TYK2 activity promotes ligand-induced IFNAR1 proteolysis

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The type I IFNR (interferon receptor) is a heterodimer composed of two transmembrane chains, IFNAR1 (interferon- α receptor 1 subunit) and IFNAR2, which are associated with the tyrosine kinases Tyk2 and Jak1 (Janus kinase 1) respectively. Ligand-induced down-regulation of the type I IFNR is a major mechanism of negative regulation of cellular signalling and involves the internalization and lysosomal degradation of IFNAR1. IFN α promotes the phosphorylation of IFNAR1 on Ser⁵³⁵, followed by recruitment of the E3 ubiquitin ligase, β -TrCP2 (β -transducin repeats-containing protein 2), 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 the function of Tyk2 in the steps that precede and succeed serine phosphorylation and ubiquitination of IFNAR1 in response to ligand binding. In the present study, we show that catalytic activation of Tyk2 is not essential for IFNAR1 internalization, but is required for ligand-induced IFNAR1 serine phosphorylation, ubiquitination and efficient lysosomal proteolysis.

Key words: degradation, interferon- α receptor 1 subunit (IFNAR1), internalization, Tyk2, type I interferon (IFN), ubiquitination.

INTRODUCTION

The 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 signalling 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 signalling proteins. Extensive studies on the down-regulation of growth factor receptors containing a tyrosine kinase domain [RTK (receptor tyrosine kinase)] have been performed [1a]. Among the best characterized system is that involving the EGFR (epidermal growth factor receptor), the intracellular fate of which 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 about 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 with tyrosine kinases of the Janus or Jak (Janus kinase) family. Binding of the ligand to the receptor complex results in the catalytic activation of the Jaks, which initiates STAT (signal transducer and activator of transcription)-mediated and other signalling events [5,6]. Recent studies performed on GHR (growth hormone receptor), EpoR (erythro-

poietin receptor) and IL-2R (interleukin-2 receptor) have provided some insights into ligand-induced trafficking events of these receptors. The activated GHR is internalized via clathrin-coated pits and is routed to lysosomes for degradation. Both processes, internalization and degradation, are dependent upon active ubiquitination machinery and on proteasomal activity [7,8]. Similar requirements were reported for EpoR. While still at the cell surface, this receptor undergoes polyubiquitination and proteasome-dependent degradation of part of its intracellular domain. After internalization, the truncated receptor is routed to lysosomes for final degradation [9]. In contrast with the GH and Epo receptors which form functional homodimers, the high affinity IL-2R is made up 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 IFNR (interferon receptor) consists of two subunits, IFNAR1 (interferon- α receptor 1 subunit) 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 of the formation of the ternary IFN–receptor complex suggests that the ligand binds first to IFNAR2, for which it displays higher affinity, and that 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

Abbreviations used: Ab, antibody; β -TrCP, β -transducin repeats-containing protein; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; Epo, erythropoietin; FCS, foetal calf serum; FERM, band 4.1, ezrin, radixin, moesin; GFP, green fluorescent protein; GHR, growth hormone receptor; IFN α , interferon α ; IFNR, IFN receptor; IFNAR1/2, interferon- α receptor 1/2 subunit; IL-2R, interleukin-2 receptor; Jak, Janus kinase; mAb, monoclonal Ab; MESNA, 2-mercaptoethanesulphonic acid; MVB, multivesicular body; NP40, Nonidet P40; RTK, receptor tyrosine kinase; STAT, signal transducer and activator of transcription; WT, wild-type.

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acids [17,18]. We have previously shown that Tyk2 binds to the juxtamembrane region of IFNAR1 via its FERM (band 4.1, ezrin, radixin, moesin) domain, and in doing so it decreases the basal internalization of IFNAR1 and enhances its level at the plasma membrane independently of catalytic activity [19].

In the present study, we have focused on the turnover of IFNAR1 after 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 Tyk2 inactivation upon internalization and subsequent degradation of IFNAR1. We have shown 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, it is the latter which contributes to efficient IFNAR1 ubiquitination and degradation.

