Site-specific ubiquitination exposes a linear motif to promote interferon-α receptor endocytosis

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Ligand-induced endocytosis and lysosomal degradation of cognate receptors regulate the extent of cell signaling. Along with linear endocytic motifs that recruit the adaptin protein complex 2 (AP2)–clathrin molecules, monoubiquitination of receptors has emerged as a major endocytic signal. By investigating ubiquitin-dependent lysosomal degradation of the interferon (IFN)-α/β receptor 1 (IFNAR1) subunit of the type I IFN receptor, we reveal that IFNAR1 is polyubiquitinated via both Lys48- and Lys63-linked chains. The SCFβTrcp (Skp1–Cullin1–F-box complex) E3 ubiquitin ligase that mediates IFNAR1 ubiquitination and degradation in cells can conjugate both types of chains in vitro. Although either polyubiquitin linkage suffices for postinternalization sorting, both types of chains are necessary but not sufficient for robust IFNAR1 turnover and internalization. These processes also depend on the proximity of ubiquitin-acceptor lysines to a linear endocytic motif and on its integrity. Furthermore, ubiquitination of IFNAR1 promotes its interaction with the AP2 adaptin complex that is required for the robust internalization of IFNAR1, implicating cooperation between site-specific ubiquitination and the linear endocytic motif in regulating this process.

Introduction

The extent of ligand-induced cell signaling is negatively regulated by cognate receptor endocytosis that is mediated by both clathrin-dependent and independent pathways. Cargo-specific clathrin-dependent endocytosis occurs via an interaction of clathrin lattices formed on the plasma membrane with cargo receptors and relies on adaptor complexes (e.g., adaptin protein complex 2 [AP2] and Dab), which recognize specific linear endocytic motifs present within the cytoplasmic domains of the target receptor. For example, the AP50/μ2 subunit of AP2 recognizes Tyr-based motifs and enables AP2-dependent tethering of cargo to clathrin molecules (Smythe and Warren, 1991; for review see Bonifacino and Traub, 2003).

In addition to the linear endocytic motifs, ubiquitin has emerged as an important internalization signal for numerous eukaryotic plasma membrane proteins (Bonifacino and Weissman, 1998; Hicke and Dunn, 2003). However, the nature of ubiquitination and the topology of the polyubiquitin chain as well as their requirement for internalization and/or postinternalization sorting vary widely among different cargo substrates and remain poorly understood. For example, monoubiquitination is required for the internalization of many yeast receptors but not of mammalian EGF receptor (EGFR). However, ubiquitination is essential for directing EGFR to the lysosome (Levkowitz et al., 1998; Huang et al., 2006). Furthermore, although EGFR undergoes polyubiquitination predominantly via K63-linked chains (Huang et al., 2006), multiple monoubiquitination of EGFR is sufficient for promoting its endocytosis (Haglund et al., 2003a; Mosesson et al., 2003).

The current paradigm (supported by studies on chimerical receptor-ubiquitin fusion proteins in yeast and mammalian cells) suggests that monoubiquitination promotes the internalization and sorting of cargo receptors by recruiting ubiquitin-binding proteins, which link cargo to the components of endocytic and sorting machinery (Dikic, 2003; Haglund et al., 2003a; Hicke and Dunn, 2003; Polo et al., 2003; Raiborg et al., 2003; Sigismund et al., 2004). Interpretation of this model would predict that neither
the topology of ubiquitin chains nor the site of ubiquitin conjugation should determine the efficiency of endocytosis.

However, in addition to monoubiquitination, K63-linked polyubiquitin chains are required for endocytosis of yeast uracil permease (Galan and Haguenaier-Tsapis, 1997; Blondel et al., 2004; Dupre et al., 2004). This linkage also regulates endocytosis of several mammalian proteins, including nerve growth factor receptor TrkA (Geetha et al., 2005), major histocompatibility class I and II proteins (Duncan et al., 2006; Ohmura-Hoshino et al., 2006; Shin et al., 2006; van Niel et al., 2006), aquaporin-2 water channel (Kamsteeg et al., 2006), and some chimerical ubiquitin fusion model proteins (Barriere et al., 2006; Hawryluk et al., 2006). Intriguingly, the lysosomal degradation of Deltesx, a regulator of the Notch pathway, might require K29-linked chains (Chastagner et al., 2006).

It is plausible that the polyubiquitination of receptors provides an advantage over monoubiquitination by increasing the number of interacting modules and, thus, the affinity of interaction between ubiquitinated cargos and ubiquitin-binding proteins (Hawryluk et al., 2006; Traub and Lukacs, 2007). Nevertheless, such increased interactions alone can hardly provide an explanation for how the use of various types of ubiquitination and of diverse ubiquitin chains might contribute to different stages of the endocytosis of various cargo receptors. One potential explanation for such diversity is that the unique linear determinants within the cytoplasmic tails of individual receptors may play a role in ubiquitin-mediated events to ensure the most efficient internalization and/or sorting.

We have investigated the mechanisms that regulate the turnover of IFN-α/β receptor 1 (IFNAR1), a chain of type I IFN receptor that plays a key role in antiviral defense (Constantinescu et al., 1994; Muller et al., 1994). Lysosomal degradation of IFNAR1 requires its ubiquitination (mainly on a cluster of lysine residues, including K501, K525, and K526) by the SCF$^{\beta$Trcp$}$ (Skp1–Cullin1–F-box complex) E3 ubiquitin ligase. This E3 ligase binds to the $^{\beta$Trcp$}$ binding site of IFNAR1; phosphorylation of S535 and S539 residues within this degron is stimulated by the ligand treatment (Kumar et al., 2003, 2004). In this study, we investigated the role of ubiquitination in internalization, postinternalization sorting, and lysosomal degradation of IFNAR1. We report that although polyubiquitination is sufficient for postinternalization sorting of IFNAR1, the maximal rate of IFNAR1 internalization and degradation requires not only both K48 and K63 linkages but also site-specific ubiquitination and its interaction with a linear endocytic motif within the cytoplasmic tail of IFNAR1.

Results

Both K48- and K63-linked polyubiquitin chains are required on IFNAR1 and can be formed by SCF$^{\beta$Trcp$}$

The intracellular domain of IFNAR1 contains six Lys residues, three of which (K501, K525, and K526) form a cluster that is critical for efficient ubiquitination and degradation given that substitution of these three Lys to Arg results in a ubiquitination-deficient and stable mutant, termed IFNAR1$^{KR}$ (Kumar et al., 2004). Coexpression of IFNAR1 with HA-tagged ubiquitin followed by denaturing immunoprecipitation of IFNAR1 revealed an HA-reactive smear (Fig. 1 A) whose apparent molecular mass is greater than expected from IFNAR1 that undergoes multiple monoubiquitination (110 kD + (8 kD × 6) = 158 kD), suggesting that IFNAR1 might be polyubiquitinated. Indeed, direct determination of the ubiquitinated species of IFNAR1 purified from cells using a quantitative mass spectrometry approach revealed the presence of polyubiquitin chains (that included both K48 and K63 linkages but not other types of chains) on both IFNAR1$^{WT}$ and IFNAR1$^{KR}$, in which the latter protein expectedly exhibited less overall levels of ubiquitin linkages (Fig. 1 B and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb .200706034/DC1; and not depicted).

