sequence of NS2 of HCV H77 (genotype 1a) was amplified from plasmid p90/FL (Kolykhalov et al., 1997) by PCR and cloned into an expression vector, pEF-myc, with the elongation factor-1α promoter (Invitrogen). The resultant NS2 protein had a translation initiation codon and a myc-tag at the carboxyl terminus (Fig. 1). The plasmid sequence was confirmed by DNA sequencing. To demonstrate NS2 expression, Huh-7 cells were transfected with pEF-NS2-myc and pEF-myc vector by the calcium phosphate method followed by immunoblotting as described previously (Jackel-Cram et al., 2007). As shown in Fig. 1, a myc-tag antibody (Invitrogen) recognized a protein of approximately 23 kDa after pEF-NS2-myc transfection, but not after vector transfection, indicating the expression of the myc-tagged NS2 protein.

To investigate whether HCV NS2 protein can influence SREBP-1c transcription, we measured SREBP-1c promoter activity by using an SREBP-1c promoter–luciferase reporter plasmid. For this purpose, Huh-7 cells were transfected with HCV NS2-expressing plasmid or the vector control, together with a human SREBP-1c promoter–luciferase reporter plasmid (SREBP-1c-571-Luc-WT, Fig. 2a), kindly provided by Dr Lefai (Dif et al., 2006). A plasmid encoding the Renilla luciferase gene under the control of the elongation factor-1α promoter was also included in the transfections as a control for luciferase assays as described previously (Oem et al., 2007). As shown in Fig. 2(b), expression of HCV NS2 resulted in more than fourfold induction in SREBP-1c promoter activity in comparison to vector control [Fig. 2b, RLU: 207 ± 64 and 1036 ± 143 for vector and NS2, respectively, $P=0.006$].
(Student’s t test), suggesting that HCV NS2 activates SREBP-1c promoter. The human SREBP-1c -571 promoter sequence contains two LXREs (at positions -311/-296 and -260/-245) and two SREs (at positions -228/-218 and -127/-117) (Fig. 2a) (Tarling et al., 2004; Dif et al., 2006). To determine whether the LXRE and SRE sequences are involved in SREBP-1c promoter upregulation by HCV NS2, the two LXRE and two SRE sequences were eliminated by site-directed mutagenesis as described previously (Dif et al., 2006). The primer sequences were as follows (the mutated sequences are underlined): 5'-GAG-GGCCAGAGTCCGCCAGATTCCCCGGCA-3' and 5'-GGGAGAAGTCCGCTAGATTCCCCAACCC-3' for LXRE; 5'-CCATTCAAGCGCCGAGATAAAACTCGAG-CCCC-3' and 5'-GGCCGGCCGCCTATCTCATGGCCGGCCGC-3' for SRE. These mutant SREBP-1c
promoter plasmids were confirmed by DNA sequencing and then used in co-transfection experiments to determine the SREBP-1c promoter activity after HCV NS2 expression. As shown in Fig. 2(c), elimination of either LXRE or SRE motifs in the SREBP-1c promoter resulted in significantly lower SREBP-1c promoter activity in comparison to the wild-type promoter after HCV NS2 expression (Fig. 2c, RLU: 1070 ± 63, 557 ± 31 and 432 ± 52 for wild-type, ΔLXREs and ΔSREs, respectively; wild-type versus ΔLXRE, \( P = 6.7 \times 10^{-6} \); wild-type versus ΔSRE, \( P = 2.2 \times 10^{-6} \)). Elimination of both LXRE and SRE motifs in the SREBP-1c promoter further decreased SREBP-1c promoter activation by HCV NS2 (Fig. 2c; RLU: 180 ± 21 for ΔLXRE/ΔSRE; wild-type versus ΔLXRE/ΔSRE, \( P = 4.9 \times 10^{-7} \); ΔLXRE versus ΔLXRE/ΔSRE, \( P = 3.2 \times 10^{-6} \) and ΔSRE versus ΔLXRE/ΔSRE, \( P = 1.5 \times 10^{-4} \)). These results demonstrate that the LXRE and SRE motifs in the SREBP-1c promoter are required for SREBP-1c promoter activation by HCV NS2.

