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Original Article

Hepatitis C virus core can induce lipid droplet formation in a yeast model system

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ABSTRACT — Chronic infection with the hepatitis C virus (HCV) frequently induces steatosis, which is characterized by the accumulation of lipid droplets (LDs) in hepatocytes. Steatosis is a significant risk factor for liver cancer. The HCV structural protein core is distributed on the surface of the endoplasmic reticulum (ER) and in LDs, thereby increasing LD levels. In this work, we attempt to elucidate the effect of the core protein on LD generation using yeast cells. We found that the core localized to the cytosolic surface of the ER in yeast and is able to increase LD levels when overexpressed from an inducible *GAL1* promoter for 3 hr. The effect of the core was conserved among three different HCV serotypes: 1b, 2a and 3a. While the ER stress inducer tunicamycin both elicited an unfolded stress response (UPR) and increased LD levels, the core did not induce the UPR. The RNA viral genome changes rapidly due to its high mutation rate in order to replicate under a variety of circumstances. Our observations suggest a functional analogy between core function in hepatocytes and in yeast cells and thus might be applicable to the screening of small molecules that impair the core-ER interaction.

Key words: Hepatitis C virus, HCV, Core protein, Endoplasmic reticulum, Lipid droplets, Yeast

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is a major risk factor for liver cirrhosis and hepatocellular carcinoma. Approximately 170 million individuals have been infected by HCV (Alter, 2007). More than 50% of patients chronically infected with HCV accumulate a significant lipid load in the liver (steatosis). Steatosis is a hallmark of the chronic hepatitis C pathogenesis and is characterized by the accumulation of liver lipid droplets (LDs), which form when neutral lipids, such as sterol ester and triacylglycerol, are accumulated. LDs are surrounded by a phospholipid monolayer, and are essential organelles for RNA replication and HCV particle formation (Miyanari *et al*., 2007).

HCV possesses a positive-sense single-stranded RNA genome that encodes an ~3,000 amino acid polyprotein. Translation of this polyprotein is followed by its cleavage by host signal peptidase to yield three structural proteins (core, E1 and E2) and by viral proteases to yield seven nonstructural proteins (Bartenschlager *et al*., 2004). The C-terminal region of the core protein is further processed by the host signal peptide peptidase to generate a mature core, which then enables its translocation to LDs (Okamoto *et al*., 2008). Core is thought to play an important role in HCV pathogenesis. Some core-transgenic mice progress from steatosis to hepatocellular carcinoma (Moriya *et al*., 1998), and the interactions between core and various factors involved in lipogenesis have been reported (Camus *et al*., 2014). However, the precise mechanism underlying the effect of core on the endoplasmic reticulum (ER) membrane is unknown.

Yeast *Saccharomyces cerevisiae* is a good model system for the study of neutral lipid homeostasis (Kohlwein, 2010). Previously, we established a high-throughput yeast system for screening anti-core toxic compounds and identified HSP90 inhibitors (Kubota *et al*., 2012). Expression of full-length core suppresses yeast growth, and inhibition of HSP90 reduces the stability of the nascent core protein. In this work, we attempt to elucidate the effect of core using a yeast model system.

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MATERIALS AND METHODS

Yeast strains, media, culture and strain construction

BY4742 (derived from S288C; EUROSCARF) was used as a wild-type yeast. BY4742 was transformed with *tgl2*Δ::KAN, *tgl3*Δ::KAN, *tgl4*Δ::KAN, *tgl5*Δ::KAN, *ayr1*Δ::KAN and *ldh1*Δ::KAN to disrupt each gene. Yeast cells were grown at 30°C in synthetic raffinose medium as described previously (Kubota *et al*., 2012). To induce a galactose-inducible *GAL1* promoter, we added galactose to a final concentration of 3% and cultured the cells for 3 hr.

Construction of plasmids

A cDNA region of the Core (1-177 aa) was isolated by PCR from an HCV genotype 1b (GenBank accession no. AY045702), genotype 2a (JFH1, GenBank accession no. AB047639) or genotype 3a (GenBank accession no. D17763) virus and cloned under the *GAL1* promoter of pKT10-GAL1, a multi-copy plasmid-based expression vector (Kubota *et al*., 2012). All experiments except for those indicated were carried out using genotype 1b core (pKT10-GAL-core). pRS314-UPRE-lacZ was constructed as described previously (Cox *et al*., 1993).

