UBP43 (USP18) Specifically Removes ISG15 from Conjugated Proteins

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UBP43 shows significant homology to well-characterized ubiquitin-specific proteases and previously was shown to hydrolyze ubiquitin-β-galactosidase fusions in *Escherichia coli*. In our assays, the activity of UBP43 toward Ub fusions was undetectable in *vitro* directing us to investigate the possibility of Ub-like proteins such as SUMO, Nedd8, and ISG15 as probable substrates. We consequently demonstrate that UBP43 can efficiently cleave only ISG15 fusions including native ISG15 conjugates linked via isopeptide bonds. In addition to commonly used methods we introduce a new experimental design featuring ISG15-UBP43 fusion self-processing. Deletion of the UBP43 gene in mice leads to a massive increase of ISG15 conjugates in tissues indicating that UBP43 is a major ISG15-specific protease. UBP43 is the first *bona fide* ISG15-specific protease reported. Both ISG15 and UBP43 genes are known to be strongly induced by interferon, genotoxic stress, and viral infection. We postulate that UBP43 is necessary to maintain a critical cellular balance of ISG15-conjugated proteins in both healthy and stressed organisms.

ISG15 is one of the most strongly induced genes after interferon (IFN) treatment (1, 2) and is also significantly induced by influenza B virus (3), lipopolysaccharide (4), and genotoxic stress (5). ISG15 was originally identified by Farrell et al. (1) and later characterized by Knight and co-workers (6, 7). Subsequently, the sequence of ISG15 protein was noted to possess significant homology to a diubiquitin sequence, accounting for its cross-reactivity with affinity purified anti-ubiquitin antibodies (8). Several reports demonstrate that ISG15 is released by various cell types and can act as cytokine leading to proliferation of NK cells (9–11). Most remarkably, ISG15 was found to be conjugated to intracellular proteins via an isopeptide bond in a manner similar to ubiquitin (Ub), SUMO, and Nedd8 (12). Conjugation of ubiquitin-like proteins (Ublls) involves a three-step mechanism whereby specific enzymes (or enzyme complexes) activate and covalently link Ublls to their substrates (13, 14). Narasimhan et al. (15) demonstrated that ISG15 conjugation occurs via a similar but distinct pathway compared with Ub conjugation. Yuan and Krug (3) discovered that an activating enzyme for ISG15 is UBE1L. Although the role of Ub, Nedd8, and SUMO conjugation has been assessed in numerous studies (16–20), the biological significance of ISG15 modification remains unknown and the proteins that are targeted by ISG15 have not been identified. It is unknown whether ISG15 conjugates can be targeted to proteasomes in a way similar to Ub conjugates. Alternatively, ISG15 conjugation might antagonize binding of Ub and save proteins from degradation or modify biological activities of targeted proteins as is the case with Nedd8 and SUMO modification (16, 18). Loeb and Haas (21) demonstrated that a substantial amount of ISG15 conjugates are co-localized with intermediate filaments of the cytoskeleton. It is therefore possible that one of the physiological roles of ISG15 modification is re-organization of the cytoskeleton after IFN stimulation. ISG15 sequences have been reported in fish, chick, and mammals. Despite the presence of Nedd8 and SUMO analogs in yeast, ISG15 is absent from this unicellular eukaryote and, therefore, the ISG15 regulatory pathway is suggested to be a relatively recent functional divergence (22). Our search of fully sequenced genomes of a nematode (*Caenorhabditis*), a plant (*Arabidopsis*), and an insect (*Drosophila*) also did not reveal a candidate for an ISG15 ortholog. It is therefore possible that the ISG15-conjugation system is restricted to higher animals with evolved IFN signaling.

