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TYK2 ACTIVITY PROMOTES THE LIGAND-INDUCED IFNAR1 PROTEOLYSIS

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Running title: Catalytic role of Tyk2 in IFN-induced IFNAR1 degradation

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ABSTRACT

The type I interferon receptor is a heterodimer of two transmembrane chains, IFNAR1 and IFNAR2, which are associated to the tyrosine kinases Tyk2 and Jak1 respectively. Ligand-induced down-regulation of the type I interferon receptor represents a major mechanism of negative regulation of signaling and involves internalization and lysosomal degradation of IFNAR1. Interferon α promotes phosphorylation of IFNAR1 on serine 535, followed by recruitment of the β -TrCP2 E3 ubiquitin ligase, ubiquitination of IFNAR1 and proteolysis. The non-catalytic role of Tyk2 in sustaining the steady-state IFNAR1 level at the plasma membrane is well documented, however little is known about Tyk2 function in steps that precede and succeed serine phosphorylation and ubiquitination of IFNAR1 in response to the ligand. Here we show that catalytic activation of Tyk2 is not essential for IFNAR1 internalization, but is required for the ligand-induced IFNAR1 serine phosphorylation, ubiquitination and efficient lysosomal proteolysis.

Keywords: Tyk2, type I IFN, IFNAR1, internalization, degradation, ubiquitination

The abbreviations used are : Jak, Janus kinase; IFN, interferon; Epo, erythropoietin; β -TrCP, beta-transducin repeats-containing protein; RTK, receptor tyrosine kinase; Stat, signal transducer and activator of transcription; GHR, growth hormone receptor; IL-2R, interleukin 2 receptor; EGFR, epidermal growth factor receptor; FERM, band 4.1, ezrin, radixin, moesin; CHX, cycloheximide; MVB, multivesicular body.

INTRODUCTION

Binding of hormones, growth factors and other ligands to their cognate receptors results in the delivery of signals that enable communication between cells and the environment. In order to control cellular responses and preserve homeostasis, the duration and magnitude of signaling events have to be tightly regulated. One way to terminate signal transduction is through the ligand-induced internalization and degradation of the occupied receptors and associated signaling proteins. Extensive studies have been performed on the down-regulation of growth factor receptors containing a tyrosine kinase domain (RTK¹). Among the best characterized system is the epidermal growth factor receptor (EGFR), whose intracellular fate results from the balance of multiple signals. The tyrosine kinase activity of the receptor itself, endocytic motifs located in its cytoplasmic domain, phosphorylation and ubiquitination events as well as a network of interactions with components of the endocytic machinery, all contribute to ultimately direct the activated receptor towards lysosomal degradation [1,2].

Less is known of the regulation of receptors that bind helical-bundled cytokines. These receptors form a large family of over 50 members, characterized by a structurally conserved extracellular cytokine binding module and an intracellular region that varies in length and lacks intrinsic tyrosine kinase activity [3,4]. Functional receptors are homodimers or heterodimers which are constitutively associated to tyrosine kinases of the Janus or Jak family. Ligand binding to the receptor complex results in the catalytic activation of the Jaks which initiate Stat-mediated and other signaling events [5,6]. Recent studies performed on the growth hormone receptor (GHR), the erythropoietin receptor (EpoR) and the interleukin-2 receptor (IL-2R) have provided some insights into ligand-induced traffic events of these receptors. The activated GHR internalizes *via* clathrin-coated pits and is routed to lysosomes for degradation. Both processes, internalization and degradation, are dependent on an active ubiquitination machinery and on proteasomal activity [7,8]. Similar requirements were reported for the EpoR. While still at the cell surface, this receptor undergoes polyubiquitination and proteasome-dependent degradation of part of the intracellular domain. After being internalized, the truncated receptor is routed to lysosomes for final degradation [9]. In contrast to the GH and Epo receptors which form functional homodimers, the high affinity IL-2R is made of three subunits that are differentially sorted after internalization through a clathrin-independent pathway: the α subunit recycles back to the plasma membrane, while the β and γ subunits are sorted to lysosomes [10-12]. Notably, the β subunit requires ubiquitination and proteasomal activity in order to be efficiently degraded [13].

The type I interferon receptor (IFN-R) consists of two subunits, IFNAR1 and IFNAR2, belonging to the cytokine receptor superfamily [4,14,15]. This heterodimeric complex is able to bind all type I IFN subtypes (14 IFN α and one IFN β) and to evoke phosphorylation of Tyk2 and Jak1, bound to IFNAR1 and IFNAR2, respectively [6]. The current model on the formation of the ternary

IFN:receptor complex envisions that the ligand binds first to IFNAR2, for which it displays higher affinity, and this is followed by the transient recruitment of IFNAR1 [16]. Although the affinity of IFNAR1 for the ligand is weak, its tethering to the IFN:IFNAR2 complex is critical for the efficient assembly of the ternary complex. IFNAR1 possesses a large N-glycosylated ectodomain with two potential cytokine binding modules and an intracellular region of 100 amino acids [17,18]. We have previously shown that Tyk2 binds to the juxtamembrane region of IFNAR1 *via* its FERM domain, and in doing so it reduces the basal internalization of IFNAR1 and enhances its level at the plasma membrane independently of catalytic activity [19].

Here, we have focused on the study of the turnover of IFNAR1 following stimulation of cells with IFN α . Since the first measurable event after ligand binding to the receptor is the activation of Jaks, we have also examined the effect of inactivating Tyk2 on internalization and subsequent degradation of IFNAR1. We show that IFN α binding to the receptor complex decreases the surface half-life of IFNAR1 and promotes its degradation in lysosomes. Our functional studies indicate that, although ligand-induced IFNAR1 internalization does not require the kinase activity of Tyk2, this latter contributes to efficient IFNAR1 ubiquitination and degradation.

EXPERIMENTAL

Cells, plasmids, antibodies and other reagents - The human fibrosarcoma Tyk2-deficient 11,1 cells and derived WT-5 and KR-2 clones were described [20,21]. Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 37°C in 6% CO₂. Recombinant Epo was kindly provided by P. Mayeux (Institut Cochin, Paris). Recombinant IFN α 2b was a kind gift of D. Gewert. pRC-Tyk2 and pGFP-Tyk2 were described [22,23]; pSV-SPORT EpoR/R1 Flag was kindly provided by J. Tavernier. Cycloheximide (Sigma) was used at 20 μ g/ml. The lysosomal inhibitor NH₄Cl (Sigma) was used at 20 mM. Phospho-Ser535 specific IFNAR1 Abs were described in [24]. Tyk2 phospho-specific Abs were from Calbiochem. The anti-Tyk2 mAb T10-2 was described [21]. Stat1 and Stat3 tyrosine phospho-specific Abs were from BioLabs and Stat2 tyrosine phospho-specific Abs from UBI. The mAb directed to the Flag epitope was from Sigma. The anti-ubiquitin FK2 mAb was from Biomol. The anti-actin mAb was from Sigma.

