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Negative regulation of ISG15 E3 ligase EFP through its autoISGylation

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Abstract

The function of ubiquitin-like protein ISG15 and protein modification by ISG15 (ISGylation) has been an enigma for many years. Recently, the research of ISGylation has been accelerated by the identification of the enzymes involved in the ISG15 conjugation process. Our previous study identified the interferon inducible protein EFP as an ISG15 isopeptide ligase (E3) for 14-3-3σ. In this study, we show that ISG15 E3 ligase EFP can be modified by ISG15. Two ubiquitin E2 conjugating enzymes, UbcH6 and UbcH8, can support ISGylation of EFP. The Ring-finger domain of EFP is important for its ISGylation. Full-length EFP can enhance the ISGylation of Ring domain deleted EFP, indicating EFP can function as an ISG15 E3 ligase for itself. We also determined the ISGylation site of EFP and created its ISGylation resistant mutant EFP-K117R. Compared to the wild-type EFP, this mutant further increases the ISGylation of 14-3-3σ. Thus we propose that autoISGylation of EFP negatively regulates its ISG15 E3 ligase activity for 14-3-3σ.

Keywords: EFP; 14-3-3σ; ISGylation; ISG15 isopeptide ligase (E3)

ISG15, which encodes a 17 kDa ubiquitin-like protein, is one of IFN induced proteins [1,2]. Among ubiquitin-like proteins, ISG15 is the oldest member and was reported only four years after the discovery of ubiquitin [3]. However, relative to ubiquitin and certain ubiquitin-like proteins, little is known about ISG15. Recently, ISG15 has been shown as an antiviral protein against Sindbis virus and HIV-1 [4,5], however, the molecular mechanism remains unknown.

Like ubiquitin, ISG15 can be conjugated to target proteins via an isopeptide bond between its C-terminal carboxyl group and the lysine ε-amino group of a targeted protein [6] and the conjugation can be reversed by a de-ISGylation enzyme (UBP43/USP18) [7]. ISG15 E1 (UBE1L), E2 (UbcH8), and E3 have been identified as sequential enzymes involved in the ISG15 conjugation process [8–13]. Our previous study reported that the UbcH8-interacting protein, EFP (estrogen-responsive finger protein), could function as an ISG15 E3 ligase for 14-3-3σ [11]. In the ubiquitin system, some Ring-finger domain E3 ligases, such as Mdm2, Parkin, and cIAP, have a potent activity of autoubiquitination [14–16]. However, there is very little information available on the automodification of E3 ligase of other ubiquitin-like proteins.

Our previous study showed that EFP has ISG15 E3 ligase activity and that the Ring domain is important for this activity. Here, we report that EFP is modified by ISG15 and that the Ring-finger domain is important for its ISGylation. Furthermore, we show that EFP facilitates the ISGylation of each other and autoISGylation of EFP negatively regulates its ISG15 E3 ligase activity for 14-3-3σ. These data demonstrate that there is a feedback regulation in the ISGylation system at the level of E3 ligase, further supporting the important function of ISG15 system.

Materials and methods

Cell culture and transfections. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA)

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with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and 2 mM L-glutamine (Invitrogen). Cell transfection has been described previously [11].

Plasmid construction. The mammalian expressing plasmids pCAGGS-mISG15, pCAGGS-6× His-mISG15, pCAGGS-HA-UBE1L, pFlagCMV2-UbcH8, pcDNA3-UBE1L, pcDNA3-UbcH8, pFlag-CMV2-14-3-3σ, pFlagCMV2-EFP, and pcDNA3-HA-EFP have been described previously [11]. Deletion and point mutants of EFP were generated by PCR and confirmed by sequencing.

Ni–NTA agarose purification. Forty-eight hours post-transfection, cells were washed with PBS and were lysed in PBS containing 1% NP-40 and 10 mM imidazole. Ni–NTA agarose beads (20 μl) (Qiagen) were then added to cell extracts (~1000 μg) and rotated at 4 °C (RT) for 4 h. Precipitates were washed three times with PBS containing 1% NP-40 and 20 mM imidazole, and then boiled in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 100 mM DTT).

