ISG15, a ubiquitin-like interferon-stimulated gene, promotes hepatitis C virus production in vitro: implications for chronic infection and response to treatment

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Upregulation of interferon (IFN)-stimulated genes (ISGs), including IFN-stimulated gene 15 (ISG15) and other members of the ISG15 pathway, in pre-treatment liver tissue of patients chronically infected with hepatitis C virus (HCV) is associated with subsequent treatment failure (pegylated IFN-α/ribavirin). This study assessed the effect of ISG15 on HCV production in vitro. The levels of ISG15 and of its conjugation to target proteins (ISGylation) were increased by plasmid transfection, but ISGylation was inhibited by small interfering RNA directed against the E1 activating enzyme, Ube1L, in Huh7.5 cells. Cells were infected with HCV FL-J6/JFH virus, and HCV RNA and viral titres were determined. Levels of both HCV RNA and virus increased when levels of ISG15 and ISGylation were increased, and decreased when ISGylation was inhibited. The effects of ISGylation on HCV were independent of upstream IFN signalling: IFN-α-induced ISG expression was not altered by Ube1L knockdown. Thus, although ISG15 has antiviral activity against most viruses, ISG15 promotes HCV production. HCV might exploit ISG15 as a host immune evasion mechanism, and this may in part explain how increased expression of ISGs, especially ISG15, correlates with subsequent IFN-based treatment failure.

INTRODUCTION

Hepatitis C virus (HCV) is adept at evading host antiviral mechanisms and is often resistant to the current standard of care – combination treatment with pegylated interferon (IFN)-α and ribavirin (PegIFN/Rib). This regimen eradicates the virus in only 50% of cases. A number of mechanisms contribute to evasion and treatment resistance, including cleavage of the RIG-I adaptor protein IPS1/MAVS/Cardif by the HCV NS3/NS4A protease and modulation of the host response by the HCV core protein (Li et al., 2005; Loo et al., 2006). However, none of these mechanisms has consistently been demonstrated to play a role in the clinical disease and thus cannot explain the ability of the virus to escape the host response in patients.

Response to treatment can be predicted by levels of expression of IFN-stimulated genes (ISGs) in the liver prior to initiation of PegIFN/Rib treatment. Non-responders have increased expression of a number of ISGs (Chen et al., 2005; Feld et al., 2007; Asahina et al., 2008; Asselah et al., 2008; Sarasin-Filipowicz, et al., 2008). Three of these ISGs are components of the ISG15 ubiquitin-like pathway. ISG15 was the first ubiquitin-like protein to be described and, like its homologue ubiquitin, is conjugated to proteins in a tightly regulated process called ISGylation. The ISG15 E1 activating protein, Ube1L, coordinates with the E2 conjugating enzyme (UbcH8) and the E3 ligase (CEB1) to join the C terminus of ISG15 to a wide variety of proteins (Loeb & Haas, 1992). ISG15 can be removed from its target proteins by USP18, an ISG15 protease (Malakhov et al., 2002). ISG15, CEB1 and USP18 are upregulated in the liver tissue of patients infected with HCV who have not responded to treatment with PegIFN/Rib (Chen et al., 2005; Feld et al., 2007; Asahina et al., 2008; Asselah et al., 2008; Sarasin-Filipowicz, et al., 2008). Three of these ISGs are components of the ISG15 ubiquitin-like pathway. ISG15 was the first ubiquitin-like protein to be described and, like its homologue ubiquitin, is conjugated to proteins in a tightly regulated process called ISGylation. The ISG15 E1 activating protein, Ube1L, coordinates with the E2 conjugating enzyme (UbcH8) and the E3 ligase (CEB1) to join the C terminus of ISG15 to a wide variety of proteins (Loeb & Haas, 1992). ISG15 can be removed from its target proteins by USP18, an ISG15 protease (Malakhov et al., 2002). ISG15, CEB1 and USP18 are upregulated in the liver tissue of patients infected with HCV who have not responded to treatment with PegIFN/Rib (Chen et al., 2005; Feld et al., 2007; Asahina et al., 2008; Asselah et al., 2008; Sarasin-Filipowicz, et al., 2008). Three of these ISGs are components of the ISG15 ubiquitin-like pathway. ISG15 was the first ubiquitin-like protein to be described and, like its homologue ubiquitin, is conjugated to proteins in a tightly regulated process called ISGylation. The ISG15 E1 activating protein, Ube1L, coordinates with the E2 conjugating enzyme (UbcH8) and the E3 ligase (CEB1) to join the C terminus of ISG15 to a wide variety of proteins (Loeb & Haas, 1992). ISG15 can be removed from its target proteins by USP18, an ISG15 protease (Malakhov et al., 2002). ISG15, CEB1 and USP18 are upregulated in the liver tissue of patients infected with HCV who have not responded to treatment with PegIFN/Rib (Chen et al., 2005; Feld et al., 2007; Asahina et al., 2008; Asselah et al., 2008; Sarasin-Filipowicz, et al., 2008).
A functional association between this pathway and the regulation of HCV production has been established: knockdown of USP18 increases the anti-HCV potency of IFN-α (Randall et al., 2006), and ISG15 protein expression is highly upregulated in the hepatocytes of treatment non-responders, but is increased in the macrophages of treatment responders. The ISG15 pathway is thus likely to be important in clinical HCV disease and in determining treatment outcomes.

