Suppression of short interfering RNA-mediated gene silencing by the structural proteins of hepatitis C virus

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Viruses have evolved strategies to overcome the antiviral effects of the host at different levels. Besides specific defence mechanisms, the host responds to viral infection via the interferon pathway and also by RNA interference (RNAi). However, several viruses have been identified that suppress RNAi. We addressed the question of whether hepatitis C virus (HCV) suppresses RNAi, using cell lines constitutively expressing green fluorescent protein (GFP) and inducibly expressing HCV proteins. It was found that short interfering RNA-mediated GFP gene silencing was inhibited when the entire HCV polyprotein was expressed. Further studies showed that HCV structural proteins, and in particular envelope protein 2 (E2), were responsible for this inhibition. Co-precipitation assays demonstrated that E2 bound to Argonaute-2 (Ago-2), a member of the RNA-induced silencing complex, RISC. Thus, HCV E2 that interacts with Ago-2 is able to suppress RNAi.

Hepatitis C virus (HCV), first identified in 1989, has been estimated to have infected 2–4 % of the world population; a large proportion of these infected individuals have chronic hepatitis (Choo et al., 1989; Moradpour et al., 2007). Chronic carriers are at risk of developing cirrhosis and hepatocellular carcinoma. The HCV genome consists of a positive-sense RNA and encodes a polyprotein precursor, which is co- and post-translationally cleaved into structural proteins (core, envelope 1 and 2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The interplay between HCV and the host is very complex. Known as the first line of defence against viral infection, interferons are key to host defence, by initiating a cascade of intracellular signalling pathways that result in the expression of a large number of interferon-stimulated genes, as well as antiviral micro RNA (miRNA) (Pedersen et al., 2007). In turn, HCV has evolved different strategies to overcome the antiviral effects of interferons (reviewed by Chung et al., 2007; Katze et al., 2002).

Normally, double-stranded RNA (dsRNA) in cells will evoke the activation of RNA interference (RNAi), first reported by Fire et al. (1998). In this process, long dsRNA will be cut by Dicer into short (21–25 nt) interfering RNAs (siRNAs); these are then incorporated into a multiprotein RNA-induced silencing complex (RISC), where the siRNA duplex is unwound, leaving the antisense strand to guide RISC to its homologous mRNA target for endonucleolytic cleavage (Collins & Cheng, 2006; Morris & Rossi, 2006). As HCV forms dsRNA during the replication process, the question of how HCV protects itself from the host’s RNAi arises. Studies with other RNA viruses showed that some are capable of counteracting the antiviral RNAi. For example, suppressors of RNAi have been identified in plant viruses (e.g. Tomato spotted wilt virus) (Takeda et al., 2002), while an animal adenovirus has been found to inhibit RNAi by binding and blocking both Dicer and RISC (Andersson et al., 2005). Moreover, viral proteins identified in flock house virus, vaccinia virus and influenza viruses can block antiviral RNAi (Li et al., 2004). Concerning HCV, a recent study showed that the core protein can function as an inhibitor of antiviral RNAi by interacting with Dicer (Wang et al., 2006). Here, we show that the envelope protein 2 (E2) of HCV may also act as an inhibitor of RNAi by interacting with Argonaute-2 protein (Ago-2) of RISC.

We used cell lines derived from U-2 OS human osteosarcoma cells (Table 1), inducibly expressing the entire HCV polyprotein or individual HCV proteins under the regulation of a tetracycline-controlled gene expression system. Cell lines that inducibly express the E1–E2 envelope glycoprotein complex were prepared as described by Moradpour et al. (1996, 1998) and Schmidt-Mende et al. (2001). In brief, the sequence corresponding to aa 170–746 of the HCV H77 consensus clone was amplified by PCR
from pBRTM/HCV1-3011con (Kolykhalov et al., 1997) (kindly provided by Dr Charles M. Rice, The Rockefeller University, New York) using primers E1E2con-fwd (5’-GCACggaattcACCATGCGTTGCTCTTTCTATC-3’) and E1E2con-rev (5’-GCTGtctagaTTACGCCTCCGCTTGGGATATGAG-3’), followed by cloning into pUHD10-3 (Gossen & Bujard, 1992) via EcoRI and XbaI, yielding plasmid pUHDE1E2con. This construct allowed expression of HCV E1 and E2 with their genuine signal sequences under the control of a tetracycline-controlled transactivator (tTA)-dependent promoter. To obtain inducible cell lines, the constitutively tTA-expressing founder cell line UTA-6 (Englert et al., 1995) was cotransfected with pUHDE1E2con and pBabePuro (Morgenstern & Land, 1990), followed by double selection with G418 and puromycin. Clones were characterized by immunofluorescence microscopy and immunoblot in the presence or absence of tetracycline. Clone UE1E2con-7 was selected based on tightly regulated expression of a correctly processed E1-E2 glycoprotein complex (data not shown).