Immunoprecipitation (IP) and Western blot analyses. IP and Western blot analyses have been described previously [11]. Antibodies against Flag (Sigma), HA (Covance, Denver, PA), Myc (Sigma), and EFP (BD Biosciences Pharmingen, San Jose, CA) were purchased from the respective manufacturer's. Mouse anti-human ISG15 monoclonal antibody and rabbit anti-mouse ISG15 polyclonal antibodies have been described previously [11]. Mouse IgG1 κ (MOPC-21) antibody was from Sigma.

Results

EFP can be modified by ISG15 in 293T cells

Our previous study showed Ring-finger type ubiquitin isopeptide ligase (E3) EFP can also function as ISG15 E3 ligase for 14-3-3σ. To determine whether EFP is a potential substrate for ISG15 conjugation, we used a well-established conjugation system to examine whether conjugation of EFP to ISG15 can be detected [10]. 293T cells were transfected with His-ISG15, HA-UBE1L, and Flag-EFP expressing plasmids. His-ISG15 conjugated proteins were purified on Ni–NTA agarose beads and the samples were analyzed by Western blotting using Flag antibody. As shown in Fig. 1A, additional higher molecular weight bands of EFP were detected from the cell extracts in which EFP was co-transfected with ISG15 (lane 2). UBE1L increased the intensity of these additional bands (lane 4). To further demonstrate the ISGylation of EFP, we also performed an opposite immunoprecipitate–Western blot. Flag-EFP was immunoprecipitated with Flag antibodies and samples were analyzed by Western blotting using

