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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-α/β on HCV replication is not clear yet. In the current study, we demonstrated that replication of HCV was inhibited by overexpression of ISG15 or ISG15-conjugation enzymes in the HCV subgenomic replicon cells. Among various nonstructural proteins of HCV, NS5A was identified as the substrate for ISGylation. Furthermore, protein stability of NS5A was decreased by overexpression of ISG15 or ISG15-conjugating enzymes. The inhibitory effect of ISG15 or ISGylation on NS5A was efficiently blocked by substitution of lysine at 379 residue to arginine within the C-terminal region, suggesting that ISGylation directly controls protein stability of NS5A. Finally, the inhibitory effect of IFN-α/β on HCV replication was further enhanced by ISGylation, suggesting ISG15 as a therapeutic tool for combined therapy with IFN against HCV.

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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-NSSA expression vector, coding regions for NSSA of HCV genotype 1b were amplified by PCR and cloned into pGEX10-NSS and pGX10-NSS plasmids as templates, respectively (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 immunofluorescence staining, cells on glass cover slips were fixed with 4% paraformaldehyde solution and permeabilized with 0.2% Triton X-100. Cells were blocked with 3% normal IgG and protein A/G agarose beads. Lysates were incubated for 2 h. For immunoblotting, commercially available anti-Myc (Roche, Basel, Switzerland), anti-HA (Roche), and GAPDH (Chemicon International, Temecula, CA), anti-HA (Roche), and anti–HCV-NSSA Abs (Virotech, Watertown, MA) were used. For immunoprecipitation, cells were harvested in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS) containing 1 mM DTT, 0.57 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 5 μg/ml aprotinin, and 1 mM benzamidine. Total-cell lysates (15–40 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Immunoblot analysis

Cells were harvested in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS) containing 1 mM DTT, 0.57 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 5 μg/ml aprotinin, and 1 mM benzamidine. Total-cell lysates (15–40 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). For immunoblotting, commercially available anti-Myc (Roche, Basel, Switzerland), anti-Flag (Sigma-Aldrich, St. Louis, MO), anti-V5 (Invitrogen), anti-GAPDH (Chemicon International, Temecula, CA), anti-HA (Roche), and anti–HCV-NSSA Abs (Virotech, Watertown, MA) were used.

Luciferase assay

Cells carrying the Huh-luc/neo-ET HCV subgenomic replicon construct were transfected with empty vectors or expression vectors for the ISGylation-conjugation system. Cells were lysed 48 h posttransfection, and luciferase activity was measured using a dual-luciferase assay kit (Promega, Madison, WI). To ensure that equal amounts of cell lysates were used for luciferase assay, protein quantification using the Bradford method was performed prior to the assay.

**Statistics**

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

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 UbcM8 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.

**Immunofluorescence staining**

Cells on glass cover slips were fixed with 4% paraformaldehyde solution and permeabilized with 0.2% Triton X-100. Cells were blocked with 3% goat serum/2.5% BSA and incubated with anti–HCV-NSSA (Virotech) or anti–human 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.
HCV-NS5A is a target protein for ISG15 conjugation

To understand the molecular mechanism of the inhibitory effect of ISG15 on HCV replication, we examined whether ISG15 conjugation exhibited a direct effect on HCV viral proteins. We cloned individual nonstructural proteins of HCV (HCV-NS3, -NS4, -NS5A, and -NS5B) and HCV-E2 structural protein in the mammalian expression vectors, and the result of ISG15 overexpression was investigated in the transfected COS7 monkey epithelial cells. In the cells overexpressing ISG15, cellular levels of HCV-E2, -NS3, and -NS4 protein were not significantly altered compared with equal amounts of empty vector-transfected cells (Fig. 2A). In contrast, protein levels of HCV-NS5A were dramatically reduced with cotransfection of ISG15 (Fig. 2B) or with components of ISG15-conjugation system: Ube1L and UbcM8 (Supplemental Fig. 1). To mimic the effect of intracellular RNA virus infection, which enhances the expression of ISG15-conjugation system, poly-IC was transfected into cells previously transfected with empty vector or expression vectors for ISGylation component. Stimulating cells with intracellular poly-IC further decreased the cellular levels of HCV-NS5A protein in the ISG15-transfected COS7 cells (Fig. 2C).

