Note

Unconjugated interferon-stimulated gene 15 specifically interacts with the hepatitis C virus NS5A protein via domain I

Short running title: Unconjugated ISG15-HCV NS5A interaction

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ABSTRACT

Interferon-stimulated gene 15 (ISG15) is a ubiquitin-like protein induced by type I interferon. Although several groups have reported ISGylation of the HCV NS5A protein, it is still unclear whether ISGylation of NS5A has anti-viral or pro-viral effects in HCV infection. In the present study, we examined the role of ISGylation-independent, unconjugated ISG15 in HCV infection. Immunoprecipitation analyses revealed that ISG15 specifically interacted with NS5A domain I. The ISG15 mutants lacking a C-terminal glycine residue essential for ISGylation still interacted with NS5A protein. Taken together, these results suggest that unconjugated ISG15 may affect the functions of HCV NS5A through protein-protein interaction.

Keywords: HCV, ISG15, NS5A

List of Abbreviations: Apolipoprotein A1 (ApoA1), Chikungunya virus (CHIKV), FK506-binding protein (FKBP8), Heat shock protein 27 (HSP27), Hepatitis C virus (HCV), Hepatocyte nuclear factor 1α (HNF-1α), Interferon (IFN), Interferon-stimulated gene 15 (ISG15), 2'-5'oligoadenylate synthase (2'-5'OAS), ovarian tumor protein 7B (OTUD7B), Proximity ligation assay (PLA), Virus-like particle (VLP).

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MAIN TEXT

Hepatitis C virus (HCV) is a major cause of chronic liver disease affecting 130-170 million people worldwide (1). Chronic HCV infection causes steatosis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (2). HCV belongs to the Hepacivirus genus of the Flaviviridae family. The positive sense single-strand RNA genome encodes a polyprotein that is cleaved by cellular signalases and viral proteases into 10 viral proteins: three structural proteins (core, E1 and E2), the p7 polypeptide, and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (3). Unlike other HCV non-structural proteins such as viral protease or polymerase, HCV NS5A lacks any enzymatic motif. Instead, it has been well established that NS5A is an essential viral component for efficient viral replication through the recruitment and interaction with various cellular host factors (4).

The interferon-stimulated gene 15 (ISG15) is a type I interferon (IFN)-inducible gene and a ubiquitin-like protein containing two ubiquitin-like domains (5). Because the modification of protein by ISG15 is similar to that by ubiquitylation, protein conjugation by ISG15 is termed ISGylation. ISGylation requires three distinct enzymes: an ATP-dependent activating enzyme for ISG15 (E1, UbE1L), an ISG15-specific carrier protein/conjugating enzyme (E2, UbcH8) and several ISG15-specific conjugating enzymes (E3, Herc5). 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 (6). In addition to existing in its conjugated form, ISG15 is present in an unconjugated form intracellularly and is also released into the extracellular space (7).

HCV infection triggers intrahepatic synthesis of several ISGs in patients (8, 9). ISG15 is one of the ISGs which are highly induced by HCV infection (10). ISG15 has

already been shown to function as an antiviral factor against numerous virus infections, but to act as a proviral factor in HCV infection (11-14). Conversely, other groups have reported that ISG15 mediates antiviral activity against HCV infection, and that this activity may occur through the proteasomal degradation pathway and in a Herc5-independent manner (15, 16). Therefore, the potential roles of ISG15 in HCV infection are still under debate. Evidence is accumulating that unconjugated ISG15 may modulate viral infection independent of ISGylation (17-19). In the present study, we attempted to gain further insight into the role of ISGylation-independent, unconjugated ISG15 in HCV infection.