ISGylation is implicated in different cellular processes, but its role in viral biology is the one that is best established (Dao & Zhang, 2005). ISG15 is targeted by a number of viruses in animal model and cell culture systems. For example, non-structural protein 1B of influenza B virus binds to the free form of ISG15, preventing ISGylation (Yuan & Krug, 2001). Overexpression of ISG15 in IFN-α/β receptor knockout mice protects them from Sindbis virus-induced lethality and decreases Sindbis virus replication in multiple organs (Lenschow et al., 2005). ISG15−/− mice are more susceptible to influenza A and B viruses, herpes simplex virus type 1 (HSV-1), Sindbis virus and murine gammaherpesvirus 68 infection; for Sindbis virus, this effect is dependent on ISGylation (Lenschow et al., 2007). Whilst these studies suggest a general role for ISG15 as an antiviral agent, a recent report found that ISG15 can inhibit IFN responses after infection by Newcastle disease virus (Kim et al., 2008a). ISGylation of the antiviral RIG-I enzyme inhibited IFN signalling in MEF cells (Kim et al., 2008b). Thus, ISG15 inhibits virus production for many viruses, but may promote production of some.

In this study, we examined the role of ISG15 and ISGylation in HCV production in vitro, using the FL-J6/JFH HCV infectious model. Unexpectedly, increasing the level of ISG15/ISGylation promoted HCV production, whilst blocking ISGylation decreased HCV RNA and viral titres. This work therefore suggests a new context for the host ISG response to HCV: some aspects of the host ISG response to HCV foster viral production, rather than inhibiting it.

RESULTS

Increasing and decreasing ISGylation in Huh7.5 cells

In order to test whether ISG15 conjugation plays a role in HCV replication/production, we developed ways of increasing and decreasing ISG15 conjugation (ISGylation). Inducing ISGylation can be difficult in certain cells: for example, in HeLa cells, ISGylation could only be induced by overexpression of ISG15 in combination with its E1 activating enzyme Ube1L and its E2 conjugating enzyme UbcH8 (Zhao et al., 2005). However, in Huh7.5 cells, overexpression of ISG15 alone led to pronounced protein ISGylation in Huh7.5 cells (Fig. 1a). Combining overexpression of ISG15 with overexpression of Ube1L and/or UbcH8 did not appreciately increase protein ISGylation beyond that observed with overexpression of ISG15 alone (data not shown). In order to inhibit ISGylation, the ISG15 E1 Ube1L enzyme was knocked down with small interfering RNA (siRNA). Ube1L mRNA was successfully knocked down, even in the presence of increasing concentrations of IFN-α (0–1 U ml⁻¹) (Fig. 2). This method abolished ISGylation, even in the presence of high levels of IFN-α (100 IU ml⁻¹) (Fig. 1b). Thus, in Huh7.5 cells, ISGylation could be increased and decreased relatively easily.

