Basal ubiquitin-independent internalization of interferon alpha receptor is prevented by Tyk2-mediated masking of a linear endocytic motif

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Running head: basal endocytosis of IFNAR1

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SUMMARY

Linear endocytic motifs of signaling receptors as well as their ubiquitination determine the rate of ligand-induced endocytosis that mediates downregulation of these receptors and restricts the magnitude and duration of their respective signal transduction pathways. We previously hypothesized that, in the absence of its cognate ligand, type I interferon (IFN), the IFNAR1 receptor chain is protected from basal endocytosis by a hypothetical masking complex that prevents the Tyr-based endocytic motif within IFNAR1 from interacting with components of the adaptin protein complex 2 (AP2). Here we identify a member of the Janus kinase (Jak) family, Tyk2, as a component of such a masking complex. In the absence of ligand or of receptor chain ubiquitination, binding of Janus kinase Tyk2 within the proximity of the Tyr-based linear motif of IFNAR1 from interacting with components of the adaptin protein complex 2 (AP2). Here we identify a member of the Janus kinase (Jak) family, Tyk2, as a component of such a masking complex. In the absence of ligand or of receptor chain ubiquitination, binding of Janus kinase Tyk2 within the proximity of the Tyr-based linear motif of IFNAR1 is required to prevent IFNAR1 internalization and to maintain its cell surface expression. Furthermore, interaction experiments revealed that Tyk2 physically shields this Tyr-based motif from the recognition by the AP50 sub-unit of AP2. These data delineate a long sought ligand- and ubiquitin-independent mechanism by which Tyk2 contributes to both the regulation of total IFNAR1 levels as well as of the and the cell surface density of this receptor chain.

INTRODUCTION

Cells react to diverse environmental stimuli by expressing specific receptors that recognize these stimuli and initiate specific signaling pathways that enable a cell or a tissue to cope with an altered environment. Downregulation of these signaling receptors represents the most specific mode of limiting the magnitude and duration of given signal transduction pathways. For transmembrane receptors displayed at the cell surface, ligand-stimulated endocytosis is a major mechanism by which the ability of a cell to react to a ligand is restricted. In addition, basal ligand-independent internalization may determine how responsive a naïve cell could be to a subsequent encounter with a particular ligand.

Mechanisms mediating internalization of signaling receptors involve a dynamic plasma membrane exchange resulting in bulk endocytosis and a cargo-specific clathrin-dependent endocytosis. Interaction of clathrin lattices formed on the plasma membrane with cargo receptors relies on adaptor complexes such as the adaptin protein-2 complex (AP2), which recognizes specific linear endocytic motifs present within the cytoplasmic domains of the target receptor. For example, the μ2 subunit of AP2 (also termed AP50) is known to recognize Tyr-based linear endocytic motifs, and to enable the AP2-dependent tethering of cargo to clathrin molecules (reviewed in (1-4)).
Receptor ubiquitination has also emerged as a key endocytic signal for numerous eukaryotic cell surface receptors (reviewed in (5-8)). For several receptors (such as epidermal growth factor receptor (EGFR), growth hormone receptor (GHR), interferon alpha receptor chain 1 (IFNAR1)), ubiquitination is stimulated by the ligand. This stimulation is mediated by the ligand-induced transduction of a signal initiated by a kinase activity that either is intrinsic to the receptor itself (as for EGFR, (9)), or by a receptor-associated kinase (as for GHR and IFNAR1 (10,11)). Ligand-induced ubiquitination of the receptor promotes its interaction with clathrin and stimulates internalization of receptor. Furthermore, ubiquitination plays an important role in post-internalization sorting of cargo receptors to the late endosomes and in subsequent lysosomal degradation (reviewed in (12-14)).

We have recently found that ligand-stimulated site-specific ubiquitination of IFNAR1 promotes IFNAR1 internalization and is required for interaction of the Tyr466-based linear endocytic motif within IFNAR1 with AP2, which is essential for efficient IFNAR1 endocytosis (15). Based on this evidence, we hypothesized that ubiquitination of IFNAR1 may expose the Tyr-based endocytic motif and allow for its interaction with the components of AP2 (e.g., AP50). This hypothesis also implies that in the absence of ubiquitination, the Tyr466-based motif is masked either by an intramolecular folding of the IFNAR1 cytoplasmic tail or by a putative IFNAR1-interacting protein. Under these scenarios, ligand-induced ubiquitination of IFNAR1 would either alter the conformation of the intracellular tail or displace the interacting protein in order to expose the Tyr466-based motif to AP50.

