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ScienceDirect
Virus Research 125 (2007) 79–87
Virus Research
www.elsevier.com/locate/virusres

Mapping of the interacting domains of hepatitis C virus core protein and the double-stranded RNA-activated protein kinase PKR

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Received 10 July 2006; received in revised form 12 December 2006; accepted 14 December 2006
Available online 30 January 2007

Abstract

Hepatitis C virus (HCV) core protein has been shown to exhibit several biological properties which suggest an important role in liver pathogenesis and carcinogenesis. During a previous study, we showed that core mutants, isolated from tumour, could directly interact with PKR and maintain it in an activated form. In the present report, we have further investigated this interaction and mapped the core and PKR domains involved. Using glutathion *S*-transferase fusion protein harbouring the different domains of core or PKR, we determined that the N-terminal 1–58 amino acid (aa) of core protein and the N-terminal 1–180 aa of PKR are responsible for this direct interaction. Using this system we also confirmed that the core–PKR interaction induced PKR autophosphorylation. Furthermore, we found that core protein co-localized and co-immunoprecipitated with PKR in cells expressing a full-length HCV replicon, thus confirming that this interaction occurs when all HCV proteins are expressed. Considering that the activation of PKR has been observed in some cancer cell lines and tissues, it suggests that, depending on the cellular context, PKR may stimulate or inhibit cell proliferation. The precise mapping of core–PKR interaction provides new data to study the molecular mechanism underlying HCV pathogenesis.

Keywords: Hepatitis C virus; Core protein; Protein kinase R

1. Introduction

Hepatitis C virus (HCV) is one of the major etiological agents of chronic hepatitis leading to liver cirrhosis and hepatocellular carcinoma (HCC). Globally, 0.25–1.2 million new cases of HCC are reported each year. Thus, HCV is responsible for high morbidity and mortality worldwide (Chen and Morgan, 2006; Wietzkebetaraun et al., 2001). At present, pegylated type I interferon (IFN) combined with Ribavirin is the only approved anti-HCV therapeutic treatment, but it is only effective in around 50% of HCV-infected individuals, depending on the genotype. A sustained response is observed in 70% of patients with genotypes 2 and 3, but only 30–50% of patients with genotypes 1a and 1b respond to this treatment. The HCV genome is a single, positive-stranded RNA of 9600 nucleotides with a unique open reading frame encoding a long polyprotein of 3010 amino acids (aa) which is processed by both cellular and viral proteases. There are three to four putative structural and at least six non-structural proteins. Of these, the viral nucleocapsid, core protein, exhibits pleiotropic features. Core protein has been shown to regulate transcriptionally, either positively or negatively, several cellular and viral genes, including *c-myc*, *c-fos* and p53. HCV core protein has also been reported to modulate apoptosis induced by cisplatin, *c-myc* (Ray et al., 1996b), Fas antigen or tumour necrosis factor-alpha (TNF-α) (Marusawa et al., 1999). Furthermore, in cooperation with the *ras* oncogene, HCV core protein may transform rodent cells into a tumorigenic phenotype (Ray et al., 1996a; Tsuchihara et al., 1999), and some transgenic mice expressing core protein in their liver develop HCC (Moriya et

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doi:10.1016/j.virusres.2006.12.010

al., 1998). The structure of HCV core protein is divided into several domains associated with different functions (Jin et al., 2000; Sabile et al., 1999). The N-terminal domain, from aa 1 to 50, contains RNA and DNA binding domains and three putative nuclear localization signals (NSL). The C-terminal domain from aa 91 to 191 binds and inactivates LZIP (Jin et al., 2000) and from aa 160 to 194, it binds to apolipoprotein II and modifies lipid metabolism (Sabile et al., 1999). The C-terminal domain is markedly hydrophobic and required for endoplasmic reticulum anchorage. Taken together, these properties strongly support the possibility that HCV core protein may affect cellular functions, such as cell proliferation and cell death, and contributes either directly or indirectly to HCV-induced hepatocarcinogenesis.

Of the many proteins activated by HCV replication, interferon-induced double-stranded RNA-activated protein kinase R (PKR) has been the subject of considerable attention, as it is a key arm of the antiviral and anti-proliferative effects of interferon (Clemens and Elia, 1997; Meurs et al., 1993). PKR binds to dsRNA, resulting in a conformational change that leads to autophosphorylation on several serine and threonine residues (Taylor et al., 1996). PKR then dimerizes and phosphorylates serine residue 51 on the alpha-subunit of the eukaryotic initiation factor 2 (eIF-2a). Phosphorylated eIF2α inhibits translation initiation and decreases the rate of protein synthesis. PKR activity is regulated by the presence of double-stranded RNA and more recently, a cellular protein, PACT, has been identified as a PKR activator important for its activity.

