Hepatitis C Virus Core Protein Down-Regulates Transcription of Interferon-Induced Antiviral Genes

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**Background.** Hepatitis C virus (HCV) proteins interfere with the interferon (IFN)–α–induced Jak/signal transducer and activator of transcription (STAT) pathway. Which protein is responsible for this effect and whether this interference results in down-regulation of IFN-induced genes remain controversial. We analyzed the effect of HCV core (HCV-Co) protein on expression of IFN-induced antiviral genes.

**Methods.** HepG2 cells were transfected with the plasmid pHCV-Co, and, after treatment with IFN-α, levels of MxA, protein kinase R (PKR), and 2′–5′ oligoadenylate synthetase (2′–5′OAS) mRNA were determined. Chloramphenicol acetyl transferase (CAT) analysis was performed on cells cotransfected with pHCV-Co and pMx4CAT (containing the MxA gene promoter) and treated with IFN. Electrophoretic mobility shift assays were used, and Western-blot analysis of STAT 1 and 2 was performed.

**Results.** Levels of MxA mRNA in pHCV-Co–transfected cells decreased in a dose-dependent manner, by down-regulation of the MxA gene promoter. HCV-Co protein inhibits binding of IFN-stimulated gene factor 3 (ISGF3) to the IFN-stimulated response element (ISRE). Intracellular distribution of STAT 1 and 2 was not modified after treatment with IFN. Expression of HCV-Co protein also results in down-regulation of expression of PKR and 2′–5′OAS genes.

**Conclusion.** HCV-Co protein inhibits IFN-α–induced transcription of antiviral genes by decreasing binding of ISGF3 to the ISRE.

Chronic hepatitis C virus (HCV) infection is a progressive disease that may end in liver cirrhosis and, eventually, in hepatocellular carcinoma [1]. Current treatment for chronic HCV infection consists of administration of interferon (IFN)–α alone or in combination with ribavirin [2–4]. However, resolution of infection occurs in only 40%–60% of cases [5], indicating that HCV may possess molecular mechanisms of resistance to the action of IFN-α.

IFNs are pleiotropic cytokines with antiproliferative, immunomodulatory, and antiviral activities [6]. Binding of IFNs to their membrane receptors promotes activation of 2 receptor-associated tyrosine kinases, Jak1 and Tyk2. These kinases recruit and activate a set of proteins termed signal transducer and activator of transcription (STAT) 1, 2, and 3, which form homo- and heterodimers [7]. IFN-α promotes formation of heterodimers between STAT 1 and 2, which further binds to a third protein, p48, to form the IFN-stimulated gene factor 3 (ISGF3) complex, which is translocated to the cell nucleus, where it binds to the IFN-stimulated response element (ISRE) sequence in IFN-α–stimulated gene promoters. The antiviral activity of type I IFNs (α/β) is mediated by effector proteins, such as double-stranded RNA-activated protein kinase (PKR), 2′–5′oligoadenylate synthetase (2′–5′OAS), and MxA [8]. Different viruses, such as hepatitis B virus, cytomegalovirus, and mumps virus, interfere with Jak/STAT signaling, as a persistence mechanism [9–11], and there is molecular evidence indicating that HCV may also have evolved mechanisms to counteract the antiviral action of IFNs. In this sense, it has been reported that the structural protein E2 and the nonstructural protein 5A repress the activity of PKR
[12–14]. Furthermore, it has also been reported that expression of HCV proteins in cell lines and in transgenic animals interferes with the IFN-induced Jak/STAT signaling pathway [15, 16]. With regard to the question of which protein is responsible for this effect, HCV core (HCV-Co) protein is the best candidate, because it has been shown that this protein, apart from its ability to interact with genomic HCV-RNA to form nucleocapsids [17, 18], is capable of modulating several cellular signaling pathways [19–22]. In fact, it has been reported that HCV-Co protein modulates the Jak/STAT signaling pathway, although it does not affect activation of IFN-responsive genes [23]. In contrast, other authors have reported HCV-Co protein–mediated activation of 2′–5′OAS gene transcription [24]. So whether this viral structural protein interferes with the Jak/STAT signaling pathway and whether this interference modifies expression of antiviral IFN–induced genes remain to be demonstrated. For these reasons, in the present study, we analyzed the effect of HCV-Co protein on the IFN-induced Jak/STAT signaling pathway and on the downstream antiviral genes.

