Protein ISGylation modulates the JAK-STAT signaling pathway

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ISG15 is one of the most strongly induced genes upon viral infection, type I interferon (IFN) stimulation, and lipopolysaccharide (LPS) stimulation. Here we report that mice lacking UB4P3, a protease that removes ISG15 from ISGylated proteins, are hypersensitive to type I IFN. Most importantly, in UB4P3-deficient cells, IFN-β induces a prolonged Stat1 tyrosine phosphorylation, DNA binding, and IFN-mediated gene activation. Furthermore, restoration of ISG15 conjugation in protein ISGylation-defective K562 cells increases IFN-stimulated promoter activity. These findings identify UB4P3 as a novel negative regulator of IFN signaling and suggest the involvement of protein ISGylation in the regulation of the JAK-STAT pathway.

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Interferons [IFNs] are important modulators of immune, inflammatory, and antiviral functions, as well as cell survival and proliferation [Stark et al. 1998]. They exert signals through the activation of the JAK-STAT pathway that mediates rapid induction of IFN-stimulated genes [ISGs; Darnell et al. 1994; O’Shea et al. 2002]. ISG15 (or UCRP) encodes a 15-kD protein which is strongly induced after IFN treatment and has significant sequence homology to ubiquitin [Blomstrom et al. 1986; Haas et al. 1987]. ISG15 can also be conjugated to intracellular proteins via an isopeptidase bond in a manner similar to ubiquitin and other ubiquitin-like modifiers [ubls], SUMO and Ned8. In contrast to other ubls, ISG15 has not been found in lower organisms, such as yeast, nematode, insects, and plants, indicating that it may be associated with specialized functions in vertebrates. Protein modification by ubiquitin, SUMO, and Ned8 has been demonstrated to play important roles in various cellular functions, including cell cycle regulation, signal transduction, transcription, and antigen presentation [Hochstrasser 2000; Jentsch and Pyrowolakis 2000; Hicke 2001; Pickart 2001]. However, little is known about the function of protein modification by ISG15 (ISGylation). Furthermore, despite the obvious relevance of its role in IFN signaling, this function has not been explored.

We previously cloned an ISG15-specific protease, UB4P3 (USP18; Liu et al. 1999; Malakhov et al. 2002), which belongs to the ubiquitin-specific protease [UBP or USP] family [Hochstrasser 1996; Wilkinson 1997]. UB4P3 expression is strongly activated by IFN or lipopolysaccharide [LPS] treatment [Zhang et al. 1999; Li et al. 2000, 2001; Kang et al. 2001; Malakhova et al. 2002]. In an effort to explore the function of protein ISGylation, we generated UB4P3 knockout mice [Ritchie et al. 2002]. UB4P3−/− mice were viable at birth but gradually manifested neurological disorders associated with the development of hydrocephalus [Ritchie et al. 2002]. Despite the potent relevance of UB4P3 to the development of the hematopoietic system [Liu et al. 1999], analyses of peripheral and bone marrow blood cells of UB4P3-null mice did not reveal any significant defect [data not shown]. Both UB4P3 expression and conjugation of ISG15 are strongly induced by IFN [Loeb and Haas 1992; Li et al. 2000], indicating that the level of ISG15 conjugates is tightly controlled. We hypothesized that ISGylation may affect the cellular response to IFN. To test this hypothesis, we studied the phenotype of UB4P3 knockout mice in response to IFN stimulation. Here, we report that UB4P3-deficient cells are hypersensitive to type I IFN and undergo apoptosis upon IFN stimulation. Lack of UB4P3 activity results in a profound increase in the level of protein ISGylation that is associated with the enhanced and prolonged JAK-STAT signaling. Furthermore, the role of ISGylation in positive regulation of IFN signaling is also confirmed in reconstitution assays of UBE1L-deficient K562 cells. These data suggest that UB4P3 may act as a negative feedback in the IFN-activated signaling pathway by decreasing levels of protein ISGylation.

Results and Discussion

UBP43-null mice are hypersensitive to potent IFN inducer-poly I-C

Injection with synthetic double-stranded RNA, polyinosinic acid–polycytidyl acid [poly(I-C), has been commonly used to induce endogenous IFN production in mice [Kuhn et al. 1995]. Therefore, we used poly(I-C) to assess the differences in response of UB4P3−/− and UB4P3+/+ mice. After daily poly(I-C) injections, UB4P3−/− mice (n = 7) survived the course of treatment. In contrast, all UB4P3−/− mice (n = 7) died within 72 h posttreatment [Fig. 1A]. We also analyzed the effect of poly(I-C) treatment on hematopoietic cells. As shown in Figure 1B and C, UB4P3−/− mice responded to poly(I-C) by exhibiting a 20% decrease in number of total peripheral white blood cells and a 30% decrease in total bone marrow nucleated cells. A much stronger response was observed in UB4P3−/− mice. There were dramatic decreases (>90%) in the total number of white blood cells in peripheral blood and nucleated cells in bone marrow.
of the UBP43−/− mice. These results indicate that UBP43-deficient mice are hypersensitive to polyI-C.