Transfections - Stable clones expressing EpoR/R1 were obtained by co-transfecting 11,1 cells with 10 μ g of pSV-SPORT EpoR/R1 Flag and 2 μ g of pcDNA1/puro. Single puro^R colonies were analyzed by anti-Flag western blot. A clone with moderate EpoR/R1 expression was transfected with 15 μ g of pRC-Tyk2 or pRC-K930R with calcium phosphate and neo^R-puro^R clones were selected. Cl.14 and cl.20 were chosen on the basis of comparable Tyk2 levels. For transient transfection, 293T cells were plated at 1.5x10⁶ in 100 mm plates. Next day, 6 μ g of plasmid DNA were transfected using FuGene (Roche). Cells were processed for FACS or western blot 24 hr later.

¹²⁵I-Epo internalization studies - ¹²⁵I-Epo was kindly provided by P. Mayeux (Institut Cochin, Paris). 1 nM (2 U/ml) ¹²⁵I-Epo was used. Non-specific binding, determined by incubating 11,1 cells with ¹²⁵I-Epo, was less than 10%. All reported data represent specific binding. Cells were incubated with ¹²⁵I-Epo in DMEM, 10% FCS, 25 mM HEPES at 37°C. At the indicated times, aliquots of 1.3x10⁶ cells were withdrawn, cooled on ice and washed twice with PBS to remove unbound ligand. Surface bound ¹²⁵I-Epo was released by subjecting the cells to acid wash (150 mM NaCl, 50 mM sodium acetate, pH 3.5) for 3 min at 4°C. After centrifugation, radioactivity in the supernatant (cell surface bound) and in the cell pellet (internalized) were measured.

Internalization of biotinylated IFNAR1 - The protocol was as in [25]. Briefly, cells were seeded at 1.5x10⁶ in 100mm dishes. The next day, cells were starved in serum free medium for 2 hr, chilled on ice and washed three times with PBS, 0.7 mM CaCl₂, 0.5 mM MgCl₂. Surface biotinylation was performed with 300 µg/ml EZ-Link-Sulfo-NHS-S-S-biotin (Pierce) for 30 min at 4°C. Unbound biotin was removed by three washes with PBS at 4°C, and then once with PBS, 0.1% BSA to block remaining active sites. Biotinylated cells were then stimulated with prewarmed 500 pM IFNα for the desired times at 37°C to allow internalization and then rechilled on ice and washed with PBS. Surface-bound biotin was stripped with three 20 min incubation with 100 mM sodium 2-mercaptoethanesulfonic acid (MESNA) (Sigma). Cells were washed and residual MESNA was quenched by a 10 min incubation with ice-cold 120 mM iodoacetamide. Cells were washed again twice and lysed in RIPA buffer. Biotinylated internalized proteins were recovered by overnight incubation of lysates with 30 µl of ImmunoPure immobilized streptavidin (Pierce) at 4°C. After 3 washes, the recovered material was boiled, subjected to SDS-PAGE and blotted for IFNAR1.

Flow cytometry - Surface IFNAR1 levels were monitored by incubating cells with 10 µg/ml of AA3 mAb [26], followed by incubation with 10 µg/ml of biotinylated anti-mouse IgG Abs and with streptavidin-phycoerythrin (Jackson Immuno Research Laboratories). Cells were analyzed with a FACScan flow cytometer (Becton Dickinson).

Protein analysis - To analyze IFNAR1 serine phosphorylation and ubiquitination, cells were lysed in NP40 buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1% NP40, 0.5 mM EDTA, 1 mM orthovanadate, 10 mM N-ethylmaleimide and a cocktail of antiproteases from Sigma). For all other experiments, lysates were made in RIPA buffer (50 mM Tris-HCl pH 8, 200 mM NaCl, 1% NP40, 0.5% DOC, 0.05% SDS, 2 mM EDTA, 1 mM orthovanadate and antiproteases). IFNAR1 was immunoprecipitated using 1 µg of EA12 mAb and detected with 64G12 mAb. In Fig. 2B Tyk2 was immunoprecipitated with R5-7 Tyk2 antiserum and analyzed by immunoblotting with the anti-phosphotyrosine 4G10 mAb.

RESULTS

IFN α decreases the surface half-life of IFNAR1 and promotes its degradation

The surface half-life of IFNAR1 was measured in 293T cells unstimulated or stimulated with IFN α for different times. 293T cells were chosen since their level of IFNAR1 is sufficiently high to be monitored by flow cytometry (FACS). Cells were treated with cycloheximide (CHX) to prevent new receptor synthesis making it possible to follow the decay of surface receptors. In unstimulated cells, IFNAR1 localized at the cell surface had a half-life of approximately 4 h, which was shortened to about 60 min in cells stimulated with IFN α (Fig. 1A). To assess the contribution of new protein synthesis to receptor surface replenishment, cells were incubated with IFN α for 90 min to maximally reduce surface IFNAR1. Within this time the kinetics of IFNAR1 disappearance is unaffected by CHX (data not shown). Cells were then either washed or replenished with IFN α in the presence or the absence of CHX. As shown in Fig. 1B, at around 120 min post-treatment surface IFNAR1 resumed in cells where CHX was omitted, suggesting that newly synthesized molecules were delivered on the cell surface. Moreover, in washed cells IFNAR1 level resumed to nearly 100% within 3 h (Fig. 1B). These results suggest that in 293T cells IFN α accelerates about 3 fold the internalization rate of IFNAR1, but it does not affect receptor delivery to the cell surface.

The overall content of IFNAR1 was also analyzed by western blot. IFNAR1 migrates in SDS-PAGE as a minor 85 kDa Endo-H-sensitive form and a major, mature and highly glycosylated form of around 90-110 kDa [19]. In cells left untreated, approximately 50% of mature IFNAR1 was degraded within 4 h of CHX treatment, whereas in IFN α stimulated cells IFNAR1 degradation was consistently accelerated (Fig. 1C). To assess whether the observed degradation involved a lysosomal pathway, cells were treated with IFN α in the presence of the lysosomotropic alkalinizing agent NH₄Cl and total IFNAR1 levels were monitored. When lysosomal function was blocked, IFNAR1 degradation was impaired (Fig. 1D), whereas blocking the proteasome had no effect (data not shown). Altogether, these data indicate that ligand stimulation shortens the half-life of cell surface IFNAR1 and robustly accelerates its lysosomal degradation.