HCV RNA and virus are increased in parallel with ISGylation

We next asked whether ISGylation (and ISG15) modulates HCV production. As seen in Fig. 3, increasing ISGylation by overexpression of ISG15 significantly increased the production of both HCV RNA and virus, even in the presence of increasing IFN-α, suggesting that increased ISG15/ISGylation decreases IFN-α anti-HCV activity (Fig. 4) in the FL-J6/JFH HCV in vitro culture system. In contrast, silencing of the ISG15 Ube1L E1 enzyme decreased the levels of HCV RNA and virus both at baseline (in the absence of IFN-α) and in the presence of IFN-α, an effect that was more pronounced for HCV viral titres (Fig. 5). To ensure that the siRNA was selective, we tested the effects of four individual Ube1L siRNAs and compared these with the effects of pooled Ube1L siRNA. As shown in Fig. 6, all four individual siRNAs had a similar inhibitory effect on HCV viral particle secretion when compared with the pooled siRNA, indicating that the effect...
we observed was specific to the knockdown of Ube1L. Taken together, these data suggested that ISGylation is important for baseline HCV production.

Silencing Ube1L does not affect upstream IFN-α signalling

Increased ISGylation has been shown to prolong STAT1 phosphorylation, suggesting that ISGylation might play an important role in IFN signalling (Malakhova et al., 2003). To test this hypothesis in the HCV model, we assessed the effect of decreasing ISGylation by Ube1L knockdown on downstream ISG expression in the presence of IFN-α. As shown in Fig. 7, the expression of a number of ISG transcripts was not affected following Ube1L siRNA knockdown in the presence of IFN-α.

DISCUSSION

ISG15 is one of the most abundant ISGs induced after virus infection and type I IFN treatment, and we and others have found that increased pre-treatment ISG15 expression in the livers of HCV-infected patients predicts subsequent treatment failure (Chen et al., 2005; Feld et al., 2007; Asahina et al., 2008; Asselah et al., 2008; Sarasin-Filipowicz et al., 2008). Although ISG15 is generally considered to be antiviral, we have presented evidence here that ISGylation promotes HCV production, decreases the anti-HCV effect of IFN-α and is particularly relevant for steps downstream of HCV RNA replication. Thus, aspects of the host ISG response favour, rather than inhibit, HCV persistence. The action of ISG15 is a novel mechanism for virus persistence, and ISGylation is a possible target for therapy of HCV infection.

As noted in the Introduction, the effect of ISG15 on virus production may be specific to the virus. For example, ISG15 can have an antiviral effect on Sindbis virus, influenza virus, HSV, human immunodeficiency virus (HIV) and Ebola virus (Lenschow et al., 2005, 2007; Okumura et al., 2006; Zhang et al., 2007), but ISGylation does not contribute to murine susceptibility to lymphocytic choriomeningitis virus and vesicular stomatitis virus (Knobeloch, et al. 2005), nor to hepatitis B virus replication in ISGylation-deficient mice (Ube1L−/−) (Kim et al., 2008a). Although ISG15 may also promote viral production by acting as a negative regulator of the innate immune response through its conjugation to RIG-I (Kim et al., 2008b), this mechanism is unlikely to contribute to our observed effects, as Huh7.5 cells are deficient in RIG-I (Sumpter et al., 2005). Our data suggest that HCV exploits the ISG15/ISGylation pathway to increase HCV production: overexpression of ISG15, which increases ISGylation in Huh7.5 cells (Fig. 1), increased HCV RNA 3-fold and viral titres 2.2-fold (Fig. 3). Blocking ISGylation by knockdown of Ube1L decreased HCV RNA and largely abolished the production of infectious virus (Fig. 5).

