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ISG15 modification of ubiquitin E2 Ubc13 disrupts its ability to form thioester bond with ubiquitin

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Abstract

ISG15 was the first ubiquitin-like modifier to be identified. However, the function of ISG15 modification has been an enigma for many years. At present, no data are available about the function of ISGylation for any target. In this paper, we report the identification of Ubc13, which forms a unique ubiquitin-conjugating enzyme (Ubc) complex with ubiquitin enzyme variant Mms2 and generates atypical Lys63-linked ubiquitin conjugates, as one of the targets of ISG15 modification. Furthermore, we identify Lys92 as the only ISG15 modification site in Ubc13, which is the first report about the ISG15 modification site. Using the "covalent affinity" purification assay, we found that unmodified Ubc13 can bind to the ubiquitin–agarose, whereas ISGylated Ubc13 cannot. This result indicates that ISGylation of Ubc13 disrupts its ability to form thioester bond with ubiquitin.

Keywords: Ubc13; ISG15; ISGylation; Thioester bond

The expression of a ubiquitin-like protein ISG15 is upregulated by type I interferon (IFN) treatment, forming covalent conjugates to cellular proteins, a process similar to ubiquitin modification (ubiquitination or ubiquitylation) [1–3]. Although ISG15 was the first identified ubiquitin-like modifier [1], the function of ISG15 modification has been a mystery for many years [3–6]. Since the primary and tertiary structures of ISG15 are similar to those of ubiquitin, it is predicted that protein ISGylation will follow a mechanism similar to those of ubiquitin and the other ubls [7,8]. As with the ubiquitin system, there are a series of distinct enzymes involved in the process of protein ISGylation, including ISG15 activating enzyme (E1)-UBE1L [9], conjugating enzyme (E2)-Ubc8 [10,11], protein ligase (E3) [12], and ISG15 protease UBP43 [13,14]. In the ubiquitin system, an isopeptide bond is formed between the C-terminus of ubiquitin and the lysine ε-amino group of a target protein [15]. Until now, there have been no data about the ISG15 modification site or whether any consensus sequences exist for the recognition of ISG15 substrate proteins. Because of the high homology between the enzymes in ubiquitin system and ISG15 system, it is hypothesized that an isopeptide bond is formed between the C-terminus of ISG15 and the lysine ε-amino group of the target protein.

As a first step for understanding the ISGylation effect, it is important to identify the ISG15 modification targets. Recently, a significant number of ISG15-targeted proteins came to light starting with serpin2a [4], Jak1, Stat1, Erk1, and PLC-γ1 [5], followed by over 200 new proteins that function in diverse cellular pathways,

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including RNA splicing, chromatin remodeling, transcription, cytoskeletal organization, stress responses, and translation [16,17]. We also used a 293T-based conjugation system and identified the human ubiquitin-conjugating enzyme Ubc13 as one of the substrates of ISG15 modification. Ubc13 is a ubiquitin-conjugating enzyme, which can interact with a Ubc enzyme variant (Uev) Mms2 and form a heterodimeric ubiquitin-conjugating enzyme (Ubc) complex, which generates an atypical Lys63-linked ubiquitin chain [18,19]. Such conjugates are attached to specific targets that modulate the activity of various cellular processes including DNA repair [20], mitotic progression [21], and nuclear factor-κB signaling [22].

Here, we report Ubc13 as the target of ISG15 modification. More importantly, we find that Lys92 in Ubc13 is the only site for ISG15 modification. Using the affinity purification assay, we demonstrate that ISG15-modified Ubc13 cannot be purified by ubiquitin–agarose, indicating that ISGylation of Ubc13 disrupted its ability of forming the thioester bond with ubiquitin. To our knowledge, this is the first report about the ISG15 modification site and the effect on a specific target. The importance of Ubc13 in the various cellular processes makes it as an attractive target to further study the biological consequences of such modification.

