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細胞因子與 C 型肝炎 NS5B 蛋白質交互作用機制的探討

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中文摘要

關鍵字：C 型肝炎病毒、非結構蛋白質、細胞因子PTB，複製起始複合物

C 型肝炎病毒(hepatitis C virus, HCV) 為輸血後非A 非B 型肝炎的重要致病原，感染後易轉變成慢性肝炎，並與肝癌形成具有密切的關係。有關C 型肝炎病毒的致病機轉尚不清楚，臨床上也還未找到有效的治療及預防策略，因此瞭解其複製機轉為一重要課題。C 型肝炎病毒的基因體為一正向單股線狀的RNA 分子，約9.5 kb，除5’端及3’端非轉譯區外，只含有一個開放編閱架構，可轉譯出一條約3010 個胺基酸的多蛋白質先驅分子，經由宿主和病毒自身蛋白酶切割成結構性蛋白質(core, E1, E2, p7)與非結構性蛋白質(NS2, NS3, NS4A, NS4B, NS5A, NS5B)。其中NS5B 蛋白質已證實為C 型肝炎病毒進行複製的主要酵素，可與病毒基因體 3’端結合，進行病毒起始複製。細胞因子PTB 曾被證實可結合於病毒基因體5’端及3’端，而本實驗室則證明，細胞因子PTB可與NS5B 蛋白質結合。本研究進一步發現細胞因子PTB 可抑制HCV蛋白質及RNA的合成。

英文摘要

Key words: Hepatitis C virus, NS proteins, PTB, replication initiation complex

Hepatitis C virus (HCV) is a single-stranded RNA virus and is a major pathogen for non-A, non-B hepatitis. Infection with HCV may sequentially develop chronic hepatitis and is closely related to the formation of hepatocellular carcinoma. At present, the mechanism involved in the pathogenic effect of HCV is still not clear and no protective vaccine and efficacy treatment strategy are available. Structural analysis of the 9.5 kb HCV RNA genome indicates that, in addition to the 5’- and 3’- untranslation regions, there is only one open reading frame encoding an approximate 3010 amino acid polyprotein precursor that can be cleaved by the proteases originated from HCV and host cells into structural (core, E1, E2, and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. Among the non-structural proteins, the NS5B protein has been shown recently to serve as the HCV replicase that binds to the 3’-end of the HCV genome and initiates virus replication. We have demonstrated recently NS5B binds to a cellular protein PTB. In this study, we found that PTB inhibit HCV translation and RNA synthesis.

Introduction

Hepatitis C virus (HCV), a member of the family *Flaviviridae*, is a positive single-stranded RNA virus, which is the primary causative agent of parenterally transmitted non-A/non-B hepatitis (Choo et al.,1989). It is estimated that there are 170 million chronically infected HCV carriers worldwide (2 to 3% of the global population), and many of these individuals are expected to develop serious HCV-related liver diseases, including hepatocellularcarcinoma (Cohen, 1999). The HCV genome comprises approximately 9,600 nucleotides, which encode a polyprotein precursor of about 3,000 amino acid residues. Co- and posttranslational proteolytic cleavage of this polypeptide by cellular and viral enzymes yields the specific HCV proteins required for virus replication and assembly (Rosenberg, 2001.). NS5B is a 66-kDa membrane- associated protein containing motifs shared by all RdRps, including the active site Gly-Asp-Asp sequence, which is believed to bind magnesium ions and is essential for enzymatic activity (Behrens, 1996). The nonstructural protein NS5B has been characterized as an RNA-dependent RNA polymerase (RdRp) based on in vitro experiments using recombinant expression protein (Lohmann, et al., 1997, Yuan, et al., 1997). The NS5B protein could bind 3'HCV genome and was suggested to initiate the replication of HCV (Cheng et al., 1999). The previous data showed that no template specificity for NS5B RNA binding activity. Recombinant NS5B proteins purified from insect cells (Behrens, 1996) or *Escherichia coli* (Yamashita et al., 1998) can synthesize RNA by using various natural or artificial RNAs as templates *in vitro*. It was reported that NS5A could bind with NS5B and modulate the RNA polymerase activity (Shirota et al 2002). In addition, there are some cellular factors was shown to bind with NS5B and inhibit RNA polymerase activiy (Hirano et al., 2003, Gao et al., 2003). All of these findings suggest that NS5B protein may interact with viral or cellular factors to regulate HCV RNA replication. PTB (p57 or hnRNP-1) has been shown to exist as a homodimer in solution and presents an oligomeric array of eight RNA recognition motifs (four in each monomer) (Perez et al., 1997). Structural analysis suggests that PTB possesses unusual features of RNP-1 and RNP-2 motifs within its RNA recognition motifs (Conte et al., 2000). The protein binds polypyrimidine tract near the 3'-splice site of many introns and acts as a repressor of splicing. In this paper, we demonstrate that the polypyrimidine binding