EXPERIMENTAL

Cells, plasmids, Abs (antibodies) and other reagents

The human fibrosarcoma Tyk2-deficient 11.1 cell line and previously derived WT-5 and KR-2 clones have been described [20,21]. Cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10 % FCS (foetal calf serum) at 37 °C in 6 % CO₂. Recombinant Epo was provided by Dr P. Mayeux (Department of Hematology, Institut Cochin, Paris, France). Recombinant IFN α 2b was a gift from Dr D. Gewert (Biolauncher, Cambridge, U.K.). pRC-Tyk2 and pGFP-Tyk2 were described previously [22,23]; pSV-SPORT EpoR-R1 FLAG was provided by Professor J. Tavernier (Department of Medical Protein Research, Ghent University, Ghent, Belgium). CHX (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 obtained from Calbiochem. The anti-Tyk2 mAb (monoclonal Ab) T10-2 was described previously [21]. STAT1 and STAT3 tyrosine phospho-specific Abs were obtained from BioLabs and STAT2 tyrosine phospho-specific Abs were obtained from UBI. The mAb directed against the FLAG epitope was purchased from Sigma. The anti-ubiquitin FK2 mAb was obtained from Biomol. The anti-actin mAb was obtained 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^R. Single Puro^R colonies were analysed by an anti-FLAG Western blot analysis. 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 a comparable level of Tyk2 expression. For transient transfection, HEK-293T (where T stands for large T antigen) cells were plated at 1.5×10^6 in 100 mm plates. Next day, 6 μ g of plasmid DNA was transfected using FuGENETM reagent (Roche). Cells were processed for FACS or Western blot analysis 24 h later.

¹²⁵I-Epo internalization studies

¹²⁵I-Epo was provided by P. Mayeux (Institut Cochin, Paris). ¹²⁵I-Epo [1 nM (2 units/ml)] 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 % (w/v) FCS and 25 mM

Hepes at 37 °C. At the indicated times, aliquots of 1.3×10^6 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 an 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 the same as that described in [25]. Briefly, cells were seeded at 1.5×10^6 in 100 mm dishes. The next day, cells were starved in serum free medium for 2 h, chilled on ice and washed three times with PBS, 0.7 mM CaCl₂ and 0.5 mM MgCl₂. Surface biotinylation was performed with 300 μ g/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 % (w/v) FCS to block remaining active sites. Biotinylated cells were then stimulated with pre-warmed 500 pM IFN α for the desired times at 37 °C to allow internalization, and were then re-chilled on ice and washed with PBS. Surface-bound biotin was stripped by 3 $\times$ 20 min of incubation with 100 mM sodium MESNA (2-mercaptoethanesulphonic acid) (Sigma). Cells were washed and residual MESNA was quenched by a 10 min incubation with ice-cold 120 mM iodoacetamide. Cells were washed twice and lysed in RIPA buffer [50 mM Tris/HCl (pH 8), 200 mM NaCl, 1 % NP40 (Nonidet P40), 0.5 % DOC (deoxycholate), 0.05 % SDS, 2 mM EDTA, 1 mM orthovanadate and antiproteases]. Biotinylated internalized proteins were recovered by overnight incubation of lysates with 30 μ l of Immuno-Pure immobilized streptavidin (Pierce) at 4 °C. After 3 washes, the recovered material was boiled, subjected to SDS/PAGE and blotted for IFNAR1.

Flow cytometry

The level of surface IFNAR1 was monitored by incubating cells with 10 μ g/ml AA3 mAb [26], followed by incubation with 10 μ g/ml biotinylated anti-(mouse IgG) Ab and with streptavidin-phycoerythrin (Jackson ImmunoResearch Laboratories). Cells were analysed using a FACScan flow cytometer (Becton Dickinson).

Protein analysis

To analyse the serine phosphorylation and ubiquitination of IFNAR1, 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 up in RIPA buffer. IFNAR1 was immunoprecipitated using 1 μ g of the anti-EA12 mAb and was detected using the 64G12 mAb. In Figure 2(B), Tyk2 was immunoprecipitated using R5-7 Tyk2 antiserum and analysed 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 HEK-293T cells that were unstimulated or stimulated with IFN α for various times. HEK-293T cells were chosen since their level of IFNAR1 is sufficiently high to be monitored by flow cytometry (FACS).