First, we sought to determine whether ISG15 physically interacts with any HCV proteins in Huh-7 cells. For this purpose, Huh-7 cells were co-expressed with a series of myc-His_6-tagged HCV proteins together with HA-ISG15 (Fig. 1). Immunoprecipitation analysis revealed that NS5A-myc-His_6 was co-immunoprecipitated with HA-ISG15 (Fig. 1a, upper panel, lane 3). On the other hand, FLAG-ISG15 was co-immunoprecipitated with HA-NS5A (Fig. 1b, upper panel, lane 3). Although only NS3-myc-His_6 was co-immunoprecipitated with HA-ISG15 (Fig. 1a, upper panel, lane 5), HA-NS3 was not co-immunoprecipitated with FLAG-ISG15 (Fig. 1b, upper panel, lane 5) when NS3 was expressed as NS3/4A in the cells (Fig. 1b). NS4A is known to be responsible for the appropriate subcellular localization of the NS3-4A complex at the organelle's membranes (20). Thus, we concluded that NS3 may not interact with ISG15 in the proper subcellular localization. In addition, neither myc-His_6-tagged NS4B, NS5B or p7 protein was co-immunoprecipitated with HA-ISG15 (Fig.1a, upper panel, lane 6; Fig. 1c, upper panel, lane 5; Fig. 1e, upper panel, lane 3). As shown in Fig. 1d, the HCV core protein was not co-immunoprecipitated with HA-ISG15 (Fig. 1d, upper

panel, lane 3). These results suggest that ISG15 specifically interacts with the HCV NS5A protein.

To determine whether endogenous ISG15 interacts with HCV NS5A in HCV-replicating cells, the HCV subgenomic replicon cells, Huh 9-13 (21), were treated with 1.5 x 10^3 IU/mL IFN-α (Sigma-Aldrich, St. Louis, MO) for 18 hr. Endogenous ISG15 was co-immunoprecipitated with NS5A protein using anti-NS5A monoclonal antibody (mAb) but not with control mouse IgG (Fig. 1f, top panel, lanes 4 and 3). These results suggest that endogenous ISG15 indeed interacts with the HCV NS5A protein expressed from HCV-replicating cells.

To determine whether the interaction between unconjugated ISG15 and HCV NS5A protein is ISGylation-independent, we constructed ISG15 mutants with amino acid substitutions in the C-terminal LRLRGG motif (mutated from LRLRGG to LRLRAA, referred to as ISG15 G156/157A) or deleting LRLRGG motif (referred to as ISG15 Δ152-157) (Fig. 2a). These two ISG15 mutants are incapable of forming ISG15-conjugates (22).

To determine whether these two ISG15 mutants can interact with the NS5A protein, HA-NS5A was co-expressed with either FLAG-ISG15, FLAG-ISG15 Δ152-157, or FLAG-ISG15 G156/157A in Huh-7 cells, respectively. The cell extracts were subjected to immunoprecipitation with anti-FLAG mAb or anti-HA polyclonal antibody (pAb). As shown in Fig. 2b, HA-tagged NS5A was co-immunoprecipitated with FLAG-ISG15 Δ152-157 and FLAG-ISG15 G156/157A (Fig. 2b, top panel, lanes 3 and 4). These results suggest that unconjugated ISG15 interacts with HCV NS5A in an ISGylation-independent manner.

To examine the subcellular localization of ISG15 and NS5A using a confocal

microscope, the HCV 1b full-length replicon (RCYM1) (23) was expressed with a vector control, FLAG-ISG15, FLAG-ISG15 Δ152-157, or FLAG-ISG15 G156/157A, respectively. The immunofluorescence staining revealed that unconjugated ISG15 was colocalized with NS5A in the perinuclear region in HCV replicon cells (Fig. 2c, merged panels). Moreover, to verify the colocalization of ISG15 and NS5A, we performed an *in situ* proximity ligation assay (PLA) using Duolink In Situ PLA (Sigma-Aldrich) (24). Red fluorescent spots were observed in HCV replicon cells expressing FLAG-ISG15, FLAG-ISG15 G156/157A, or FLAG-ISG15 Δ152-157, whereas no signal was detected in the vector control (Fig. 2d). These results suggest that unconjugated ISG15 colocalizes with the HCV NS5A protein in an ISGylation-independent manner.