Subcellular fractionation of yeast organelles and trypsin treatment

Subcellular fractionation of yeast organelles was performed as described previously (Kaiser *et al*., 2002). Trypsin sensitivity was determined as follows: 50 μg P13 fraction was treated with 2 μg of trypsin for 10 min at 37°C in the presence or absence of Triton X-100, which solubilizes the membrane.

Preparation of cell lysates and western blotting

Identification of core by western blotting was performed as described previously (Kubota *et al*., 2012). We used primary antibodies specific for actin (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Kar2 (Sc-33630; Santa Cruz Biotechnology), Core (515S) and Pyk1 (our stock; Irokawa *et al*., manuscript in preparation).

β-Galactosidase assay

β-galactosidase activity was assayed using 4-methylumbelliferyl β-D-galactopyranoside (Sigma-Aldrich, St. Louis, MO, USA) as the substrate. We determined the fluorescence (450 nm) of 4-methylumbelliferone by excitation at 360 nm.

LD detection and quantitation

After induction of core expression for 3 hr, cells were collected by centrifugation (3,000 rpm for 5 min at room temperature) and fixed with Fix buffer (0.67% Difco™ yeast nitrogen base w/o amino acids, 1% formaldehyde and 1 M sorbitol) for 40 min. The cells were then washed twice with wash buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA and 1 M sorbitol). The cells were then stained with 2 μg/mL 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene (BODIPY 493/503; Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) in wash buffer for 20 min at room temperature to visualize lipid droplets and washed with phosphate buffered saline (PBS) before flow cytometry or fluorescence microscopy. Flow cytometry was performed using BD FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The LD level was represented by the mean relative fluorescent value of 10,000 cells.

Fluorescence microscopy

The samples were fixed on microscope slides in PBS. Fluorescent images of LDs were captured using GFP.S filter sets (DMIRE2; LEICA, Wetzlar, Germany) as described previously (Kubota *et al*., 2012).

Statistical analysis

Multiple independent replicates (at least three) were performed for each experiment, and data are presented as the mean of four independent experiments with the standard error of the mean (SEM). We used Student's *t*-test to determine statistical significance.

RESULTS

In mammalian cells, core pre-protein is cleaved from the HCV polyprotein by signal peptidase to yield full-length core (1-199 aa) and other HCV proteins; then, core is further processed to yield mature core (1- 177aa; hereafter we refer to mature core as "core") by signal peptide peptidase (McLauchlan *et al*., 2002) (Okamoto *et al*., 2008). In yeast cells, the latter processing step is ineffective (Kubota *et al*., 2012). Thus, we expressed cDNA encoding mature core protein. Our previous results suggested that core is colocalized with the ER protein Hmg2 in a typical ER-like shape (Kubota *et al*., 2012). To further characterize the intracellular distribution of core, we expressed core driven by the *GAL1* promoter in raffinose/galactose-containing medium for 3 hr, and prepared P13 fractions from homogenates, and the supernatants (S13) were further precipitated at 10,000 g to generate P100 and cytosol S100 fractions. As shown in Fig. 1, core was

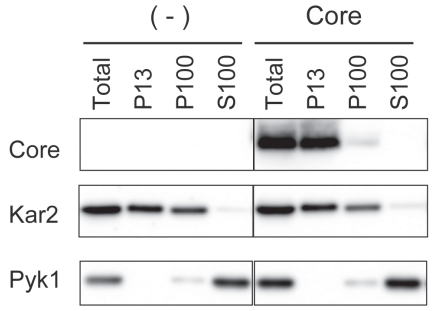


Fig. 1. Core is found in the P13 fraction. Subcellular fractionation of yeast cells expressing core as described in the text. Equal volumes of cell equivalents from crude lysate (total) and the P13, P100 and S100 fractions were mixed with SDS sample buffer. We obtained similar results from three independent experiments, and a representative result is shown. Western blotting was performed using antibodies against core, Kar2 and Pyk1. Pyk1 encodes a cytosolic pyruvate kinase.

