J. Biochem. **138**, 711–719 (2005)
doi:10.1093/jb/mvi172

Link between the Ubiquitin Conjugation System and the ISG15 Conjugation System: ISG15 Conjugation to the UbcH6 Ubiquitin E2 Enzyme

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Received August 13, 2005; accepted September 4, 2005

ISG15 is a ubiquitin-like protein that is upregulated on treatment with interferon. ISG15 is considered to be covalently conjugated to cellular proteins through a sequential reaction similar to that of the ubiquitin conjugation system consisting of E1/E2/E3 enzymes: UBE1L and UbcH8 have been reported to function as E1 and E2 enzymes, respectively, for ISG15 conjugation. Several cellular proteins have been identified as targets for ISG15 conjugation, but the roles of ISG15 conjugation remain unclear. In this study, we found that UbcH6 and UbcH8, E2 enzymes for ubiquitin conjugation, are covalently modified by ISG15. We also found that UbcH6 is capable of forming a thioester intermediate with ISG15 through Cys131. We determined that the Lys136 residue near the catalytic site Cys131 is the ISG15 conjugation site in UbcH6. We isolated ISG15-modified and unmodified UbcH6 proteins, and analyzed their abilities to form thioester intermediates with ubiquitin. A ubiquitin thioester intermediate was detected in the case of unmodified UbcH6, but not in that of ISG15-modified UbcH6, strongly suggesting that ISG15 conjugation to UbcH6 suppresses its ubiquitin E2 enzyme activity. Thus, we provide evidence for a link between the ubiquitin conjugation system and the ISG15 conjugation system.

Key words: interferon, ISG15, posttranslational modification, ubiquitin, ubiquitin-conjugating enzyme.

Abbreviations: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; GST, glutathione S-transferase; IFN, interferon; ISG, interferon-stimulated gene; SCF, Skp1-cullin-F box protein.

Interferons (IFNs) are pleiotropic cytokines that play essential roles in innate immune responses. Upon infection with viruses, cells produce IFNs, and the IFNs are secreted and bind with their receptors. Binding of IFNs with the receptors activates the IFN signaling pathway, leading to the expression of interferon-stimulated genes (ISGs). Thus, IFNs exert their antiviral ability via ISGs (*1*, *2*). One of the ISGs, ISG15 (an ISG with a molecular mass of 15 kDa), is classified as a ubiquitin-like protein, similar to SUMO-1 or NEDD8 (*3*). It contains two ubiquitin-like domains and is strongly upregulated on IFN treatment (*4*, *5*). Upon IFN stimulation, ISG15 is known to be secreted and to act as a cytokine (*6*, *7*). Alternatively, ISG15 becomes conjugated to diverse proteins (*8*). As ISG15 exhibits sequence similarity with ubiquitin, ISG15 is considered to be covalently conjugated to cellular proteins through a sequential reaction similar to that of the ubiquitin conjugation system consisting of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). To date, two components of the system for ISG15 conjugation (ISGylation), UBE1L and UbcH8, which function as E1 and E2 enzymes, respectively, for ISGylation, have been identified, while serpin 2a, Jak1, Stat1, PLCγ1, and Erk1/2 have been identified as target proteins for ISGylation (*9*–*13*).

Recently, mice carrying a null mutation of the gene for UBP43 (USP18, a de-ISGylation enzyme) were generated, and a series of analyses on the mice revealed that UBP43 plays an essential role in innate immunity against viral infection (*14*–*16*). This finding implies that protein ISGylation plays an important role in innate immunity against viral infection. To date, several target proteins for ISGylation have been identified, as described above, but the biological consequences of the protein ISGylation remain unclear.

In this study, we addressed the question of whether some of the E2 enzymes in the ubiquitin conjugation system, which are upregulated on IFN treatment, could function as E2 enzymes in the ISG15 conjugation system, since UBE1L and UBP43, as well as ISG15, are IFN-inducible proteins (*5*). We cloned several IFN-inducible ubiquitin E2 enzymes (*17*) and examined whether these enzymes participate in ISG15 conjugation. We found that UbcH6 and UbcH8 are modified by ISG15 when they are co-expressed with both ISG15 and UBE1L. In addition, we obtained several lines of evidence that not only UbcH8 but also UbcH6 is able to form an E2-ISG15 thioester intermediate, indicating that both UbcH6 and UbcH8 function as E2 enzymes in the ISG15 conjugation