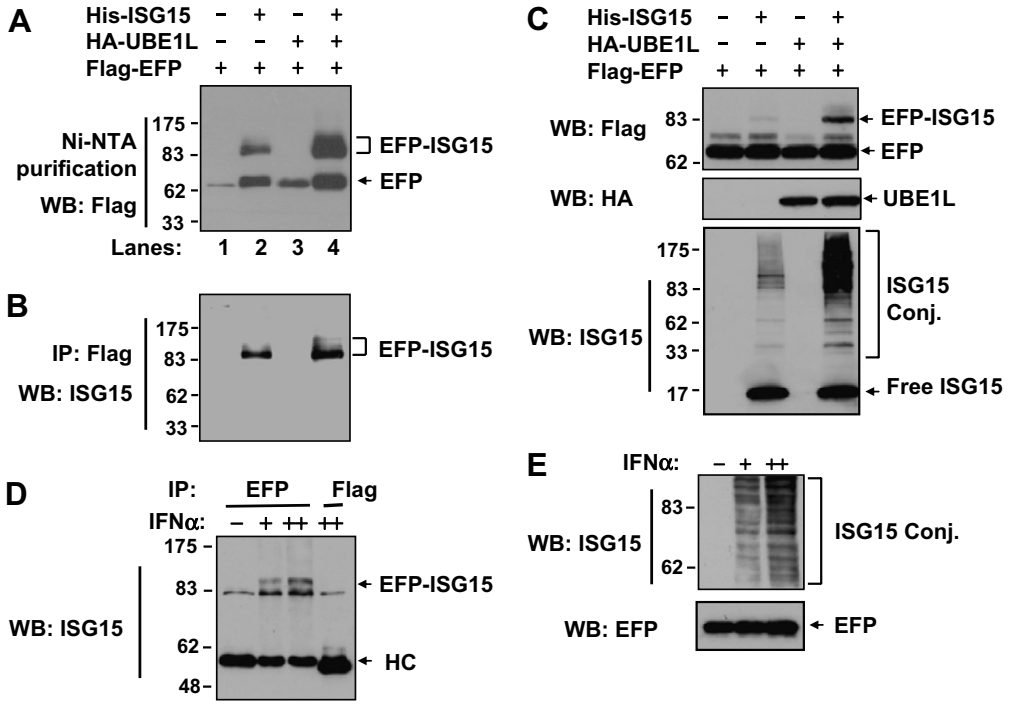

Fig. 1. EFP can be ISGylated in a 293T cell conjugation system. (A) 293T cells were transfected with Flag-EFP, HA-UBE1L, and His-ISG15 expression constructs as indicated. Cells were harvested 48 h after transfection. Protein of cell extracts (500 μg) was subjected to Ni–NTA pull down and Western blotted (WB) with Flag antibody. (B) Protein of cell extracts (500 μg) was subjected to immunoprecipitation with Flag antibody and Western blotted with ISG15 antibody. The positions of unmodified EFP and ISGylated EFP are indicated. (C) Twenty micrograms of protein from transfected cells was separated by SDS–PAGE and analyzed by Western blotting with antibodies specific for Flag (EFP), HA (UBE1L), and ISG15. (D) MCF-7 cells were treated with 0 (–), 500 (+), and 2000 (++) U/ml of human IFN-α for 48 h. Cell extracts (1000 μg) were immunoprecipitated (IP) with EFP antibody and Western blotted with antibodies specific for ISG15. The proteins from 2000 U/ml IFN-α treated cells were also immunoprecipitated with Flag antibody as a negative control. The positions of ISGylated EFP and the immunoglobulin heavy chain (HC) are shown. (E) The expression of EFP and protein ISGylation was analyzed by Western blotting with antibodies specific for EFP and ISG15. The positions of molecular weight markers (kDa) are shown on the left side of each panel.

ISG15 antibodies, showing the conjugation of EFP with ISG15 (Fig. 1B). Agreeing with the above data, the expression of UBE1L increased the ISGylation of EFP. Furthermore, we also detected ISG15 modified forms of EFP from direct Western blots in the same set of experiments (Fig. 1C). The total levels of Flag-EFP protein were determined by direct Western blots in the same set of experiments. Although the total levels of EFP protein were quite equal (Fig. 1C), higher levels of unmodified EFP were pulled down by Ni–NTA accompanied with higher levels of ISGylated EFP (Fig. 1A, lanes 2 and 4). There are two possible explanations for that, one is that EFP can interact with each other, and ISGylated EFP can still interact with unmodified EFP, which was also pulled down by Ni–NTA resin through the interaction with His-ISG15–EFP. Another possibility is that after Ni–NTA pull down, the ISG15 of ISGylated EFP is removed by some unknown reason.