Hypersensitivity to IFN is intrinsic in UBP43-deficient hematopoietic cells

After birth, UBP43−/− mice gradually show defects associated with the central nervous system. To confirm that the hypersensitivity of hematopoietic cells to IFN is not due to other metabolic and physiological defects in UBP43−/− mice, we performed bone marrow transplantation experiments. The UBP43−/− mice used in this study were initially generated by crossing 129 and C57BL/6 strains, and back-crossing to the C57BL/6 strain. Therefore, their hematopoietic cells are CD45.2+. C57BL/6 strains, and back-crossing to the C57BL/6

The main reasons for such decreases were explained by a <10% reduction in colony number. These results directly related to type I IFN stimulation, whereas UBP43−/− bone marrow cells showed only a 40% reduction in colony formation upon IFN stimulation, whereas UBP43+/+ bone marrow cells formed no colonies at all (data not shown). Such a difference in colony formation clearly indicated an abnormal sensitivity of UBP43-deficient cells to IFN-β. Furthermore, this sensitivity appeared to be dose-dependent: 50 units/mL of IFN-β resulted in an >80% reduction of UBP43−/− bone marrow colony numbers (Fig. 2B) as well as a marked decrease in colony size (data not shown). Similar CFU assays using UBP43+/+ cells showed the appearance of regular-sized colonies and a <10% reduction in colony number. These results directly demonstrate the hypersensitivity of UBP43−/− cells to type I IFN stimulation. Furthermore, consistent with UBP43 enzymatic specificity (Malakhov et al. 2002), a significantly higher level of protein ISGylation was detected in UBP43−/− bone marrow cells relative to UBP43+/+ bone marrow cells (Fig. 2C).

Augmented apoptosis of UBP43-deficient cells is specific to type I IFN stimulation

A number of previous studies showed a reduced number of cells subsequent to IFN treatment (Stark et al. 1998). The main reasons for such decreases were explained by an IFN-mediated antiproliferative effect and/or apoptosis (Stark et al. 1998). To determine whether the hypersensitivity of UBP43-deficient bone marrow cells to IFN is associated with augmented apoptosis, we conducted a TUNEL assay on liquid bone marrow culture of UBP43+/+ and UBP43−/− cells following 48-h culture in the absence or the presence of 100 units/mL of IFN-β. As presented in Figure 3A, the percentage of apoptotic cells in UBP43+/+ bone marrow cell culture remained constant with the addition of IFN-β. However, a significant increase in the number of apoptotic cell was observed in UBP43−/− bone marrow cells. To confirm that the observed increase of apoptosis in UBP43−/− cells was due
to a lack of UBP43, we also reintroduced UBP43 into UBP43−/− cells using a retroviral expression vector MigR1 [MSCV-ires-EGFP, Pear et al. 1998]. Because UBP43 and EGFP were translated from the same transcript, EGFP+ cells also ectopically expressed UBP43. Compared to EGFP− cells, EGFP+ cells [UBP43-expressing] displayed significantly reduced apoptosis upon IFN-β treatment [Fig. 3B]. MigR1 vector alone infection did not rescue cells from IFN-induced apoptosis [data not shown]. These results demonstrate that an augmented rate of apoptosis correlates with the hypersensitive response of UBP43-deficient cells to type I IFN.

In the next experiment we investigated whether other proapoptotic cytokines could have a similar effect on UBP43−/− cells. TNF-α and the type II IFN, IFN-γ are commonly used to study bone marrow cell growth and apoptosis and in the analysis of various knockout mouse models. Therefore, we performed liquid bone marrow cell culture assays to study the effect of TNF-α, IFN-γ, and IFN-β on UBP43+/− and UBP43−/− cells. As expected, IFN-β treatment resulted in a substantial increase of apoptosis in UBP43−/− cells, whereas IFN-γ and TNF-α did not cause a significant difference in cell death between UBP43+/− cells and UBP43−/− cells [Fig. 3C]. This result indicates a specific role of type I IFN in the induction of apoptosis in UBP43-deficient cells.