Epo-induced internalization of an EpoR/R1 chimera does not require active Tyk2

Having shown that IFN α affects IFNAR1 surface half-life and degradation, we addressed the question whether IFNAR1-associated Tyk2 contributes catalytically to these events. Since, upon ligand binding, Tyk2 and Jak1 are both activated by cross-phosphorylation, we initially analyzed the role of Tyk2 using a simplified receptor system that involves neither IFNAR2 subunit nor Jak1. A chimeric receptor was used that contains the ectodomain of the EpoR fused to the transmembrane and the intracellular regions of IFNAR1 (EpoR/R1). In SDS-PAGE, the EpoR/R1 chimera gives rise to two bands, an abundant 45 kDa band, corresponding to the calculated unprocessed form, and a minor

slower migrating 50 kDa band, corresponding to the mature form (see Fig. 3B). It is likely that the poor processing of the chimera is due, to a large extent, to the ectodomain, since inefficient processing of the native EpoR is well described [27].

This homodimeric-type receptor (EpoR/R1) was studied in a wt and a kinase inactive Tyk2 cellular backgrounds (Fig. 2A) For this, the EpoR/R1 construct was transfected in Tyk2-deficient 11,1 cells and one clone was chosen based on moderate expression of the chimera. From this clone we derived subclones expressing either wt Tyk2 or the kinase inactive K930R mutant, bearing a substitution in the ATP binding cleft [21]. Two clones were chosen for further studies on the basis of their comparable levels of wt Tyk2 (cl.14) and K930R (cl.20) (Fig. 2B). To characterize the activity of the chimera in these two clones, we first analyzed phosphorylation events in response to Epo. HT-1080 cells exposed to a single saturating dose of IFN α were used as control. In cells reconstituted with wt Tyk2 (cl.14), Epo induced the phosphorylation of Tyk2, Stat1 and Stat3 (Fig. 2B and C). Stat2 was not phosphorylated, pointing to the need for other components of the native IFN receptor complex, most likely IFNAR2 which constitutively binds Stat2 [28]. Intriguingly, phosphorylation on Ser535, which is required for ubiquitination of native IFNAR1 triggered by IFN α [29], was also stimulated by Epo (Fig. 2D) indicating that IFNAR2 is likely dispensable for the activation of this pathway. In all, the Epo-driven homodimerization of the chimera led to efficient activation of the associated Tyk2 and downstream substrates. As predicted, no Epo-induced tyrosine phosphorylation events were detected in cl.20 cells expressing the inactive K930R mutant (Fig. 2B). Furthermore, phosphorylation of Ser535 as well was impaired in these cells (Fig. 2D), suggesting that Tyk2 catalytic activity may be required for ubiquitination of IFNAR1.

This model system recapitulated the ligand-induced phosphorylation events and was therefore suitable to investigate the catalytic role of Tyk2 in the down-regulation of the chimeric receptor. First, the internalization rate of the ligand/chimera complex was measured in the two clones, using 125 I-Epo. To avoid plasma membrane replenishment with newly synthesized receptors, CHX was added 15 min before ligand addition. The amount of bound radioactivity was equivalent in the two clones. The amount of internalized 125 I-Epo was calculated as the percentage of total cell-associated radioactivity as a function of time. At both early and late time points after the addition of 125 I-Epo, the amount of internalized radioactivity increased comparably in two clones (Fig. 3A). These results demonstrate that the internalization of the chimera in the presence of Epo occurs independently of the catalytic activity of Tyk2.

Epo-induced degradation of the EpoR/R1 chimera requires active Tyk2

Next, the steady-state level of the chimera was monitored by anti-Flag western blot in the two clones at different times after ligand addition. In cl.14 cells, expressing wt Tyk2, the addition of Epo led to a noticeable time-dependent decrease of the level of only the mature band. Conversely, in cl.20 cells, expressing K930R, no evidence of degradation of this band was observed (Fig. 3B). Overall, these

results suggest that the tyrosine kinase activity of Tyk2 is dispensable for the internalization of the chimeric receptor that occurs in the presence of the ligand. However, catalytic activity of Tyk2 is essential for the serine phosphorylation (Fig. 2D) as well as for the proteolysis of the chimeric receptor.

IFN α -induced degradation of endogenous IFNAR1 in 293T cells requires catalytically active Tyk2

In a complementary approach to study the contribution of Tyk2 to ligand-induced IFNAR1 down-modulation, we asked whether the overexpression of wt or kinase inactive Tyk2 affected the regulation of the endogenous IFNAR1. GFP-fused versions of wt Tyk2 and K930R were transfected in 293T cells and surface IFNAR1 decay was monitored in GFP-positive cells by FACS. As shown in Fig. 4A, upon IFN α treatment the decrease of IFNAR1 was comparable in the two GFP-gated populations. Total IFNAR1 levels were analyzed by western blot in cells transfected with Tyk2 constructs lacking GFP. Degradation of IFNAR1 occurred in cells transfected with wt Tyk2, but it was noticeably impaired in cells transfected with K930R (Fig. 4B). Tyk2 activation was monitored in the two transfected populations, using phospho-specific antibodies directed to the activation loop tyrosines. Wild type Tyk2, but not K930R, was basally phosphorylated and a further increase in its phosphorylation level was detected following IFN α addition (Fig. 4B). As expected from a previous study [21], weak phosphorylation of Tyk2, initiated by Jak1, was observed in cells expressing the K930R mutant (Fig. 4B).

Given that endogenous Tyk2 is yet present in 293T cells and may dampen down the effect of the mutant K930R protein, to further demonstrate the role of Tyk2 kinase activity in IFNAR1 down-regulation, we turned to the analysis of IFNAR1 in two clones derived from Tyk2-deficient cells. One clone (WT-5) stably expresses wt Tyk2 and the other clone (KR-2) expresses K930R. The low level of IFNAR1 in these cells precluded cytofluorimetric analysis of the surface pool and the extent of IFNAR1 internalization was therefore analyzed by reversible biotinylation of cell surface. As shown in Fig. 5A, the internalization of biotinylated IFNAR1, measured within the first 60 min of IFN addition, did not differ between the cells that harbor either wild type or catalytically inactive Tyk2. These results corroborate the conclusion from our analysis of the EpoR/R1 chimera (Fig. 3A) that Tyk2 kinase activity is not required for the initial steps of IFNAR1 endocytosis. In addition, we assessed the total IFNAR1 level in these cells. As shown in Fig. 5B, the ligand-induced degradation of IFNAR1 was impaired in KR-2 cells, confirming the need for active Tyk2. The blot was re-probed with anti-phospho-Tyk2 antibodies (Fig. 5B). A robust and transient Tyk2 phosphorylation was detected in IFN α -treated WT-5 cells, while the induced phosphorylation of K930R on the activation loop was weaker and sustained, reflecting Jak1-initiated action. Altogether, these results are in line with those obtained for the EpoR/R1 chimeric receptor (Fig. 4A and 4B) and suggest that Tyk2 activation is required for the efficient degradation of IFNAR1, but not for IFNAR1 internalization.

