Role of seipin in lipid droplet morphology and hepatitis C virus life cycle

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Infectious hepatitis C virus (HCV) particle assembly starts at the surface of lipid droplets, cytoplasmic organelles responsible for neutral fat storage. We analysed the relationship between HCV and seipin, a protein involved in lipid droplet maturation. Although seipin overexpression did not affect the total mean volume occupied by lipid droplets nor the total triglyceride and cholesterol ester levels per cell, it caused an increase in the mean diameter of lipid droplets by 60%, while decreasing their total number per cell. The latter two effects combined resulted in a 34% reduction of the total outer surface area of lipid droplets per cell, with a proportional decrease in infectious viral particle production, probably due to a defect in particle assembly. These results suggest that the available outer surface of lipid droplets is a critical factor for HCV release, independent of the neutral lipid content of the cell.

Hepatitis C virus (HCV) is a major human pathogen whose life cycle is tightly dependent on the hepatocyte lipid metabolism (Syed et al., 2010). Lipid droplets (LDs) are neutral lipid storage organelles required for efficient HCV production (Miyanari et al., 2007). The HCV non-structural protein 5A (NS5A) is associated with the core protein at the LD surface and is supposed to coordinate virion assembly (Bartenschlager et al., 2011). Recently, DGAT1, an essential enzyme for LD formation, has been described as a key factor for HCV assembly (Camus et al., 2013; Herker et al., 2010). On the other hand, HCV often leads to the appearance of large cytoplasmic LDs, a pathological feature known as steatosis (Rubbia-Brandt et al., 2000). LDs store triglycerides (TG) and cholesterol esters (CE). Fatty acids can stimulate or inhibit HCV replication, depending on the extent of their saturation: saturated and mono-unsaturated fatty acids increase while poly-unsaturated fatty acids impair HCV replication (Kapadia & Chisari, 2005), although these data have not been confirmed in all studies (McPherson et al., 2008; Wu et al., 2011). FAS inhibition by small molecules can block HCV replication (Yang et al., 2008). In contrast, nystatin, which activates SREBP, induces a dose-dependent increase of viral replication (Su et al., 2002). Lipids are involved in several steps of the viral life cycle, e.g. as prosthetic groups of membrane proteins, as structural components of membrane microdomains where replication takes place, and as constituents of secreted lipoviroparticles (Bartenschlager et al., 2011). However, whether the occurrence of large LDs, such as seen in steatosis, benefits the viral life cycle remains to be proven.

Seipin, encoded by BSCL2, has been associated with LD maturation (Fei et al., 2008; Magré et al., 2001). The anomalous expression of this protein is associated with various diseases including congenital generalized lipodystrophy type 2 (Agarwal & Garg, 2004). Seipin is a resident protein of the endoplasmic reticulum (ER), located at the junction between ER and LDs (Szymanski et al., 2007).

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Although the exact function of seipin remains unclear, studies have suggested a role in cell differentiation of adipocytes as well as in controlling the LD morphology, i.e. in regulating their size (Fei et al., 2008; Szymanski et al., 2007). We hypothesized that seipin may play a role in the biogenesis of HCV particles. Therefore, we first investigated the effect of seipin overexpression on LD morphology and lipid content in hepatocytes. Lentivectors expressing the seipin isoform 2 (BSCL2, GenBank accession number BC093048) were constructed as previously described (Clément et al., 2010) using pSPORT6.1-BSCL2 (Openbiosystem) as a template. The lentiviral particles, produced by transient transfection in HEK293T cells (Dull et al., 1998) and concentrated 120-fold by ultracentrifugation (20000 g for 90 min at 4 °C) were used to transduce the Huh-7 hepatoma cell line. Untransduced cells or cells transduced with a GFP-encoding lentivector were used as controls. Overexpression of seipin was verified at the mRNA level by real-time quantitative PCR (RT-qPCR) (Fig. 1a, primers in Table S1, available in JGV Online) and at the protein level by both immunoblot and immunofluorescence on paraformaldehyde-fixed cells using an anti-seipin antibody (kind gift of M. Daisuke Ito, Japan) (Fig. 1b, c). Seipin mRNA was detectable in untransfected

![Seipin overexpression decreases HCV replication](http://vir.sgmjournals.org)
(a) 

(b) 

(c) 

(d) 

(e) 

(f) 

(g) 

(h) 

(i) 

