Phosphorylation and Specific Ubiquitin Acceptor Sites Are Required for Ubiquitination and Degradation of the IFNAR1 Subunit of Type I Interferon Receptor*

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Ubiquitination, endocytosis, and lysosomal degradation of the IFNAR1 (interferon α receptor 1) subunit of the type I interferon (IFN) receptor is mediated by the SCF (Skp1-Cullin1-F-box) protein β-transducin repeat-containing protein (β-Trcp). This E3 ubiquitin ligase functions in a phosphorylation-dependent manner. In addition, stability of IFNAR1 is regulated by its binding to Tyk2 kinase. Here we characterize the determinants of IFNAR1 ubiquitination and degradation. We found that the integrity of two Ser residues at positions 535 and 539 within the specific destruction motif present in the cytoplasmic tail of IFNAR1 is essential for the ability of IFNAR1 to recruit β-Trcp as well as to undergo efficient ubiquitination and degradation. Using an antibody that specifically recognizes IFNAR1 phosphorylated on Ser535 we found that IFNAR1 is phosphorylated on this residue in cells. This phosphorylation is promoted by treatment of cells with IFNa. Although the cytoplasmic tail of IFNAR1 contains seven Lys residues that could function as potential ubiquitin acceptor sites, we found that only three (Lys501, Lys525, and Lys526), all located proximal to the destruction motif, are essential for ubiquitination and degradation of IFNAR1. Expression of Tyk2 stabilized IFNAR1 in a manner that was dependent neither on its binding to β-Trcp nor IFNAR1 ubiquitination. We discuss the complexities and specifics of the ubiquitination and degradation of IFNAR1, which is a β-Trcp substrate that undergoes degradation via a lysosomal pathway.

Ubiquitin-mediated proteolysis plays an important regulatory role in many biological processes including cell cycle progression, transcription, and signal transduction (1). The process of protein ubiquitination is catalyzed by three distinct enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligases (E3). The E1 enzyme activates ubiquitin in an ATP-dependent reaction and then transfers ubiquitin to an E2. The E2 enzyme then cooperates with an E3 ligase to catalyze the formation of an isopeptide bond between ubiquitin and substrate. E3 ligases, which bind both cognate E2 and the substrates, are responsible for conferring substrate specificity in the ubiquitination reaction. Proteins polyubiquitinated by these enzymes are degraded by the 26 S proteasome (1, 2).

Alternately, oligo- or monoubiquitinated transmembrane proteins are endocytosed and degraded via the lysosomal pathway (3–6). E3 ligases that mediate the ubiquitination of cell surface receptors include c-Cbl/Hakai and Rsp5/NEDD4 family members (7, 8). Recently we have shown that the SCFβ-Trcp E3 ubiquitin ligase mediates ligand-induced ubiquitination of the IFNAR1 subunit of the type I interferon receptor complex. SCFβ-Trcp-mediated ubiquitination of IFNAR1 leads to endocytosis of this receptor complex, followed by its degradation via the lysosomal pathway, ultimately resulting in the down-regulation of the Type I IFN receptor. This mechanism plays a pivotal role in the negative regulation of IFN signaling (9).

SCF (Skp1-Cul1-F-box) E3 ligases represent the best studied family of E3s known so far (10). Members of the F-box protein family β-transducin repeat-containing proteins (β-Trcp, including β-Trcp1 and β-Trcp2/HOS) interact with Skp1 via the 42–48-amino acid F box motif and bind phosphorylated substrates through C-terminal WD40 repeats to mediate ubiquitination of their substrates, including inhibitors of NF-κB (IκB), β-catenin, and others that contain the DSGXX(S/T)_2_S destruction motif (Fig. 1, reviewed in Ref. 11). Substrates of β-Trcp-dependent ubiquitination are characterized by phosphorylation of Ser residues within the destruction motif (11). Whereas the integrity of Ser535 within the IFNAR1 destruction motif is essential for its ubiquitination and degradation (9), the importance of the second Ser residue (Ser539) remains obscure. Our previous findings suggest that down-regulation of IFNAR1 in response to type I IFN is promoted by phosphorylation of IFNAR1. However, there is no definitive report that IFNAR1 is actually phosphorylated on Ser535 in a ligand-dependent manner.