**JAK-STAT signaling is extensively activated in UBP43-null cells upon IFN stimulation**

Tremendous efforts have been made over the past years to understand the molecular basis of IFN action. The binding of type I IFN to its receptor leads to the activation of tyrosine kinases Jak1 and Tyk2, and the subsequent recruitment and tyrosine phosphorylation of Stat1 and Stat2 [Stark et al. 1998; Levy 1999; Ihle 2001; O'Shea et al. 2002]. Phosphorylated Stat1 and Stat2 associate with p48/IRF9 to form a multimeric ISGF3 complex that plays an essential role in the induction of ISG expression [Fu et al. 1990; Darnell et al. 1994]. Defective JAK-STAT signaling was found to cause either sensitivity or resistance to IFN, confirming its absolute requirement for adequate response to this cytokine. To further analyze the IFN-hypersensitivity of UBP43-deficient cells, we asked whether increased protein ISGylation in these cells alters type I IFN signaling. We therefore analyzed DNA binding properties of ISGF3 complex in gel shift.
assays using protein extracts prepared from UBP43+/+ and UBP43−/− bone marrow cells following stimulation with IFN-β for a maximum of 48 h. In UBP43+/+ cells, ISGF3 DNA binding was rapidly and transiently induced upon addition of IFN-β and became undetectable by 12 h of IFN-β stimulation [Fig. 4A]. In contrast, strong ISGF3 DNA binding was still detectable in protein extracts from IFN-β-treated UBP43−/− cells at 48 h. Addition of antibodies against Stat1 and p48 also supershifted the ISGF3 complex [data not shown]. Consistent with this observation, we identified prolonged Stat1 phosphorylation in UBP43−/− cells by Western blot using antibodies specifically recognizing tyrosine-phosphorylated Stat1 [Fig. 4B]. We also evaluated the expression pattern of IFN target genes, including ISG15, 2′-5′OAS, and IRF7 [Fig. 4C]. Upon IFN-β stimulation of bone marrow cells, the expression of these genes was clearly activated in both UBP43+/+ and UBP43−/− cells. However, the level and the timing of activation were much higher and extended in UBP43−/− cells compared to UBP43+/+ cells. Indeed, not only was there a higher level of protein ISGylation, but also an increase of free ISG15 was observed in UBP43−/− cells upon IFN-β stimulation compared to controls [Fig. 2C]. These results suggest that an augmented level of ISG15 conjugates and/or the absence of their turnover in UBP43-deficient cells may enhance and prolong the signaling of type I IFN.

Protein ISGylation enhances IFN signaling

We also analyzed the effect of protein ISGylation on IFN signaling in additional assays. It was reported that K562 human leukemic cells do not have protein ISGylation upon type I IFN treatment (Loeb and Haas 1992). UBE1L is an ISG15-activating enzyme [E1] that is crucial for ISG15 conjugation and was originally cloned from the study of chromosome 3p deletion associated with small cell lung cancer (Kok et al. 1993; Yuan and Krug 2001). We investigated whether missing functional UBE1L was responsible for the lack of protein ISGylation in K562 cells. HA-tagged UBE1L was transiently transfected into K562 cells. After culturing cells in the presence or absence of IFN-α for 24 h, protein extracts were subjected to Western blot analysis with anti-ISG15 antibodies. UBE1L-expressing cells in contrast to controls showed a strong induction of protein ISGylation [Fig. 5A]. This result demonstrates that lack of functional UBE1L expression is the major reason for the absence of ISGylation upon IFN stimulation in K562 cells. To further analyze whether protein ISGylation is involved in the enhanced and prolonged IFN signaling as observed in UBP43-deficient cells, cotransfected empty vector or UBE1L expression construct into K562 cells, together with luciferase reporter construct under the control of interferon-responsive elements [ISRE; Malakhova et al. 2002]. The luciferase activity in the absence of exog-
enes UBE1L expression started to decline 24 h post-IFN-stimulation (Fig. 5B). In the presence of exogenous UBE1L expression, significantly higher luciferase activity was detected at all three time points measured, reaching the maximum by 48 h. These results further support the conclusion from the analysis in UBP43-deficient cells that protein ISGylation enhances and prolongs type I IFN signaling.