protein (PTB) which was reported to interact with 5' or 3' untranslation region of HCV (Anwar et al., 2000, Gontarek. et al., 1999) could inhibit HCV proteins translation in replicon cells and may caused by it specific interact with HCV NS5B RNA dependent RNA polymerase.

Material and Methods

Transient transfection, preparation of cell extracts, and Western blot analysis

Huh7 and its subline replicon cells that barely expressed HCV subgenome were cultured as described previously(Choi J et al, 2004). For transfection, Replicon cells were plated at a density of 2×10^6/35 mm plate and were applied with 6 μg DNA premixed with 10 μ LF2000. Fourty-eight hours later, the transfected cells were split and cultured at a density of 1.5×10^4/ml followed by theindicated treatments. The preparation of cell extracts and Western blot analysis and flow cytometer analysis were performed as described previously( ).

RT-PCR and real-time PCR detection

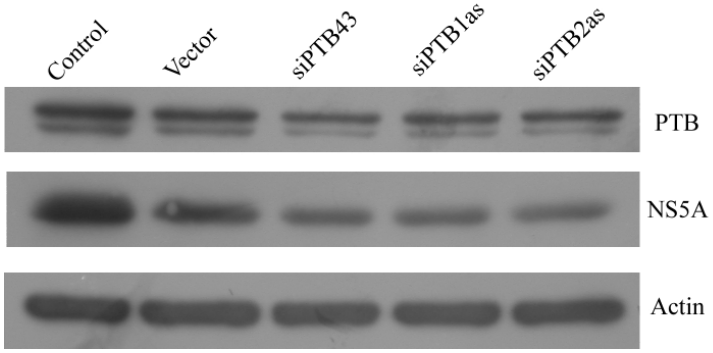
The negative strand of HCV detection was modified by the previous study (Lin L et al, 2002). The positive starand of HCV was detected by the real-time RT-PCR method. Briefly, the one-step real-time quantitative RT-PCR was performed with the ABI7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Briefly, indicated amount of RNA was added into a reaction mixture containing 25 μl of 2X RT-PCR universal master mix, 2.5 μl of 40X reverse transcriptase mixture, 5μl of forward primer, 5 μl of reverse primer in a final volume of 50 μl. The condition for RT-PCR was 1 cycle of 48^0C for 30 min; 1 cycle of 95^0C for 10 min; 40 cycles of 95^0C for 15 sec and 60^0C for 1 min. After amplification was complete, a melting curve analysis for HCV genotyping was performed by cooling the reaction to 60^0C and then heating slowly until 95^0C according to the instruction of manufacturer (Applied Biosystems). The cycle threshold value (C_T) used to assess the quantity of target gene was determined by which the fluorescence exceeds a preset limit. On the other hand, the SYBR Green I fluorescence (F) measured continuously during the heating period and the signal was plotted against temperature (T) to produce melting curves for each sample. The melting peaks were then generated by

plotting the negative derivative of the fluorescence with respect to temperature against temperature (-dF/dT versus T).