Tyk2 kinase activity is required for serine phosphorylation and ubiquitination of IFNAR1

Next, we investigated the mechanism by which Tyk2 promotes IFNAR1 degradation. Recent data showed that upon ligand binding IFNAR1 is phosphorylated on serine residues (Ser535 and 539) located in the cytoplasmic tail. This modified motif is recognized by the F-box containing β -TrCP2 (also termed HOS), a protein known to recruit the core SCF E3 ligase complex which ubiquitinates and targets for degradation numerous proteins. Ligand-induced ubiquitination of IFNAR1 was shown to precede receptor degradation [29]. In the present study, we observed that serine phosphorylation of the EpoR/R1 chimera was impaired in the cells that harbor catalytically inactive Tyk2 (Fig. 2D). On the basis of these results, we tested whether the overexpression of K930R in 293T cells affected serine phosphorylation and/or ubiquitination of endogenous IFNAR1. This latter was immunoprecipitated from cells overexpressing Tyk2 or K930R and the blot was revealed first with anti-ubiquitin antibodies and subsequently with anti-phospho-Ser535 antibodies. As shown in Fig. 4C, ubiquitination and serine phosphorylation of IFNAR1 were affected in cells overexpressing K930R as compared to cells overexpressing wt Tyk2. The residual ligand-induced modifications of IFNAR1 in K930R transfected cells could be attributed either to the presence of endogenous Tyk2 or to the existence of Tyk2-independent alternative pathways that trigger Ser535 phosphorylation in 293T cells. Thus, to further substantiate these findings we analyzed the ligand-induced events in the WT-5 and KR-2 clones, which lack endogenous Tyk2. As seen in Fig. 5C, neither serine phosphorylation nor ubiquitination of IFNAR1 were detected in KR-2 cells. These data provide the genetic evidence that Tyk2 activity is required for IFNAR1 serine phosphorylation. Overall the results presented here suggest that the catalytic activation of Tyk2 contributes to early events leading to ligand-induced IFNAR1 ubiquitination and degradation.

DISCUSSION

Recent studies have highlighted a chaperon-like, non catalytic role of Jak proteins in cytokine receptor traffic. Tyk2 was found to increase IFNAR1 surface level by reducing its basal internalization rate [19]. Tyk2 and Jak2 were shown to promote surface expression of the thrombopoietin receptor by enhancing its recycling [30]. Similar findings were reported for Jak1 and the oncostatin M receptor and for Jak2 and the EpoR. In these latter contexts, the Jak protein enhances the surface level of the cognate receptor by facilitating its delivery to the plasma membrane [31,32]. A number of groups have also analyzed the function of Jaks in the ligand-induced trafficking of cytokine receptors. It was shown that a mutant EpoR unable to activate Jak2 internalized efficiently after Epo binding [33]. In line with this observation, Walrafen *et al.* demonstrated that the pharmacological inhibition of Jak2 kinase activity did not interfere with ligand-induced EpoR internalization [9]. A mutant GHR bearing mutations in the box1, and thus unable to bind Jak2, was shown to internalize with the same kinetics as the wt receptor. Furthermore, the internalization rate of the GHR was not impaired in cells deficient

for Jak2 [34]. Similar uncoupling of receptor internalization and signaling has been reported for two other cytokine receptors, gp130 and the bipartite IL-4 receptor [35,36].

Here, we have initiated studies of the intracellular fate of the heterodimeric IFN-R and focused on the IFNAR1 subunit. In 293T cells, IFN α binding accelerates about 3 fold the internalization rate of IFNAR1. While the analyses reported here were performed using IFN α 2 as the ligand, our conclusions can be extended to the IFN β subtype. The basal and ligand-induced down-regulation of IFNAR1 was also investigated in Jurkat and Daudi cells. Despite anticipated cell type differences in both the surface level and the basal turnover of IFNAR1, a ligand-induced effect, ranging from 3 to 6 fold, was consistently observed on both internalization and degradation.

To evaluate the catalytic contribution of Tyk2 to ligand-induced IFNAR1 down-regulation, we monitored internalization, post-translational modifications and degradation of the receptor in two different genetic contexts, harboring either wt or kinase inactive Tyk2. The internalization of iodinated Epo bound to the homodimeric EpoR/R1 coupled with either wt Tyk2 or K930R was studied. We also analyzed the effect of overexpressed wt or K930R on the internalization of endogenous IFNAR1 and lastly we measured internalization of biotinylated IFNAR1 in Tyk2-deficient cells stably expressing wt or K930R. Altogether, the results of these analyses provide evidence that catalytic activation of Tyk2 is not required for IFNAR1 internalization, similarly to what was reported for Jak2 coupled to the EpoR and the GHR.

It has been demonstrated that the F-box containing β -TrCP2 E3 ligase is involved in early steps of the down-regulation of the IFN-R and of at least one other member of the cytokine receptor superfamily, the prolactin receptor [29,37]. The existence of an IFN-activated Ser/Thr kinase is supported by the need for phosphorylation of IFNAR1 on serine in order to recruit β -TrCP2. The ability of the homodimerizing EpoR/R1 to be serine phosphorylated suggest that the IFNAR2 subunit is dispensable for activation of this yet to be identified kinase. Furthermore, we have analyzed the role of Tyk2 on membrane-proximal IFNAR1 modifications and shown that Tyk2 activation is required for the serine phosphorylation and the ubiquitination of IFNAR1 in response to the ligand. Our finding raises the question of the mechanism by which Tyk2, once activated, affects serine phosphorylation and ubiquitination of IFNAR1. One possibility is that Tyk2 activation generates conformational changes in the receptor/kinase complex which have repercussions on the cytoplasmic tail of IFNAR1 rendering it more accessible to post-translational modifications including serine phosphorylation. Future studies will identify kinase(s) that are involved in the phosphorylation of IFNAR1 on Ser535 and will determine how these activities are regulated by Tyk2.

While required for serine phosphorylation and ubiquitination of IFNAR1, catalytic activity of Tyk2 is not essential for IFNAR1 internalization. Our data are concordant with the conclusion of the Mayeux group that Jak2 activity, although dispensable for internalization of the EpoR, is required for its ubiquitination [9]. These data together suggest that IFNAR1 ubiquitination is not required for its

internalization. However, given that either inhibition of β -TrCP2 or mutation in Ser526 of murine IFNAR1 - corresponding to Ser535 in human IFNAR1- decreases the efficiency of the uptake of radioactively labeled IFN α [29], it cannot be ruled out that ubiquitination of IFNAR1 may yet contribute to the maximal efficiency of IFNAR1 internalization. Another possibility is that more than one internalization pathway exists, the choice of which may be influenced by the level of ubiquitination, as recently proposed for the EGFR [38]. Nevertheless, the data presented here indicate that efficient ligand-induced degradation of both the EpoR/R1 chimera and the native IFNAR1 relies on active Tyk2. It is likely that Tyk2 dependent IFNAR1 ubiquitination contributes to the post-internalization sorting steps that ultimately lead to proteolysis.

