Cyclophilin A-independent recruitment of NS5A and NS5B into hepatitis C virus replication complexes

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The mechanisms by which cyclophilin A (CypA) governs hepatitis C virus (HCV) replication remain unknown. Since CypA binds two essential components of the HCV replication complex (RC) – the polymerase NS5B and the phosphoprotein NS5A – we asked in this study whether CypA regulates their RC association. We found that CypA, via its isomerase pocket, locates in a protease-resistant compartment similar to that where HCV replicates. CypA association with this compartment is not mediated by HCV. Moreover, CypA depletion of RC does not influence NS5A and NS5B RC association, arguing against a model where CypA governs HCV replication by recruiting NS5A or NS5B into RC.

We and others recently found that CypA and NS5A form a stable complex (Hanoule et al., 2009; Chatterji et al., 2010). The CypA-NS5A interaction is conserved among genotypes and is interrupted by CsA. CypA, devoid of its isomerase activity, fails to bind NS5A, suggesting that CypA, via its isomerase pocket, binds directly to NS5A (Hanoule et al., 2009; Chatterji et al., 2010). Since NS5A, like NS5B, is vital for HCV replication, resides in RC (Appel et al., 2006; Penin et al., 2004) and binds CypA (Hanoule et al., 2009; Chatterji et al., 2010; Liu et al., 2009), we questioned in this study whether CypA enhances HCV replication by recruiting both NS5A and NS5B into RC, providing an alternate mechanism of action for the CsA-mediated inhibition of HCV.

We first questioned whether CypA associates with a compartment similar to where HCV RNA replicates. We isolated a crude RC membrane fraction (CRCMF) from Con1 Huh-7 cells using the elegant protocol developed by the Lohmann lab (Quinkert et al., 2005) and analysed its content in CypA, NS5B and NS5A [Fig. 1a, lane 3 for PNF (subgenomic, sg); lane 7 for S-68.5 (sg); lane 11 for CRCMF (sg)]. Calnexin – a component of the ER membrane – exclusively associates with the CRCMF (sg) (lane 11), but not with the S-68.5 (sg) cytosolic fraction (Fig. 1a, lane 7), suggesting that the CRCMF (sg) is sufficiently pure. NS5A and NS5B, like calnexin, associate with the CRCMF (sg) (Fig. 1a, lane 11). Although the bulk of CypA resides in the cytosolic fraction (Fig. 1a, lane 7), a CypA subset associates with the CRCMF (sg) (Fig. 1a, lane 11).
We then asked whether CypA association with the CRCMF (sg) is mediated by HCV. Importantly, CypA also associates with the MF isolated from parental (P) Huh-7 cells (Fig. 1a, lane 9), suggesting that CypA is not recruited into this membrane-rich fraction via viral components. Similar CypA levels were found in PNF (P) (Fig. 1a, lanes 1 and 3), S-68.5 (P) (Fig. 1a, lanes 5 and 7) and MF (P) (Fig. 1a, lanes 9 and 11) isolated from parental and Con1 Huh-7 cells.

We then examined the effect of CsA on CypA association with the MF (P) or CRCMF (sg). Parental and Con1 Huh-7 cells were treated for 2 h with CsA before MF (P) and CRCMF (sg) subcellular fractionation. CsA has no effect on CypA in PNF (Fig. 1a, lanes 2 and 4) and S-68.5 (Fig. 1a, lanes 6 and 8) fractions isolated from both parental (P) or Con1 (sg) cells. In contrast, CsA significantly depleted CypA from the MF from parental (P) and the CRCMF from Con1 cells (sg) (Fig. 1a, lanes 10 and 12). To our surprise, NS5B levels remained unchanged after CsA treatment (Fig. 1a, lane 12). This suggests that NS5B association with the CRCMF (sg) is CypA-independent. Similarly, CsA does not affect NS5A association with the CRCMF (sg) (Fig. 1a, lane 12), suggesting that CypA depletion of the CRCMF (sg) does not influence CRCMF (sg) association of NS5A and NS5B. We obtained similar results when parental (P) and Con1 (sg) cells were treated with CsA for 22 h (Fig. 1b) rather than 2 h (Fig. 1a).

We then examined whether the CypA subset associated with the CRCMF (sg) is resistant to protease treatment.
The CRCMF (sg) was treated with proteinase K and analysed for CypA content. No full-length calnexin was detectable after protease treatment (Fig. 1c), indicating that the enzyme cut the C-terminal domain of calnexin that is exposed to the cytosolic side. Importantly, the CypA subset associated with the CRCMF (sg) remains intact after proteinase K exposure (Fig. 1c), further supporting the existence of a CypA subset sheltered in an ER compartment. We obtained similar results after nuclelease treatment (data not shown).

To more accurately confirm that CsA decreases the association of CypA, but not NS5A and NS5B with the CRCMF (sg), we developed ELISAs for CypA, NS5A and NS5B. Parental (P) and Con1 (sg) Huh-7 cells were treated with CsA for 2 h, and subsequently, MF (P) and CRCMF (sg) were isolated and solubilized with detergent. CypA, NS5A and NS5B levels in MF (P) and CRCMF (sg) were then quantified by ELISA. Data of six independent experiments are presented in Fig. 1(d). Importantly, we constantly found that CsA has only a minor effect on the association of NS5A and NS5B with CRCMF (sg), but has a significant effect on the association of CypA with either the MF (P) or CRCMF (sg) (Fig. 1d). Thus, the ELISA data (Fig. 1d) correlate well with the Western blot data (Fig. 1a, b).