The presence of the K63-linked ubiquitin chains on IFNAR1 was surprising given that (1) the SCF$^{\beta$Trcp$}$ E3 ubiquitin ligase is a major regulator of IFNAR1 ubiquitination (Kumar et al., 2003) and (2) this ubiquitin ligase generally targets its substrates for proteasomal degradation (Harper et al., 2002; Fuchs et al., 2004; Petroski and Deshaies, 2005), which preferentially utilizes the K48-linked chains (Pickart and Fushman, 2004). Therefore, we determined the ability of SCF$^{\beta$Trcp$}$ to catalyze the ligation of polyubiquitin chains using ubiquitin mutants that lack all Lys residues except either Lys48 (R48K) or Lys63 (R63K). In the presence of either UbcH5c or Cdc34 (as an E2 ubiquitin–conjugating enzyme), purified SCF$^{\beta$Trcp$}$ indeed facilitated the formation of both types of chains (Fig. 1 C), indicating that linkages of both types could be formed by this E3 ubiquitin ligase in vitro.

Both K48- and K63-linked polyubiquitin chains are required for efficient IFNAR1 degradation

We next sought to investigate the role of polyubiquitination and different ubiquitin chain topologies in the degradation of IFNAR1. Currently, the only approach available to address this question in mammalian cells is the forced expression of various ubiquitin mutants to overcome the functions of endogenous ubiquitin. This approach is a standard methodology for delineating the role of diverse ubiquitin chains in receptor internalization and turnover (Haglund et al., 2003b; Mosesson et al., 2003; Duncan et al., 2006). We have used this approach together with an IFNAR1 degradation assay (measured by IFNAR1 levels in cells treated with protein synthesis inhibitor cycloheximide; further termed cycloheximide chase), in which treatment with the ligand robustly increases IFNAR1 turnover (Fig. 2 A).

Expression of a lysine-less ubiquitin mutant (K0) interfered with the formation of high molecular weight bands that react with antiubiquitin antibody and represent polyubiquitinated proteins (Fig. S1 B). Cells transfected with K0 exhibited a delayed degradation of either endogenous or cotransfected IFNAR1 in comparison with cells expressing wild-type ubiquitin (Fig. 2, B and C) or empty vector (Fig. S1 C). Expression of K0 mutant also decreased the efficiency of the ubiquitination of endogenous (Fig. 2 D) or exogenously expressed (not depicted) IFNAR1 in cells. Although these results are consistent with the idea that polyubiquitination of IFNAR1 is required for its turnover,
the expression of K0 might interfere with upstream events essential for IFNAR1 ubiquitination such as IFNAR1 phosphorylation within the phosphodegron or with β-transducin repeats–containing protein (βTrcp) recruitment. However, the expression of K0 did not inhibit IFNAR1 phosphorylation on Ser535. To the contrary, this ubiquitin mutant’s expression resulted in the accumulation of phosphorylated IFNAR1 in untreated cells, further indicating the role of polyubiquitination in the degradation of IFNAR1 (Fig. 2 E). Furthermore, the K0 mutant did not inhibit the recruitment of βTrcp to either IFNAR1 (Fig. 2 F) or to other SCF components (Fig. 2 G). Together, these results indicate that IFNAR1 is polyubiquitinated and that K0 might directly inhibit IFNAR1 polyubiquitination (and polyubiquitination-dependent turnover) by capping (i.e., interfering with the elongation of polyubiquitin chains on IFNAR1).

We next tested whether interfering with a specific type of polyubiquitin chain would affect the proteolysis of IFNAR1 (measured by a cycloheximide chase assay) using the expression of various point or reverse ubiquitin mutants (Fig. S1 B). Although the expression of K48R but not of K63R ubiquitin mutant stabilized a proteasomal substrate c-Jun, either of these ubiquitin mutants decreased the rate of degradation of both endogenous (Fig. 3 A) and exogenously expressed (Fig. 3 B) IFNAR1. Consistent with more abundant K48 linkages found on Flag-tagged IFNAR1 (Fig. 1 B), K48R mutant was more efficient in stabilizing exogenous IFNAR1 than K63R mutant, indicating that a fraction of exogenous IFNAR1 might undergo intracellular K48-dependent proteasomal degradation that may occur in the biosynthetic pathway. Given that K29R mutant did not affect IFNAR1 turnover (Fig. 3 B) while being expressed at similar levels as K48R and K63R (Fig. S1 B), it is likely that both K48- and K63-linked (but not K29 linked) ubiquitin chains are important for the efficient degradation of IFNAR1. Remarkably, the expression of neither of these ubiquitin point mutants substantially affected the overall extent of IFNAR1 ubiquitination that was noticeably inhibited by K0 (Fig. 3 C) despite its relatively low level of expression (Fig. S1 B). These data suggest that formation of indiscriminate types of polyubiquitin chains may not suffice for efficient IFNAR1 degradation under conditions in which either K48 or K63 linkage is inhibited.

To corroborate these conclusions, we tested the effects of expression of the reverse ubiquitin mutants, in which all lysine residues except either K48 (R48K) or K63 (R63K) were mutated to arginines. When expressed separately, these mutants inhibited IFNAR1 ubiquitination that was noticeably inhibited by K0 (and to a lesser extent than that produced by point K48R or K63R mutants, most likely as a result of the difference in expression levels; Fig. S1 B). These data suggest that formation of indiscriminate types of polyubiquitin chains may not suffice for efficient IFNAR1 degradation under conditions in which either K48 or K63 linkage is inhibited.