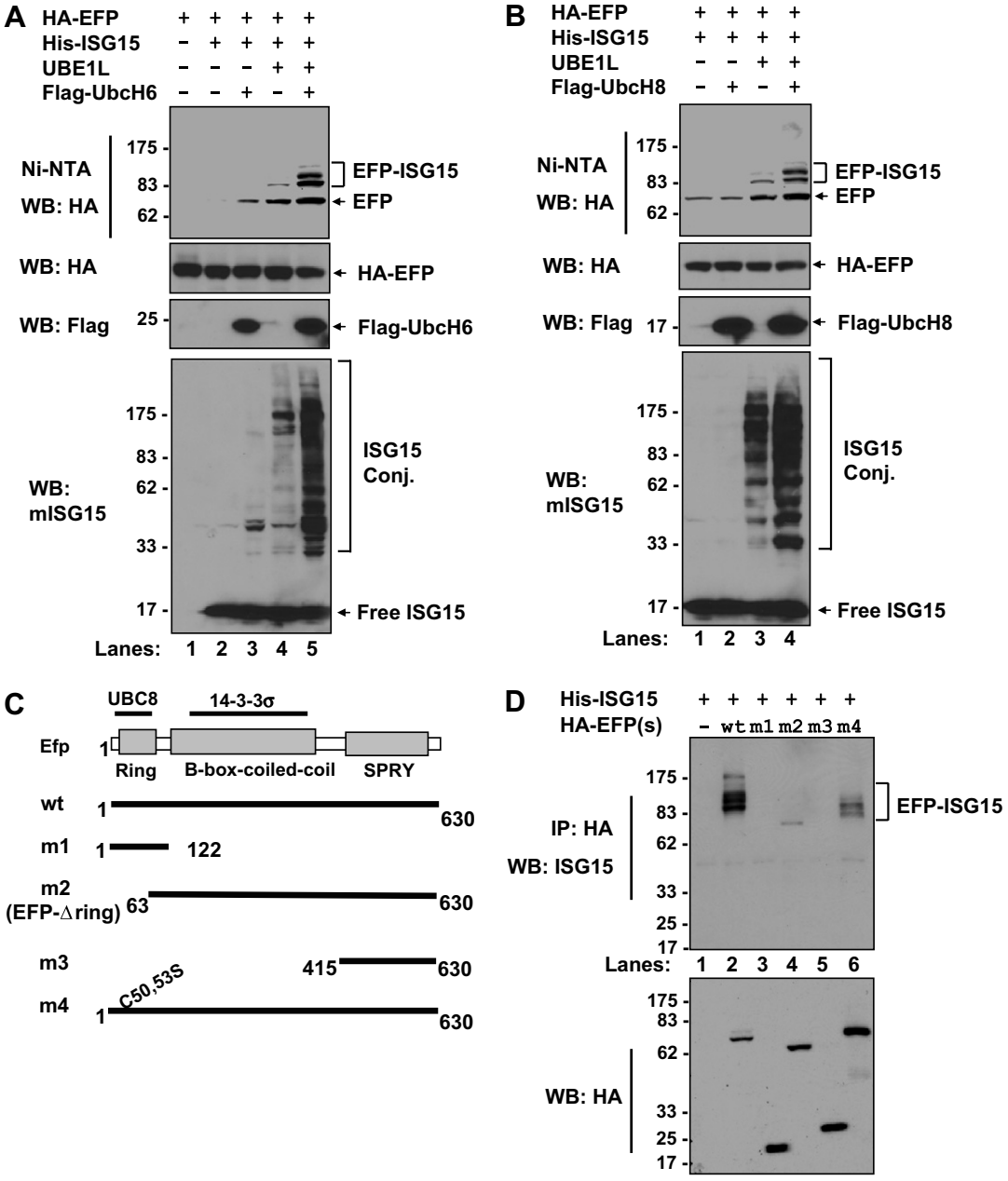

Fig. 2. The effects of UbcH6, UbcH8, and Ring-finger domain on EFP autoISGylation. 293T cells were transfected with expression plasmids encoding His-ISG15, HA-EFP, UBE1L, UbcH6 (A), and UbcH8 (B). Cells were harvested 48 h after transfection. Proteins (500 μg) were subjected to Ni–NTA pull down and Western blotted with HA. (C) Schematic representation of wild-type EFP and EFP mutants. The reported domains interacting with UbcH8 and 14-3-3σ are shown. (D) 293T cells were transfected with plasmids encoding His-ISG15, HA-EFP, and HA-EFP mutants as indicated. Protein of cell extracts (500 μg) was subjected to immunoprecipitation with HA antibody and Western blotted with ISG15 antibody.

Endogenous EFP can be modified by ISG15 after interferon treatment

To determine whether EFP is subject to ISG15 modification at endogenous levels, we performed immunoprecipitations with EFP antibody and control antibody (anti-Flag) using extracts from MCF7 cells treated with human IFN-α. The immunoprecipitates were analyzed by Western blotting using antibodies against human ISG15. As shown in Fig. 1D, a species of the predicted molecular weight for EFP modified with a single ISG15 moiety was observed in the IFN treated samples but not in the untreated one. Importantly, EFP–ISG15 conjugation was not observed in the immunoprecipitates of anti-Flag control. The molecular weight of the EFP–ISG15 conjugate in Fig. 1D corresponds to the predominant EFP species detected in the 293T ISG15 conjugating system described in Fig. 1A and B. The total levels of EFP protein and ISGylation level were determined by direct Western blots in the same set of experiments (Fig. 1E).

UbcH6 and 8 can support the ISGylation of EFP

UbcH8 is reported as an ISG15 conjugating enzyme [9,10]. Using the siRNA approach, it has been shown that UbcH8 is a predominant ISG15 E2 for IFN-induced protein ISGylation in HeLa cells. Interestingly, an ISG15 thioester intermediate was also detected with UbcH6, suggesting that UbcH6 would be another ISG15 conjugating enzyme [17]. We separately tested the effect of UbcH8 and UbcH6 on the ISGylation of EFP. As shown in Fig. 2A and B, additional higher molecular weight bands of EFP were detected from the cell extracts in which EFP was co-transfected with ISG15 and UBE1L. Co-transfection of UbcH8 or UbcH6 expression plasmids increased the ISG15 modified form of EFP (Fig. 2A, lane 5 and Fig. 2B, lane 4). The total levels of HA-EFP and ISG15 conjugation were determined by direct Western blots. As expected, both UbcH8 and UbcH6 increased the total ISGylation.

