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Interaction of HCV core protein with 14-3-3ε protein releases Bax to activate apoptosis

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Abstract

Through protein–protein binding assays, we found that HCV core protein interacted with 14-3-3ε protein. Interestingly, the expression of HCV core protein induced apoptosis in 293T cells. The apoptosis induced by core expression is accompanied by translocation of Bax from cytosol to mitochondria, disruption of mitochondrial membrane potential, cytochrome *c* release, and activation of caspase-9 and caspase-3. Furthermore, over-expression of 14-3-3ε inhibited the core-induced apoptosis and Bax translocation to mitochondria. These results indicate that HCV core protein induces the Bax-mediated apoptosis by interacting with 14-3-3ε protein in 293T cells. As a mechanism of apoptosis induction by HCV core, we propose that the interaction of HCV core with 14-3-3ε causes the dissociation of Bax from the Bax/14-3-3ε complex in cytosol, and the free Bax protein provokes activation of the mitochondrial apoptotic pathway.

Keywords: HCV core protein; 14-3-3ε; Apoptosis; Bax; Mitochondrial membrane potential; Cytochrome *c*; Caspase-3

Hepatitis C virus (HCV) is a major causative pathogen of chronic liver diseases including hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. The core protein of HCV is known as a multifunctional regulator of cellular events including apoptosis. HCV core protein inhibits apoptosis mediated by cisplatin [2], TNFα [3], and Fas [4], but pro-apoptotic effects have been also presented. The HCV core protein triggered apoptosis by inducing ER stress and ER calcium depletion [5], and protein kinase R (PKR) activation [6]. It also sensitizes the Fas- and TNFα-mediated apoptosis [7–10].

The dimeric form of the 14-3-3 proteins binds a number of proteins mostly with phospho-Ser/Thr motifs leading to many changes in biological processes. These proteins are involved in regulation of cell survival through interaction with pro-apoptotic proteins: Bad, Bax, FKHRL1, ASK1, and Nur77 [11–16]. The 14-3-3 protein binds phosphorylated target proteins at the consensus 14-3-3 binding motifs (RSXpSXP or RXXXpSXP) [17,18]. Exceptionally, Bax interacts with 14-3-3 proteins in a phosphorylation-independent manner [14]. The 14-3-3θ, σ, ζ, and ε proteins interact with Bax protein in cytoplasm [14,15], and Bax is dissociated from the 14-3-3 proteins in response to stress, and translocates to mitochondria to stir up the caspase-dependent and -independent apoptotic pathways [14].

On the other hand, caspases cleave 14-3-3θ within its carboxyl terminal region and promote its dissociation from Bax [14] and Bad [13], respectively. Deletion of 14-3-3 also induces Bax translocation to mitochondria in response to cellular stimuli [19]. Phosphorylation of 14-3-3ζ and 14-3-3σ by JNK also leads to the release of several 14-3-3 ligands from 14-3-3, including Bax and FOXO3a [14,15]. These together indicate that the 14-3-3 and its ligand interaction is regulated by both cleavage and phosphorylation of 14-3-3 proteins.

With known more than 200 ligands of 14-3-3, HCV core was documented as one of 14-3-3 ligand. In this study, we report that HCV core interacts with 14-3-3ε to induce

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apoptosis in 293T cells and activates the mitochondrial apoptotic pathway. In addition, HCV core-induced apoptosis was inhibited by over-expression of 14-3-3ε. The results indicate that HCV core protein induces apoptosis by dissociating Bax from the 14-3-3ε/Bax complex.

Materials and methods

Cells and reagents. Human embryonic kidney 293T cells were maintained in DMEM (Jeil Biotech services, Daegu, Korea) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. Anti-HCV core antibody (c7-50) was from Affinity BioReagents (Golden, CO), anti-tubulin antibody (T5186) was from Sigma (St. Louis, MO). Antibodies to 14-3-3ε (sc-1020), Bax (sc-493), HRP-conjugated goat anti-mouse antibody (sc-2005), and HRP-conjugated goat anti-rabbit antibody (sc-2004) were from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies to cytochrome *c* (556433), caspase-9 (51-8127KC), and Heat-shock protein (HSP) 60 (611562) were from BD Biosciences (San Diego, CA). Benzyloxycarbonil-Val-Ala-DL-Asp fluoromethylketone (Z-VAD-fmk) was purchased from AG scientific (San Diego, CA).

Plasmid constructs. For expression of EGFP-fused HCV core protein in mammalian cells, the coding region of the core protein was amplified by PCR from the CMV-C980 vector containing HCV genotype 1b using a forward primer, 5′-AGTCGAATTCATGAGCACAAATCCTAAA-3′, and a reverse primer, 5′-AGTCCTCGAGAGCGGAAGCTGGGA TGGT-3′, respectively. The PCR product was subcloned into *Eco*RI and *Sal*I sites of the pEGFP-C2 vector (Clontech, Palo Alto, CA). The glutathione *S*-transferase (GST) fusion constructs were made with amplified cDNA encoding full-length 14-3-3ε. The PCR products were subcloned into *Eco*RI and *Xho*I sites of pGEX4T-1 bacterial expression vector.

