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Regulation of hepatitis C virus replication by the core protein through its interaction with viral RNA polymerase

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ABSTRACT

The hepatitis C virus (HCV) core protein is a structural component of the nucleocapsid and has been shown to modulate cellular signaling pathways by interaction with various cellular proteins. In the present study, we investigated the role of HCV core protein in viral RNA replication. Immunoprecipitation experiments demonstrated that the core protein binds to the amino-terminal region of RNA-dependent RNA polymerase (RdRp), which encompasses the finger and palm domains. Direct interaction between HCV RdRp and core protein led to inhibition of RdRp RNA synthesis activity of *in vitro*. Furthermore, over-expression of core protein, but not its derivatives lacking the RdRp-interacting domain, suppressed HCV replication in a hepatoma cell line harboring an HCV subgenomic replicon RNA. Collectively, our results suggest that the core protein, through binding to RdRp and inhibiting its RNA synthesis activity, is a viral regulator of HCV RNA replication.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis and liver cirrhosis, the latter often leading to hepatocellular carcinoma [1]. HCV is an enveloped RNA virus in the *Flaviviridae* family, with a positive-sense single-stranded RNA genome of ~9.6 kb. The viral genome consists of one long open reading frame (ORF) that is flanked by untranslated regions (UTRs) at both the 5′ and 3′ ends [2,3]. The ORF encodes a single polyprotein of 3010 amino acids that is proteolytically processed by cellular and viral proteases into at least 10 polypeptides, including structural proteins and non-structural (NS) proteins [4]. The 65-kDa HCV NS5B protein carrying RNA-dependent RNA polymerase (RdRp) activity is the key player in HCV RNA replication [5]. It is a compact globular protein consisting of three sub-domains called the palm, finger, and thumb, and resembles a right hand, similar to other related enzymes [6].

HCV core protein is a viral structural protein that packages the viral RNA genome [7]. Its binding to the 5′-UTR of the HCV genome has been suggested to be important for specific encapsidation of the viral genome [8]. In addition to its roles as a structural protein, HCV core protein also displays pleiotropic functions though its interaction with cellular proteins [9,10]. Besides its roles in virus particle assembly and in pathogenesis, HCV core protein has been postulated to be involved in regulation of HCV RNA replication. It has been suggested, based on comparison of the RNA replication level of a selectable full-length HCV replicon with that of an HCV subgenomic replicon lacking HCV core/E1/E2 structural protein expression, that the decreased level of HCV RNA in cells containing the full-length replicon might be due to the presence of viral structural proteins [11]. Consistent with this hypothesis, other groups have reported that the efficiency of colony formation by the full-length replicon is about 3–4 logs lower than that of subgenomic replicons [12]. In addition, the subcellular localization of core protein is likely to overlap with that of viral RdRp, since both proteins were shown to be associated with intracellular lipid-raft structures [13,14]. Together, the results described above suggest that HCV core protein might indeed be involved in regulation of HCV RNA replication. To date, however, the precise role of core protein in HCV replication remains unknown.

In the present study, we addressed the question of how HCV core protein regulates viral RNA replication. We performed a comprehensive biochemical analysis of the interaction between HCV core protein and NS5B RdRp, and demonstrated the role of direct interaction between these proteins in modulating the function of RdRp using an *in vitro* RdRp assay and an HCV subgenomic replicon cell line.

Materials and methods

Cells and cell culture. The human hepatoma cell line, Huh7, was cultured in RPMI-1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin under standard culture conditions (5% CO_2, 37 °C). The human hepatoma stable cell lines Huh7TR-4, which expresses the

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tetracycline repressor, and Huh7TR-core, which expresses HCV core protein in a tetracycline-inducible manner [15], were maintained in the presence of blasticidin S (10 μg/ml) and Zeocin (100 μg/ml). Core protein expression in Huh7TR-core was induced by addition of 1 μg/ml of tetracycline for 48 h, unless otherwise specified. The Huh7 cell line R-1, which supports stable, autonomous replication of genotype 1b HCV subgenomic replicon RNA, was described previously [16].

