The Interferon-inducible Ubiquitin-protein Isopeptide Ligase (E3) EFP Also Functions as an ISG15 E3 Ligase*

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The expression of the ubiquitin-like protein ISG15 and protein modification by ISG15 (ISGylation) are strongly activated by interferons. Accordingly, ISG15 expression and protein ISGylation are strongly activated upon viral and bacterial infections and during other stress conditions, suggesting important roles for the ISG15 system in innate immune responses. Here, we report the identification of the ubiquitin-protein isopeptide ligase (E3) EFP (estrogen-responsive finger protein) as the ISG15 E3 ligase for 14-3-3σ protein. Like other known components of the protein ISGylation system (ISG51, UBE1L, UBP43, and UBC8), EFP is also an interferon-inducible protein. Expression of EFP small interfering RNA decreased the ISGylation of 14-3-3σ in the 293T cell ISGylation system as well as in MCF-7 cells upon interferon treatment. Furthermore, the ISGylation enzyme activity of EFP was RING domain-dependent. These findings indicate that EFP is an ISG15 E3 ligase for 14-3-3σ in vivo. The fact that both UBC8 and EFP are common components in the ubiquitin and ISG15 conjugation pathways suggests a mechanism whereby a limited set of enzymes accomplishes diverse post-translational modifications of their substrates in response to changes in environmental stimulations.

ISG15 is one of several known Ub ubiquitin-like modifiers (1, 2). Upon type I interferon (IFN)3 treatment, ISG15 forms covalent conjugates with cellular proteins, a process similar to ubiquitination (ubiquitination or ubiquitylation) (1). Ubiquitination is now understood to be the dominant mechanism whereby cellular proteins are marked for degradation (3, 4). However, unlike ubiquitination, the role of ISG15 modification (ISGylation) has not been clearly defined, although ISG15 has been known since 1979 (5, 6).

ISG15 is composed of two domains, each of which bears sequence identity to ubiquitin (33 and 32% for the N- and C-terminal domains, respectively) (7). The process of ISGylation is expected to be similar to that of protein ubiquitylation. Mass spectrometry analysis confirmed that an isopeptide bond can be formed between the C terminus of ISG15 and the lysine ε-amino group of the target protein (8). There are a series of distinct enzymes involved in the process of protein ubiquitylation and deubiquitylation, viz. the ubiquitin-activating enzyme (E1), ubiquitin carrier protein (E2), ubiquitin-protein isopeptide ligase (E3), and the Dub deubiquitinating proteases (9, 10). An E1 enzyme for ISGylation (UBE1L) has been cloned and shows high homology to the E1 enzyme UBE1 (11). UBE1L-deficient cells express only free ISG15 and do not show ISGylation, indicating that UBE1L is necessary for ISGylation (12, 13). UBP43 (USP18) has been identified as a de-ISGylating isopeptidase and is highly specific in removing ISG15, but not Nedd8 and SUMO (small ubiquitin-like modifier) from their conjugates (14). UBP43 is a member of the UBP (USP) family of ubiquitin-specific proteases and contains conserved Cys and His domains, which are common in all members of this family (15).