To test this hypothesis we sought to identify the putative protein(s) that mask the Tyr-based endocytic linear motif within IFNAR1 and protect the receptor from basal ubiquitination-independent endocytosis in cells that are yet to encounter ligand. Here we report that IFNAR1-associated Janus kinase Tyk2 (previously shown to be required for maintaining the cell surface levels of IFNAR1 independently of its catalytic activity (16)) functions as such a masking component. We demonstrate that Tyk2 is required for suppression of ligand- and ubiquitination-independent endocytosis of IFNAR1 in a manner that depends on both the integrity of the Tyr-based linear endocytic motif and the proximity of this motif to the Tyk2 binding site. Furthermore, interaction between IFNAR1 and AP2 is regulated by Tyk2 expression levels. We conclude that in cells that did not encounter type I interferon (IFN), Tyk2 inhibits the ubiquitination-independent internalization of IFNAR1. This inhibitory effect is mediated by physical interaction between IFNAR1 and Tyk2 that shields IFNAR1 from binding to AP2 components, which facilitate receptor endocytosis via recognition of linear endocytic motifs.

**EXPERIMENTAL PROCEDURES**

**Cells, transfection and plasmids**

Human embryo kidney 293T cells (a gift from Z. Ronai), Tyk2-null human fibrosarcoma 11,1 cells, and an 11,1-derived clone expressing the kinase-inactive Tyk2 K930R mutant (“KR” cells) were maintained as described elsewhere (10,17). Transfection was carried out using Lipofectamine Plus (Invitrogen, Carlsbad, CA) or FuGene (Roche, Palo Alto, CA). Respective empty vectors were added to transfection mixtures to keep the amount of transfected DNA constant in each case. Vectors for the expression of Tyk2 (wild type or the Y103A/F104A double mutant (Tyk2YFAA) (16,18)), as well as C-terminally (19) or N-terminally (15) Flag-tagged human (wild type or S535A) IFNAR1 were described previously. Mutant IFNAR1SP, in which the Tyk2 binding site was separated from the Tyr466-based linear endocytic motif was generated by an introduction of a 10 amino acid spacer (SSSIDEYFSE). This spacer sequence was introduced between Pro-486 and Lys-487 of human IFNAR1 using an overlap PCR method. All the plasmid constructs were sequenced to confirm the desired mutations. The vector for the expression of HA-tagged AP50 (20) was a kind gift from V. Haucke. ShRNA constructs for knocking down Tyk2 were constructed in a modified version of pSuper-retro that lacks the puromycin marker (pRSP, a kind gift from J. Wade Harper, (21)) using 5’-gtctgcatccacattgcacat-3’ (for shTyk2-1) and 5’-agttggtatcactcctccttg-3’ (for shTyk2-2) sequences. Control shRNA vector contained the sequence against GFP (21). Retroviral shRNAs validated against the expression of human Jak2 were purchased from Sigma. Flag-tagged pCDNA3-based vector for
expression of prolactin receptor has been previously described (22).

**Antibodies and immunotechniques**

Antibodies specific for Flag (M2, Sigma), HA (Y-11, Covance, Princeton, NJ), AP50 (μ2, BD Transduction Laboratories, San Diego, CA), and β-actin (Santa Cruz, CA) were purchased. Antibodies against Tyk2 (23), as well as antibodies that recognize endogenous IFNAR1 (AA3, EA12, and GB8, (24)) were described previously. Secondary antibodies conjugated to horseradish peroxidase were purchased from Chemicon (Temecula, CA) and Pierce Biotechnology (Rockford, IL). Immunoprecipitation and immunoblotting procedures are described elsewhere (25). Densitometry data were obtained and analyzed using Scion Image Software (version Beta 4.0.2) and the digital images were prepared using Adobe Photoshop 7.0 software.

**Cell surface biotinylation and internalization assays**

Cell surface biotinylation was carried out as described elsewhere (26). Briefly, 36 hours after transfection, cells were starved in serum-free DMEM for 2 hours, chilled on ice and washed with phosphate buffered saline. Surface biotinylation was performed with 0.25 mg/mL EZ-Link-Sulfo-NHS-S-S-biotin (Pierce, Rockland, IL) for 15-30 min at 4°C. Unbound biotin was removed by washing with ice-cold HEPES-buffered saline (HBS; 10 mM HEPES-NaOH pH 7.4, 150 mM NaCl) and cells were harvested and lysed with 60 mM n-octyl β-d-glucopyranoside, 0.1% SDS in the same buffer containing protease inhibitor cocktail (Sigma) for 10-20 min and then centrifuged at 14,000g for 15 min. Biotinylated proteins were recovered by incubating with immobilized NeutrAvidin (Pierce) overnight, washed, and analyzed by immunoblotting with indicated antibodies.

