Virus Research 172 (2013) 24–34

Contents lists available at SciVerse ScienceDirect

Virus Research

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

The p7 protein of the hepatitis C virus induces cell death differently from the influenza A virus viroporin M2

Jude Juventus Aweya [a], Tze Minn Mak [b], Seng Gee Lim [c], Yee-Joo Tan [a,b,d,*]

[a] Department of Microbiology, Yong Loo Lin School of Medicine, National University Health System (NUHS), National University of Singapore, Singapore
[b] NUS Graduate School for Integrative Sciences and Engineering, Singapore, National University of Singapore, Singapore
[c] Department of Medicine, Yong Loo Lin School of Medicine, National University Health System (NUHS), National University of Singapore, Singapore
[d] Institute of Molecular and Cell Biology, A*STAR (Agency for Science, Technology and Research), Singapore

ARTICLE INFO

Article history:
Received 28 September 2012
Received in revised form 4 December 2012
Accepted 4 December 2012
Available online 12 December 2012

Keywords:
HCV
p7 protein
Cell death
Ion channel activity

ABSTRACT

Most viruses encode proteins that modulate cell-death signaling by the host. For hepatitis C virus (HCV) infection, apoptosis and other forms of cell-death have been observed in vitro and in vivo but the detailed understanding of this intricate viral-host interplay is unclear. This study examined the role played by the HCV p7 protein in the induction of cell-death. By measuring caspase-3/7 activation and cleavage of endogenous PARP, two hallmarks of apoptosis, the overexpression of p7 protein was shown to induce apoptosis in Huh7.5 cells. Furthermore, p7-induced apoptosis is caspase-dependent and involves both the intrinsic and extrinsic pathways. Similar to the M2 protein of influenza A virus, p7-induced apoptosis is independent of its ion channel activity. Coimmunoprecipitation experiments further showed that both M2 and p7 interact with the essential autophagy protein Beclin-1. However, only the M2 protein could cause an increase in the level of LC3-II, which is an indicator of autophagic activity. Thus, although the p7 protein is functionally similar to the well-characterized M2 protein, they differ in their activation of autophagic cell-death. Taken together, these results shed more light on the relationship between the HCV p7 ion channel protein and cell-death induction in host cells.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Hepatitis C virus (HCV), a small positive strand RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family, is one of the major causes of liver disease such as cirrhosis, steatosis, and hepatocellular carcinoma (Chen and Morgan, 2006). In fact, HCV infection is the most frequent indication for liver transplantation in developed countries (Brown, 2005). Currently, about 130–170 million or 3% of the world population are estimated to be infected with HCV; with about 3–4 million new infections per year (WHO, 2012). HCV has a high degree of genetic diversity, so it is classified phylogenetically into six major genotypes and several subtypes (Simmonds, 2004). The HCV genome of ~9.6 kb encodes a unique open reading frame that is translated into a precursor polyprotein of ~3000 residues. Co- and post-translational processing of the polyprotein by both viral and cellular proteases yields three structural (core, E1 and E2), six non-structural proteins (NS2–NS5), and a small membrane ion channel protein (p7) (Lin et al., 1994); the protein of interest in this study.

The HCV p7 protein, a small (63 amino acid) hydrophobic protein located between the structural and nonstructural region of HCV, is not clearly identified as structural or nonstructural protein, although it has been shown to be a transmembrane protein (reviewed in Khaliq et al., 2011). HCV p7 is classified as a viroporin, a group of small hydrophobic proteins encoded by a variety of RNA viruses that oligomerize to form pores (ion channels) in host-cell membranes through which viruses can enter/exit as well as contribute to virus assembly and pathology of disease by altering membrane permeability and disrupting ion homeostasis in cells (Gonzalez and Carrasco, 2003). The precise role of p7 in the HCV life-cycle has been hard to define. However, some recent papers have implicated p7 in the assembly and release of virus particles, mostly acting in concert with other viral proteins (reviewed in Khaliq et al., 2011; Steinmann and Pietschmann, 2010), and in a genotype-specific manner (Steinmann et al., 2007). For example, a strain-specific tripartite relationship between core, p7 and NS2 has been reported to be responsible for modulating the subcellular localization of core (Boson et al., 2011) and NS2 (Tedbury et al., 2011), which is independent of p7's ion channel activity. Similarly, it has been shown that mutations in the p7 protein, either singly or in combination with E2 glycoprotein enhances several-fold

* Corresponding author at: MD4, 5 Science Drive 2, Singapore 117597, Singapore. Tel.: +65 65163692; fax: +65 67766872.
E-mail addresses: Yee_Joo_TAN@NUHS.edu.sg, mcbtanyj@imcb.a-star.edu.sg (Y.-J. Tan).

0168-1702/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2012.12.005

production of infectious virus particles in cell culture (Kim et al., 2011). Thus, p7 unlike other viroporins such as M2 of influenza A virus and Vpu of HIV-1, is absolutely essential for HCV replication in vitro (Brohm et al., 2009; Steinmann et al., 2007).

Cell death regulation is an important determinant in the survival of most viruses, therefore, many viruses encode proteins that interfere with cell death signaling pathways, skewing it in their favor (Chen et al., 2006). For HCV, both proapoptotic and prosurvival properties have been attributed to different HCV proteins (reviewed in Aweya and Tan, 2011; Fischer et al., 2007). HCV p7 like other members of the viroporins (e.g., HIV-1 Vpu, human T-cell lymphotropic virus-1 p13II, hepatitis B virus X protein, and influenza virus PB1 ORF2), might be targeted to the mitochondrial membranes where it modulates apoptosis by altering the mitochondrial membrane permeability. However, there is currently limited information on the apoptotic activity of p7 except that reported by Madan et al. (2008) who demonstrated that the p7 protein of genotype 1b HCV induces caspase-dependent apoptosis via the mitochondria in baby hamster kidney cells. Since the genotype 2a JFH1 strain is the only one that can replicate efficiently in permissive cells, such as Huh7.5 cells, without adaption, this study sought to examine how p7 protein of this strain modulates cell-death and how similar it is to the M2 ion channel protein of influenza A virus, which is a well characterized viroporin. We were able to show that HCV p7 protein induces caspase-dependent apoptosis which is independent of its ion channel activity, and although p7 protein shares a couple of functional properties with M2 protein of influenza A virus, they seem to differ in their induction of autophagic cell death.