Figure 1 Surface disappearance, replenishment and degradation of IFNAR1 in HEK-293T cells

(A) IFNAR1 surface half-life in control (dashed line) and in IFN α -treated (solid line) HEK-293T cells was measured by flow cytometry. Cells were incubated with 20 μ g/ml CHX in the presence or absence 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 (closed arrow), cells were either washed (●) or replenished with IFN α in the presence (■) or absence of CHX (▲) and incubated for an additional 150 min. The level of IFNAR1 was measured by flow cytometry. (C) Constitutive and IFN α -induced degradation of endogenous IFNAR1. HEK-293T cells were incubated with CHX in the presence or absence of IFN α for the indicated times. Total lysates (50 μ g) were resolved by SDS/PAGE. The membrane was incubated with an anti-IFNAR1 mAb. *, non-specific band; WB, Western blot. (D) Lysosomal degradation of endogenous IFNAR1. HEK-293T cells were incubated with IFN α in the presence or absence of 20 mM NH $_4$ Cl for the indicated times. An aliquot of total lysates (50 μ g) was resolved by SDS/PAGE and the membrane was incubated with an anti-IFNAR1 mAb. *, non-specific band; WB, Western blot.

Cells were treated with CHX to prevent new receptor synthesis, making it possible to follow the decay of surface receptors. In unstimulated cells, IFNAR1 localized to the cell surface had a half-life of approx. 4 h, which was shortened to approx. 60 min in cells stimulated with IFN α (Figure 1A). To assess the contribution of new protein synthesis to surface receptor replenishment, cells were incubated with IFN α for 90 min to maximally decrease surface IFNAR1. Within this time the kinetics of IFNAR1 disappearance is unaffected by CHX (results not shown). Cells were then either washed, or replenished with IFN α in the presence or absence of CHX. As shown in Figure 1(B), at approx. 120 min post-treatment, cell surface IFNAR1 expression was resumed in cells in which CHX treatment had been omitted, suggesting that newly synthesized molecules were delivered to the cell surface. Moreover, in washed cells the level of IFNAR1 resumed to nearly 100 % within 3 h (Figure 1B). These results suggest that in HEK-293T cells IFN α accelerates the internalization rate of IFNAR1 approx. 3-fold, but it does not affect receptor delivery to the cell surface.

The overall IFNAR1 content in cells was also analysed by Western blotting. IFNAR1 migrates on SDS/PAGE gels as a minor 85 kDa Endo-H-sensitive form and a major, mature and highly glycosylated form of approx. 90–110 kDa [19]. In cells left untreated, approx. 50 % of mature IFNAR1 was degraded within 4 h of CHX treatment, whereas in IFN α -stimulated cells IFNAR1 degradation was consistently accelerated (Figure 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, NH $_4$ Cl, and total IFNAR1 levels were monitored. When lysosomal function was blocked, IFNAR1 degradation was impaired (Figure 1D), whereas blocking the proteasome had no effect (results not shown). Taken together, 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 chimaera does not require active Tyk2

Having shown that IFN α affects IFNAR1 surface half-life and degradation, we addressed the question of whether IFNAR1-associated Tyk2 contributes catalytically to these events. Since, upon ligand binding, Tyk2 and Jak1 are both activated by cross-phosphorylation, we initially analysed the role of Tyk2 using a simplified receptor system that involves neither IFNAR2 subunit nor Jak1. A chimaeric receptor was used that contains the ectodomain of the EpoR fused to the transmembrane and the intracellular regions of IFNAR1 (EpoR–R1). On SDS/PAGE gels, the EpoR–R1 chimaera gave rise to two bands, an abundant 45 kDa band, corresponding to the calculated unprocessed form, and a minor more slowly migrating 50 kDa band, corresponding to the mature form (see Figure 3B). It is likely that the poor processing of the chimaera is due, to a large extent, to the ectodomain, since inefficient processing of the native EpoR has been well described [27].