Ring-finger domain is important for EFP ISGylation

EFP protein is a Ring-finger type ubiquitin isopeptide ligase. We reported that the Ring-finger domain is important for EFP ISG15 E3 ligase activity toward 14-3-3σ [11]. To determine whether Ring-finger domain is important for EFP autoISGylation, we used a number of EFP mutants (Fig. 2C) to examine their autoISGylation. As shown in Fig. 2D, Ring-finger domain alone and the C-terminal of EFP did not show the auto-ISGylation (Fig. 2D, lanes 3 and 5). Ring-finger-deleted EFP showed very weak ISGylation (Fig. 2D, lane 4). Replacement of conserved cysteins in EFP Ring-finger domain with serines also

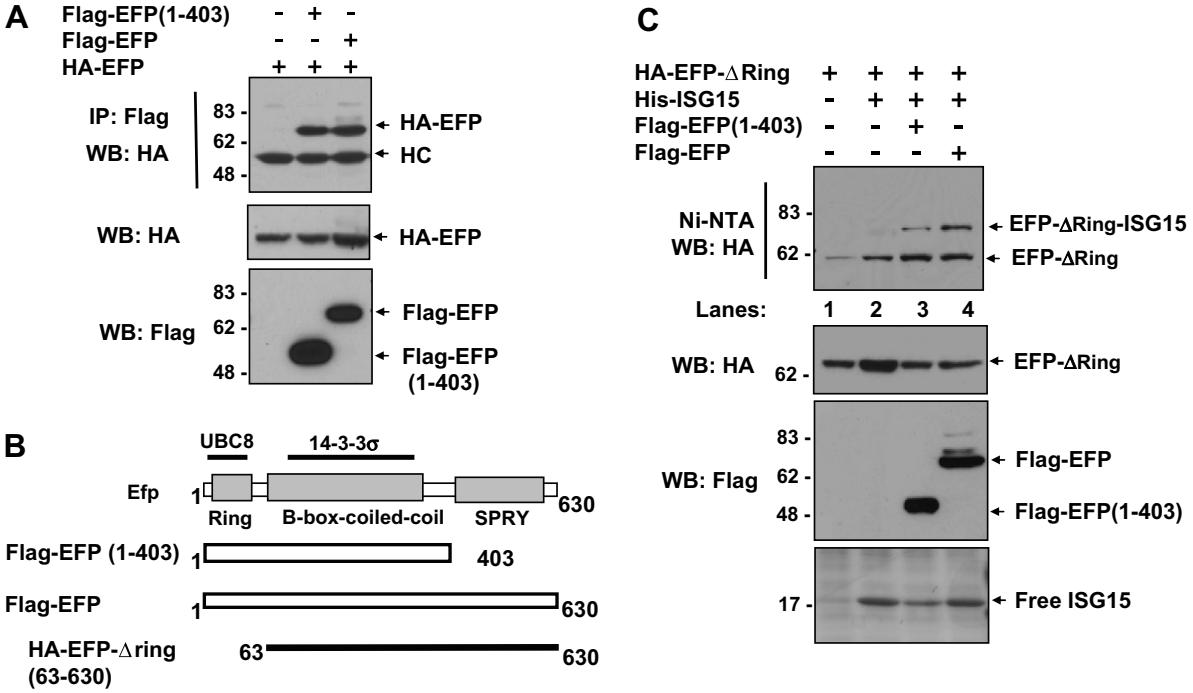

Fig. 3. EFP can enhance the ISGylation of Ring domain deleted EFP. (A) 293T cells were transfected with plasmids encoding Flag-EFP, Flag-EFP(1–403), and HA-EFP constructs as indicated. Protein of cell extracts (500 μg) was subjected to immunoprecipitation with Flag antibody and Western blotted with HA antibody. (B) Schematic representation of different EFP constructs. The reported domains interacting with UbcH8 and 14-3-3σ are shown. (C) 293T cells were transfected with plasmids encoding His-ISG15, Flag-EFP, Flag-EFP(1–403), and HA-EFP-ΔRing constructs as indicated. Forty-eight hours later, protein of cell extracts (500 μg) was subjected to Ni–NTA pull down and Western blotted with anti-HA antibody. Protein expression was analyzed by direct western blot with HA and Flag antibody. The amount of Ni–NTA enriched free ISG15 was shown by Ponceau staining.

decreases the ISGylation of EFP (Fig. 2D, lane 6). These results indicate that the Ring-finger domain is important for EFP autoISGylation.

EFP functions as ISG15 E3 ligase for itself

The Ring domain dependence in the ISGylation of EFP raised the question whether EFP can function as an E3 ligase for itself. First of all, we checked whether different tagged EFP can interact with each other. As shown in Fig. 3A, HA-EFP can bind to Flag-EFP, indicating that EFP could interact with each other. At the same time, the deletion of C-terminal SPRY domain did not affect Flag-EFP's interaction with HA-EFP (Fig. 3A and B).