UBP43 (USP18 in standard nomenclature proposed by Baker et al. (23)) was initially cloned in our laboratory (24, 25) and later, independently by three other groups; cDNA coding for porcine UBP43 was cloned in a differential screen from lung macrophages of virus-infected swine (26), Li et al. (27) identified mRNA for human UBP43 in a screen for RNase L substrates, and Kang et al. (28) cloned human UBP43 in a screen for genes induced by IFN in melanoma cell lines. Significant up-regulation of the UBP43 gene by viral infection (or double-stranded RNA), by IFN and lipopolysaccharide suggests that UBP43 might be involved in a number of processes including the control of cell proliferation, inflammation, stress, and immune response. Several regions in the UBP43 sequence exhibit homology to catalytic domains of Ub-specific proteases (USPs) which remove Ub from conjugated proteins. The USP family may include more than 100 proteins which differ in size and...
amino acid sequence, yet all share several highly homologous patches around the residues required for catalytic activity (29–32). Using a conventional assay that involves co-transformation of two plasmids into Escherichia coli (first coding for USP enzyme and the second for Ub-β-galactosidase fusion as a substrate), UBP43 was demonstrated to have proteolytic activity against Ub fusion, and was thought to work on Ub conjugates (24, 25). However, our subsequent experiments failed to detect activity of UBP43 toward Ub fusions in vitro. Similarity in the expression patterns of ISG15 and UBP43 genes lead us to hypothesize that ISG15 conjugates may be the preferred substrates for UBP43. We consequently investigated the ability of UBP43 to cleave ISG15 and other known Ub-like proteins (namely SUMO, Ned8, and Ub) from artificial substrates.

Here we demonstrate that UBP43, a member of the Ub-specific protease family, cleaves both artificial and native ISG15 conjugates. No other USPs were specific to ISG15. Therefore, UBP43 is the first ISG15-specific protease reported. Significantly, in vivo mouse data also demonstrate that UBP43 is a major ISG15-specific protease and activity of this enzyme is crucial for proper cellular balance of ISG15-conjugated proteins.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—pcDNA6-UBP43 construct for expression UBP43-V5-6His fusion in mammalian cells has been previously described (23). Plasmids expressing the ubiquitin-specific proteases UBP43 (as GST fusion), UBP1 (33), Unp, and Unp(mut) (34) as well as pUb-GSTP1 (35) construct were provided by Dr. R. Baker (Australian National University, Canberra). pGEX-Ned8-gsPESTc and pGEX-SUMO-gsPESTc plasmids were from Dr. K. Tanaka (The Tokyo Metropolitan Institute of Medical Science, Japan) (36). pGEX-ISG15-Recp and pRSV-ISG17 plasmids were received from Dr. A. L. Haas (Medical College of Wisconsin, Milwaukee, WI). To produce pGEX-ISG15-gsPESTc and pGEX-ISG17-gsPESTc expressing GST-UBP43-His6 fusions produced less than 30% of full-length UBP43. Therefore the gene was expressed in nutrient-poor medium M9 and induction of expression occurred as either GST-UBP43 or UBP43-6His fusions (both wild-type and mutant versions). One liter of E. coli culture was transformed with pGEX-UBP43-H plasmid that had been grown in LB broth to Optical Density 0.8 and protein expression was induced with isopropyl-1-thio-β-D-galactopyranoside. After 3 h of induction at 21 °C, cells were harvested by centrifugation, washed with PBS, and lysed by sonication in PBS adjusted to 10 mM imidazole, 300 mM NaCl, 2 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride. Lysates were cleared by 15 min centrifugation at 16,000 x g and UBP43-V5–6His fusions were absorbed to 40 µl of Ni-NTA-agarose (Qiagen, Valencia, CA). After elution with PBS containing 300 mM NaCl, 250 mM imidazole, and 0.1% n-octyl β-D-glucopyranoside, the fusions were absorbed to 0.6 ml of GSH-agarose. The resin was washed with PBS, 0.1% n-octyl β-D-glucopyranoside, resuspended in 10 ml Tris, pH 7.5, 30% glycerol and stored at −20 °C until use. All purification procedures were performed at 4 °C.