Materials and methods

Plasmid construction and mutagenesis. Plasmids pCAGGS-6×His-mISG15, pFlagCMV2-Ubc8, and pCAGGS-HA-UBE1L have been described previously [10]. pcDNA3-Ubc8 and pcDNA3-UBE1L were constructed by subcloning cDNA of murine Ubc8 and human UBE1L into pcDNA3.1(+) vector. Human Ubc13 cDNA was subcloned into pcDNA3.0 containing a 5′-end HA tag sequence generating pcDNA3-HA-Ubc13 and subcloned into FlagCMV2 vector generating pFlagCMV2-Ubc13. Single-point mutation to convert lysine 92 to arginine (K92R) was introduced into wild-type (wt) Ubc13 using pcDNA3-HA-Ubc13 as template and was performed by using a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, resulting in pcDNA3-HA-Ubc13-K92R. Mutated construct was confirmed by DNA sequencing.

Cell culture and transfections. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and 2 mM L-glutamine (Invitrogen). For small-scale transfections, cells were grown in 6-well plates and transfected using PolyFect reagent (Qiagen, Germany). For large-scale transfections, cells were plated in 10-cm dishes and transfected using calcium phosphate precipitation as described previously [23].

Ni–NTA-agarose purification. Forty-eight hours post-transfection, cells were washed with PBS and lysed in PBS containing 1% NP-40 and 10 mM imidazole. Ni–NTA agarose beads (20 μl) (Qiagen) were then added to cell extracts (500 μg) and rotated at room temperature for 4 h. Precipitates were washed three times with PBS containing 1% NP-40 and 20 mM imidazole, and then boiled in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 100 mM DTT).

Mass spectrometry of mISG15 conjugates. Ni–NTA-purified protein was separated by 6–18% gradient SDS–PAGE gel. Individual gel bands were excised from SDS–PAGE gels and manipulated (washed, reduced with DTT, and alkylated by iodoacetamide, followed by overnight in-gel tryptic digestion) using a MassPrep robotic station (Bio-Rad). Tryptic peptides were separated on a PicoFrit fused silica column (New Objective) manually packed with Magic C_{18} stationary phase (Michrom) to provide a 75 μ ID nanocolumn approximately 15 cm long. The peptides were loaded directly on the column and eluted with a standard gradient of increasing acetonitrile containing 0.3% formic acid at a flow rate of approximately 250 nl/min. Peptides were ionized by electrospray ionization and analyzed using a QTOF-2 quadrupule time-of-flight mass spectrometer (Micromass) to obtain the peptide *m/z* values. Eluting peptides detected by the QTOF-2 mass spectrometer were subjected to collisional-induced dissociation (CID) to obtain MS/MS spectra for establishing the amino acid sequence and any post-translational modifications. MS and MS/MS peptide spectra were acquired using standard data-dependent scanning methods. The mass spectral data were searched against the NCBInr database using the Mascot database search engine (Matrix Science).

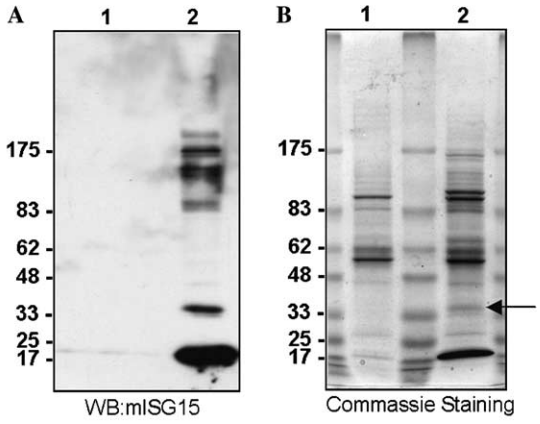
Immunoprecipitation (IP) and Western blot analyses. Forty-eight hours post-transfection, cells were lysed in modified RIPA buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, and 0.1% SDS). Immunocomplexes were precipitated with a mixture of protein-A– and -G–agarose (Amersham Biosciences, Piscataway, NJ). Immunoprecipitates were washed with the same buffer for three times and boiled in SDS–PAGE sample buffer. Antibodies against Flag (Sigma), HA (Covance, Denver, PA), and Ubc13 (Zymed, San Francisco, CA) were purchased from the respective manufacturers. Rabbit anti-mouse ISG15 polyclonal antibodies have been described previously [24]. Western blotting was performed as described previously [5].

