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A Novel Protein to Bind HCV Core Protein: The Carboxyl Terminus-Truncated $Core_{120}$ Protein of HCV Interacts with E7 Antigen of Human Papilloma Virus Type 18

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Abstract In order to analyze the cellular proteins which interact with core protein of hepatitis C virus (HCV), a yeast two-hybrid screening technique was employed. A carboxyl terminus truncated core protein, which contained amino acid residues from the 1st to 120th, was used as a bait to screen cellular proteins. The expression library prepared from HeLa cell was screened and 400 positive clones were selected. The 75 clones from the positive clones were sequenced and analyzed by undergoing the Blast search. Interestingly, 7 out of the 75 clones encoded E7 antigen of human papilloma virus (HPV). We studied in detail the interaction between the truncated version of HCV core and E7 antigen *in vitro*. The $core_{120}$ protein expressed in chimeric form with GST was able to bring down the E7 protein of HPV type 18 expressed in bacteria. It is therefore suggested that the core of HCV might affect the interaction between E7 and a normal cellular tumor suppressor, known as Rb protein.

Key words: Hepatitis C virus, core protein, human papilloma virus, E7

Hepatitis C virus (HCV) is recognized as a positive strand RNA virus belonging to the *Flaviviridae* family [4], and is considered to be the major etiological agent of posttransfusion non-A, non-B hepatitis (NANB). It has been identified as the causes of most cases of post-transfusion viral hepatitis, which result in cirrhosis and eventually hepatocellular carcinoma [21]. The genomic RNA of Hepatitis C virus contains a single open reading frame for its precursor, which is proteolytically processed into at least 10 polypeptides [5, 22]. The large open reading frame encodes collinearly structural and nonstructural proteins; the core protein, the glycoprotein envelope 1 (E1) and 2 (E2), the 7 kDa peptide (p7), along with the nonstructural proteins 2–5 (NS2, 3, 4a, 4b, 5a, 5b).

The core protein of HCV is a multifunctional protein involved in several processes. It has several phosphorylation sites for protein kinases A and C [24], and activates NF-κB signaling pathway through TNF receptor-associated factor [29, 31], repress the cyclin-dependent kinase inhibitor p21 gene expression in cooperation with hepatitis B virus (HBV) X protein [6], and modulates Rb pathway through Rb down-regulation [3]. It activates c-myc, Rous sarcoma virus LTR, and SV40 early promoter, but suppresses the c-fos promoter, HIV-1 LTR activity [19], and HBV gene expression with replication [25]. It also interacts with the cytoplasmic tail of the LT-β receptor [2, 13], and associates with cellular lipid storage droplets [1, 23]. The effect of the core protein in apoptosis is implicated in the transforming activity as well as in pathogenesis. The core protein is also known to inhibit the cisplatin- and c-myc-mediated apoptosis [20].

The direct oncogenic activity of the HCV core protein has been demonstrated *in vitro* and *in vivo*. The embryonic rat fibroblasts are transformed in cooperation with the ras oncogene [18]. The transgenic mice which express core are prone to hepatocellular carcinoma [14]. Furthermore, HCV infection has been associated with increased risk of non-Hodgkin's lymphomas [15, 17]. All of the above mentioned suggest that the core protein is actually involved in pathogenesis of HCV-infected human hepatocellular carcinoma.

In the present study, we screened cellular proteins interacting with HCV core protein by the yeast two-hybrid

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system. Interestingly, the E7 oncogene of the human papilloma virus (HPV) type 18 was found to interact with the truncated $core_{120}$ protein, which corresponded to the 16 kDa form of core protein, suggesting that HCV core protein may influence the transforming process of HPV E7 oncogene via Rb down-regulation.