Control Seipin Seipin

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Control Seipin Seipin

NS

Control Free cholesterol Cholesterol esters Oleic acid (50 μm) Seipin

Control Seipin

Oleic acid (50 μm)
Huh-7 cells using cDNA synthesized from 1 µg total RNA as starting material. Seipin expression was 10 times higher in liver samples of normal individuals as compared to untransfected Huh-7 cells (Table S2, Fig. 1a), corroborating the results previously published by Windpassinger et al. (2004) demonstrating, by Northern blot, that the liver expresses seipin transcripts. Both RT-qPCR and immunoblot analyses showed an increase in seipin expression in transduced cells. By immunoblot, we detected a ~50 kDa band corresponding to the molecular mass expected for seipin and several additional higher molecular mass bands (highlighted in Fig. 1b by the brace), probably corresponding to previously reported post-translationally modified forms of the protein (Ito & Suzuki, 2007; Windpassinger et al., 2004). However, seipin was not detected in control Huh-7 cells at the protein level (probably below the sensitivity threshold of the method, Fig. 1b, c). Double staining with anti-calreticulin (Abcam), an ER marker, showed partial colocalization of seipin with calreticulin, as demonstrated by the presence of yellow pixels on the overlay image (Fig. 1d). Since an overload of the ER membrane can cause ER stress, we investigated whether seipin overexpression could activate the unfolded protein response. Seipin overexpression did not induce the upregulation of the chaperones BiP and CHOP (antibodies from Cell Signalling). Similarly, the amplification of XBP-1 mRNA by PCR (94 °C for 30 s; 58 °C for 30 s and 72 °C for 1 min, 30 cycles) using specific primers (provided by F. Duong, Basel, Switzerland, leading to the amplification of two fragments of 398 bp and 424 bp representing, respectively, spliced and unspliced XBP-1) indicated that seipin overexpression did not induce the splicing of XBP-1 mRNA (Fig. S1a). In addition, the overexpression of seipin had no impact on cell proliferation (Fig. S1b).

Then, the effect of seipin overexpression on LD morphology was assessed by staining of neutral lipids with oil-Red-O (ORO). As shown in Fig. 2(a), seipin overexpression induced a dramatic increase in the size of the LD (characterized by the presence of adipose differentiation-related protein (ADRP) at their periphery; Fig. S1c), while decreasing the overall number of LDs per cell. Detailed morphometric analyses, using the Metamorph software, showed that seipin overexpression induced (i) a ~60% decrease in the number of LDs per cell (Fig. 2b), and (ii) a ~50% increase of the maximum radius of each LD (Fig. 2c). As a result of these two modifications combined, we estimated that the overall outer surface area of LDs per cell was significantly decreased by ~34% (Fig. 2d); while the total volume occupied by LDs in each cell was not modified, a small, non-significant increase was seen (Fig. 2e). We then investigated the expression of three different members of the PAT family (perilipin 5, ADRP and TIP47) in both control and seipin-overexpressing cells (Fig. 2f, g). While seipin overexpression had no effect on perilipin 5 mRNA level, it had opposing effects on TIP47 and ADRP, i.e. it induced an increase of TIP47 mRNA and a decrease of ADRP mRNA (Fig. 2f), in agreement with the observation that a synthetic peroxisome proliferator-activated receptor (PPAR)-α agonist increased ADRP gene expression, but not that of TIP47 (Dalen et al., 2006), indicating that these two genes are not regulated by the same transcription factors. In this context, it is noteworthy that seipin appeared to be a negative regulator of cyclic AMP (cAMP)-dependent protein kinase A (PKA) (Chen et al., 2012), known to activate some transcription factors such as the PPAR subfamily (Lazenec et al., 2000). These changes in mRNA expression were only confirmed for ADRP at the protein level (Fig. 2g), nicely matching the fact that, in seipin-overexpressing cells, the LD surface area is decreased. Indeed, it has been demonstrated that ADRP is degraded through the proteasome pathway when not associated with the LD membrane (Masuda et al., 2006). More importantly, measurement of cellular TGs and CEs, using respectively the GPO/PAP kit (Roche) and the cholesterol/cholesteryl ester quantification kit (Calbiochem), did not show any significant difference in seipin-overexpressing vs control cells, whereas, as expected, oleic acid treatment significantly increased TGs (Fig. 2h, i). Altogether, these data are consistent with the potential role of seipin in LD fusion, as suggested by observations on fibroblasts isolated from a patient with a nonsense mutation of seipin (Szymanski et al., 2007). However, the effect of seipin overexpression in Huh-7 cells seemed to vary with respect to that recently observed in 3T3 or HeLa cells (Fei et al., 2011), where seipin appeared to interfere with both TG biosynthesis and LD formation. This discrepancy may be explained by the fact that seipin has multiple functions depending on the cell type (Cartwright & Goodman, 2012). Another important difference may relate to the culture media conditions used, since we investigated the effect of seipin overexpression in the absence of exogenous fatty acids in the medium. Indeed, contribution of medium composition has also been demonstrated in yeast (Fei et al., 2008; Wolinski et al., 2011).

To evaluate whether changes in LD morphology had an impact on the HCV life cycle, control and seipin-overexpressing...
Huh-7 cells were infected with HCV particles at an m.o.i. of 1, produced by electroporation of Huh-7.5 cells (kind gift of C. Rice, New York) with HCV RNA transcribed from pFK-J6/C3 (Jc1) (from R. Bartenschlager, Heidelberg, Germany) in cytomix using a Gene Pulser II electroporator (260 V, 950 μF; Bio-Rad Laboratories) and titrated by infecting naive Huh-7.5 cells as described (Pietschmann et al., 2006). Forty-eight hours post-infection, we assessed both the intracellular content of HCV core protein by immunoblot and the relative number of intracellular HCV RNA copies by RT-qPCR (primers in Table S1). Seipin overexpression did not significantly modify the intracellular HCV core protein level (Fig. 3a, b). In addition, both core and NS5A displayed a punctated pattern (Fig. S2), in agreement with previous reports (Boson et al., 2011).