Apart from phosphorylation, the efficiency of protein substrate ubiquitination depends on the availability of ubiquitin acceptor sites. Unlike many substrates of the ubiquitin-proteasome pathway, which can be promiscuously ubiquitinated on various Lys residues (e.g. c-Jun, Ref. 12), substrates of the SCFβ-Trcp E3 ligases are preferentially ubiquitinated on Lysines that are located proximal to the destruction motif. Data from recent structural studies of this complex indicate the requirement for such Lys residues as ubiquitin acceptor sites and predict that the lysine destruction motif spacing serves as a determinant of ubiquitination efficiency (13). Indeed, in

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Ubiquitination and Degradation of the IFNAR1 Subunit

Cell Culture and Transfections—293T and HeLaS3 cells were kindly provided by Z. Ronai. Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C, 5% CO₂, and high humidity. Transfections were performed with LipofectAMINE Plus (Invitrogen) according to the manufacturer's recommendations 36 h prior to harvesting. The amount of plasmid DNA was equalized in all transfection reaction by adding empty vector (pCDNA3, Invitrogen).

Antibodies and Immunoenzymes—Antibodies against HA (Roche Applied Science), FLAG (M2, Sigma), and IFNAR1 (H-11, Santa Cruz) were purchased. Co-immunoprecipitation assays were carried out as previously described (21). A phospho-Ser³³⁵ antibody that specifically recognizes phosphorylated Ser³³⁵ within the DSGNYS motif of human IFNAR1 was raised in rabbits using synthetic monophosphopeptide EDHKKYSSQTSQP5GNSYNEDESE (522-546) in collaboration with PhosphoSolutions Inc. (Golden, CO). Antibody was further affinity purified using monophosphopeptide affinity columns and tested for specificity by immunoprecipitation and immunoblotting reactions.

To determine phosphorylation of endogenous IFNAR1, 293T cells were treated with IFNα (500 units/ml) for the indicated time, harvested, and subjected to denaturing lysis by boiling in 1% SDS-containing Tris-buffered saline, followed by sonication. After 10-fold dilution with Tris-buffered saline containing 1% Triton X-100, lysates were incubated with protein A beads for 1 h at 4 °C. Supernatants were used for immunoprecipitation with phospho-Ser³³⁵ antibody and immunoblotting analysis with IFNAR1 antibody. Digital images were processed with Adobe Photoshop 7.0 software.

In Vivo Ubiquitination Assays—293T cells were co-transfected with HA-tagged ubiquitin and FLAG-tagged IFNAR1 plasmids. Cells were subjected to denaturing lysis as described previously (16) and analyzed by immunoprecipitation with M2 antibody followed by immunoblotting with HA antibody to detect ubiquitinated IFNAR1 proteins. The blot was stripped and re-probed with M2 antibody to detect IFNAR1.

Pulse-Chase Analysis—Pulse-chase analysis was carried out with 293T cells as described elsewhere (9, 16). Briefly, cells were grown in 100-mm diameter dishes and transfected with the indicated plasmids. After starvation in Dulbecco's modified Eagle's medium lacking methionine, cysteine and metabolic labeling with a [³⁵S]cysteine mixture (PerkinElmer Life Sciences), cells were harvested at various time points of a chase with complete Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%) and unlabeled methionine and cysteine (2 mM). Lysates from harvested cells were immunoprecipitated with M2 antibody, separated on 10% SDS-PAGE, and analyzed by autoradiography.

RESULTS

Ser Residues within the Destruction Motif of IFNAR1 Are Required for β-Trcp Binding, Ubiquitination, and Degradation—We have recently found that β-Trcp mediates the ubiquitination, endocytosis, and lysosomal degradation of IFNAR1. Our findings implicated the integrity of Ser³³⁵ within the putative destruction motif DSGNYS³³⁵ in the recognition of IFNAR1 by β-Trcp and suggested that phosphorylation of Ser residues within this motif is required for this recognition (9). A similar extracellular stimuli-induced phosphorylation of Ser³²³,³³⁶ within IκBα induces its interaction with β-Trcp (23).

Thus, we investigated whether analogous Ser and Ser³³⁵ residues are required for interaction between IFNAR1 and β-Trcp.

To test this possibility we generated the IFNAR1⁵³⁵⁵⁹⁰A mutant and compared its binding to β-Trcp with the wild-type receptor by co-immunoprecipitating FLAG-tagged IFNAR1 and HA-tagged β-Trcp2 that had been co-expressed in 293T cells. We found that, consistent with our earlier data (9), β-Trcp2 interacted with IFNAR1⁵³⁵⁵⁹⁰ but did not bind to the IFNAR1⁵³⁵⁵⁹⁰ mutant (Fig. 2A, upper panel, lane 2 versus 3). Similar to the IFNAR1⁵³⁵⁵⁹⁰ protein, the IFNAR1 mutant that harbors the Ser⁵³⁹ substitution to Ala also failed to bind to β-Trcp (Fig. 2A, upper panel, lane 4). These results suggest that both Ser³³⁵ and Ser³³⁹ are important for β-Trcp binding to IFNAR1.