Although the subject of numerous studies, the precise role of ubiquitin conjugation in receptor internalization is still a matter of debate [39]. On the other hand, ubiquitination of activated RTK was shown to be required for their efficient endosomal sorting towards degradation. Conjugated ubiquitin represents a recognition signal for several sub-complexes of Vps class E proteins (including Hrs/STAM and the ESCRT system) that transiently localize on the cytosolic face of the early endosomal/multivesicular body membrane and relay in a sequential manner ubiquitinated cargoes towards internal vesicles (reviewed in [40]). Further studies will address the role of Tyk2-promoted ubiquitination in IFNAR1 endosomal sorting.

Our present and past studies highlight a dual role of Tyk2 in controlling the turnover of the IFNAR1 receptor chain. In unstimulated cells, Tyk2 exerts a positive effect on IFNAR1 cell surface expression, by anchoring it to the plasma membrane. This function of Tyk2 does not require its kinase activity. In IFN-stimulated cells, Tyk2 contributes catalytically to the degradation of the internalized IFNAR1. Our future work will investigate the basal and ligand-induced turnover of IFNAR2, the other subunit of the type I IFN receptor, and the potential contribution of Jak1. Of note, IFNAR2 was reported to undergo regulated proteolysis in response to treatment of cells with IFN α and phorbol esters [41]. It remains an open question whether IFNAR2, once internalized in response to IFN only, is routed towards degradation.

FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Surface disappearance, replenishment and degradation of IFNAR1 in 293T cells.

- (A) IFNAR1 surface half-life in control (dashed line) and in IFN α treated (solid line) 293T cells was measured by flow cytometry. Cells were incubated with 20 μ g/ml CHX in the absence or the presence of 500 pM IFN α for the indicated times.
- (B) Surface replenishment of IFNAR1. Cells were incubated with 500 pM IFN α for 90 min to maximally decrease surface IFNAR1 levels. At 90 min post-treatment (arrow), cells were either washed (circle) or replenished with IFN α in the presence (square) or the absence of CHX (triangle) and incubated for additional 150 min. The level of IFNAR1 was measured by flow cytometry.
- (C) Constitutive and IFN α -induced degradation of endogenous IFNAR1. 293T cells were incubated with CHX in the absence or the presence of IFN α for the indicated times. Total lysates (50 μ g) were resolved by SDS-PAGE. The membrane was incubated with anti-IFNAR1 mAb.
- (D) Lysosomal degradation of endogenous IFNAR1. 293T cells were incubated with IFN α in the absence or the presence of 20 mM NH $_4$ Cl for the indicated times. Fifty μ g of total lysates were resolved by SDS-PAGE and the membrane was incubated with anti-IFNAR1 mAb.

In C and D, the asterisk points to a non specific band.

Fig. 2. Epo-induced signaling of the EpoR/R1 chimera.

- (A) Schematic representation of the EpoR/R1 chimera containing the ectodomain of Epo-R fused to the transmembrane and intracellular regions of IFNAR1 associated with wt Tyk2 (WT) or K930R (KR). The black circle represents the Flag epitope tag added at the C-terminus of the chimera.
- (B) Tyk2 phosphorylation in response to Epo. Two EpoR/R1 clones expressing either wt Tyk2 (cl.14) or K930R (cl.20) were treated with 10 U/ml of Epo for 20 min. Tyk2 was immunoprecipitated from cell lysates and resolved on 7% SDS-PAGE. Tyrosine phosphorylation was revealed with anti-phosphotyrosine 4G10 mAb. The membrane was stripped and incubated with a Tyk2-specific mAb.
- (C) Phosphorylation of Stat proteins in response to Epo. Control HT-1080 cells were treated for 20 min with 500 pM IFN α . Cells expressing wt Tyk2 (cl.14) were treated with 10 U/ml of Epo for the indicated times. Forty μ g of lysates were resolved on 7% SDS-PAGE. Stat phosphorylation was revealed with phosphotyrosine-specific Stat1, Stat2 and Stat3 Abs.
- (D) Phosphorylation of the EpoR/R1 chimera on Ser535 within the IFNAR1 cytoplasmic tail was analyzed in the two clones by immunoprecipitation using P-Ser535-IFNAR1-specific Abs [29] and immunoblotting with anti-Flag mAb. Ig, immunoglobulin.

Fig. 3. Epo-induced internalization and degradation of the EpoR/R1 chimera

- (A) Kinetics of ^{125}I -Epo internalization in cl.14 (solid line) and cl.20 cells (dashed line). Cells were incubated for 15 min with 20 $\mu\text{g/ml}$ of CHX prior stimulation with 2 U/ml of ^{125}I -Epo. Cells were sampled for the determination of internalized radioactivity at early (left panel) or late (right panel) time intervals. The results represent the mean of four independent experiments.
- (B) Epo-induced degradation of the chimeric EpoR/R1 receptor in cl.14 and cl.20 cells. Cells were treated with 10 U/ml of Epo for the indicated times. Forty μg of lysates were resolved on 8% SDS-PAGE. EpoR/R1 was revealed with anti-Flag mAb. The lysates were run on a separate blot which was detected with anti-actin mAb to control for protein content. Right panel, the bands revealed with anti-Flag mAb were quantified. The ratios of mature (top band) to immature (bottom band) EpoR/R1 were plotted for each clone (cl.14, solid line; cl.20, dashed line).

Fig. 4. Surface disappearance, serine phosphorylation, ubiquitination and degradation of IFNAR1 in 293T cells overexpressing wt Tyk2 or K930R.

- (A) Tyk2 kinase activity is dispensable for IFN α -induced IFNAR1 disappearance from the cell surface. Surface levels of IFNAR1 were determined by flow cytometry on cells transfected with Tyk2/GFP or K930R/GFP. Similar basal IFNAR1 levels were observed in the two GFP-gated cell populations (thick black line). IFN α treatment for 90 min (gray line) or 180 min (thin black line) induced a comparable decrease in surface IFNAR1 in the two populations.
- (B) IFN α -induced IFNAR1 degradation requires active Tyk2. Cells expressing wt Tyk2 or K930R were treated with 500 pM IFN α for the indicated times. Fifty μg of total lysates were resolved on 7% SDS-PAGE and IFNAR1 was revealed with anti-IFNAR1 mAb. The membrane was stripped and reprobed with anti-phospho-Tyk2 Abs or anti-Tyk2 mAb.
- (C) Tyk2 activation promotes IFN α -induced serine phosphorylation and ubiquitination of IFNAR1. Cells expressing wt Tyk2 or K930R were treated with 500 pM IFN α for the indicated times. Five mg of total lysates were used to immunoprecipitate endogenous IFNAR1. The immunoprecipitates were resolved on 7% SDS-PAGE and ubiquitinated IFNAR1 was revealed using anti-ubiquitin Abs. The membrane was stripped and reprobed with phosphoserine-IFNAR1-specific Abs or anti-IFNAR1 mAb. Migration of molecular weight markers is indicated on the right. Bands in the middle and bottom panels were quantified and the ratio of phosphorylated to total IFNAR1 is plotted. Tyk2 transfected cells, solid line. K930R transfected cells, dashed line.