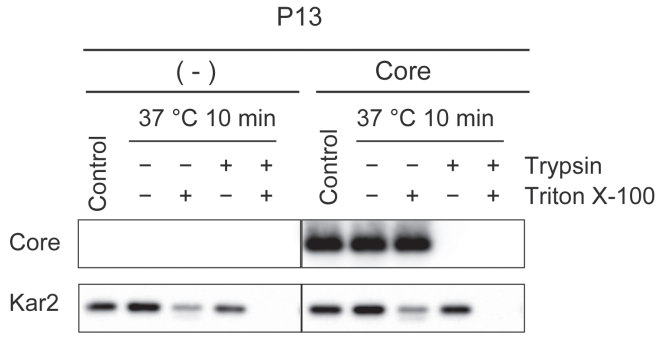
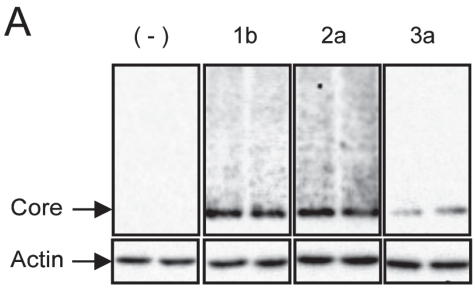
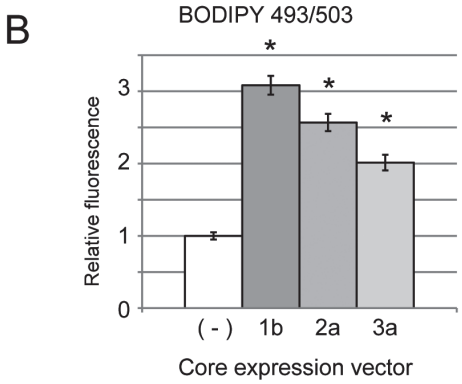


Fig. 2. Core localizes to the cytosolic surface of the ER. The P13 fraction was incubated with (+) or without trypsin (−) and in the presence (+) or absence (−) of Triton-X100. Lysates were not incubated (Control) or were incubated for 10 min at 37°C.

found mainly in the P13 fraction, which also contains the ER luminal protein Kar2. To examine whether core localized to the outside or inside of the membrane, we treated the P13 fraction with trypsin. As shown in Fig. 2, core was sensitive to trypsin. Because the ER luminal protein Kar2 was resistant to the trypsin digest in the absence of Triton-X100 but was sensitive in the presence of Triton-X100, we concluded that core in the P13 fraction localized outside the membrane. In addition to our previous result suggesting that core colocalized with Hmg2 (Kubota *et al*., 2012), this result suggests that core is distributed on the cytosolic side of ER membrane.

Next, we examined whether core could increase LD levels in yeast cells by using BODIPY 493/503, a





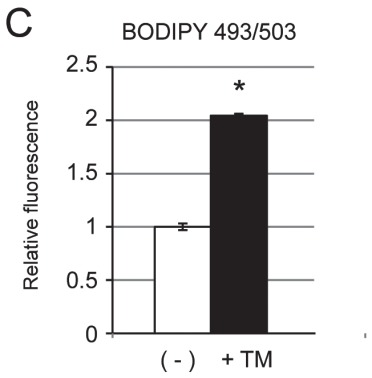


Fig. 3. Core increases LD levels in a UPR-independent manner. (A) Expression of core from HCV genotypes 1b, 2a and 3a. Western blotting was performed using anti-core and anti-actin antibodies. (N = 2). (B) LDs in fixed yeast cells were stained with BODIPY 493/503 and analyzed by flow cytometry. Wild-type yeast cells carrying an empty vector (−) and pKT-GAL1 containing each genotype of core (1b, 2a or 3a) were cultured in medium containing raffinose/galactose for 3 hr. (C) Wild-type cells carrying control vector were treated with 2 µg/mL tunicamycin (TM) for 3 hr. The relative fluorescence of BODIPY 493/503 is shown. The data in B and C are represented as the mean +/– the standard error of the mean (N = 4). "*" indicates significance ($p < 0.01$) vs. (−).