Ubiquitin covalent purification analyses. Forty-eight hours post-transfection, cells were harvested and sonicated in the buffer which contains 50 mM Tris–HCl, pH 7.2, 10 mM $MgCl_2$, 5 mM ATP, and 0.2 mM DTT. Proteins (1000 μg) were incubated with ubiquitin–agarose (Boston Biochem, Cambridge, MA). Ubiquitin is covalently coupled to agarose beads via primary amines allowing for a fully functional C-terminus and can be used for affinity binding of Ubc13. After 4 h, the beads were washed with the same buffer for three times and boiled in SDS–PAGE sample buffer. The flowthrough of the purification is also harvested.

Results

Identification of Ubc13 as predicted ISGylated target by mass spectrometry

In order to understand the biological roles of ISGylation, we sought to identify proteins that were ISG15 targets using a proteomics approach. Taking advantage of the identification of Ubc8 as an ISG15 E2 enzyme, we have established an ISG15 conjugation system by transfecting 293T cells with His-ISG15, UBE1L, and UbcM8 [10]. ISG15 conjugates were enriched by Ni–NTA purification from this system, with proteins from untransfected 293T cells used as the control. The Ni–NTA-purified proteins were separated by SDS–PAGE followed by Coomassie blue staining (Fig. 1B). Compared to the 293T cells control lane (lane 1), there are additional bands in the lane of ISG15 system-transfected cells (lane 2). A duplicate set of samples were run in the same gel



C 1 MAGLPRRIIK ETQR**LLAEPV PGIK**AEPDES NARYFHVVIA GPQDSPFEGG
51 TFK**LELFLPE EYPMAAPK**VR FMTK**IYHPNV DK**LGR**ICLDI LKDK WSPALQ**
101 **IR**TVLLSIQA LLSAPNPDDP LANDVAEQWK **TNEAQAIETA R**AWTRLYAMN
151 NI

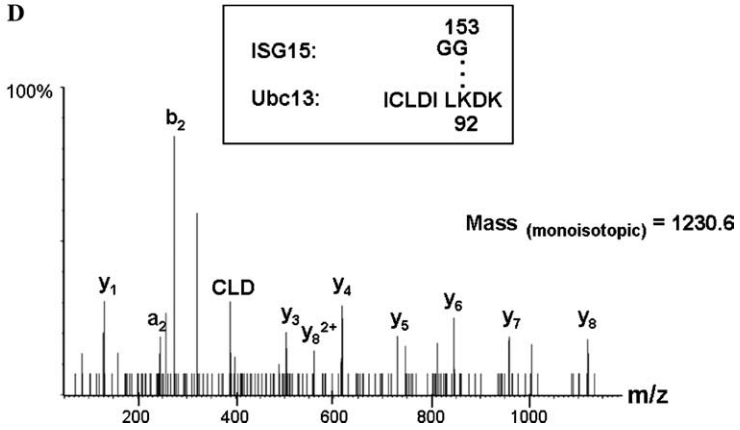


Fig. 1. Identification of Ubc13 as predicted ISGylated target by mass spectrometry. 293T cells were transfected with His-mISG15, UBE1L, and Ubc8, after 48 h, the cell lysates were purified by Ni–NTA pull-down as in Materials and methods. 293T cells without any transfection were used as the control (lane 1). The purified proteins were separated by 8–18% gradient gel and subjected to anti-ISG15 Western blot (A) and Coomassie blue staining (B). The arrow indicates a band, which showed strong signal of anti-ISG15 Western blot, was excised for mass spectrometric analysis. As a control, gel slices of the same molecular weight were excised from lane 1. Molecular weight markers are indicated in kilodaltons (kDa). (C) The identified peptide matched with Ubc13 is shown in bold. (D) MS/MS spectrum of the Ubc13 peptide 86–94 (linear sequence) with the ISG15 site of attachment. The observed peptide mass was 114.1 Da higher due to the additional Gly-Gly residues and the nearly complete y ion series (see text) supports the predicted isopeptide bond between the COOH-terminal glycine 153 of ISG15 and Lys92 of Ubc13 shown at the top of this figure.

and then transferred onto a PVDF membrane for an ISG15 Western blot (Fig. 1A). Western blots showed that the major ISG15 signals matched with the extra bands of the Coomassie blue staining, confirming that these extra bands are true ISG15 targets. One extra band of about 35 kDa showing a strong ISG15 signal was excised from the SDS–PAGE gel and subjected to mass spectrometry analysis. As expected, in this band, we detected several peptides of ISG15. At the same time, we detected six different peptides of ubiquitin E2 enzyme-Ubc13 (Fig. 1C, the matched peptides shown in bold). On the other hand, there were no peptides from Ubc13 and ISG15 detected in the corresponding band in the control lane. Ubc13 is a 152 amino acid protein with a calculated molecular weight of 17 kDa. The addition of a single ISG15 moiety (17 kDa) to Ubc13 would create a conjugate ISG15-Ubc13 protein with a total molecular weight of ~34 kDa, which is consistent with the approximate molecular weight observed on the gel for the analyzed band.