Fig. 5. Surface disappearance, serine phosphorylation, ubiquitination and degradation of IFNAR1 in WT-5 and KR-2 cells.

- (A) Internalization of biotinylated IFNAR1 was assessed in cells expressing wild-type Tyk2 (WT-5) or kinase-dead Tyk2 (KR-2). Cells were biotinylated at 4°C with a disulfide-cleavable biotin treated with IFN α at 37°C for the times indicated to allow internalization of surface IFNAR1. The remaining cell surface biotinylated material was stripped by incubation with MESNA (see Exp. Procedures). After lysis internalized proteins were recovered with streptavidin beads, subjected to SDS-PAGE and immunoblotting with anti-IFNAR1 Abs (lanes 3-5 and 8-10). The surface biotinylation at time zero (lanes 1 and 6) and the efficiency a MESNA stripping (lanes 2 and 7) were controlled.
- (B) IFN α -induced degradation of IFNAR1 requires active Tyk2. Cells were treated with 500 pM IFN α for the indicated times. Forty μ g of total lysates were resolved on 7% SDS-PAGE and IFNAR1 was revealed with anti-IFNAR1 mAb. The membrane was stripped and reprobed with anti-phospho-Tyk2 Abs or anti-Tyk2 mAb.
- (C) IFN α -induced serine phosphorylation and ubiquitination of IFNAR1 requires Tyk2 activation. Cells were treated with 500 pM IFN α for the indicated time. Five mg of total lysates were used to immunoprecipitate IFNAR1. Ubiquitinated IFNAR1 was revealed with anti-ubiquitin Abs. The membrane was stripped and reprobed with P-Ser535-IFNAR1-specific Abs or anti-IFNAR1 mAb. Tyk2 levels were measured by direct western blot on 50 μ g of lysate. Migration of molecular weight markers is indicated on the right.

