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ISG15 is a ubiquitin-like molecule whose expression is induced by type I IFN (IFN-α/β) or in response to virus or bacterial infection. ISG15 or conjugation of ISG15 to target proteins was reported to play critical roles in the regulation of antiviral responses. IFN restricts replication of hepatitis C virus (HCV). However, molecular mechanism of IFN-infection. ISG15 or conjugation of ISG15 to target proteins was reported to play critical roles in the regulation of antiviral infection, combined treatment of pegylated IFN-α and ribavirin is the most favorable choice for therapy. However, resistance to IFN is often observed in patients with HCV infection (12, 13). Interestingly, mutations in HCV-NS5A were reported to correlate with responsiveness to IFN therapy in patients, although its precise mechanism is not conclusive (14). To overcome IFN resistance in patients with HCV infection, the development of chemicals that inhibit HCV-specific enzymatic activities or antiseNSE oligomers that inhibit HCV replication has been undertaken (15–17).

Pathogen-associated molecular patterns of replicating HCV RNA genomes, such as 5′-triphosphate RNA or dsRNA, are recognized by the product of RIG-I in the cytoplasm. It triggers the activation of intracellular signaling pathways and transcription factors, such as NF-κB and IFN regulatory factors (18–22). An outcome of these events is the production of type I IFN (IFN-α/β) (23, 24). Secreted IFN-α/β induces the activation of the IFN-stimulated gene factor 3 complex, which translocates to the nucleus, where it binds to the IFN-stimulated response element regions of target genes to direct the expression of IFN-stimulated genes (ISGs). Among the genes induced by IFN, IFN-induced, dsRNA-activated protein kinase, MxA, and ISG15 function as effector molecules in the host cell response to viral infection (24). Multiple studies have shown a clear absence or low level of IFN-α/β in the hepatocytes of patients with chronic HCV infection. In addition, patients with chronic HCV infection show no significant level of ISG induction upon treatment with IFN (25). Based on these findings, it has been suggested that HCV viral proteins disturb the innate immune response of the host and that altered hepatic ISG expression is associated with liver pathology.

ISG15 is a type 1 IFN-inducible, ubiquitin-like protein. Most components for ISG15 conjugation (ISGylation), including UbeIL. ISG15 is activated by a kinasedependent mechanism and activation of the IFN-stimulated gene factor 3 complex, which translocates to the nucleus, where it binds to the ISG15-stimulated response element regions of target genes to direct the expression of IFN-stimulated genes (ISGs). Among the genes induced by IFN, IFN-induced, dsRNA-activated protein kinase, MxA, and ISG15 function as effector molecules in the host cell response to viral infection (24). Multiple studies have shown a clear absence or low level of IFN-α/β in the hepatocytes of patients with chronic HCV infection. In addition, patients with chronic HCV infection show no significant level of ISG induction upon treatment with IFN (25). Based on these findings, it has been suggested that HCV viral proteins disturb the innate immune response of the host and that altered hepatic ISG expression is associated with liver pathology.

ISG15 is a type 1 IFN-inducible, ubiquitin-like protein. Most components for ISG15 conjugation (ISGylation), including UbeIL, E1, ISG15-activating enzyme), UbcH8 (E2, ISG15-conjugating enzyme), and HERC (E3, ISG15 ligase), are highly responsive to IFN-α/β-induced signal transduction (26, 27). Expression of ISG15 and the conjugation of ISG15 to target proteins are strongly promoted by IFN-α/β treatment, dsRNA, and viral or bacterial infection (28). Production of ISG15 or ISGylation is believed to play an important role in establishing the antiviral state of an infected cell. ISG15-deficient mice are highly susceptible to influenza, herpes, and Sindbis viral infections (29), whereas the protective role of overexpressed ISG15 in Sindbis virus-infected IFN-α/βR-deficient mice has been reported (30). It was also reported that ISGylation of cellular proteins...
can be suppressed by infection with influenza B virus through the interaction of ISG15 with the NS1B viral protein (31). In the current study, we investigated the role of ISGylation on HCV-NS5A and its implication for viral replication in subgenomic HCV replicon cells.