Finally, to determine the ISG15-binding site on NS5A protein, we used a series of NS5A-deletion mutants constructed previously (25, 26). The series of NS5A-deletion mutants were co-expressed with FLAG-ISG15 in Huh-7 cells (Fig. 3a). Immunoprecipitation analysis was performed with anti-FLAG antibody, followed by immunoblotting with anti-HA PAb, anti-Myc MAb, or anti-ISG15 MAb, respectively (Fig. 3b and 3c). Among the HA-tagged NS5A mutants shown in Fig. 3a, the N-terminal-deletion mutants lacking the region from aa 357 to 447, aa 250 to 447, or aa 214 to 447 were not co-immunoprecipitated with FLAG-tagged ISG15 (Fig. 3, upper panel, lanes 7-9), whereas other C-terminal-deletion mutants were co-immunoprecipitated with FLAG-tagged ISG15 (Fig 3b, upper panel, lanes 2-6). Co-immunoprecipitation analysis using the Myc-His_6-tagged NS5A 1-126, NS5A 1-146, or NS5A 1-447 revealed that these NS5A mutants were co-precipitated with FLAG-ISG15 (Fig. 3c, upper panel, lanes 6-8). These results suggest that the region from aa 1 to 126 within the domain-I of NS5A is responsible for the interaction with

unconjugated ISG15.

Taken together, these findings suggest that unconjugated ISG15 interacts with NS5A in transfected cells and in HCV-replicating cells. Moreover, the immunofluorescence staining and the *in situ* PLA assay also demonstrated the colocalization of unconjugated ISG15 with the HCV NS5A protein in an ISGylation-independent manner. We mapped the region ranging from aa 1 to 126 within the domain I of NS5A, which was the region found to be responsible for the interaction between unconjugated ISG15 and NS5A. As far as we know, this is the first report to show the potential role of unconjugated ISG15 in HCV infection.

After the discovery of ISG15, much effort was invested in studying ISGylation. Recently, however, it was reported that ISG15-/- mice are more susceptible to Chikungunya virus (CHIKV) infection, whereas UbE1L-/- mice display similar susceptibility to CHIKV as wild-type mice, suggesting a conjugation-independent role for ISG15 on the CHIKV infection (17). Other studies have also showed that an unconjugated ISG15 interacts with E3 ubiquitin ligase Nedd4 and blocks the Nedd4-mediated ubiquitylation of Ebola virus VP40 for the efficient viral particle release (18, 19) . These results may imply that viral infection is modulated through unconjugated ISG15. Our results raise the possibility that unconjugated ISG15 may have some roles in the HCV life cycle and in viral pathogenesis.

HCV NS5A is a proline-rich, hydrophilic phosphoprotein that functions as a key regulator of HCV RNA replication and assembly (27). NS5A can interact with a wide variety of host cell proteins, such as HNF-1α, ApoA1, 2’-5’OAS, La, HSP27, PKR, FKBP8, and OTUD7B (25, 26, 28-34). Here we demonstrated a specific interaction between unconjugated ISG15 and NS5A via the domain I of NS5A (Fig. 3). To elucidate

the physiological significance of the unconjugated ISG15-NS5A interaction, it will be necessary to examine whether unconjugated ISG15 is involved in the viral replication, assembly, or immune evasion from the host innate immune response. Our results suggest that unconjugated ISG15 as well as ISGylation forms of NS5A are present in HCV-replicating cells. Co-existence of unconjugated- and conjugated-ISG15 interactions with NS5A may affect versatile functions of NS5A protein in HCV replication and in HCV pathogenesis. Further analysis will be required to elucidate the biological significance of the interplay between unconjugated- and conjugated-ISG15.