Another approach to examining the role of ISG15 in HCV production would be to decrease ISG15 mRNA using specific siRNA. We have not employed this method in the current study – in preliminary work, we found that ISG15-specific siRNA was not able to decrease ISG15 mRNA consistently in Huh7/7.5 cells, particularly in the presence of IFN-α (data not shown). However, others have demonstrated that knockdown of ISG15 in two HCV

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**Fig. 2.** Ube1L mRNA expression was silenced by siRNA. Huh7.5 cells were electroporated with irrelevant siRNA (shaded bars) or siRNA specific to Ube1L (filled bars). Thirty hours after electroporation, cells were treated with increasing amounts of IFN-α for 15 h before being harvested to determine the levels of Ube1L mRNA by real-time PCR (normalized to β-actin). Data represent the means ± SD of three replicates; the results shown are representative of three similar experiments. *P < 0.05 (versus irrelevant siRNA).

**Fig. 3.** ISG15 promotes HCV production in vitro. Huh7.5 cells were transfected with empty vector (V) or ISG15 plasmid DNA for 48 h before the cells were infected with FL-J6/JFH as described in Methods. HCV RNA was quantified by real-time PCR (a), and HCV virus particle titres were assessed by serial dilution of culture supernatants (b). Data represent the means ± SD of three replicates; the results shown are representative of three similar experiments. U, Untreated control. ***P < 0.001; *P < 0.05 (versus empty vector).
replicon models (Con1 and murine MH1 cells) resulted in decreased HCV production both with and without IFN-α (Broering et al., 2008). Although this study does not directly address the role of ISGylation, it adds evidence for the permissive role of the ISG15 pathway in the HCV life cycle.

Our data provide a mechanistic insight into how ISG15 affects HCV production. ISG15 exists in three forms: (i) a free, unconjugated intracellular protein, (ii) conjugated to viral and/or host target proteins, and (iii) an extracellular cytokine (Recht et al., 1991; D’Cunha et al., 1996a, b; Lai et al., 2009). All three forms could potentially affect HCV viral production. In other systems, the free form of ISG15 has been shown to inhibit the release of Ebola virus-like particles by interfering with the activity of Nedd4 (Malakhova & Zhang, 2008; Okumura et al., 2008). ISGylation is critical to the effect of ISG15 on Sindbis virus, and ISGylation is targeted by the human influenza virus NS1 protein (Yuan & Krug, 2001; Lenschow et al., 2007). As a cytokine, purified ISG15 can activate natural killer and cytotoxic T-cells, stimulate IFN-γ production, and induce dendritic cell maturation and neutrophil recruitment (Recht et al., 1991). Our data argue that ISGylation is the predominant mechanism through which ISG15 affects HCV production.

Blocking ISGylation by Ube1L knockdown did not decrease free ISG15 but dramatically reduced HCV viral titres and significantly reduced HCV RNA levels. In order to test for a direct cytokine role of ISG15, we exposed Huh7.5 cells to a high dose of purified ISG15 (2 μg ml⁻¹) for 36 h before cells were infected with FL-J6/JFH virus (m.o.i. = 0.3) as before. Although the dose we used is considerably higher than that used by D’Cunha et al. (1996b) (100 ng ml⁻¹) to define the cytokine effect of ISG15, we were unable to find any inhibition of HCV production, nor did we find any reduction in the ability of IFN-α to stimulate ISG expression (data not shown).

