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Identification of a Cellular Protein Interacting with RNA Polymerase of Hepatitis C Virus

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Hepatitis C virus (HCV) nonstructural 5B (NS5B) protein is an RNA-dependent RNA polymerase (RdRp). To determine whether it can contribute to viral replication by interaction with cellular proteins, the yeast two-hybrid screening system was employed to screen a human liver cDNA library. Using the HCV NS5B as a bait, we have isolated positive clones encoding a cellular protein. The NS5B interacting protein, 5BIP, is a novel cellular protein of 170 amino acids. Interaction of the HCV NS5B protein with 5BIP was confirmed by a protein-protein blotting assay. Recently, we have demonstrated that NS5B possesses an RdRp activity and thus it is possible that 5BIP, in association with NS5B, plays a role in HCV replication.

Keywords: Celluar factors, Hepatitis C virus, RNA polymerase, Viral replication, Yeast two-hybrid system

Introduction

Hepatitis C virus is the major cause of post-transfusion- and community-acquired non-A, non-B hepatitis (Choo *et al.*, 1989; Aach *et al.*, 1991; Alter et al., 1992), which often leading to liver cirrhosis and hepatocellular carcinoma (Saito *et al.*, 1990; Di-Bisceglie *et al.*, 1994; Shimotohno, 1995). HCV belongs to the *Flaviviridae* family (Francki *et al.*, 1991). The virion is enveloped and contains a single-stranded, positive-sense RNA genome of approximately 9.5 kilobases (Kato *et al.*, 1990; Inchauspe *et al.*, 1991). The genome encodes a long polyprotein of approximately 3,000 amino acids, which is cleaved by both cellular and viral proteases into multiple proteins (Hijikata *et al.*, 1991; Grakoui *et al.*, 1993; Lin *et al.*, 1994; Manabe *et al.*, 1994). The N-terminal one third of the protein is composed of structural proteins. These include the nucleocapsid (core) protein and envelope proteins (E1, E2, and p7) which are followed by six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B).

A workable vaccine strategy against HCV has been hampered due to continuous emergence of escape mutant viruses, even in the same inoculum (Farci *et al.*, 1992; Shimizu *et al.*, 1994; Wyatt *et al.*, 1998). For this reason, NS3 protease, RNA helicase, and NS5B RNA-dependent RNA polymerase (RdRp) have become the major target proteins for the development of anti-viral inhibitors. NS5B is believed to be the most important target because it is the key enzyme in HCV genome replication.

NS 5B encodes an RdRp. Indeed, several groups including our laboratory have demonstrated that the NS5B protein expressed in both eucaryotic and procaryotic cells possesses an RdRp activity (Behrens *et al.*, 1996; Lohmann *et al.*, 1997; Hwang *et al.*, 1999; Oh *et al.*, 1999). Since NS5B is the key enzyme responsible for HCV replication, its biochemical properties and the cellular factors which interact with NS5B are of particular interest. Previously, we reported that NS5B is membrane associated and localized in the perinuclear region (Hwang *et al.*, 1997). To explore the HCV replication mechanism, the yeast two-hybrid system was used to screen a human liver cDNA library for cellular factors that can interact with NS5B protein. In this study, we have identified a novel protein that may be involved in viral replication.

Materials and Methods

Plasmid Construction The HCV cDNA encoding NS5B (aa 2419 to aa 3011) of the Korean strain (Cho *et al.*, 1993) was generated by PCR and was cloned into the *Sal*1 site of the yeast plasmid pGBT9. The human liver cDNA library fused with the GAL4-activation domain in pGAD10 was purchased from Clontech company. The plasmid used for *in vitro* translation of the NS5B was constructed as described previously (Hwang *et al.*, 1997). To construct a plasmid expressing Glutathione S-transferase (GST) fusion protein, cDNA encoding 5BIP was