Figure 1. Both K48- and K63-linked polyubiquitin chains are present on IFNAR1 in cells and can be formed by SCFβTrcp in vitro. (A) In vivo ubiquitination of IFNAR1 expressed in 293T cells as indicated, purified via denaturing immunoprecipitation and analyzed by immunoblotting using the indicated antibodies. (B) Amount of IFNAR1-conjugated polyubiquitin chain links (in pmoles) and relative percentage of K48 and K63 linkages found in these preparations were measured by the absolute quantification of ubiquitin adducts technique. (C) In vitro formation of polyubiquitin chains by the SCFβTrcp E3 ligase. Recombinant SCFβTrcp (absent in lane 1) was incubated with E1, E2 [UbcH5C or Cdc34 as indicated], biotinylated ubiquitin (wt, lanes 1–3 and 7–8; R48K, lanes 4, 6, and 9; R63K, lanes 5, 6, and 10), and ATP. The reaction was analyzed by immunoblotting using HRP-conjugated streptavidin.
Nonspecific polyubiquitination is necessary and sufficient for postinternalization sorting of IFNAR1 toward lysosomes

Given that ubiquitination is essential for postinternalization sorting of many signaling receptors, including EGFR (Levkowitz et al., 1998; Huang et al., 2006), we tested whether IFNAR1 sorting also requires ubiquitination. Immunocytochemical analysis showed that internalized wild-type IFNAR1 mostly colocalized with fluorophor-conjugated dextran (which is targeted to the late endosomes/lysosomes; Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200706034/DC1) but not with transferrin (that is localized in early/recycling endosomes). However, an opposite pattern was seen for the partially ubiquitination-deficient IFNAR1KR mutant, who colocalized predominantly...
with transferrin and not with dextran (Fig. 4 A). This result suggests that ubiquitination might be required for efficient sorting of IFNAR1 into the lysosomes.

To verify this conclusion quantitatively, we used fluorescence ratio image analysis (FRIA; see Materials and methods; Sharma et al., 2004) that determines the sorting efficiency of internalized receptors from early endosomes to the lysosomal compartment. This analysis is based on monitoring the luminal pH of cargo-containing vesicles (pH<sub>v</sub>) and relies on the distinct pH<sub>v</sub> of early/recycling endosomes (pH<sub>v</sub> of 6.4–6.5) and late endosomes/lysosomes (pH < 5.5; Mukherjee et al., 1997). Additional control experiments verified that the antibody used did not dissociate from the receptor in a moderately acidic environment (pH of ~5.0; Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200706034/DC1), thus allowing these measurements.

Although a mean pH<sub>v</sub> of internalized wild-type IFNAR1-containing vesicles was 5.01 ± 0.01, indicating its efficient delivery to the lysosomes, the ubiquitination-deficient mutant IFNAR1<sup>KR</sup> remained in vesicles, exhibiting a markedly higher pH<sub>v</sub>. Remarkably, sorting of ubiquitination-competent IFNAR1<sup>KR-RPT</sup> mutant (see Fig. 6, A and D) was more efficient than that of IFNAR1<sup>KR</sup> (Fig. 4 B). These data suggest that the ubiquitination of IFNAR1 is required for routing this receptor to the lysosomes.

Expression of the lysine-less K0 ubiquitin mutant noticeably increased the number of less acidic endogenous IFNAR1-containing vesicles (Fig. S3 B), indicating that polyubiquitination is required for IFNAR1 sorting. Although expression of either
Figure 4. Role of IFNAR1 polyubiquitination in mediating the efficient postinternalization sorting of IFNAR1. (A) Localization of internalized Flag-tagged IFNAR1<sup>wt</sup> and IFNAR1<sup>KR</sup>. IFNAR1 proteins were labeled for 1 h in the presence of IFN-α, anti-Flag antibody, and TRITC-conjugated secondary Fab and were washed and chased (allowed to internalize) for an additional 30 min. Lysosomes and recycling endosomes were labeled with FITC-dextran and FITC-transferrin (Tfr), respectively. Dextran completely colocalized with Lamp1 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200706034/DC1). Single optical sections of representative cells obtained by laser confocal fluorescence microscopy are depicted. A similar localization of IFNAR1<sup>KR</sup> was also observed when chase time was increased to 90 min. Bars, 10 μm. (B) Vesicular pH (pH<sub>v</sub>) of Flag-tagged IFNAR1-containing endosomes/lysosomes in transiently transfected HEK293T cells. Endosomal pH was measured by FRIA upon labeling and 30-min chase, and the frequency distribution of the pH<sub>v</sub> among the indicated number of analyzed vesicles (n) was plotted. After calibration, the ratios are expressed in pH values. A similar distribution for IFNAR1<sup>KR</sup> was also observed at 90-min chase. The IFNAR1<sup>KR-RPT</sup> mutant contains an additional ubiquitination repeat module and is ubiquitination competent (see Fig. 6). (C) Postendocytotic sorting of Flag-tagged IFNAR1<sup>wt</sup> in cells that coexpress ubiquitin constructs (as indicated, upon labeling and chase for 30 min) was monitored by pH<sub>v</sub> measurements as described in B. Data are expressed as the frequency of pH<sub>v</sub> and mean ± SEM (n = 3).
R48K or R63K alone was capable of partially reverting the sorting phenotype of K0, a combination of R48K and R63K proteins expressed together restored the efficient lysosomal sorting of IFNAR1 (Fig. 4 C).

This result could be explained by at least two possibilities. One is that, similar to IFNAR1 degradation, the sorting of IFNAR1 also requires polyubiquitin chains of both K48- and K63-linked topologies. Alternatively, given that the coexpression of R48K and R63K modestly increased IFNAR1 ubiquitination in cells compared with either of these mutants alone (Fig. 3 E), it is plausible that increased efficiency of IFNAR1 sorting could simply result from a higher overall ubiquitination of the cargo. Additional experiments revealed that expression of either K48R or K63R point ubiquitin mutants (ineffective in impeding IFNAR1 ubiquitination; Fig. 3 C) did not inhibit the efficiency of IFNAR1 sorting (Fig. 4 C) despite being expressed at high levels (Fig. S1 B), suggesting that (1) polyubiquitination via either type of chain suffices for efficient IFNAR1 delivery to lysosomes and (2) degradation defects caused by K48R or K63R ubiquitin mutants are likely caused by impaired IFNAR1 internalization.

Polyubiquitination via both K48- and K63-linked chains is required for the efficient internalization of IFNAR1

We previously demonstrated that either inhibition of βTrcp by expression of its dominant-negative βTrcp<sup>DN</sup> mutant or the S535A mutation within IFNAR1 that abrogates βTrcp recruitment decreased the efficiency of endocytosis of radiolabeled IFN-α (Kumar et al., 2003). To directly determine a role of the ubiquitination of IFNAR1 in its internalization, we established a model system for assessment of the ligand-stimulated internalization of endogenous (Fig. 5 A) or exogenous (not depicted) IFNAR1 using two independent endocytosis assays (fluorescence-based and cell surface biotinylation; see Materials and methods).