This homodimeric-type receptor (EpoR–R1) was studied in a WT (wild-type) and a kinase inactive Tyk2 cellular background (Figure 2A). For this, the EpoR–R1 construct was transfected into Tyk2-deficient 11.1 cells, and one clone was chosen based on moderate expression of the chimaera. From this clone we derived subclones expressing either WT Tyk2 or the kinase inactive mutant, K930R, that bears 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 the K930R mutant protein (cl.20) expression (Figure 2B). To characterize the activity of the chimaeric protein in these two clones, we first analysed phosphorylation events in response to Epo. HT-1080 cells exposed to a single saturating dose of IFN α were used as a control. In cells reconstituted with WT Tyk2 (cl.14), Epo induced the phosphorylation of Tyk2, STAT1 and STAT3 (Figures 2B and 2C). STAT2 was not phosphorylated, highlighting the need for

Figure 2 Epo-induced signalling of the EpoR-R1 chimaera

(A) Schematic representation of the EpoR-R1 chimaera containing the ectodomain of Epo-R fused to the transmembrane and intracellular regions of IFNAR1 associated with WT Tyk2 (WT) or the K930R mutant (KR). ●, FLAG epitope tag added at the C-terminus of the chimaera. (B) Tyk2 phosphorylation in response to Epo. Two EpoR-R1 clones expressing either WT Tyk2 (cl.14) or the K930R mutant (cl.20) were treated with 10 units/ml Epo for 20 min. Tyk2 was immunoprecipitated from cell lysates and resolved by SDS/PAGE. Tyrosine phosphorylation was revealed using an 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 IFNα. Cells expressing WT Tyk2 (cl.14) were treated with 10 units/ml Epo for the indicated times. Lysates (40 µg) were resolved by SDS/PAGE. STAT phosphorylation was revealed using phosphotyrosine-specific STAT1, STAT2 and STAT3 Abs. (D) Phosphorylation of the EpoR-R1 chimaera on Ser⁵³⁵ within the IFNAR1 cytoplasmic tail was analysed in the two clones by immunoprecipitation using P-Ser⁵³⁵-IFNAR1-specific Abs [29] and immunoblotting using an anti-FLAG mAb. Ig, immunoglobulin; WB, Western blot.

other components of the native IFNR complex, most probably IFNAR2 which constitutively binds STAT2 [28]. Intriguingly, phosphorylation of Ser⁵³⁵, which is required for the ubiquitination of native IFNAR1 triggered by IFNα [29], was also stimulated by Epo (Figure 2D) indicating that IFNAR2 is probably dispensable for the activation of this pathway. In conclusion, the Epo-driven homodimerization of the chimaera 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 (Figure 2B). Furthermore, phosphorylation of Ser⁵³⁵ was also impaired in these cells (Figure 2D), suggesting that Tyk2 catalytic activity may be required for ubiquitination of IFNAR1.

This model system recapitulated ligand-induced phosphorylation events and was therefore suitable for investigating the catalytic role of Tyk2 in the down-regulation of the chimaeric receptor. First, the internalization rate of the ligand-chimaera complex was measured in the two clones, using ¹²⁵I-Epo. To avoid plasma membrane replenishment with newly synthesized receptors, CHX was added 15 min before the addition of the ligand. The amount of bound radioactivity was equivalent in these two clones. The amount of internalized ¹²⁵I-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 ¹²⁵I-Epo, the amount of internalized radioactivity was comparably increased in the two clones (Figure 3A). These results demonstrate that the internalization of the chimaera in the presence of Epo occurs independently of the catalytic activity of Tyk2.

Epo-induced degradation of the EpoR-R1 chimaera requires active Tyk2

Next, the steady-state expression of the chimaera was monitored by anti-FLAG Western blotting of 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 only in the level of the mature band. Conversely, in cl.20 cells expressing the K930R mutant, no evidence for degradation of this band was observed (Figure 3B). Overall, these results suggest that the tyrosine kinase activity of Tyk2 is dispensable for the internalization of the chimaeric receptor that occurs in the presence of the ligand. However, catalytic activity of Tyk2 is essential for the serine phosphorylation (Figure 2D), as well as for the proteolysis of the chimaeric receptor.