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inserted in frame into the *Bam*H1 site of pGEX2T expression vector (Novagen, Madison, USA). GST or GST-5BIP expression plasmids were grown in *Escherichia coli* BL21 (DE) (Novagen) and protein expression was induced at 30°C for 3 hr by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Yeast two-hybrid library screening *Saccharomyces cerevisiae* HF7C or SFY526 was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic minimal medium (0.67% yeast nitrogen base, 2% dextrose, and appropriate auxotrophic supplements). HF7C cells were cotransformed with both GAL4 DNA-binding domain plasmid and GAL4 activation domain library plasmid by the lithium acetate method. Colonies grown on tryptophan-, leucine-, and histidine- lacking plates were selected. Four or 5 days after transformation, the β-galactosidase activity was assayed by the colonies lifted on the nitrocellulose filter replicas of yeast transformants. Briefly, filters were placed in liquid nitrogen for 20 to 30s to permeabilize the cells. Following brief drying, filters were incubated for 2 to 6 h with buffer containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL, Clontech, PoloAlto, USA). Yeast clones containing library DNA were isolated from the blue positive colonies by tryptophan selection. These positive plasmids were further confirmed by testing for β-gal acivity in yeast strain SFY526.

Sequence analysis cDNAs isolated from the positive clones were sequenced by the dideoxynucleotide chain termination sequencing method (Sanger *et al.*, 1977). DNA sequences were compared against the database of the National Center for Biotechnology Information by the BLAST program.

***In vitro* transcription and translation** HCV NS5B encoding region was cloned into the *Bam*H1 site of the plasmid pcDNA3 (Invetrogen) followed by digestion with *Eco*R1. The linearized plasmid was used for synthesis of *in vitro* transcripts by using T7 RNA polymerase (Promega, Madison, USA) as described previously (Hwang *et al.*, 1997). RNA aliquots were translated in rabbit reticulocyte lysate (Promega) for 1 hr at 30°C using [^{35}S]methionine (Amersham).

Immunoprecipitation Aliquots of ^{35}S-labeled NS5B were incubated either with rabbit preimmune serum or with anti-NS5B rabbit serum with gentle rocking at 4°C for 2 hr. The immune complex was further incubated with protein-A Sepharose for 1 hr and precipitated by centrifugation in a microfuge. Pellets were washed four times with RIPA buffer [1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 0.1 mM PMSF] and resuspended in Laemmli sample buffer (Laemmli, 1970). Proteins were analyzed by electrophoresis on a 12% SDS-PAGE gel and detected by autoradiography.

Protein-protein blotting assay Both GST and GST-5BIP proteins expressed in *E. coli* were lysed by Laemmli sample buffer and then separated by SDS-PAGE on 12.5% polyacrylamide gels. One protein gel was stained with Coomassie brilliant blue and the other gel was electrotransferred to a nitrocellulose membrane. The membrane was briefly washed with buffer A [10 mM HEPES-KOH (pH 7.5), 60 mM KCl, 1 mM EDTA and 1 mM 2-mercaptoethanol] and incubated with 6 M guanidine HCl for 30 min. Further incubation was followed with sequentially diluted guanidine HCl to renature the proteins. The membrane was blocked for 1 hr with 5% nonfat dry milk in buffer A containing 0.05% Nonidet P-40. [^{35}S]methionine-labeled NS5B protein was then incubated with the membrane in buffer A containing 3% nonfat dry milk and 0.05% Nonidet P-40 overnight at 4°C. Unbound proteins were removed by vigorous washing in buffer A containing 1% nonfat dry milk and 0.05% Nonidet P-40. Protein binding was detected by autoradiography.

Results and Discussion

To identify possible cellular proteins interacting with HCV RNA polymerase (NS5B), the yeast two-hybrid system was employed to screen a human liver cDNA library using the NS5B as a bait. The NS5B open reading frame was fused in frame into the GAL4 DNA-binding domain of pGBT9 yeast vector, as shown in Fig.1. A human adult liver cDNA library fused to the GAL4 activation domain of pGAD10 yeast vector was used. These two plasmids were cotransformed into the HF7C yeast strain. We screened approximately 4 million yeast transformants which had grown in the absence of tryptophan, histidine, and leucine. Ten clones were obtained by β-Gal assay. Eight library cDNA plasmids (pGAD10 hybrid) were segregated from the pGBT9 hybrid plasmids. All contained the inserts. Of these, 3 clones retained β-Gal activity (blue colonies) when re-transformed into the yeast host only in the presence of the pGBT9-NS5B plasmid, but not the empty plasmid, indicating that 5BIP clone specifically interacts with NS5B protein. DNA sequence analysis showed that all 3 clones were identical. Sequence comparision using the BLAST program revealed that 5BIP encodes 170 aa and is an unknown protein (Fig. 2). Computer research showed that this protein shared no sequence homology with any other known proteins in the database, indicating that this is a novel protein. To demonstrate direct interaction between NS5B and novel