Plasmid construction. The pcDNA3.1-Flag-Core plasmid expressing Flag-epitope-tagged full-length HCV core protein was described previously [15]. The pcDNA3.1-Flag-NS5B plasmid was constructed by inserting the cDNA encoding Flag-epitope-tagged full-length HCV NS5B protein into the EcoRI and XhoI sites of pcDNA3.1 (Invitrogen, Carlsbad, CA). cDNAs encoding the finger domain (amino acids 1–187), the finger–palm domain (amino acids 1–371), the palm–thumb domain (amino acids 188–591), the palm domain (amino acids 188–371), and the thumb domain (amino acids 372–591) of HCV NS5B were amplified by PCR from the pThNS5B plasmid [5] and inserted at the NheI/EcoRI site of pET-28a(+) (Novagen, Madison, WI) to obtain pET-28a(+)/NS5B(1–187), pET-28a(+)/NS5B(1–371), pET-28a(+)/NS5B(188–591), pET-28a(+)/NS5B(188–371), and pET-28a(+)/NS5B(372–591), respectively. For expression of the enhanced green fluorescence protein (EGFP)-fused HCV core protein and its deletion derivatives, pEGFP-C(1–191), pEGFP-C(1–75), pEGFP-C(1–121), pEGFP-C(1–173), pEGFP-C(76–191), and pEGFP-C(99–191) were used [10]. The cDNA encoding the full-length SARS coronavirus (SARS–CoV) capsid protein was synthesized by RT-PCR using total RNA extracted from SARS–CoV (Urbani strain)-infected Vero E6 cells, and cloned into the pTrcHisB vector (Invitrogen).

Expression and purification of recombinant proteins. Recombinant HCV NS5B protein with a hexahistidine tag at the N-terminus was expressed in *Escherichia coli* and purified as described previously [16]. HCV core protein was expressed in *E. coli* and purified from inclusion bodies as described previously [15]. SARS–CoV capsid protein (N) was expressed in Top10 *E. coli* cells (Invitrogen) at 25 °C overnight by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The recombinant N protein was purified by metal affinity chromatography using Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen, Hilden, Germany), heparin–Sepharose (Amersham Biosciences, Piscataway, NJ), and SP-Sepharose columns (Amersham Biosciences) sequentially.

Western blot analysis and immunoprecipitation. In vitro immunoprecipitation of HCV core and NS5B proteins was performed using 1 μg of each recombinant protein. The two proteins in buffer A (50 mM Tris–HCl, pH 8.0; 1 mM dithiothreitol [DTT]; 50 mM NaCl; 5 mM $MgCl_2$; 10% glycerol) were incubated with an anti-HCV core antibody (Virogen, Watertown, MA) for 1 h on a rotator at 4 °C. For co-immunoprecipitation experiments, Huh7TR-4 or Huh7TR-core cells were transfected with pcDNA3.1-Flag-NS5B using FuGENE6 (Roche, Switzerland) and treated with 1 μg/ml of tetracycline for induction of core protein expression. At 48 h post-transfection, cells were harvested and washed twice with cold PBS, resuspended in lysis buffer A (100 mM Tris–HCl, pH 8.0; 150 mM NaCl; 1% Triton X-100; 10 mM NaF; 1 mM Na_3VO_4; 17.5 mM β-glycerophosphate) supplemented with an EDTA-free protease inhibitor cocktail (Roche) and incubated on ice for 30 min. After centrifugation, cell lysates was incubated with an anti-Flag antibody (Sigma–Aldrich, Saint-Louis, MO) for immunoprecipitation of the Flag-tagged NS5B protein. For domain mapping of the NS5B-interacting region of HCV core protein, the subgenomic replicon cell line (R-1) was transfected with pEGFP, pEGFP-C(1–191), pEGFP-C(1–75), pEGFP-C(1–121), pEGFP-C(1–173), pEGFP-C(76–191), or pEGFP-C(99–191) using FuGENE6 (Roche) and harvested 48 h post-transfection. Cell lysates were incubated with an anti-GFP monoclonal antibody (BD Biosciences, Palo Alto, CA). Immunocomplexes were recovered by adsorption to Protein G-Sepharose or Protein A-Sepharose (Qiagen). Cell lysates and immunoprecipitates were subjected to SDS–PAGE and electroblotted onto nitrocellulose membrane (Amersham Biosciences). Western blot analysis was performed as described previously [15] using an anti-core antibody (Virogen), a Penta-His antibody (Qiagen), an anti-Flag antibody (Sigma–Aldrich), an anti-NS5B monoclonal antibody (provided by S.B. Hwang at Hallym University, Korea), an anti-GFP antibody (Santa Cruz Biotechnology), or an HCV patient serum (provided by K.H. Han at Yonsei University College of Medicine). Proteins associated with antibodies were visualized using appropriate peroxidase-conjugated secondary antibodies and a chemiluminescent substrate (ECL, GE Healthcare Life Sciences, Piscataway, NJ).