IFNα-induced degradation of endogenous IFNAR1 in HEK-293T cells requires catalytically active Tyk2

In a complementary approach to study the contribution of Tyk2 to ligand-induced IFNAR1 down-regulation, we asked whether the overexpression of WT or kinase inactive Tyk2 affects the regulation of endogenous IFNAR1. GFP (green fluorescent protein)-fused versions of WT Tyk2 and the K930R mutant were transfected into HEK-293T cells, and surface IFNAR1 decay was monitored in GFP-positive cells by FACS. As shown in Figure 4(A), upon treatment with IFNα the decrease in IFNAR1 was comparable in the two GFP-gated populations. Total IFNAR1 levels were analysed by Western blotting of cells transfected with Tyk2 constructs lacking GFP. The degradation of IFNAR1 occurred in cells transfected with WT Tyk2, but it was noticeably impaired in cells transfected with the K930R mutant (Figure 4B). Tyk2 activation was monitored in the two transfected populations, using phospho-specific antibodies directed against the activation loop tyrosines. WT Tyk2, but not the K930R mutant, was basally phosphorylated and a further increase in its phosphorylation level was detected after the addition of IFNα (Figure 4B). As expected from a previous study [21], weak phosphorylation of Tyk2, initiated by Jak1, was observed in cells expressing the K930R mutant (Figure 4B).

Given that endogenous Tyk2 is still present in HEK-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 the K930R mutant protein. The low level of IFNAR1 in these cells precluded cytofluorimetric analysis of the cell-surface pool, and the extent of IFNAR1 internalization was therefore analysed by reversible biotinylation of the cell surface. As shown in Figure 5(A), the internalization of biotinylated IFNAR1, measured within the first 60 min after the addition of IFN, did not differ between the cells that harboured either WT or catalytically inactive Tyk2. These results corroborate the conclusion from our analysis of the EpoR-R1 chimaera (Figure 3A), that Tyk2 kinase activity is not required for the initial steps of IFNAR1 endocytosis. In addition, we assessed the total level of IFNAR1 in these cells. As shown in Figure 5(B), 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 (Figure 5B). A robust and transient phosphorylation of Tyk2 was detected in IFNα-treated WT-5 cells, whereas the induced phosphorylation of the K930R mutant on the activation loop was weaker and sustained, reflecting Jak1-initiated action.

Figure 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 μ g/ml CHX before stimulation with 2 units/ml 125 I-Epo. Cells were sampled for the determination of internalized radioactivity at early (left panel) or late (right panel) time intervals. The results are the means for four independent experiments. (B) Epo-induced degradation of the chimaeric EpoR-R1 receptor in cl.14 and cl.20 cells. Cells were treated with 10 units/ml Epo for the indicated times. Lysates (40 μ g) were resolved by SDS/PAGE. EpoR-R1 was revealed using an anti-FLAG mAb. The lysates were run on a separate blot and were detected with an anti-actin mAb to control for protein content. Right panel, the bands that were revealed with an anti-FLAG mAb were quantified. The ratio of mature (top band) to immature (bottom band) EpoR-R1 was plotted for each clone (cl.14, solid line; cl.20, dashed line). WB, Western blot.

Figure 4 Surface disappearance, serine phosphorylation, ubiquitination and degradation of IFNAR1 in HEK-293T cells overexpressing WT Tyk2 or the K930R mutant