We next examined the ubiquitination of these mutant receptors. Co-expression of HA-tagged ubiquitin and FLAG-tagged IFNAR1 in 293T cells followed by denaturing lysis

FIG. 1. Schematic depiction of the cytoplasmic domain (100 amino acids) of IFNAR1. Scheme indicates the destruction motif (DSGNYS), which is recognized by β-Trcp and the lysine residues (upper panel). TM, transmembrane domain. Lower panel depicts the N-terminal part of IκBα that contains the destruction motif and Lys²¹,²² ubiquitin acceptor sites.

the case of IκBα, a pair of lysines (Lys²¹,²²) have been shown to act as ubiquitin acceptor sites. These are necessary and sufficient to support ubiquitination and degradation of IκBα (Fig. 1) (14). Similarly positioned Lys in the Epstein-Barr virus latent membrane protein 1, which is a pseudosubstrate of β-Trcp, converts this protein into a substrate for β-Trcp, converts this protein into a substrate for β-Trcp. Furthermore, introduction of analogous Lys residues into the Epstein-Barr virus latent membrane protein 1, which is a pseudosubstrate of β-Trcp, converts this protein into a substrate for β-Trcp. In human cells, internalization and lysosomal degradation of IFNAR1 is also regulated by its binding to the Janus kinase family member Tyk2. The interaction between IFNAR1 and Tyk2 sustains the cell surface localization of IFNAR1 and is required for maintaining the overall expression levels of IFNAR1. Interestingly, the kinase activity of Tyk2 is not required for this function (17–20). Although it is tempting to speculate that Tyk2 may affect the IFNAR1 turnover either by inhibiting β-Trcp-mediated ubiquitination or independently of β-Trcp, these possibilities remain to be investigated.

Because of its membrane location, IFNAR1 is expected to impose potentially novel structural constraints for ubiquitination by SCF-β-Trcp E3 ligase; such constraints might not be present in the cytoplasmic or nuclear substrates of β-Trcp. Therefore, it is of major importance to understand the structural determinants that contribute to the ubiquitination and degradation of IFNAR1. In this study, we characterize IFNAR1 as a substrate for the SCF-β-Trcp E3 ligase and identify such determinants. We show that both Ser³³⁵ and Ser³³⁵ residues within the DSGNYS destruction motif are required for recognition of IFNAR1 by β-Trcp as well as for ubiquitination and degradation of IFNAR1. Phosphorylation of Ser³³⁵ is induced by treatment of cells with IFNα. Furthermore, we identify the specific Lys residues that are essential for IFNAR1 ubiquitination and proteolysis. In addition, we demonstrate that Tyk2 binds to IFNAR1 and stabilizes this receptor subunit without interfering with β-Trcp-mediated IFNAR1 ubiquitination and in a manner that is independent of IFNα.

EXPERIMENTAL PROCEDURES

Plasmids—Constructs for expression of hemagglutinin (HA)-tagged β-Trcp2/HOS, HA-tagged Tyk2, and FLAG-tagged human IFNAR1 (wild-type and S535A mutant) were previously described (9, 21, 22). HA-tagged ubiquitin (12) was a generous gift from D. Bohmann. Site-directed mutagenesis of FLAG-IFNAR1 cDNA was carried out using QuikChange site-directed mutagenesis kit (Stratagene). The integrity of all resulting mutants was confirmed by dideoxy sequencing.

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and immunoprecipitation allowed us to detect ubiquitination of IFNAR1 \textit{in vivo} (Fig. 2B). In agreement with our earlier observations (9), the extent of ubiquitination of IFNAR1$_{S535A}$ was decreased compared with that of IFNAR1$_{wt}$ (Fig. 2B, lane 4 versus 5). In addition, we observe a noticeable decrease in the extent of ubiquitination of IFNAR1$_{S539A}$ (Fig. 2B, lane 6).

Given an important role of \(\beta\)-Trcp in the ubiquitination of IFNAR1 (9), these results further indicate that both Ser$_{S535}$ and Ser$_{S539}$ residues within the destruction motif of IFNAR1 are required for E3 ligase recruitment and \(\beta\)-Trcp-mediated ubiquitination of IFNAR1.

