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NS5A-ISGylation via Lysine 26 Has a Critical Role for Efficient Propagation of Hepatitis C Virus Genotype 2a

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We previously reported that hepatitis C virus (HCV) NS5A (1b, Con1) protein accepts covalent ISG15 conjugation at specific lysine (Lys) residues (K44, K68, K166, K215 and K308), exhibiting proviral effects on HCV RNA replication. Here we investigated a role of NS5A-ISGylation via Lys residues in HCV propagation using HCV infectious clone. The alignment of amino acid sequences revealed that 5 Lys residues (K20, K26, K44, K139, and K166) of the 13 Lys residues within NS5A (genotype 2a, JFH1 strain) were conserved compared to those of HCV (genotype 1b, Con1 strain). The cell-based ISGylation assay revealed that the K26 residue in the amphipathic helix (AH) domain and the K139 residue in domain I of NS5A (2a, JFH1) had the potential to accept ISGylation. Use of the HCV replicon carrying luciferase gene revealed that the K26 residue but not K139 residue of NS5A (2a, JFH1) was important for HCV RNA replication. Furthermore, cell culture HCV revealed that the mutation with the K26 residue in combination with K139 or K166 on NS5A (2a, JFH1) resulted in complete abolishment of viral propagation, suggesting that the K26 residue collaborates with either the K139 residue or K166 residue for efficient HCV propagation. Taken together, these results suggest that HCV NS5A protein has the potential to accept ISGylation via specific Lys residues, involving efficient viral propagation in a genotype-specific manner.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent causing chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma and still remains a public health burden worldwide. For a long term, interferon (IFN)-based therapy has been used as a standard therapy for patients with chronic HCV infection. However, the IFN-based therapy is restrictive and can cure only approximately 50% of the patients, especially for the patients infected with genotype 1 strains (29, 33). Recent basic and clinical advances in HCV research have developed novel anti-HCV therapeutics, direct-acting antivirals (DAAs) therapy, and achieved sustained virological response rates over 95 % in patients with several HCV genotypes (7). Several approved DAAs targeted to nonstructural proteins, including NS3/4A protease, NS5A, and NS5B polymerase, are currently available for the treatment of chronic hepatitis C.

HCV is an enveloped, single-stranded positive-sense RNA virus classified into the Hepacivirus genus of the Flaviviridae family. HCV is divided into seven major genotypes (genotype 1-7) and dozens of subtypes. Among the HCV genotypes, genotypes 1 and 3 are the most prevalent globally, while the other genotypes are typically geographically restricted. HCV genotypes 1 and 2 are widely spread in Asia, with the subtypes 1b and 2a common in Japan (18). The HCV genome consists of 9.6-kb RNA encoding a single polyprotein which is processed by viral proteases and cellular signalases to produce three structural proteins (Core, E1, and E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (21). HCV replication occurs on the endoplasmic reticulum (ER)-derived double membrane vesicles associated with lipid droplet (LD) and requires the involvement of NS5A protein through the recruitment of several cellular host factors in the viral replication complexes (RCs) (15, 20, 21).

NS5A protein is a membrane-associated RNA-binding protein that is involved in HCV replication and virus assembly. Although NS5A protein does not possess any enzymatic motifs like NS3 and NS5B protein, NS5A protein plays an essential role in viral replication. NS5A is divided into three distinct domains (Domain I, II, and III) in addition to the N-terminal membrane anchor domain forming an amphipathic alpha-helix (AH). Domain I (DI) contains the binding site for daclatasvir, which is an NS5A inhibitor, and various host factors required for

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HCV RNA replication (9, 11). Domain II (DII) is involved in the viral replication (8, 23), while Domain III (DIII) is required for the process of viral release (2, 10, 12).

IFN-stimulated gene 15 (ISG15) is a ubiquitin-like protein that is induced by IFN production upon stimulation with viral and bacterial infection (28). ISG15 is covalently conjugated to the target substrate proteins via specific lysine (Lys) residues by three enzymes: E1 (UBE1L), E2 (UbcH8), and E3 ligase (HERC5). This process is called 'ISGylation' and is a post-translational protein modification that is similar to ubiquitylation. The function of ISG15 has been suggested to be antiviral function to several viruses (31). However, there were conflicting observations, suggesting that ISG15 has proviral function in HCV infection (1, 3, 4, 5, 22). We previously reported that HCV NS5A (1b, Con1) protein accepts ISGylation to promote HCV RNA replication through the recruitment of cyclophilin A (CypA), which is the critical host factor for HCV RNA replication (1).

In the present study, we sought to elucidate the role of NS5A-ISGylation in HCV genotype 2a replication. We demonstrate that the K26 residue in the AH domain and the K139 residue in DI of NS5A (2a, JFH1) have the potential to accept ISGylation. Furthermore, we demonstrate that the K26 residue in combination with the K139 residue or K166 residue is required for efficient HCV propagation.