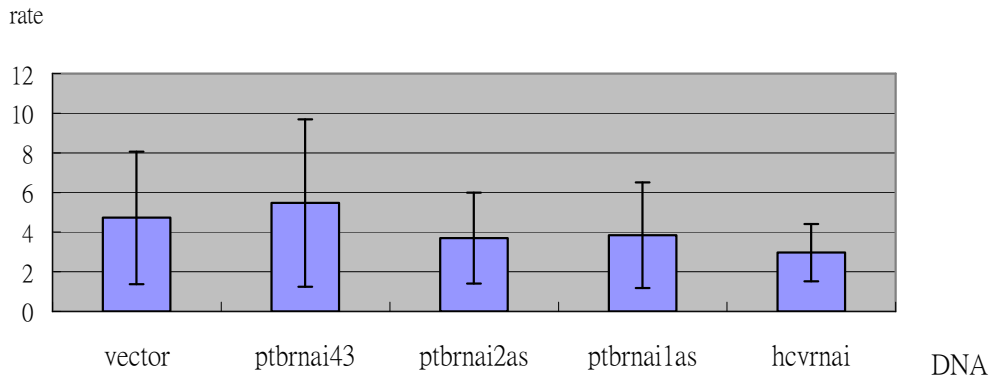
Results and Discussion

Modulation of HCV protein expression by polypyrimidine binding protein small interfering RNA.

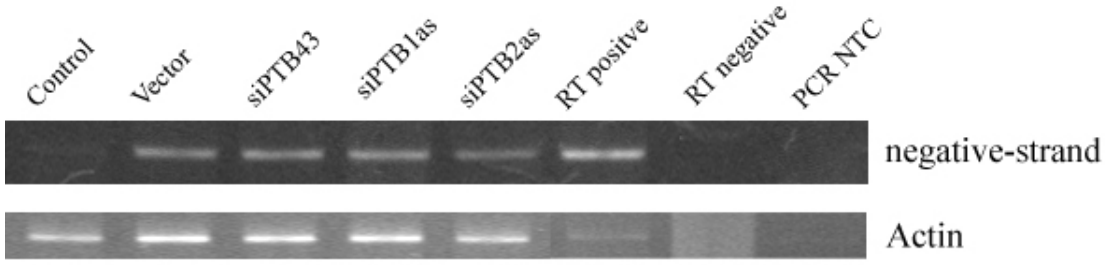
Polypyrimidine binding protein (PTB) was thought to bind HCV IRES to inhibit HCV traslation (Anwar A et al., 2000). The depleted PTB expression by siRNA in replicon cells reulted in the decreased expression of HCV NS5A as shown in the below.



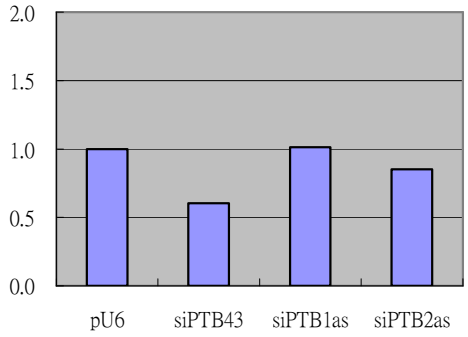
When we examined the effects of PTB on HCV IRES, the PTB-RNAi plasmids and HCV-IRES-GFP reporter plasmid were cotransfected into HEK-293 cells. Comparison with the decreased level of NS5A on replicon cells and GFP expression on HEK-293 cells, the expression level on NS5A is lower than on GFP. It implied that the decreased effect on NS5A by PTB may not only from inhibition on IRES region.



In addition, the replication and transcription level of HCV on the PTB RNAi transfected cells were also evaluated. The amount of negative strand of HCV was not significant changed to vector only control.



On the other hand, the HCV positive strand was estimated by real-time RT PCR analysis. The data showed that the trasncriptional level of HCV was also lower than the vector only control.



Taken together, the decreased level of HCV NS5A on PTB RNAi transfected replicon may involved with other factors other than IRES element. We have defined that PTB could interact with HCV NS5B *in vitro* and *in vivo*. Whether or not the PTB-NS5B interaction is involved in

the post-replication regulation of viral protein expression is under investigation in our laboratory.

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