Hepatocytes That Express Variants of Cyclophilin A Are Resistant to HCV Infection and Replication

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BACKGROUND & AIMS: Hepatitis C virus (HCV) uses several host factors to infect and replicate in human hepatocytes. Cyclophilin A (CypA) is required for viral replication, and CypA inhibitors are in development. We investigated the effects of nonsynonymous single nucleotide polymorphisms (SNPs) in the region of *peptidyl-prolyl isomerase A* (*PPIA*) that encodes CypA on HCV infection and replication of human hepatocytes. **METHODS:** We used a combination of virologic, biochemical, and genetic approaches to investigate the effects of *PPIA* variants on HCV replication in cultured Huh-7.5 cells. We reduced levels of CypA in these cells using small hairpin RNAs (shRNAs). **RESULTS:** Using shRNAs, we showed that CypA was required for replication of HCV in Huh-7.5 cells and identified 3 SNPs in *PPIA* that protected cells from HCV entry or replication. Levels of HCV RNA were reduced 3–4 log in cells homozygous for the variant alleles; release of new particles was also reduced, but viral entry was not affected. The effects of the variant alleles were recessive and stronger for preventing replication of full-length HCV genomes than subgenomes. CypA inhibitors prevented replication of residual HCV in hepatocytes. The variants appeared to destabilize the CypA protein; the single amino acid changes led to rapid degradation of the protein. **CONCLUSIONS: We identified variants in *PPIA* that destabilize its product, CypA, and prevent HCV infection and replication. These findings indicate mechanisms by which some cells might be resistant to HCV infection and that CypA is a good therapeutic target.**

Keywords: Cell Culture System; In Vitro Model; Virology; Liver.

Currently, there are about 160 million individuals worldwide chronically infected with hepatitis C virus (HCV), a frequent cause of chronic hepatitis, cirrhosis, and end-stage liver disease.[1] Clinically, hepatitis C is a highly variable disease; acute infection resolves spontaneously or in about one-fourth of cases, and the remaining 75% become chronically infected.[2] Treatment of chronic infection with a combination of pegylated interferon, ribavirin, and, for genotype 1 infection, an inhibitor of the viral NS3/4A protease clears the infection in approximately 75%.[3] Finally, among those who remain chronically infected, some will be asymptomatic for life while others will develop cirrhosis and end-stage liver disease. Variations in genes in the interleukin-28B gene and receptors present on natural killer cells influence spontaneous and treatment-induced resolution of infection.[4–9] What genetic factors modulate disease progression is poorly defined. Variants in factors directly involved in the HCV replication cycle, such as the occludin coreceptors, have been described, but the impact on the viral replication cycle seems to be limited.[10,11] Nonetheless, additional genetic factors are likely involved in determining the course of HCV infection.

HCV is a highly variable small enveloped virus with a single plus-strand RNA genome and belongs to the *Flaviviridae* family.[2] Its natural host range is limited to humans and chimpanzees, and its primary reservoir is in hepatocytes. Cyclophilin A (CypA), a member of a family of cellular peptidyl-prolyl-isomerases, is a host encoded factor that is essential for HCV genome replication and possibly particle assembly.[12–14] Although the exact mechanism is unclear, the function of several viral nonstructural proteins (NS2, NS5A, and NS5B) has been reported to be CypA dependent.[15] Moreover, CypA mutants lacking isomerase activity do not sustain viral replication.[13,14,16] For these reasons, CypA is deemed a promising target for antiviral therapy[17] and a CypA inhibitor, alisporivir, is currently in phase 3 clinical testing, making it the currently most advanced anti-HCV drug targeting a host factor.[18]

Abbreviations used in this paper: CypA, cyclophilin A; GFP, green fluorescent protein; HCVcc, cell culture–grown hepatitis C virus; HIV, human immunodeficiency virus; PPIA, peptidyl-prolyl isomerase A; shRNA, small hairpin RNA; SNP, single nucleotide polymorphism.

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Single nucleotide polymorphisms (SNPs) in the promoter region of peptidyl-prolyl isomerase A (PPIA) have been reported to modulate progression to acquired immunodeficiency syndrome (AIDS) in individuals infected with the human immunodeficiency virus (HIV),[19] but the impact of PPIA variants on HCV infection or its treatment with CypA inhibitors has not been evaluated. Six coding nonsynonymous SNPs, each expected to result in the exchange of a single amino acid in the CypA protein, have been deposited in the National Center for Biotechnology Information dbSNP database (www.ncbi.nlm.nih.gov/snp), but there is no published information on their frequency and they have not been linked to any human disease.

We found that 3 of these 6 variants present in human populations render host cells refractory to HCV replication in vitro. Moreover, we were able to show that the underlying mechanism is a destabilization of the CypA protein, resulting in near-complete intracellular CypA depletion. Thus, the stability of CypA can become rate limiting for HCV RNA replication.