The rate of IFNAR1 endocytosis was sensitive to RNAi against the clathrin heavy chain but not to treatment with the caveolae inhibitor filipin (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200706034/DC1). This rate was decreased either by knocking down βTrcp2 (short hairpin RNAs [shRNAs] against βTrcp [shBTR]; Fig. 5, B and C) or by expressing βTrcp<sup>DN</sup> mutant (not depicted). Given that this delay in IFNAR1 endocytosis is already observed at 2 min (Fig. 5 B), it is likely that it reflects changes in the initial internalization receptor rather than in recycling, although the latter possibility cannot be formally excluded. This result indicates that ubiquitination of IFNAR1 might be required for the maximal rate of its endocytosis. To confirm this possibility, we used ubiquitination-deficient IFNAR1 mutants (Fig. 6 A). In line with our data, which were obtained using radiolabeled IFN-α, Ser to Ala substitution within the phosphodegron of IFNAR1 produced a mutant protein (IFNAR1<sup>SA</sup>) that exhibited a reduced rate of internalization (Figs. 5 D and 6 E). Furthermore, another partially ubiquitination-deficient IFNAR1<sup>SR</sup> was also internalized less efficiently compared with wild-type IFNAR1 (Figs. 5 E and 6 F). In all, these data suggest that ubiquitination of IFNAR1 is required for its efficient internalization.

The expression of a lysine-less K0 mutant dramatically reduced the efficiency of the internalization of IFNAR1 (Fig. 5 F and Fig. S4 B) but not of transferrin receptor (not depicted), which is known to undergo ubiquitin-independent clathrin-dependent endocytosis (Hoeller et al., 2006). The latter result indicates that K0 expression does not indiscriminately affect the function of clathrin-based endocytic machinery. Intriguingly, the expression of either K48R, K63R, R48K, or R63K but not of K29R decreased the rate of IFNAR1 internalization observed in the presence of wild-type ubiquitin (Fig. 5 F and Fig. S4 C), indicating that both types of chains are essential for promoting the efficient endocytosis of IFNAR1. The higher inhibitory efficiency of K48R and K63R observed (in comparison with that of K0 or of reverse R48K or R63K mutants) is most likely due to the higher levels of their expression (Fig. S1 B). Furthermore, although the expression of reverse R48K or R63K mutant alone also led to the inhibition of IFNAR1 internalization, the combined expression of these mutants improved the efficiency of IFNAR1 internalization almost to the level observed in cells transfected with wild-type ubiquitin (Fig. 5 F). Collectively, these results suggest that both K48- and K63-linked polyubiquitin chains are required for the efficient internalization of IFNAR1.

Both site-specific ubiquitination of IFNAR1 and integrity of a linear endocytic motif are required for efficient internalization of IFNAR1

We noted that IFNAR1<sup>SR</sup> mutant lacking the K501/525/526 cluster (but harboring several other lysines within its cytoplasmic tail) undergoes ubiquitination to some extent (Figs. 1 B and 6 D; Kumar et al., 2004). Nevertheless, this mutant exhibited an impaired internalization (Figs. 5 D and 6 F) and turnover (Kumar et al., 2004). Therefore, we investigated the possibility that a specific position of ubiquitin acceptor sites might play a role in IFNAR1 internalization.

The SCF<sup>Trcp</sup><sup>E3</sup> ubiquitin ligase is known to preferentially conjugate ubiquitin to the lysine residues located 9–13 amino acids upstream of the ligase recognition site (Tang et al., 2003; Wu et al., 2003). These characteristics enabled us to generate IFNAR1 mutant proteins in which ubiquitination-conferring sequences (including phosphodegron and ubiquitin-accepting lysines) were copied and fused to the distal part of ubiquitination-and endocytosis-deficient IFNAR1<sup>SA</sup> and IFNAR1<sup>SR</sup>, resulting in the repeat mutants, including IFNAR1<sup>SA-RPT</sup> and IFNAR1<sup>SR-RPT</sup> (Fig. 6 A). Introduction of such repeat sequence restored the ability of IFNAR1<sup>SA</sup> to undergo Ser phosphorylation within the phosphodegron (Fig. 6 B), to recruit βTrcp (Fig. 6 C), and to get ubiquitinated to an extent no less than that of wild-type IFNAR1 (Fig. 6 D). However, the internalization of IFNAR1<sup>SA-RPT</sup> mutant did not proceed faster than that of IFNAR1<sup>SA</sup> protein (Figs. 5 D and 6 E). Furthermore, although another IFNAR1<sup>SR-RPT</sup> mutant exhibited an increased Ser phosphorylation, βTrcp binding, ubiquitination, and lysosomal targeting as compared with the IFNAR1<sup>SR</sup> protein (Figs. 4 B and 6, B–D), the internalization rates of these mutants did not differ much and were lower compared with wild-type IFNAR1 (Fig. 6 F). Together, these results...
Figure 5. **Role of ubiquitination in IFNAR1 internalization.**

(A) Effect of IFN-α (black squares) on the internalization of endogenous IFNAR1 measured by a fluorescence assay. All other experiments were performed in the presence of IFN-α. (B and C) Effect of βTrcp2 knockdown (shBTR) on IFNAR1 internalization observed by fluorescence assay (B; white squares) or biotinylation assay (C). (C) The internalized, biotinylated IFNAR1 analyzed by immunoblotting using anti-Flag antibody appears at the time points indicated in minutes on the top. 100% indicates the sample that represents the total amount of biotinylated IFNAR1. Quantification of these panels was calculated as [signal at time point X – signal at time point 0]/signal at 100%.

(D and E) Comparison of the internalization of wild-type IFNAR1 (black squares) and its ubiquitination-deficient mutants IFNAR1SA and IFNAR1SA-RPT (D; white squares and diamonds, respectively) and IFNAR1KR (E; triangles) that was assessed by biotinylation assays. NB, nonbiotinylated control. (F) Effect of expression of the indicated ubiquitin mutants on the internalization of IFNAR1 assessed by fluorescent assay. Data are depicted as the percentage of IFNAR1 internalization detected in cells expressing wild-type ubiquitin (100%) at 15 min. *, P < 0.05 (t test; differences compared with wild-type ubiquitin). Error bars represent SEM.
suggest that the ubiquitination of IFNAR1 alone is not sufficient for conferring a maximum rate of its internalization and that the site of IFNAR1 ubiquitination may play an important role in this process.

