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.

There is a growing body of evidence that cyclosporine A (CsA) and CsA analogues exert their potent anti-hepatitis C virus (HCV) effect both in vitro (Coelmont et al., 2009; Fernandes et al., 2007; Goto et al., 2006; Ma et al., 2006; Mathy et al., 2008; Paeshuyse et al., 2006; Robida et al., 2007) and in vivo (Flisiak et al., 2008, 2009; Hopkins et al., 2009) by neutralizing the enzymic activity of cyclophilins (Cyp) (Chatterji et al., 2009; Gallay, 2009; Goto et al., 2009; Ishii et al., 2006; Kaul et al., 2009; Liu et al., 2009; Nakagawa et al., 2004; Watashi et al., 2003, 2005; Yang et al., 2008). Supporting this notion, several studies including ours showed that stable knockdown of cyclophilin A (CypA) expression in Huh-7 cells decreases HCV replication (Chatterji et al., 2010; Kaul et al., 2009; Yang et al., 2008), suggesting that CypA is the main member of the Cyp family that assists HCV replication. However, the mechanisms by which CypA enhances HCV replication remain to be fully elucidated. To date, no less than four distinct models have been proposed for the role of CypA in the HCV life cycle (Gallay, 2009).

Formation of a membrane-associated replication complex (RC), composed of viral proteins, replicating RNA and altered cellular membranes, is a hallmark of positive-strand RNA viruses (Appel et al., 2006; Penin et al., 2004). The current hypothesis is that HCV RC resides in protected spherules in the ER membrane. It is thought that multiple copies of HCV non-structural (NS) protein complexes encompassing NS3 to NS5B build up a vesicular membrane structure, which mediates the protection against nucleases and proteases. An attractive model was recently proposed to explain the requirement for CypA in HCV RNA replication. This model proposes that CypA mediates the recruitment of NS5B into the RC (Liu et al., 2009). Specifically, Liu et al. (2009) presented evidence that CsA reduces CypA and NS5B association with RC. Based on these findings, the authors proposed that CypA, by recruiting NS5B into the RC, mediates proper assembly and function of RC.

We and others recently found that CypA and NS5A form a stable complex (Hanoulle 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 (Hanoulle 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 (Hanoulle 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.

**Fig. 1.** Specific CypA association with a proteinase K-resistant membrane-rich subcellular compartment. (a) Parental (P) and subgenomic (sg) Con1 Huh-7 cells (2 \( \times \) 10^6) pre-treated with or without CsA (1 \( \mu \)g ml\(^{-1} \)) for 2 h were washed with PBS, scraped and pelleted by centrifugation at 800 g for 10 min at 4 °C. Cells were resuspended (2.5 \( \times \) 10^6 cells ml\(^{-1} \)) in hypotonic buffer [10 mM Tris/HCl (pH 7.5), 10 mM KCl, 1.5 mM MgCl\(_2\), 0.5 mM PMSF, 2 \( \mu \)g aprotinin ml\(^{-1} \)] and lysed by 75 strokes with a Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation at 1000 g for 10 min at 4 °C. The intracellular membranes in the resulting supernatant (called PNF, post-nuclear fraction) were then sedimented on 300 \( \mu \)l 60% (w/w) sucrose in 10 mM Tris/HCl (pH 7.5), 10 mM KCl, 1.5 mM MgCl\(_2\) in an ultracentrifuge at 68500 g for 1 h at 4 °C. The resulting supernatant (called S-68.5) corresponding to the cytosolic fraction deprived of membranes was carefully removed, and the pelleted MF containing the Con1 RC (called CRCMF) was resuspended in lysis buffer. After protein content standardization, lysates were loaded onto an SDS-gel and analysed for calnexin, NS5B, NS5A and CypA content by Western blotting. (b) Same as (a), except that the cells were treated for 22 h with CsA instead of 2 h. (c) Same as (a), except that the isolated CRCMF from Con1 Huh-7 cells (2 \( \times \) 10^6) was incubated with or without proteinase K (50, 200 and 800 \( \mu \)g ml\(^{-1} \) final concentrations) for 60 min at 25 °C. Enzymic activity was neutralized by the addition of 1 mM PMSF final concentration. Proteinase K efficiency was monitored using an antiserum directed against the N-terminal part of calnexin, which is located in the lumen of the ER and thus should be protected from proteinase K in an intact ER structure, whereas the C-terminal part should be accessible and sensitive to the enzyme (Quinkert et al., 2005). (d) Levels of CypA, NS5A and NS5B in detergent-solubilized MF (P) and CRCMF (sg) were quantified by ELISA. Nunc MaxiSorb eight-well strip plates were coated with control, anti-CypA, anti-NS5A or anti-NS5B IgG (10 \( \mu \)g ml\(^{-1} \)) for 16 h at 4 °C. MF and CRCMF lysates (dilutions 1 : 5 and 1 : 25) were added to wells for 16 h at 4 °C. Captured CypA, NS5A or NS5B was detected using horseradish-peroxidase (HRP)-labelled anti-CypA, anti-NS5A or anti-NS5B IgG. Wells were washed and HRP substrate was added. Wells (triplicates) were read on a plate reader at A\(_{490}\). Results of six independent experiments are presented.
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 nuclease 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 (Hanouelle 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.
Fig. 3. CypA associates with HCV CRC via its isomerase hydrophobic pocket. (a) Con1 Huh-7 cells (2×10⁶) pre-transfected for 3 days with wild-type (WT) and isomerase-deficient H126Q CypA, S-68.5 (sg) and CRCMF (sg) were isolated and analysed for CypA content using anti-HA antibodies as described in Fig. 1(a). (b) CsA toxicity was assessed by measuring ¹⁴C-leucine incorporation. Con1 cells (100 000 cells per 50 µl per 96-well) 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) are expressed in percentage of trypan blue-positive cells. The results of (a) and (b) are representative of three independent experiments.