Collectively, we provided evidence indicating that unconjugated ISG15 interacts with HCV NS5A in an ISGylation-independent manner. Understanding the role of unconjugated ISG15 in HCV infection may shed new light on the molecular mechanisms of the HCV life cycle and HCV pathogenesis.

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DISCLOSURE

The authors declare that they have no conflicting interests.

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FIGURE LEGENDS

Fig. 1. ISG15 specifically interacts with HCV NS5A protein.

HA-ISG15 was co-expressed with NS3-myc-His_6, NS4B-myc-His_6, or NS5A-myc-His_6 (a), NS5B-myc-His_6 (c), P7-myc-His_6 (e) or FLAG-Core (d) in Huh-7 cells, and subjected to immunoprecipitation (IP) and immunoblotting (IB) using the indicated antibodies. FLAG-ISG15 was co-expressed with HA-NS5A or HA-NS3/4A (b), and subjected to IP and IB using the indicated antibodies. (f) HCV subgenomic replicon cells (Con1, genotype 1b) were treated with $1.5x10^3$ IU/ml recombinant IFN-α for 18 hr, and then the cell lysates were subjected to IP with anti-NS5A mAb or control mouse IgG, followed by IB with anti-NS5A mAb or anti-ISG15 mAb, respectively.

Fig. 2. ISG15 interacts with HCV NS5A protein in an ISGylation-independent manner.

(a) Schematic representation of ISG15 and its mutants (indicated as ISG15 Δ152-157 and ISG15 G156/157A). UBL, Ubiquitin-like domain; UBL, LRLRGG, Leu-Arg-Leu-Arg-Gly-Gly. (b) HA-NS5A was co-expressed with FLAG-ISG15, FLAG-ISG15 Δ152-157, and FLAG-ISG15 G156/157A in Huh-7 cells, followed by immunoprecipitation (IP) with anti-FLAG mAb, coupled with immunoblotting (IB) with anti-HA rabbit pAb or anti-FLAG mAb, respectively. The arrow indicates co-immunoprecipitated HA-NS5A. The asterisk indicates a non-specific band. (c) HCV full-genome replicon cells (Con1, genotype 1b, termed as RCYM1) were transfected with pCAG-FLAG-ISG15, pCAG-FLAG-ISG15 Δ152-157, or pCAG-FLAG-ISG15 G156/157A. The cells were fixed, permeabilized, and stained with anti-FLAG rabbit pAb and anti-NS5A mouse mAb, followed by incubation with anti-rabbit Alexa Fluor®

488 and anti-mouse Alexa Fluor® 594, respectively. Nuclei were stained with Hoechst 33342. (d) *In situ* proximity ligation assay (PLA). HCV replicon RCYM1 cells were transfected with either FLAG-ISG15, FLAG-ISG15 Δ152-157, or FLAG-ISG15 G156/157A. Cells were fixed, permeabilized, and incubated with anti-NS5A mouse mAb and anti-FLAG rabbit pAb, followed by staining with PLA. The red fluorescent spots indicate the interactions with NS5A and ISG15 or mutant ISG15. Nuclei were stained with Hoechst 33342.

Fig. 3. Mapping of ISG15-binding domain on the NS5A protein.

(a) Schematic representation of the NS5A and deletion mutants. The N-terminal- and C-terminal-deletion mutants of NS5A are fused to the HA-tag (gray region, a to h) or myc-His_6-tag (lattice region, i and j), respectively. Closed boxes represent proteins that are bound specifically to the unconjugated ISG15 protein and open boxes represent those that are not bound. (b and c) FLAG-ISG15 was co-expressed with one of the above-described HA-NS5A-deletion mutants in Huh7 cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody coupled with immunoblotting (IB) with anti-HA rabbit pAb, anti-c-myc mouse mAb, or anti-ISG15 mouse mAb, respectively.