As shown in Fig. 2D, Ring domain deleted EFP showed very weak ISGylation. We suspected endogenous EFP could function as an ISG15 E3 ligase and support the ISGylation of Ring domain deleted EFP. Meanwhile, Ring domain deleted EFP can still interact with Flag-EFP and Flag-EFP(1–403) (data not shown). We checked whether EFP can promote the ISGylation of Ring domain deleted EFP. As shown in Fig. 3C, ISGylated EFP-ΔRing was weakly detected when HA-EFP-ΔRing was co-expressed with His-ISG15; however, the amount of ISGylated EFP-ΔRing was dramatically increased by Flag-EFP expression (Fig. 3C, lane 4). Strikingly, C-terminal truncated Flag-EFP(1–403) also enhanced the ISGylation of EFP-ΔRing. We also checked the effect of Flag-EFP(1–403) on the ISGylation of 14-3-3σ, indicating Flag-EFP(1–403) also promoted the ISGylation of 14-3-3σ (data not shown). From these data, we concluded that EFP can promote the ISGylation of EFP-ΔRing, indicating that EFP can function as an ISG15 E3 ligase for each other.

ISGylation of EFP negatively regulates its ISG15 E3 ligase activity for 14-3-3σ

Our previous study identified Lysine 92 in Ubc13 as the ISG15 modification site [6]. Recently, we also observed that UbcH6 and HSPC150 (UBE2T) can be modified by ISG15 at a similar site (unpublished data, Zou and Zhang). However, there is no similar site in EFP. From our above data, C-terminus of EFP is not important for its ISG15 E3 ligase activity. Another interesting point is that there are only sporadic lysine residues present in the N-terminal region of EFP. We mutated four lysine residues at N-terminus of EFP (which cover EFP N-terminal 155 amino acids) to arginines one by one and checked their ISGylation level. We found that the mutation of the fourth lysine (K117) abolished the ISGylation of EFP. However, there is no effect for the mutations of other 3 lysines (K21, K65, and K112) (data not shown). As shown in Fig. 4A, ISGylated