(A) Tyk2 kinase activity is dispensable for IFN α -induced IFNAR1 disappearance from the cell surface. The surface level of IFNAR1 was determined by flow cytometry of cells transfected with Tyk2-GFP or K930R-GFP. A similar basal level of IFNAR1 expression was observed in the two GFP-gated cell populations (thick black line). IFN α treatment for 90 min (grey line) or 180 min (thin black line) induced a comparable decrease in surface IFNAR1 expression in the two populations. (B) IFN α -induced IFNAR1 degradation requires active Tyk2. Cells expressing WT Tyk2 or the K930R mutant were treated with 500 pM IFN α for the indicated times. Total lysates (50 μ g) were resolved by SDS/PAGE, and IFNAR1 was revealed using an anti-IFNAR1 mAb. The membrane was stripped and reprobed with anti-phospho-Tyk2 Abs or an anti-Tyk2 mAb. (C) Tyk2 activation promotes IFN α -induced serine phosphorylation and ubiquitination of IFNAR1. Cells expressing WT Tyk2 or the K930R mutant were treated with 500 pM IFN α for the indicated times. Total lysates (5 mg) were used to immunoprecipitate endogenous IFNAR1. The immunoprecipitates were resolved by SDS/PAGE, and ubiquitinated IFNAR1 was revealed using anti-ubiquitin Abs. The membrane was stripped and reprobed with P-Ser⁵³⁵-IFNAR1-specific Abs or an anti-IFNAR1 mAb. The migration of molecular-mass markers is indicated on the right. Bands shown 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. WB, Western blot.

Taken together, these results are in line with those obtained for the EpoR-R1 chimaeric receptor (Figures 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 residue phosphorylation and ubiquitination of IFNAR1

Next, we investigated the mechanism by which Tyk2 promotes IFNAR1 degradation. Previous data have shown that upon ligand

Figure 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 WT Tyk2 (WT-5) or kinase-dead Tyk2 (KR-2). Cells were biotinylated at 4 °C with a disulphide-cleavable biotin and 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 (as described in the Experimental section). After lysis, internalized proteins were recovered using streptavidin beads, and 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 of MESNA stripping (lanes 2 and 7) were controlled. WB, Western blot. (B) IFN α -induced degradation of IFNAR1 requires active Tyk2. Cells were treated with 500 pM IFN α for the indicated times. Total lysates (40 μ g) were resolved by SDS/PAGE, and IFNAR1 was revealed using an anti-IFNAR1 mAb. The membrane was stripped and reprobed with anti-phospho-Tyk2 Abs or an 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. Total lysates (5 mg) were used to immunoprecipitate IFNAR1. Ubiquitinated IFNAR1 was revealed using anti-ubiquitin Abs. The membrane was stripped and reprobed with P-Ser⁵³⁵-IFNAR1-specific Abs or an anti-IFNAR1 mAb. Tyk2 levels were measured by direct Western blot analysis of 50 μ g of lysate. Migration of molecular-mass markers is indicated on the right. IP, immunoprecipitate.

binding, IFNAR1 is phosphorylated on serine residues (Ser⁵³⁵ and Ser⁵³⁹) located in the cytoplasmic tail [29]. This modified motif is recognized by the F-box containing β -TrCP2 [β -transducin repeats-containing protein 2/HOS (homologue of Slimb)], a protein known to recruit the core SCF (Skp1-Cullin-F-box) E3 ligase complex which ubiquitinates and targets numerous proteins for degradation. Ligand-induced ubiquitination of IFNAR1 has been shown to precede receptor degradation [29]. In the present study, we observed that phosphorylation of the serine residue in the EpoR–R1 chimera was impaired in the cells that harboured catalytically inactive Tyk2 (Figure 2D). On the basis of these results, we tested whether the overexpression of the K930R mutant in HEK-293T cells affected serine residue phosphorylation and/or ubiquitination of endogenous IFNAR1. The latter was immunoprecipitated from cells overexpressing Tyk2 or the K930R mutant and the blot was revealed first with anti-ubiquitin Abs and subsequently with anti-phospho-Ser⁵³⁵ Abs. As shown in Figure 4(C), ubiquitination and serine residue phosphorylation in IFNAR1 were affected in cells overexpressing the K930R mutant as compared with cells overexpressing WT Tyk2. The residual ligand-induced modifications of IFNAR1 in the K930R mutant transfected cells could be attributed either to the presence of endogenous Tyk2 or to the existence of alternative Tyk2-independent pathways that trigger phosphorylation of Ser⁵³⁵ in HEK-293T cells. Thus to further substantiate these findings we analysed the ligand-induced events in the WT-5 and KR-2 clones, which lack endogenous Tyk2. As seen in Figure 5(C), neither serine residue phosphorylation nor ubiquitination of IFNAR1 were detected in KR-2 cells. These data provide the genetic