Internalization of IFNAR1 was determined by measuring the levels of cell-surface immunoreactivity of endogenous or Flag-epitope-tagged receptors using an ELISA assay as described previously (15,27). Briefly, 293T or 11,1 cells transfected (or not) with the various N-terminally FLAG-epitope-tagged IFNAR1 plasmids were plated into 24-well plates. Cells were starved for 2 hours in serum free DMEM and chilled on ice for 15 min. Internalization was initiated by incubation of cells with warm (37°C) serum free DMEM (with or without IFNα, 3000U/ml) for 2.5-10 min at 37°C, and terminated by placing the plate on ice. Cells were washed, blocked, and incubated with anti-FLAG antibody (monoclonal Flag M2 from Sigma) or anti-IFNAR1 antibody (AA3 for endogenous IFNAR1) for 1 h, washed, and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Molecular Probes) for 1.5 h. After extensive washing, cells were incubated with AmplexRed Ultra Reagent (10-acetyl-3,7-dihydroxyphenoxazine, Molecular Probes). Aliquots were transferred to black 96-well plates, and fluorescence was measured using a Bioscan Chameleon fluorescence plate reader (Washington, DC) with excitation at 530 nm and emission monitored at 590 nm. Results are expressed after subtracting the value obtained with mock-transfected cells as a percentage of fluorescence registered before internalization. Average data from at least three independent experiments (each in triplicate or quadruplicate) ± SEM are presented.

**RESULTS**

A putative protein preventing basal IFNAR1 internalization by shielding the Tyr-based motif from recognition by AP2 is expected to fit the following criteria: (i) it should interact with IFNAR1 in cells not exposed to IFN; (ii) the site of interaction should map in proximity to the Tyr466 residue, (iii) it should regulate basal internalization of IFNAR1 in a manner that depends on its binding to IFNAR1 and on the integrity of the Tyr-based motif, and (iv) it should prevent the interaction between IFNAR1 and AP2. Close examination of the primary sequence of IFNAR1 surrounding the Tyr466-based linear endocytic motif (Figure 1) revealed the presence of a stretch of near thirty amino acids that were previously implicated in mediating the recruitment of Tyk2 (28).

Thus, we considered the possibility of Tyk2 being the masking component. We have previously described that the level of IFNAR1 is decreased in a Tyk2-deficient human fibrosarcoma cell line (mutant 11,1 or U1A) (29). Re-expression of Tyk2 in 11,1 cells restores IFNAR1 levels. We also reported that Tyk2 sustains IFNAR1 by slowing down its basal internalization and this function is independent of catalytic activity (16). The role of Tyk2 in sustaining IFNAR1 at the cell
surface was further supported by the cytofluorimetric analysis of surface IFNAR1 level in T lymphocytes from a Tyk2-deficient patient suffering from hyper IgE syndrome (30). On the other hand, the level of the murine IFNAR1 is hardly affected in cells from mice in which Tyk2 was genetically ablated by the knockout technique (31-33). Intriguingly, all sequenced IFNAR1 orthologs possess the Tyr-based linear endocytic motif (YVFF) sequence, with the sole exception of the murine IFNAR1 (Figure 1). Furthermore, concordant with the role of this motif in endocytosis of the human chain (15), the levels of the murine IFNAR1 were only modestly affected by Tyk2 expression (data not shown).

These observations led us to test the hypothesis that Tyk2 protects human IFNAR1 from basal (i.e., ligand- and ubiquitination-independent) endocytosis via masking of the Tyr-based linear motif and thereby preventing binding of AP2. First, we sought to corroborate the role of Tyk2 in regulating IFNAR1 levels using the RNAi approach. To this end, we developed short hairpin RNAs that efficiently knocked down Tyk2 in 293T human embryonic kidney cells (Figure 2A). Transfection of these shRNAs led to a noticeable decrease in the level of endogenous IFNAR1 (Figure 2B). Remarkably, knockdown of another member of Janus kinase family, Jak2, which does not associate with IFNAR1, did not affect the levels of this receptor indicating that physical interaction between the receptor chain and the cognate kinase is required for such regulation.

We next investigated whether Tyk2 played a role in regulating the basal endocytosis of IFNAR1. Knockdown of Tyk2, but not of Jak2, dramatically increased the initial rate of internalization of endogenous IFNAR1 in cells that did not undergo treatment with IFNα (Figures 2C and 3A). In line with our previous observations (15), treatment of 293T cells with IFN induced endocytosis of endogenous IFNAR1. However, this effect was no longer observed in cells in which Tyk2 was knocked down, where basal IFNAR1 internalization proceeded at a high rate (Figure 3A). These results provide further evidence that Tyk2 negatively regulates the basal internalization of IFNAR1.