GST pull-down experiment. To prepare GST-14-3-3ε fusion proteins, *Escherichia coli* BL21 strain was transformed with pGEX4T-1/14-3-3ε(F) or pGEX4T-1/14-3-3ε(T) plasmids encoding the full-length (1–255) or a truncated form (127–255) of 14-3-3ε, induced by 0.25 mM IPTG for 3 h at 30 °C, and cells were lysed with a lysis buffer (1% Triton X-100 in PBS) with sonication on ice. The soluble GST fusion protein was immobilized on a glutathione-agarose bead (Sigma, St. Louis, MO). [^{35}S]Methionine-labeled HCV core protein was prepared *in vitro* by using the TNT reticulocyte lysate system (Promega, Madison, WI). Ten microliters of the ^{35}S-labeled core protein was incubated with the immobilized GST fusion proteins (GST, GST-14-3-3ε (F or T)) in 0.5 ml of binding buffer (50 mM Tris–Cl [pH 7.4], 125 mM NaCl, 5 mM EDTA, 10% [v/v] glycerol, 0.5% [v/v] NP-40, 1 mM PMSF) for 4 h at 4 °C. The mixtures were analyzed by SDS–PAGE, and with a Molecular Imager FX analyzer (Bio-Rad, Hercules, CA).

Cell fractionation, immunoblotting, and co-immunoprecipitation. Cell fractionation was performed using a mitochondria isolation kit (Pierce, Rockford, IL). Briefly, cells (1×10^7 cells/ml) were washed with ice-cold PBS twice, re-suspended in reagent A containing protease inhibitors. After adding buffer B, the mixture was vortexed for 5 min and added with reagent C. The nuclei and unbroken cells were removed by centrifugation at 700*g* for 10 min. The supernatant was centrifuged at 12,000*g* for 15 min to collect the cytosolic and mitochondrial fraction.

For Western blot, cells were harvest at 12, 24 h post-transfection, and lysed in RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 0.5% NP-40, 0.1% SDS, 0.1% deoxycholate, 1 mM PMSF), respectively. The samples (2–10 μg) were subjected to SDS–PAGE and Western blot using primary antibodies and HRP-conjugated secondary antibodies followed by the West-Pico chemiluminescence substrates (Pierce, Rockford, IL) in dark condition. For co-immunoprecipitation, 400 μg of the total lysate precleared with Protein A agarose beads and normal rabbit serum was incubated with 1–2 μg of anti-14-3-3ε antibody at 4 °C for 1 h, and added with the protein A agarose bead. After 1 h incubation at 4 °C, the samples were analyzed by immunoblot.

Confocal microscopy. The cells grown on coverslips were fixed with ice-cold 4% paraformaldehyde at RT for 15 min and permeabilized with a buffer (0.5% Triton X-100 in PBS) for 20 min, and followed by a staining with 20 μg/ml Hoechst dye (Molecular Probes, Eugene, OR) for 5 min in dark condition. After washing with PBS, coverslips were mounted on glass slides and analyzed by a laser scanning microscope (LSM 510, Carl Zeiss, Oberkochen, Germany).

Caspase activity assay. All experiments were performed using a caspase fluorescent assay kit (Peptron, Daejeon, Korea) by manufacturer's instruction. Briefly, cells (1×10^6 cells/ml) were resuspended in lysis buffer on ice for 10 min. After microcentrifugation at maximal speed for 3 min, supernatants were mixed with the provided caspase-3 substrate at 37 °C for 1 h. Intensity of fluorescence was detected at 460 nm in the 360 nm excitation wave length using a VICTOR^3 fluoroluminometer (Perkin-Elmer, Boston, MA).

Flow cytometry analysis. Cells (1×10^6 cells/ml) were fixed with 70% ice-cold ethanol at 4 °C and stained with 50 μg/ml propidium iodide (PI) containing 100 μg/ml RNase at 37 °C for 1 h and subgenomic content was evaluated by FACSCalibur (Becton–Dickinson, San Jose, CA). To measure mitochondrial membrane potential (MMP), cells (1×10^6 cells/ml) stained with 500 μl of 1 μg/ml JC-1 solution at 37 °C for 20 min were analyzed by the FACSCalibur using the Cell Quest software. The JC-1 emits red fluorescence (intact mitochondria, 590 nm) and green fluorescence (uncoupled mitochondria, 527 nm).

Results

HCV core protein interacts with 14-3-3ε in vitro and in vivo

We isolated a partial sequence of 14-3-3ε (corresponding to amino acids from 127 to 255) as a candidate protein interacting with HCV core protein through a yeast two-hybrid screening of a HeLa cell library using a processed form of core protein (amino-terminal 120 amino acids) as bait [20]. *In vitro* translated ^{35}S-labeled core protein (amino-terminal 191 amino acids) was incubated with GST-14-3-3ε fusion protein containing the full-length (1–255) or a truncated form (127–255), respectively. We found that the core protein was precipitated with both the full-length and truncated form of GST-14-3-3ε protein (Fig. 1A). To confirm their interaction in mammalian cells, we performed a co-immunoprecipitation experiment using 14-3-3ε-specific antibody in 293T cells transiently transfected with the full-length core expressing vector. The core protein was co-immunoprecipitated with 14-3-3ε (Fig. 1B). Furthermore, in the transfected cells with both GFP-core and RFP-14-3-3ε expression vectors, the GFP-core protein was co-localized with the RFP-14-3-3ε protein at perinuclear region of the cells (Fig. 1C). These results clearly indicate that the HCV core protein physically interacts with 14-3-3ε protein.

HCV core protein induces apoptosis in 293T cells

The 14-3-3ε protein was known as a substrate of the caspase-3, and associated with Bad in cytosol [13]. The cleaved form (D238) of 14-3-3ε has lower affinity to Bad than wild-type, so that 14-3-3ε dissociates from Bad/14-3-3ε complex, and the dissociated Bad protein interacts with Bcl-xL to translocate to mitochondria in apoptotic cells. Since HCV core protein interacts with the 14-3-3ε protein, we hypothesized that an ectopic expression of HCV core may affect cell survival.