**MATERIALS AND METHODS**

**Plasmids.** The plasmids pHCV-Co, in which the complete coding region of HCV-Co protein (1b genotype) was cloned in the plasmid pcI (Promega) under the control of cytomegalovirus immediate early promoter, and pMx4CAT, in which chloramphenicol acetyl transferase (CAT) gene was cloned under the control of a 969-bp fragment of the MxA gene promoter containing 3 ISRE sequences, have been described elsewhere [25, 26]. The irrelevant plasmid pGEM3Z (Promega) was also used.

**Cell line and transfection.** The human hepatoblastoma cell line HepG2, which produces MxA protein on induction with IFN-α [26], was used in the present study. Cells were propagated in Dulbecco’s modified Eagle medium (Imperial) supplemented with 10% heat-inactivated fetal bovine serum (Imperial) and antibiotics (0.1 mg/mL streptomycin and 100 U/mL penicillin), at 37°C in a humidified atmosphere with 5% CO₂.

HepG2 cells were transfected by electroporation of 3 × 10⁶ cells at 0.21 V and 250 μF (Gene Pulser; Bio-Rad). After transfection, cells were cultured for 24 h before treatment with IFN-α (1000 IU/mL; Roferon; Roche). Twenty-four hours later, cell lysates were collected for CAT analysis. In single-transfection experiments, cells were transfected with increasing amounts of pHCV-Co (1, 3, and 5 μg). As a control, cells were transfected with 5 μg of pcI. To maintain a constant total amount of DNA transfected, pGEM3Z was used as filler. In cotransfection experiments, cells were electroporated with 1 μg of pMx4CAT and increasing amounts of pHCV-Co (1, 3, and 5 μg). All transfection experiments were performed at least 3 times, to verify the reproducibility of the results.

**Detection of MxA mRNA by reverse-transcription polymerase chain reaction (RT-PCR).** Total mRNA from HepG2 cells transfected with pcI or pHCV-Co was isolated 24 h after treatment with IFN-α, by use of Trizol LS reagent (Invitrogen). One microgram of mRNA was reverse-transcribed and amplified by use of the Superscript One-Step RT-PCR System (Invitrogen). The MxA-specific primers used in the amplification reaction (5′-CCCTTTCCAGGGCCAGGGG-3′ and 5′-CTGATTGCCACAGCCACTC-3′) produce a PCR product of 290 bp. The RT-PCR was performed for 45 min at 48°C, followed by 2 min at 95°C and 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 68°C and a final extension step of 7 min at 68°C. For the detection of PKR mRNA, we used the primers 5′-CCAGTGATGATTCTCTTGAGAGC-3′ and 5′-CCCGAAAAGGTAGAGGTGCTCCA-3′, which amplify a PCR product of 419 bp. The primers used in the amplification of 2′–5′OAS mRNA were 5′-GGTGATGAAAAAGGTGGCTCTC-3′ and 5′-TCTGCA-GGTTAGGTGACCTC-3′, which produce a PCR product of 374 bp. RT-PCR conditions for the amplification of PKR and 2′–5′OAS mRNA were the same as those for the amplification of Mx mRNA, except that the annealing temperature was 60°C in both cases. As an internal control, β-actin mRNA was coamplified in the same reaction, by use of the primers 5′-AGCGGGGAAATCGGTGGGTG-3′ and 5′-CAGGTACATGGTGTTGCCC-3′, which amplify a PCR product of 311 bp. The RT-PCR products were resolved by 1.5% agarose gel electrophoresis. After ethidium bromide staining, quantitation of the amplified products was performed by densitometric analysis, by use of a laser densitometer (Molecular Dynamics). Levels of Mx mRNA were normalized with respect to levels of β-actin mRNA.

**CAT assay.** Cell lysis and determination of CAT activity was performed by use of the CAT Enzyme Assay System (Promega), in accordance with the instructions supplied by the manufacturer. Equal amounts of protein were used and adjusted to ensure that enzyme activity remained within the linear (<50% conversion) range. Quantitation was performed by densitometric analysis of spots on thin-layer chromatography plates. Data are presented as fold inhibition with respect to CAT activity in HepG2 cells transfected with pMxCAT and treated with IFN-α.