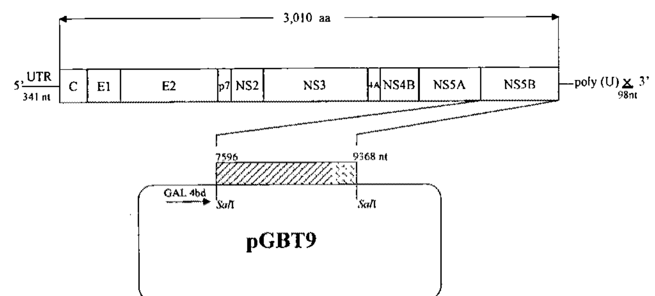

Fig. 1. Schematic diagram of HCV NS5B (RNA polymerase) construct in a yeast vector. HCV cDNA fragment representing the NS5B protein-coding region was cloned into the unique *Sal*1 site of the yeast plasmid pGBT9. The NS5B protein-coding sequence was fused in frame with the GAL4 DNA-binding domain of pGBT9 vector.

```
    atg ata agc ata cat aat gaa gaa gaa aat gct ttt ata ctg gat act ttg aaa aag caa
1   M I S I H N E E E N A F I L D T L K K Q

    tgg aaa ggc cca gat gat atc cta cta ggc atg ttt tat gac aca gat gat gcg agt ttc
21  W K G P D D I L L G M F Y D T D D A S F

    aag tgg ttt gat aat tca aat atg aca ttt gat aag tgg aca gac caa gat gat gat gag
41  K W F D N S N M T F D K W T D Q D D D E

    gat tta gtt gac acc tgt gct ttt ctg cac atc aag aca ggt gaa tgg aaa aaa gga aat
61  D L V D T C A F L H I K T G E W K K G N

    tgt gaa gtt tct tct gtg gaa gga aca cta tgc aaa aca gct atc cca tac aaa agg aaa
81  C E V S S V E G T L C K T A I P Y K R K

    tat tta tca gat aac cac att tta ata tca gca ttg gtg att gct agc acg gta att ttg
101 Y L S D N H I L I S A L V I A S T V I L

    aca gtt ttg gga gca atc att tgg ttc ctg tac aaa aaa cat tct gat tct cgt ttc acc
121 T V L G A I I W F L Y K K H S D S R F T

    aca gtt ttt tca acc gca ccc caa tca cct tat aat gaa gac tgt gtt ttg gta gtt gga
141 T V F S T A P Q S P Y N E D C V L V V G

    gaa gaa aat gaa tat cct gtt caa ttt gac taa
161 E E N E Y P V Q F D
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Fig. 2. Nucleotide and deduced amino acid sequence of 5BIP cDNA. cDNA isolated from a human cDNA library was sequenced and compared with the data base using the BLAST program.

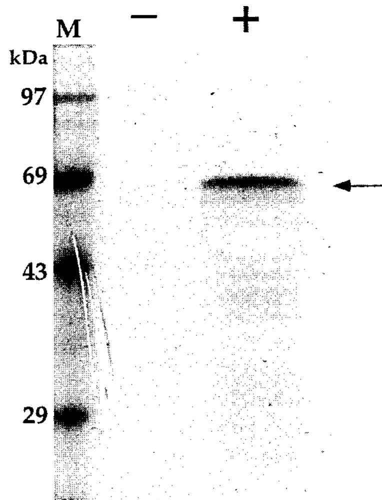

Fig. 3. Immunoprecipitation of HCV NS5B protein. The NS5B protein was translated *in vitro* in rabbit reticulocyte lysate and precipitated either with rabbit preimmune serum (-) or with an rabbit antibody raised against the baculovirus-expressed NS5B protein (+). Proteins were separated by SDS-PAGE and detected by autoradiography.

5BIP, we performed a Far Western protein-protein blotting assay as described previously (Hsieh *et al.*, 1998). Both GST and GST-5BIP fusion protein were expressed in *E. coli*, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with *in vitro*-translated NS5B. The

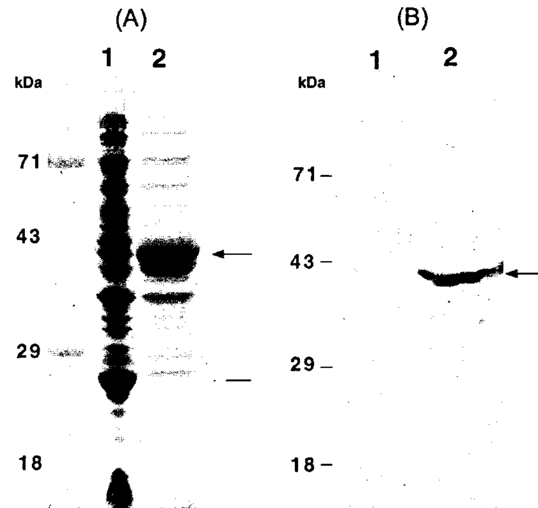

Fig. 4. Association of HCV RNA polymerase (NS5B) with 5BIP in a Far Western protein blotting assay. *E. coli*-expressed GST and GST-5BIP proteins were separated by SDS-PAGE on 12% polyacrylamide gels and either stained with Coomassie brilliant blue (A) or electrotransferred to a nitrocellulose membrane (B). The proteins on the membrane were renatured and incubated with *in vitro* translated, [^{35}S]methionine-labeled HCV RNA polymerase. Bound protein was detected by autoradiography (lane 2 in B). GST is indicated by an open arrow (lane 1 in A) and GST-5BIP fusion protein is indicated by a solid arrow (lane 2 in B).

nature of the [^{35}S]methionine-labeled NS5B was determined by immunoprecipitation with rabbit antibody raised against baculovirus-expressed NS5B protein (Fig. 3). The preimmune serum did not precipitate any proteins, confirming the specificity of the rabbit antibody against *in vitro* translation product of the NS5B ORF in rabbit reticulocyte lysates. As shown in Fig. 4A, GST-5BIP was expressed as a 41 kDa protein. Although there are many proteins in cells, the blotting results showed that NS5B was bound to the 5BIP protein, but not to GST or other cellular proteins, indicating that the interaction between HCV RNA polymerase and 5BIP is specific (Fig. 4B).