**Purification of UBP43 from 293T Cells**—2 × 10^9 293T cells were seeded on 60-mm cell culture plates and transfected with pcDNA6-UBP43 plasmids using Polyfect reagent (Qiagen) according to the manufacturer’s instructions. Thirty-six hours after transfection cells were washed with PBS and harvested into 1 ml of lysis buffer (PBS adjusted to 10 mM imidazole, 0.1% n-octyl β-D-glucopyranoside, 300 mM NaCl, 2 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin) and then lysed by a 5-s pulse of sonication. Lysate was cleared by a 5-min centrifugation at 16,000 x g and UBP43-V5–6His fusions were absorbed to 40 µl of Ni-NTA-agarose for 1 h. Beads were washed once with 1 ml of PBS adjusted to 300 mM NaCl, 20 mM imidazole, and 0.1% n-octyl β-D-glucopyranoside, twice with the same buffer containing 4 M urea, and twice with the buffer with no urea. Beads were resuspended in 80 µl of 10 mM Tris, pH 7.5, 5 mM EDTA, 5 mM dithiothreitol, 20 mM imidazole, and 0.1% n-octyl β-D-glucopyranoside. The suspensions (15 µl per enzymatic reaction, 1 µg of total protein, and 0.1 µg of UBP43 fusion) were immediately used for enzymatic assay. Equal efficiency of expression, and purification between wild-type and mutant UBP43s was confirmed by Western blot with anti-V5 antibodies. To assess the ability of UBP43 and ISG15-UBP43 fusions to undergo self-hydrolysis, E. coli were transformed with pET-Ub-UBP43-H and pET-ISG15-UBP43-H carrying wild-type or inactive versions of UBP43. Production of UBP43 in E. coli grown in rich media, such as LB, resulted in high yield but massive (more than 70%) degradation of UBP43. On the contrary, growth of E. coli in nutrient-poor medium M9 and induction of expression at low temperature, resulted in production of small quantities of full-length UBP43. Therefore, in this experiment, E. coli cultures were grown in 5 ml of M9 minimal medium to Optical Density 0.8 and induced with isopropyl-1-thio-β-D-galactopyranoside for 4 h at 21 °C. Cells were harvested, lysed by sonication, and after removal of insoluble material at 15,000 x g for 5 min, supernatants were resolved on 10% SDS-PAGE, electroblotted, and then probed with antibodies against the NH2-terminus of UBP43.

**Mouse Lung Protein Extracts and Western Blotting**—Lungs were surgically removed from euthanized wild-type and UBP43−/− mice (3.5-week old) washed in cold PBS and kept on ice. For preparation of total protein extracts a pair of lungs was homogenized by sonication (four 8-s pulses) in 1 ml of 20 mM Tris, pH 7.5, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Sigma, product number...
Triton X-100 and β-mercaptoethanol were added to 1% and 2 mM, respectively, and suspensions were incubated on ice for 10 min. Particulate material was removed by centrifugation at 18,000 g for 5 min and supernatants were stored at 80 °C until use.

For preparation of cytoskeleton-enriched fraction, lungs combined from three mice were homogenized using a tissue homogenizer in 5 ml of 20 mM Tris, pH 7.5, 2 mM EGTA, 100 mM NaCl, 2 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Particulate was collected by centrifugation for 10 min at 16,000 g and thoroughly washed twice with 7 ml of the same buffer. The final pellet was then resuspended in 1 ml of 10 mM Tris, pH 8.0, 5 mM EDTA, 100 mM NaCl, 5 mM β-mercaptoethanol, 2 mM CaCl₂, and 0.5% n-octyl β-D-glucopyranoside. The suspension was then briefly sonicated and any insoluble material removed by 10 min centrifugation at 15,000 × g. Supernatant designated as “cytoskeleton-enriched fraction” was stored at −80 °C.

Rabbit polyclonal IgGs against human ISG15 were kindly provided.