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system. We identified the Lys136 residue near the catalytic site Cys131 as the ISG15 conjugation site in UbcH6. We compared the ability of ISG15-modified UbcH6 to form a ubiquitin thioester intermediate with that of the unmodified form, and found that ISG15 conjugation to UbcH6 suppresses its ubiquitin E2 enzyme activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—A549 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 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, UbcH2, UbcH5c, UbcH6, UbcH7, and UbcH8 were amplified by PCR. Point mutants of UbcH6 were generated by PCR. All constructs were verified by DNA sequencing. To generate mammalian expression plasmids, PCR fragments were subcloned into pCI-neo-6His, pCI-neo-3T7, pCI-neo-2S, and pCI-neo-3Flag vectors, which had been generated by inserting oligonucleotides encoding the histidine tag sequence (6His), three repeats of the T7 tag sequence, two repeats of the S peptide sequence, and three repeats of the Flag tag sequence, respectively, into the pCI-neo mammalian expression vector (Promega). To generate a yeast expression plasmid, the open reading frame of human UBE1L was subcloned into the pYF3 vector, which had been generated by inserting the oligonucleotide encoding the Flag tag sequence into the pYES3 yeast expression vector (Invitrogen). To express GST fusion proteins, the open reading frames of ISG15 and UbcH6 were subcloned into the pGEX6P1 vector (Amersham Biosciences). The pGEX-2TK-ubiquitin, pGEX-2TK-SUMO-1, and pGEX-2TK-NEDD8 plasmids were generous gifts from Dr. K. Tanaka of the Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Isolation of Recombinant Proteins and Preparation of Anti-ISG15 Antibody—Escherichia coli BL21 (DE3) cells transformed with the pGEX6P1-ISG15, pGEX6P1-UbcH6, pGEX-2TK-ubiquitin, pGEX-2TK-SUMO-1, or pGEX-2TK-NEDD8 plasmid were cultured overnight at 37°C in LBA medium (Luria-Bertani medium supplemented with 50 μg/ml ampicillin), transferred to a 20-fold volume of LBA medium, and then incubated at 37°C for 2 h. After the cells had been chilled in water, isopropyl-1-thio-β-D-galactopyranoside was added to the cells to the final concentration of 0.1 mM and the cells were further incubated at 20°C for 18 h. The cells were then harvested and lysed with recombinant buffer comprising 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 5% glycerol, and 1 mM dithiothreitol (DTT). After the lysate had been centrifuged, the resulting supernatant was mixed with glutathione-immobilized agarose beads (Amersham Biosciences) and incubated at 4°C for 2 h, and then the beads were extensively washed with recombinant buffer. The GST fusion protein was then eluted from the beads with glutathione. Alternatively, PreScission protease (Amersham Biosciences) was added to the beads and the mixture was incubated at 4°C for 16 h to allow the protease to cleave the specific site between GST and the respective proteins.

To prepare human UBE1L protein, we transformed *Saccharomyces cerevisiae* KA31a cells (*18*) with pYF3-UBE1L. Yeast cells were cultured at 30°C in a synthetic minimal medium consisting of 0.67% yeast nitrogen base, 2% raffinose, 0.5% casamino acid and 20 μg/ml uracil, but lacking tryptophan to select a strain containing the pYF3-UBE1L plasmid, and then the cells were grown to an optical density at 600 nm of 2.0. Galactose was then added to the cells to the final concentration of 1%, and the cells were further incubated at 30°C for 15 h, harvested, and washed once with ice-cold water and once with buffer comprising 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10% glycerol. The cells were suspended in buffer comprising 20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 5% glycerol, and then lysed with glass beads in a Bead-Beater (Biospec Products). Nonidet P-40 and DTT were then added to the cell lysate to the final concentrations of 0.2% and 1 mM, respectively. The mixture was centrifuged and the resulting supernatant was incubated with anti–Flag M2 antibody–immobilized agarose beads (Sigma). The beads were extensively washed with buffer comprising 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Nonidet P-40, and 5% glycerol. The materials bound to the beads were eluted with 3× Flag peptide (200 μg/ml) (Sigma). Yeast *S. cerevisiae* E1 (Uba1) protein was prepared as described previously (*19*).

To prepare anti-ISG15 polyclonal antibody, the recombinant ISG15 protein was emulsified with Freund's complete adjuvant and then injected subcutaneously into a rabbit, followed by several booster injections. The antiserum was subjected to 40% ammonium sulfate precipitation, followed by chromatography on a column of protein A–Sepharose (Amersham Biosciences). The resulting IgG fraction was successively passed through three columns of the beads, for which GST-ubiquitin, GST-SUMO-1, and GST-NEDD8, respectively, had been coupled to HiTrap NHS-activated beads (Amersham Biosciences), and was then affinity-purified with ISG15-immobilized beads, for which the ISG15 protein had been coupled to HiTrap NHS-activated beads. The resulting antibody was used as a highly specific antibody against ISG15: This antibody was found to be immunoreactive with ISG15 but not with ubiquitin, SUMO-1, or NEDD8 (data not shown).

Immunoprecipitation and Western Blotting—A549 cells that had been transiently transfected with the indicated plasmids using Metafectene and cultured for 30–36 h were washed with ice-cold phosphate-buffered saline, and then lysed with RIPA buffer including 0.1% SDS. The cell lysate was sonicated for 3 s and the debris was removed by centrifugation. The resulting supernatant was incubated with anti–Flag tag M2 antibody–immobilized agarose beads, and then the immunoprecipitate was washed five times with RIPA buffer. To investigate the effect of IFN treatment, A549 cells were first treated with IFNβ (1,000 units/ml) (Pepro Tech) for 24 h and then a cell lysate was prepared as described above. After centrifugation, the resulting supernatant was incubated with both protein G–immobilized agarose beads (Santa Cruz) and anti-UbcH6 antibody (Chemicon), and the immunoprecipitate formed was washed with RIPA buffer.

For Western blotting, the whole cell lysate and the immunoprecipitate were separated by SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad). The membranes were immunoblotted with anti-Flag tag M2 (Sigma), anti-T7 tag (Novagen), anti-ISG15, and anti-UbcH6 antibodies, and then incubated with horseradish peroxidase-conjugated antibodies against mouse or rabbit immunoglobulin (Amersham Biosciences), followed by detection with ECL immunoblotting detection reagents (Amersham Biosciences).

Measurement of In Vitro *ISG15 Conjugation*—GST-ISG15 (2 μg), UBE1L (125 ng), and UbcH6 (1 μg) were incubated at 37°C for 1 h in a reaction mixture (20 μl) comprising 25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM DTT, and 0.2 unit of inorganic pyrophosphatase (Sigma). The reaction was stopped by adding SDS-PAGE loading buffer with or without 200 mM DTT, and then the reaction mixture was subjected to Western blotting with anti-UbcH6 antibody.