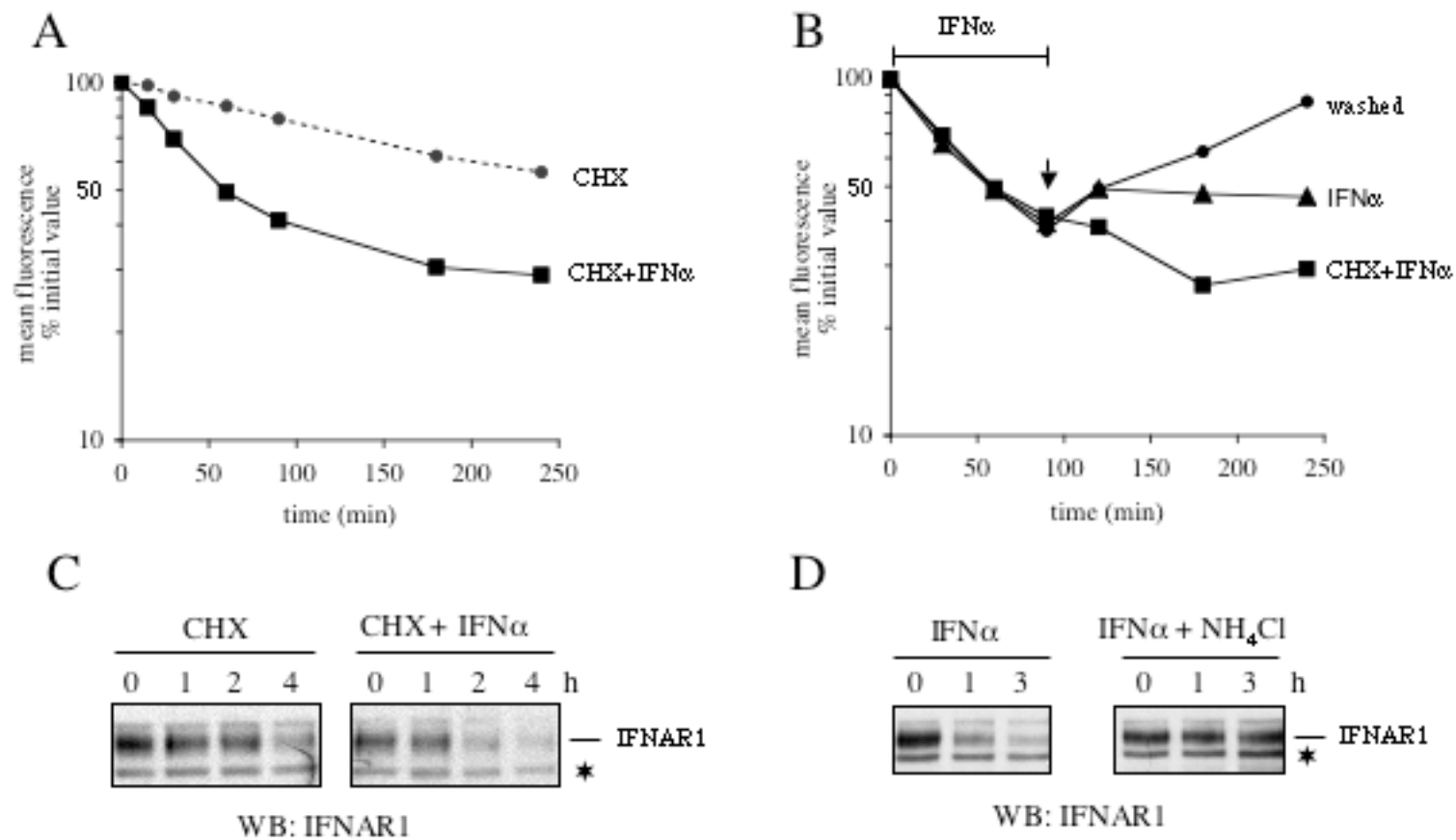


Figure 1

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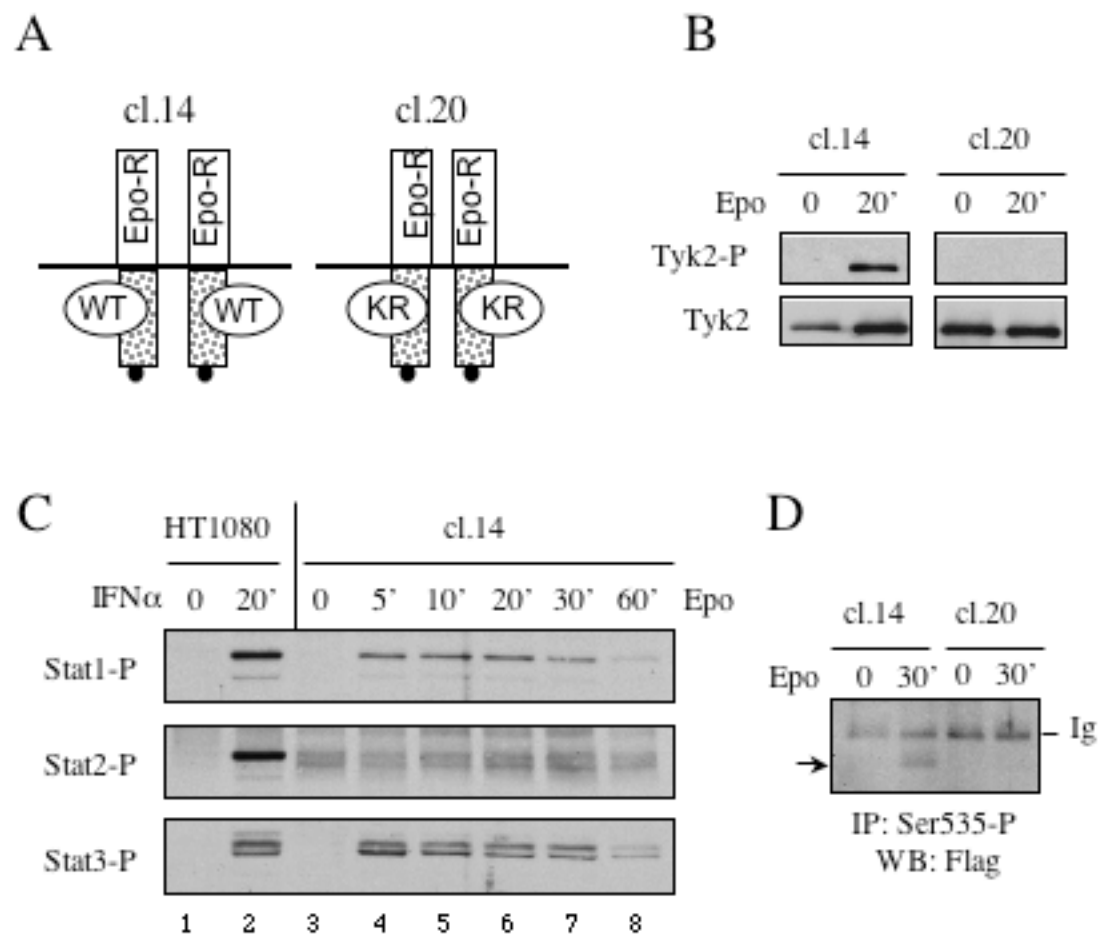
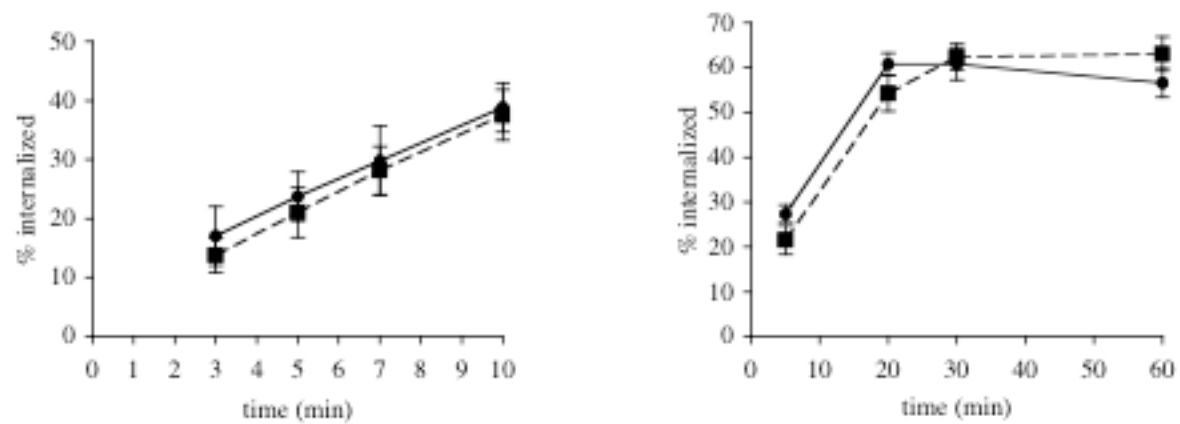