Ubiquitination of IFNAR1 in a \(\beta\)-Trcp-dependent manner

\[\text{Ubiquitination and Degradation of the IFNAR1 Subunit}\]
was shown to target IFNAR1 for degradation (9). To examine whether mutation of Ser539 within the DSGNYS motif would affect the stability of IFNAR1, we carried out pulse-chase analysis. Consistently with our previous studies, IFNAR1 WT was degraded efficiently whereas degradation of IFNAR1 S535A was noticeably delayed. Remarkably similar to IFNAR1 S535A, IFNAR1 P539A exhibited substantial stabilization compared with IFNAR1 WT (Fig. 2C). These results, together with the co-immunoprecipitation data and in vivo ubiquitination experiments suggest that, similar to the requirement of both Ser residues in IκB and β-catenin, the integrity of both Ser535 and Ser539 is crucial for the interaction of β-Trcp with IFNAR1, as well as for ubiquitination and degradation of IFNAR1.

Phosphorylation of IFNAR1 on Ser535 Is Promoted by Ligand Treatment—It has been well documented that phosphorylation of Ser residues within the DSGNYS2 motif is a prerequisite for the binding of β-Trcp to its substrates such as IκB, β-catenin, ATF4, Emi1, and others (11). We previously found that the interaction between IFNAR1 and β-Trcp also depends on IFNAR1 phosphorylation and that this interaction is promoted by treatment of cells with IFNa. We hypothesized that recruitment of β-Trcp by IFNAR1 (followed by its ubiquitination and down-regulation) is a result of IFNAR1 phosphorylation on Ser residues within the destruction motif by an as yet to be identified IFNa-activated kinase.

To investigate whether ligand-induced phosphorylation of Ser residues within the DSGNYS motif of IFNAR1 indeed occurs in cells, we used a polyclonal antibody that would specifically recognize IFNAR1 phosphorylated on Ser535. This antibody was raised against a synthetic phosphopeptide encompassing the DpSGNYS motif and then affinity purified. We carried out experiments to characterize this reagent using FLAG-tagged IFNAR1 proteins expressed in 293T cells and purified with anti-FLAG antibody. These experiments showed that the antibody indeed specifically recognizes IFNAR1 phosphorylated on Ser535 but not on Ser539 in both ubiquitinating and immunoprecipitation assays (Fig. 3A).

We then investigated the phosphorylation status of endogenous IFNAR1. HeLaS3 (chosen for these experiments because they express higher levels of endogenous IFNAR1 than 293T cells) were stimulated with IFNa (1000 units/ml) for the indicated times, and immunoprecipitation was carried out on the cell lysates using the antiphospho-Ser535 antibody, followed by immunoblotting with an antibody against IFNAR1. We found that IFNa promotes the phosphorylation of IFNAR1 within 15–30 min of treatment (Fig. 3B). This result (along with the data indicating that integrity of Ser535 is essential for IFNAR1 binding to β-Trcp as well as ubiquitination and degradation of IFNAR1, see Fig. 2) suggests a mechanism for the ligand-dependent down-regulation of IFNAR1 that involves IFNa-stimulated phosphorylation of IFNAR1 within the destruction motif followed by recruitment of β-Trcp and ubiquitination of IFNAR1.

Three Lys Residues Located Proximally to the Destruction Box Are Essential for IFNAR1 Ubiquitination and Degradation—Previous studies on cytoplasmic substrates of the SCFβ-Trcp E3 liga demonstrated the requirement for acceptor lysines that are positioned appropriately from the recognition motif (11). In the case of IκBα, two adjacent lysines (Lys21 and Lys25) positioned 10 and 9 residues upstream of the destruction motif have been shown to be necessary and sufficient for ubiquitination and degradation (14). Also, structural studies using a β-catenin-derived phosphopeptide as a substrate, which contains acceptor lysines positioned similarly to IκBα, indicated that the rate of ubiquitination depends on the proper positioning of the lysines in proximity for the ubiquitin transfer from E2 enzymes (13).

However, the identity of the ubiquitin acceptor lysis residues and the precise mechanism of ubiquitination of membrane receptor substrates such as IFNAR1 remain to be determined. The cytoplasmic tail of IFNAR1 consists of 100 amino acids with a total of seven lysine residues including Lys458, Lys473, Lys488, Lys501, Lys525, Lys526, and Lys547 (Fig. 1). We noted that Lys458 and Lys525 within the cytoplasmic tail of IFNAR1 are located approximately at the same position from the destruction motif as seen in IκBα (Fig. 1) and β-catenin. Thus, we investigated whether these lysines act as ubiquitin acceptor sites in IFNAR1. To this end, we created a FLAG-tagged IFNAR1 K501R/K525R/K526R mutant by site-directed mutagenesis and analyzed the effect of this mutation on receptor stability and ubiquitination. FLAG-tagged IFNAR1 proteins were expressed in 293T cells and their stability was analyzed by pulse-chase assay. Surprisingly, the IFNAR1 K501R/K525R/K526R mutant protein did not exhibit any change in stability and was degraded as efficiently as the wild-type receptor (Fig. 4A). However, an additional mutation of Lys501 stabilized the triple IFNAR1 K501R/K525R/K526R mutant whereas the single IFNAR1 K501R mutant underwent efficient degradation (Fig. 4A). This result suggests that three Lys residues at positions 501, 525, and 526 are essential for IFNAR1 proteolysis.