This site specificity also indicates that some other determinants within IFNAR1 cytoplasmic tail might contribute to the rate of IFNAR1 endocytosis. Interestingly, the key lysine residues (mutated in IFNAR1KR) are located within the proximity of a
Tyr-based linear endocytic motif (Y466VFF), which we found to play a role in a basal ubiquitin-independent endocytosis of IFNAR1 via interacting with AP2 (our unpublished data). Single amino acid substitution of Tyr466 to Phe within this motif resulted in an IFNAR1YF protein (Fig. 6 A) that exhibited grossly impaired internalization (Figs. 6, E and F; and 7 A) despite being competent in Ser535 phosphorylation, recruitment of βTrcp, and ubiquitination (Fig. 6, B–D). Furthermore, while undergoing efficient ubiquitination, IFNAR1YF mutant degraded much slower than wild-type IFNAR1 (Fig. 7 B). These results confirm that ubiquitination is not sufficient for rapid IFNAR1 internalization and turnover, suggesting that the linear endocytic motif plays a key role in these processes.

Tyr-based linear endocytic motifs are known to serve as a recognition site for the AP50 subunit of the AP2 complex (for review see Bonifacino and Traub, 2003). Immunoprecipitated from ligand-treated cells, the IFNAR1YF mutant did not efficiently interact with coexpressed AP50. These data indicate that binding of AP50 to IFNAR1 is mainly mediated by a linear motif rather than by receptor ubiquitination. Remarkably, endocytosis-deficient IFNAR15A and IFNAR1KR mutants that contain the unmodified Tyr-based motif exhibited a noticeably decreased interaction with AP50 (Fig. 7 C) that was proportional to the extent of their ubiquitination (Fig. 6 D). This result suggests that IFNAR1 ubiquitination might be required for exposure of the Tyr-based motif to AP2 components.

Indeed, although a brief treatment of cells with IFN-α stimulated the interaction between endogenous IFNAR1 and AP50, inhibition of IFNAR1 ubiquitination by expressing either dominant-negative βTrcp52 or shRNA against βTrcp2 decreased this interaction (Fig. 7 D). Furthermore, the expression of either K0, K63R, or K48R (but not K29R) ubiquitin mutants also decreased the ligand-induced recruitment of AP50 to endogenous IFNAR1 (Fig. 7 E). Finally, RNAi-mediated knockdown of an AP2 subunit (α2) led to a noticeable decrease in the rate of internalization of endogenous IFNAR1 (Fig. 7 F). Together, these data suggest that ubiquitination of IFNAR1 is required for efficient exposure of the Tyr-based linear endocytic motif within IFNAR1 to the interaction with AP2, which is essential for achieving the maximum rate of IFNAR1 internalization.

**Discussion**

**Interaction between ubiquitination and the linear endocytic motif in internalization of IFNAR1**

Current paradigm on the role of ubiquitination in the endocytosis of cell surface receptors proposes that ubiquitinated cargo (often monoubiquitinated) is recognized by the ubiquitin-binding domain–containing adaptor proteins. These proteins tether this cargo to the components of endocytic and sorting machinery, thereby enabling receptor internalization and postinternalization sorting. Within the original model, internalization or sorting could be triggered simply by a linear fusion of ubiquitin to the intracellular tail of a receptor. This suggests that neither polyubiquitination, the topology of ubiquitin chains, nor the site of ubiquitin conjugation should be important for the efficiency of endocytosis (Dikic, 2003; Haglund et al., 2003a; Hicke and Dunn, 2003; Polo et al., 2003; Raiborg et al., 2003; Sigismund et al., 2004).

In this study, we report that IFNAR1 undergoes polyubiquitination via both K48- and K63-linked chains. Although an efficient internalization of IFNAR1 requires both types of these chains, once the receptor is internalized, its postinternalization fate depends on polyubiquitination that does not discriminate between K48- and K63-linked chains. This diverse ubiquitination type requirement indicates that IFNAR1 internalization and IFNAR1 postinternalization sorting use differential mechanisms by which ubiquitination regulates these events.

Remarkably, a conjugation site-specific polyubiquitination of IFNAR1 is required but not sufficient for the maximum rate of IFNAR1 internalization, which also depends on the integrity of a linear Tyr-based endocytic motif. Interfering with either K48/K63 linkages or the ubiquitination of IFNAR1 decreases the interaction between this receptor and components of the adaptin complex, which is essential for efficient IFNAR1 internalization. Collectively, our data suggest that ubiquitination of IFNAR1 on specific ubiquitin-acceptor lysines might unmask a Tyr-based linear endocytic motif for its interaction with the adaptin components under conditions permitting both K48- and K63-linked ubiquitin chains. Such ubiquitination-dependent exposure of a linear endocytic motif to increase the endocytic rate represents a novel paradigm on a role of ubiquitin in regulating the endocytosis of signaling receptors.

**Putative mechanisms for ubiquitin-dependent exposure of an endocytic motif within IFNAR1 and its significance for endocytosis**

Several models might explain how site-specific ubiquitination contributes to interaction between the linear motif within IFNAR1 and the adaptin complex, which is required for the efficient internalization of IFNAR1. In the absence of the ligand, either an intramolecular interaction or a putative IFNAR1-interacting protein complex might shield a Tyr-based linear motif and prevent it from interacting with the AP50 subunit. Upon IFN addition, a yet unknown protein kinase phosphorylates IFNAR1 within its phosphodegron on Ser535 and Ser539 (Kumar et al., 2004; Marijanovic et al., 2006). This phosphorylation enables recruitment of the SCFβTrcp E3 ubiquitin ligase that facilitates the ubiquitination of IFNAR1 on various lysines, including a cluster of K501, K525, and K526 (Kumar et al., 2003, 2004).

This site-specific ubiquitination of IFNAR1 results in exposure of the linear motif to AP50. This exposure might occur via altering the conformation of IFNAR1 intracellular tail or/and rearranging a putative masking protein complex. The latter scenario is based on exciting discoveries that many proteins can interact with ubiquitin moieties either via bona fide specialized ubiquitin-interacting domains (French et al., 2005; Seet et al., 2006) or via specific subtypes of other domains such as coiled-coil, LZ, novel zinc finger (Kanjyama et al., 2004; Ee et al., 2006), and SH3 domains (Stamenova et al., 2007). Whereas additional mechanisms leading to an exposure of a linear endocytic motif cannot be ruled out, ubiquitination-stimulated
interaction of the AP2 adaptin complex with such a motif on one hand and clathrin lattices on the other hand should promote the efficient internalization of IFNAR1.

Future studies will identify the nature of masking the linear endocytic motif in the absence of ubiquitination. A candidate regulator of such a phenomenon is a Janus kinase, Tyk2, whose interaction with IFNAR1 prevents its ligand-independent endocytosis in a catalytic-independent manner (Ragimbeau et al., 2003). Although we found that Tyk2 inhibits ligand/ubiquitination-independent internalization of IFNAR1 via masking the linear endocytic motifs (including a Tyr-based motif; our unpublished data), a dissociation of Tyk2 from IFNAR1 under the conditions of ligand treatment described in Fig. 7 (D and E) was not observed in this study (our unpublished data).

