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# ISG15 modification of Ubc13 suppresses its ubiquitin-conjugating activity ☆

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**Abstract**

ISG15 is one of the interferon-stimulated genes and is classified as a ubiquitin-like protein. Upon interferon stimuli, ISG15 is upregulated and becomes conjugated to various cellular proteins (ISGylation). Several target proteins for ISGylation have recently been identified, but the biological consequence of protein ISGylation remains unclear. In the course of our study to identify components of the ISGylation system, we found that Ubc13, an E2 enzyme for ubiquitin conjugation, is covalently modified with ISG15. To determine the meaning of ISGylation of Ubc13, we isolated ISG15-modified Ubc13 protein and compared its ubiquitin-conjugating activity with that of an unmodified one. We found that ISGylation of Ubc13 suppresses its ability to form a thioester intermediate with ubiquitin.


*Keywords:* ISG15; Interferon; Ubiquitin; Ubiquitin-conjugating enzyme; Ubc13

ISG15 is one of the interferon-stimulated genes and it contains two ubiquitin-like domains [1–3]. Upon interferon stimuli, ISG15 is upregulated and becomes conjugated to diverse cellular proteins (ISGylation) [4]. It has been proposed that ISGylation is mediated by a sequential reaction similar to the ubiquitin conjugation system that consists of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase), and E1 and E2 enzymes but not E3 enzyme for ISGylation have been identified [5–7]. Recently, mice carrying null mutation of the gene for UBP43 (de-ISGylating enzyme) have been generated, and a series of analyses of the mice has suggested that protein ISGylation has an important role in innate immunity against viral infection [8–11]. To date, several target proteins for ISGylation have been identified, but the biological consequence of protein ISGylation remains unclear [12,13].

Ubc13 is an exceptional E2 enzyme for ubiquitin conjugation because it forms a heterodimer with Mms2, an E2-like protein that lacks the catalytic Cys residue, and the Ubc13–Mms2 complex catalyzes the assembly of a K63-linked polyubiquitin chain [14–17]. Unlike the K48-linked polyubiquitin chain, a signal for proteasome-mediated protein degradation, the K63-linked polyubiquitin chain is not involved in proteasome-mediated degradation and functions in DNA repair and IκB kinase activation [18–21].

In this study, we found that Ubc13 is covalently modified with ISG15 and that ISGylation of Ubc13 suppresses its formation of a thioester intermediate with ubiquitin.

## Materials and methods

*Cell culture and transfection.* A549 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10%

☆ *Abbreviations:* ISG, interferon-stimulated gene; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; IκB, inhibitor of nuclear factor-κB; PCR, polymerase chain reaction; DTT, dithiothreitol; GST, glutathione *S*-transferase.

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heat-inactivated fetal bovine serum (Invitrogen) and 10% heat-inactivated calf serum (Hyclone), respectively. Transfection in A549 cells was performed using Metafectene (Biontex) according to the manufacturer's protocol, while that in HeLa cells was performed according to the standard calcium precipitation protocol.

*Plasmid construction.* The open reading frames of human ISG15, UBE1L (an E1 for ISGylation), Ubc13 (UBE2N), and Mms2 (UBE2V2) were amplified by PCR. Point mutants of Ubc13 were generated by PCR. All constructs were verified by DNA sequencing. To generate the expression plasmids, the PCR fragments were subcloned into pCI-neo-6His, pCI-neo-3Flag, pCI-neo-3T7, and pCI-neo-2S vectors that had been generated by inserting oligonucleotides encoding a histidine tag sequence (6His), three repeats of a Flag tag sequence, three repeats of a T7 tag sequence, and two repeats of an S peptide sequence, respectively, into the pCI-neo mammalian expression vector (Promega).

*Immunoprecipitation and Western blotting.* A549 cells that had been transiently transfected with the indicated plasmids and cultured for 30 h were washed with ice-cold phosphate-buffered saline and lysed with RIPA buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 0.5% sodium deoxycholate. The cell lysate was sonicated for 3 s and the debris was removed by centrifugation. The resulting supernatant was incubated with anti-Flag M2 antibody-immobilized agarose (Sigma), and the immunoprecipitate was washed five times with RIPA buffer.

For Western blotting, the whole cell lysate and the immunoprecipitate were separated by SDS–PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were immunoblotted with anti-Flag tag M2 (Sigma), anti-T7 tag (Novagen), and anti-S peptide (Santa Cruz) antibodies and were subsequently incubated with horseradish peroxidase-conjugated antibodies against mouse or rabbit immunoglobulin (Amersham Biosciences), followed by detection with ECL immunoblotting detection reagents (Amersham Biosciences).