Measurement of In Vitro *Formation of an E2-Ubiquitin Thioester Intermediate*—To investigate the effect of ISGylation of UbcH6 on its formation of a thioester intermediate with ubiquitin, ISG15-modified and unmodified UbcH6 proteins were isolated as follows. First, to isolate ISGylated UbcH6 protein, HeLa cells were transiently transfected with the Flag-tagged UbcH6, S-tagged UBE1L and His-tagged ISG15 expression plasmids according to the standard calcium precipitation protocol, and then cultured for 42 h. The cells were washed with ice-cold phosphate-buffered saline, and 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 beads (BD Biosciences), and the beads were extensively washed with phosphate-buffered saline containing 0.2% Nonidet P-40. The materials bound to the beads were eluted with 300 mM imidazole. The eluted materials were diluted with phosphate-buffered saline containing 0.2% Nonidet P-40 and 1 mM DTT, and 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 buffer comprising 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 the ISGylated UbcH6 preparation. Second, to isolate unmodified UbcH6 protein, HeLa cells were transiently transfected with the Flag-tagged UbcH6 expression plasmid and then cultured for 48 h. The cells were washed with ice-cold phosphate-buffered saline and then lysed with buffer comprising 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, 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 anti–Flag M2 antibody–immobilized agarose beads. The resulting immunoprecipitate was extensively washed with buffer comprising 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40 and 10% glycerol, followed by washing three times with buffer comprising 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 as described above and used as the unmodified UbcH6 preparation.

To analyze the *in vitro* formation of an E2-ubiquitin thioester intermediate, 1 μg of GST-ubiquitin, 100 ng of Uba1, and 2 ng of ISGylated or unmodified UbcH6 were incubated in a reaction mixture (20 μl) comprising 25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM DTT, and 0.2 unit of inorganic pyrophosphatase. The reaction was stopped by adding SDS-PAGE loading buffer without DTT, and then the reaction mixture was subjected to Western blotting with anti-Flag tag antibody.

RESULTS

Identification of Ubiquitin E2 Enzymes Susceptible to ISG15 Conjugation—In the course of our study to identify components of the ISG15 conjugation system and its target proteins, we assumed that some of the ubiquitin E2 enzymes, which are upregulated on IFN treatment, could function as E2 enzymes in the ISG15 conjugation system. We cloned several E2 enzymes, including IFN-inducible ubiquitin E2 enzymes, UbcH5c, UbcH6 and UbcH8, and expressed the Flag-tagged respective E2 enzymes together with T7-tagged ISG15 and S-tagged UBE1L in A549 cells. Extracts of the transfected cells were subjected to Western blotting with anti–Flag tag and anti–T7 tag antibodies (Fig. 1). In the cases of UbcH6 and UbcH8, bands exhibiting slower mobility (Fig. 1, open arrowheads) than those of the intact forms (closed arrowheads) were detected: The same bands were detected on Western blotting with anti–T7 tag antibody (data not shown). On the other hand, in the cases of UbcH2, UbcH5c and UbcH7, corresponding bands exhibiting slower mobility were undetectable (Fig. 1). These results suggest that UbcH6 and UbcH8 are covalently modified by ISG15.

To obtain definitive evidence that UbcH6 and UbcH8 are covalently modified by ISG15, we expressed Flag-tagged UbcH6 and UbcH8 together with T7-tagged ISG15 and S-tagged UBE1L in A549 cells, and extracts of the transfected cells were subjected to immunoprecipitation with anti-Flag tag antibody, and then to Western blotting with anti-T7 tag and anti-Flag tag antibodies (Fig. 2). UbcH6 and UbcH8 were again found to be covalently modified by ISG15 (Fig. 2, A and B, open arrowheads). In the case of UbcH6, modification by ISG15 was detected even in the absence of exogeneous UBE1L and was enhanced on co-expression of UBE1L. Although the expression of UBE1L is induced by IFN treatment, ISG15 conjugates are detected in A549 cells without IFN treatment (*8*), indicating the existence of endogenous UBE1L without IFN treatment. As shown in Fig. 2, A and B, both UbcH6 and UbcH8 were found to be not only covalently modified by ISG15 but also co-immunoprecipitated with free ISG15 (closed arrowheads). Since the lysis and immunoprecipitation buffers used included 0.1% SDS, it is unlikely that UbcH6 and UbcH8 are able to bind with ISG15 through non-covalent bonds. In addition, UbcH8 was recently identified as a major E2 enzyme for ISG15 conjugation (*10*, *11*). Taken together, the results suggest that co-immunoprecipitated ISG15 in the case of either UbcH6 or UbcH8 is derived from the E2-ISG15 thioester intermediate.

