HSC90 is required for nascent hepatitis C virus core protein stability in yeast cells

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ABSTRACT

Hepatitis C virus core protein (Core) contributes to HCV pathogenicity. Here, we demonstrate that Core impairs growth in budding yeast. We identify HSP90 inhibitors as compounds that reduce intracellular Core protein level and restore yeast growth. Our results suggest that HSC90 (Hsc82) may function in the protection of the nascent Core polypeptide against degradation in yeast and the C-terminal region of Core corresponding to the organelle-interaction domain was responsible for Hsc82-dependent stability. The yeast system may be utilized to select compounds that can direct the C-terminal region to reduce the stability of Core protein.

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1. Introduction

Chronic hepatitis C virus (HCV) infection causes liver cirrhosis and hepatocellular carcinoma [1]. Approximately 170 million individuals have been infected by HCV and are at risk for viral hepatitis, cirrhosis, and hepatocellular carcinoma [2]. HCV has a positive-strand RNA genome that encodes a polyprotein of ~3000 amino acids. The polyprotein is cleaved to yield the structural core, E1 and E2 polypeptides and seven non-structural poly-peptides [3]. HCV core protein exhibits RNA-binding activity and is the major component of the viral nucleocapsid [4]. During HCV polyprotein synthesis, the core protein (Core, found at the N-terminus) is cleaved by a signal peptidase, releasing a protein 191 amino acids in length (Fig. 1A). The C-terminus of Core is further cleaved by a signal peptide peptidase at the endoplasmic reticulum (ER) membrane [5], which is required for virus production [6]. Generation of the mature Core (aa 1–177, Core177) enables it to translocate to lipid droplets [7], where virion assembly is thought to occur. In addition to the direct role of the Core in viral production, expression of the Core induces lipid accumulation in hepatocytes and may be responsible for HCV-associated steatosis [8]. The Core has been implicated in the modulation of cellular processes that include lipid droplet reorganization, apoptosis, transformation, and host cell gene expression [9]. Thus, compounds that inhibit Core function or that reduce the stability of Core may also prevent the progression of HCV pathogenesis and viral production.

In this study, we established a system to screen compounds that reduce the stability of Core in the budding yeast Saccharomyces cerevisiae. We identified HSP90 inhibitors as compounds that reduce intracellular Core protein level and restore yeast growth. Our results suggest that HSC90 (Hsc82) may function in the protection of the nascent Core polypeptide against degradation in yeast and the C-terminal region of Core corresponding to the organelle-interaction domain was responsible for Hsc82-dependent stability. The yeast system may be utilized to select compounds that can direct the C-terminal region to reduce the stability of Core protein.

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2. Materials and methods

2.1. Yeast strains, media, reagents and yeast transformation

Yeast cells were grown in a synthetic raffinose (SR dropout) medium [1.67% Bacto™ yeast nitrogen base lacking aminoacids (Difco) with 2% raffinose] supplemented with 0.04 mg/ml adenine and amino acids [SR dropout; [10]] at 30 °C. S. cerevisiae strains used in this study are indicated in the Supplemental Information.

2.2. Construction of plasmids for expression of HCV Core in yeast

To induce expression of the Core in yeast, we utilized a multicycopy plasmid containing the GAL1 promoter and the GAPDH terminator region (pKT10-GAL1) and a pRS425 [11] derivative containing the same GAL1 promoter-GAPDH terminator region (pRS425-GAL1). The Core of HCV (1b) (hepatitis C virus isolate HCR6; GenBank accession no. AY045702) and pJFH1 (hepatitis C virus isolate JFH1; GenBank accession no. AB047639) were used. There are 11 amino acid differences within the D2 and the signal peptide between JFH1 and HCR6.