In addition, the catalytic function of Jak kinases and counterpart protein phosphatases that determine the phosphorylation status of the Tyr residues within IFNAR1 (including Y466; Yan et al., 1996) might add another level of regulation complexity. Phosphorylation of Y466 is expected to reduce the affinity of AP50 for the Tyr-based endocytic motif as described for CTLA-4.
(Chuang et al., 1997; Shiratori et al., 1997; Zhang and Allison, 1997). Thus, it is possible that tyrosine phosphatase activities play a role in regulating IFNAR1 endocytosis (indeed, the over-expression of tyrosine phosphatase PTP1B stimulates IFNAR1 internalization; unpublished data). Further investigation is underway of this modification as well as of proteins that interact with Tyk2 and ubiquitinated versus nonubiquitinated IFNAR1 and that might regulate IFNAR1 endocytosis and degradation.

**Diverse topology of ubiquitin chains and their requirement for the internalization of IFNAR1**

Whereas the SCFβTrcp E3 ubiquitin ligase is well known to facilitate the K48-linked polyubiquitination of numerous proteasomal substrates (Harper et al., 2002; Fuchs et al., 2004; Petroski and Deshaies, 2005), our data demonstrate that IFNAR1 is ubiquitinated by K63-linked chains as well. Although we cannot rule out that an additional E3 produces the latter chains on IFNAR1 in vivo, purified SCFβTrcp in combination with Cdc34 can actually produce both types of linkages in vitro (Fig. 1 C), and the efficiency of these reactions is proportional to the levels of specific linkages present on IFNAR1 in cells (Fig. 1 B). Intriguingly, other ubiquitin ligases of both HECT and RING superfamilies (such as yeast Rsp5 and mammalian c-Cbl) were implicated in the degradation of both lysosomal (such as uracil permease and EGFR; Hicke and Dunn, 2003) and proteasomal (such as Mga2p120 [Shcherbik et al., 2003] and Syk [Rao et al., 2001]) substrates.

It remains unclear what factors would determine the specificity of polyubiquitin linkages present on IFNAR1. It is plausible that recruitment of specific E2 in cells would serve as such a determinant. Although some of our in vitro studies were performed using a notoriously promiscuous UbcH5c (Brzovic and Klevit, 2006), which is known to produce non–K48-linked chains when combined with recombinant Cul1 and Roc1 in vitro (Wu et al., 2002), we observed a similar result using a more specific Cdc34, albeit the latter formed K63-linked chains with lesser efficiency (Fig. 1 C). Other contributing factors may include special substrate constraint or a lack thereof (for example, cytoplasmic- or membrane-bound localization of a substrate) as well as the extent of Cul1 modification by Nedd8.

Future studies will determine why both types of polyubiquitin chains are needed for efficient IFNAR1 internalization and turnover. Although our mass spectrometry data are consistent with analyses using ubiquitin mutants, the current state of technology does not allow us to directly address this issue because we cannot distinguish whether the effect of the ubiquitin mutants is mediated solely by the altered ubiquitination of IFNAR1 itself or by other potential regulators. On one hand, the possibility that mixed topology K48/K63 chains are needed for efficient IFNAR1-binding complex. Another possibility is that although the K63-linked chain (conjugated to IFNAR1) titrates away a putative masking protein, the expression of K48R interferes with the proteasomal degradation of such a protein, thereby saturating K63-dependent binding and preventing exposure of the linear motif to AP50. Intriguingly, although a decrease in internalization of growth hormone receptor in cells treated with proteasomal inhibitors has been previously reported (van Kerkhof et al., 2000), such treatment did not affect IFNAR1 internalization (our unpublished data).

**Role of ubiquitination in receptor internalization**

As per the role of ubiquitination, linear motifs, or both in clathrin-mediated internalization of cell surface receptors, three groups of receptors could be envisioned. The first group includes receptors whose linear motifs are required and sufficient for rapid internalization (for example, transferrin receptor). The second group includes those receptors whose ubiquitination is required and sufficient for the maximal rate of internalization. In keeping with the well-established fact that any kind of ubiquitination should provide a substrate receptor with an endocytic signal (Hicke and Dunn, 2003), a properly ubiquitinated IFNAR1YF mutant still undergoes internalization at a noticeable rate (Figs. 6 F and 7 A). These results indicating that the ubiquitination of IFNAR1 is sufficient for some extent of internalization are in line with reports on chimerical proteins that harbor ubiquitin fusion instead of a cytoplasmic tail (Haglund et al., 2003b; Barriere et al., 2006). Further studies will determine other endogenous proteins that undergo clathrin-dependent internalization solely via the ubiquitin-mediated pathway. Additional complexity is constituted by the existence of ubiquitin-dependent but clathrin-independent mechanisms of receptor internalization (Sigismund et al., 2005).

Cooperation between ubiquitination and linear endocytic motifs might serve as a mechanism for promoting internalization of the third group of cell surface receptors, including IFNAR1. Whereas ubiquitination appears to affect the function of the linear motif within IFNAR1, these two pathways could work simply in an additive manner for some other receptors to ensure their robust internalization. Given that different receptors interacting with their own binding proteins may harbor different linear motifs within diverse structural contexts, it is not surprising that various individual receptors might require different types of ubiquitination (multiple mono- versus polyubiquitination of various chain topologies) for maximal efficiency of endocytosis as reported above (see Introduction).

Our observations presented here are limited to the role of protein ubiquitination in the recognition of linear determinants (within a substrate of ubiquitination) that occurs during endocytosis. However, given that ubiquitination is also implicated in regulating other types of protein trafficking (e.g., in the nuclear export of p53; Li et al., 2003), it is tempting to speculate about a possible interaction between ubiquitination and linear trafficking signals that might use a similar mechanism to modulate the efficiency of protein transport between different subcellular compartments. Future studies are required to test this possibility.
Materials and methods

Cells and constructs

HEK293T cells were maintained and transfected as described previously (Fuchs et al., 1999), Vectors for the expression of HA- or myc-tagged βTrCP2 (wild-type or dominant-negative βTrCP2; Fuchs et al., 1999; Winston et al., 1999), C-terminal Flag-tagged human IFNAR1 (Kumar et al., 2003), HA-ubiquitins (Masesson et al., 2003), and HA-APSO (Rohde et al., 2002) were previously described. Human IFNAR1 with Flag tag inserted after the signal sequence at the N terminus was constructed by an overlap PCR method. All IFNAR1 mutations created by PCR and site-directed mutagenesis were verified by DNA sequencing. Reagents for RNAi approach, including shRNA constructs for knocking down the heavy chain of clathrin (Royle et al., 2005) or βTrCP2 (Li et al., 2004), as well as siRNA oligonucleotides for knocking down the α subunit of the AP2 complex (Barriere et al., 2006) were characterized and described previously. Control shRNA and siRNA were targeted against GFP (Jin et al., 2003) and luciferase (Kumar et al., 2003), respectively.