evidence that Tyk2 activity is required for serine phosphorylation of IFNAR1. Overall the results presented in the present study, 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 chaperone-like, non-catalytic role for Jak proteins in cytokine receptor traffic. Tyk2 was found to increase the surface level of IFNAR1 by decreasing 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 EpoR. In the latter context, the Jak protein enhances the surface level of the cognate receptor by facilitating its delivery to the plasma membrane [31,32]. A number of research groups have also analysed the function of Jaks in the ligand-induced trafficking of cytokine receptors. It has been shown that a mutant EpoR that is unable to activate Jak2 was efficiently internalized after Epo binding [33]. In line with this observation, Walrafen et al. [9] demonstrated that the pharmacological inhibition of Jak2 kinase activity did not interfere with ligand-induced EpoR internalization. A mutant GHR bearing mutations in box1, and thus unable to bind Jak2, has been shown to be internalized with the same kinetics as the WT receptor. Furthermore, the internalization rate of GHR was not impaired in cells deficient in Jak2 [34]. Similar uncoupling of receptor

internalization and signalling has been reported for two other cytokine receptors, gp130 and the bipartite IL-4 receptor [35,36].

In the present study, we have initiated studies on the intracellular fate of the heterodimeric IFNR and focused on the IFNAR1 subunit. In HEK-293T cells, IFN α binding accelerates the internalization rate of IFNAR1 approx. 3-fold. Although the analyses reported in the present study, 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, leading to a 3- to 6-fold increase in IFNAR1 internalization and degradation, was consistently observed.

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, cells harbouring either WT or kinase inactive Tyk2. The internalization of iodinated Epo bound to the homodimeric EpoR–R1 complex, coupled with either WT Tyk2 or the K930R mutant was studied. We also analysed the effect of overexpressed WT Tyk2 or the K930R mutant on the internalization of endogenous IFNAR1, and lastly we measured internalization of biotinylated IFNAR1 in Tyk2-deficient cells stably expressing WT Tyk2 or the K930R mutant. Taken together, the results of these analyses provide evidence that catalytic activation of Tyk2 is not required for IFNAR1 internalization, as has been reported for Jak2 coupled to the EpoR and 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 IFNR 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 Ser⁵³⁵ in order to recruit β -TrCP2. The ability of the EpoR–R1 homodimer to be serine phosphorylated suggests that the IFNAR2 subunit is dispensable for activation of this yet to be identified kinase. Furthermore, we have analysed 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 has repercussions in the cytoplasmic tail of IFNAR, 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.

Although 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 Walrafen et al. [9] that Jak2 activity, although dispensable for internalization of EpoR, is required for its ubiquitination. Together, these data suggest that IFNAR1 ubiquitination is not required for its internalization. However, given that either inhibition of β -TrCP2 or mutation of Ser⁵²⁶ of murine IFNAR1 – corresponding to Ser⁵³⁵ in human IFNAR1 – decreases the efficiency of the uptake of radioactively labelled 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]. Never-

theless, the data presented here indicate that efficient ligand-induced degradation of both the EpoR–R1 chimera and native IFNAR1 rely upon 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 RTKs has been shown to be required for their efficient endosomal sorting towards degradative pathways. Conjugated ubiquitin represents a recognition signal for several sub-complexes of Vps (vacuolar protein sorting) class E proteins [including Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate)–STAM (signal-transducing adaptor molecule) and the ESCRT (endosomal sorting complex required for transport) system] that transiently localize to the cytosolic face of the early endosomal/multivesicular body membrane and relay ubiquitinated cargoes towards internal vesicles in a sequential manner (reviewed in [40]). Further studies will address the role of Tyk2-promoted ubiquitination in endosomal sorting of IFNAR1.

Our present and previous studies [19] 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 IFNR, and the potential contribution of Jak1. Of note, IFNAR2 was reported to undergo regulated proteolysis in response to cells treated with IFN α and phorbol esters [41]. It remains an open question as to whether IFNAR2, once internalized in response to IFN α only, is routed towards degradative pathways.

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