Research Article

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HCV resistance to cyclosporin A does not correlate with a resistance of the NS5A–cyclophilin A interaction to cyclophilin inhibitors

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Background & Aims: The cyclophilin (Cyp) inhibitors – cyclosporine A (CsA), NIM811, Debio 025, and SCY 635 – block HCV replication both *in vitro* and *in vivo*, and represent a novel class of potent anti-HCV agents. We and others showed that HCV relies on cyclophilin A (CypA) to replicate. We demonstrated that the hydrophobic pocket of CypA, where Cyp inhibitors bind, and which controls the isomerase activity of CypA, is critical for HCV replication. Recent studies showed that under Cyp inhibitor selection, mutations arose in the HCV nonstructural 5A (NS5A) protein. This led us to postulate that CypA assists HCV by acting on NS5A.

Methods: We tested this hypothesis by developing several interaction assays including GST pull-down assays, ELISA, and mammalian two-hybrid binding assays.

Results: We demonstrated that full-length NS5A and CypA form a stable complex. Remarkably, CsA prevents the CypA–NS5A interaction in a dose-dependent manner. Importantly, the CypA–NS5A interaction is conserved among genotypes and is interrupted by CsA. Surprisingly, the NS5A mutant protein, which arose in CsA-resistant HCV variants, behaves similarly to wild-type NS5A in terms of both CypA binding and CsA-mediated release from CypA. This latter finding suggests that HCV resistance to CsA does not correlate with a resistance of the CypA–NS5A interaction to Cyp inhibitors. Moreover, we found that CypA, devoid of its isomerase activity, fails to bind NS5A.

Conclusions: Altogether these data suggest that CypA, via its isomerase pocket, binds directly to NS5A, and most importantly, that disrupting this interaction stops HCV replication.

Keywords: HCV; Cyclophilin A; NS5A.

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Introduction

HCV is the main contributing agent of acute and chronic liver diseases worldwide [1]. Primary infection is often asymptomatic or associated with mild symptoms. However, persistently infected individuals develop high risks for chronic liver diseases such as hepatocellular carcinoma and liver cirrhosis [1]. The combination of IFN alpha and ribavirin that serves as current therapy for chronically HCV-infected patients not only has a low success rate (about 50%) [2], but is often associated with serious side effects [2]. There is thus an urgent need for the development of novel anti-HCV treatments [2].

Cyclosporine A (CsA) was reported to be clinically effective against HCV [3]. Controlled trials showed that a combination of CsA with IFN alpha is more effective than IFN alpha alone, especially in patients with a high viral load [4,5]. Moreover, recent *in vitro* studies provided evidence that CsA prevents both HCV RNA replication and HCV protein production in an IFN alpha-independent manner [6–10]. CsA exerts this anti-HCV activity independently of its immunosuppressive activity because non-immunosuppressive CsA derivates – more recently termed Cyp inhibitors [11] – also block HCV RNA and protein production [9,12–15]. Recent clinical data demonstrated that these Cyp inhibitors profoundly decreased HCV viral load in HCV-infected patients [16,17]. More recently, the anti-HCV effect of Debio 025 in combination with peginterferon alpha 2a (peg-IFNα2a) was demonstrated in patients with chronic hepatitis C [17]. These findings are critical because they suggest that Cyp inhibitors represent a novel class of anti-HCV agents.

Although there was a growing body of evidence that Cyp inhibitors exert their antiviral effect by targeting Cyps, a disagreement existed on the respective roles of Cyp members in HCV replication. One study suggested that CypB, but not CypA, is critical for HCV replication [18], another suggested that CypA, but not CypB and CypC, was critical for HCV replication [19], and a third study suggested that three Cyps – CypA, B and C – are all required for HCV replication [9]. In order to attempt to clarify this apparent controversy, we recently re-analyzed the respective contribution of Cyp members to HCV replication by specifically and stably knocking down their expression by small RNA interference (sRNAi). We found that only the CypA knockdown drastically decreased HCV

replication [20]. The re-expression of an exogenous CypA escape protein, which contains escape mutations at the sRNAi recognition site, restored HCV replication, demonstrating the specificity for the CypA requirement [23]. We also mutated residues, which reside in the hydrophobic pocket of CypA where proline-containing peptide substrates and CsA bind, and which are vital for the enzymatic or the hydrophobic pocket binding activity of CypA [20]. Remarkably, these CypA mutants fail to restore HCV replication, suggesting that HCV exploits the isomerase activity of CypA to replicate in hepatocytes and that CypA is the principal mediator of the Cyp inhibitor anti-HCV activity [20]. These results have now been confirmed by two independent studies from the Tang lab and from the Bartenschlager lab [21,22].

Since recent studies demonstrated that NS5A mutations arose when HCV were grown under CsA selection, we postulated for the existence of an interplay between CypA and NS5A. We thus tested this hypothesis and found that full-length NS5A and CypA directly associate. Remarkably, CsA prevents the CypA–NS5A interaction in a dose-dependent manner. The CypA–NS5A interaction is conserved among HCV genotypes and is prevented by CsA. Surprisingly, the interaction between CypA and the NS5A mutant protein identified in CsA-resistant HCV variants remains sensitive to CsA. Moreover, we found that CypA, devoid of its isomerase activity due to the introduction of a mutation in its enzymatic pocket, fails to bind to full-length NS5A. Altogether these data suggest that CypA, via its isomerase pocket, binds directly to NS5A, and most importantly, that disrupting this interaction stops HCV replication.