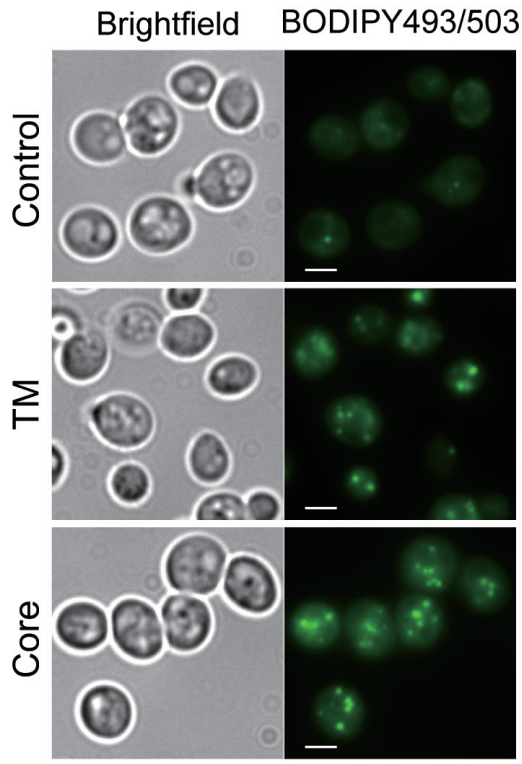


Fig. 4. Induction of punctuated LD-like structures by core. Yeast cells carrying an empty vector without (Control) and with 2 μg/mL tunicamycin (TM) as well as core expression vector (Core) were fixed and stained by BODIPY 493/503 and examined by fluorescence microscopy. Bright field images are also shown. Scale bars: 5 μm.

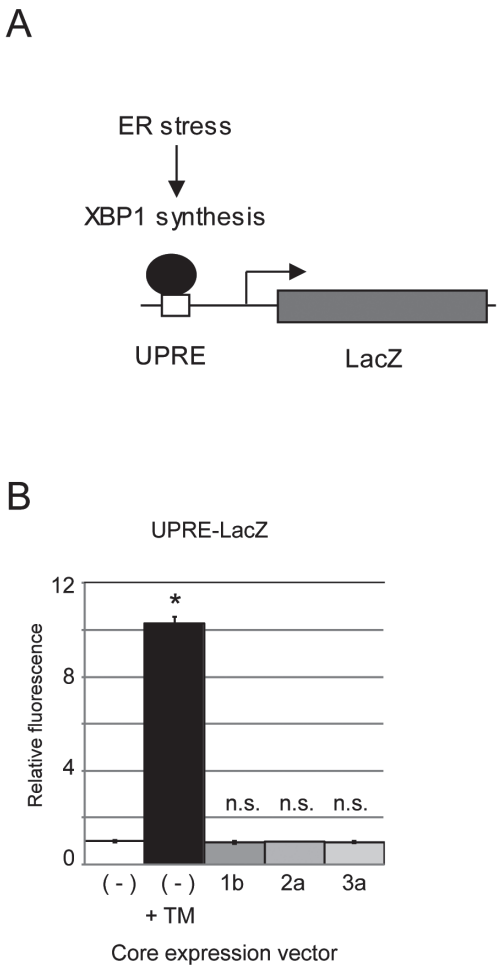


Fig. 5. Core does not induce UPRE-LacZ reporter gene expression. (A) Scheme of the UPRE-LacZ reporter gene, which is ER stress-dependent. (B) Activation of UPRE-LacZ by tunicamycin (TM) but not by core (1b, 2a, 3a). The same culture conditions used in Fig. 3 were used for cells carrying pRS314-UPRE-LacZ plasmid. The data are presented as the mean +/– the standard error of the mean (N = 4). "*" indicates significance ($p < 0.01$) vs. (–), and "n.s." indicates no significant change ($p > 0.5$).

lipophilic dye that specifically stains neutral lipids, and flow cytometry. We found that core proteins from different genotypes (1b, 2a and 3a) that were successfully expressed in yeast (Fig. 3A) induced BODIPY 495/503 fluorescence (Fig. 3B). Tunicamycin also increased the fluorescence level under our experimental conditions (Fig. 3C). We also examined LDs by fluorescence microscopy. As shown in Fig. 4, we observed an increase in punctate fluorescence.