When we applied green fluorescent protein (GFP)-specific siRNA (siRNA-G) to induce GFP gene silencing (Ji et al., 2003, 2005), it was observed that the silencing activity of siRNA-G was decreased in the HCV polyprotein-expressing UHCV/ER-GFP-15-2 cells, compared with the same cells cultured in the presence of tetracycline and not expressing HCV proteins (Fig. 1b, cell line 1). This observation suggested that certain HCV proteins may function as inhibitors of siRNA-mediated gene silencing. Thus, we tested the influence of siRNA-mediated gene silencing in cell lines transfected with different portions of the HCV genome (Table 1; Fig. 1). As shown in Fig. 1(b), all four HCV E2-expressing cell lines demonstrated inhibition of siRNA-G-induced GFP gene silencing, whereas all non-E2-expressing cell lines (cell line 5, UTH-28; cell line 6, UNS3-5Bcon-27; cell line 7, UNS5Bcon-5), except for cell line 8 (UCcon-39), did not show inhibition. UE1E2con-7 cells, expressing E1 and E2 only (cell line 4), exhibited the strongest suppression (Fig. 1c), while UTH-28 cells expressing core and part of E1 but no E2 (cell line 5) showed little inhibitory activity of siRNA-G-induced GFP gene silencing. These data indicate that HCV E2 may be a major inhibitor of siRNA-mediated gene silencing. The testing of HCV E2 alone is not possible as E1 and E2 form a functional unit and assist each other for proper folding (Op De Beeck et al., 2001). Interestingly, UCcon-39 cells (cell line 8, high level of HCV core expression) also showed inhibition, whereas UCcon-18 cells (data not shown) and UTH-28 which express low to medium levels of HCV core protein did not inhibit at all. Thus, our data are in agreement with the observation of Wang et al. (2006), that HCV core protein can act as an RNAi inhibitor by interacting with Dicer.

Further studies showed that the inhibition of GFP gene silencing observed in HCV envelope glycoprotein-expressing cells (cell line 4, UE1E2con-7) depends on the amount of viral proteins available (Fig. 2a). As the level of HCV envelope proteins expressed by the cells increases, so does suppression of the siRNA-mediated gene silencing. Varying levels of expression of HCV envelope proteins were obtained by varying the concentration of tetracycline in the culture medium (Fig. 2a). To confirm that siRNA-mediated RNAi is involved in the inhibition of GFP gene silencing, the level of GFP mRNA was investigated. Quantitative real-time RT-PCR (qRT-PCR) (Fig. 2b) showed that the envelope proteins inhibited the cleavage of GFP reporter mRNA, thereby ruling out a miRNA-mediated mechanism, which would only inhibit protein translation but not affect mRNA levels (Ji et al., 2003, 2005). In addition, comparing UCcon-39 cells, which inducibly express high levels of core protein, cultured in the presence or absence of tetracycline, did not reveal any significant change of GFP reporter mRNA levels (data not shown).

Kapadia et al. (2003) showed that labelled siRNA co-localized with HCV proteins in the perinuclear area. Moreover, it has been shown that transfected siRNA duplexes may ‘activate’ RISC for gene silencing (Schwarz et al., 2003). Thus, we investigated whether HCV E2 interacts with RISC in order to suppress RNAi. Although not all elements of RISC have been identified so far, Ago-2 represents one important component and is considered to be the catalytic core of RISC (Collins & Cheng, 2006). Pull-