MATERIALS AND METHODS

Cell culture and transfection

Huh-7.5 cells (kindly provided by Dr. C.M. Rice, The Rockefeller University, NY), which are highly permissive for the culture-adapted HCV of genotype 2a JFH1 strain propagation (25, 26). Huh-7.5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (High Glucose) with L-glutamine (Wako, Osaka, Japan) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Gibco, Grand Island, NY), and 10% heat-inactivated fetal bovine serum (Biowest, Nuaillé, France) at 37°C in a 5% CO_2 incubator.

Cells were transfected with plasmid DNA using FuGene 6 transfection reagents (Promega, Madison, WI). The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, JFH1 (14), was kindly provided by Dr. C.M. Rice. The HCV genome RNA was synthesized *in vitro* using pFL-J6/JFH1 as a template and was transfected into Huh-7.5 cells by electroporation. The virus produced in the culture supernatant was used for the titration of virus infectivity.

Plasmids

The cDNA fragment of NS5A (2a, JFH1) was inserted into the *Not*I site of pCAG-HA using the In-Fusion HD-Cloning kit (Clontech, Mountain View, CA). The HCV (2a, JFH1) reporter subgenomic replicon (SGR) plasmid encoding the firefly luciferase gene (pSGR-luc) was kindly provided by Dr. T. Wakita (NIID, Japan). The cDNA fragments of NS5A (2a, JFH1) with the Lys residue mutated to Arg residue were generated by the mutagenesis PCR method using pCAG-HA-NS5A, pSGR-luc, or pFL-J6/JFH1 as a template. The specific primers used for the PCR were as follows: sense primer (K26R), 5'-TGGCTGACCTCTAGATTGTTCCCC-3'; antisense primer (K26R), 5'-GGGGAACAATCTAGAGGTCAGCCA-3', sense primer (K44R), 5'-CAAAAGGGGTACAGGGGTGTGTGG-3'; antisense primer (K44R), 5'-CCACACACCCCTGTACCCCTTTTG-3', sense primer (K139R), 5'-ACTGACAATCTGAGAATTCCTTGC-3'; antisense primer (K139R), 5'-GCAAGGAATTCTCAGATTGTCAGT-3', sense primer (K166R), 5'-GCACCCACACCAAGGCCGTTTTC-3'; antisense primer (K166R), 5'-GAAAAACGGCCTTGGTGTGGGTGC-3'. The expression plasmid for pCAG-FLAG-ISG15 was previously described (19). The cDNA fragments encoding UBE1L and UbcH8, or HERC5 were cloned into the *Not*I/*Bgl*II or *Sma*I/*Kpn*I site of pCAG-MCS2, respectively using the In-Fusion HD-Cloning kit.

The various genotypes of HCV subgenomic replicon plasmids (SG-Feo) possessing a chimeric gene encoding the firefly luciferase and neomycin resistance gene, including H77c (L+8), S52 (AII), ED43 (VYG), and SA1 (SKIP), were kindly provided by Dr. C.M. Rice (24, 34). The insertions of NS5A with the Lys26 residue mutation (K26R) from each of HCV genotypes were generated by the mutagenesis PCR method using SG-Feo as a template. The specific primers used for the PCR were as follows: sense primer (H77c, K26R), 5'-TGGCTGAAAGCCAGGCTCATGCCA-3'; antisense primer (H77c, K26R), 5'-TGGCATGAGCCTGGCTTTCAGCCA-3', sense primer (S52, K26R), 5'-CTCTCTGCTAGGATTATGCCAGCA-3'; antisense primer (S52, K26R), 5'-TGCTGGCATAATCCTAGCAGAGAG-3', sense primer (ED43, K26R), 5'-ACGTGGCTAAAAGCCAGGTTGCTG-3'; antisense primer (ED43, K26R),

5'-CAGCAACCTGGCTTTTAGCCACGT-3', sense primer (SA1, K26R), 5'-TGGTTGCAGGCAAGACTCCTCCCG-3'; antisense primer (SA1, K26R), 5'-CGGGAGGAGTCTTGCCTGCAACCA-3'. The sequences of the inserts were extensively confirmed by sequencing (Eurofins Genomics).

Antibodies and reagents

The mouse monoclonal antibodies (mAbs) used in this study were anti-FLAG (M2) mAb (F-3165, Sigma-Aldrich, St. Louis, MO), anti-NS3 (MAB8691, Millipore), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mAb (MAB374, Millipore), and anti-Core mAb (2H9 clone, kindly provided by Dr. T. Wakita, NIID, Japan). The rabbit polyclonal antibodies (pAbs) used in this study were anti-HA pAb (H-6908, Sigma-Aldrich) and anti-NS5A pAb (2914-1 clone; kindly provided by Dr. Wakita). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibody (Cell Signaling Technology, Beverly, MAR), and HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) were used as secondary antibodies. CsA was purchased from Sigma-Aldrich.