Based on the information on UBE1L and UBP43, it is logical to hypothesize that ISG15 E2 and E3 are also members of the ubiquitin E2 and E3 families. Recently, Kim et al. (16) and Zhao et al. (17) reported the identification of the ubiquitin E2 enzyme UBC8 (UBCH8/UbcM8) as an ISG15-conjugating enzyme. Using the small interfering RNA (siRNA) approach, both groups indicated that UBC8 is a predominant ISG15 E2 enzyme for IFN-induced protein ISGylation in HeLa cells. Interestingly, UBC8 also functions as a ubiquitin E2 enzyme (18), indicating the possible cross-talk of protein ISGylation and ubiquitylation. In the ubiquitin system, the transfer of ubiquitin from E2 enzymes to target proteins is mediated by E3 enzymes, which interact with both E2 and its target proteins and function as a bridge between E2 and the target protein (19). UBC8 has been shown to interact with a number of ubiquitin E3 ligases to mediate protein ubiquitylation, such as E6AP, HHARI, Parkin, cIAP, Dorfin, EFP, RLIM, and Staring (18, 20–26). The fact that UBC8 functions as a dual E2 enzyme for both ISG15 and ubiquitin raises the possibility that some UBC8-interacting ubiquitin E3 ligases can function as the ISG15 E3 ligase, mediating the interaction between UBC8 and the ISG15 target proteins and helping in the transfer of ISG15 from UBC8 to target proteins. Here, we report that a UBC8-interacting protein, EFP (estrogen-responsive finger protein), can help in the ISG15 modification of 14-3-3σ protein both in transfection experiments and in vivo. EFP depends on its RING finger domain to support the ISGylation of 14-3-3σ. These data indicate that EFP is an ISG15 E3 ligase for 14-3-3σ. This is the first study reporting the identification of ISG15 E3 in vivo, and we provide evidence that EFP protein is a common component in the ubiquitin and ISG15 conjugation pathways.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmids pCAGGS-mISG15, pCAGGS-His6-mISG15, pFLAG-CMV2-UBC8, and pCAGGS-HA-UBE1L have been described previously (16). pcDNA3-UBC8 and pcDNA3-UBE1L were constructed by subcloning the cDNAs of UBC8 and UBE1L into the pcDNA3.1(+) vector. Human EFP cDNA (Open Biosystems, Huntsville, AL) was subcloned into the pFLAG-CMV2 vector (Sigma), generating pFLAG-CMV2-EFP, and into the pcDNA3 vector containing...
EFP is an ISG15 E3 Ligase

ing an N-terminal hemagglutinin (HA) tag, generating pCDNA3-HA-EFP. 14-3-3-σ cDNA (Open Biosystems) was subcloned into the pFLAG-CMV2 vector, generating pFLAG-CMV2–14-3-3-σ. Human HHARI cDNA (Open Biosystems) was subcloned into the pFLAG-CMV2 vector, generating pFLAG-CMV2-HHARI. pcDNA3.1(+)-FLAG-Parkin was a kind gift from Dr. Haian Fu (Emory University School of Medicine, Atlanta, GA). Mouse IgG1 ISG15 polyclonal antibody has been described previously (13). Anti-FLAG (Sigma), and 14-3-3-σ/HHARI were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Hyclone, Logan, UT), and 2 mM L-glutamine were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen). For small-scale transfection, cells were grown in 6-well plates and transfected using PolyFect reagent (Qiagen Inc.). For large-scale transfection, cells were plated in 10-cm dishes and transfected by calcium phosphate precipitation as described previously (27). The MCF-7 breast cancer cell line (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), and 2 mM L-glutamine (Invitrogen). For large-scale transfection, cells were plated in 10-cm dishes and transfected by calcium phosphate precipitation as described previously (27). The MCF-7 breast cancer cell line (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Invitrogen). WM-19 melanoma cells (kindly provided by Dr. Meenhard Herlyn, The Wistar Institute, Philadelphia, PA) were cultured in the 2% tumor medium, which contains MCDB 153 (80%; Sigma) and Leibovitz L-15 medium (20%; Invitrogen) with 2% fetal bovine serum and 1 μg/ml insulin (Sigma) (28). Human IFN-α (Roferon-A) was from Roche.

Northern Blotting—Total RNA from WM-19 and MCF-7 cells was isolated using RNA-Bee reagent (Tel-Test) according to the manufacturer’s instructions. Ten micrograms of total RNA from each time point was separated on a formaldehyde-agarose gel (0.22 M), blotted onto Hybond N* membrane (Amersham Biosciences), and probed with 32P-labeled cDNAs.