<table>
<thead>
<tr>
<th>Cell line</th>
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<th>HCV segment</th>
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<tr>
<td>1</td>
<td>UHCV/ER-GFP-15-2</td>
<td>Entire HCV polyprotein</td>
<td>Schmidt-Mende et al. (2001); B. Wölk and D. Moradpour, unpublished data</td>
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<tr>
<td>2</td>
<td>UTHCNS3-43-GFP</td>
<td>5′ NCR to partial NS3</td>
<td>Moradpour et al. (1998)</td>
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<tr>
<td>3</td>
<td>UCp7con-9-GFP</td>
<td>Core to p7</td>
<td>D. Moradpour, unpublished data</td>
</tr>
<tr>
<td>4</td>
<td>UE1E2con-7-GFP</td>
<td>E1 and E2</td>
<td>This study</td>
</tr>
<tr>
<td>5</td>
<td>UTH-28-GFP</td>
<td>5′ NCR to partial E1</td>
<td>Moradpour et al. (1996)</td>
</tr>
<tr>
<td>6</td>
<td>UNS3-5Bcon-27-GFP</td>
<td>NS3 to NS5B</td>
<td>D. Moradpour, unpublished data</td>
</tr>
<tr>
<td>7</td>
<td>UNS5Bcon-5-GFP</td>
<td>NS5B</td>
<td>Schmidt-Mende et al. (2001)</td>
</tr>
<tr>
<td>8</td>
<td>UCcon-39-GFP</td>
<td>Core</td>
<td>D. Moradpour, unpublished data</td>
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Down experiments were performed to test whether HCV E2 and Ago-2 interact with each other. When anti-E2 antibodies (A11 or H52, kindly provided by Dr Jean Dubuisson, Institute Pasteur de Lille) were used to immunoprecipitate the HCV E2 in the protein extracts of cells cultured in the absence of tetracycline, Ago-2 was also pulled down (Fig. 2c). Reciprocally, the anti-Ago-2 antibody (kindly provided by Dr Witek Filipowicz,}

Fig. 1. Expression of HCV proteins affects the gene silencing activity of siRNA. (a) Schematic representation of the HCV genome and the segments contained in cell lines 1–8 (full explanations given in Table 1). (b) The difference in GFP gene silencing in the presence or absence of tetracycline (HCV proteins are only expressed in cells cultured in the absence of tetracycline). Inhibition of siRNA-mediated gene silencing was calculated using the following formula: (% gene silencing in the absence of tetracycline)− (% gene silencing in the presence of tetracycline). Cells were cultured in 24-well plates in the presence or absence of tetracycline and transfected with 20 nM siRNA-G or negative control siRNA (siRNA-N) (Qiagen) in the presence of Lipofectamine 2000 (Invitrogen). GFP expression 2 days post-transfection was determined by flow cytometry. Results are expressed as the mean (±sd) change in GFP gene silencing from five independent experiments for each cell line. (c) UE1E2con-7 cells (cell line 4) continuously expressing GFP were transfected with siRNA-G or siRNA-N, as described in (b), or were not transfected (no siRNA). Twenty thousand cells were investigated for GFP expression by flow cytometry. The proportion of gene silencing (%) was calculated as 100− (% GFP expression). In this representative example, GFP expression is 23.91%, corresponding to siRNA-G-mediated gene silencing of 76.09%, in the absence of HCV envelope glycoproteins (Tet+), and 61.13% in the presence of envelope proteins (Tet−), corresponding to 38.87% specific gene silencing. Thus, inhibition of gene silencing is 37.22% (76.09−38.87%). Five independent experiments of this type have been performed and representative examples are shown. Cell counts (vertical axes) are given from 0–200 on each chart.
Friedrich Miescher Institute for Biomedical Research, Switzerland) specifically immunoprecipitated HCV E2, whereas the control isotype IgG did not pull down Ago-2 or HCV E2 in protein extracts from cells cultured in the presence or absence of tetracycline (Fig. 2d). We have, however, been unable to demonstrate co-localization of Ago-2 and HCV E2, as the available anti-Ago-2 antibodies produced too much background in immunofluorescence microscopy. Altogether, the co-immunoprecipitation experiments indicate that Ago-2 of RISC is targeted by HCV E2 to inhibit RNAi. We could not demonstrate an interaction between HCV E2 and Dicer (data not shown). Thus, our study differs from that of Wang et al. (2006), who showed that HCV core protein can act as an RNAi inhibitor by interacting with Dicer. The reason that cells expressing both core and envelope proteins (Fig. 1b, cell line 1) do not show an additive inhibition of siRNA-mediated gene silencing can be explained by the fact that different targets and different steps of RNAi are involved.