The current study demonstrates that increasing ISGylation promotes HCV production and decreases the anti-HCV effect of IFN-α. However, previous work from our group has demonstrated that decreasing the expression of USP18, the ISG15 protease, increases ISGylation yet potentiates IFN-α anti-HCV activity (Randall et al., 2006). These data initially appear conflicting, but only if one assumes that USP18 and ISG15 work entirely through the same pathway. In fact, USP18 clearly has additional targets beyond ISG15, and manipulating USP18 expression has effects on protein expression that are independent of ISG15. For example, epidermal growth factor receptor synthesis is regulated by USP18 (Dux & Sorkin, 2009). USP18 has both protease-
dependent and -independent functions (Malakhova et al., 2006). Our preliminary data would support USP18 having a role in HCV production that is independent of ISG15 and ISG15 protease activity (Chen et al., 2008). Taken together, the data from our group suggest that ISGylation is necessary but not sufficient for HCV production.

Blocking ISGylation enhances the anti-HCV effect of IFN-α (Fig. 5). This effect is not likely to be mediated at the level of IFN signalling, as Ube1L knockdown did not promote (or inhibit) IFN-dependent ISG expression, which is the indicator of activation of the IFN pathway (Fig. 7). Although ISGylation may play an important role in regulation of the JAK/STAT pathway and IFN signalling in some cells (Malakhov et al., 2002; Malakhova et al., 2002; Ritchie et al., 2002), the IFN signalling pathway is intact in ISG15−/− and Ube1L−/− mice (Osiak et al., 2005; Kim et al., 2006). In our work, IFN signalling appeared to be unaffected, despite knockdown of Ube1L, and Ube1L knockdown inhibited HCV production, even in the absence of IFN-α. These data suggest that ISGylation of viral (or host) proteins is directly important for the viral life cycle. The conjugation of ISG15 to cellular or viral proteins might alter their function, or compete for ubiquitination. For example, in order for HIV to be secreted from infected cells, the Gag protein must be ubiquitinated and then recruited to the endosomal transport complex. ISG15 conjugation to Gag prevents its ubiquitination and thus inhibits HIV release (Okumura et al., 2006). In another example, ISG15 conjugation to interferon regulatory factor 3 (IRF3), a key signal-transducing factor for IFN-dependent immune responses,
protects IRF3 from ubiquitin-mediated degradation (Lu et al., 2006). Thus, ISGylation of HCV proteins or host proteins important for the HCV life cycle may alter their function or protect them from degradation; the specific steps involved in the ISG15 effect remain to be defined.

In summary, our *in vitro* data strongly argue that ISG15, and specifically ISGylation, is important to the HCV life cycle in an infectious cell culture model of HCV. This study offers one explanation for how increased baseline expression of some ISGs, including ISG15, correlates with treatment failure in HCV-infected patients. Targeting ISGylation or specific targets of ISGylation may identify new antiviral therapies for HCV.

**METHODS**

**Cells and HCV FL-J6/JFH virus.** Huh7.5 cells and HCV FL-J6/JFH were kindly provided by Dr Charles Rice (Rockefeller University, NY, USA) (Lindenbach et al., 2005). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with non-essential amino acids, 10% fetal bovine serum, 100 U ampicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ at 37 °C in a 5% CO₂ humidified incubator.

**ISG15 expression plasmid.** Human full-length ISG15 was generated using pOT-B7-ISG15 plasmid DNA (MGC clones; Open Biosystems) as template, and the resulting PCR product was cloned into a pcdNA4/HisMax TOPO TA expression vector (Invitrogen). The primers used were 5'-ATGGGCTGGGACCTGACGGTG-3' (forward) and 5'-TTAGCTCCGCCCGCCAGGCTC-3' (reverse). Plasmid DNA for transfection studies was prepared using a Plasmid Maxiprep kit (Qiagen).