**Fig. 1. Identification of substrate and cleavage site for UBP43 enzyme.** SUMO-gsPESTc, Nedd8-gsPESTc, ISG15-gsPESTc, and Ub-gsPESTc were expressed in *E. coli*, purified, and gsPESTc extensions were radiolabeled as described under “Experimental Procedures.” A, GST-UBP43-H fusions carrying wild-type or inactive versions of UBP43 were expressed in *E. coli* and purified by affinity chromatography. Ubl-gsPESTc fusions were incubated with either reaction buffer (mock), or with 200 ng of either GST-UBP43(wt)-H (WT) or GST-UBP43(mut)-H (mut) and separated on 20-cm long Tris-Tricine SDS-PAGE (16% acrylamide) to allow detection of gsPESTc (38). Note that ISG-gsPESTc migrates at a rate slower than expected in this gel system. B, wild-type or inactive versions of UBP43 were expressed in transiently transfected 293T mammalian cell line as His-tagged proteins and purified from cell lysates by affinity chromatography on Ni-agarose beads. Reaction was carried out with either reaction buffer (mock), with Ni-agarose beads incubated with lysates from untransfected 293T cells (293T), with Ni-agarose beads incubated with lysates from 293T cells transfected with the plasmids carrying either wild-type (WT) or inactive (mut) UBP43. The reactions were separated on a 4-cm long minigel using standard SDS-PAGE (15% acrylamide) (39). Note that gsPESTc co-migrates with SDS in this gel system and therefore is undetectable. C, GST-pro-ISG15-gsPESTc was expressed in *E. coli* and purified on GSH-agarose. Large scale reactions with wild-type or inactive versions of UBP43 were carried out as in B. Inset shows Coomassie-stained minigel of 20% of each reaction. Products of the reaction (UBP43-wt) were selectively precipitated with trichloroacetic acid, and molecular mass of recovered carboxyl-terminal peptide was determined by MALDI-TOF. The predicted molecular mass of the released peptide is 3058.17 Da. The minor peak at 1530 Da corresponds to double-charged peptide.

**Fig. 2. ISG15-gsPESTc cannot be cleaved by other USPs.** Ub-gsPESTc and ISG15-gsPESTc were expressed in *E. coli*, purified, and radiolabeled as described under “Experimental Procedures.” Total cell lysates (10 μg of protein) of either untransformed *E. coli* (*E. coli*) or expressing UBP41, UBP1, Unp (Unp-wt), inactive Unp (Unp-mut), UBP43 (UBP43-wt), or inactive UBP43 (UBP43-mut) were incubated with Ubl-gsPESTc fusions and resolved on minigels as in Fig. 1B.
by Dr. E. Borden (Cleveland Clinic Foundation, OH) and were used at a final concentration of 0.5 μg/ml (11). Rabbit anti-serum against Ub was purchased from Sigma and used at 1:300 dilution.

For anti-UBP43 antibodies the NH2-terminal part (amino acids 1–120) of ubiquitin (Ub) was produced in E. coli as a GST fusion and purified on GSH-agarose. Immunization of rabbits with GST-UBP43(1–120) attached to GSH-agarose was performed at Charles River Pharmaceuti-
services (Southbridge, MA). Total IgGs were purified on a protein A column (Amersham Biosciences, Inc.) and GST-specific antibodies were removed by passing the total IgG fraction through GST-agarose (Fierce). The resulting IgG fraction was subjected to purification of UBP43-specific antibodies on Affi-Gel resin (Bio-Rad, Hercules, CA) to which GST-UBP43 fusion protein was coupled. All chromatographic procedures were performed according to the instructions of the respective manufacturers.

Proteins were electrophoretically transferred onto nitrocellulose membranes (Amersham Biosciences Inc.). Where indicated, membranes were stripped after the first blot in 50 mM Tris, pH 7.0, 2% SDS, and 50 mM dithio-
reitol at 55°C for 30 min. Chemiluminescence system was from PerkinElmer Life Sciences (Boston, MA).

RESULTS

UBP43 Exhibits Specificity Toward ISG15 In Vitro—Despite significant homology of UBP43 to well characterized Ub-specific proteases and detection of activity in E. coli using Ub-β-
galactosidase fusion protein (24), we were not able to detect activity of UBP43 in vitro using a variety of Ub fusions. This fact prompted us to analyze activity of UBP43 toward other major UbIs, namely SUMO, Nedd8, and ISG15. A sensitive assay originally developed by Woo et al. (37) was selected for this purpose. Each Ub produced in E. coli had a cleavable carboxyl-terminal extension of the following sequence GSMHISPESEEEEEHYC (referred to as gsPESTc) (36). To avoid covalent modifications to the backbones of Ubls caused by 125I attachment and possible artifacts in the assay we used conditions under which 125I exclusively labels the tyrosine in gsPESTc extension. As a consequence, Ubl-gs-
PESTc fusion (detectable when intact) after hydrolysis loses the labeled gsPESTc extension and appears as unlabeled and therefore undetectable on autoradiograms. gsPESTc due to its low molecular mass could be detected on Tris-Tricine (38) but not on regular SDS-PAGE (39). As evidenced by disappearance of the ISG15-gsPESTc band and appearance of the gsPESTc band, UBP43 hydrolyzed only ISG15-gsPESTc and not the Ub-, Nedd8-, or SUMO-gsPESTc fusions (Fig. 1A). The mutant version of UBP43, in which the cysteine residue critical for activity is converted to serine, was inactive toward any substrate. To confirm this observation with mammalian-expressed UBP43 we transfected 293T cells with either wild-type or mutant His6-
tagged UBP43. The wild-type version of UBP43-H purified on Ni-agarose efficiently digested ISG15-gsPESTc fusion while mutated UBP43-H did not (Fig. 1B).