To directly demonstrate whether HCV NS2 could activate transcription driven by LXRE or SRE motifs, we used luciferase reporter plasmids containing two copies of the LXRE sequences (TGACCGGCAGTAACCC, pLXRE-Luc) (Amemiya-Kudo et al., 2002) or three copies of the SRE sequences (ATTCACCCAC, pSRE-Luc) (Amemiya-Kudo et al., 2002). As shown in Fig. 2(d), expression of HCV NS2 was associated with 2.8- or 5.2-fold induction of LXRE- or SRE-driven luciferase expression in comparison to vector control. Taken together, these results indicate that HCV NS2 increases SREBP-1c transcription through SRE and LXRE elements in the SREBP-1c promoter.

To demonstrate whether increased SREBP-1c transcription by HCV NS2 resulted in enhanced SREBP-1c protein expression and proteolytic cleavage, the amount of SREBP-1c protein was determined by immunoblotting using the lysates of Huh-7 cells transfected by plasmid expressing HCV NS2 or the plasmid vector control and an SREBP-1c protein was determined by immunoblotting using the lysates of Huh-7 cells transfected by plasmid expressing HCV NS2 or the plasmid vector control and an SREBP-1c-specific antibody (Santa Cruz Biotechnology). As protein loading controls, the levels of \( \beta \)-actin were also determined using a \( \beta \)-actin-specific antibody (Cell Signaling Technology). Quantification of the density ratio of the precursor and mature SREBP-1 proteins to \( \beta \)-actin within the same sample was performed using an Odyssey Infrared Imaging System (LI-COR Biosciences). As shown in Fig. 2(e), HCV NS2 expression was associated with increased levels of both precursor and mature SREBP-1 proteins. These results demonstrate that HCV NS2 enhances SREBP-1 protein expression and proteolytic cleavage.

Since FAS is a target gene of SREBP-1c, we were interested in determining whether HCV NS2 could also activate FAS transcription. For this purpose, the FAS transcript level was determined by RT-PCR. Total RNA was extracted from Huh-7 cells 48 h after transfection with a plasmid expressing HCV NS2 or the plasmid vector by Trizol (Invitrogen) followed by a clean-up with RNaseasy mini-columns (Qiagen). After digestion with an RNase-free DNase I (Ambion), cDNA was synthesized by reverse transcription and subjected to PCR amplification using FAS-specific primers (forward, 5'-GGCCGAGAGAGTGGCTTGAG-3' and reverse, 5'-AATTGGCAGGCGTAGTTG-3'). As a control, \( \beta \)-actin was amplified with specific primers (forward, 5'-ACGGGTACATGTTGGTGCCG-3' and reverse, 5'-CAGGGTACATGTTGGTGCCG-3'). The PCR products were resolved in agarose gels and analysed by densitometry using the software Quantity One (Bio-Rad). As shown in Fig. 3(a), NS2 expression was associated with threefold induction of FAS transcripts in comparison to vector control. This was confirmed by luciferase assay results when a FAS promoter–luciferase reporter plasmid (Swinnen et al., 1997; Jackel-Cram et al., 2007) was used to measure FAS promoter activity after NS2-expressing vector plasmid transfection (Fig. 3b; RLU: 93 ± 4 and 205 ± 8 for vector and NS2, respectively, \( P = 0.002 \). Furthermore, when the SREBP-1c-binding element (SRE) was deleted from the FAS promoter, HCV NS2 was no longer able to activate the FAS promoter (Fig. 3b; RLU: 205 ± 8 and 16 ± 1 for NS2 + FAS-wild-type and NS2 + FAS-ΔSRE, respectively, \( P = 0.0007 \)), suggesting FAS promoter upregulation by HCV NS2 is through the SRE sequence. To further confirm the role of SREBP-1c, we used a dominant-negative (DN) SREBP-1 plasmid in a co-transfection experiment as described previously (Jackel-Cram et al., 2007). As shown in Fig. 3(c), while plasmid vector did not have an effect on FAS activation by HCV NS2, transfection of DN-SREBP-1 significantly abolished FAS activation by HCV NS2. These results indicated that HCV NS2 enhances FAS transcription in an SREBP-1-dependent manner, probably as a consequence of SREBP-1c activation.