We next examined the ubiquitination of various IFNAR1 lysine mutants. In vivo ubiquitination of exogenously expressed IFNAR1 receptors was assessed as described under “Experimental Procedures.” Mutation of lysine 501 alone did not result in noticeable changes in the efficiency of ubiquitination of this mutant (Fig. 4B). Whereas the extent of ubiquitination of the other mutants was increased, the efficiency of ubiquitination of the triple mutant was less compared with the wild-type receptor (Fig. 4B). This result suggests that multiple lysine residues within the cytoplasmic tail of IFNAR1 are required for efficient ubiquitination.

2 K. G. S. Kumar and S. Y. Fuchs, unpublished observations.
IFNAR1K501R/K525R/K526R mutant using a co-immunoprecipitation-pulse-chase analysis and Ability of IFNAR1 to Recruit degradation (17, 18). The mechanisms of stabilization of IFNAR1 by counteracting its internalization and lysosomal degradation (17, 18). We sought to investigate whether the IFNAR1 stabilization effect of Tyk2 is mediated by inhibition of β-Treph and thereby result in reduced ubiquitination and degradation. To this end, we assessed the binding of β-Treph to the IFNAR1K501R/K525R/K526R mutant using a co-immunoprecipitation assay. As shown in Fig. 4C, efficiency of β-Treph binding to the IFNAR1K501R/K525R/K526R mutant was similar to the interaction between β-Treph and the wild-type IFNAR1. This finding indicates that an inhibitory effect of the Lys-Arg substitutions on IFNAR1 ubiquitination and turnover does not arise from the lack of binding to β-Treph. Together with results of the pulse-chase analysis and in vivo ubiquitination assays, these data suggest a role for Lys at positions 501, 525, and 526 as potential ubiquitin acceptor sites in ubiquitin conjugation to IFNAR1 mediated by β-Treph.

**Fig. 4.** Identification of Lys residues that are essential for IFNAR1 ubiquitination and degradation. A, pulse-chase analysis of the FLAG-tagged IFNAR1 proteins (wild type or indicated Lys-Arg substitution mutants) that were expressed in 293T cells. Autoradiograph of 35S-labeled proteins immunopurified using M2 antibody and separated on SDS-PAGE is shown. B, in vivo ubiquitination of the FLAG-tagged IFNAR1 proteins co-expressed with HA-tagged ubiquitin in 293T cells as indicated was assessed as described under “Experimental Procedures.” Position of ubiquitinated IFNAR1 species is depicted (Ub−/IFNAR1). C, interaction between FLAG-tagged IFNAR1 proteins and HA-tagged β-Treph expressed in 293T cells was assessed by co-immunoprecipitation with M2 antibody and analysis by immunoblotting with HA antibody (upper panel) and M2 antibody (middle panel). Levels of β-Treph were determined by immunoblotting with HA antibody (lower panel).

Tyk2 Increases the Level of IFNAR1 Independently of the Ability of IFNAR1 to Recruit β-Treph or Undergo Ubiquitination—Previous studies have clearly established that the Tyk2 Janus kinase plays an important role in sustaining the levels of IFNAR1 by counteracting its internalization and lyosomal degradation (17, 18). The mechanisms of stabilization of IFNAR1 by Tyk2 remain to be determined. Constitutive association of Tyk2 with IFNAR1 is essential for IFN signaling; we have previously mapped the Tyk2-binding domain (amino acids 465–511) within the intracellular tail of IFNAR1 (22). Interestingly, this region is adjacent to IFNAR1 ubiquitination sites (Fig. 4B). This result suggests that the three Lys residues at positions 501, 525, and 526 are essential for ubiquitination of IFNAR1 in vivo.