Antibodies, immunotechniques, and ubiquitination assays

Antibodies were purchased against Flag (M2; Sigma-Aldrich), HA (HA11; Covance), myc-tag, β-actin, and c-Jun (Santa Cruz Biotechnology, Inc.). AP50- and β-2adrenoceptor (BD Transduction Laboratories) and IFNAR1 (Thermo Fisher Scientific) antibodies were generated using synthetic peptides. Antibodies were purified against the predicted epitopes of recombinant proteins. Other antibodies were purchased against Flag (M2), HA (HA11), and ubiquitin (FK2; BIOMOL International, L.P.). Antibodies that recognize endogenous IFNAR1 (Goldman et al., 1999) and IFNAR1 phosphorylated on Ser535 (pS535; Kumar et al., 2004) were described previously. Secondary antibodies conjugated to HRP were purchased from Chemicon and Invitrogen. Immunoprecipitation and immunoblotting procedures were described previously (Fuchs et al., 1999). Cytosolic cell lysates were obtained, analyzed, and quantitated using Image software (version Beta 4.0.2; Scion), and the digital images were prepared using Photoshop 7.0 software (Adobe).

In vitro ubiquitin ligation assay was performed as previously described (Tan et al., 1999) using recombinant SCF and either Ub-H5c or Cdc34 (gifts of Z.-Q. Pan, Mount Sinai School of Medicine, New York, NY) as E2. In brief, SCF was incubated with E1, E2, ATP, and biotin-labeled ubiquitins (wild-type, R48K, and R63K as indicated; Boston Biochem). After 1-h incubation at 37°C, the reaction was terminated by adding SDS-PAGE loading buffer, and ligase was boiled, separated on 10% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. The blot was blocked with 3% BSA in TBS, incubated with Avidin-D-Biotin, and developed by chemiluminescence.

In vivo ubiquitination was performed as described previously (Kumar et al., 2003). In brief, 293T cells in 10-cm dishes were cotransfected with 1 μg Flag-tagged IFNAR1 or empty vector with 4 μg of various HA-tagged ubiquitin plasmids (where indicated). 36 h after transfection, cells were harvested and lysed under denaturing conditions (1% SDS). Upon neutralization of SDS with 10 vol of 1% Triton X-100, IFNAR1 was immunoprecipitated with anti-Flag antibody (when cotransfected with Flag-IFNAR1 plasmids) or with anti-IFNAR1 antibody (when cotransfected with empty vector) and analyzed by immunoblotting with the indicated antibodies. Where indicated, the relative percentage of ubiquitination was calculated by setting the ratio of ubiquitination with wild-type IFNAR1 or wild-type ubiquitin as 100%.

Mass spectrometric analysis of polyubiquitin chains linked to IFNAR1

Flag-tagged IFNAR1 proteins (wild type or KR mutant) were linked to IFNAR1 293T cells and purified using M2 agarose (Sigma-Aldrich) followed by sequential stringent washes with buffers containing 1% Nonidet P-40, 1 M NaCl, and 0.1% SDS. Subsequent washes were performed with the same buffer containing 200 mM NaCl, 100 mM NaCl, and, finally, TBS. These washes were performed with the aim to remove any detectable Flag from non-precipitated IFNAR1. The precipitated proteins were eluted with 0.1 M glycine, pH 3.0 and resolved by SDS-PAGE. Approximately 1% of the eluate was analyzed by immunoblotting with anti-ubiquitin antibody (FK2; BIOMOL International, L.P.) to determine the apparent molecular weight of ubiquitinated receptor and anti-Flag M2 antibody. 80% of eluate was analyzed by SDS-PAGE followed by colloidal Coomassie (Invitrogen) staining.

Cell surface biotinylation was performed as described previously (Hammond et al., 2003). This assay uses immunoblotting analysis to detect biotinylated proteins that were protected from debiotinylation as a result of their internalization. In brief, 293T cells in 60-mm dishes were transfected (where indicated) and plated onto 24-well plates. Transfection of shRNAs against clathrin heavy chain (2 μg of plasmids) or the α-adaptin subunit of AP2 complex (100 nM oligonucleotides) were performed twice in 24-h intervals and analyzed after 72 h. Cells were starved for 2 h in serum-free DMEM and chilled on ice for 15 min. Internalization was initiated by the incubation of cells with warm (37°C) serum-free DMEM containing IFN-α for the indicated time periods at 37°C, or cells were kept on ice without IFN-α for the same time period by placing the plate on ice. Cells were washed, blocked, and incubated with anti-IFNAR1 antibody (AA3 for endogenous IFNAR1) or anti-FLAG antibody (monoclonal Flag M2 for exogenous IFNAR1 and its mutants; Sigma-Aldrich) for 1 h, washed, and incubated with HRP-conjugated goat anti-mouse secondary antibody (Invitrogen) for 1 h. After extensive washing, cells were incubated with AmplexRed Ultra Reagent (10-acetyl-3,7-dihydroxyphenoxazin; Invitrogen). Aliquots were transferred to black 96-well plates, and fluorescence was measured by reading with a fluorescence plate reader (Chameleon; BIOSCAN) using 530-nm excitation and 590-nm filters for excitation. Results were calculated using the following formula: % Internalized = 1 - (V0 - Vf)/Vf at t0 × 100, in which V0 is the value of background (mock transfected or nonspecific antibody), t0 is time point 0, and tf is time point f. Average results of at least four experiments (each in triplicates) ± SEM are presented.