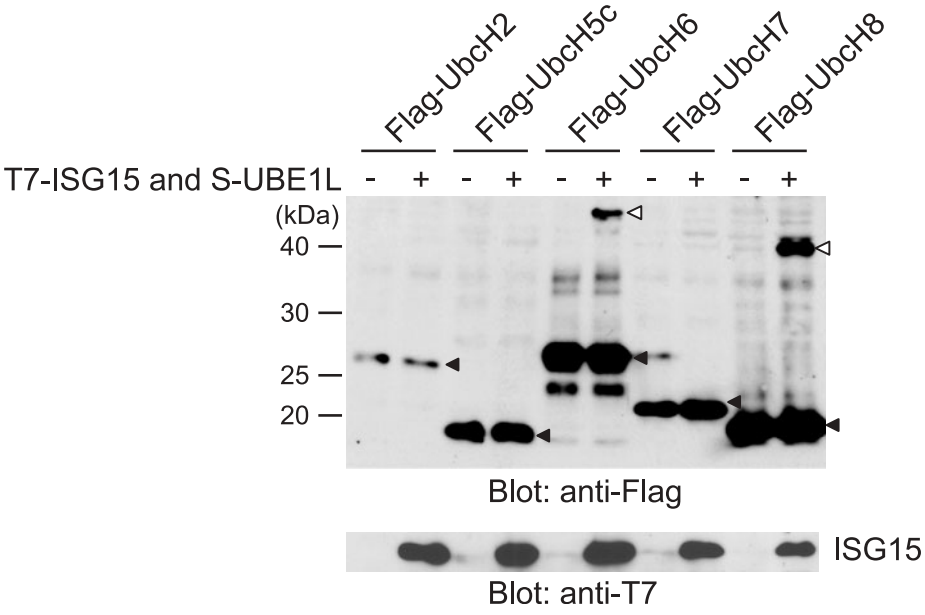

Fig. 1. **The two ubiquitin E2 enzymes UbcH6 and UbcH8 are conjugated with ISG15.** An extract of A549 cells that had expressed either Flag-tagged UbcH2, UbcH5c, UbcH6, UbcH7 or UbcH8 together with T7-tagged ISG15 and S-tagged UBE1L, as indicated, was subjected to Western blotting with anti–Flag tag and anti–T7 tag antibodies. The original E2 enzymes and their ISG15-conjugated forms are indicated by closed and open arrowheads, respectively.

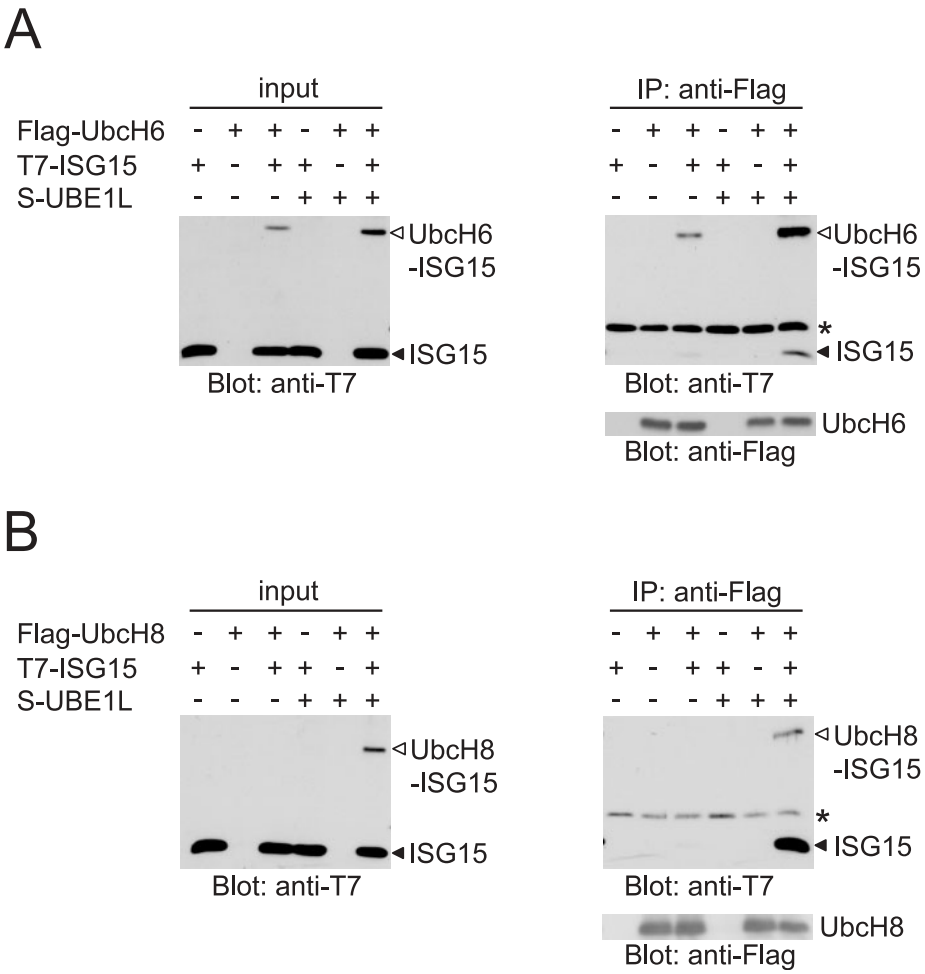

Fig. 2. **The two ubiquitin E2 enzymes UbcH6 and UbcH8 are co-immunoprecipitated with ISG15.** An extract of A549 cells that had expressed either Flag-tagged UbcH6 (A) or UbcH8 (B) together with T7-tagged ISG15 and S-tagged UBE1L, as indicated, was subjected to immunoprecipitation (IP) with anti–Flag tag antibody. The resulting immunoprecipitates were subjected to Western blotting with anti–T7 tag and anti–Flag tag antibodies under reducing conditions. The ISG15-conjugated E2 and ISG15 are indicated by open and closed arrowheads, respectively. Nonspecific bands are indicated by asterisks.