We next investigated the possibility that these Lys-Arg substitutions bring about gross structural changes in the receptor that might affect its interaction with β-Treph, and thereby result in reduced ubiquitination and degradation. To this end, we assessed the binding of β-Treph to the IFNAR1K501R/K525R/K526R mutant using a co-immunoprecipitation assay. As shown in Fig. 4C, efficiency of β-Treph binding to the IFNAR1K501R/K525R/K526R mutant was similar to the interaction between β-Treph and the wild-type IFNAR1. This finding indicates that an inhibitory effect of the Lys-Arg substitutions on IFNAR1 ubiquitination and turnover does not arise from the lack of binding to β-Treph. Together with results of the pulse-chase analysis and in vivo ubiquitination assays, these data suggest a role for Lys at positions 501, 525, and 526 as potential ubiquitin acceptor sites in ubiquitin conjugation to IFNAR1 mediated by β-Treph.

**Fig. 5.** Tyk2 interacts with IFNAR1 and elevates IFNAR1 levels in a manner that depends neither on the presence of a ligand nor on ability of IFNAR1 to interact with β-Treph or undergo ubiquitination. A and B, interaction between FLAG-tagged IFNAR1 proteins and HA-tagged Tyk2 expressed in 293T cells was assessed by co-immunoprecipitation with M2 antibody and analysis by immunoblotting with HA antibody (upper panel) and M2 antibody (middle panel). Levels of IFNAR1 (middle panel) and Tyk2 (lower panel) were determined by immunoblotting with M2 antibody and HA antibodies, respectively. C, experiment was performed as in A except that 293T cells were treated with IFNα (1000 units/ml) for indicated time periods before harvested. D, in vivo ubiquitination of the FLAG-tagged IFNAR1 in 293T cells co-transfected with Tyk2 was assessed by co-immunoprecipitation with M2 antibody and analysis by immunoblotting with HA antibody (upper panel) and M2 antibody (middle panel). Levels of HA antibody (upper panel) and M2 antibody (middle panel) were determined by immunoblotting with HA antibody (lower panel).
cient binding to Tyk2. However, we found that the interaction between Tyk2 and the IFNAR1<sup>5535A</sup> mutant was comparable to the binding of this kinase to the wild-type receptor (Fig. 5A, upper panel, lane 3 versus lane 5). Furthermore, co-expression of Tyk2 led to an increase in the levels of this IFNAR1 mutant as well (Fig. 5A, middle panel, lane 4 versus lane 5). This result suggests that stabilization of IFNAR1 by Tyk2 is not likely to be mediated by interfering with recruitment of E3 ubiquitin ligase by IFNAR1.

Co-expression of Tyk2 also increased the levels of the IFNAR1<sup>K501R/K525R/K526R</sup> mutant protein (Fig. 5B, middle panel, lane 4 versus 5). Furthermore, this mutant interacted with Tyk2 with efficiency similar to IFNAR1<sup>wt</sup> (Fig. 5B, upper panel, lane 3 versus 5). Given that ubiquitination of IFNAR1<sup>K501R/K525R/K526R</sup> is substantially impaired (Fig. 4, panel, lane 4 versus 5), this finding indicates that the stabilizing effects of Tyk2 on IFNAR1 are independent of ubiquitination status of IFNAR1.

Since IFNα promotes β-Trcp binding as well as ubiquitination and subsequent endocytosis of IFNAR1 (9), we investigated whether treatment with the ligand may result in dissociation of Tyk2 from IFNAR1. Such dissociation could hypothetically expose an otherwise unavailable endocytosis dissociation of Tyk2 from IFNAR1. Such dissociation could hypothetically expose an otherwise unavailable endocytosis

If the IFNα-induced down-regulation of its receptor. Such a mechanism requires activation of a yet to be identified kinase in response to IFNα treatment, phosphorylation of IFNAR1 within the destruction motif, recruitment of β-Trcp to phosphorylated IFNAR1, and ubiquitination of IFNAR1 followed by its endocytosis and lysosomal degradation. Future studies will be required to identify the nature of the IFNα-activated kinase that mediates phosphorylation of IFNAR1 within the destruction Ser<sup>535</sup>, and to explore the mechanisms by which IFNα activates such a kinase.

Ubiquitination of plasma membrane receptors on their intracellular domains leads to internalization of these proteins (4, 5, 7, 8). The cytoplasmic tail of IFNAR1 contains seven Lys residues, however, mutation of only three is sufficient to dramatically inhibit ubiquitination and degradation of IFNAR1 (Fig. 4, A and B). Recent structural studies indicate that SCF E3 ligases that complex with a phosphorylated substrate and recruit the E2 ubiquitin-conjugating enzyme exhibit a fairly rigid conformation. This suggests that ubiquitin-accepting lysine residues within a substrate should be optimally aligned toward E2 (29–31). This positioning model predicts an optimal spacing between a degradation motif and a lysine residue as a key characteristic of a bona fide SCF substrate. Indeed, E2b contains specific Lys residues in a position 9–10 amino acid residues proximal to the destruction motif (Fig. 1). Integrons of these Lys residues are essential for degradation of Ib (14). Moreover, it has been recently shown that efficient ubiquitination of a β-catenin-derived phosphopeptide by SCF<sup>β-Trcp</sup> requires such specific spacing (13). Our data showing that a mutation in similarly spaced Lys<sup>526</sup> and Lys<sup>526</sup> substantially decreased the extent of IFNAR1 ubiquitination (Fig. 4B) is consistent with the positioning model and its prediction of the preferred use of specific ubiquitin-acceptor sites by the SCF<sup>β-Trcp</sup> E3 ligase.