Immunoprecipitation and immunoblot analysis

Cells were transfected with the plasmids using FuGene 6 (Promega), harvested at 48 h post-transfection, and suspended in 0.5 ml of RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.1% SDS, 1% NP-40, 0.5% deoxycholate (DOC), and protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were incubated for 2 h at 4°C and centrifuged at 20,400 × g for 30 min at 4°C (TOMY centrifuge MX-307, Rotor Rack AR015-SC24, TOMY, Tokyo). The supernatant was immunoprecipitated with protein G Sepharose 4 fast flow (GE Healthcare, Buckinghamshire, UK) and incubated with appropriate antibodies at 4°C overnight.

After being washed with the RIPA buffer five times, the samples were boiled in 15 μl of sodium dodecyl sulfate (SDS) sample buffer and then subjected to SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF) (Millipore). The membranes were blocked with Tris-buffered saline containing 20 mM Tris-HCl (pH 7.6), 135 mM NaCl, and 0.05% Tween 20 (TBST) containing 5% skim milk at room temperature for 2 h and incubated with corresponding antibodies. The membranes were then incubated with HRP-conjugated secondary antibody at room temperature for 2 h. The immune complexes and cell lysates were visualized with ECL Western blotting detection reagents (GE Healthcare) and detected by the LAS-4000 image analyzer system (GE Healthcare). The band intensities were quantified using ImageQuant TL software (ver.7.0).

Quantification of extracellular core protein

The HCV core protein in the culture supernatants was quantified by highly sensitive enzyme immunoassay using the Ortho HCV core antigen ELISA test (Ortho-Clinical Diagnostics, Raritan, NJ).

HCV infectivity assay

Briefly, culture supernatants were serially diluted 10-fold and used to infect duplicate 24-well cultures of Huh-7.5 cells. At 24 h post-inoculation, the cultures were overlaid with complete DMEM containing a final concentration of 0.25% methylcellulose (Sigma-Aldrich). At 72 h of incubation, the cells were fixed in 4% paraformaldehyde (PFA) and immunohistochemically stained using anti-Core mAb (2H9 clone). The infectious HCV titers were determined based on the focus-forming units (FFU)/ml (32).

***In vitro* transcription of HCV RNA, electroporation into the cells, and reporter analysis**

The HCV replicon plasmid pSGR (2a, JFH1)-Luc, and pFL-J6/JFH1 plasmids were digested with *Xba*I, respectively, and transcribed *in vitro* using a MEGAscript T7 kit (Ambion, Austin, TX). Then, 10 μg of *in vitro*-transcribed HCV RNA was electroporated at 270 V and 960 μF by a GenePulser Xcells™ (Bio-Rad, Hercules, CA) into 4×10^6 Huh-7.5 cells treated with BTXpress buffer (BTX, Holliston, MA). The electroporated cells were seeded into 24-well plates and harvested at the indicated time points, and luciferase activity was determined in triplicate using a GloMax™ 96 microplate luminometer (Promega). The luciferase activity at 4 h after electroporation was used for normalization to account for the varying transduction efficiency of HCV RNA.

Statistics

Results are expressed as the mean ± standard error. The statistical significance was determined by Dunnett's test for the results shown in Fig. 3A, 3B, 4A, and 4B. P-values <0.05 was considered significant.

RESULTS

The Lys residues K26 and K139 on NS5A from genotype 2a are acceptor sites for ISGylation

The alignment of amino acid sequences revealed that 5 Lys residues (K20, K26, K44, K139, and K166) of the 13 Lys residues within NS5A (2a, JFH1) are conserved compared to those of genotype 1b (Con1) (Fig. 1A). In contrast, the K308 residue within domain II (DII) of NS5A (1b, Con1), which is important for both NS5A-ISGylation and HCV RNA replication (1), is not conserved in genotype 2a due to lack of Lys residues in DII of NS5A (2a, JFH1) (Fig. 1A). On the other hand, three Lys residues, including K20 and K26 in the amphipathic helix (AH) domain and K139 in domain I (DI), are well conserved among the HCV genotypes examined in this study (Fig. 1A). These results imply the possibility that there are distinct roles of Lys residues in NS5A-ISGylation to regulate HCV RNA replication between genotype 1b and 2a.