**ISG15 transfection and detection of ISG15 expression by Western blotting.** ISG15 plasmid DNA (8 µg) or empty vector was transfected into Huh7.5 cells (2.5 × 10⁵ ml⁻¹, 5 ml per 6 cm culture dish) with Lipofectamine 2000 (Invitrogen) following the kit protocol. At 48 h post-transfection, the cells were harvested, washed in PBS and lysed in 200 µl lysis buffer [50 mM HEPES (pH 7.8), 500 mM NaCl, 1% Triton X-100, 1 mM EDTA, protease inhibitor cocktail (Sigma)]. Proteins were separated by NuPAGE 4–12% Bis-Tris gels and transferred onto nitrocellulose membrane using a Trans-Blot SD Semi-dry transfer cell (Bio-Rad). ISG15 expression was assessed using a polyclonal anti-ISG15 antiserum raised in rabbits (as a control, human ISG15 protein (Cedarlane Laboratories). Blots were developed using an OptiBlaze WEST femtoLUCENT kit (G-Biosciences).

For studies of the effect of ISG15/ISGylation on HCV production, 0.3 µg ISG15 plasmid DNA or empty vector was transfected into Huh7.5 cells in each well of a 96-well plate for 48 h, after which the cells were treated in the absence or presence of IFN-α (0–1 U ml⁻¹) for 16 h, followed by infection with HCV FL-J6/JFH virus (m.o.i. = 0.3) for 6 h. The cells were then washed and overlaid with fresh medium. At 2 days post-infection (p.i.), the cells were harvested for assessment of HCV replication (RNA and infectious particles, see below).

**Ube1L siRNA knockdown.** Four Ube1L siRNAs were designed as follows (the sense strand sequence is described): siUbe1L#1, 5'-CAUCUUUGCUAGUAACUA-3'; siUbe1L#2, 5'-CGAAUUGUGGCGCAGAAU-3'; siUbe1L#3, 5'-AUAAGGCGCCUCAAUCUC-3'; and siUbe1L#4, 5'-GCAUGAGUUUGCUUUCUG-3'. The irrelevant siRNA was 5'-GGCCGCUUGGCAUAUCUGT-3'. Chemically synthesized RNA oligonucleotides were prepared as recommended by the manufacturer (Dharmacon). One nanomole of RNA duplexes was electroporated into 2.5 × 10⁵ Huh-7.5 cells as described previously (Randall et al., 2006). Thirty hours after electroporation, cells were treated with IFN-α (0–1 U ml⁻¹) for 15 h, and then either harvested for determination of siRNA knockdown efficiency (Ube1L mRNA by real-time PCR) or washed, infected with HCV FL-J6/JFH (m.o.i. = 0.3) for 6 h, rinsed and overlaid with fresh medium. At 48 h p.i., cells were harvested for assessment of HCV production (RNA and infectious particles). A similar experiment was performed using higher dosages of IFN-α (0–1000 U ml⁻¹) to investigate whether silencing Ube1L and/or decreased ISGylation had any effect on IFN downstream ISG mRNA expression (real-time PCR).

**Quantification of infectious HCV virion and HCV RNA.** Viral titres were determined by limiting dilution analysis of culture supernatants as described previously (Lindenbach et al., 2005). For HCV RNA quantification, total cellular RNA was harvested and purified with 96-well RNAeasy columns (Qiagen), reverse transcribed (Superscript II; Invitrogen) and the cDNA constructed (AnCt primer; Invitrogen). Real-time PCR was performed using SYBR Green mix and either the primers listed in Supplementary Table S1 (available in JGV Online) or the HCV-specific primers 5'-TGAAGTCTGACAGCCCTCA-3' and 5'-ACGCATCTGGCTAGCAGTC-3' (Platinum Quantitative RT-PCR ThermoScript One-Step System; Invitrogen, Life Technologies) as described previously (Randall et al., 2006).

**Statistics.** Where appropriate, Student’s *t*-test was used to compare two categorical values and one-way analysis of variance was used to compare more than two categorical values. For Western blot studies, the experiments were repeated at least three times.

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