2.3. Screening for anti-Core chemicals

We examined the effects of various compounds isolated from microorganisms and known antibiotics (a library constructed in-house at the Kitasato Institute) as described in the Supplemental Information.
Yeast cells expressing core proteins carrying pKT10-GAL1-GFP-core177 were analyzed using confocal microscopy (Olympus FV1000). Core could be restored by treatment with certain compounds, it might provide clues regarding the cytotoxic effect of Core on yeast. The C-terminal region of Core includes a domain (D2: aa 118–171) (Fig. 1A) that is responsible for the core protein’s effect on yeast cells. The C-terminal region of Core might provide clues regarding the cytotoxic effect of Core on yeast cells. We screened various antibiotics isolated from fungi and actinomycetes and found that inhibitors of HSP90 were able to suppress Core toxicity. The compounds determined to exhibit this suppressive activity included geldanamycin (300 and 400 μg/ml), radicicol (0.5, 1 and 10 μg/ml), herbimycin A (100–400 μg/ml), and herbimycin C (100 and 200 μg/ml), but not herbimycin B (Fig. 2A). We observed that treatment with radicicol (>50 μg/ml) and herbimycin C (>300 μg/ml) had cytotoxic effects, even under galactose-free conditions, when no Core protein was produced (Fig. 2B). HSP90 inhibitors inhibit HSP90/HSC90 ATPase activity by competing with ATP for binding and thereby eliminate HSP90/HSC90 chaperone activity [19]. This chaperone activity may affect expression of the Core. The level of the Core in HCV core structure from different strains may be responsible for the core protein’s effect on yeast cells. The C-terminal region of Core includes a domain (D2: aa 118–171) (Fig. 1A) that is responsible for association with the ER and lipid droplets [5] and a Core-E1 signal peptide (aa 178–191) [17]. We examined the distribution of Core on ER in yeast. Immunofluorescent microscopy indicated that Core localized on the periphery of the nucleus (Fig. 1C). The D2 domain fused to GFP (GFP-Core) co-localized with Hmg2 as an ER marker [18] (Hmg2-mCherry; Fig. 1D) in live yeast cells (Fig. 1E) and showed a reticular fluorescence pattern and cell periphery.

2.4. Preparation of cell lysates and western blotting

We examined the Core by western blotting as described previously [12] and in the Supplemental Information. We used primary antibodies specific for actin [sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA, USA], Hsp90 [K41110, from Dr. Nemoto] [13,14], and Core (5155S) [15].

2.5. Analysis of the distribution of the Core in yeast cells using GFP-core proteins and immunofluorescence

Core in yeast were examined by indirect immunofluorescence as described previously [16] and in the Supplemental Information. Yeast cells expressing core proteins carrying pKT10-GAL1-GFP-core191 and pKT10-GAL1-GFP-core177 were analyzed using confocal microscopy (Olympus FV1000).

2.6. Statistical analysis

Multiple independent replicates (n = 3, except as indicated) were performed for each experiment, and data are presented as the mean of three independent experiments with the standard deviation (SD).

3. Results

3.1. Inhibition of yeast cell growth by expressing Core

Yeast cell growth was significantly inhibited when the full-length core of HCV 1b (aa 1–191; Core) was expressed under the control of the inducible GAL1 promoter (Fig. 1B). Core of the HCV 2a (JFH1) genotype had a similar effect, suggesting that the conserved core proteins and immunofluorescence

2.5. Analysis of the distribution of the Core in yeast cells using GFP-core proteins and immunofluorescence

Yeast cells expressing core proteins carrying pKT10-GAL1-GFP-core177 and pKT10-GAL1-GFP-core191 were analyzed using confocal microscopy (Olympus FV1000).

Fig. 2. HSP90 inhibitors reverse Core-induced growth inhibition in yeast cells. (A) Yeast cells carrying the pRS425-GAL1-core plasmid were cultured with (+) and without (−) 3% galactose plus geldanamycin, herbimycin A, herbimycin B, herbimycin C (0.1–400 μg/ml) or radicicol (0.1–100 μg/ml) as indicated for 72 h at 30 °C. As a control, cells were cultured without the antibiotics (0). (B) Growth recovery in the presence of 10 μg/ml radicicol for 36 h at 30 °C. The percentage of the growth recovery was 35%. The data shown are the mean ± S.D. of three independent experiments. (C) Effect of radicicol on the level of Core protein in yeast cells. Yeast cells carrying the pRS425-GAL1-core plasmid were cultured with (+) or without (−) 1 μg/ml radicicol in the presence of 3% galactose for 72 h at 30 °C. Photographs of immunoblots using Core-specific and actin-specific antibodies. (D) Effects of radicicol (1, 5 and 10 μg/ml) on the level of Core RNA as determined by northern blotting. Total RNA was prepared from yeast cells 2 h after induction of Core protein expression. Effects of radicicol on galactose-induced Gal4-dependent transcription of Core (Core) and GAL1 (GAL1) were examined by hybridization with probes to detect Core, GAL1 and ACT1 RNAs. Ribosomal RNA was visualized by staining with ethidium bromide.
radicicol-treated yeast cells (1 μg/mL) was 36% of control levels (Fig. 2C) whereas the level of Core mRNA did not change (Fig. 2D).