2. Materials and methods

2.1. Cell culture and cell lines

Huh7.5 cells (subclone of the Huh-7 human hepatoma cell line; Apath, Brooklyn, NY) and 293FT cells (human embryonic kidney cell line with the temperature sensitive gene for SV40 T-antigen; Invitrogen, Karlsruhe, Germany) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Utah, USA), nonessential amino acids and antibiotics (10 units/ml penicillin and 10 μg/ml streptomycin) (Invitrogen, Carlsbad, CA). All cells were maintained in a 37 °C incubator with 5% CO_2.

2.2. Plasmid construction

Expression plasmids for the HCV p7 proteins were generated by PCR cloning using Titanium Taq DNA polymerase (Clontech Laboratories Inc., Palo Alto, CA). Two plasmids containing the full-length HCV genome of the genotype 1b (S1) cloned in Singapore ((Soo et al., 2002) GenBank accession number AF356827) and genotype 2a JFH-1 strain ((Wakita et al., 2005), GenBank accession number AB047639) were used as templates. The PCR products were digested with restriction enzymes BamHI and XhoI and then ligated into the pXJ40flag vector which is a flag-tagged plasmid derived from pXJ40 (Xiao et al., 1991). The pXJ40flag vector was used so that a flag epitope is fused to the N-terminus of the p7 protein to allow the comparison of protein expression levels with an anti-flag antibody. Similarly, the p7 mutants were generated by 2 rounds of PCR: the first round of PCR was used to generate 5′ and 3′ fragments of p7 containing the appropriate alanine or glutamine substitutions; these were then amplified into full-length p7 using end primers for the p7 gene and cloned into pXJ40flag vector using BamHI and XhoI sites. The M2 gene of influenza A virus (A/Puerto Rico/8/34/Mount Sinai/Wi (H1N1), GenBank accession number AY768951.1) was produced by gene synthesis (GenScript USA Inc., Piscataway, NJ, USA) and cloned into the pXJ40flag vector in a similar manner. All sequences were confirmed by sequencing performed by the DNA Sequencing Facility at the Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore.

2.3. Transient transfections and Western blot analysis

Transient transfections of cells were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Approximately 24 h after transfection, the cells were harvested by scrapping into the media, spun down in a bench-top centrifuge and washed twice with cold PBS. The cell pellets were then resuspended in RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP40, 0.5% deoxycholic acid, 0.005% SDS and 1 mM phenylmethylsulfonyl fluoride) and subjected to freeze–thawing five times before being spun down at 13,000 rpm to remove cellular debris. The cell lysate was then used for Western blot analysis and quantification of apoptosis (see next section below).

Primary antibodies used in the study included anti-actin monoclonal, anti-flag monoclonal and polyclonal (Sigma, St. Louis, MO), anti-poly[ADP-ribose] polymerase [PARP] polyclonal, and anti-LC3B polyclonal (Cell Signaling Technology Inc., Beverly, MA) antibodies. Secondary antibodies used included horseradish peroxidase (HRP)-conjugated goat anti-mouse, and goat anti-rabbit antibodies (Pierce, Rockford, IL).

2.4. Apo-One fluorometric and TUNEL assay

Apoptosis was quantified in the form of caspase-3/7 activation using the Apo-One fluorometric assay system from Promega Corporation (Madison, WI) according to the manufacturer's protocol. To further confirm the induction of apoptosis by cells overexpressing p7 protein, the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was carried out after 24 h of transfection using the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, cells were plated in Lab-Tek™ Chamber Slides, transfected with plasmids expressing p7 protein followed by treatment with cell permeable caspase inhibitors as described below. After 24 h, cells were fixed in 4% paraformaldehyde at 4 °C for 25 min. Fixed cells were then permeabilized in 0.2% Triton X-100 and labeled with fluorescein-12-dUTP using terminal deoxynucleotidyl transferase. After rinsing with PBS, slides were mounted with VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). The TUNEL-positive cells (green fluorescence) corresponding to the nuclei location (DAPI) were captured with Olympus FluoView FV1000 (Olympus, Japan) laser scanning confocal microscope using a 60x/1.45 oil objective, with 543 nm HeNe laser as the excitation source.

2.5. Treatment of cells with caspase inhibitors and ammonium chloride

Cells were transfected (as above) followed by treatment with 2.5 μM of caspase-3 (Z-DEVD-fmk), caspase-8 (Z-IETD-fmk), caspase-9 (Z-LEHD-fmk) or a negative caspase inhibitor (Z-FA-fmk) (BD Biosciences). Briefly, 6 h post-transfection, the transfection media was changed and replinished with fresh media plus 2.5 μM of the respective cell permeable caspase inhibitors which are reported to have a very short half-life (Ekert et al., 1999; Garcia-Calvo et al., 1998). After about 24 h post-transfection, the cells were harvested by scrapping into the media, spun down in a bench-top centrifuge and washed three times with 1× cold PBS to remove any residual inhibitor found in the media. The

Download English Version:

https://daneshyari.com/en/article/3428566

Download Persian Version:

https://daneshyari.com/article/3428566

Daneshyari.com