UbcH6 Is Capable of Forming a Thioester Intermediate with ISG15—To confirm the idea that co-immunoprecipitated free ISG15 is derived from the E2-ISG15 thioester intermediate, we carried out the same experiments as those for which the results are shown in Fig. 2, A and B, except for Western blotting with anti-T7 tag and anti-Flag tag antibodies under non-reducing conditions (Fig. 3, A and B). A band corresponding to free ISG15 was not detected under non-reducing conditions (Fig. 3, A and B, closed arrowheads), indicating that UbcH6, as well as UbcH8, is capable of forming a thioester intermediate with ISG15. Thus, we concluded that both UbcH6 and UbcH8 function as E2 enzymes for ISG15 conjugation. On the basis of the amount of co-immunoprecipitated free ISG15 (see Fig. 2, A and B) that is derived from the thioester intermediate, it can be inferred that UbcH8 is a major E2 enzyme for ISG15 conjugation, as previously reported (*10*, *11*), while UbcH6 also functions as an E2 enzyme for ISG15 conjugation.

To determine the catalytic site in UbcH6 that is thioester-linked with ISG15, we generated a C131A mutant of UbcH6 in which the Cys131 residue had been changed to Ala because the catalytic site in UbcH6 for ubiquitin is considered to be Cys131 on the basis of the similarity to other ubiquitin E2 enzymes. Flag-tagged UbcH6 or UbcH6 C131A was expressed together with T7-tagged ISG15 and S-tagged UBE1L in A549 cells, and then an extract of the transfected cells was subjected to immunoprecipitation with anti-Flag tag antibody, and then to Western blotting with anti-T7 tag and anti-Flag tag antibodies (Fig. 3C). The UbcH6 C131A mutant was found to be no longer co-immunoprecipitated with free ISG15, indicating that UbcH6 forms a thioester intermediate with ISG15 through Cys131. In addition, we noticed that the UbcH6 C131A mutant was no longer conjugated with ISG15. This finding confirms the above conclusion that UbcH6 is not only an E2 enzyme for ISG15 conjugation but also a target protein for ISG15 conjugation.

Since auto-ubiquitination of UbcH6 was recently reported (*20*), we next addressed the question of whether ISG15 conjugation to UbcH6 requires an additional factor such as an E3-like factor. To examine this hypothesis, we established an *in vitro* reconstitution system for ISG15 conjugation. UbcH6, UBE1L, and GST-ISG15 proteins were purified, incubated, and then subjected to Western

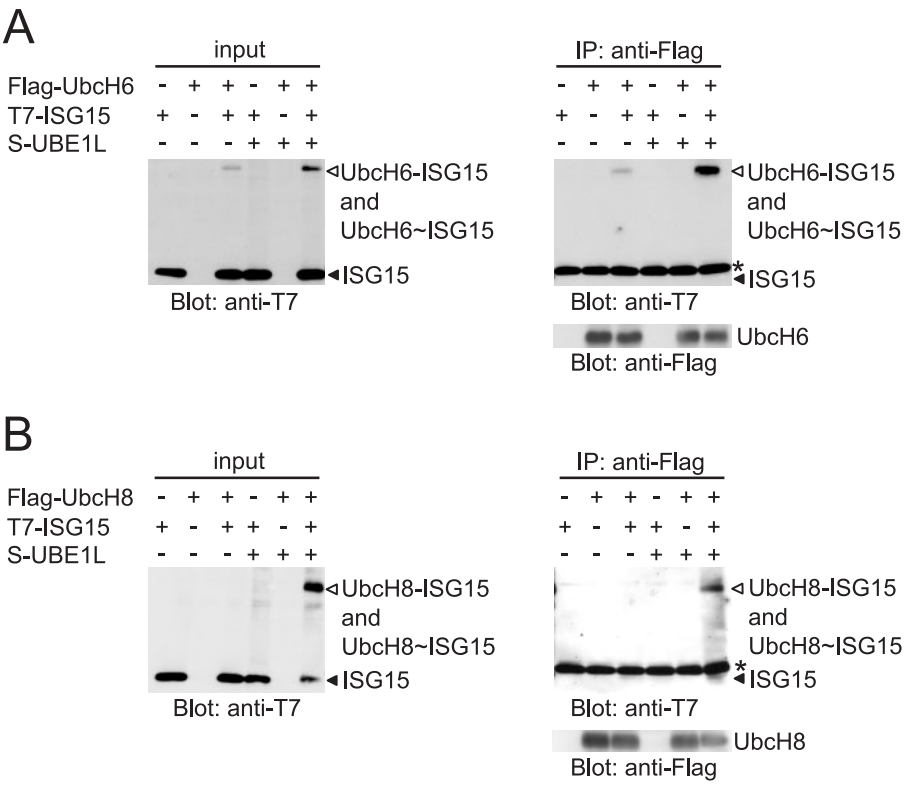

Fig. 3. **UbcH6 functions as an E2 enzyme for ISG15 conjugation.** (A and B) The extracts of A549 cells in Fig. 2, A and B, were subjected to immunoprecipitation (IP), and to Western blotting as in Fig. 2, A and B, respectively, except for the non-reducing conditions. Note that the ISGylated UbcH6/UbcH8 and UbcH6-ISG15/UbcH8-ISG15 thioester intermediates migrated to the same positions, as indicated by open arrowheads: "~" is used to indicate a thioester bond. ISG15 is indicated by a closed arrowhead, and nonspecific bands are indicated by asterisks. (C) An extract of A549 cells that had expressed either Flag-tagged UbcH6 or UbcH6 C131A together with T7-tagged ISG15 and S-tagged UBE1L, as indicated, was subjected to immunoprecipitation (IP) with anti–Flag tag antibody and to Western blotting with anti–T7 tag or anti–Flag tag antibody. A nonspecific band is indicated by an asterisk. (D) The UbcH6, UBE1L, and GST-ISG15 proteins isolated were incubated as indicated for 1 h and then subjected to Western blotting with anti-UbcH6 antibody under reducing (+DTT) or non-reducing (–DTT) conditions.

blotting with anti-UbcH6 antibody under reducing or non-reducing conditions (Fig. 3D). A band corresponding to an ISG15-bound form of UbcH6 was detected under non-reducing conditions (Fig. 3D, upper panel) but not under reducing conditions (lower panel), indicating that the above ISG15-bound form of UbcH6 is a UbcH6-ISG15 thioester intermediate, not ISGylated UbcH6. Formation of ISGylated UbcH6 was undetectable even when the reaction time was increased (24 h) (data not shown). Based on these results, it can be inferred that ISG15 conjugation to UbcH6 in A549 cells requires an additional factor, perhaps an E3-like factor.