In the ubiquitin system, an isopeptide bond is formed between the C-terminus of ubiquitin and the lysine ε-amino group of a targeted protein. The similarity between the ubiquitin and ISG15 systems leads to the possibility that ISG15 is also conjugated to the lysine ε-amino group of the targeted protein in a similar fashion to ubiquitin. If this hypothesis were correct, a two-residue remnant (Gly-Gly) derived from the C-terminus

of ISG15 would remain covalently attached to the target lysine residue via the isopeptide bond following tryptic digestion [25]. In addition, the lysine attached to GG remnant from ISG15 would result in a missed proteolytic cleavage since the trypsin proteolysis does not occur at the modified lysines [25]. In the 35 kDa band described above, one of the six peptides derived from Ubc13, detected as a doubly charged ion at m/z 616.3, was observed to have a (monoisotopic) mass of 1230.6 Da. This mass is consistent with the Ubc13 peptide sequence 'ICLDILKDK' with a mass shift at the lysine residue of 114.1 Da, which would be expected by the addition of 2 Gly. The MS/MS spectrum of the peptide ion m/z 616.3 is shown in Fig. 1D. Despite the overall weak signal-to-noise ratio, the mass spectrum clearly exhibits a nearly complete y ion series [26], thereby corroborating the assignment of the peptide sequence as shown at the top in Fig. 1D. Further support for the location of the modification is the fact that this peptide contains an internal lysine residue (Lys92) which, when modified by ISG15, should be resistant to cleavage by trypsin. In summary, the mass spectrometric data strongly suggest that ISG15 modifies Ubc13 via an isopeptide bond between the carboxyl group of glycine of ISG15 and the ε-amino group of Lys92 of Ubc13, as depicted in Fig. 1D.

Ubc13 can be modified by ISG15

Mass spectrometry analysis showed that Ubc13 could be detected in the band of ~35 kDa, suggesting that Ubc13 is a target of ISG15 modification (Fig. 1). To further examine whether Ubc13 is a substrate for ISG15 conjugation, we used a conjugation system to examine whether conjugation of Ubc13 to ISG15 can be detected [10]. 293T cells were transfected with His-ISG15, UBE1L, Ubc8, and HA-Ubc13 expressing plasmids. Cells were lysed and His-ISG15-conjugated species were purified on Ni–NTA agarose beads. The samples were analyzed by Western blotting using HA antibody. As shown in Fig. 2, a ~35 kDa band of Ubc13 was detected from the cell extracts in which Ubc13 was co-transfected with ISG15 and UBE1L (Fig. 2, lane 4 of top panel). Co-transfection of UBE1L and Ubc8 increased the intensity of this band (Fig. 2, lane 8 of top panel). Furthermore, we also detected ISG15-modified forms of Ubc13 from direct Western blots in the same set of experiments (Fig. 2, lane 8 of low panel).

To determine whether Ubc13 is subjected to ISG15 modification at endogenous levels, we transfected 293T cells with His-ISG15, UBE1L, and Ubc8, and carried