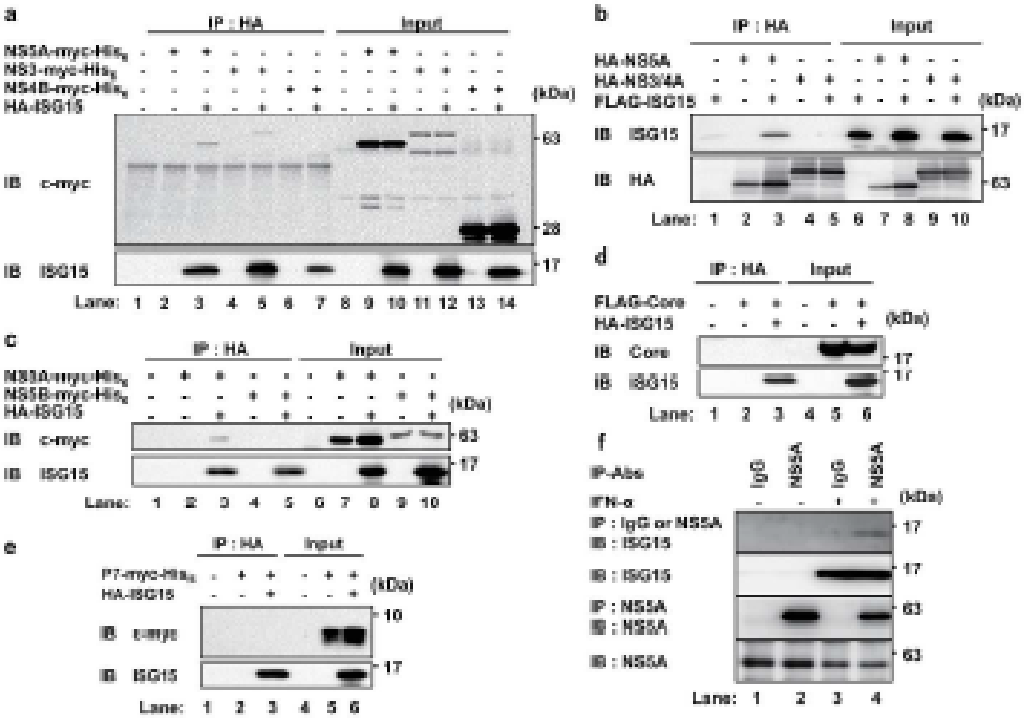

Figure 1. Minami et al.

Figure 1

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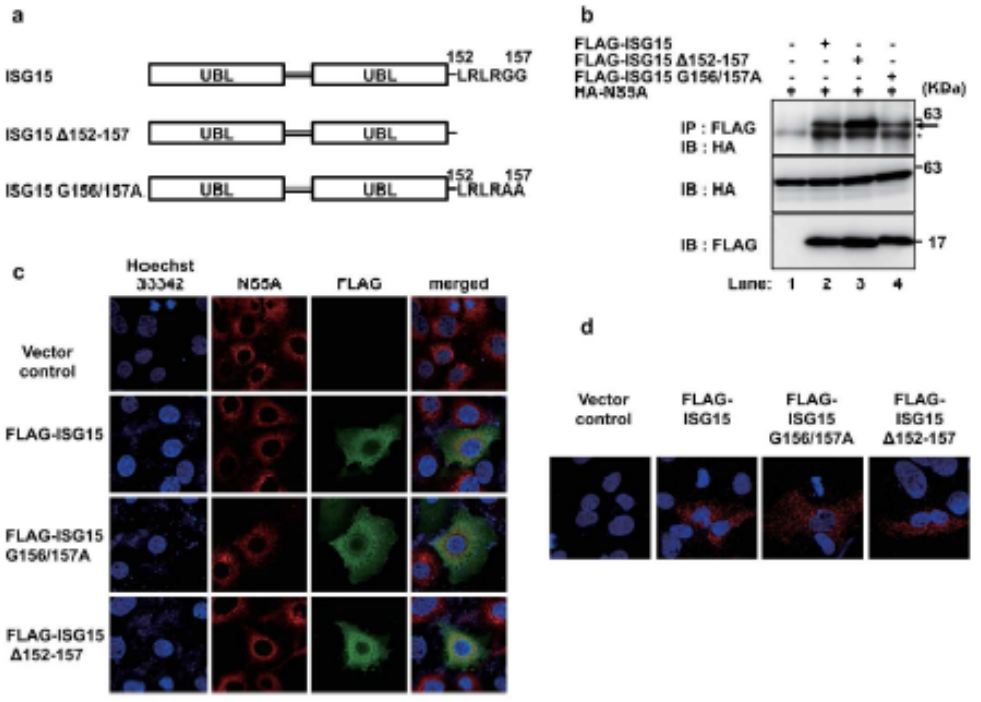