ISG15 Conjugation to UbcH6 in Response to IFN—To confirm the modification of UbcH6 by ISG15 under physiological conditions, A549 cells were treated with IFNβ, and then an extract of the stimulated cells was subjected to Western blotting with anti-ISG15 and anti-UbcH6 antibodies (Fig. 4A). Smear bands with high molecular masses, corresponding to ISG15 conjugates, were detected when the cells were stimulated with IFNβ. Next, an extract of the IFN-stimulated cells was subjected to immunoprecipitation with anti-UbcH6 antibody and then to Western blotting as described above (Fig. 4B). Formation of ISGylated UbcH6 was only detected in the case of IFN treatment. Thus, ISG15 conjugation to UbcH6 occurs in response to the IFN signal.

UbcH6 Is Modified by ISG15 through Lys136—Since ubiquitin and ubiquitin-like modifiers such as SUMO-1 are covalently linked to the Lys residues of target proteins (*21*), it is reasonable to assume that ISG15 is also covalently linked to the Lys residue of target proteins. To determine the Lys residue in UbcH6 that is covalently linked with ISG15, we constructed various UbcH6 mutants in which the Lys residues had been changed to Arg, and then expressed the Flag-tagged respective mutants together with T7-tagged ISG15 and S-tagged UBE1L in A549 cells. An extract of the transfected cells was subjected to Western blotting with anti–T7 tag and anti–Flag tag antibodies (Fig. 5). It was found that the K136R and K136/147R mutants, in which the Lys136 or/and Lys147 residues had been changed to Arg, are substantially insusceptible to ISG15 conjugation (Fig. 5, lanes 7 and 9). Thus, we concluded that UbcH6 is modified by ISG15 at least through Lys136.

Modification of UbcH6 by ISG15 Suppresses Its Formation of a Thioester Intermediate with Ubiquitin—We found that UbcH6 is covalently linked with ISG15 through Lys136 near the catalytic site Cys131. Based on

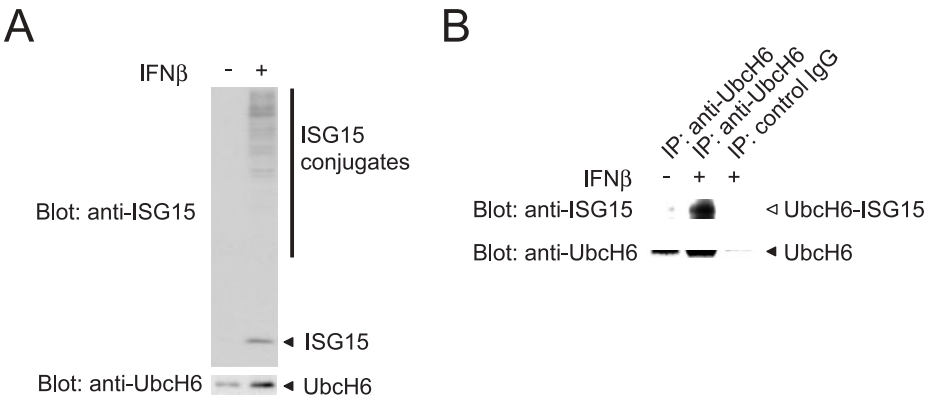

Fig. 4. **Interferon treatment induces ISG15 conjugation to UbcH6.** (A) An extract of A549 cells, which had been stimulated by treatment with IFNβ (1,000 units/ml) for 24 h, was subjected to Western blotting with anti-ISG15 or anti-UbcH6 antibody. (B) The extract in A was subjected to immunoprecipitation (IP) with anti-UbcH6 antibody or control IgG, and to Western blotting as in A.