*Isolation of ISGylated and unmodified Ubc13 proteins.* To prepare ISGylated Ubc13 protein, HeLa cells were transiently transfected with the pCI-neo-3Flag-Ubc13, pCI-neo-2S-UBE1L, and pCI-neo-6His-ISG15 plasmids and cultured for 48 h. The cells were washed with ice-cold phosphate-buffered saline and were then lysed with phosphate-buffered saline containing 0.2% Nonidet P-40 and 1 mM DTT. The cell lysate was sonicated for 3 s and the debris was removed by centrifugation. The resulting supernatant was incubated with Talon resin (BD Biosciences) and the resins were extensively washed with phosphate-buffered saline containing 0.2% Nonidet P-40. The materials bound to the resins were eluted with 300 mM imidazole. The eluted ISGylated proteins were diluted with phosphate-buffered saline containing 0.2% Nonidet P-40, and 1 mM DTT and were then incubated with anti-Flag M2 antibody-immobilized agarose beads. The resulting immunoprecipitate was extensively washed with phosphate-buffered saline containing 0.2% Nonidet P-40, followed by washing three times with a buffer containing 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.2% Nonidet P-40, and 10% glycerol. The materials bound to the beads were eluted with 3× Flag peptide (200 μg/ml) and used as ISGylated Ubc13 that had been Flag-tagged. On the other hand, to prepare unmodified Ubc13 protein, HeLa cells were transiently transfected only with the pCI-neo-3Flag-Ubc13 plasmid, and the lysate was prepared as described above. The cell lysate was sonicated for 3 s and the debris was removed by centrifugation. The resulting supernatant was incubated with anti-Flag M2 antibody-immobilized agarose beads and Flag-tagged Ubc13 was eluted as described above.

*In vitro ubiquitination and binding assays.* GST–ubiquitin and Uba1 (an E1 for ubiquitination) were prepared as described previously [22]. To analyze in vitro formation of the Ubc13–ubiquitin thioester intermediate, 2 μg GST–ubiquitin, 200 ng Uba1, and 2 ng ISGylated or unmodified Ubc13 that had been Flag-tagged were incubated in a reaction mixture (20 μl) containing 25 mM Tris–HCl, pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM DTT, and 0.2 U of inorganic pyrophosphatase. The reaction was stopped by adding SDS–PAGE loading buffer without DTT, and the reaction mixture was subjected to Western blotting with anti-Flag tag antibody.

To investigate the effect of ISGylation of Ubc13 on its binding with Mms2, we first prepared S-tagged Mms2 bound to S protein-immobilized agarose beads. HeLa cells were transiently transfected with the pCI-neo-2S-Mms2 plasmid and cultured for 36 h. The cells were washed with ice-cold phosphate-buffered saline and were then lysed with a buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, and 10% glycerol. The cell lysate was sonicated for 3 s and the debris was removed by centrifugation. The resulting supernatant was incubated with S protein-immobilized agarose beads (Novagen). The beads were extensively washed with a buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, and 10% glycerol, followed by washing three times with the same buffer, except for 500 mM NaCl. Next, the thus-prepared S-tagged Mms2 bound to the beads was incubated with the isolated ISGylated or unmodified Ubc13 in a buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, and 10% glycerol. After the beads had been extensively washed with the same buffer, the materials bound to the beads were subjected to Western blot analysis as described above.

## Results and discussion

### *Ubc13 is covalently modified with ISG15*

In the course of our study to identify components of the ISG15 conjugation system and its target proteins, we identified Ubc13, an E2 enzyme for the ubiquitin conjugation system, as a candidate target protein for ISG15 conjugation. We expressed Flag-tagged Ubc13 together with T7-tagged ISG15 and S-tagged UBE1L (the E1 for the ISG15 conjugation system) in A549 cells, and the extract of transfected cells was subjected to Western blotting with anti-Flag tag antibody (Fig. 1A). In the case of co-expression of Ubc13 with both ISG15 and UBE1L, one band with slower mobility (Fig. 1A, open arrowhead) than that of the intact one (closed arrowhead) was detected. On the other hand, when we expressed Ubc13 only, the corresponding band with slower mobility was not detected. These results suggest that Ubc13 is covalently modified with ISG15. To obtain definitive evidence for this, we next carried out immunoprecipitation experiments. The above extract of transfected cells was subjected to immunoprecipitation with anti-Flag tag antibody in a buffer containing 0.1% SDS, followed by Western blotting with anti-T7 tag antibody (Fig. 1B). Ubc13 was again found to be modified with ISG15 in the case of co-expression of Ubc13 with both ISG15 and UBE1L (Fig. 1B, open arrowhead). These results indicate that Ubc13 is covalently modified with ISG15 in A549 cells.

### *Ubc13 is modified with ISG15 through Lys92*

To study the biological consequence of ISG15 conjugation to Ubc13, it is important to determine the Lys