Materials and Methods

Plasmids

Plasmids constructed in this study are listed in Table 1. The cDNA encoding HCV $core_{120}$ (amino acid residues 1–120 with truncated C-terminal hydrophobic region) and the $E2_{715}$ (amino acid 384–715 with truncated C-terminal transmembrane region) were amplified by PCR from the plasmid CMV-C980 containing core, E1, E2, and p7 of HCV genotype 1b [12]. For the HCV $core_{120}$ amplification, oligonucleotides 5' AGTCGAATTCATGAGCACAAATCCTAAA 3' (forward) and 5' AGTCCTCGAGTACCCAAATTACGCGACC 3' (reverse) were used. For the HCV $E2_{715}$ amplification, olignucleotides 5' GATCGAATTCCACACCCACGTGACAG 3' (forward) and 5' GATCCCGAGTTTGATTACGACGGAG 3' (reverse) were used. The pLexA/$core_{120}$ and pLexA/$E2_{715}$ were constructed by making an insertion of the PCR products into the pLexA vector (Clontech, Palo Alto, U.S.A.) after *Eco*RI and *Xho*I digestion.

The E7 cDNA of HPV type 18 was amplified by PCR by using a plasmid pRcCMV/HPV18-E7 as a template and oligonucleotides 5' GATCGAATTCATGCATGGACCTAA 3' (forward) along with 5' GATCCTCGAGTTACTGCTGGGATG 3' (reverse) as primers [9]. pJG4-5/HPV E7 was constructed by making an insertion of the PCR product into the *Eco*RI and *Xho*I digested pJG4-5 vector (Clontech, Palo Alto, CA, U.S.A.).

For the production of GST fusion protein, pGEX4T-1/$core_{120}$ vector was constructed by inserting *Eco*RI and *Xho*I fragments of pLexA/$Core_{120}$ into the corresponding sites of pGEX4T-1 (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). For the production of N-terminal histidine-tagged protein, pET28/HPV E7 vector was constructed by subcloning the *Bam*HI and *Xho*I fragments of pGEX4T-1/HPV E7 into the corresponding sites of pET-28a(+) (Novagen, Madison, WI, U.S.A.).

Two-Hybrid Screening

We used the LexA BD-$core_{120}$ fusion protein as "bait" to screen a human HeLa cDNA library (from Dr. Jeong Keun Ahn, Chungnam National University). Thus, *Saccharomyces cerevisiae* reporter strain EGY48/pSH18-34 (Clontech, Palo Alto, CA, U.S.A.) was co-transformed with pLexA/$core_{120}$ and pJG4-5/human HeLa cDNA library by using the lithium acetate method. Primary transformants were plated onto the synthetic medium lacking uracil, histidine, tryptophan, and leucine, but containing 2% galactose and 1% raffinose, and isolated clones with leucine prototropy. After 5 days, all colonies were transferred to Whatmann filter paper and incubated in X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) containing solution for 8 h.

For carrying out the β-galactosidase activity assay in liquid culture, yeast cells were grown overnight to the midlog phase. Cells were then frozen in a liquid nitrogen, thawed at 37°C, and then assayed for β-galactosidase activity with o-nitrophenylgalactoside (Sigma, St. Louis, MO, U.S.A.) as the substrate.

Plasmid DNAs from LEU2, lacZ positive clones, were isolated and *E. coli* strain KC8 cells were transformed with the DNAs and cultured in a M9 medium. The plasmid DNAs were then isolated from the bacteria and analyzed by sequencing. DNA sequences were compared with the database from the National Center for Biotechnology Information by the BLAST program.

Bacterial Expression and Purification of HCV $Core_{120}$ and E7 of HPV Type 18

To prepare GST-$core_{120}$ fusion protein, *E. coli* BL21 strain was transformed with pGEX4T-1/$core_{120}$ plasmid, and the transformed *E. coli* BL21(DE3) was grown to 0.5 OD_{600} and induced by adding 0.1 mM IPTG for 4 h at 25°C. Cells were harvested, washed with phosphate buffered saline (PBS), resuspended in lysis buffer (1% triton X-100 in

Table 1. Description of plasmids.