Nickel-Nitrilotriacetic Acid (Ni-NTA)-Agarose Purification—Forty-eight hours post-transfection, cells were washed with phosphate-buffered saline (PBS) and lysed in PBS containing 1% Nonidet P-40 and 10 mM imidazole. Ni-NTA-agarose beads (20 μl; Qiagen Inc.) was then added to cell extracts (500 μg) and rotated at room temperature for 4 h. Precipitates were washed three times with PBS containing 1% Nonidet P-40 and 20 mM imidazole and then boiled in SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 100 mM dithiothreitol).

Immunoprecipitation and Western Blot Analyses—Forty-eight hours post-transfection, cells were lysed in modified radioimmune precipitation assay buffer (50 mM Tris-HEC (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.25% deoxycholate, and 0.1% SDS). Immunocomplexes were precipitated with a mixture of protein A/G-agarose (Amersham Biosciences). Immunoprecipitates were washed three times with the same buffer and boiled in SDS-PAGE sample buffer. Antibodies against FLAG (Sigma), HA (Covance Inc., Denver, PA), Myc (Sigma), EFP (BD Biosciences), and 14-3-3-σ (Upstate, Chicago, IL) were purchased from the indicated manufacturers. Mouse anti-human ISG15 monoclonal antibody (clone 5.1) was kindly provided by Dr. Ernest Borden (The Cleveland Clinic Foundation, Cleveland, OH). Rabbit anti-mouse ISG15 polyclonal antibody has been described previously (13). Mouse IgG1 antibody (clone MOPC-21) was from Sigma. Western blotting was performed as described previously (29).

RNA Interference for EFP—The mammalian expression vector pSUPER.retro.puro (OligoEngine, Seattle, WA) was used for constructing small hairpin RNA (shRNA) for EFP. The targeted sequences for siRNA are EFP cDNA regions 1158–1176 (ggaagaataaagaaa; siRNA1) and 711–729 (ggagggacatcaaca; siRNA2). The synthesized oligonucleotides for siRNA1 are 5’-Gatcccgggttagacagatctcaatcagagattttcttggagttcatcctattttta-3’ and 5’-Agcttaaaaggggggagctcaatcagagattttgttgagttcctc-3’. The annealed oligonucleotides were digested with BglII and HindIII and inserted into the pSUPER.retro.puro vector. To test the efficiency of EFP shRNA in the cotransfection experiments, 293T cells were transfected with pSUPER-EFP shRNA and mammalian expression plasmids encoding FLAG-14-3-3-σ and His-ISG15. The expression of EFP and the ISGylation of 14-3-3-σ were analyzed 48 h post-transfection. To test the effect of EFP shRNA on IFN-induced 14-3-3-σ ISGylation in vivo, we packaged retrovirus by cotransfection of pSUPER-EFP shRNAs with the amphotropic retroviral packaging vector pCL-10A1 into 293T cells. MCF-7 cells seeded in 6-well plates were infected with control pSUPER.retro.puro vector retrovirus or pSUPER-EFP shRNA retrovirus. Twenty-four hours post-infection, cells were selected with 6 μg/ml puromycin. Dead cells were washed out with PBS, and attached live cells were amplified, resulting in stable shRNA-expressing cell pools. MCF-7 cells and different cell pools were treated with 1000 units/ml human IFN-α. After 48 h, the expression of EFP and the ISGylation of 14-3-3-σ were analyzed.

RESULTS

EFP Is Up-regulated upon IFN Treatment—Because the primary and tertiary structures of ISG15 are similar to those of ubiquitin (30), the mechanism of ISGylation is expected to be similar to that of protein ubiquitylation and is predicted to involve the activity of the E1, E2, and E3 enzymes. Notably, both ISG15 expression and protein ISGylation are highly induced upon IFN stimulation. Furthermore, all known components of the protein ISGylation system (i.e. ISG15, UBE1L, UBP43, and UBC8) are IFN-inducible genes (6, 31). We hypothesized that ISG15 E3 ligases are UBC8-interacting ubiquitin E3 ligases that may also be encoded by IFN-inducible genes. Based on published reports (24, 28) and available information from Web sites, we identified one candidate protein (EFP) that fully matched the hypothesized criteria.