Consistent with the important role of PKR in controlling viral replication, several viruses, including HCV, have developed strategies to modulate PKR functions in terms of their persistence. PKR is a key protein which mediates the apoptotic response in the host cell (Der et al., 1997; Proud, 1995) and inhibits cell growth and proliferation (Barber et al., 1995; Donze et al., 1995; Koromilas et al., 1992). In this regard, it has been proposed that PKR may function as a tumour suppressive protein (Gale et al., 1999; Meurs et al., 1993). However, the role of PKR in cell growth remains controversial. In fact, it has been demonstrated that PKR may stimulate growth through the p38 MAP kinase and nuclear factor-kB (NF-kB) pathways (Bonnet et al., 2000; Goh et al., 2000). PKR has been observed to be over-expressed in breast cancer cell lines (Kim et al., 2000). In HCV-related HCC, we previously observed that HCV core protein isolated from tumour tissue interacted with and activated PKR. Furthermore, high levels of PKR expression have been found in HCV-related HCC in moderately to well-differentiated carcinomas, when compared with poorly differentiated HCC or liver cirrhosis (LC) (Shimada et al., 1998). Comparing PKR and related protein expression in paired tumour (T) and surrounding non-tumour (NT) tissue, it has also recently been found that PKR was strongly expressed and activated in human HCC compared with LC (Hiasa et al., 2003). More recently, another study showed that HCV-core protein facilitated the transport of PKR into nucleoli, and that this nuclear translocation of PKR seemed to correlate with its activation (Realdon et al., 2004). This suggests that HCV core protein may also be associated with PKR to some extent, and may constitute a viral activator of PKR. Our previous work has shown that the interaction between PKR and natural tumour core variants increases PKR autophosphorylation and phosphorylation of its eIF2-α substrate, these two processes being markers of PKR activity (Delhem et al., 2001). The present study further characterizes the direct interaction between HCV core protein and PKR and maps the regions involved in this interaction leading to PKR autpophosphorylation. Furthermore, using full-length HCV replicon cell line, we also provide evidence of a cytoplasmic co-localization of core and PKR when all HCV proteins are expressed. The results of this study thus further confirm that core interacts with PKR and may therefore modulate its activity during liver cell carcinogenesis.

2. Materials and methods

2.1. Plasmid constructs

Different glutathione-*S*-transferase (GST)-core or core-truncated fusion proteins were constructed using the pGEX-4T-1 vector. The different core sequences were amplified from the pDP18 vector containing tumour (T) or non-tumour (NT) core sequences from patient B (BT and BNT), previously described in Delhem et al. (2001), or HCV core genotype 1b, C191, isolated from serum (Fig. 1A). The following primers were designed according to the different core sequences and the various sizes desired.

HCV BT 1–172: 5′-CGCGGATCC ATGAGCACGAATCC-3′ sense, 5′-CCGGAATTCGCAACCGGGCAG-3′ anti-sense. HCV BT 1–126: 5′-CGCGGATCC ATGAGCACGAATCC-3′ sense, 5′-CCGGAATTCGAGGGTATCG-3′ anti-sense sense, 5′-HCV BT 1–58: 5′-CGCGGATCCATGAGCACGAATCC-3′ CCGGAATTCAGGTTGTGACCGC-3′ anti-sense, HCV BT 59–126: 5′-CGCGGATCCCGTGGAAGGCG-3′ sense, 5′-CCGGAATTCGAGGGTATCG-3′ anti-sense HCV BT 127–172: 5′-CGCGGATCC AC ATGCGGCTTCG-3′ sense, 5′-CCGGAATTCGCAACCGGGCAG-3′ anti-sense HCV BNT 1–172: 5′-CGCGGATCCATGAGCACGAATCC-3′ sense, 5′-CCGGAATTCGCAACCGGGCAG-3′ anti-sense, HCV C191 1–172: 5′-CGCGGATCC ATGAGC AC AAATCC-3 sense, 5′-CCGGAATTCGCAACCGGGC-3′ anti-sense.

PCR products were cloned at the *Eco*R-I and *Bam*H-I sites in the pGEX-4T-1 vector to obtain various GST-core fusion proteins. Full length and different truncated GST-PKR expression plasmids were generous gifts from Dr. Meurs (Bonnet et al., 2000) (Pasteur Institute, France): full-length PKR corresponded to amino acids (aa) 1–551, and truncated forms to PKR aa 1–265, PKR aa 1–180, and PKR aa 265–551 (Fig. 2A).

2.2. Cell culture

The human hepatoma cell lines HepG-2, Huh-7 or Huh-7.5 were grown in Dulbecco's modified eagle medium (DMEM, Invitrogen, France) supplemented with 10% foetal bovine serum (FBS, Invitrogen, France), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml plasmocin. Cells from the full-length HCV Replicon cell line (A kind gift from C. Rice, Rockefeller University, New York City, USA), were cultured in DMEM containing 10% FBS, 100 U/ml penicillin,