However, unlike Ib, despite noticeable decrease in ubiquitination of the IFNAR1<sup>K525R/K526R</sup> mutant, degradation of this mutant remains efficient. Mutation of another Lys residue within the DSG sequence is critically important for degradation of IFNAR1 (9).

DISCUSSION

SCF E3 ligases consist of Skp1 tethering protein, the Cul51-Roc1/Rbx1 complex that enables core ligase activity and an F box protein that acts as the substrate recognition subunit. Phosphorylation-dependent structural determinants that are present in a substrate confer recognition of this substrate by specific F-box proteins (10). In addition, availability of convenient Lys residues, which serve as acceptor sites for ubiquitin conjugation, often determines whether a recruited protein will be defined as a pseudo-substrate or will indeed undergo ubiquitination (15, 24).

SCF<sup>β-Trcp</sup> E3 ubiquitin ligase facilitates ubiquitination of phosphorylated substrates such as Ib and β-catenin, which undergo subsequent degradation via the 26 S proteasome (10, 11, 25). Identification of a membrane receptor as a substrate for β-Trcp adds to the structural complexity of substrate recognition and ubiquitination facilitated by these F box proteins. In the present study, we investigated the structural characteristics required for β-Trcp-dependent ubiquitination of the IFNAR1 subunit of the Type 1 IFN receptor that undergoes degradation via the lysosomal pathway (9).

We found that, similar to Ib and β-catenin, the integrity of both Ser<sup>535</sup> and Ser<sup>539</sup> residues within the DSG(X)2,S destruction motif located in the cytoplasmic tail of IFNAR1 is required for recruitment of β-Trcp. Interestingly, whereas the Ser residue within the DSG sequence is critically important for recruitment of β-Trcp, the requirement for the second Ser residue is less evident in some β-Trcp-interacting proteins including Vpu (26) and Cdc25A (27), although the compo- pose of the destruction motif within Cdc25A requires further clarification (28). Similar to Ib and β-catenin, the recruitment of β-Trcp depends on both Ser<sup>535</sup> and Ser<sup>539</sup>, which are essential for ubiquitination and degradation of IFNAR1 (Fig. 2). However, unlike Ib and β-catenin, which are degraded by 26 S proteasome (10, 11), ubiquitination of IFNAR1 leads to its endocytosis and lysosomal degradation (9).

Extracellular signal-induced phosphorylation of serines within the destruction motif of Ib by Ib kinases provides the hallmark signature recognized by β-Trcp (23). Using phosphospecific antibody, we found that IFNAR1 is similarly phosphorylated within the DSGNY5S motif. Our current data indicate that phosphorylation of IFNAR1 on Ser<sup>535</sup> within the destruction motif is promoted by ligand treatment (Fig. 3). This finding suggests a mechanism for IFNα-induced down-regulation of its receptor. Such a mechanism requires activation of a yet to be identified kinase in response to IFNα treatment, phosphorylation of IFNAR1 within the destruction motif, recruitment of β-Trcp to phosphorylated IFNAR1, and ubiquitination of IFNAR1 followed by its endocytosis and lysosomal degradation. Future studies will be required to identify the nature of the IFNα-activated kinase that mediates phosphorylation of IFNAR1 within the destruction Ser<sup>535</sup>, and to explore the mechanisms by which IFNα activates such a kinase.