**Electrophoretic mobility shift assays (EMSA).** To obtain nuclear extracts, 24 h after transfection and 12 h after treatment with IFN-α, cells were lysed in buffer A (10 mmol/L Hepes-KOH [pH 7.4], 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.5 mmol/L dithiothreitol [DTT], and 0.2 mmol/L phenylmethylsulfonyl fluoride [PMSF]) for 10 min at 4°C. After centrifugation at 12,000 g for 10 s, the pellets were incubated with 20 μL of buffer C (20 mmol/L Hepes-KOH [pH 7.4], 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, and 25% glycerol) for 20 min at 4°C. Samples were cleared by centrifugation at 4000 g for 2 min at 4°C, were
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Figure 1. Decreased levels of MxA mRNA induced by hepatitis C virus core (HCV-Co) protein. Levels of MxA and β-actin mRNA were determined in HepG2 cells transfected with pCI or pHCV-Co and treated for 24 h with 1000 IU/mL interferon (IFN)-α. Lane 1, Untreated HepG2 cells transfected with 3 μg of pCI; lane 2, IFN-treated HepG2 cells transfected with 3 μg of pCI; lane 3, IFN-treated HepG2 cells transfected with 1 μg of pHCV-Co; lane 4, IFN-treated HepG2 cells transfected with 3 μg of pHCV-Co; lane 5, no-RNA control.

Figure 2. Down-regulation of MxA gene promoter activity, by hepatitis C virus core (HCV-Co) protein. CAT analysis was performed on HepG2 cells cotransfected with pMx4CAT and increasing amounts of pHCV-Co and treated with 1000 IU/mL IFN-α. Lane 1, HepG2 cells transfected with pMx4CAT and 5 μg of pCI; lane 2, HepG2 cells transfected with pMx4CAT and 5 μg of pCI and treated with IFN-α; lanes 3–5, HepG2 cells transfected with pMx4CAT and 1, 3, and 5 μg of pHCV-Co, respectively, and treated with 1000 IU/mL IFN-α.

RESULTS

Effect of HCV-Co protein on levels of MxA mRNA. To study the effect of HCV-Co protein on IFN-induced transcription of the MxA gene promoter, HepG2 cells were transfected with pHCV-Co or pCI and were treated for 24 h with 1000 IU/mL IFN-α. After that, total mRNA was isolated, and levels of MxA mRNA were semiquantitated by RT-PCR. As shown in figure 1, treatment of pCI-transfected HepG2 cells with IFN-α results in induction of transcription of the MxA gene promoter. However, levels of MxA mRNA were strongly reduced in cells transfected with pHCV-Co.

Inhibition of IFN-α–induced activation of the MxA gene promoter, by HCV-Co protein. To analyze whether the reduction in levels of MxA mRNA observed in IFN-α–treated HepG2 cells transfected with pHCV-Co was due to inhibition of MxA gene promoter activity, HepG2 cells were cotransfected with pMx4CAT and increasing amounts of pHCV-Co and were treated with IFN-α. CAT analysis showed that transfection with pHCV-Co results in inhibition of IFN-α–induced MxA gene promoter activity in a dose-dependent manner (figure 2).
**Inhibition of binding of the ISGF3 complex to the ISRE sequences, by HCV-Co protein.** Since activation of transcription of IFN-α–inducible genes depends on binding of the ISGF3 complex to the ISRE sequences in IFN-α–responsive promoters, we next tested whether HCV-Co protein inhibits this binding. For this purpose, an EMSA was performed by use of nuclear extracts isolated from IFN-α–treated HepG2 cells either transfected with pHCV-Co or untransfected. This analysis showed that there was inhibition of binding of the ISGF3 complex to the ISRE sequence in HepG2 cells transfected with pHCV-Co, with respect to untransfected cells (figure 3A). The decrease in ISGF3 binding observed in HepG2 cells expressing HCV-Co protein was not due to competition for binding to the ISRE between the ISGF3 complex and HCV-Co protein, because recombinant HCV-Co protein does not bind to the oligonucleotide containing this sequence (figure 3B).