Materials and Methods

Cell culture and transfection

Huh-7 and COS7 cells were maintained in DMEM (Invitrogen, Grand Island, NY), supplemented with 10% FBS (HyClone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). Huh-5-15 and Huh-luc.neo-ET HCV (genotype 1b) subgenomic replicon cells (gift of Y. C. Sung, Pohang University of Science and Technology [POSTECH], Pohang, South Korea), and were cloned into pDEST mammalian expression vectors (Invitrogen). For the HCV-NS5A expression vector, coding regions for NS5A of HCV genotype 1b were amplified by PCR using pGX10-NS5 as the template (gift of Y. C. Sung, POSTECH), digested with KpnI and XhoI, and inserted into the pDNA3.1/myc or subsequently cloned into the pFlag/CMV plasmid. Deletion mutants of HCV-NS5A were generated by PCR and were cloned into the pFlag/CMV plasmid. Point mutations in NS5A-NS5B were obtained by PCR amplification using pGX10gDs ST, pGX10-NS3, pGX10-NS4, and pGX10-NS5 plasmids as templates, respectively (gift of Y. C. Sung, POSTECH, Pohang, South Korea), and were cloned into pDEST mammalian expression vectors (Invitrogen).

Plasmid construction

The coding regions for HCV-E2, -NS3, -NS4, and -NS5B of HCV genotype 1b were obtained by PCR amplification using pGX10gDs ST, pGX10-NS34, and pGX10-NS5 plasmids as templates, respectively (gift of Y. C. Sung, POSTECH). DNA sequences of the constructed plasmids were verified by automated sequencing. The expression plasmids (2 μg plasmids/35-mm dish) were transfected using Lipofectamine 2000 (Invitrogen).

Generation of mutant HCV subgenomic replicon cells

The K379R point mutation was introduced into NS5A of NK5.1 plasmid construct (provided by S. K. Jang, POSTECH) using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. DNA sequences of the constructed plasmids were verified by automated sequencing. The expression vectors for HA-Ube1L and Flag-UbcM8 were provided by Dr. D.-E. Zhang (The Scripps Research Institute, La Jolla, CA), and HA-ubiquitin plasmid was provided by Chin Ha Chung (Seoul National University, Seoul, Korea). Myc-ISG15 plasmid was cloned, as described (32).

Immunoblot analysis

Cells were harvested in lysis buffer, and 1 mg total-cell lysates was precleared with normal IgG and protein A/G agarose beads. Lysates were incubated for 12 h at 4°C with the appropriate Ab, followed by an additional 2 h of incubation with protein A/G agarose beads.

Immunofluorescence staining

Cells on glass cover slips were fixed with 4% parafomaldehyde solution and permeabilized with 0.2% Triton X-100. Cells were blocked with 3% goat serum/2.5% BSA and incubated with anti-HCV-NS5A (Virogen) or anti-Flag (Sigma-Aldrich, St. Louis, MO) Abs for 2 h. Cells were then incubated with Alexa Fluor 568- or Alexa Fluor 488-conjugated secondary Abs (both from Invitrogen). Slides were mounted and analyzed using a fluorescence microscope.

Results

Replication of HCV was inhibited by the overexpression of ISG15-conjugation components in the HCV subgenomic replicon cells

ISG15 is an IFN-inducible gene that is strongly induced by virus infection in immune and nonimmune cells. ISG15 was shown to negatively regulate the replication, assembly, and budding of Sindbis virus, HIV, and Ebola virus, respectively, which strongly suggests that it functions as an antiviral agent (30, 33). We wondered whether ISG15 or ISG15 conjugation had similar inhibitory effect on the replication of HCV. To explore the effect of ISGylation, we transfected Huh-luc.neo-ET replicon cells with ISG15, Ube1L, and UbeM8 expression plasmids and monitored the replication of subgenomic HCV replicons. Huh-luc.neo-ET replicon cells harbor the luciferase reporter gene in the HCV replicon construct (34). Therefore, it can be used to quantitatively analyze the effect of ISG15 conjugation on HCV replication (Fig. 1). Quite surprisingly, the overexpression of ISG15 and ISG15-conjugation components significantly inhibited the replication of the HCV subgenomic replicon to up to 65% of that observed in the empty vector-transfected control cells.

Statistics

Data are represented as mean ± SD, and differences between groups were analyzed by the t test.