Next, to identify the acceptor sites for ISGylation on NS5A (2a, JFH1) protein, we constructed a series of NS5A mutants, containing a point mutation of Arginine (R) at a corresponding Lys (K) (K to R mutant series). We then co-transfected HEK293T cells with pCAG-HA-NS5A (2a) or its Lys (K) mutants and pCAG-FLAG-ISG15 together with E1 (UBE1L), E2 (UbcH8), and E3 (HERC5), followed by immunoprecipitation with anti-HA antibody and detection with anti-NS5A (2a, JFH1) specific rabbit pAb. The immunoprecipitation analysis coupled with immunoblotting revealed that the slowly migrating forms of HA-NS5A (indicated as NS5A-ISGylation) were clearly detected in the co-transfected cells compared to the cells expressed with HA-NS5A alone (Fig. 1B, upper panel, lanes 1 and 2). On the other hand, ISGylation of NS5A (2a) protein was markedly reduced by the mutations of K26R and K139R but not K44R, K166R compared to HA-NS5A (2a) (WT) (Fig. 1B, upper panel, lanes 2 to 6). These results suggest that K26 and K139 residues are acceptor sites for ISGylation on NS5A (2a) protein.

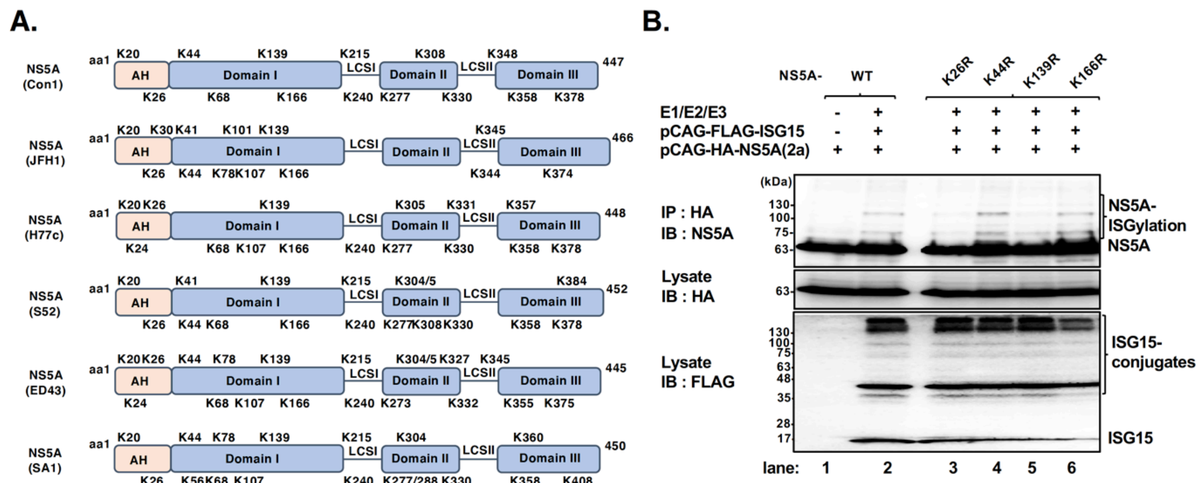

Fig. 1. Schematic diagrams of the distribution of Lys residues on NS5A protein from HCV genotypes and the cell-based ISGylation assay for HA-NS5A (2a) WT and mutants.

A: Schematic diagrams of the distribution of Lys (K) residues on NS5A protein from HCV genotypes (1b: Con1, 1a: H77c, 2a: JFH1, 3a: S52, 4a: ED43, 5a: SA1). AH: amphipathic helix. **B:** The expression vector encoding HA-NS5A (2a, JFH1) or HA-NS5A mutants, in which the K26, K44, K139, and K166 residues were mutated to Arg (R) (indicated as K26R, K44R, K139R, and K166), was co-transfected with FLAG-ISG15 together with E1 (UBE1L), E2 (UbcH8), and E3 ligase (HERC5) in HEK293T cells, followed by immunoprecipitation with anti-HA rabbit pAb and detection with anti-NS5A specific rabbit pAb. Input samples (indicate as Lysate) were detected with anti-HA rabbit pAb and anti-FLAG mouse mAb.

The K26 residue on NS5A from genotype 2a is involved in the positive regulation of HCV replication

To determine the role of the K26 and K139 residues on NS5A (2a, JFH1) in HCV RNA replication, we constructed plasmids expressing firefly luciferase (F-luc) reporter subgenomic RNA replicons (indicated as SGR-luc RNA), with a point mutation of NS5A that replaces one of the 13 Lys residues with Arg (Fig. 2A). We then electroporated *in vitro*-transcribed RNAs into Huh-7.5 cells to evaluate the viral replication by measuring the luciferase activity. As shown in Fig. 2B, the electroporation of SGR-Luc RNA with the mutation of K26R

but not K139R exhibited undetectable luciferase activities at 48 h compared to that of WT. The luciferase activities of the other mutants, except for the K44R, were comparable to that of the WT (Fig. 2B). Immunoblot analysis revealed undetectable NS3 protein levels in the cells electroporated with SGR-Luc RNA with the mutations of K26R and K44R on NS5A protein and the polymerase-inactive mutant (GND) on NS5B protein (Fig. 2C) (16). These results suggest that the K26 residue is involved in the positive regulation of HCV replication via the NS5A-ISGylation, while the K44 residue may contribute to HCV RNA replication in the NS5A-ISGylation-independent manner.