In gene expression profiling studies using oligonucleotide array, Leaman et al. (28) showed that EFP is one of the IFN-stimulated genes in human melanoma cell lines WM-19 and WM35. To determine whether EFP mRNA is truly up-regulated by IFN treatment, Northern blot analysis was performed with total RNA prepared from WM-19 human melanoma cells and MCF-7 human breast cancer cells. EFP has been reported to be an estrogen-inducible gene in MCF-7 cells (32). Northern blotting showed that EFP mRNA could be up-regulated by IFN treatment at 2 h, reaching a maximum at ~5 h. EFP mRNA levels at 8 and 24 h were still higher than the basal level (Fig. 1A). ISG15 mRNA levels were also analyzed by Northern blotting as a positive control (Fig. 1A).

To determine whether the induction of EFP is at the level of protein expression, Western blot analysis was performed with lysates prepared from WM-19 human melanoma cells and MCF-7 human breast cancer cells. Immunodetection using EFP-specific antibody showed that IFN increased the protein level of EFP, reaching a maximum at ~8 h (Fig. 1B). As a positive control, ISG15 protein was also induced by IFN treatment.

The promoter regions of genes related to ISG15 modification, including ISG15, UBE1L, UBP43, and UBC8, contain the IFN-stimulated response element (ISRE) (6, 16), which is responsible for promoter activation by type I IFN via its interaction with the IFN-stimulated gene factor-3 complex containing phosphorylated Stat1, Stat2, and IRF9 (33, 34). As shown in Fig. 1C, the 5′-flanking region of the human EFP gene (32) also contains an ISRE sequence. The facts that the EFP promoter

**EFP Is an ISG15 E3 Ligase**
contains the ISRE sequence and that EFP protein is induced by IFN treatment support the involvement of EFP protein in IFN-stimulated ISGylation.

**14-3-3σ Can Be Modified by ISG15 in the 293T Transfection System**—EFP protein is a RING finger-type ubiquitin E3 ligase that directs 14-3-3σ ubiquitination and promotes 14-3-3σ proteolysis in a proteasome-dependent manner (24). EFP can interact with UBC8 via its RING finger domain and mediates the transfer of ubiquitin from UBC8 to 14-3-3σ (24). Recently, UBC8 has been identified as an ISG15 E2-conjugating enzyme (16, 17). These facts support the possible role of EFP in the ISG15 modification of 14-3-3σ protein. To determine whether 14-3-3σ could be modified by ISG15, we cotransfected plasmid DNA expressing His-ISG15, UBE1L, UbcM8, and FLAG-14-3-3σ into 293T cells. His-ISG15 and covalently linked proteins were enriched by Ni-NTA pull down, and antibody against the FLAG epitope was used in 293T cells. His-ISG15 and covalently linked proteins were enriched by Ni-NTA resin through the interaction with His-ISG15/14-3-3σ.

**Figure 1.** EFP Is an IFN-inducible protein. A, WM9 and MCF-7 cells were treated with 1000 units/ml human IFN-α for the indicated times. Gene expression for ISG15 and EFP was detected by Northern blotting. The 28S rRNA signal is shown as the loading control. B, WM9 and MCF-7 cells were treated with 500 units/ml human IFN-α for the indicated times. ISG15 and EFP expression was detected by Western blotting. Molecular mass markers (in kilodaltons) are indicated on the left. Tubulin-α indicates the relative amounts of protein in the samples. C, the promoter region of the human EFP gene contains a conserved sequence for the interferon regulator factor-binding element/ISRE-binding site (boxed).