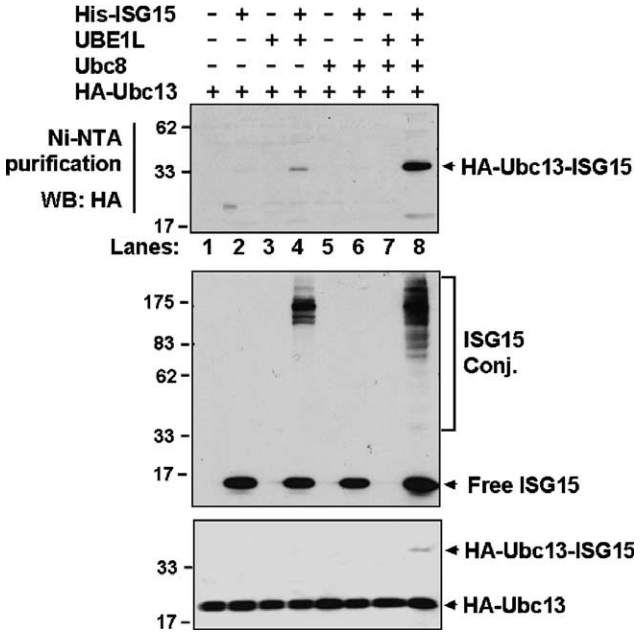


Fig. 2. Ubc13 can be ISGylated in a 293T cell conjugation system. 293T cells were transfected with HA-Ubc13, UBE1L, Ubc8, and His-ISG15 expression constructs as indicated. Forty-eight hours after transfection, 1000 μg of proteins in cell lysates was subjected to Ni–NTA pull-down and Western blotted with HA antibody. The positions of unmodified and ISGylated Ubc13 are shown. The expression of HA-Ubc13 and protein ISGylation were analyzed by Western blot with antibodies specific for HA and mISG15.

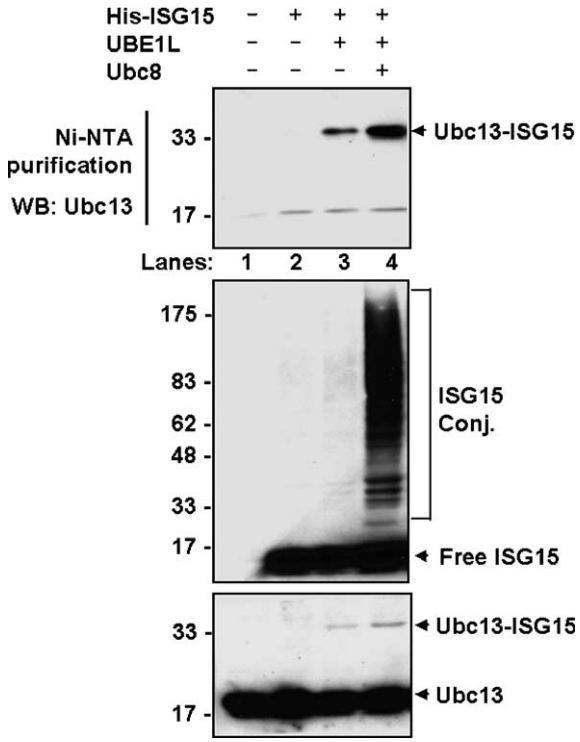


Fig. 3. ISGylation of endogenous Ubc13 in 293T cells. 293T cells were transfected with UBE1L, Ubc8, and His-ISG15 expression constructs as indicated. Ni–NTA pull-down is the same with Fig. 2. Pulled-down protein was Western blotted with Ubc13 antibody. The positions of unmodified and ISGylated Ubc13 are shown. The expression of endogenous Ubc13 and protein ISGylation were analyzed by Western blot.

out Ni–NTA purification. The immunoprecipitates were analyzed by Western blotting using antibodies against Ubc13. As shown in Fig. 3, a band of the predicted molecular weight for Ubc13 modified with a single ISG15 moiety was observed in the cell extracts in which Ubc13 was co-transfected with ISG15 and UBE1L (Fig. 3, lane 3 of top panel). Co-transfection of Ubc8 increased the intensity of this band (Fig. 3, lane 4 of top panel). ISG15-modified forms of endogenous Ubc13 can also be detected by Western blots in the same set of experiments (Fig. 3, lanes 3 and 4 of low panel). The data further confirm that Ubc13 can be modified with ISG15.