Figure 2. Minami et al.

Figure 2

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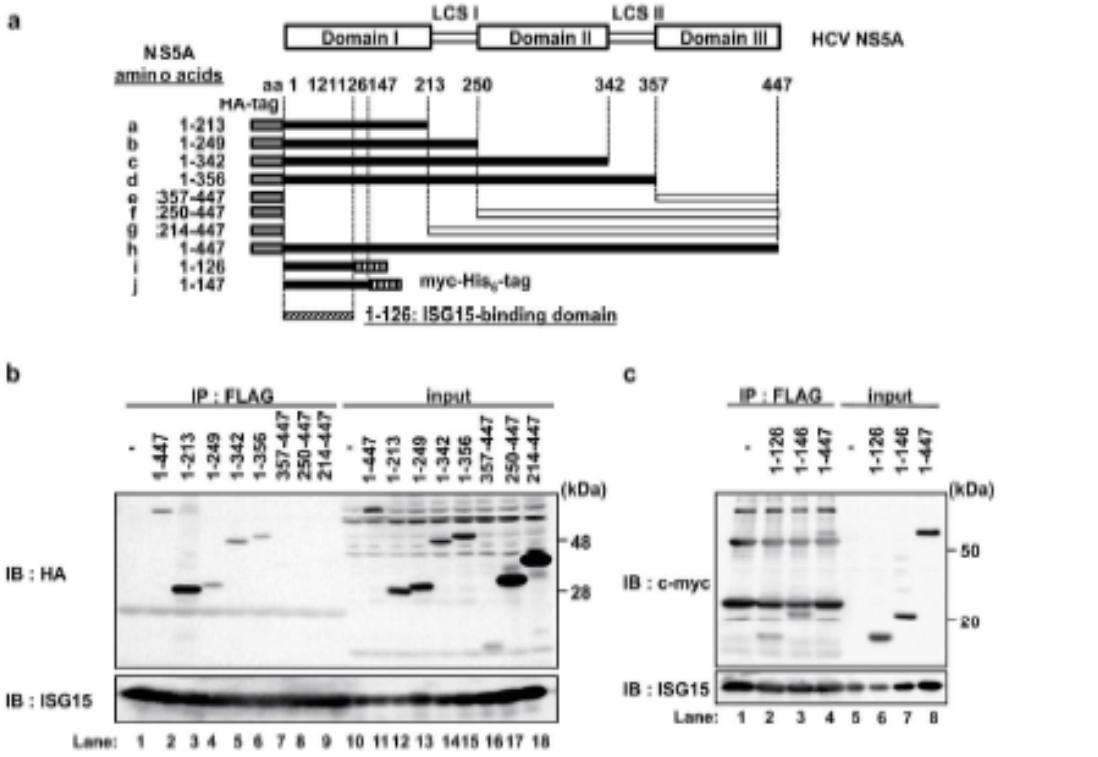

Figure 3. Minami et al.

Figure 3

192x145mm (300 x 300 DPI)