FIGURE 1. Overexpression of the ISGylation system inhibits HCV replication in the HCV subgenomic replicon cells. Huh-luc.neo-ET replicon construct used in this study (34) (top panel). Cells were transfected with the indicated expression plasmids, and replication was measured by luciferase assay (bottom panel). Graph shows mean ± SD of triplicate experiment set. Representative data repeated in at least three independent experiments.
**FIGURE 2.** HCV-NS5A is a target protein for ISG15 conjugation. COS7 cells were cotransfected with expression vectors for ISG15 along with HCV-E2-V5, -NS3-V5, or -NS4-V5 (A) or with Flag-HCV-NS5A (B). Total-cellular lysates (40 μg) were analyzed 48 h after transfection by immunoblot assay. C. Transfected COS7 cells were stimulated with intracellular poly-IC (25 μg/ml) for 12 h before analysis. D, Myc-ISG15–transfected COS7 cells were stimulated with poly-IC (25 μg/ml) or IFN-β (100 U/ml) for 24 h. ISGylation in total-cell lysates (15 μg) was analyzed by immunoblot using anti-Myc Abs. E and F, COS7 cells were transfected with the indicated expression plasmids. Total lysates (1 mg) were subjected to immunoprecipitation using anti-Flag (E) or anti-V5 (F) Abs and were analyzed using the indicated Abs. For whole-cell extracts (WCE), 1/20th volume of total-cell lysates was analyzed. *H/L chains of IgG, **ISGylated HCV-NS5A. Arrowheads mark NS3 and NS5B proteins.
diverse HCV isolates (Fig. 3C). Among the tested KR mutants of HCV-NS5A, only the K379R mutant remained unchanged after the overexpression of ISGylation components (Fig. 3D). Furthermore, ISGylation on HCV-NS5A was completely blocked in the K379R mutant compared with normal ISGylation in the wild-type (WT) or other KR mutants (Fig. 3E), suggesting that lysine 379 on HCV-NS5A is critical for protein regulation by ISGylation.

Reduction of HCV-NS5A protein by ISG15 conjugation in the subgenomic HCV replicon cells

We demonstrated that HCV-NS5A was a target protein of ISG15 conjugation, which led to decreased protein levels in COS7 cells. Based on these observations, we hypothesized that the reduction of HCV-NS5A by ISG15 conjugation may be responsible for the decreased replication of HCV observed in the Huh-luc/neo-ET replicon cells (Fig. 1). To investigate the molecular mechanism of HCV replication controlled by ISG15, we examined whether NS5A was directly controlled by ISG15 conjugation in the HCV subgenomic replicon cells. With good agreement with the previous results, overexpression of ISG15/E1/E2 restrained protein levels of endogenous HCV-NS5A in the Huh-5-15 or Huh-luc/neo-ET replicon cells (Fig. 4A, B). The inhibitory effect of ISG15 conjugation on HCV-NS5A became evident when protein levels of NS5A were visualized by immunofluorescence staining at the single-cell level. Immunoreactive HCV-NS5A proteins were diminished in a cell with high levels of UbcM8 (E2) but remained unchanged in a nontransfected cell (Fig. 4C). Finally, we confirmed that NS5A in the HCV replicon cells was subject to ISG15 conjugation (Fig. 4D). Taken together, our results strongly suggest that ISG15 induced by type I IFNs may suppress replication of HCV, presumably because of the reduced cellular levels of HCV-NS5A protein mediated by ISG15 conjugation.