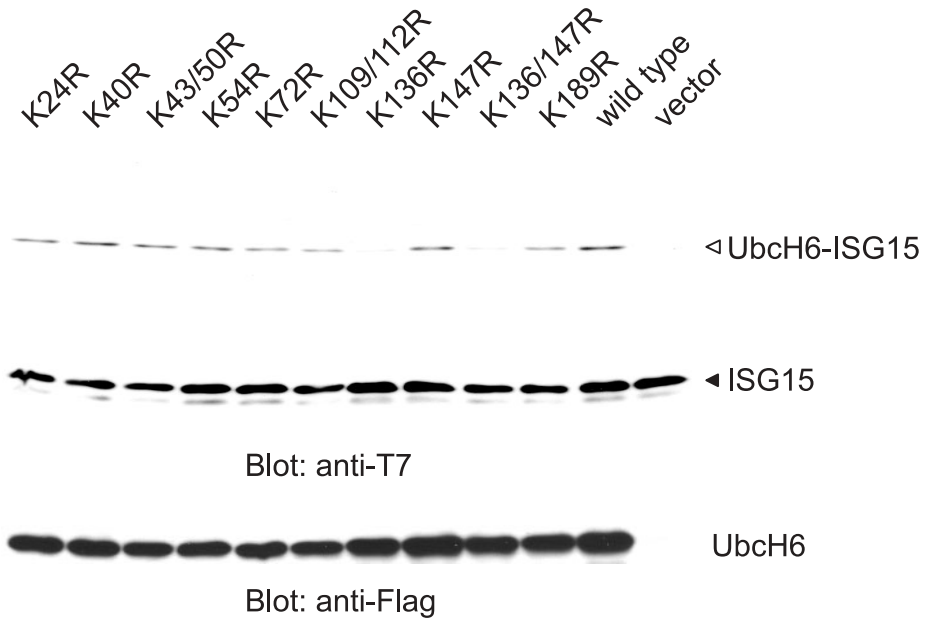

Fig. 5. **UbcH6 is conjugated with ISG15 through Lys136.** An extract of A549 cells that had expressed either Flag-tagged wild-type UbcH6 or its mutant, in which the Lys residue had been changed to Arg, together with T7-tagged ISG15 and S-tagged UBE1L was subjected to Western blotting with anti–T7 tag and anti–Flag tag antibodies.

this finding, we hypothesized that the modification of UbcH6 by ISG15 could prevent its formation of a thioester intermediate with ubiquitin. To examine this hypothesis, we isolated ISG15-modified and unmodified UbcH6 proteins, both of which were Flag-tagged, and then examined their abilities to form ubiquitin thioester intermediates. Either ISG15-modified or unmodified UbcH6 was incubated with GST-ubiquitin and E1 (Uba1, yeast E1 for ubiquitin conjugation), and then the reaction mixture was subjected to Western blotting with anti-Flag tag antibody under non-reducing conditions (Fig. 6A). In the case of unmodified UbcH6, a band (63 kDa) corresponding to a ubiquitin thioester intermediate was detected in the presence of GST-ubiquitin and E1 (Fig. 6A, lane 2, open arrowhead), while in the case of ISG15-modified UbcH6, a band corresponding to a ubiquitin thioester intermediate, the molecular mass of which was estimated to be approximately 80 kDa, was not detected under the same conditions (Fig. 6A, lane 7), suggesting that ISGylation of UbcH6 suppresses its ubiquitin-conjugating activity. The 63 kDa band detected under non-reduced conditions in the former case was not detected under reducing conditions (data not shown). It should be noted that the isolated ISG15-modified and -unmodified UbcH6 preparations contained ubiquitin-modified forms because a band exhibiting slower mobility than that due to the original one was detected in both cases. In the experiment shown in Fig. 6A, we used ISG15-modified and unmodified UbcH6 isolated from transfected cells. The construction of an *in vitro* ISG15 conjugation system is necessary to obtain pure ISG15-modified and -unmodified UbcH6 proteins.

Next, we analyzed the time-dependence of the ubiquitin thioester intermediate formation in the case of either ISG15-modified or -unmodified UbcH6 (Fig. 6B). In the case of unmodified UbcH6, the level of its ubiquitin thioester intermediate increased in a time-dependent manner (lanes 1–4), while in the case of ISG15-modified one, the pattern remained unchanged throughout the incubation time (lanes 5–8). These results support the above assumption that ISG15 conjugation to UbcH6 suppresses its ubiquitin E2 enzyme activity.

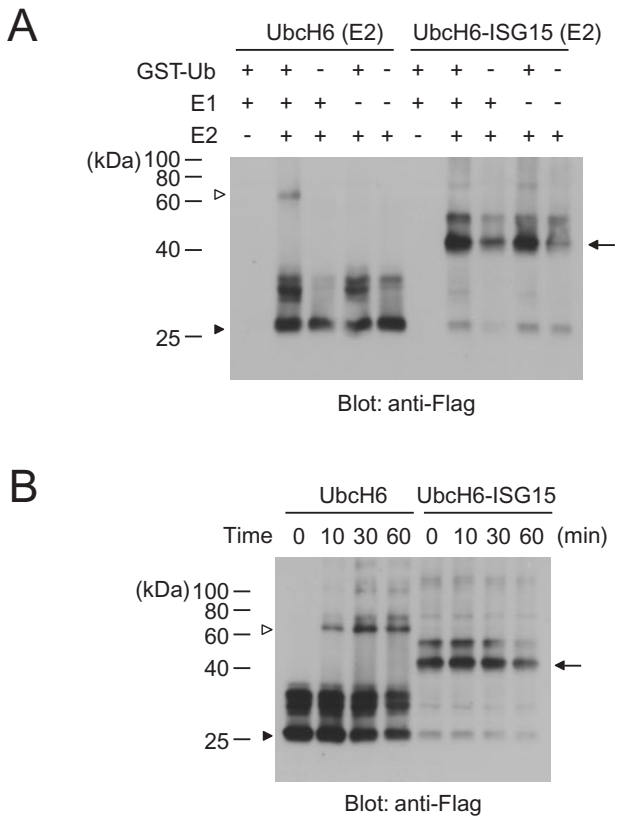

Fig. 6. **ISG15 modification of UbcH6 suppresses its formation of a thioester intermediate with ubiquitin.** (A) GST-ubiquitin (GST-Ub), Uba1 (E1), and either unmodified UbcH6 [UbcH6 (E2), lanes 1–5] or ISGylated UbcH6 [UbcH6-ISG15 (E2), lanes 6–10] were incubated as indicated at 30°C for 30 min, and then subjected to Western blotting with anti–Flag tag antibody under non-reducing conditions. (B) GST-ubiquitin, Uba1, and either unmodified UbcH6 (UbcH6, lanes 1–4) or ISGylated UbcH6 (UbcH6-ISG15, lanes 5–8) were incubated at 30°C for various times, and then subjected to Western blotting as in A. The original unmodified (lanes 1–5 in A and lanes 1–4 in B) and ISGylated UbcH6 (lanes 6–10 in A and lanes 5–8 in B) are indicated by a closed arrowhead and an arrow, respectively. The ubiquitin thioester intermediate in the case of unmodified UbcH6 is indicated by an open arrowhead.