We found that CsA mediates the depletion of CypA, but not NS5A and NS5B from the proteinase K-treated CRCMF (sg) (Fig. 2a, lane 8). This further suggests that NS5A and NS5B association with the CRCMF (sg) is CypA-independent. Proteinase K treatment of the CRCMF (sg) does not fully digest CypA, NS5A and NS5B (Fig. 2a, lanes 5 and 7), suggesting that the majority of these molecules reside in protected membrane compartments. In contrast, CypA molecules associated with the PNF (sg) are partially digested by proteinase K (Fig. 2a, lanes 3 and 4), suggesting that a population of CypA molecules are unprotected, probably exposed to the cytosolic compartment. CsA, probably by dissociating CypA molecules from a protected compartment, renders CypA more susceptible to proteinase K digestion. We also verified that CypA, NS5A and NS5B, protected from proteinase K in the CRCMF (sg), are sensitive to proteinase K degradation by solubilizing the CRCMF (sg) with detergent before the enzymic treatment (Fig. 2b). Together these data argue against a model in which CypA assists HCV replication by recruiting NS5A and NS5B into RC.

Our finding that CsA depletes CRC of CypA suggests that CypA associates with this protected compartment via its isomerase pocket, where proline-containing peptide substrates and CsA bind (Fernandes et al., 2007; Fischer et al., 1989; Zydowsky et al., 1992). To test this hypothesis, we used the isomerase-deficient H126Q CypA mutant, which fails to support HCV replication (Chatterji et al., 2009; Kaul et al., 2009; Liu et al., 2009), bind NS5B (Liu et al., 2009) and NS5A (Hanouille et al., 2009; Chatterji et al., 2010). Wild-type and H126Q CypA were equally expressed (Fig. 3a, lanes 5–8). In contrast to wild-type CypA (Fig. 3a, lane 1), H126Q CypA poorly associates with CRC [proteinase K-treated CRCMF (sg) (Fig. 3a, lane 3)]. This is the first direct demonstration that residues, which reside in the enzymic pocket of CypA, are critical to CypA association with a protected compartment similar to that where HCV RNA replication occurs.

In this study, we found that a CypA subset pre-exists in a protected compartment similar to that where HCV initiates the formation of its RC. First, we identified a CypA subset associated with a proteinase K-resistant compartment. Second, CsA depletes this compartment of CypA. Third, an isomerase-deficient CypA mutant fails to associate with this compartment. This CypA subset resides in this protected compartment even in the absence of the virus. This suggests that a CypA subset pre-exists in a protected compartment rather than being recruited subsequently into RC by HCV. Our observation that CsA depletes this compartment of CypA and that an isomerase-deficient CypA mutant fails to associate with it suggests that this CypA subset is bound to ER components via its enzymic pocket. In this scenario, CsA, by dissociating CypA from its ER ligand, triggers the release of CypA into the cytosol. Our data may also suggest that HCV exploits this compartment enriched with CypA to initiate the formation of its RC. In this putative model, HCV would be in an ideal position to exploit the isomerase activity of CypA to modulate NS5A- and/or NS5B-mediated functions vital for HCV replication. It is expected that only a very limited part of all produced HCV proteins is used in the assembly of the

**Fig. 2.** NS5A and NS5B remain associated with HCV CRC in the absence of CypA. (a) Same as Fig. 1(a) except that some PNF sg and CRCMF sg samples were treated with or without proteinase K. (b) CRCMF sg were treated with or without 1% Triton X-100 prior to proteinase K treatment and Western blot analysis.
subset of CypA that cannot be differentiated from the particular mechanism in RC assembly involving a micro-RC. Thus, one cannot exclude the existence of some C-leucine incorporation. Con1 cells (100,000 cells per 50 ml) were exposed to increasing concentrations of CsA (1–16 μg ml⁻¹) in the presence of [¹⁴C]leucine for 4 days. At the indicated time points, cells (20,000 cells) were washed and lysed. Radioactivity incorporation into trichloroacetic acid (TCA) precipitable material was used to measure protein synthesis. TCA precipitates were centrifuged, washed, dissolved in 1 M KOH, neutralized with 1 M HCl, and transferred into Aquasol liquid scintillation. Counting (¹⁴C) was performed on a Packard Tri-Carb model 3375. Data (triplicates) were expressed in d.p.m. per culture per 20,000 cells. (b) CsA toxicity was assessed by trypan blue uptake. Con1 cells (100,000 cells per 50 μl per 96-well) were incubated with CsA (1–16 μg ml⁻¹). At the indicated time points, cells (20,000 cells) were washed, resuspended in 20 μl PBS and 20 μl trypan blue (0.8 mM in PBS) and transferred to a counting chamber. After 2 min precisely, numbers of total and stained cells were counted. Viable cells exclude trypan blue, while dead cells stain blue due to trypan blue uptake. Data (triplicates) were expressed in percentage of trypan blue-positive cells. The results of (a) and (b) are representative of three independent experiments.

In conclusion, this study shows that NS5A and the NS5B polymerase remains associated with CRC in the presence of CsA, that CypA associates with a protected intracellular compartment independently of HCV proteins, and that NS5A and NS5B recruitment into CRC is CypA-independent. This study also provides a putative mechanism of antiviral action for Cyp inhibitors, which consists of depleting CRC of CypA, leading to abortive HCV replication. Moreover, this study may suggest that HCV exploits a protected compartment enriched with CypA to initiate the formation of its RC. In this attractive model, HCV would be ideally located in this ER sanctuary to exploit the isomerase activity of CypA to enhance NS5A and/or NS5B functions within the RC.

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