Cell surface bioirritation was performed as described previously (Hammond et al., 2003). This assay uses immunoblotting analysis to detect biotinylated proteins that were protected from debiotinylation as a result of their internalization. In brief, 293T cells in 60-mm dishes were transfected with 0.1 μg of various C-terminal 3×-Flag-tagged IFNAR1 plasmids and 1.9 μg pCDNA3 or other indicated plasmids. 36 h after transfection, cells were starved in serum-free DMEM for 2 h, chilled on ice, and washed with PBS. Surface bioirritation was performed with 0.25 mg/ml EZ-Link-Sulfosuccinimidyl-NHS-S-biotin (Thermo Fisher Scientific) in 150 mM Na2B4O7, pH 8.0, for 15–30 min on ice. After washing the excess unreacted biotin by washing three times with DMEM containing 0.1% BSA followed by an additional wash with ice-cold Heps-buffered saline (HBS; 10 mM Heps-NaOH, pH 7.4, 150 mM NaCl) containing 0.7 mM CaCl2 and 0.5 mM MgCl2 (HBS−), cells were either kept on ice or incubated at 37°C after adding warm HBS containing IFN-α or IFN-γ to allow internalization at the indicated time points. After internalization, cells were returned to ice and were either left untreated (100%) or subjected to three 20-min incubations with 100 mM sodium 2-mercaptoethanesulfonic acid (MESNA; Sigma-Aldrich) in 50 mM Tris-HCl, pH 8.6, 100 mM NaCl, 1 mM EDTA, and 0.2% BSA to selectively remove biotin remaining at the cell surface. Time point 0 was set using cells that were biotinylated and kept on ice and treated with MESNA to remove surface biotin. Cells that were not biotinylated were used as a negative control. Cells were then rinsed twice with HBS− and residual MESNA quenched by 10-min incubation with ice-cold 120 mM iodoacetamide in HBS−. After two additional washes with ice-cold HBS, cells were lysed with 60 mM n-octyl β-glucopyranoside (Thermo Fisher Scientific) and 0.1% SDS in the same buffer containing protease inhibitor cocktail for 10–20 min and were centrifuged at 15,000 rpm for 15 min. Biotinylated proteins were recovered by incubating with immobilized Neut-Avidin (Thermo Fisher Scientific) overnight, and proteins were washed, boiled with SDS-PAGE loading buffer, separated on 10% SDS-PAGE, and analyzed by immunoblotting with anti-Flag M2 antibody to detect Flag-tagged IFNAR1. Denaturing data were obtained and analyzed using Image software. Results were expressed as the percentage internalized to total biotinylated receptor (after subtracting the value at time point 0). Representative results of three independent experiments are shown.
For detecting endogenous AP50 interaction with IFNAR1, four 10-cm plates of 293T cells at 60–70% confluence were either mock transfected or transfected with various constructs (shRNA against β-Trcp2 or GPP, pEF-βTrcp2M, ubiquitin constructs, or empty vector; 4 μg/plate). 48 h after transfection, cells were starved in serum-free DMEM containing 20 mM methylimidazole hydrochloride for 3 h. Cells were chilled on ice for 15 min and stimulated with 1,500 μg/ml IFN-α for 2.5 min at 37°C. Cells were washed with ice-cold PBS, harvested, and collected by centrifugation at 3,000 rpm for 5 min, and the pellets were lysed using TGH lysis buffer (1% Triton X-100, 10% glycerol, 50 mM Hepes, pH 7.4, 1 mM EGTA, 50 mM NaCl, 1 mM sodium orthovanadate, 1 μM PMSF, 544 μM β-mercaptoethanol, 1% Triton X-100, 10% glycerol, 50 mM Hepes, pH 7.4, 1 μM EGTA, 50 mM NaCl, 1 mM sodium orthovanadate, 1 mM PMSF, 544 μM β-mercaptoethanol, and protease inhibitor cocktail) by rotating at 4°C for 1 h. After centrifugation at 14,000 rpm for 30 min, supernatants were preclarified with 30 μl of protein A beads for 2 h. Lysates were incubated with EA12 antibody for 3 h followed by 50 μl of protein A beads overnight at 4°C, and lysates were washed with lysis buffer four times, boiled with SDS-PAGE loading buffer, separated by 10% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The blot was probed with AP50 antibody to detect AP50 and with G8 antibody to detect IFNAR1. Whole cell lysates were analyzed for the expression of AP50. Representative results of four independent experiments are shown.

For interactions of exogenous IFNAR1 with AP50, 293T cells were transfected with 3 μg N-terminal Flag-tagged IFNAR1, 2 μg HA-tagged AP50. and 0.25 μg HA-tagged Tyk2 (to prevent ligand-independent internalization of IFNAR1, Rangbe et al., 2003). 48 h after transfection, cells were starved in serum-free DMEM containing 20 mM methylimidazole hydrochloride for 3 h. Cells were harvested using cell dissociation buffer (enzyme-free Hank’s based; Invitrogen), incubated with anti-Flag antibody (M2; Sigma-Aldrich) for 3–4 h, and incubated with 1,500 IU/ml IFN-α for 2.5 min by incubating at 37°C. Cells were washed thoroughly with 1× PBS and pelleted by centrifugation at 3,000 rpm for 5 min. Pellets were lysed with TGH lysis buffer. Lysates were incubated with protein A beads overnight, and the beads were washed with lysis buffer. After boiling with SDS-PAGE loading buffer, an aliquot from each sample was separated on 10% SDS-PAGE and analyzed for IFNAR1 levels by Flag immunoblotting to normalize the receptor levels. Based on normalization, adequate amounts of samples were separated on 10% SDS-PAGE and analyzed by immunoblotting with anti-HA antibody to detect AP50 and anti-Flag antibody to detect IFNAR1. Whole cell lysates were analyzed for the expression of HA-AP50. Representative results of three independent experiments are shown.

Vesicular pH (pHv) measurement, microscopy, and image acquisition

For transient transfection of 293T cells, cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations and analyzed after 48 h. For cotransfection, the cDNA ratio of Ub/IFNAR was 2000 (Invitrogen) according to the manufacturer’s recommendations and was added to the transfection mixture before transfection. Cells were plated on poly-L-lysine-coated coverslips 24 h before experiments. Lysosomes were labeled overnight in the presence of 50 μg/ml FITC-dextran (mol wt of 10 kD, Invitrogen) and were chased for >3 h. Recycling endosomes were visualized with 5 μg/ml FITC-Tf (3-h labeling at 37°C) and chased for 15 min. Cells expressing IFNAR1 were allowed to internalize Flag M2 antibody (1/100) complexed to TRITC-conjugated goat anti–mouse IgG (for 45 min at 37°C) in DME supplemented with 1% FBS and were chased as described for pH measurement. Single optical sections were collected by a laser confocal fluorescence microscope (LSM510; Carl Zeiss, Inc.) equipped with a plan Apochromat 63× NA 1.4 objective (Carl Zeiss, Inc.) under conditions described for pH measurement. Images were processed with Photoshop software.

Online supplemental material

Fig. S1 shows purification of IFNAR1 for analysis of ubiquitin chains, expression of ubiquitin mutants, and effect of the lysine-less mutant on IFNAR1 degradation. Fig. S2 shows that dextran colocalized with lysosomal markers. Fig. S3 shows the effect of pH on the binding of anti-IFNAR1 antibody to the receptor chain and effect of the lysine-less ubiquitin mutant on IFNAR1 sorting. Fig. S4 shows the effect of the expression of ubiquitin mutants on the clathrin-dependent internalization of IFNAR1. Table S1 presents a comparison of relative protein abundance in samples from cells expressing different IFNAR1 mutants. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200706034/D1C1.

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