**14-3-3σ Can Be Modified by ISG15 upon Interferon Treatment in MCF-7 Cells**—To determine whether endogenous 14-3-3σ is subjected to ISG15 modification, we performed immunoprecipitation with anti-14-3-3σ antibody and anti-mouse IgG1 control antibody using MCF-7 cells extracts with or without stimulation by human IFN-α. The immunoprecipitates were analyzed by Western blotting using antibody against human ISG15 or 14-3-3σ. As shown in Fig. 3, a species with the predicted molecular mass of 14-3-3σ modified with a single ISG15 moiety (28 + 17 kDa) was detected in the IFN-treated sample, but not in the untreated one. Notably, 14-3-3σ/ISG15 conjugation was not observed in the immunoprecipitates of the anti-mouse IgG1 control antibody. The immunoprecipitates were also detected by anti-14-3-3σ antibody (Fig. 3B). As shown in Fig. 3B, besides unmodified 14-3-3σ, an additional band was detected in the IFN-treated samples, but not in the untreated one. The size of this band corresponds to the ISG15 band detected in Fig. 3A. The molecular mass of the 14-3-3σ/ISG15 conjugate in Fig. 3 corresponds to that of the predominant 14-3-3σ species detected in the 293T ISG15-conjugating system described in Fig. 2. We could also detect the ISGylation of 14-3-3σ in IFN-treated HeLa cells and A549 cells (data not shown).

**EFP Promotes ISGylation of 14-3-3σ**—The observation that 14-3-3σ is a target of ISG15 modification prompted us to investigate whether EFP can function as an ISG15 E3 ligase for 14-3-3σ. We first used the 293T system to examine the effect of EFP on 14-3-3σ ISGylation. FLAG-14-3-3σ was expressed in 293T cells along with the His-ISG15, UBE1L, and UBC8 expression constructs in the absence or presence of HA-EFP. The ISGylated proteins were enriched with Ni-NTA-agarose and detected by Western blotting. As described above, ISGylated 14-3-3σ was detected when it was coexpressed with His-ISG15, UBE1L, and UBC8 without the addition of HA-EFP (Fig. 4A, lane 4); however, the amount of ISGylated 14-3-3σ was dramatically increased by HA-EFP expression (lane 5). On film with longer exposure times, the ISGylated 14-3-3σ signal was also detected when FLAG-14-3-3σ was coexpressed with His-ISG15, and EFP expression increased this signal (Fig. 4A, lanes 2 and 3) (data not shown). As in Fig. 2, higher levels of unmodified 14-3-3σ were pulled down by Ni-NTA accompanied by higher levels of ISGylated 14-3-3σ. The whole cell lysates were analyzed...
EFP Is an ISG15 E3 Ligase

FIGURE 3. ISGylation of endogenous 14-3-3-3r in MCF-7 cells upon IFN treatment.
MCF-7 cells were incubated for 48 h in the absence (−) or presence (+) of 2000 units/ml human IFN-α. Cell extracts (1000 μg) were immunoprecipitated (IP) with anti-14-3-3-3r antibody and then Western-blotted (WB) with antibodies against ISG15 (A) and 14-3-3-3r (B). The proteins were also immunoprecipitated with anti-mouse IgG1 antibody as a negative control. The positions of unmodified 14-3-3-3r, ISGylated 14-3-3-3r, and the immunoglobulin heavy (HC) and light (LC) chains are shown. 14-3-3-3r expression and protein ISGylation in cellular protein extracts were analyzed by Western blotting with antibodies specific for 14-3-3-3r and ISG15 (C). Conj., conjugate.

FIGURE 4. EFP enhances the ISGylation of 14-3-3-3r. A, 293T cells were transfected with FLAG-14-3-3-3r, HA-EFP, UBE1L and UBC8, and His-ISG15 expression constructs as indicated. Proteins (500 μg) were subjected to Ni-NTA pull down and Western-blotted (WB) with anti-HA antibody. The expression of these proteins via transfection was examined by Western blotting. Conj., conjugate.

