Virology 511 (2017) 1–8

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Treatment with PTEN-Long protein inhibits hepatitis C virus replication

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ARTICLE INFO

Keywords:
PTEN-Long
Hepatitis C virus
Viral replication
Core
Protein - protein interaction

ABSTRACT

Hepatitis C virus (HCV) infection is a confirmed risk factor for hepatocellular carcinoma (HCC). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) possesses tumor suppression function that is frequently defective in HCC tumors. PTEN-Long, a translation isoform of PTEN, functions in a cell non-autonomous manner. In this study, we demonstrated that intracellular overexpression of PTEN-Long inhibits HCV replication. More importantly, we showed that treatment with extracellular PTEN-Long protein inhibits HCV replication in a dose-dependent manner. Furthermore, we showed that PTEN-Long interacts with HCV core protein and this interaction is required for HCV replication inhibition by PTEN-Long. In summary, we demonstrated, for the first time, that PTEN-Long protein, an isoform of the canonical PTEN and in the form of extracellular protein treatment, inhibits HCV replication. Our study offers an opportunity for developing additional anti-HCV agents.

1. Introduction

Despite the approval of anti-viral drugs, hepatitis C virus (HCV) infection continues to be a significant public health problem with hepatocellular carcinoma (HCC) as the most deadly clinical outcome (Messina et al., 2015). HCV has a single-stranded RNA genome encoding a polyprotein that is processed by cellular and viral proteases to generate structural and non-structural proteins (Scheel and Rice, 2013). Although it is a structural protein, HCV core has been reported to possess many regulatory functions including tumorigenesis (Kao et al., 2016; Moriya et al., 1998).

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual phosphatase with lipid and protein phosphatase activities (Song et al., 2012; Leslie et al., 2008). These phosphatase activities render PTEN to function as a tumor suppressor (Song et al., 2012). PTEN has been characterized as one of the most frequently mutated or deleted genes in various tumors including HCC (Zhang and Yu, 2010; Song et al., 2012). For instance, three single amino acid mutations in the phosphatase domain have been identified: C124S (lipid and protein phosphatase defective), G129E (lipid phosphatase activity defective), and Y138L (protein phosphatase defective) (Davidson et al., 2010). As protein products translated from alternative start codons upstream of the ATG initiation sequence of PTEN, four longer isoforms of PTEN, termed PTEN-Long/PTENα, PTEN-M/PTENβ, PTEN-N, and PTEN-O, respectively, were discovered (Malaney et al., 2017). Whether these translation isoforms have similar or different functions in comparison to canonical PTEN has not been well characterized. PTEN-Long, the focus of the current study, has 173 N-terminal extra amino acids with a poly-arginine region. This region enables PTEN-Long to be exported into extracellular compartments, enter neighboring cells, and induce signaling events in recipient cells (Hopkins et al., 2013; Wang et al., 2015). PTEN-Long possesses comparable or even higher constitutive lipid phosphatase activity than PTEN (Masson et al., 2016; Hopkins et al., 2013; Johnston and Raines, 2015). Similar to the canonical PTEN, PTEN-Long can suppress PI3K-Akt activation (Hopkins et al., 2013; Wang et al., 2015; Liang et al., 2017). Interestingly, a G to R point mutation in PTEN-Long (G302R) has the same phenotype as the corresponding G129R mutation in canonical PTEN in regulating Akt activity (Wang et al., 2015; Hopkins et al., 2013), suggesting a conserved functional role for the amino acid residues amongst different PTEN isoforms. Hence, other point mutations of PTEN, such as C124S and Y138L, have also been transferred to the corresponding residues in longer PTEN isoforms in functional studies (Liang et al., 2017).

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http://dx.doi.org/10.1016/j.virol.2017.08.002
Received 23 June 2017; Received in revised form 26 July 2017; Accepted 2 August 2017

We recently demonstrated that PTEN can inhibit HCV replication through interacting with HCV core protein (Wu et al., 2017). Whether PTEN-Long affects HCV replication has not been investigated. In this study, we showed that intracellular expression of or extracellular treatment with PTEN-Long protein can inhibit HCV replication. Furthermore, we showed that PTEN-Long - HCV core protein interaction is involved in the inhibitory effect of PTEN-Long on HCV replication.

2. Materials and methods

2.1. Plasmids and in vitro transcription

Plasmids containing HCV-2a J6/JFH-1(p7-RLuc-2A) or HCV-2a J6/JFH-1(p7-RLuc-2A) ΔE1E2 sequences were provided by Charles Rice (Jones et al., 2007). Plasmid expressing Flag-PTEN was provided by Jack Dixon (Maehama and Dixon, 1998). Plasmids expressing PTEN-Long-V5-His_6 (Addgene plasmid #49417) and PTEN-V5-His_6 (Addgene plasmid #49420) in the bacterial JpExpress404 vector or in the pcDNA3 vector were obtained from Addgene or Ramon Parsons (Hopkins et al., 2013). Bacterial or eukaryotic expression plasmids encoding red fluorescent protein (RFP) fusion proteins with PTEN or PTEN-Long were constructed as described (Hopkins et al., 2013). Plasmids expressing PTEN-Long mutants with single amino acid substitutions C297S, G302E, or Y311L were generated in reference to the corresponding mutations of PTEN (Peyrou et al., 2013). His_6-blue fluorescent protein (BFP)-expressing plasmid in the pT7 His_6-SUMO vector (Lucigen) was described previously (Hoffman et al., 2015). Plasmid expressing a fusion protein between HCV-2a J6 core and enhanced green fluorescent protein (EGFP) was also constructed. All plasmids were generated using standard methods and confirmed by DNA sequencing when necessary. HCV RNA was generated by in vitro transcription using the MEGAscript T7 *In Vitro* Transcription reagents (Thermo Fisher Scientific).