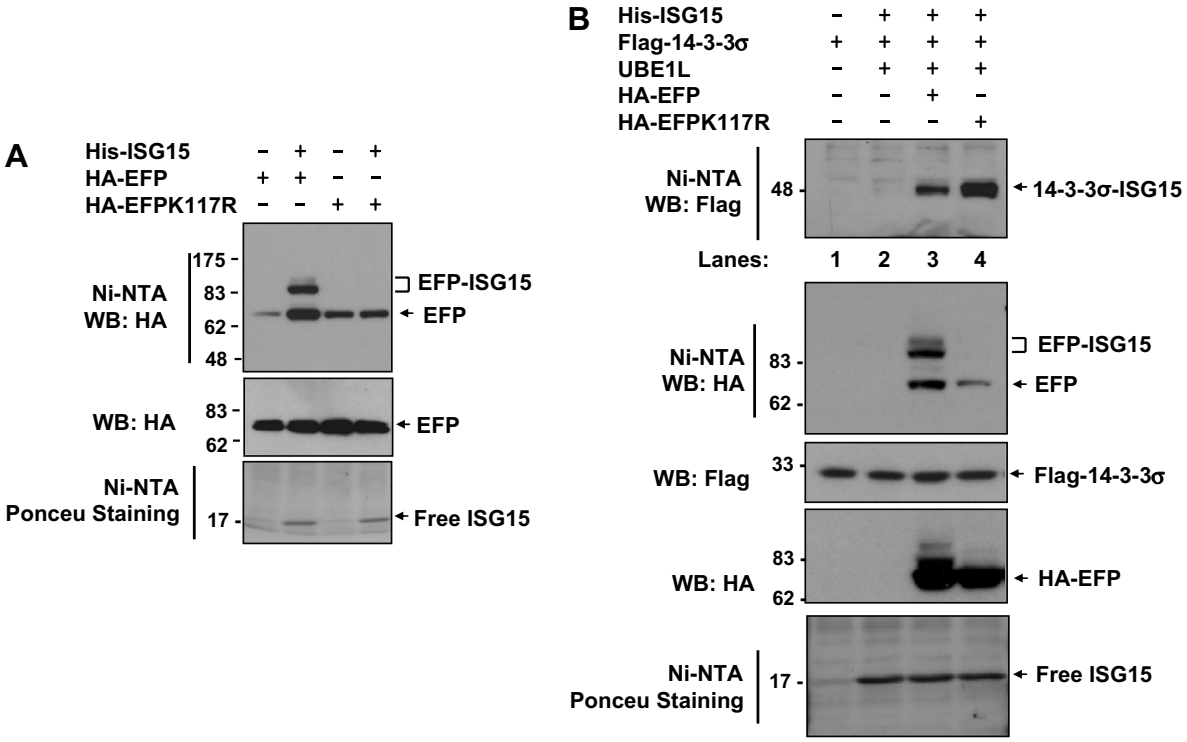

Fig. 4. ISGylation of EFP inhibits its ISG15 E3 ligase activity for 14-3-3σ. (A) 293T cells were transfected with HA tagged wt EFP (lanes 1 and 2) and mutant EFP-K117R (lanes 3 and 4) in the absence or presence of His-ISG15 as indicated. Forty-eight hours after transfection, 500 μg of protein in cell lysates was subjected to Ni–NTA pull down and Western blotted with HA antibody. (B) 293T cells were transfected with plasmids encoding His-ISG15, HA-EFP, HA-EFP-K117R, and Flag-14-3-3σ as indicated. Forty-eight hours later, protein of cell extracts (500 μg) was subjected to Ni–NTA pull down and Western blotted with anti-Flag and anti-HA antibody. Protein expression was analyzed by direct Western blotting with HA and Flag antibody. The amount of Ni–NTA enriched free ISG15 was shown by Ponceau staining.

EFP was detected from the cell extracts in which wt HA-EFP was co-transfected with His-ISG15 (Fig. 4A, lane 2). In contrast, we did not detect this signal when mutant HA-EFP-K117R was co-transfected with ISG15 (Fig. 4A, lane 4). In 293T co-transfection artificial system, ISGylated EFP appears to constitute multiple bands (Figs. 1 and 2). From the molecular weight, it is possible that EFP can be modified by multiple ISG15 conjugates. Given that EFP-K117R abolish the total modification, it is possible that the single lysine can be polyISGylated. The report that ISG15 itself can be modified by ISG15 supports the exits of polyISGylation [18]. Using different tagged of ISG15, we have shown that ISG15 can form polybands in 293T artificial system (data not shown).

The identification of the ISGylation site in EFP facilitates the investigation of the effect of ISG15 system on EFP. Using the EFP-K117R mutant as an ISGylation-resistant mutant, we checked EFP ISG15 E3 ligase activity for 14-3-3σ. As described previously, Flag-14-3-3σ was expressed in 293T cells along with the His-mISG15 constructs in the absence or presence of HA-EFP or HA-EFP-K117R. The ISGylated proteins were enriched with Ni–NTA agarose and detected by Western blotting. Agreeing well with our previous data, HA-EFP expression increased the ISGylated 14-3-3σ (Fig. 4B, lanes 2 and 3). Interestingly, the level of ISGylated 14-3-3σ was more dramatically increased by the expression of HA-EFP-K117R (Fig. 4B, lane 4). We also did Western blot with HA and checked the ISGylation level of EFP, confirming that EFP is ISGylated and EFP-K117R is resistant to ISGylation. The amount of Ni–NTA enriched free ISG15 is shown by Ponceau staining (Fig. 4B, bottom panel), which is quite equal among different lanes. Direct Western blot was done in the same experiment to determine the total level of Flag-14-3-3σ and HA-EFP level, which were quite equal, suggesting that the different level of ISGylated 14-3-3σ is not due to a difference of protein expression level. In summary, the level of ISGylated 14-3-3σ protein is opposite to the level of ISGylated EFP protein. These results suggest that ISGylation of EFP inhibits its ISG15 E3 ligase activity for 14-3-3σ.