Plasmid	Description	Source
pLexA	*LexA*, *HIS3*, Amp^r	Clontech
pLexA/$core_{120}$	HCV $core_{(1-120)}$ in pLexA, *HIS3*, Amp^r	This study
pLexA/$core_{178}$	HCV $core_{(1-178)}$ in pLexA, *HIS3*, Amp^r	This study
pLexA/$E2_{715}$	HCV $E2_{(384-715)}$ in pLexA, *HIS3*, Amp^r	This study
pJG4-5	Acidic activator B42, *TRP1*, Amp^r	Clontech
pJG4-5/HPV E7	HPV type18 E7 in pJG4-5, *TRP1*, Amp^r	This study
pSH18-34	*LacZ* under control of $LexA_{op(x8)}$, *URA3*, Amp^r	Clontech
pGEX4T-1	GST gene fusion, Amp^r	Amersham
pGEX4T-1/$core_{120}$	HCV $core_{(1-120)}$ in pGEX4T-1, Amp^r	This study
pET-28a(+)	His tag sequence, T7 promotor, Kan^r	Novagen
pET-28a/HPV E7	HPV type 18 E7 in pET-28a(+), Kan^r	This study

PBS), and sonicated on ice. Cleared lysates were loaded onto an glutathione agarose beads column (Sigma, St. Louis, MO, U.S.A.). After extensive washing with lysis buffer, GST-Core$_{120}$ fusion proteins were eluted with elution buffer (10 mM reduced glutathione, 50 mM Tris-Cl, pH 7.5).

N-terminus histidine tagged HPV 18 E7 protein was expressed in *E. coli* BL21(DE3) strain that was transformed with plasmid pET28/E7. *E. coli* BL21(DE3) was grown to 0.8 OD_{600} and induced by adding 1 mM IPTG for 4 h at 25°C. Cells were harvested, resuspended in lysis buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0), and sonicated on ice. Cleared lysates were loaded onto a Ni-NTA column (Qiagen, Valencia, CA, U.S.A.). After extensive washing with washing buffer (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, pH 8.0), histidine tagged HPV E7 protein was eluted with elution buffer (300 mM NaCl, 50 mM NaH_2PO_4, 250 mM imidazole, pH 8.0).

In Vitro Binding Assay

Glutathione-agarose beads (20 μl of 50% slurry) bound to the GST fusion protein was incubated at 4°C overnight with histidine tagged proteins (6 μg) in a binding buffer (300 mM NaCl, 50 mM NaH_2PO_4, 0.1% of Nonidet P-40, pH 8.0). Beads were washed three times with binding buffer, and the bound proteins were released by boiling in an SDS-sample buffer and separated by 10% SDS-polyacrylamide gel electrophoresis. The protein was transferred onto PVDF membrane and detected by Western blot analysis using an anti-6x His antibody (Roche, Mannheim, Germany).

Western Blot Analysis

Yeast extract was prepared by lysing cells with the cracking buffer (8 M Urea, 5% SDS, 40 mM Tris-Cl, 0.1 mM EDTA, 0.02% Bromophenol blue, 1% 2-mercaptoethanol, 5 mM PMSF) and an equal volume of acidic washed glass beads (425–600 μm) [16]. Western blot was performed according to the method described by Shim *et al.* [26]. Cell lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis, and the protein was then transferred onto PVDF membrane (Millipore, Bedford, MA, U.S.A.). The membrane was incubated with polyclonal anti-HCV core antibody (from Dr. Chul Joong Kim, Chungnam National University) and developed by ECL (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

RESULTS

Identification of HPV E7 Protein Interacting with Truncated HCV Core$_{120}$ Protein by a Yeast Two-Hybrid System

We used a yeast two-hybrid system in order to study potential HCV core-interacting cellular proteins from a HeLa cDNA library. A truncated form of core protein (containing amino acid residues of 1 to 120), which lacked the carboxyl-terminal hydrophobic part of the HCV core protein, was used as the bait. The deleted carboxy-terminal hydrophobic part is known to interfere with nuclear transportation of the bait protein in yeast. First of all, the expression of bait protein was detected by Western blot analysis in the EGY48/pSH18-34 yeast cells after transformation with pLexA/HCV core$_{120}$ construct. A 40 kDa protein band of predicted molecular size was detected with the HCV core polyclonal antibody in the extracts of yeast cells transformed with pLexA/HCV core$_{120}$, but not in the extracts of the pLexA vector-transformed cells (Fig. 1).