2.2. Cell culture, transfection, fluorescent microscopy, and HCV infection

Huh-7 and Huh-7.5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Huh-7 cells with replicating HCV-2a J6 core-Flag/JFH-1(p7-RLuc-2A) or HCV-2a J6 core R50A-Flag/JFH-1(p7-RLuc-2A) RNAs were maintained with culture medium plus G418 (Enzo Life Sciences) as described previously (Wu et al., 2017). Cells were transfected with plasmid DNA or HCV RNA using the calcium phosphate precipitation method (Jackel-Cram et al., 2007) or the jet-PEI transfection reagent (Polyplus Transfection) (Shi et al., 2016). Cells transfected with fluorescent protein-expressing plasmids were visualized with appropriate settings under a Leica TCS SP5 confocal microscope (Leica Microsystems). HCV-2a JFH-1 rLuc subgenomic replicon cells and infection with HCV-2a J6 core-Flag/JFH-1(p7-RLuc-2A) virus were described previously (Wu et al., 2017).

2.3. Luciferase and RT-qPCR assays

Cells were lysed in Passive Lysis Buffer (Promega) and the firefly or renilla luciferase activities were measured by Luciferase Assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs). Luciferase levels were normalized to the protein concentrations determined by the Bradford assay (Bio-Rad Laboratories). To determine the HCV RNA levels, total RNA was extracted by Trizol (Thermo Fisher Scientific) and reverse transcribed into cDNA by SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). Real time PCR was performed using primers HCV-FD (5`-AGAGCCATAGTGGTCTGCGGAAC-3`) and HCV-rev (5`-CCTTTCGCAACCCAACGCTACTC-3`) (Lim and Hwang, 2011). The transcript levels of GUSB, a house keeping gene, were used for normalization (Qiao et al., 2013). Relative changes in RNA levels were analyzed by the $2^{-\Delta\Delta ct}$ method using the iQ5 program (Bio-Rad Laboratories). The results were analyzed for statistical differences by the Student's *t*-test. A p value of ≤ 0.05 was considered statistically significant.

2.4. Co-immunoprecipitation (Co-IP) and Western blotting

For Flag co-IP experiments, cells were harvested by radioimmunoprecipitation assay (RIPA) buffer and incubated with anti-Flag (Sigma-Aldrich) antibody at 4 ℃ overnight and then incubated with Protein G Sepharose (GE Healthcare) at 4 ℃ for 4 h. The mixtures were centrifuged at 10,000 rpm for 10 min and the supernatants were removed. The pellets were resuspended in SDS lysis buffer and boiled for 10 min to elute the proteins. For Western blotting, proteins were subjected to SDS-PAGE and then blotted onto nitrocellulose membranes. The membranes were blocked in 5% skim milk in PBS and incubated with a primary antibody overnight at 4 ℃. Membranes were washed and incubated with a secondary antibody for 1 h at room temperature. After a wash with PBST (PBS+0.1% Tween 20), membranes were scanned using Li-Cor Odyssey scanner (ODY-CLx) and band intensities were determined by Quantity One software (Bio-Rad Laboratories). The primary antibodies used were HCV core (Anogen), Flag (Sigma-Aldrich), PTEN and β-actin (Cell Signaling Technology), and PTEN-Long (Gly-2) provided by Ramon Parsons (Hopkins et al., 2013). The secondary antibodies used were IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (Li-Cor Biosciences).

2.5. Purification of recombinant proteins

The expression of recombinant proteins in *E. coli* was induced by IPTG (Thermo Fisher Scientific). His_6-tagged PTEN, PTEN-Long, BFP, PTEN-RFP, PTEN-Long-RFP, and RFP proteins were purified by Ni-NTA agarose (Qiagen) as previously described (Hoffman et al., 2015).

3. Results

3.1. Intracellular PTEN-Long inhibits HCV replication

We recently showed that ectopic expression of PTEN inhibits HCV replication (Wu et al., 2017). Whether PTEN-Long, a longer isoform of PTEN, can also regulate HCV replication has not been studied. To investigate the effect of PTEN-Long on HCV replication, we transfected Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1(p7-rLuc-2A) replicating RNA with vector, plasmids expressing PTEN or PTEN-Long and measured HCV replication by luciferase assay. We also used a set of plasmids expressing fusion proteins with RFP, which will enable subsequent experiments. Consistent with our previous results, overexpression of PTEN significantly inhibited HCV replication in comparison to vector (Fig. 1A). Expression of the fusion protein PTEN-RFP also inhibited HCV replication as effectively as PTEN itself, while RFP expression had no effect (Fig. 1A). Overexpression of PTEN-Long or PTEN-Long-RFP could reduce HCV replication to the same extent as PTEN (Fig. 1A). The intracellular expression of PTEN and PTEN-Long proteins was demonstrated by Western blotting using a PTEN-specific antibody (Fig. 1B). It is interesting to point out that while the endogenous PTEN protein could be readily detected in the Huh-7 cells as shown in Fig. 1B, the endogenous PTEN-Long protein was not visible in Western blotting using a PTEN-Long-specific antibody (Gly-2) (Hopkins et al., 2013) (data not shown). These results indicated that both PTEN and PTEN-Long can inhibit HCV replication when expressed intracellularly.

Because Huh-7 cells harboring HCV-2a J6 /JFH-1(p7-rLuc-2A) or HCV-2a J6 core-Flag/JFH-1(p7-rLuc-2A) replicating RNA have been shown to produce infectious virus particles (Jones et al., 2007; Wu