Ubiquitination of plasma membrane receptors on their intracellular domains leads to internalization of these proteins (4, 5, 7, 8). The cytoplasmic tail of IFNAR1 contains seven Lys residues, however, mutation of only three is sufficient to dramatically inhibit ubiquitination and degradation of IFNAR1 (Fig. 4, A and B). Recent structural studies indicate that SCF E3 ligases that complex with a phosphorylated substrate and recruit the E2 ubiquitin-conjugating enzyme exhibit a fairly rigid conformation. This suggests that ubiquitin-accepting lysine residues within a substrate should be optimally aligned toward E2 (29–31). This positioning model predicts an optimal spacing between a degradation motif and a lysine residue as a key characteristic of a bona fide SCF substrate. Indeed, Ib contains specific Lys residues in a position 9–10 amino acid residues proximal to the destruction motif (Fig. 1). Integrity of these Lys residues is essential for degradation of Ib (14). Moreover, it has been recently shown that efficient ubiquitination of a β-catenin-derived phosphopeptide by SCF<sup>β-Trcp</sup> requires such specific spacing (13). Our data showing that a mutation in similarly spaced Lys<sup>526</sup> and Lys<sup>526</sup> substantially decreased the extent of IFNAR1 ubiquitination (Fig. 4B) is consistent with the positioning model and its prediction of the preferred use of specific ubiquitin-acceptor sites by the SCF<sup>β-Trcp</sup> E3 ligase.

However, unlike Ib, despite noticeable decrease in ubiquitination of the IFNAR1<sup>K525R/K526R</sup> mutant, degradation of this mutant remains efficient. Mutation of another Lys residue within the DSG sequence is critically important for degradation of Ib (14). Moreover, it has been recently shown that efficient ubiquitination of a β-catenin-derived phosphopeptide by SCF<sup>β-Trcp</sup> requires such specific spacing (13). Our data showing that a mutation in similarly spaced Lys<sup>526</sup> and Lys<sup>526</sup> substantially decreased the extent of IFNAR1 ubiquitination (Fig. 4B) is consistent with the positioning model and its prediction of the preferred use of specific ubiquitin-acceptor sites by the SCF<sup>β-Trcp</sup> E3 ligase.
that elimination of all ubiquitin acceptor sites, including those that are ubiquitinated less efficiently, is required for stabilization of IFNAR1. Nevertheless, considering that a single ubiquitin moiety is sufficient to trigger endocytosis of plasma membrane proteins (4, 6, 7), the selectivity of ubiquitin acceptor sites (three out of seven) within the intracellular domain of IFNAR1 that are essential for its degradation is intriguing. Since well characterized substrates of the SCF\(^{\beta-Trcp}\), such as IxB and \(\beta\)-catenin, also exhibit preferential ubiquitination of a subset of Lys residues, these data further corroborate our previous results suggesting that IFNAR1 is a \textit{bona fide} substrate of the SCF\(^{\beta-Trcp}\) E3 ligase. Future studies are needed to determine whether IFNAR1 undergoes mono- or polyubiquitination and the relative importance of these processes for regulation of the endocytosis and stability of IFNAR1.

Tyk2, a member of the Janus tyrosine kinase family, is constitutively associated with the cytoplasmic domain of IFNAR1 and is important for Type I IFN signaling. Previous studies have demonstrated that, apart from exhibiting kinase activity that regulates IFN\(\alpha\) signaling, Tyk2 plays a key role in sustaining the level of IFNAR1 (17). It has been shown that Tyk2 expression prevents internalization and lysosomal degradation of IFNAR1 thereby stabilizing it at the cell surface (18). We investigated potential cross-talk between Tyk2- and \(\beta\)-Trcp-dependent mechanisms in the regulation of IFNAR1 stability. As evident from Fig. 5, Tyk2 does not act upstream of \(\beta\)-Trcp-mediated ubiquitination of IFNAR1. Expression of Tyk2 did not interfere with ubiquitination of IFNAR1 (Fig. 5D). Furthermore, whereas \(\beta\)-Trcp-dependent ubiquitination relies on IFN\(\alpha\)-inducible phosphorylation of IFNAR1 (Fig. 2 and Ref. 9), regulation of IFNAR1 levels by Tyk2 seems to be independent of the presence of ligand (18). Consistent with the latter data, we did not find any appreciable changes in the binding of Tyk2 to IFNAR1 upon treatment of cells with IFN\(\alpha\) (Fig. 5C).

It is plausible that Tyk2 stabilizes IFNAR1 in a \(\beta\)-Trcp-independent manner. However, the possibility that Tyk2 might act downstream of \(\beta\)-Trcp-mediated ubiquitination cannot be ruled out. Such a possibility would predict that Tyk2 binding to IFNAR1 facilitates recruitment of other factor(s), which might prevent endocytosis of the receptor by masking endocytosis motifs within IFNAR1 (including ubiquitated lysines) and impeding recognition by the endocytic machinery. These hypothetical factors, in turn, could be released from the IFNAR1-Tyk2 complex in response to IFN\(\alpha\) treatment, thereby allowing internalization and lysosomal degradation of IFNAR1. Further studies are required to address this scenario as well as to delineate how Tyk2 regulates the endocytosis and degradation of IFNAR1.

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