Members of USP family including pro-ISG15 processing en-
zyme (22) perform cleavage of substrates immediately after LRRG motif (identical in Ub and ISG15) yielding functional carboxyl-terminal di glycine available for conjugation. To con-
firm that hydrolysis catalyzed by UBP43 occurs at the same site, a larger processing reaction similar to that of Fig. 1B was carried out on unlabeled GST-pro-ISG15-gsPESTc. As evi-
denced by band shift (Fig. 1C, inset), the incubation of GST-
pro-ISG15-gsPESTc with wild-type UBP43-H resulted in re-
moval of the carboxyl-terminal octapeptide extension with gsPESTc peptide (TGETPGGRSGMHSIPPESEEEEEHYC) leaving mature ISG15. The released peptide was recovered from the reaction by selective precipitation with trichloroacetic acid (22, 37) and its exact molecular mass was determined by MALDI analysis. The molecular mass obtained in this experiment (3059 Da; Fig. 1C) corresponded with the predicted mass (3058.17 Da) of the released peptide suggesting that UBP43 cleaves ISG15 at the expected site following diglycine.

The use of ISG15-gsPESTc fusion has never been reported before and UBP43 is the first enzyme to hydroyze ISG15-
gsPESTc. Therefore, to eliminate a possibility of experimental artifacts we tested a yeast (UBP1) and two mammalian USPs (UBP41 and Unp) in an identical assay. None of these three USPs could hydrolyze ISG15-gsPESTc (nor could they hydro-
lyze Nedd8- or SUMO-gsPESTc, not shown) while, as expected, all three efficiently hydrolyzed Ub-gsPESTc (Fig. 2). The re-
sults presented in Figs. 1 and 2 strongly suggest that UBP43 is an ISG15-specific protease that exhibits no proteolytic activity toward other Ubls.

UBP43 Preferentially Hydrolyzes ISG15 Fusions in E. coli—Detection of Ub specific activity in E. coli co-transformed with Ub-β-galactosidase and UBP43 expressing constructs contra-
dicted the ISG15-specific activity presented in Fig. 1. In a standard co-transformation assay, Ub-β-galactosidase is ex-
pressed from low-copy number pACYC plasmid, whereas USPs are expressed from high-copy number plasmids. Such a differ-
ence in expression is likely to generate significant excess of enzyme over the substrate which may permit proteolytic activ-
ity on Ub fusions even though they are not preferred sub-
strates. We therefore redesigned the E. coli assay to allow one copy of UBP43 to be synthesized per one copy of Ub or ISG15 by making Ub-UBP43 and ISG15-UBP43 fusions. Only ISG15-
UBP43 (wt) fusion could be efficiently hydrolyzed while ex-
pessed in E. coli (Fig. 3). No hydrolysis could be observed when ISG15-UBP43 (mut) was expressed and, consistent with in vitro experiments, no hydrolysis was observed when either wild-type or mutated versions of UBP43 were fused to Ub.

UBP43 Hydrolyzes Native ISG15 Conjugates in Vitro—Use of artificial substrates such as Ub fusions (Ub-carboxyl-termi-
nal extension proteins) in vitro is a commonly used technique, yet, even when an excess of enzyme is used and incubation times are extended, not all USPs that are able to hydrolyze Ub fusions are able to cleave an isopeptide bond, i.e. the bond between the Ub and the side chain on the lysine residue of the targeted protein (40). Activity of UBP43 in vitro and in E. coli was detected against ISG15 carboxyl-terminal extension pro-
teins and not against isopeptide bonds. Therefore, to demon-
strate activity of UBP43 against isopeptide bonds and to con-
firm specificity of UBP43 observed in vitro we used cytoskeletal-enriched fractions (CEF) of UBPR21/C mouse lungs. It has been reported that a substantial portion of cy-
toskeletal proteins (or proteins associated with cytoskeleton) are modified by ISG15 (21). We found that, CEF contained no detectable proteolytic activity (not shown) and only trace amounts of unconjugated ISG15. Incubation of purified GST-
UBP43–6His fusion with CEF lead to an obvious decrease of