Figure 2

A



B

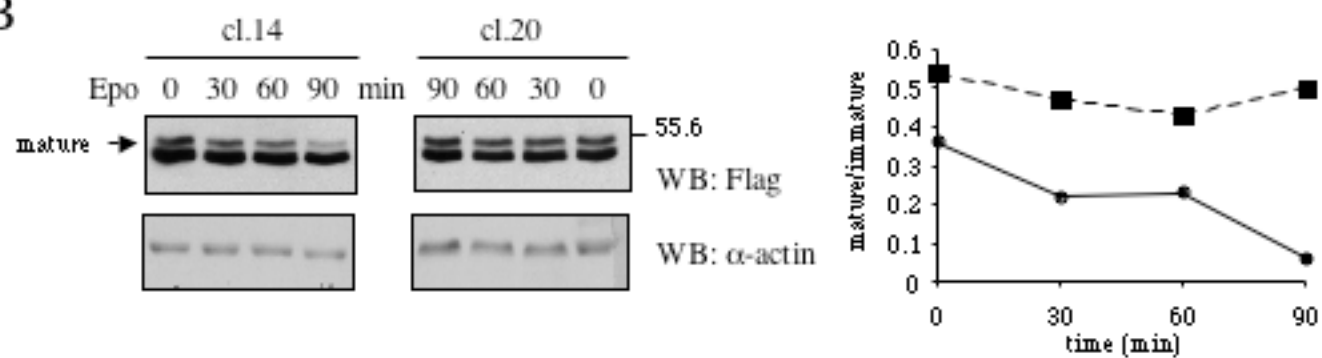


Figure 3

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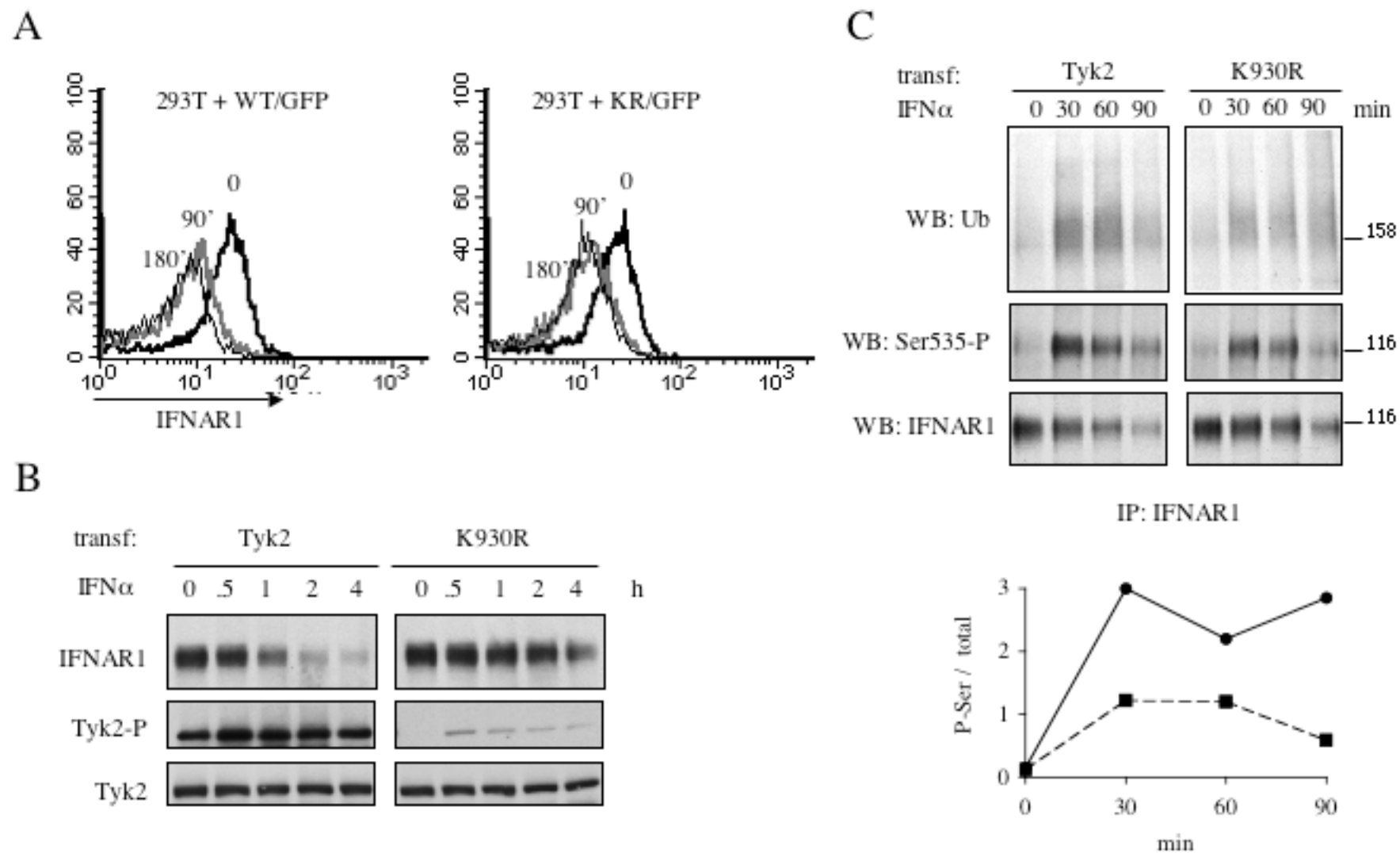


Figure 4

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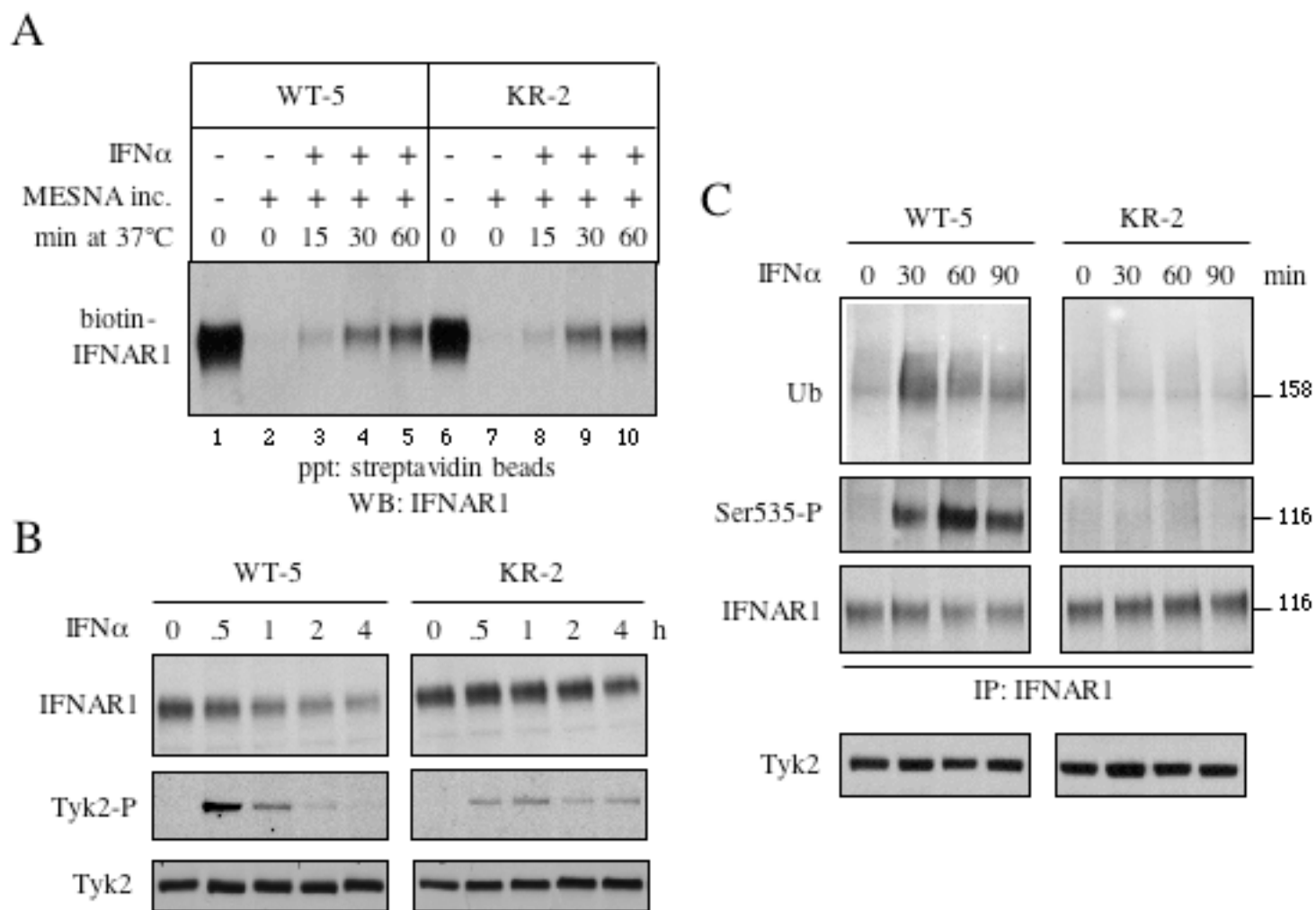


Figure 5

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