**No blocking effect of HCV-Co protein on translocation of the ISGF3 complex from the cytoplasm to the cell nucleus.** Finally, to test whether the decrease in ISGF3 binding activity observed in pHCV-Co–transfected HepG2 cells was due to inhibition of translocation of this complex from the cytoplasm to the cell nucleus, Western-blot analysis of STAT 1 and 2 was performed by use of nuclear and cytoplasmic extracts obtained from IFN-α–treated HepG2 cells transfected with pHCV-Co and from untransfected cells. As shown in figure 4, in untreated HepG2 cells, most of STAT 1 and 2 localize in the cytoplasm. After 5 min of treatment with IFN-α, both proteins were mostly detected in the cell nucleus independently, whether or not the cells had been transfected with pHCV-Co. Similar results were obtained after 30 min of treatment with IFN-α.

**Inhibition of transcription of PKR and 2′–5′OAS genes, by HCV-Co protein.** To determine whether the reduction in formation of the ISGF3 complex induced by the HCV-Co protein affects transcription of other IFN-activated antiviral genes, levels of PKR and 2′–5′OAS mRNA were determined in IFN-α–treated HepG2 cells transfected with increasing amounts of pHCV-Co. In these experiments, it was shown that, in transfected cells, levels of mRNA of both genes decreased in a dose-dependent manner (figure 5).

**DISCUSSION**

Approximately 85% of patients acutely infected with HCV are not able to clear the virus and, therefore, become chronically infected [27]. To cause persistent infections, HCV may have evolved mechanisms to circumvent the antiviral state induced by IFN-α. This hypothesis is reinforced by the fact that only 10%–20% of patients with chronic HCV infection respond to monotherapy with IFN-α [5]. In relation to this, in vitro studies have revealed that the viral structural protein E2 and the nonstructural protein 5A repress the activity of PKR, one of the effector proteins that mediate the antiviral activity of IFN-α.
Figure 4. Intracellular distribution of signal transducer and activator of transcription (STAT) 1 and 2 in HepG2 cells transfected with hepatitis C virus core plasmid (pHCV-Co) after treatment with interferon (IFN)-α. Western blot analysis was performed by use of cytosolic and nuclear extracts from HepG2 cells transfected with pCI or pHCV-Co and treated for 0, 5, or 30 min with 1000 IU/mL IFN-α. Lanes 1–3, Cytosolic (C) extracts from HepG2 cells transfected with 5 μg of pCI (lane 1), 3 μg of pHCV-Co (lane 2), and 5 μg of pHCV-Co (lane 3). Lanes 4–6, nuclear (N) extracts from HepG2 cells transfected with 5 μg of pCI (lane 4), 3 μg of pHCV-Co (lane 5), and 5 μg of pHCV-Co (lane 6).

[12–14]. Apart from this, it has also been reported that expression of HCV proteins in an osteosarcoma cell line and in transgenic mice inhibits the IFN-induced Jak/STAT signaling pathway [15, 16], which is essential for production of antiviral effector proteins [8]. Related to this finding, Busu et al. have reported that expression of HCV-Co protein induces a reduction in formation of the ISGF3 complex, although it does not affect activation of the IFN-sensitive genes IRF-1 and 561 [23]. In contrast, Naganuma et al. have shown that HCV-Co protein activates transcription of the inducible 2′-5′OAS gene [24]. Thus, whether HCV-Co protein interferes with the Jak/STAT signaling pathway and whether this interference results in a modification of transcription of IFN-inducible genes remain controversial. For this reason, in the present study, we have sought to determine whether HCV-Co protein inhibits expression of the antiviral IFN–sensitive MxA gene and whether this inhibition is caused by interference of this viral protein with the Jak/STAT signaling pathway.