A single lysine residue (Lys92) in Ubc13 is modified by ISG15

The MS/MS spectrum of the Ubc13 peptide 'ICLDILKDK' strongly suggests that the Lys92 of Ubc13 is functioning as the acceptor site for ISG15 (Figs. 1C and D). To provide further support for Lys92 as the acceptor site, we mutagenized the Ubc13 cDNA at a single base to convert Lys92 to arginine, and expressed wt Ubc13 and mutated Ubc13-K92R as HA-tagged proteins in 293T cells. We compared the ISGylation of these two proteins using an 293T conjugation system. Cells were lysed and His-ISG15-conjugated species were purified on Ni–NTA agarose beads. The samples were analyzed by Western blotting using HA antibody. As shown in Fig. 4, ~35 kDa bands of Ubc13 were detected from the cell extracts in which wt Ubc13 was co-transfected with ISG15 system, which contains His-ISG15, UBE1L, and Ubc8 (Fig. 4, lane 2 of top panel). In contrast, we did not detect this signal when mutant Ubc13-K92R was co-transfected with ISG15 system (Fig. 4, lane 4 of top panel). Direct Western blot analysis confirmed the modification of wt Ubc13, whereas Ubc13-K92R was not modified (Fig. 4, low panel). These data indicated not only that Lys92 is the site of modification by ISG15, but that it is the only site in Ubc13 capable of being modified in this assay.

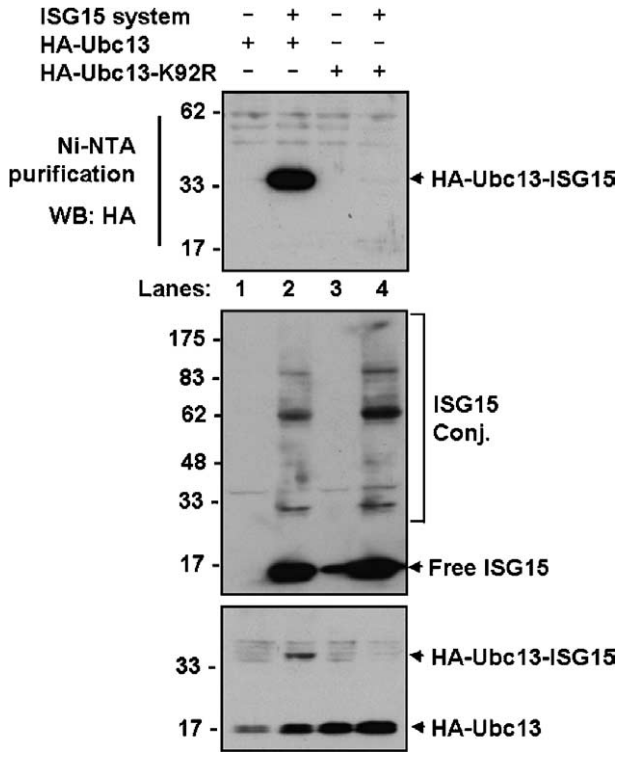


Fig. 4. A single lysine residue (Lys92) in Ubc13 is modified by ISG15. 293T cells were transfected with HA-tagged wt Ubc13 (lanes 1 and 2) and mutant Ubc13-K92R (lanes 3 and 4) in the absence and presence of ISG15 system, which includes His-ISG15, UBE1L, and Ubc8 as indicated. Forty-eight hours after transfection, 1000 μg of proteins in cell lysates was subjected to Ni–NTA pull down and Western blotted with HA antibody. The expression of wt and mutant Ubc13 was analyzed by Western blot with antibodies specific for HA. The ISGylation level was analyzed by mISG15 Western blot.

ISGylation of Ubc13 disrupts its ability to form thioester bond with ubiquitin

Ubc13 interacts with Mms2 and mediates catalysis of Lys63 Ub chains [18]. In this Ubc13–Mms2 heterodimer complex, Ubc13 is the catalytically active ubiquitin-conjugating enzyme and Mms2 is the catalytically inactive paralogue. The active site (cysteine 87) of Ubc13 can form a thioester bond with the donor ubiquitin [18]. Ubc13 and Mms2 can orient the Lys63 residue of an acceptor Ub to the donor Ub at the Ubc13 active site. An isopeptide bond can then form between the two molecules. Ubc13 is modified by ISG15 on Lys92, which is very close to the active site of Ubc13 (cysteine 87), raising the question of whether ISG15 modification of Ubc13 affects its capacity to form thioester bond with ubiquitin. We used ubiquitin–agarose beads to pull down Ubc13 and ISGylated Ubc13. This ubiquitin has fully functional C-terminus and can be covalently linked to Ubc13 via the thioester bond. The formation of this bond is dependent on the Mg^{2+} and ATP (data not shown and see reference [27]). To determine the ability of ISGylated Ubc13 to form thioester bond with ubiquitin, 293T cells were transfected with Flag-Ubc13 expressing plasmids with or without the ISG15 system containing ISG15, UBE1L, and Ubc8. As expected, ISGylated Ubc13 was detected by direct Western blot when it was co-transfected with the ISG15 system (Fig. 5, lane 2). When we performed covalent affinity purification with ubiquitin–agarose, Western blot analysis showed that only unmodified Ubc13 can be pulled down, but ISGylated Ubc13 cannot. Western blot analysis of supernatants confirmed that the presence of ISGylated Ubc13 (Fig. 5, lane 6). These data indicated that ISGylated Ubc13 cannot be linked to the ubiquitin via a thioester bond, suggesting that ISGylation of