The expression of core in human hepatoma cells induces ER stress (Benali-Furet *et al.*, 2005). Previous reports have indicated that the ER-stress inducer tunicamycin increases LD levels in yeast (Fei *et al.*, 2009) and in human hepatoma cells (Lee *et al.*, 2012). These implicate core in increasing the number of LDs in response to ER stress in a manner similar to tunicamycin. Thus, we examined whether core induces ER stress in yeast using an ER stress-dependent reporter gene (Fig. 5A; an unfolded protein response element-dependent lacZ reporter: UPRE-LacZ). As shown in Fig. 5B, tunicamycin successfully induced significant levels of ER stress, whereas all genotypes of core failed to induce ER stress in yeast cells. These results suggest that the increase in LD levels by core might be the result of an ER-stress-independent mechanism.

In mammalian cells, core impairs LD turnover by inhibiting the activity of adipose triglyceride lipase (ATGL) (Harris *et al.*, 2011). Thus, we examined the effect of core

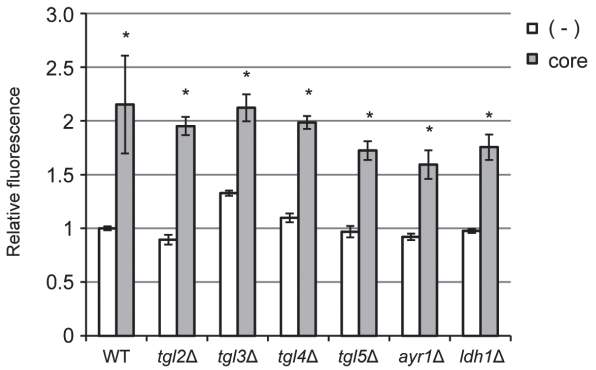


Fig. 6. Triacylglycerol lipase genes are not responsible for the core-dependent increase in LDs. Disruption yeast mutants of *TGL2*, *TGL3*, *TGL4*, *TGL5*, *AYR1* and *LDH1* were fixed, stained with BODIPY 493/503, and analyzed by flow cytometry. The data are presented as the mean +/– the standard error of the mean (N = 3). "*" indicates significance vs (−) ($p < 0.01$).

on yeast disruption mutants for each of the triacylglycerol lipase family members. As shown in Fig. 6, none of the triacylglycerol lipase (TGL) mutants affected the core-induced level of LDs.

DISCUSSION

We have utilized yeast cells as a model system to study the mechanism by which the HCV protein core increases LD levels. We found that the core localized to the cytosolic surface of the ER in yeast and is able to increase LD levels when overexpressed. The ER localization of core in yeast resembles that observed in mammalian cells. All three genotypes of core protein tested in this study caused an increase in the level of LDs but not in UPR-dependent reporter gene activation. While Benali-Furet *et al*. (2005) reported that the expression of core induces ER stress in hepatoma cells, other report indicated that the induction of core-induced ER stress is dependent on specific amino acid substitution in core from some isolates of HCV (Funaoka *et al*., 2011). RNA viruses such as HCV evolve rapidly due to their high mutation rate in order to accommodate changes in cellular function. Thus, our results suggest that the interaction between core and the ER membrane might be conserved between yeast and mammalian cells. Because we failed to isolate the lipid droplet fraction from core-expressing yeast cells – perhaps due to an insufficient level of LDs (data not shown) – we are unsure whether core localized to the surface of LDs. In mammalian cells, the localization of ATGL on the surface of LDs is perturbed by core (Camus *et al*., 2014). Thus, we examined the effect of core on triacylglycerol lipase activity. However, no TGL gene loss affected the LD level. We are currently working to identify the gene responsible for core activity.

Our result suggests that a possible core-ER interaction could result in an increase in LDs in yeast cells. Core may affect the ER membrane without affecting intra-ER protein homeostasis because the interaction did not result in ER stress. This system may be useful for the evaluation of the core-ER membrane interaction. Screening small molecules that perturb this interaction may inhibit HCV replication and pathogenesis.

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Author's contribution

Most of the experiments were performed by S. I. The initial stage of the study was completed by N. S. and J. K. Yeast disruption mutants were constructed by H. I. The initial experimental systems were established by J. O. and M. N. G-W, H. and A. N supported the experimental systems. S. K. conducted all of the experiments and wrote the paper.

Conflict of interest---- The authors declare that there is no conflict of interest.

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