Materials and methods

Production of recombinant CypA and NS5A proteins

Recombinant GST-CypA was produced and purified as we described previously [23], whereas full-length NS5A Con1 (pET-Ub-NS5A Con1-His) was expressed as described previously [24]. GST-CypA H126Q and NS5A D320E mutants were created by PCR mutagenesis. The NS5A genes from genotype 1a (H77), 1b (Con1), 2a (JFH-1), and 2b (MD2b-1) were cloned and expressed as described previously [24].

CypA–NS5A pull-down studies

Glutathione beads were incubated for 2 h in dialysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.5% NP-40, 1 mM DTT) with 5 mg/ml BSA and washed twice at 4 °C in binding buffer (20 mM Tris pH 7.9, 0.5 M NaCl, 10% glycerol, 10 mM DTT and 1% NP-40). Meanwhile, 100 ng of GST-CypA or GST was mixed with 10 ng of NS5A-His in a total volume of 200 µl of binding buffer for 3 h at 4 °C on wheel. Glutathione beads (25 µl) were added to the GST-CypA/NS5A mixture for 30 min at 4 °C, washed three times with 400 µl of binding buffer. Beads were pelleted for 30 s at 2000*g* in a microfuge and bound material was eluted with 25 µl of 2× SDS sample buffer, heated for 5 min, and frozen at −20 °C. Bound material was then analyzed by Western blotting using anti-GST, anti-CypA, and anti-His antibodies as described previously [20].

CypA–NS5A ELISA

Nunc MaxiSorb 8-well strip plates were coated with GST, GST-CypA, and GST-H126Q CypA for 16 h at 4 °C and blocked as we described previously [26]. Recombinant NS5A-His (1 ng/ml) was added to wells in 50 µl of binding buffer (20 mM Tris pH 7.9, 0.5 M NaCl, 10% glycerol, 10 mM DTT and 1% NP-40) for 16 h at 4 °C. Captured NS5A-His was subsequently detected using mouse anti-His antibodies (1 µg/ml) (anti-6×His, Clontech) and rabbit anti-mouse-horseradish peroxidase phosphatase (HRP) antibodies (1:1000 dilution) as we described previously [23].

Scatchard analyses

Recombinant NS5A proteins were labeled with [^{125}I] (New England Nucle, Boston) using Iodogen (Pierce Chemical Co., Rockford) to a specific radioactivity of 400 cpm/fmol. Nunc MaxiSorb 8-well strip plates were coated with GST or GST-CypA for 16 h at 4 °C and blocked as we described previously [23]. For K_D measurements, ^{125}I-NS5A was added to GST or GST-CypA coated wells for 2 h at 4 °C in the presence of increasing concentrations of unlabeled NS5A. Wells were then washed three times and bound ^{125}I-NS5A was solubilized by incubating wells for 30 min at 50 °C with 2% SDS, collected, and measured in a liquid scintillation counter. The amount of ^{125}I-NS5A bound to GST-coated wells was used as nonspecific binding and was subtracted from all values. K_D values were analyzed by the Scatchard plot procedure [25].

Mammalian two-hybrid system

Evaluation of intracellular CypA–NS5A interactions using the two-hybrid screening technology was accomplished using the Checkmate Mammalian Two-Hybrid System according to the manufacturer's instructions (Promega). Briefly, pACT- and pBIND-based plasmids were co-transfected (using Genejuice) with the pG5*luc* reporter construct into Huh7 cells and incubated for 37 °C. After 72 h, cell lysates were assessed for luciferase activity using the "Steady-Glo Luciferase Assay System" according to the manufacturer's instructions (Promega).

Results

Specific and direct interaction between CypA and full-length NS5A

Since recent studies demonstrated that NS5A mutations arose when HCV was grown under CsA selection, we postulated for the existence of an interplay between CypA and NS5A. To test this hypothesis, we asked whether CypA possesses the capacity to interact with HCV NS5A. Specifically, we conducted binding studies between recombinant CypA and full-length wild-type NS5A. Importantly, we found that NS5A efficiently binds to GST-CypA (Fig. 1A, lane 2), but not GST (Fig. 1A, lane 1). This demonstrates that full-length NS5A possesses the ability to bind CypA directly. To further demonstrate a direct contact between NS5A and CypA, we developed an ELISA using CypA as solid phase to capture NS5A. Specifically, plates were coated with BSA, GST or GST-CypA and incubated with NS5A Con1-His. CypA-captured NS5A proteins were detected using anti-His antibodies. Importantly, NS5A binds to wells coated with GST-CypA, but not to those coated with GST or BSA (Fig. 1B). The ELISA data are perfectly in accordance with those of the GST-CypA pull-down assay (Fig. 1A). Thus, full-length NS5A binds directly and specifically to CypA.

The isomerase hydrophobic pocket of CypA contains the NS5A-binding site

We and others demonstrated that the introduction of mutations in the active enzymatic pocket of CypA, where proline-containing peptide substrates bind, blocks HCV replication [20–22]. This led us to postulate that HCV requires the isomerase activity of CypA to replicate in human hepatocytes [20]. In this study, we asked whether CypA binds to NS5A via its isomerase hydrophobic active pocket. To address this issue, we created in the context of our bacterial expression GST-CypA construct, a CypA mutant deprived of its isomerase activity. Specifically, we replaced the histidine located at position 126 (H126) in the hydrophobic pocket of CypA by a glutamine, creating the H126Q CypA mutant. This mutation diminishes CypA isomerase activity by more than