**Fig. 3.** Effect of seipin overexpression on HCV replication and particle production. Control and seipin-overexpressing cells were infected with Jc1 viral particles. Intracellular proteins and RNA were collected 48 h after infection to assess, respectively, the level of HCV core protein expression by immunoblot (a, b) and the relative number of intracellular HCV RNA copies by RT-qPCR (c). Virion secretion was measured by RT-qPCR (c); in parallel, viral infectivity (as TCID<sub>50</sub> ml<sup>–1</sup>) of both intracellular and extracellular HCV particles was measured (d). (e) Cells were transduced with HCVpp. Forty-eight hours post-transduction, a luciferase assay was performed. VSV-Gpp entry was evaluated as control. (f) Control and seipin-overexpressing cells were transfected with the subgenomic replicon in vitro transcript. Luciferase activity was measured at 48 h post-transfection. Results are represented as mean ± SD *P<0.05; **P<0.01; ***P<0.001.
Colocalization analysis, using the colocalization module of the Zen software (Zeiss) demonstrated no colocalization of the core protein with LDs in both cell types [colocalization coefficients (mean ± SEM): 0.058 ± 0.015 for control cells and 0.055 ± 0.037 for seipin-overexpressing cells]. However, NS5A was significantly more colocalized with LDs in control cells as compared to seipin-positive cells (0.15 ± 0.041 vs 0.030 ± 0.005, P = 0.049), although the colocalization coefficients were rather low (value range: 0–1; 0: no colocalization, 1: all pixels colocalized). The intracellular HCV RNA level decreased (Fig. 3c). Intracellular particles were collected by three cycles of freeze and thaw and used to investigate their infectivity by TCID₅₀ ml⁻¹ calculation. Intracellular infectivity levels were also reduced (Fig. 3d), suggesting that seipin overexpression has a negative impact on HCV assembly. The effect of seipin overexpression on HCV viral particle egress was investigated by measuring the number of virions released by control and seipin-transduced cells. We thus measured the relative number of Jc1 RNA copies in the culture supernatants of Jc1-infected cells. In parallel, the supernatants were titrated as described above. Seipin overexpression decreased viral particle secretion and HCV infectivity by 40% and 60%, respectively (Fig. 3c, d). Overall, this effect was independent of the conditions used for the infection assay (m.o.i. used to infect cells; time points post-infection) (Fig. S3). However, we did not observe any effect of seipin overexpression on either JFH1 viral particle production (Wakita et al., 2005) or JFH1 core localization, despite the fact that this viral strain is believed to rely more on LD (Fig. S4). One explanation for this unexpected phenomenon may be that in the case of JFH1, the core protein localization at the LD surface could result in its sequesteration, thus explaining why JFH1 produces fewer infectious particles than Jc1, as previously suggested (Shavinskaya et al., 2007). We then assessed the entry of HCV pseudoparticles (HCVpp) (produced with the construct pFK_i389LucNS3 construct, supplementary methods), which are lentivectors harbouring HCV entry proteins and containing luciferase as a reporter gene. Seipin overexpression had no effect on HCVpp entry (Fig. 3e). In accordance with this observation, the transcription level of receptors implicated in HCV entry, measured by RT-qPCR (primers in Table S1), was not modified (Fig. S5). The effect of seipin overexpression on HCV replication was studied by transfecting cells with an HCV subgenomic replicon containing a luciferase reporter gene driven by the HCV IRES and allowing the determination of the efficiency of replication without any formation of viral particles (in vitro transcript produced from the pFK_i389LucNS3 construct, supplementary methods). Luciferase activity measured at 48 h post-transfection was not modified in seipin-overexpressing cells as compared to control cells (Fig. 3f), suggesting that seipin has no impact on the replication step of HCV life cycle. The apparent discrepancy between the latter result and the observation that the intracellular HCV Jc1 RNA level decreased at 48 h post-infection could be assigned to the reduced secretion of viral particles, leading to a decreased viral spread to adjacent cells, in keeping with the latent period of HCV in culture recently estimated at ~12 h (Keum et al., 2012).

In conclusion, we characterized the effect of overexpression of seipin on LD morphology in a hepatoma cell line. Our data allowed us to corroborate the hypothesis that this protein is involved in LD fusion, leading to the appearance of large cytoplasmic LDs. However, since LD fusion decreases the surface-to-volume ratio of the overall LD pool per cell, in keeping with the current model of LD physiopathology (Ohsaki et al., 2009), seipin overexpression in HCV-infected cells has a deleterious effect on HCV particle egress. This is consistent with the notion that the surface area of LDs is a critical factor influencing the efficiency of infectious virion production (Miyanari et al., 2007).

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References