![Fig. 3. ISG15-specific activity of UBP43 in E. coli.](image-url)
UBP43 removes ISG15 from native ISG15 conjugates in vivo. Proteins of cytoskeleton-enriched fraction (CEF) from lungs of UBP43<sup>−/−</sup> mouse were either incubated with reaction buffer (CEF), or with 4 μg of either GST-UBP43(WT-H) (CEF+WT) or GST-UBP43(mut)-H (CEF+mut) purified from E. coli. Lanes WT and mut contain only respective GST-UBP43-H proteins. Reaction products were separated on 15-cm long, 8% SDS-PAGE. The proteins were visualized on the membrane by Ponceau S staining (Sigma). After probing with anti-ISG15 the membrane was stripped and reprobed with anti-Ub antibodies. Positions of molecular mass standards are indicated on the left. Note that heavy nonspecific bands correspond to GST-UBP43-H and its degradation products.

ISG15 conjugates which resulted in an increase of free ISG15 (Fig. 4). Consistent with our experiments in Figs. 1–3, no Ub specific activity was detected in this assay. This result demonstrates that UBP43 can cleave isopeptide bonds of ISG15 and not Ub conjugates.

Deletion of the UBP43 Gene in Mice Increases the Level of Intracellular ISG15 Conjugates—to assess UBP43 specificity in vivo and to comprehensively characterize the biological function of UBP43, our laboratory has produced a Knockout mouse model in which the UBP43 gene has been deleted by homologous recombination. Western blot analysis demonstrates a massive increase of ISG15 conjugates in lung (Fig. 5) and other tissues (not shown) of UBP43<sup>−/−</sup> mice. The absence of an increase of Ub conjugates in the same tissues provides additional support for UBP43 specificity toward ISG15 conjugates. Significantly, this massive increase of ISG15 conjugates suggests that UBP43 is a major ISG15-specific protease in mammals.

**DISCUSSION**

UBP43 shows significant homology to well characterized ubiquitin-specific proteases and initially was thought to work on Ub conjugates. In this work, using several experimental approaches we unambiguously demonstrate that UBP43 can efficiently cleave only ISG15 conjugates. A massive increase of ISG15 conjugates in tissues of UBP43<sup>−/−</sup> mice is consistent with the results of our enzymatic assays and indicates that UBP43 is a major ISG15-specific protease.

The ubiquitin-specific protease family has been characterized and may include more than 100 members (29–32, 41). Although the overall degree of homology between individual enzymes is low, all of them share high homology in several active site regions. Sequence analysis of UBP43 clearly identifies this protein as a member of this family (24–26). The deubiquitinating activity previously observed in E. coli (24, 25) using Ub-β-galactosidase fusion protein, however, was not supported by our subsequent attempts to detect activity in vitro and was attributed to high lability of UBP43. In this work we developed a construct and purification strategy allowing production of UBP43 that is functional in vitro. UBP43 produced either in E. coli or in mammalian cells was able to cleave only ISG15-gsPESTc fusion and not the Ub-, Nedd8-, or SUMO-gsPESTc fusions (Fig. 1). The previously reported Ub specific activity of UBP43 in E. coli may be attributed to an improper enzyme to substrate ratio. In conventional deubiquitination assays that utilize E. coli (42), Ub-β-galactosidase is expressed under the control of weak promoter from low-copy number pACYC plasmid (pA15 origin of replication; one plasmid copy per cell). At the same time, USPs are expressed under the control of strong promoters from high-copy number plasmids (ColE1 origin of replication; 50 plasmid copies per cell). Such differences in expression are likely to generate a significant excess of enzyme over substrate (more than 50:1), which may explain the previously observed proteolytic activity of UBP43 on Ub fusions even though they are not optimal substrates. To obtain a stoichiometric substrate/enzyme ratio we produced substrate-enzyme fusions thus ensuring one copy of UBP43 was synthesized per one copy of Ub or ISG15. Evidently from results presented in Fig. 3, only ISG15-UBP43 fusion can be self-hydrolyzed (most likely via intermolecular hydrolysis) supporting the results of the in vitro assays. Finally, we demonstrate that UBP43 can hydrolyze the native ISG15-isopeptide bond conjugates (Fig. 4). To the best of our knowledge, UBP43 is the only second enzyme in the USP family that has been demonstrated to hydrolyze native Ub-isopeptide bond conjugates in vitro. UBP-t1 and its isomorph UBP-t2 have been reported to hydrolyze protein extracts from testes (43). The reason for difficulties in detection of enzymatic activity toward native isopeptide-bond Ub conjugates is likely to be due to instability of USPs and associated problems in purification of enzyme in the active form.