Materials and Methods

Drugs

Alisporivir (formerly DEB025) was provided by Novartis (Basel, Switzerland). Atazanavir, lopinavir, ritonavir, and bortezomib were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Cycloheximide and lactacystin were purchased from Sigma (Sigma-Aldrich, Munich, Germany). MG-132 was purchased from Merck-Millipore (Darmstadt, Germany).

Cell Culture and Cell Lines

Huh-7.5 cells and subclones were maintained in Dulbecco's modified Eagle medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum, L-glutamine, nonessential amino acids, penicillin, and streptomycin. Cells harboring small hairpin RNA (shRNA) constructs were kept in the presence of blasticidin 5 μg/mL. Cells harboring pWPI-encoded CypA variants were additionally kept in the presence of G418 750 μg/mL.

DNA Constructs

The CypA open reading frame was cloned into a lentiviral pWPI vector (pWPI-GUN) using *Pme*I and *Spe*I restriction sites. In addition, pWPI-GUN encodes a green fluorescent protein (GFP)-neomycin-resistance fusion protein for selection and tracking purposes from a separate cistron.[20] CypA variants were created in this context by standard polymerase chain reaction–based mutagenesis. Detailed cloning strategies are available on request. The DNA sequences of all newly modified constructs were confirmed by direct sequencing (Eurofins MWG Operon, Ebersberg, Germany). Plasmids used for the generation of lentiviral pseudoparticles have previously been described.[10] The HCV reporter genome F-luc Jc1, an intragenotypic HCV chimera consisting of JFH1 (genotype 2a; GenBank accession no. AB047639) and J6/CF (genotype 2a; GenBank accession no. AF177036) genome segments, has been described.[21] The intergenotypic chimera genomes were a kind gift from Jens Bukh (Copenhagen University Hospital, Copenhagen, Denmark).[22]

shRNAs targeting cyclophilin A were constructed in the context of the shRNA expression vector pLenti-3′-U6-EC-EP7, a blasticidin-selectable lentigenome vector expressing the shRNA of interest from an internal U6 promoter.[23] The target-specific sequence used for the creation of a CypA^{low} cell line was 5′-CTGGATTGCAGAGTTAAGTTTA-3′, which is contained in the 5′-untranslated region of the messenger RNA transcript encoding CypA in humans.[13]

Patient Samples

After approval from the respective institutional review boards, anonymized human DNA samples (100 each) from healthy volunteers were collected at Hannover Medical School (Hannover, Germany), Cairo University (Cairo, Egypt), German University in Cairo (Cairo, Egypt), and Charité Berlin (Berlin, Germany). These were used to determine allele frequencies.

Genotyping

EDTA blood was used to isolate human DNA by using a commercial DNA isolation kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Genotyping was performed using custom-made LightSNiP assays that were purchased from TIB Molbiol (Berlin, Germany) and run on a LightCycler 480 II (Roche Diagnostics, Mannheim, Germany). Briefly, LightSNiP assays use fluorescently labeled probes to create a genotype-specific melting curve pattern. More information is available on the manufacturer's web site (www.tib-molbiol.com). In addition, sequencing results were confirmed or refuted using polymerase chain reaction amplification and sequencing of target regions. Oligonucleotide sequences are available on request.

Pseudotyping of Lentiviral Particles and Transduction of Target Cells

Pseudoparticles were generated as previously described.[10] Briefly, 3 plasmids were cotransfected into 293T cells. These encoded (1) a provirus containing a firefly luciferase reporter (CSFlucW2), one of various transgenes (pWPI constructs), or an shRNA (pLenti-3′-U6-EC-EP7), (2) HIV gag-pol, and (3) either the G protein of vesicular stomatitis virus (VSV-G) or the HCV glycoproteins E1 and E2 of strain H77 preceded by the core signal sequence. Supernatants were collected at 48 and 72 hours after transfection, supplemented with Polybrene (Sigma Aldrich, Seelze, Germany) 4 μg/mL, and added to the target cells for 6 hours.

Expression and Characterization of CypA in Escherichia coli

Human CypA was expressed from the vector pQE70-Cyp18 in *Escherichia coli* M15 cells and purified as described.[24] Mutagenesis was performed according to the QuikChange site-directed mutagenesis protocol (Stratagene, San Diego, CA) using appropriate primers and pQE70-Cyp18 as template. Bacterial cells were disrupted using a French press. Samples were analyzed by 15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue. The thermodynamic characterization of CypA variants[24] and measurements of peptidyl-prolyl isomerase activity[25] were performed as previously described.

Cell Culture–Grown HCV Infection

Huh-7.5 cells were transfected with HCV genomes (F-luc Jc1 containing a firefly luciferase reporter gene or one of several intergenotypic chimeras without a reporter) by electroporation