We have shown that expression of HCV-Co protein in the HepG2 cell line provokes a decrease in levels of MxA mRNA in a dose-dependent manner, suggesting that this protein inhibits IFN-α–induced transcription of the gene. This hypothesis was confirmed in cells cotransfected with pHCV-Co and pMx4CAT, in which CAT analysis, after treatment with IFN-α, showed that expression of HCV-Co protein inhibits the activity of the MxA gene promoter. HCV-Co protein not only inhibits IFN-induced transcription of the MxA gene promoter but also down-regulates transcription of PKR and 2′-5′OAS, as shown by the dose-dependent decrease in levels of mRNA of both genes in HepG2 cells transfected with pHCV-Co. These...
results are in contrast with those reported by Basu et al. [23]. We do not have a clear explanation for this discrepancy, although it may be speculated that HCV-Co protein may differentially regulate expression of IFN-α-sensitive genes, depending on the specific ISRE sequence present in the promoter of these genes, as has been proposed elsewhere [28]. On the other hand, Naganuma et al. [24] have reported up-regulation of 2′-5′OAS gene transcription by HCV-Co protein in HepG2 cells, which is in contrast with the results obtained in the present study. This difference may be explained by the fact that the cDNA clone used by Naganuma et al. to express HCV-Co protein was isolated from in vitro–infected cells, and it presents amino acid changes, with respect to HCV-J and HCV-K isolates [29, 30], that may affect its properties. In fact, the HCV-Co proteins used by Naganuma et al. did not activate the NF-κB signal transduction pathway, which is in contrast with what has been reported by several other authors [22, 25, 31, 32].

Since IFN-α–induced gene transcription depends on binding of the ISGF3 complex to the ISRE sequences in the gene promoters, we next tested whether expression of HCV-Co protein inhibits this binding. EMSA analysis of HCV-Co–transfected cells showed that expression of the viral protein results in a decrease in binding of the ISGF3 complex after treatment with IFN-α, although the shift did not completely disappear, probably because the efficiency of transfection resulting from the use of the electroporation method that we used in the present study is 40%–50% of the cells (data not shown), and, so, in the remaining cells, the Jak/STAT signalling pathway is not affected by the HCV-Co protein. The decrease observed in the ISGF3 complex was not due to inhibition of translocation of this complex from the cytoplasm to the cell nucleus, as demonstrated by Western-blot analysis of cytoplasmic and nuclear extracts obtained from HCV-Co–transfected and untransfected cells. Furthermore, this analysis also showed that there were no differences in levels of STAT 1 and 2 proteins between HCV-Co–transfected and untransfected cells, showing that the decrease in binding of the ISGF3 complex observed in cells expressing HCV-Co was not due to inhibition of transcription of STAT 1 and 2 genes. This finding is in contrast with that reported by Basu et al. [23], who found a decrease in levels of STAT 1 protein, compared with levels in control cells. This difference may be explained by the different methods (transient vs. stable) used to express HCV-Co protein.

Thus, the effect on levels of STAT 1 protein observed by Basu et al. may be due to an accumulation of HCV-Co protein rather than to a direct effect of the viral protein on transcription of the STAT 1 gene or on the stability of the protein. The mechanism by which HCV-Co protein interferes with binding of the ISGF3 complex to the ISRE remains unknown. In relation to this, it has been reported that HCV-Co protein may counteract the antiviral effect of IFN-α by inducing synthesis of the suppressor of cytokine signaling 3 [33], which belongs to a family of proteins that inhibit cytokine signaling via the Jak/STAT signaling pathway [34]. Alternatively, HCV-Co protein may promote nuclear dephosphorylation of STAT, as has been proposed by Blindenbacher et al. [16].

Finally, it may be argued that the findings of the present study have no clinical or biological relevance, since the levels of HCV-Co protein expressed in vivo are probably lower than those in transfected cells. However, immunohistochemical studies of liver biopsy specimens from patients with chronic HCV infection have shown that the expression of viral proteins is focal with some hepatocytes containing high levels of HCV antigens [35, 36], so it is possible that, in infected patients, some hepatocytes may present levels of HCV-Co protein similar to those in transfected cells. Furthermore, even a slight interference of the IFN signalling pathway may be of relevance, in terms of persistence of the infection and response to therapy, in patients with chronic HCV infection.

In summary, in the present study, we have demonstrated that HCV-Co protein inhibits IFN-α–induced transcription of antiviral genes by decreasing binding of the ISGF3 complex to the ISRE sequence in the promoter. More studies are needed to understand the molecular mechanism by which this viral protein inhibits ISGF3 binding.

References


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