3.3. The HSC90 ortholog Hsc82 is responsible for the Core toxicity

An HSP90 family member (HSP90/HSC90) is a highly conserved molecular chaperone that can induce the maturation of various proteins, including kinases, transcription factors, and proteins, that function in signal transduction (for review, see [20]). To identify the HSP90 gene that affects Core levels, we disrupted HSP90 family member (HSP82 and HSC82) genes in yeast [21] and examined growth in the presence of Core. Disruption of HSC82 (hsc82Δ) but not of HSP82 (hsp82Δ) alleviated some of the negative effects of the Core on cell growth (Fig. 3A). The effect of HSC82 disruption was also clear in liquid culture: the growth rate of Core-expressing hsc82Δ cells was similar to that of wild-type cells and of hsc82Δ cells not expressing Core (Fig. 3B). In addition, the Core levels in hsc82Δ cells decreased to 44% of the level in control cells (Fig. 3C), but the level of Core mRNA was only slightly lower (Fig. 3D). The total levels of HSP90 family proteins (Hsp82 and Hsp82) decreased to 18% of the levels in control cells (Fig. 3C, compare lanes 1 and 2 of the left panel) after disruption of the HSC82 gene, indicating that the principal HSP90 family protein (at steady state) in yeast is Hsc82, as previously reported [21]. Furthermore, neither heat-inducible Hsp82 protein levels (compare lanes 2 and 5 in Fig. 3E) nor total HSP90 family protein levels (compare lanes 1 and 4) were increased in response to induced Core expression. There was a correlation between the effects of radicicol and disruption of HSC82, suggesting that total HSP90 family protein levels (activity) may be involved in modulating Core levels and thereby contribute to the Core protein’s growth-inhibitory effects.

3.4. An HSP90 family protein is required for stability of the nascent Core

To examine whether the Core degradation rate is affected by disruption of HSC82, we treated cells expressing the Core with cycloheximide (CHX) and examined the time-dependent decay of the core protein. As shown in Fig. 4A, the Core degradation rate was significantly greater when HSC82 was disrupted. This greater degradation rate was also observed when cells were pretreated with radicicol prior to CHX treatment (Fig. 4B). In contrast, there was no significant difference in the Core degradation rate when cells were treated with radicicol and CHX simultaneously (Fig. 4C). These results suggest that the degradation rate of nascent Core may be enhanced when the levels of HSP90 family members are reduced or the activity of HSP90 family members is inhibited. However, the stability of Core produced in the presence of a wild-type level of activity of HSP90 family members was not affected by HSP90 inhibitors. Our results suggest that HSP90/HSC90 may be essential for protecting nascent Core from degradation during protein synthesis.
3.5. The Core D2 domain contributes to HSC90-dependent stability and association with the ER

The Core-E1 signal peptide in the full-length Core is efficiently cleaved by a signal peptide peptidase to form Core177 in human hepatoma cells [17]. This Core-processing activity is significantly lower in yeast (data not shown). Thus, we expressed Core177 and examined its stability. We found that yeast cell growth was also impaired by Core177 (Fig. 5A). As shown in Fig. 5B, we discovered that the rate of Core177 degradation was accelerated in HSC82-disrupted cells. The D2 domain includes two amphipathic α-helices with the potential ability to interact with lipid droplets as well as the ER [9]. We examined whether the Core D2 domain is responsible for Hsc82-dependent stability of the Core. The degradation rate of GFP fused to a region containing the D2 domain and the signal peptide (aa 125–191; GFP-Core) and GFP fused to the D2 domain with the processed signal peptide (aa 125–177; GFP-Core177) increased twofold when Hsc82 was disrupted (Figs. 5B–D). Interestingly, the Hsc82-dependent stability of Core177 was comparable to that of GFP-Core177. However, GFP-Core was the most stable form in the absence of Hsc82 (Fig. 5E).