Confocal microscopy. The Huh7TR-core cell line was cultured in eight-well chamber slides (Nunc, Rochester, NY) to ~50% confluence and transfected with pcDNA3.1-Flag-NS5B. At 48 h post-transfection, cells were fixed and treated as described previously [15]. Cells were then incubated with a monoclonal anti-core protein antibody (Virogen) and anti-Flag antibody (Sigma–Aldrich). Primary antibodies were detected by fluorescein isothiocyanate- and Texas Red-coupled secondary antibodies. Nuclei were visualized by staining with 1 μM 4′,6′-diamidino-2-phenylindole (DAPI) in PBS for 10 min. Confocal images were obtained using a Bio-Rad Radiance 2000 laser scanning confocal microscope.

Enzyme assays. In vitro RNA polymerase activity assays were performed using the full-length $(His)_6$-tagged NS5B protein as described previously [16]. For the (−)3′-UTR template, which represents the complementary sequence of the 5′-UTR, 200 ng of RNA was used. RdRp reactions with the poly(A) template (1 μg) were conducted in the presence of 10 pmol of oligonucleotide $(U)_{20}$ as a primer. Heat-denatured RdRp products were resolved on an 8 M urea, 5% polyacrylamide gel. The gels were dried after fixing and then exposed to X-ray film for autoradiography. RdRp product levels were determined by densitometric analysis of autoradiographs using Scion Image from the National Institutes of Health or by measuring the cpm using a Perkin Elmer Topcounter.

TaqMan real-time quantitative reverse transcription-PCR. Total RNA was extracted from R-1 cells using TRIZOL LS reagent (Invitrogen) and purified according to the manufacturer's instructions. HCV RNA levels were quantified by real-time quantitative RT-PCR using a primer pair and TaqMan probe targeting a region within the HCV 5′-UTR as described previously [16]. Cellular glyceraldehyde-3-phosphate dehydrogenase mRNA from the same extracts was used as an internal control.

Results

Direct interaction between HCV core protein and NS5B RdRp

To investigate the interaction between HCV core protein and NS5B RdRp, and the effect of this protein interaction on viral RNA synthesis *in vitro*, both full-length core and NS5B proteins were expressed and purified from *E. coli* as fusion proteins with an N-terminal hexahistidine. Using highly purified full-length recombinant core and NS5B proteins, as assessed by Coomassie staining (Fig. 1A), we performed immunoprecipitation experiments to demonstrate the direct interaction between these proteins. The core protein was immunoprecipitated with an anti-core protein antibody. As seen in Fig. 1B, immunoprecipitation of core protein co-immunoprecipitated NS5B (lane 2). We further confirmed the interaction in human hepatoma cells by co-immunoprecipitation experiments. Huh7TR-core cells expressing core protein were transfected with pcDNA3.1-Flag-NS5B. Flag-tagged NS5B in detergent-solubilized cell lysates were immunoprecipitated with an anti-Flag antibody, and the resulting immunocomplexes were examined for the presence of core protein by