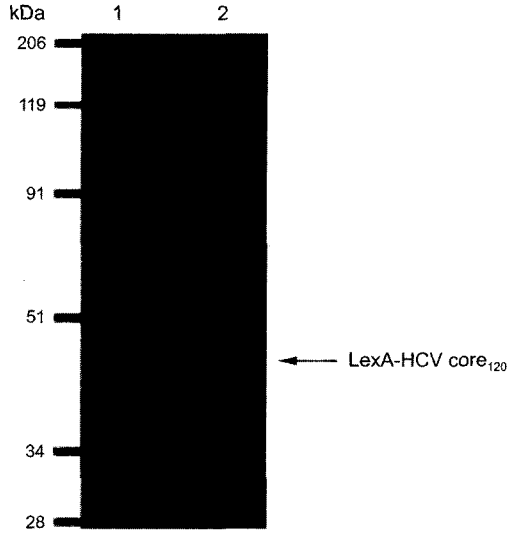

Fig. 1. Identification of LexA BD-HCV core$_{120}$ fusion protein expressed as a bait in yeast cells.
Protein extracts from pLexA vector only (lane 1) and pLexA/HCV core$_{120}$ (lane 2) transformed yeast cells were separated by SDS-PAGE (10%) and subjected to Western blot analysis with polyclonal anti-HCV core antibody.

To screen the HCV core$_{120}$ interacting cellular proteins, we co-transformed EGY48/pSH18-34 cells with the human HeLa cDNA library in pJG4-5 vector and the pLexA/HCV core$_{120}$ vector. Approximately 1×10^6 of the cotransformants were obtained and subsequently tested for leucine prototropy and β-galactosidase expression. Around 400 clones of LEU2, lacZ positive phenotype were obtained. Subsequently, 75 clones were chosen from the 400 clones, which showed high level of β-galactosidase activity in a liquid β-galactosidase assay. Sequence analysis of the 75 cDNA plasmids revealed that seven of these clones partially represented the E7 protein of HPV type 18. The frequency level was the highest when compared with other cloned genes.

To determine the specificity of the interaction between HCV core$_{120}$ and HPV E7 protein, we attempted to analyze the ability of full-length HPV E7 to interact with HCV core$_{120}$ and HCV E2$_{715}$ proteins by using the yeast two-hybrid system. The β-galactosidase activity was observed

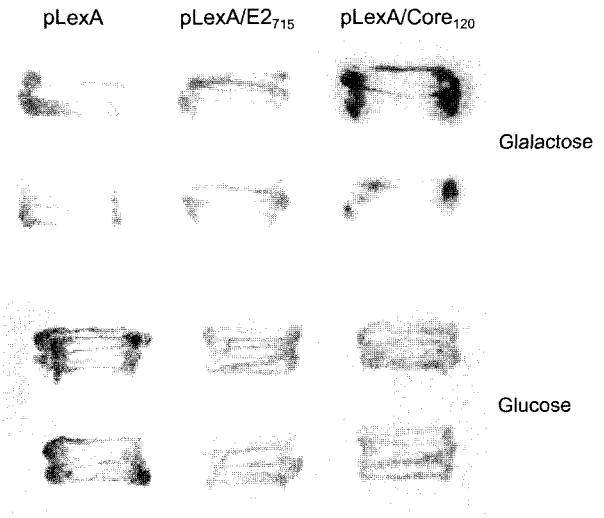

Fig. 2. Identification of the HPV E7 protein as a target of the HCV core$_{120}$ protein by the yeast two-hybrid system.
The yeast cells (EGY48/pSH18-43) were transformed with pJG4-5/HPV E7 along with either pLexA only or pLexA/HCV E2$_{715}$, or pLexA/HCV core$_{120}$. The transformants were allowed to grow in Ura/-His/-Trp glucose minimal medium which is selective for transformation. Yeast cells were then streaked out onto X-gal containing Ura/-His/-Trp/glucose or galactose minimal medium which is selective for galactose dependant β-galactosidase activity.