The massive increase of ISG15 but not Ub conjugates in tissues of UBP43<sup>−/−</sup> mice (Fig. 5) provides additional support for the specificity of UBP43 toward ISG15. The dramatic difference between wild-type and UBP43<sup>−/−</sup> mice indicates that UBP43 is the major ISG15-specific protease.

In vivo ISG15 is synthesized as an inactive 17-kDa precursor (pro-ISG15 or ISG17) from which eight COOH-terminal amino acids are removed by a processing protease. After processing, the COOH-terminal diglycine motif becomes accessible for activation and ISG15 can be conjugated (22). Here we report the use of artificial substrates in which a cleavable extension is fused in the same way as in pro-ISG15. For the experiment in Fig. 1C we used a fusion of the actual pro-ISG15. Activity of UBP43 against such fusions suggests that UBP43 may be capable of pro-ISG15 processing in vivo. However, if UBP43 was a processing enzyme for pro-ISG15 the deletion of this gene in mice...
should have lead to a block of processing and inactivation of the ISG15 conjugation mechanism. The observed accumulation of ISG15 conjugates in UBP43−/− mice suggests that pro-ISG15 is being efficiently processed and the resultant ISG15 conjugated. Furthermore, a major processing protease for pro-ISG15 has been purified and its partial sequence has been determined (22). There is no homology between the reported sequence and sequence of UBP43. These two observations argue against the role of UBP43 in maturation of pro-ISG15.

Our most recent study related to generation and characterization of UBP43-deficient mice provides solid evidence for an important biological role of UBP43 and balance of ISG15-conjugates under normal and stress conditions. Although no proteins modified by ISG15 have been identified and the physiological significance of ISG15 modification has not been established, certain speculations can be made. Both ISG15 (1–3) and UBP43 (26–28) can be significantly induced by viruses (or dsRNA) and IFN. We recently characterized transcriptional activation of UBP43 in response to lipopolysaccharide and demonstrated that the molecular mechanism of UBP43 regulation is very similar to that of ISG15. Expression of the ISG15-activating enzyme UBE1L is also IFN-dependent (3) and the promoter of UBE1L possesses IFN-stimulated response elements similar to those found in promoters of UBP43 and ISG15. Coordinated induction of ISG15, UBP43, and UBE1L suggests that ISG15 conjugation is a dynamic process and that a critical balance of ISG15 modification should be maintained at all times. Indeed, overexpression of UBP43 in a monoblast cell line M1 caused delayed of terminal differentiation into macrophages suggesting that a decrease of ISG15 conjugation may alter the differentiation program of the cell (24). The ISG15 activating enzyme UBE1L was found to be absent in 14 different lung cancer cell lines suggesting that decrease of ISG15 conjugation may contribute to carcinogenesis (44). Certain viruses can specifically block conjugation or synthesis of ISG15 (3) possibly in an attempt to suppress host-cell suicide and inflammatory response. Such reports and the fact that IFN suppresses cell proliferation may suggest that balance of ISG15 conjugation is important for the control of cell differentiation and growth suppression. The anti-proliferative activity of IFN has been utilized in cancer therapy, yet, the molecular mechanisms by which IFNs elicit their growth inhibitory and antitumor effects remain largely unknown. Current progress in identification of ISG15 activating (UBE1L (3)) and de-conjugating (UBP43, this work) enzymes should allow to address the biological role of ISG15 modification.

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