The Ubiquitin-like protein ISG15 is highly induced upon IFN stimulation (1, 5, 38) and is detected as a free protein and a target protein–ISG15 conjugate. Upon IFN stimulation, the expression of ISG15 conjugates is increased (1) and the abundance of free ISG15 protein decreases (2, 24). To determine whether the EFP E3 ligase activity is required for ISG15 conjugation, 293T cells were transfected with HA-14-3-3-3r, FLAG-EFP, FLAG-Parkin, Parkin, FLAG-HHARI, and His-ISG15 expression constructs as indicated. Proteins (500 μg) were subjected to Ni-NTA pull down and Western-blotted with anti-HA antibody. The expression of these proteins via transfection was examined by Western blotting. Conj., conjugate.

DISCUSSION

The Ubiquitin-like protein ISG15 is highly induced upon IFN stimulation (1, 5, 38) and is detected as a free protein and a target protein–ISG15 conjugate.
EFP Is an ISG15 E3 Ligase

FIGURE 5. siRNA for EFP decreases IFN-induced 14-3-3ζ ISGylation. A, increasing amounts of pSUPER-EFP shRNA were cotransfected with plasmids encoding His-ISG15 and FLAG-14-3-3ζ into 293T cells as indicated. Forty-eight hours after transfection, proteins of cell lysates were subjected to Ni-NTA pull down and Western-blotted (WB) with anti-FLAG antibody. Endogenous EFP protein was analyzed by Western blotting with anti-EFP antibody. Tubulin-α shows the loading control. The positions of unmodified and ISGylated 14-3-3ζ proteins are shown. B, MCF-7 cells were infected with the pSUPER retro.puro vector or different pSUPER-EFP shRNA retroviruses. Cells were selected with 6 μg/ml puromycin, resulting in retrovirus-positive pools of MCF-7 cells. Parental MCF-7 cells and different pools of infected cells were treated with 1000 units/ml human IFN-α for 48 h. Cell extracts (1000 μg) were immunoprecipitated (IP) with anti-14-3-3ζ antibody, and Western-blotted with anti-ISG15 and anti-14-3-3ζ antibodies. The positions of ISGylated 14-3-3ζ and the immunoglobulin heavy (HC) and light (LC) chains are shown. The expression of 14-3-3ζ and EFP was analyzed by Western blotting directly. Tubulin-α shows the loading control.

FIGURE 6. RING finger domain dependence of EFP to enhance 14-3-3ζ ISGylation. A, shown is a schematic representation of wild-type (wt) EFP and EFP mutants (mt). The domains reported to interact with UBC8 and 14-3-3ζ are shown. B, 293T cells were transfected with plasmids encoding FLAG-14-3-3ζ, HA-EFP, and HA-EFP mutants as indicated. Proteins (500 μg) were subjected to Ni-NTA pull down and Western-blotted (WB) with anti-FLAG antibody. The positions of unmodified and ISGylated 14-3-3ζ proteins are shown. The level of FLAG-14-3-3ζ expression was verified by Western blotting and is indicated in bottom panel.

The expression of 14-3-3ζ and the RING domain, which is required for the ubiquitin E3 ligase activity of EFP (24), is required for promoting 14-3-3ζ ISGylation. These results suggest that EFP acts as an ISG15 E3 ligase for 14-3-3ζ. To our knowledge, this is the first report on the identification of an ISG15 E3 ligase in vivo. These results indicate that the pathways of ubiquitin and ISG15 overlap in vivo.