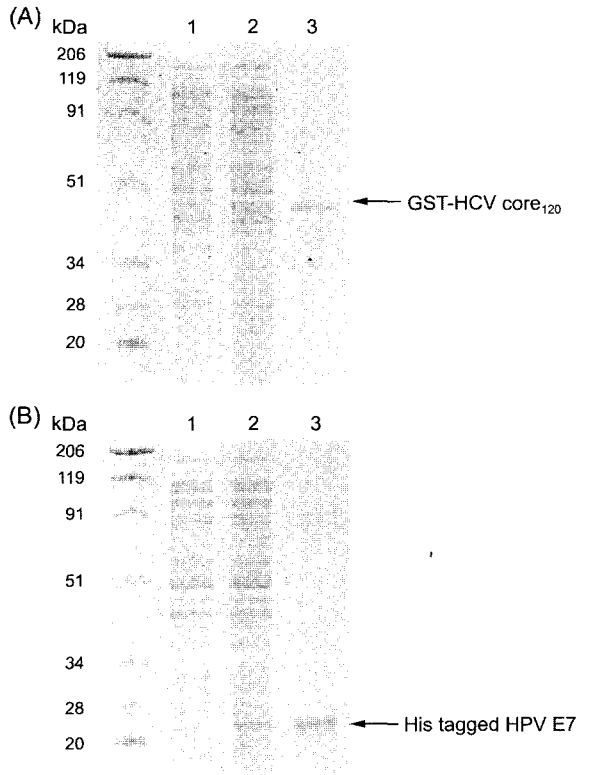

Fig. 3. Purification of GST-HCV core$_{120}$ and His tagged HPV E7.
pGEX4T-1/HCV core$_{120}$ (A) or pET28/HPV E7 (B) were transformed into E. *coli* BL21. The GST or His tagged proteins were induced by IPTG for 4 h. Cell extracts (noninduced: each lane 1; induced: each lane 2) and purified protein (each lane 3) were analyzed by SDS-PAGE (10%) and staining with Coomassie brilliant blue.

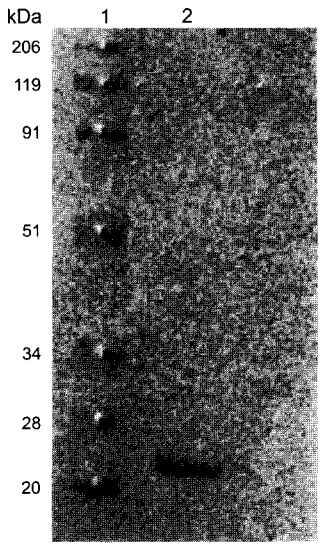

Fig. 4. *In vitro* binding analysis of HCV core$_{120}$ and the HPV E7.
Glutathione-agarose beads bound to the GST-core$_{120}$ (lane 1) or GST (lane 2) protein were incubated overnight with purified His tagged HPV 18 E7 protein. The bound protein was detected by Western blot analysis by using an anti-6x His antibody.

in the cotransformants with pJG4-5/HPV E7 and pLexA/HCV core$_{120}$ on X-gal containing galactose media, but not in the cotransformants with pLexA and pLexA/E2$_{715}$. However, on X-gal containing glucose media, the β-galactosidase actvity in any of the cotransformants was not shown. From these results, we confirmed specific interaction of HPV E7 with HCV core$_{120}$ in a yeast two-hybrid system (Fig. 2).