Discussion

Our previous study identified EFP as an ISG15 E3 ligase for 14-3-3σ [11]. Recently, two other groups showed that interferon-induced HECT domain protein Herc5 could help the ISGylation status of whole cellular proteins [12,13]. Based on the loss of activity in targeted substitution of Cys-994 to Ala in the HECT domain, Herc5 should function as an ISG15 E3 ligase [12]. Interestingly, as an ISG15 E3 ligase, Herc5 can stimulate ISGylation of a variety of cellular proteins [12,13]. However, EFP has little effect on the total ISGylation when co-transfected with ISG15, UBE1L, and UbcH8 into 293T cells [11]. The direct comparison between EFP and Herc5 are reported by a recent paper [19]. Among the tested four targets, EFP can increase the ISGylation of tubulin as well as Herc5 and weakly increase the conjugation of two other targets. However, Herc5 can increase the ISGylation of all four targets [19]. We also tested the effect of Herc5 on the ISGylation of 14-3-3σ. Interestingly, co-transfection of Herc5 can also increase the ISGylation of 14-3-3σ in 293T cells (data not shown). If Herc5 can function as an E3 ligase for all targets and directly help the transfer of ISG15 from E2 to substrates, it is likely that a motif common to target proteins might be directly recognized by Herc5. However, analysis of target proteins identified to date has not revealed this kind of motif. At the same time, there is no consensus sequence among the identified ISG15 modification sites from UbcH13 [6,20], EFP, PP2C [21]. Recently, we found that another pair of ubiquitin E3 ligase-substrate, HHAR1-4EHP, can also work as an E3 ligase-substrate in ISG15 system (data in preparation for publication). It is likely that there is E3 ligase specificity in the ISG15 conjugation system. One hypothesis is that Herc5 may work together with specific ISG15 E3 ligase and function as an accelerator for a broad range of ISG15 substrates. The exact model for the role of EFP and Herc5 in the ISG15 system may be dependent on the construction of *in vitro* system.

In the present study, we identified lysine 117 in EFP as an ISGylation site. As a next step, we directed our attention on whether ISGylation has an effect on EFP ISG15 E3 ligase activity. We found that ISGylation-resistant mutant EFP-K117R has more effect on the ISGylation of 14-3-3σ than wild-type EFP (Fig. 4). These results provide a rationale for the inhibition of EFP ISG15 E3 ligase activity via its autoISGylation. Lysine 117 is localized between Ring domain and coiled-coil domain. Through interacting with UbcH8 via Ring domain and interacting with 14-3-3σ via coiled-coil domain, EFP can mediate the transfer of ubiquitin or ISG15 from UbcH8 to 14-3-3σ [11,22]. It is reasonable to postulate that addition of a 17 kDa ISG15 protein between Ring domain and coiled-coil domain can inhibit the transfer of ISG15 from UbcH8 to 14-3-3σ and inhibit EFP E3 ligase activity. However, it might be difficult to imagine how modification of a relatively small percentage of EFP (Fig. 4) has a substantial effect on the ISGylation of 14-3-3σ and has physiological relevance. At least two different scenarios are compatible with the low steady-state levels of EFP ISGylation. First, EFP may go through constant cycles of modification and demodification, which may contribute to assembly and disassembly of E1–E2–E3. Second, this inhibition may be spatially or temporally regulated. For example, ISGylation of EFP may easily happen on the EFP molecules charged with UbcH8-ISG15. It could take place on the same molecule or 'trans' transfer to another interacted EFP. Our data that EFP activates the ISGylation of Ring-domain deleted EFP support the possibility of the transfer of ISG15 to another interacting EFP. UbcH8-ISG15 charged EFP may transfer ISG15 to EFP instead of to 14-3-3σ. From this perspective, EFP behaves as a competitor for

14-3-3σ. Regardless of the exact mechanism, our data show that EFP ISG15 E3 ligase activity could be negatively regulated by its autoISGylation.

In summary, we showed that ISG15 E3 ligase EFP can be modified by ISG15 in 293T transfection system and in IFN-treated MCF7 cells. During the preparation of this paper, Nakasato et al. reported that overexpressed EFP can be modified by ISG15 [23]. In this study, we determined the importance of Ring-domain in EFP autoISGylation and showed the support of UbcH6 and UbcH8 as ISG15 E2s for EFP. We also identified the ISG15 modification site in EFP and determined that the ISG15 E3 ligase activity of EFP can be negatively regulated by its autoISGylation. Though the biological function of ISG15 system is yet unclear, our present study that the process of ISGylation can be nicely regulated on the ISG15 E3 ligase level supports the importance of this system.

Acknowledgments

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