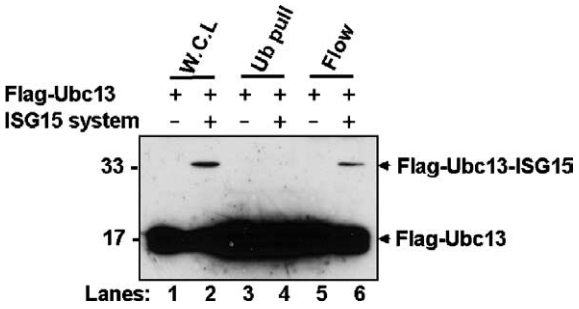


Fig. 5. ISGylation of Ubc13 disrupts its ability to form thioester bond with ubiquitin. 293T cells were transfected with Flag-Ubc13 in the absence or presence of ISG15 system, which includes ISG15, UBE1L, and Ubc8 expression constructs. After 48 h, cells were sonicated in a covalent purification assay buffer which contains 50 mM Tris–HCl, pH 7.2, 10 mM $MgCl_2$, 5 mM ATP, and 0.2 mM DTT. Proteins (1000 μg) were covalent purified using ubiquitin–Sepharose. The flowthrough of purification is harvested and run together with whole cell lysate and purified sample, and then Western blotted with Flag antibody. The positions of Ubc13 and ISGylated Ubc13 are shown.

Ubc13 disrupted its ability to form the thioester bond with ubiquitin.

ISGylation of Ubc13 does not disrupt its association with Mms2

The revelation of Ubc13–Mms2-mediated catalysis suggested the necessity of heterodimer formation between Ubc13 and Mms2 [18,19]. Our covalent affinity purification assay suggested that ISGylation of Ubc13 disrupted its thioester bond formation with ubiquitin, which lead to the question of whether ISG15 modification of Ubc13 affects its capacity to associate with Mms2. To determine the interaction between ISGylated Ubc13 and Mms2, 293T cells were transfected with Flag-Ubc13, ISG15, UBE1L, and Ubc8 expressing plasmids with or without HA-Mms2. As expected, unmodified and ISGylated Ubc13 were detected by direct Western blot (Fig. 6, lanes 1 and 4). We performed immunoprecipitations with HA antibody. When Mms2 was immunoprecipitated, both the unmodified Ubc13 and ISGylated Ubc13 were coprecipitated (Fig. 6, lane 5). As the control, Mms2 non-transfected cells could not pull down Ubc13. Western blot analysis of IP supernatants confirmed the presence of the unmodified Ubc13 and ISGylated Ubc13 (Fig. 6, lanes 3 and 6). The fact that ISGylated Ubc13 can still be co-immunoprecipitated with Mms2 indicates that ISGylation of Ubc13 does not disrupt its association with Mms2.