UBC8 and its interacting E3 ligase EFP have the capacity to catalyze both ubiquitin and ISG15 conjugation. How are the specificities of these enzymes determined for their involvement in ubiquitin or ISG15 conjugation? Ubiquitin is a highly conserved housekeeping gene. It is ubiquitously and constitutively expressed and plays fundamental roles in many cellular processes (3, 43). In contrast, ISG15 has been identified only in vertebrates, and its sequence conservation is much lower than that of ubiquitin (44), suggesting that ISG15 does not have essential housekeeping functions. ISG15 expression is almost undetectable under normal conditions and is strongly up-regulated during certain stress responses, especially type I IFN stimulation. Therefore, in the absence of ISG15, UBC8 and EFP can function as ubiquitilation enzymes. Upon the induction of ISG15 synthesis, they also serve as protein ISGylation enzymes. Many members of the ubiquitin E2 family are expressed in a given cell. These E2 enzymes can accept activated ubiquitin from UBE1. However, only a few of these members (UBC8 is the only currently identified member) can accept activated ISG15 from UBE1. ISG15 competes with ubiquitin for its E2 when it is highly expressed. Furthermore, the spatial-temporal availability of UBC8-linked ubiquitin or ISG15 in vivo determines the specific functions of EFP and possibly other UBC8-interacting E3 ligases. The fact that both UBC8 and EFP are the common components in the ubiquitin and ISG15 conjugation pathways suggests a mechanism whereby a limited set of enzymes accomplishes diverse post-translational modifications of their substrates in diverse cellular pathways, including RNA splicing, chromatin remodeling, transcription, cytoskeletal organization, stress responses, and translation (41, 42). Based on the similarity between the ubiquitin and ISG15 systems, we hypothesized that ISG15 E3 ligases are members of the ubiquitin E3 family, interact with the ISG15 E2 enzyme UBC8, and may be encoded by IFN-inducible genes. Recently, using purified Saccharomyces cerevisiae Rsp5p E3, Zhao et al. (17) showed that ISG15 conjugation can be accomplished by a ubiquitin E3 ligase in vitro. However, there are no in vivo data supporting this hypothesis. In this study, we have demonstrated that the RING finger ubiquitin E3 ligase EFP can promote ISG15 conjugation to 14-3-3ζ upon IFN treatment. We have also demonstrated that the RING domain, which is required for the ubiquitin E3 ligase activity of EFP (24), is required for promoting 14-3-3ζ ISGylation. These results suggest that EFP acts as an ISG15 E3 ligase for 14-3-3ζ. To our knowledge, this is the first report on the identification of an ISG15 E3 ligase in vivo. These results indicate that the pathways of ubiquitin and ISG15 overlap in vivo.

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response changes to environmental stimulations, resulting in distinct molecular consequences.

ISG15 expression and ISGylation are regulated by type I IFN. Expression of all known components of the ISGylation system, including ISG15, UBE1L, UBAP43, and UBC8, is inducible by IFN (6, 16, 31). The promoters of these genes contain the ISRE, which is responsible for activation by type I IFN. In this study, we have also shown that EFP expression is increased upon IFN treatment and that the promoter region of EFP contains an ISRE (Fig. 1). EFP is also an estrogen-responsive gene (45). Although EFP protein is widely expressed, its level of expression is not uniformly high in various human tissues (46). It is possible that its expression in certain types of cells is heavily dependent on type I IFN signaling and that the major function of EFP in these cells is related to ISGylation of its substrates.

The function of ISG15 modification has been an enigma for many years. Although ISG15 has been implicated in a variety of biological activities based mostly on its similarities to ubiquitin and other ubiquitin-like proteins, the generation and analysis of ISG15-deficient mice (47) and ISG15 conjugation-deficient UbEcI knockout mice (48) did not reveal any significant developmental abnormalities, suggesting that ISG15 targeting is most likely a fine-tuning process that is induced by IFN. Very thorough studies must be performed to identify the exact consequences of ISGylation for some special target proteins. In this work, we have identified 14-3-3σ as a new in vivo target of ISG15 modification. More important, we have demonstrated that EFP is an ISG15 E3 ligase for 14-3-3σ. Taking into account the large number and diversity of 14-3-3 ligands and the importance of 14-3-3 proteins in many cellular processes (49, 50), 14-3-3 is an attractive target for further study of the biological consequences of ISG15 modification. These findings will facilitate further analysis and accelerate our understanding of the biological effects of protein ISGylation.

Acknowledgments—We thank Dr. Ernest Borden for anti-ISG15 antibody. Drs. Haian Fu and Jian Feng for the DNA constructs, Dr. Meinhard Herlyn for WM9 cells, and members of the D.-E. Z. laboratory for valuable discussions and critically reading the manuscript.

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