The K26 residue on NS5A is well conserved among HCV genotypes: 1a (H77c), 1b (Con1), 2a (JFH1), 3a (S52), 4a (ED43), and 5a (SA1) (Fig. 1A). The K26 residue on NS5A (1b, Con1) has no impact on HCV RNA replication (1). To test whether the K26 residue on NS5A protein is involved in HCV RNA replication in other HCV genotypes, we electroporated HCV-SGR-Luc RNA with the mutation of K26R from other HCV genotypes into Huh-7.5 cells to evaluate viral replication. Interestingly, the levels of luciferase activity in the cells electroporated with SGR-Luc RNA (K26R) from other HCV genotypes were comparable to those of WT (Fig. 2D). These results suggest that the K26 on NS5A (2a, JFH1) plays an important role in HCV RNA replication in genotype 2a-specific manner.

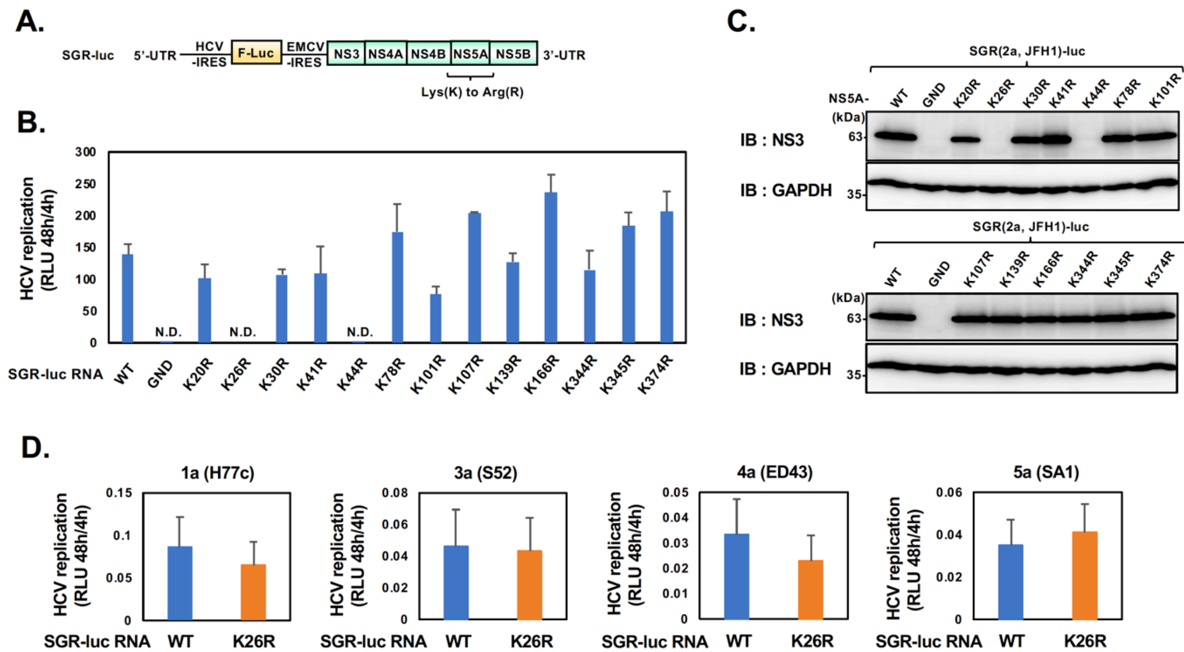

Fig. 2. HCV replication assays using subgenomic replicon RNA possessing the firefly luciferase gene.
A: Schematic diagram of HCV reporter subgenomic replicon (SGR-luc) RNA comprising NS3 to NS5B of the genotype 2a (JFH1). HCV reporter replicon RNA possesses the firefly luciferase (F-Luc) gene. IRES: internal ribosomal entry site, UTR: untranslated region, EMCV: encephalomyocarditis virus. **B:** Huh-7.5 cells were electroporated with HCV-SGR-luc RNA or the replicon possessing NS5A Lys mutants in which a Lys (K) is replaced with Arg (R) (indicated as K to R mutant series). The luciferase activity was measured at 48 h after electroporation. The luciferase activity measured at 4 h after electroporation was used to normalize for the input RNA. **C:** The cell lysates from luciferase assay were subjected to immunoblotting analysis with anti-NS3 mouse mAb or anti-GAPDH mouse mAb. **D:** Huh-7.5 cells were electroporated with either HCV SGR-luc from HCV genotypes or their mutant replicon possessing NS5A K26 mutant, in which a Lys (K) is replaced with Arg (R) (indicated as K26R). The luciferase activity was measured at 48 h after electroporation. The luciferase activity measured at 4 h after electroporation was used to normalize for the input RNA.