The functions of HCV NS2 protein are not well understood since limited studies have been performed to understand the role of NS2 in HCV–host interactions. In this study, we showed that HCV NS2 may be a contributing factor to intracellular lipid accumulation by upregulating the transcription of SREBP-1c through LXRE and SRE motifs in the SREBP-1c promoter. We further showed that HCV NS2 protein expression resulted in increased levels of precursor and processed SREBP-1 proteins. As a target gene of SREBP-1c, we showed that HCV NS2 can activate FAS transcription in an SREBP-1-dependent manner. These results indicate that HCV NS2 enhances SREBP-1c functional activity.

Since phosphorylation of SREBP-1c may also play a role in modulating its activity, we attempted to characterize whether HCV NS2 alters SREBP-1c phosphorylation. However, we did not detect any discernible changes in SREBP-1c phosphorylation after HCV NS2 expression (data not shown). Since it has been demonstrated that HCV infection increases SREBP-1c phosphorylation (Waris et al., 2007), it would be interesting to determine which HCV proteins are responsible for the enhanced SREBP-1c phosphorylation.
The exact mechanisms of how HCV NS2 activates SREBP-1c are not clear. HCV NS2 is localized in the ER with no nuclear localization (Kim et al., 1999; Franck et al., 2005). This suggests that HCV NS2 itself is unlikely to be a transcriptional factor that can directly activate SREBP-1c transcription. Further investigations are required to study this issue.

Our experimental approach was to express HCV NS2 protein in Huh-7 cells after plasmid transfection. It would be interesting to study the modulation of lipid metabolism by HCV NS2 protein in the context of HCV infection. However, this might not be readily achievable since NS2 is essential for HCV virus morphogenesis (Pietschmann et al., 2006; Yi et al., 2007).

Fig. 3. Activation of FAS transcription through SREBP-1c by HCV NS2 protein. (a) Activation of FAS transcription by HCV NS2. FAS transcript levels were determined by RT-PCR using FAS-specific primers in Huh-7 cells 48 h after transfection with a plasmid expressing HCV NS2 or the vector control. As a control, the level of β-actin was also determined. The PCR products in the upper panel were analysed by agarose gel electrophoresis followed by densitometry analysis shown in a graph in the lower panel. The statistical differences were demonstrated as * if $P \leq 0.05$. (b) Activation of FAS promoter by HCV NS2. Huh-7 cells were transfected with HCV NS2-expressing plasmid or the vector control, together with wild-type or SRE-deleted FAS promoter–luciferase reporter plasmids. Luciferase assay was performed 48 h after transfection to determine the FAS promoter activity. The statistical differences were demonstrated as ** if $P \leq 0.01$ or *** if $P \leq 0.001$. (c) DN-SREBP-1 abrogated FAS activation by HCV NS2. Huh-7 cells were transfected with a plasmid expressing HCV NS2 or the plasmid vector, a wild-type FAS promoter–luciferase reporter plasmid, together with pcDNA3.1(+) vector or a plasmid expressing DN-SREBP-1. Luciferase assay was performed 48 h after transfection. The statistical differences were demonstrated as * if $P \leq 0.05$ or ns for not significant.
In conclusion, our study identified a novel functional role of HCV NS2 protein in modulating lipid metabolism, which increased our understanding of the molecular mechanisms of HCV-associated steatosis.

Acknowledgements

We thank Drs E. Lefai and R. Bartenschlager for providing plasmids and cell lines. This work was supported by the Banting Research Foundation, John Delfrari Research Trust and Canadian Liver Foundation. C.J.-C. is a recipient of a CHF Doctoral Research Award. This paper is published as VIDO Manuscript #487.

References