Protein stability of HCV-NS5A was affected by ISGylation and ubiquitination

To understand the underlying mechanism of HCV-NS5A downregulation by ISGylation, we examined whether ISGylated NS5A can be degraded by 26S proteasome. For this purpose, we measured ISGylation of HCV-NS5A in the cells treated with 26S proteasome inhibitor MG132 (Fig. 5A). As previously reported (32), ISGylation was strongly enhanced in the MG132-treated total-cell lysates, and ISG15 conjugation to HCV-NS5A was correspondingly increased. Consequently, cellular levels of unconjugated NS5A protein were further decreased by MG132 treatment. Intriguingly, migrating patterns of ISGylated HCV-NS5A in SDS-PAGE were different from the untreated control, indicating that NS5A can be subject to further modification, such as ubiquitination. To prove this idea, ubiquitination of HCV-NS5A protein was separately assessed by immunoprecipitation in the ubiquitin-overexpressing COS7 cells (Fig. 5B). As expected, ubiquitin-conjugated forms of HCV-NS5A were observed in the MG132-treated cells. Interestingly, the ubiquitinated form of NS5A was also detected in the absence of MG132, suggesting that HCV-NS5A protein is a heavily ubiquitinated protein. It is noteworthy that zinc mesoporphyrin, which suppresses HCV
replication, exhibited enhanced ubiquitination of HCV-NS5A (39). To determine whether ISGylation of NS5A affects ubiquitination of NS5A, which leads to the protein degradation via 26S proteasome, we examined ubiquitination of HCV-NS5A (K379R) mutant protein. Although basal ubiquitination of HCV-NS5A (K379R) was diminished, ubiquitination of WT and K379R mutant NS5A protein was similarly increased when 26S proteasome activity was blocked by MG132, indicating that HCV-NS5A might be ubiquitinated on the lysine residue other than K379 (Fig. 5C, Supplemental Fig. 3). However, despite MG132’s effect on the ubiquitination of NS5A, it is noteworthy that cellular levels of HCV-NS5A and NS5A (K379R) proteins were not changed (Fig. 5B, 5C). We speculated that the protein-synthesis rate of NS5A might be fast enough to counterbalance the degradation rate. To prove this idea, cells were treated with cycloheximide to block new protein synthesis, and the effect of MG132 on protein degradation was examined (Fig. 5D, Supplemental Fig. 4). Interestingly, expression of ubiquitin alone increased cellular levels of HCV-NS5A and NS5A (K379R) proteins. However, the cellular levels of the proteins were not affected by MG132 treatment, in the absence or presence of cycloheximide treatment. These results indicate that protein stability of HCV-NS5A is subject to control that involves ISGylation and ubiquitination.

ISG15-conjugation system as a potential combined therapy with type I IFNs

Finally, we examined whether ISG15 or the ISG15-conjugation system could be used as combined treatment with type I IFN. In the Flag-HCV-NS5A–transfected COS7 cells, cellular levels of HCV-NS5A protein were slightly suppressed by treatment with IFN-β, which was dramatically reduced by cotreatment of ISG15/E1/E2 overexpression (Fig. 6A). In the HCV replicon cells, IFN-β–mediated inhibition of HCV replication was further suppressed by cotransfection of ISGylation components (Fig. 6B). In addition, equivalent levels of the inhibitory effect of IFN-β (100 U/ml) on HCV replication were obtained using combined treatment with a lower dose of IFN-β (50 U/ml) and ISG15/E1/E2 overexpression in the HCV subgenomic replicon cells (Fig. 6C). In contrast, ISGylation-mediated inhibition of HCV replication was not observed in the mutant HCV replicon cells with NS5A (K379R), demonstrating that HCV-NS5A might be the only target for ISG15 conjugation (Fig. 6D). In conclusion, effective control of HCV replication can be achieved by ISG15 or ISG15-conjugation system, in combination with IFN-β.
Discussion

In the current study, we demonstrated that the overexpression of ISG15 and ISGylation components induces ISGylation in the C-terminal regions of HCV-NS5A, reduces cellular levels of NS5A protein, and, hence, inhibits replication in the HCV subgenomic replicon cells.

The antiviral potential of ISG15 and ISGylation has been reported in various viral systems. Overexpression of ISG15 was shown to inhibit viral packing and replication of HIV, Ebola virus, and Sindbis virus in type I IFN-deficient mice (30, 33, 40). Mice with a defect in the production of ISG15 are hyperresponsive to viral infection by influenza A and B, herpes, and Sindbis viruses (29, 41). Viral proteins that specifically target ISG15 to inhibit ISGylation have been reported (31). Among the viruses that cause acute and chronic infection, HCV is notorious for evading host immune mechanisms and developing resistance to current IFN-based therapy tools. An internal domain that determines sensitivity to IFN has been identified in HCV-NS5A proteins, although the exact mechanism for regulation is not clear (14). Although there is strong evidence that ISG15 can function as an antiviral mediator, its role in regulating HCV replication has not been extensively explored. Silencing UBP43, which specifically cleaves ISGylated residues, potentiates antiviral activity of IFN against HCV replication (42), indicating that IFN-dependent ISGylation of NS5A does not require ISDR region (Supplemental Fig. 5). Instead, we identified a lysine residue, K379, in the polyproline cluster of HCV-NS5A as the target site for ISGylation. Because K379 residue on HCV-NS5A is conserved in most HCV genotypes, it is likely that the negative effect of ISG15 conjugation on HCV-NS5A may be universal. Although we now know that ISG15 conjugation of HCV-NS5A at Lys 379 is critical for ISGylation-mediated negative control of HCV-NS5A, the molecular link between ISGylation and its effect on target protein regulation is still elusive.