Two major cellular effects of IFN are the suppression of cell proliferation and the promotion of apoptosis (Stark et al. 1998). Here, we report that ISG15 protease UBP43-deficient cells are hypersensitive to type I IFN and undergo apoptosis upon IFN stimulation. Furthermore, enhanced and prolonged IFN signaling is detected in UBP43-deficient cells. Mechanisms responsible for the regulation of IFN signaling are known to operate at several levels, including the negative controls by down-regulation and degradation of receptors, and regulation of JAKs and STATs by protein tyrosine phosphatases (PTPs), SOCS, and PIAS proteins (Greenhalgh and Hilton 2001; O'Shea et al. 2002; ten Hoeve et al. 2002). In the present study, we demonstrate that UBP43 is a novel negative regulator of IFN signaling. Lack of UBP43 results in a profound increase in the level of protein ISGylation that is associated with the enhanced and prolonged type I IFN signaling. Furthermore, the role of ISGylation in the regulation of IFN signaling is also confirmed by transfection experiments in which reconstitution of ISG15 conjugation system in UBE1L-deficient K562 cells increases protein ISGylation and the activity of IFN-responsive promoter. These data suggest that UBP43 negatively regulates the IFN-activated JAK-STAT signaling pathway by decreasing levels of protein ISGylation. As shown in Figure 2C, many proteins can be modified by ISG15. The enhanced IFN signaling in UBP43-deficient cells may involve the increased ISGylation of one or a group of proteins either directly or indirectly associated with the JAK-STAT signaling pathway. Detailed biochemical studies are needed to identify and characterize proteins modified by ISG15 to understand how protein ISGylation controls the signaling pathway.

Materials and Methods

Poly(C) injection and hematopoietic cell counting
Poly(C) (Sigma) was dissolved in phosphate buffered saline and intraperitoneally injected. Percentage of nucleated cells was counted in Türk solution (3% acetic acid and 0.01% crystal violet in H2O).

Bone marrow transplantation
Bone marrow cells [CD45.2+] were collected from UBP43+/− and UBP43−/− donor mice 5 d after they were injected with 5-fluorouracil (5-FU, Sigma) at 100 µg/gbw. Blood and bone marrow nucleated cells were counted in Türk solution (3% acetic acid and 0.01% crystal violet in H2O).

In vitro bone marrow cell culture
The colony forming unit (CFU) assay was performed as described previously in the presence of the indicated concentration of IFN-β (Calbiochem, Rhoades et al. 2000). Liquid culture of bone marrow cells was performed in RPMI 1640 with 10% fetal bovine serum, 10 ng/ml IL-3 (Peprotech), 10 ng/ml IL-6 (Peprotech), and 100 ng/ml SCF (Peprotech) in the presence or absence of 100 unit/ml IFN-β, 10 ng/ml IFN-γ (Peprotech), or 10 ng/ml TNF-α (Calbiochem).

Apoptosis assay
The TUNEL assay was performed according to the manufacturer’s instructions (Boehringer Mannheim). A total of 200 cells was counted to determine the percentage of apoptotic cells. An annexin V-FITC/7-AAD (7-aminomannocycin D) apoptosis assay was performed using an apoptosis detection kit according to the manufacturer’s instructions (BD Pharmingen).

Retroviral infection
UBP43 cDNA was subcloned into Bgl II and Hap I sites of MigR1. The production of replication-defective retrovirus stock and bone marrow cell infection was performed as described (Pear et al. 1998).

Gel shift assay
Assays were performed as described (Malakhova et al. 2002). Double-stranded oligonucleotide from the ISG15 promoter that contains an ISGF3 binding site was used in the assay (Fu et al. 1990).

Plasmid construction and transfection
UBE1L cDNA was kindly provided by Dr. Charles Buys (Kok et al. 1993) and subcloned into pcDNA3 containing a 5′ end HA tag sequence generating pcDNA-HA-UBE1L. The UBP43 promoter-luciferase construct p3K-UBP43-luc was described previously (Malakhova et al. 2002). Transfection of K562 cells was performed with electroporation (220 V, 975 µF). A total of 1 × 10⁶ K562 cells were transfected with p3K-UBP43-luc (4 µg), pcDNA3 or pcDNA-HA-UBE1L (6 µg), and promoterless Renilla luciferase construct pRL-luc (200 ng) as an internal control for transfection efficiency. Twenty-four hours after the electroporation, cells were split into two flasks and cultured in the presence or absence of 1000 units/mL IFN-α for the indicated length of time. Luciferase activities were analyzed using the Dual-Luciferase assay system (Promega).

Western blotting
Antibodies against phospho-Stat1 Tyr701 (Cell Signaling), Stat1 (Santa Cruz), and HA (Rabco) were purchased from the respective manufactures. Rabbit anti-mouse ISG15 polyclonal antibodies were generated using full-length mouse ISG15. Rabbit polyclonal antibodies against human ISG15 were generously provided by Dr. E. Borden (D'Cunha et al. 1996). Western blotting was performed as described (Malakhov et al. 2002).

Northern blotting
Total RNA was isolated using RNeasy B reagent according to the manufacturer’s instructions (TEL-TEST). Ten micrograms of total RNA from each sample was separated in an agarose/formaldehyde (0.22 M) gel, blotted on Hybond N+ membrane (Amersham), and probed with 32P-labeled cDNAs.

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