DISCUSSION

In this study, we found that two ubiquitin E2 enzymes, UbcH6 and UbcH8, are susceptible to ISG15 conjugation (Figs. 1 and 4B), and that both UbcH6 and UbcH8 are capable of forming thioester intermediates with ISG15 (Figs. 2 and 3). The latter finding indicates that both the ubiquitin E2 enzymes function as E2 enzymes for ISG15 conjugation. In addition, we determined the ISG15 conjugation site in UbcH6, and its mutant, UbcH6 K136R, in which this ISG15 conjugation site, *i.e.*, Lys136, had been changed to Arg, was not susceptible to ISG15 conjugation (Fig. 5). The fact that Lys136 in UbcH6 is located near the active site Cys131 led us to assume that ISGylation of UbcH6 could suppress its formation of a ubiquitin thioester intermediate. We isolated ISG15-modified and unmodified UbcH6 proteins, and analyzed their abilities to form ubiquitin thioester intermediates. We found that a ubiquitin thioester intermediate was detected in the case of unmodified UbcH6, but not in that of the ISG15-modified form, strongly suggesting that ISG15 conjugation to UbcH6 suppresses its ubiquitin E2 enzyme activity (Fig. 6). Thus, we have obtained evidence of negative regulation by ISG15 conjugation of the ubiquitin E2 enzyme, which also functions as an ISG15 E2 enzyme.

In the ISG15 conjugation system, UBE1L has been identified as an E1 enzyme (*9*), while UbcH8 has been identified as an E2 enzyme (*10*, *11*). Both enzymes are IFN-inducible proteins. Our results clearly showed that UbcH6, an IFN-inducible protein, also functions as an E2 enzyme for ISG15 conjugation, the Cys131 residue being the catalytic site. Comparison of the amount of co-immunoprecipitated free ISG15 in the case of UbcH6 with that in the case of UbcH8 (Fig. 2, A and B, right panel) revealed that UbcH6 is a minor E2 enzyme for ISG15 conjugation, while UbcH8 is a major E2 enzyme. On the other hand, although the ISG15 E2 enzymes UbcH6 and UbcH8 themselves are conjugated with ISG15, comparison of the amount of the co-immunoprecipitated ISG15-bound form, *i.e.*, the

ISGylated one, in the case of UbcH6 with that in the case of UbcH8 (Fig. 2, A and B, right panel) revealed that UbcH6 is more susceptible to ISG15 conjugation than UbcH8 is. This ISGylation of UbcH6 increases on treatment with IFN (Fig. 4), and seems to require an additional factor present in A549 cells (Fig. 3).

Among the UbcH6 mutants in which the Lys residues had been changed to Arg, the K136R mutant is not susceptible to ISG15 conjugation (Fig. 5), clearly indicating that the Lys136 residue is the ISG15 conjugating site. To the best of our knowledge, this is the first evidence that ISG15 is covalently linked to a substrate through a specific Lys residue, and also the first construction of a non-ISGylated mutant of a substrate that is susceptible to ISG15 conjugation. The success in the above construction of a non-ISGylated UbcH6 mutant could enable us to investigate the role of ISGylation of UbcH6. Our finding that ISG15 conjugation to UbcH6 suppresses the formation of a thioester intermediate between UbcH6 and ubiquitin *in vitro* (Fig. 6) indicates that the ubiquitin-conjugating activity of UbcH6 is negatively regulated by ISGylation. With respect to UbcH6 functioning as a ubiquitin E2 enzyme, it has been reported that Topors, a ubiquitin E3 enzyme, together with UbcH6, functions in the ubiquitination of p53 (*20*). In connection with this, it should be noted that p53 is upregulated by IFN and plays a critical role in the antiviral defense of an infected host through its evoking an apoptotic response (*22*): In these circumstances, UbcH6 would be modified by ISG15 and become inactive, which would lead to suppression of the degradation of p53, resulting in the accumulation of p53 protein. In addition, UbcH6 functions in the self-ubiquitination of IAP, a ubiquitin E3 enzyme (*23*), but the link between the IAP function and protein ISGylation remains unclear.

It is well known that the ubiquitin conjugation system is linked to other ubiquitin-like protein conjugation systems. For example, SUMO-1 conjugation to IκBα stabilizes this protein by competing with ubiquitination for identical Lys residues, while NEDD8 conjugation to Cullin1, a subunit of the SCF E3 complex, is necessary for the exhibition of ubiquitin ligase activity (*3*, *21*, *24*). With respect to a link between ubiquitination and ISG15 conjugation, UBP43 functioning in de-ISGylation is ubiquitinated and degraded by the proteasome (*25*), and inhibition of the proteasome suppresses ISG15 expression but enhances ISGylation (*26*, *27*). In this study, we obtained novel evidence of a link between the ubiquitin conjugation system and the ISG15 conjugation system, *i.e.*, negative regulation of the ubiquitin E2 enzyme by ISG15 conjugation. Our recent finding that Ubc13, an IFN-non-inducible E2, is also negatively regulated by ISG15 conjugation (*28*) supports the above conclusion. Study of the control mechanism governing the E2 enzyme that has dual functions in ubiquitination and ISG15 conjugation is needed to understand the physiological role of protein ISGylation.

We thank Dr. K. Tanaka of the Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, for providing the plasmids. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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