The K26 residue on NS5A from genotype 2a is involved in the positive regulation of HCV RNA replication

To further examine the role of the K26 residue on NS5A (2a, JFH1) in HCV propagation, we constructed an infectious HCV RNA clone (indicated as J6/JFH1 RNA) carrying the K26R mutation on NS5A protein. We electroporated *in vitro*-transcribed infectious HCV RNA into Huh-7.5 cells to evaluate viral propagation by measuring several virological parameters. The extracellular core protein levels were significantly decreased in the cells electroporated with J6/JFH1 RNA carrying K26R mutation compared to those in the cells

electroporated with WT and other Lys mutations, including K44R, K139R, and K166R at 5 days post-electroporation (Fig. 3A). Consistent with this result, in the cells electroporated with J6/JFH1 RNA with K26R mutation, we found marked reduction in the extracellular virus titers (Fig. 3B) and the intracellular core protein levels (Fig. 3C). Taken together, these results suggest that the K26 residue on NS5A (2a, JFH1) plays an important role in HCV propagation.

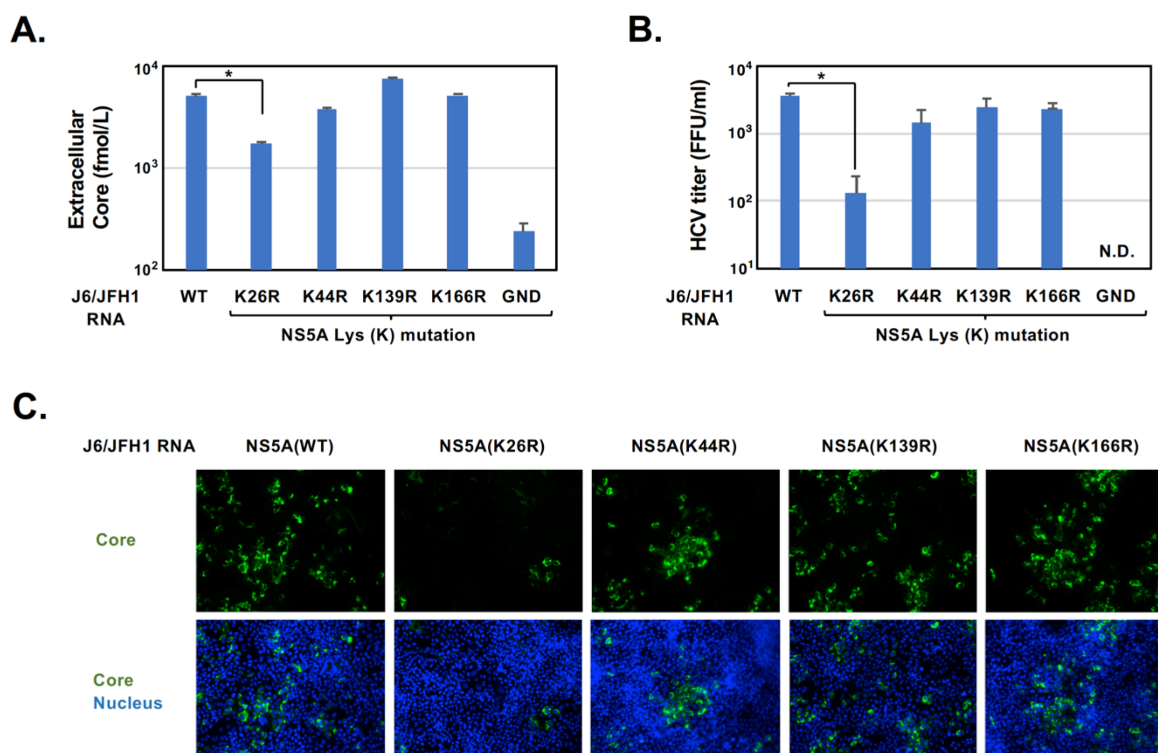

Fig. 3. The amounts of extracellular core protein, the infectious HCV titers, and intracellular core protein levels in Huh-7.5 cells electroporated with HCV J6/JFH1 RNA wild-type and NS5A Lys mutants.

HCV J6/JFH1 RNAs derived from WT, a replication-defective mutant (GND), or a series of NS5A Lys (K) mutants (indicated as K26R, K44R, K139R, and K166R) were electroporated into Huh-7.5 cells. At 5 days post-electroporation, supernatants were collected and subjected to measure the various virological parameters. The supernatants were inoculated to naïve Huh7.5 cells, and the amounts of extracellular core protein (A), the infectious HCV titer (B), and intracellular core protein levels (C) were determined at 3 days post-inoculation using core ELISA, a focus-forming assay (FFA) and an indirect immunofluorescent analysis, respectively. Results are the mean values of triplicates ± S.E. *$p<0.05$ vs. the results for cells transduced with J6/JFH1 WT RNAs. N.D.: Not detected.