Ago-2 is considered to be localized in discrete foci, so-called P-bodies, in which programmed RNA degradation takes place (Liu et al., 2005; Sen et al., 2005). It is, however, unclear whether P-bodies just serve as a storage facility for RISC components or whether RNAi takes place in P-bodies (Rossi, 2005). Nevertheless, our data show an interesting link between Ago-2 in P-bodies and HCV E2, which is believed to be localized in the lumen of the endoplasmic reticulum, although the nature of this link is unclear.

RNAi is considered to be the major adaptive defence mechanism against viruses in plants and lower invertebrates, while the role of RNAi in vertebrates and mammals

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**Fig. 2.** HCV envelope protein dose-dependent suppression of GFP gene silencing and interaction of E2 with Ago-2 of RISC. (a) GFP (grey bars) or HCV envelope protein expression (white bars) was determined by flow cytometry in cell line 4 (Table 1). HCV E2 was first stained by mAb A11, followed by anti-mouse–APC conjugate. The relative level of HCV E2 was normalized to that at 0 ng tetracycline ml⁻¹, which was set to 100 %. Suppression of GFP gene silencing was calculated as described in Fig. 1. (b) Total RNA was purified from HCV envelope protein-expressing cells (cell line 4) transfected as described in Fig. 1(b). The level of GFP mRNA was measured by qRT-PCR simultaneously with human glyceraldehyde 3-phosphate dehydrogenase as an internal control for standardization, by using the QuantiTect SYBR Green PCR kit and the QuantiTect Primer Assay (Qiagen). The relative level of GFP was normalized to that of siRNA-untreated cells, which was set to 100 %. Cells were cultured in the presence (white bars) or absence (black bars) of tetracycline. Results in (a) and (b) are expressed as the mean ± SD of more than three different experiments. *, P<0.01. (c) Two different anti-HCV E2 antibodies, A11 and H52, co-immunoprecipitated Ago-2 from the cytoplasmic extracts of U87E2con-7-GFP cells using Dynabeads (Invitrogen). Lanes: 1, anti-E2 antibody A11; 2, anti-E2 antibody H52; 3, no antibody; 4, isotype IgG control. (d) Anti-Ago-2 antibody co-immunoprecipitated HCV E2 protein from the cytoplasmic extracts of the same cells. Lanes: 1 and 3, anti-Ago-2 antibody; 2 and 4, isotype IgG control. Lanes: 1 and 2, protein extracts were obtained from cells cultured in the absence of tetracycline; 3 and 4, protein extracts were obtained from cells cultured in the presence of tetracycline. Isotype IgG was used as a control. The antibodies used for immunoprecipitation (IP) are indicated. Antibodies used for immunoblot are indicated on the right.
is still subject to debate, as they have adaptive immune defence mechanisms which might play a more important role. Recently, it has been demonstrated that HCV replication is dependent on the host RNAi machinery (Jopling et al., 2005; Cullen, 2006; Pedersen et al., 2007; Randall et al., 2007), and miRNA 122, which is highly abundant in liver cells, was found to facilitate HCV RNA replication (Jopling et al., 2005). However, RNAi could also be damaging to HCV RNA and the virus must try to prevent this. Thus, our findings that HCV structural proteins and in particular E2 can suppress siRNA activities are not in conflict with the above observations. However, as the results were obtained in U-2 OS cells expressing HCV proteins, the effect of E2 on RNAi in the context of HCV infection is not yet known. Both our data and that of Wang et al. (2006) indicate that HCV expresses proteins that inhibit RNAi at different steps. From a structural point of view, it is questionable whether HCV RNA in the replication complex is accessible to RNAi-mediated cleavage (Gosert et al., 2003). However, many laboratories have been able to inhibit HCV replication with siRNA in vitro, because its single-stranded RNA functions both as mRNA and as a replication template (Watanabe et al., 2007). Several RNA viruses, including HCV, have evolved mechanisms to overcome RNAi, perhaps with the goal of inducing persistent infection (Voinnet, 2005). A better understanding of the complex interactions between HCV and its host will be a prerequisite for the development of a siRNA-based therapy for HCV infection in the future.

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References