To test whether HCV-NS5A was a direct target of ISG15 conjugation, we next examined the ISGylation of NS5A in the COS7 cells. ISGylation of the total cellular proteins was strongly induced by poly-IC or IFN-β treatment in the COS7 cells (Fig. 2D), indicating that COS7 cells are equipped with an intact ISG15-conjugation system. For strong induction of ISGylation, cells were transfected with expression vectors for ISGylation enzymes, along with Flag-HCV-NS5A. Total cellular lysates were subjected to immunoprecipitation using an anti-Flag Ab, followed by immunoblot analysis using an anti-Myc Ab to detect ISGylated HCV-NS5A. In the cells overexpressing Myc-NS5A/NS5B/E1/E2, slowly migrating forms of ISGylated Flag-HCV-NS5A were clearly visible (Fig. 2E). In contrast, ISG15 conjugation to other HCV viral proteins, such as HCV-NS3 or -NS5B, was not detected in the transfected COS-7 cells (Fig. 2F), nor in the cells stimulated with poly-IC or IFN-β (Supplemental Fig. 2).

Lys379 in the polyproline-cluster motif of HCV-NS5A is the target site for ISG15 conjugation

In the earlier study using subgenomic replicon systems, HCV-NS5A was suggested to be involved in HCV RNA replication (8, 35–38). Because HCV-NS5A contains multiple domains with specific functions, we attempted to determine the responsible domain(s) for regulation by ISG15 conjugation. We constructed deletion mutants of HCV-NS5A and examined ISGylation effect on the protein level. Protein levels of the deletion mutant NS5A (I del) lacking an N-terminal amphipathic helix (Fig. 3A) was similarly reduced by cotransfection with ISGylation components. In contrast, deletion mutant of NS5A (IV del) lacking a carboxy-terminal domain with poly-proline cluster motifs was not affected by the coexpression of the ISGylation components, indicating that ISGylation site(s) might reside in the C-terminal regions of HCV-NS5A. To test this possibility, we conducted immunoprecipitation assay of NS5A (IV del) protein in the ISG15/E1/E2-cotransfected COS7 cells (Fig. 3B). As predicted, ISGylation of NS5A (IV del) mutant was not observed, suggesting that the C-terminal domain that spans aa 309–447 of the HCV-NS5A is required for regulation by ISG15 conjugation. To identify the responsible site(s) for ISG15 conjugation in HCV-NS5A, we subsequently constructed various KR mutants of NS5A, in which lysine residues in the C-terminal domain have been individually substituted for arginine residue. Five lysine residues (K309, K331, K349, K359, and K379) were in the C-terminal domain with poly-proline cluster motifs was not affected by the coexpression of the ISGylation components, indicating that ISGylation site(s) might reside in the C-terminal regions of HCV-NS5A. To test this possibility, we conducted immunoprecipitation assay of NS5A (IV del) protein in the ISG15/E1/E2-cotransfected COS7 cells (Fig. 3B). As predicted, ISGylation of NS5A (IV del) mutant was not observed, suggesting that the C-terminal domain that spans aa 309–447 of the HCV-NS5A is required for regulation by ISG15 conjugation. To identify the responsible site(s) for ISG15 conjugation in HCV-NS5A, we subsequently constructed various KR mutants of NS5A, in which lysine residues in the C-terminal domain have been individually substituted for arginine residue. Five lysine residues (K309, K331, K349, K359, and K379) were in the C-terminal domain of HCV-NS5A, and lysines on 309, 331, 359, and 379 were found to be well conserved in the

![FIGURE 2](http://example.com/figure2.png)

**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-NS15–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-5-15 and 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). 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, strongly inhibited ISGylation of HCV-NS5A (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, strongly inhibited ISGylation of HCV-NS5A (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.

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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 hypersensitive 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 ISGylation may play a critical role in regulating anti-HCV replication.

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 unconjugated 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.

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. 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 and IFN-β, exhibited improved efficacy in the clearance of HCV in the HCV subgenomic replicon cells. It would be interesting to see whether this combined therapy could limit HCV replication efficiently in primates. For this purpose, future studies aimed at the effective delivery of ISG15-conjugation system to the virus-infected regions in the liver are required.

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Disclosures
The authors have no financial conflicts of interest.

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