We examined the distribution of GFP-Core177 in yeast. GFP-Core177 was detected in small particles in the perinuclear region, although it also co-localized with Hmg2-mCherry (Fig. 6A). These results suggest that the ER-D2 domain interaction might affect the distribution of ER-localized Hmg2 proteins or the structure of the ER membrane itself. In addition to the observed puncta, GFP-Core177 was distributed throughout the cytoplasm (compare Fig. 6A and Fig. 1E). Next, to demonstrate the effect of Hsc82 disruption on the distribution and degradation of GFP-Core proteins, we obtained time-lapse fluorescent images of GFP-Core after suppressing its expression; the GAL4 promoter was suppressed by addition of glucose (Figs. 6B and C). Disruption of Hsc82 did not affect the localization of either GFP-Core or GFP-Core177, but the degradation rates were again enhanced. Our results suggest a contribution of the D2 domain to the ER localization and the HSP90/HSC90-dependent stability of the Core. In fact, the intracellular levels (Fig. 7A) and the stability of a truncated Core (aa 1–151; Core151; Fig. 7B), in which a half of D2 was removed, were significantly decreased (the half-life was less than 0.5 h). The yeast cell growth was not affected by expression of Core151 (Fig. 7B). Thus, the stabilities (expression levels) of core might determine the growth inhibition.

4. Discussion

Here, we demonstrate that the Core impairs growth in yeast and that HSC90 is required for Core stabilization in yeast cells. Our
previous results involving expression profiling of yeast suggest that Core induces the expression of genes involved in the ER stress response, but not the heat stress response that induces molecular chaperons [22]. Furthermore, Core did not increase the level of a heat-inducible HSP90 (Hsp82; Fig. 3E). Thus, Core and Core177 expressed in the yeast cells may fold correctly, accumulate on the ER, and induce cellular responses involved in the observed growth inhibition. Growth of yeast cells were not significantly affected by GFP-fused D2 peptides (GFP-Core191 and GFP-Core177; data not shown). Consequently, it is possible that some characteristics of N-terminal region such as oligomerization [4] also may impact on the yeast cell growth. Several reports indicate that expression of Core disturbs cellular signaling and enhances the cell growth [23,24]. It is possible that higher level of Core expression in yeast cells inhibits intrinsic functions of ER (manuscript in preparation).

We showed that treatment with HSP90 inhibitors and disruption of HSC82 reduced the stability of the nascent Core protein, but pre-existing Core protein was not affected by HSP90 inhibitors. Steady-state HSP90 family protein levels, which derive from the HSC82 gene, could determine the stability (amount) of the Core, causing growth inhibition in yeast cells. We also demonstrated that the stability of nascent Core and Core177 were increased in...
an Hsc82-dependent manner and that the half-lives of GFP-fused D2 peptides (GFP-Core, GFP-Core177) were comparable to those of Core and Core177 (Fig. 5E). Our results suggest that Hsc82 activity might play a role in ensuring that the D2 peptide folds correctly, allowing the Core and Core177 to be stabilized. Our results are consistent with the finding that the D2 domain is essential for the stability of the mature Core (Core177), conferring protection from degradation in mammalian cells [5]. We failed to detect a direct interaction between Core and Hsp82/Hsc82 in yeast cells using immunoprecipitation (data not shown). Further studies are required to elucidate the Hsc82-dependent mechanism involved in stabilization of the Core peptide chain during translation, including the potential requirement for a co-chaperone. In this aspect, one of the Hsp70 co-chaperones, Ydj1 may be such a candidate: Ydj1 can interact with HSP90-client proteins [25] and protect nascent chains against degradation [26].

Thus far, we have no direct evidence of the effect of HSC90 on the stability of the D2 domain of Core in human hepatoma Huh7 cells. It is possible that the result demonstrated here may be specific to yeast cells. Nevertheless, the D2 domain is shown to be responsible for stability of Core in mammalian cells [27], thereby the yeast system presented herein, which can be applied to high-throughput screening, may be useful for identifying (screening or validation) for compounds that can direct the nascent D2 peptide to reduce the stability of the Core. Such compounds may represent possible drug candidates for prevention of the progression of HCV pathogenesis and HCV production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.05.023.
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