NS5B is a membrane associated phosphoprotein and localized in the perinuclear region (Hwang *et al.*, 1997). NS5B has been shown to be an RNA-dependent RNA polymerase (Behrens, *et al.*, 1996; Lohnann *et al.*, 1997; Yamashita *et al.*, 1998; Oh *et al.*, 1999). Thus NS5B is believed to be responsible for genome replication of HCV. The goal of this study was to elucidate the mechanism of HCV replication. However, *in vitro* HCV culture system is not yet available, and thus the biological significance of 5BIP in HCV replication needs further studies. HCV RNA polymerase is the key enzyme responsible for HCV replication and may exist as a protein complex. Thus HCV may use NS5B to regulate cellular proteins. In this study, we have identified a cellular protein which interacts with NS5B. Although the functional role of 5BIP is not yet known, we postulate that it may be involved in HCV replication. This possibility is under

investigation.

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References

Aach, R. D., Stevens, C. E., Hollinger, F. B., Mosley, J. W., Peterson, D. A., Taylor, P. E., Johnson, R. G., Barbosa, L. H., and Nemo, G. J. (1991) Hepatitis C virus infection in post-transfusion hepatitis. An analysis with first- and second-generation assays. *N. Engl. J. Med.* **325**, 1325-1329.

Alter, M. J., Margolis, H. S., Krawczynski, K., Judson, F. N., Mares, A., Alexander, W. J., Hu, P. Y., Miller, J. K., Gerber, M. A., Sampliner, R. E., Meeks, E. L., and Beach, M. J. (1992) The natural history of community-acquired hepatitis C virus in the United States. *N. Engl. J. Med.* **327**, 1899-1905.

Behrens, S. E., Tomei, L., and De Francesco, R. (1996) Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J.* **15**, 12-22.

Cho, Y.-G., Yoon,J.-W., Jang, K.-L., Kim, C.-M., and Sung, Y.-C. (1993) Full genome cloning and nucleotide sequence analysis of hepatitis C virus from sera of chronic hepatitis patients in Korea. *Mol. Cells* **3**, 195-202.

Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B hepatitis genome. *Science* **244**, 359-362.

Di-Bisceglie A. M., Simpson, L. H., Lotze, M. T., and Hoofnagle, J. H. (1994) Development of hepatocellular carcinoma among patients with chronic liver disease due to hepatitis C viral infection. *J. Clin. Gastroenterol.* **19**, 222-226.

Farci, P., London, W. T., Wong, D. C., Dawson, G. J., Vallari, D. S., Engle, R., and Purcell, R. H. (1992) The natural history of infection with hepatitis C virus (HCV) in chimpanzees: comparison of serologic responses measured with first- and second-generation assays and relationship to HCV viremia. *J. Infect. Dis.* **165**, 1006-1011.

Francki, R. I. B., Fauquet, C. M., Knudson, D. L. and Brown, F. (1991) Classification and nomenclature of viruses. Fifth Report of the International Committee on Texonomy of Viruses. *Arch. Virol.* (suppl. 2), **223**

Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. M. (1993) Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* **67**, 2832-2843.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., and Shimotohno, K. (1991) Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. *Proc. Natl. Acad. Sci. USA* **88**, 5547-5551.

Hsieh, T.-Y., Matsumoto, M., Chou, H.-C., Schneiders, R., Hwang, S. B., Lee, A. S., and Lai, M. M. C. (1998) Hepatitis C virus core protein interacts with heterogeneous nuclear ribonucleoprotein K. *J. Biol. Chem.* **273**, 17651-17659.

Hwang, S. B. (1998) Expression and characterization of hepatitis C virus core proteins: Effects of single amino acid substitution on protein conformation and subcellular localization. *J. Biochem. Mol. Biol.* **31**, 281-286.

Hwang, S. B., Park, K.-J., Kim, Y.-S., Sung, Y. C., and Lai, M. M. C. (1997) Hepatitis C virus NS5B protein is a membrane-associated phosphoprotein with a predominantly perinuclear localization. *Virology* **227**, 439-446.

Hwang, S. B., Choi, S.-H., and Park, K.-J. (1999) Unpublished observation.

Inchauspe, G., Zebedee, S., Lee, D.-H., Sugitani, M., Nasoff, M., and Prince A. M. (1991) Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanease isolates. *Proc. Natl. Acad. Sci. USA* **88**, 10292-10296.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., and Shimotohno, K. (1990) Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc. Natl. Acad. Sci. USA* **87**, 9524-9528.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature (London)* **227**, 680-685.

Lin, C., Pragai, B., Grakoui, A., Xu, J., Rice, C. M. (1994) The hepatitis C virus NS3 serine proteinase: *trans* processing requirements and cleavage kinetics. *J. Virol.* **68**, 8147-8157.

Lohmann, V., Korner, F., Herian, U., and Bartenschlager, R. (1997) Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J. Virol.* **71**, 8416-8428.

Manabe, S., Fuke, I., Tanishita, O., Kaji, C., Gomi, Y., Yoshida, S., Mori, C., Takamizawa, A., Yoshida, I., and Okayama, H. (1994) Production of nonstructural proteins of hepatitis C virus requires a putative viral protease encoded by NS3. *Virology* **198**, 636-644.

Oh, J.-W., Ito, T., and Lai, M. M. C. (1999) A recombinant hepatitis C virus RNA-dependent RNA polymerase capable of copying the full-length viral RNA. *J. Virol.* **73**, 7694-7702.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta,Y., Choo, Q.-L., Houghton, M., and Kuo, G. (1990) Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* **87**, 6547-6549.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminàting inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Shimizu, Y. K., Hijikata, M., Iwamoto, A., Alter, H. J., Purcell, R. H., and Yoshikura, H. (1994) Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. *J. Virol.* **68**, 1494-1500.

Shimotohno, K., Tanji, Y., Hirowatari, Y., Komoda, Y., Kato, N., and Hijikata, M. (1995) Processing of the hepatitis C virus precursor protein. *J. Hepatology* **22**, 87-92.

Wyatt, C. A., Andrus, L., Brotman, B., Huang, F., Lee, D. H., and Prince, A. M. (1998) Immunity in chimpanzees chronically infected with hepatitis C virus: role of minor quasispecies in reinfection. *J. Virol.* **72**, 1725-1730.

Yamashita, T., Kaneko, S., Shirota, Y., Qin, W., Nomura, T., Kobayashi, K., and Murakami, S. (1998) RNA-dependent RNA polymerase activity of the soluble recombinant hepatitis C virus NS5B protein truncated at the C-terminal region. *J. Biol. Chem.* **273**, 15479-15486.