RC. Thus, one cannot exclude the existence of some particular mechanism in RC assembly involving a micro-subset of CypA that cannot be differentiated from the overall pools of CypA.

Liu et al. (2009) also found a CypA subset associated with the CRCMF isolated from GS5 Huh-7.5 cells; however, no CypA subset was detected in parental Huh-7.5 cells (Liu et al., 2009). How could we reconcile these apparent conflicting results? The use of different cell lines and methodologies to isolate CRC may explain the discrepancy. Another possibility is that different amounts of material were analysed. Supporting this possibility, total CypA levels in parental cells were considerably lower than those in GS5 cells (Liu et al., 2009). This reduced amount of analysed material may explain why no CypA subset was detected in the CRCMF isolated from parental Huh-7.5 cells (Liu et al., 2009). Importantly, Liu et al. (2009) standardized their loading material per number of cells, whereas here it was standardized by protein content. Total CypA and calnexin levels in CRC isolated from parental and Con1 cells were equal (Figs 1 and 2), demonstrating that similar amounts of material were analysed in this study. We obtained similar results using parental and JFH-1 Huh-7.5 cells (data not shown).

We demonstrated that CypA depletion by CsA does not affect NS5A and NS5B association with CRC. In contrast, Liu et al. (2009) showed that CsA significantly reduces the amounts of NS5B associated with CRC isolated from G5 cells. Interestingly, Liu et al. (2009) used higher concentrations of CsA (4 µg ml⁻¹) than in the present study (1 µg ml⁻¹). Because high CsA concentrations may disturb cell viability and membrane integrity (Azouzi et al., 2010; Epand et al., 1987; Zydowsky et al., 1992), one could envision that NS5B association with CRC could be destabilized independently of CypA. To test this possibility, we examined the effect of increasing concentrations of CsA on the viability of Huh-7 cells. Importantly, we found that CsA decreases both protein synthesis (monitored by leucine incorporation) (Fig. 3b), and the number of living Huh-7 cells (monitored by trypan blue uptake) in a time- and dose-dependent manner. Nevertheless, our current study clearly shows that CsA, used at a dose (1 µg ml⁻¹) that totally blocks HCV replication, does not influence NS5A and NS5B association with CRC, suggesting that NS5A and NS5B remain associated with CRC even in the absence of CypA. This finding somehow argues against the recruitment of NS5A and NS5B by CypA into CRC.

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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