We did not observe a similar inhibitory effect of HCV replication by ISG15 or ISGylation in the Huh-7.5 cells infected with the JFH strain of HCV (Supplemental Fig. 6). Similarly, ISG15 was recently reported to promote HCV replication in the HCV J6/JFH1-infected Huh-7.5 cells (47). There are several possible explanations for this difference. First, the effect of ISG15 or ISGylation on HCV replication may be specific to the genotypes. To confirm the inhibitory effect of ISGylation, we examined two HCV subgenomic replicon constructs, with all of the HCV genome sequence derived from genotype 1b, whereas the HCV J6/JFH1 strain used for cellular infection was derived from genotype 2a. HCV-NS5A from genotypes 1b and 2a showed only 64% amino acid identity derived from genotype 2a. We showed that treating cells with intracellular poly-IC and overexpressed ISG15 synergistically suppressed the cellular levels of HCV-NS5A protein, suggesting that IFN-induced ISG15 conjugation may be involved in the negative regulation of HCV replication. Although ISGylation does not directly target substrates for degradation by 26S proteasome complex (43), it inhibits normal functions of target protein by reducing the amounts of un conjugated forms (32). Alternatively, it is possible that ISG15 conjugation could lead to altered subcellular localization of target proteins, thereby inhibiting their physiological functions. HCV viral genomes are replicated in the vicinity of ER membranes and form an lipid droplet-associated replication complex (10). ER membrane association mediated by the N-terminal amphipathic helix domain of HCV-NS5A is a critical step in HCV RNA replication, and a mutant form of HCV-NS5A lacking the N-terminal domain does not support HCV viral replication (8). Recently, it was reported that E2-conjugating enzymes for ISGylation suppress ubiquitination of cellular protein (44), presenting a competition mechanism between ISGylation and ubiquitination or cross-talk between ubiquitination and ISGylation (45). Therefore, future studies addressing the regulatory role of ubiquitinated NS5A and its cross-talk to ISGylation will be a key to our understanding of the underlying mechanism of ISGylation-mediated regulation of HCV-NS5A protein.

HCV-NS5A is composed of four functional regions: an N-terminal amphipathic helix domain, a hyperphosphorylation cluster, the interferon sensitivity-determining region (ISDR), and a polyproline cluster in the C-terminal region (46). Although internal ISDR in the HCV-NS5A is known to determine sensitivity to IFN therapy (14), mutant forms of NS5A lacking ISDR exhibited similar levels of ISGylation, indicating that IFN-dependent ISGylation of NS5A does not require ISDR region (Supplemental Fig. 5). Instead, we identified a lysine residue, K379, in the polyproline cluster of HCV-NS5A as the target site for ISGylation. Because K379 residue on HCV-NS5A is conserved in most HCV genotypes, it is likely that the negative effect of ISG15 conjugation on HCV-NS5A may be universal. Although we now know that ISG15 conjugation of HCV-NS5A at Lys 379 is critical for ISGylation-mediated negative control of HCV-NS5A, the molecular link between ISGylation and its effect on target protein regulation is still elusive.
acid identity. In addition, it is possible that accumulated mutations in the HCV J6/FH1 RNA genome during replication provides an evasion mechanism against ISGylation. Finally, the effect of ISG15 or ISGylation on HCV-NS5A may be compromised by HCV viral proteins that are not present in the replicon construct. The HCV subgenomic replicon used in this study contained coding sequences covering NS3, NS4A/B, NS5A, and NS5B. Therefore, it lacked core, E1, E2, p7, and NS2 proteins.

Currently available treatment of HCV infection is restricted to pegylated IFN-α and ribavirin (48, 49). However, limited efficacy and side effects in patients treated with IFN-α demand the development of new therapeutic tools. Based on the focal replication of virus-infected regions in the liver of patients, focal treatment has been suggested to substitute for the classical systematic management of IFNs and to increase HCV clearance with fewer side effects (50). In this study, combined treatment, consisting of enhanced ISG15 conjugation of HCV in the liver of patients, focal treatment has been suggested for genotype 1 nonresponders. Gastroenterology 132: 1270–1278.