In Vitro Binding Assay; the Association of the Truncated HCV Core$_{120}$ Protein with HPV 18 E7 Protein

To confirm the above result from the yeast two-hybrid system, the *in vitro* binding properties of the HCV core$_{120}$ and HPV E7 proteins were examined. Thus, GST-HCV core$_{120}$ fusion protein was expressed in *E. coli* and purified through a glutathione-agarose bead column. N-terminal histidine tagged HPV 18 E7 protein was also expressed in *E. coli* and purified through the Ni-NTA agarose beads column (Fig. 3). The GST-HCV core$_{120}$ fusion protein that was immobilized onto glutathione-agarose beads was then incubated with histidine tagged HPV E7. After an extensive washing, the bound proteins were eluted from the beads, and separated by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4, the histidine tagged HPV E7 protein was retained to the GST-HCV core$_{120}$ fusion protein, but not to the GST alone.

Discussion

In the present study, we demonstrated the interaction of HCV core protein with HPV E7 oncogene. The HCV

infectivity on epithelium-originated cells such as HeLa cells, and the interaction of HCV core and E7 of other type of HPV (for example, type 16 which is prevalent in Korea) remain to be determined, even though the cooperation of the two oncogenes may be involved in the transforming processes.

The cellular processing of HCV core protein is not well elucidated [8, 10, 30]. Among the several processed products, the p21 kDa core protein that consists of 1–173 amino acids seems to translocate into the nucleus, which lacks the hydrophobic part in the carboxy terminus. We used 1–120 amino acids core protein as bait in a yeast two-hybrid system, which corresponds to the p16 kDa protein that translocates into the nucleus definitely [10].

Many evidences suggest that the core protein is involved in pathogenesis and hepatocellular transformation. HCV infection is associated with the mixed cryoglobulinemia [7], a benign monoclonal lymphoproliferation that leads to B cell non-Hodgkin's lymphoma [15], and increased risk of B cell lymphoproliferative disorders [17]. The direct relationship between these disorders is not yet clear, but many of the pathogenic activities of the HCV core protein have been well documented. For example, HCV core protein reduces HBV gene expression and replication [25]. The phosphorylation of serine 99 and serine 116 in the HCV core protein by protein kinases A and C has been shown to be an essential element for the suppressive activity on HBV gene expression and replication in HuH-7 human hepatoma cells [24]. Core proteins also repress the cyclin-dependent kinase inhibitor p21 gene expression in cooperation with HBV X protein [6], and also interacts with the cytoplasmic tail of the LT-β receptor to modulate the signaling pathway of LT-β receptor, which is involved in the development of peripheral organs, and in triggering cytolytic activity [29]. In addition, HCV core protein activates the NF-κB pathway through I-κB kinase (IKKβ) [2, 13]. The activation of the NF-κB pathway is interpreted to be a role of the core protein in the inflammatory reaction of "hepatitis" that is induced by HCV [31], which is probably a proceeding step leading to carcinogenesis. The core protein may have an effect on lipid metabolism inside of the cells by associating with cellular lipid storage droplets [1, 23]. All of these suggest that the core protein is involved in pathogenesis of hepatocellular carcinoma among HCV-infected humans.

The high-risk human HPVs are etiologically linked to human cervical and oral cancers. The E6 and E7 oncogenic proteins actually affect the host cell tumor suppressor proteins. E6 induces p53 proteolysis, and E7 induces Rb proteolysis [28]. The malignant transformation by HPV is associated with the loss of Rb function [27]. Interestingly, in our study to screen cellular proteins interacting with the HCV core protein, E7 of HPV type 18 and EID-1 (E1A-like inhibitor of differentiation) were found to be positive in the yeast two-hybrid screening. These two proteins share the EnLXCXE motif, which is the consenus sequence of the proteins interacting with the Rb protein [11]. The HCV core protein may also affect the Rb activity in an indirect way, involving the Rb-interacting proteins such as BRG1, Elf-1, SV40, and E1A of adenovirus type 5. Sung and his group earlier showed that the HCV core protein decreased the level of Rb protein by down-regulating the Rb mRNA and also by an uncharacterized indirect way [3]. We suggest that the HCV core interacts with the proteins with the EnLXCXE motif, which subsequently decreases the level of Rb activity indirectly. This mechanism may be involved in hepatocellular carcinoma formation.

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