The Lys residues K26, K139, and K166 on NS5A from genotype 2a have an essential role in HCV propagation

Because a single mutation of the K26 residue on NS5A (2a, JFH1) resulted in the decrease of HCV propagation (Fig. 3), we sought to determine the effect of other Lys mutations in combination with K26R mutation on HCV propagation. We constructed an infectious HCV RNA clone (J6/JFH1 RNA) with K26R mutation in combination with the each of K44R, K139R, and K166R mutation (indicated as K26R/K44R, K26R/K139R, and K26R/K166R). Consistently, we observed reduction of the extracellular core protein levels in cells electroporated with J6/JFH1 RNA with K26R mutation compared to that of WT (Fig. 4A). Importantly, the cells electroporated with J6/JFH1 RNA with both K26R/K139R and K26R/K166R mutations exhibited undetectable extracellular core protein levels (Fig. 4A). On the other hand, the extracellular core protein levels in the cells electroporated with J6/JFH1 RNA with K26R/K44R mutation was comparable to that of K26R mutant (Fig. 4A). In consistent with this result, in the cells electroporated with J6/JFH1 RNA with both K26R/K139R and K26R/K166R mutations but not K26R/K44R mutation, we observed undetectable extracellular virus titers

(Fig. 4B) and intracellular core protein levels (Fig. 4C). Taken together, these results suggest that the K26 residue may collaborate with the K139 or K166 residues to facilitate efficient HCV propagation.

Collectively, these results suggest that HCV NS5A (2a, JFH1) protein has the potential to accept ISG15-conjugation via the specific Lys residues, leading to the facilitation of HCV propagation.

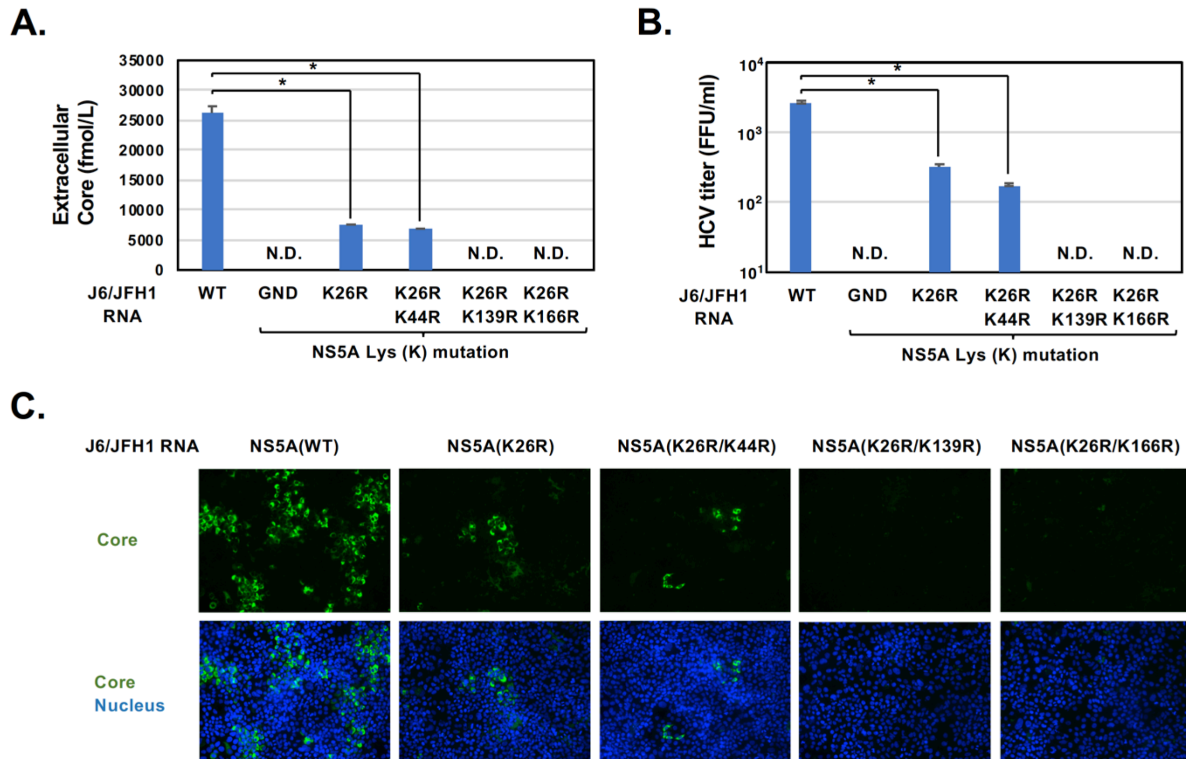

Fig. 4. The amounts of extracellular core protein, the infectious HCV titers, and intracellular core protein levels in Huh-7.5 cells electroporated with HCV J6/JFH1 RNA wild-type and NS5A Lys double mutants.