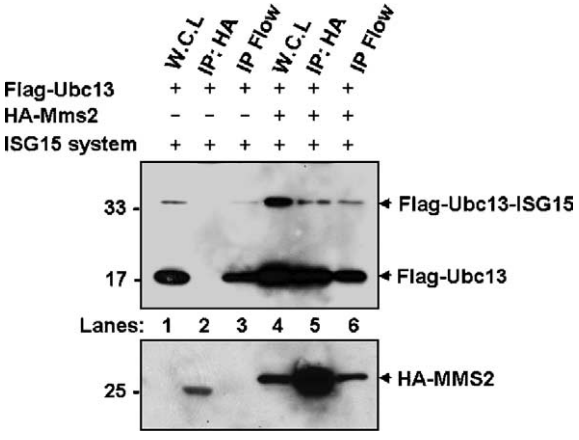


Fig. 6. ISGylation of Ubc13 does not disrupt its interaction with Mms2. 293T cells were transfected with Flag-Ubc13, mISG15, UBE1L, and Ubc8 expression constructs together with or without HA-Mms2. After 48 h, proteins (1000 μg) were lysed and precipitated with HA antibody. The flowthrough of IP is harvested and run together with whole cell lysate and IP sample, and then Western blotted with Flag antibody. The positions of Ubc13, ISGylated Ubc13, and Mms2 are shown. The signal in lane 2 of lower panel is from the immunoglobulin light chain.

Discussion

While being known for a quarter of a century, IFN-inducible ubiquitin-like protein ISG15 has not been associated with a single known function [7]. Recently, the research of ISGylation has accelerated. Key components of ISG15-conjugation/deconjugation system were found as well as a significant number of ISG15-targeted proteins were identified (see the Introduction). Though ISG15 has been implicated in a variety of biological activities mostly based on its similarities to ubiquitin and other ubiquitin-like proteins, generation and analysis of ISG15-conjugation-deficient Ube1L KO [28] or ISG15-deficient mice [29] did not reveal any significant developmental abnormalities. There was also no effect on the composition of the main cellular compartments, suggesting that ISG15-targeting is most likely a fine-tuning process that is induced by IFN. Very thorough studies must be performed to identify the exact consequences of ISGylation for some special target proteins.

In the present report, we initially used a proteomics approach with mass spectrometry to identify Ubc13, which is the major ubiquitin-conjugating enzyme involved in the formation of Lys63-chains, as a target of ISG15 with a single ISG15 modification at Lys92. Further mutational analysis revealed that only a single ISG15 moiety could be added onto Lys92 of Ubc13. Taking into account the importance of Ubc13-related atypical Lys63-linked ubiquitin conjugates in the activity of various cellular processes (e.g., DNA repair [20], mitotic progression [21], and nuclear factor-κB signaling [22]), Ubc13 is an attractive target to further study the biological consequences of ISG15 modification.

The same lysine residue of Ubc13 is also targeted by monoubiquitination [30]. However, the effect of this modification is not known. Previous reports have suggested that the mutation of Lys92 to arginine affected neither the ability of Ubc13 to interact with Mms2 nor

its ability to generate Lys63 chains [30]. However, the addition of 15 kDa of ISG15 at this site may have different effects. Therefore, we directed our attention on whether ISG15-modified Ubc13 could still function as an active E2. We checked the effect of ISGylation of Ubc13 on its interaction with Mms2. Pull-down assays for the Mms2 protein showed that both modified and unmodified forms of Ubc13 could be successfully co-precipitated (Fig. 6), suggesting that the presence of ISG15 did not interfere with the formation of Ubc13/Mms2 complexes. These results are also in accordance with previously reported crystal structure data [18], where the Lys92 surrounding area lies solely on the surface of Ubc13 and should not make any contacts with Mms2. At the same time, the very same area provides an interacting interface for the positioning of donor ubiquitin for a subsequent transfer onto Lys63 of the acceptor Ub-molecule. Thus far, the proximity of ISG15-modified Lys92 to the active site Cys87 of Ubc13 suggests that the addition of ISG15 at this position could possibly interfere with the thioester bond formation between Ubc13 and donor ubiquitin. Indeed, in our pull-down assays using ubiquitin-conjugated beads, thioester bonds could only be formed between ubiquitin and the unmodified form of Ubc13 (Fig. 5). Covalent attachment of ISG15 at the position Lys92 of Ubc13 interfered with the formation of thioester intermediate complex with ubiquitin, thereby inhibiting the enzymatic activity of Ubc13. The inhibitory effect could also be achieved by masking the active site of Ubc13, thereby preventing recognition by E1 and further transfer of ubiquitin from E1 to E2, or by the inability to properly position Ub onto Ubc13. Our results indicate that ISG15 can modulate the activity of Lys63 ubiquitin-conjugating pathway by modifying the E2 function of Ubc13.

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