HCV J6/JFH1 RNAs derived from WT, a replication-defective mutant (GND), or a series of NS5A Lys (K) mutants (indicated as K26R, K26R/K44R, K26R/K139R, and K26R/K166R) were electroporated into Huh-7.5 cells. At 5 days post-electroporation, supernatants were collected and subjected to measure the various virological parameters. The supernatants were inoculated to naïve Huh7.5 cells, and the amounts of extracellular core protein (A), the infectious HCV titer (B), and intracellular core protein levels (C) were determined at 3 days post-inoculation by core ELISA, a focus-forming assay (FFA) and an indirect immunofluorescent analysis, respectively. Results are the mean values of triplicates ± S.E. *$p<0.05$ vs. the results for cells transduced with J6/JFH1 WT RNAs. N.D.: Not detected.

DISCUSSION

We previously reported that HCV NS5A (1b, Con1) protein accepts ISGylation to promote HCV RNA replication through the recruitment of cyclophilin A (CypA), which is the critical host factor for HCV RNA replication (1). In the present study, we examined the role of ISGylation in HCV genotype 2a (J6/JFH1). We demonstrated that the K26 residue in the AH domain of NS5A (2a, JFH1) plays an important role in NS5A-ISGylation to regulate efficient HCV propagation. The present results suggest the mechanistic differences between genotype 1b and genotype 2a. These results suggest the possibility that NS5A-ISGylation has distinct roles in efficient viral replication between genotype 1b and 2a of HCV. Although the detailed contributions of the individual Lys residues on NS5A protein from each HCV genotype remain to be elucidated, the present study is the first to demonstrate the involvement of NS5A-ISGylation via the specific Lys residues on HCV 2a propagation using an infectious HCV clone.

Interestingly, the residue K26 in the AH domain of NS5A from several HCV genotypes except genotype 2a did not show any impact on HCV RNA replication (Fig. 2D), although it was well conserved among HCV genotypes (Fig. 1A). We thus speculate that the Lys residue (e.g., K20) in the AH domain of NS5A may

compensate for the role of the K26 residue in genotype 2a of HCV. Further studies are needed to clarify the detailed contribution of the K20 residue on NS5A from other HCV genotypes.

Crystal structure of NS5A DI was analyzed (13, 17, 27), while other structures, including DII and DIII, remain to be analyzed. The N-terminal AH domain of NS5A protein are believed to mediate its membrane anchoring (6). The recent report by Zhang and colleagues suggests that the AH domain of NS5A protein may be involved in the facilitation of both the viral polyprotein cleavage and the viral replicase assembly formation (30). These results suggest that the AH domain of NS5A has potential as a functional regulatory domain for efficient HCV propagation. Our results indicate that the mutation of the K26 residue in the AH-domain results in reduction of HCV propagation (Fig. 2B, Fig. 3 and Fig. 4). We thus speculate that the ISGylated NS5A within the AH domain may facilitate both viral polyprotein cleavage and viral replicase assembly formation.

Additionally, we found that the K26 residue may collaborate with the K139 residue or K166 residue for efficient HCV propagation (Fig. 4). The Lysine residue K139 but not K166 exhibited the involvement of NS5A-ISGylation, which is similar to the K26 residue (Fig. 1B). These results may imply the role of ISGylation-dependent and -independent manners in the collaboration of their Lys residues on HCV propagation. Further studies are needed to clarify the intermolecular interactions of the K26 residue together with K139 and K166 residues for efficient HCV propagation.

There was discrepancy in the role of the K44 residue between HCV subgenomic replicon and cell culture HCV (Fig. 2B, Fig. 3, and Fig. 4). We speculate that the role of the K44 residue in the cell culture HCV might be compensated by another lysine residue, as we discussed the role of the K26 residue in HCV genotypes.

In summary, using an HCV infectious clone, we demonstrated that NS5A (2a, JFH1) protein accepts ISGylation via the K26 residue in AH domain and participates in the positive regulation of HCV propagation in genotype 2a-specific manner. The targeting of the ISGylation machinery may lead to development of novel therapeutics for HCV infection.

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AUTHOR CONTRIBUTIONS

R.G.B., T.A., and I.S. conceived and designed the experiments. R.G.B., Y.S. and T.A. carried out most of the experiments. C.M., and L.D. assisted the constructions and the data analysis. T.A. and I.S. wrote the manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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