Previous studies demonstrated that the catalytic activity of Tyk2 contributes to both IFNα signaling (31,32) and to IFNAR1 ubiquitination and degradation observed in response to ligand (10). Conversely, catalytic activity of Tyk2 is not required for maintenance of IFNAR1 in non-stimulated cells (16,29). We therefore investigated whether the latter function of Tyk2 is ubiquitination-dependent or not. To this end, we compared the rates of basal internalization of exogenously expressed wild type IFNAR1 (that is still ubiquitinated even in untreated cells via the ligand-independent pathway, ((34) and Figure 3B) with that of the ubiquitination-deficient IFNAR1SA mutant ((19) and Figure 3B). In keeping with an important role of ubiquitination of IFNAR1 in internalization (15), the mutant IFNAR1SA was internalized at a slower rate than the wild type receptor in control cells that received irrelevant shRNA. However, knockdown of Tyk2 eliminated this difference and robustly increased the endocytic rate of both proteins (Figure 3C). These results suggest that adequate expression of Tyk2 prevents basal internalization of IFNAR1 in a ligand- and ubiquitination-independent manner.

We next investigated whether the effects of Tyk2 are dependent on its ability to interact with the cognate receptor. To this end, we used the Tyk2YFAA double mutant that exhibits defective interaction with thrombopoietin receptor (TpoR, (18)). Under conditions where the levels of immunoprecipitated IFNAR1 were normalized to evaluate the extent of Tyk2 binding, this Tyk2YFAA mutant showed a weaker ability to interact with IFNAR1 as compared to the wild type Tyk2 protein (Figure 4A). In Tyk2-null 11,1 cells, co-expression of wild type Tyk2 led to an increase in both total and cell surface levels of IFNAR1 (assessed using cell surface biotinylation; Figure 4B), but not of prolactin receptor (data not shown), which is known to interact predominantly with Jak2 (35). Under these conditions, co-expression of Tyk2YFAA mutant was inefficient in increasing the surface level of IFNAR1 (Figure 4B). Furthermore, whereas co-expression of wild type Tyk2 in 11,1 cells slowed down the initial internalization of IFNAR1, the Tyk2YFAA double mutant was not capable of such inhibition (Figure 4C). These data suggest that Tyk2 prevents basal...
endocytosis of IFNAR1 by directly interacting with this receptor.

We next investigated the role of the Tyr-based motif in the basal ubiquitin-independent endocytosis of IFNAR1. To this end, we substituted the Tyr466 residue with a Phe in the context of the ubiquitination-deficient IFNAR1\textsuperscript{SA} mutant. The combined mutations (Y466F/S535A) did not affect the ability of IFNAR1 to interact with Tyk2 (Figure 5B). However, they almost abolished the requirement for Tyk2 co-expression to protect IFNAR1 from basal endocytosis in Tyk2-null 11,1 cells (Figure 4D). These results suggest that Tyk2 suppresses ligand- and ubiquitin-independent internalization of IFNAR1 in a manner that depends on integrity of the Tyr466-based endocytic motif. Modest residual effects of Tyk2 expression on the endocytic rate of IFNAR1\textsuperscript{SAYF} protein could be attributed to secondary di-leucine- and/or di-isoleucine-based endocytic motifs that are still present in the IFNAR1 intracellular tail (Figure 1).

Given that the Tyk2 binding site within IFNAR1 is situated in close proximity to Tyr466 (Figure 1), it is plausible that Tyk2 prevents basal internalization of IFNAR1 by physically shielding the Tyr-based motif from the components of AP2. To test this hypothesis, while avoiding manipulating Tyk2 levels or generating IFNAR1 deletion mutants that may lose other important endocytic determinants, we designed a more subtle IFNAR1 mutant that would still bind Tyk2 but would unmask the Tyr466-based endocytic motif. Such an IFNAR1 mutant was generated by inserting a 10 amino acid “spacer” sequence between Tyr466 and the Tyk2 binding site, in order to move the Tyk2 binding site away from the Tyr466-based motif (Figure 5A). Although this spacer mutant (IFNAR1\textsuperscript{SP}) retained the ability to co-immunoprecipitate with Tyk2 (Figure 5B), its rate of basal internalization was noticeably greater than that of wild type IFNAR1. This increased endocytosis was likely due to an exposed Tyr-based motif, because introduction of the Y466F mutation in the context of IFNAR1\textsuperscript{SP} (SPYF) reduced the internalization rate (Figure 5C). These results indicate that Tyk2 protects IFNAR1 from basal endocytosis by physically masking the Tyr466-based linear endocytic motif.

Tyr466 is a major determinant for the recruitment of AP2 which is required for IFNAR1 internalization (15). Thus, we tested whether the presence of Tyk2 affects the interaction of IFNAR1 with AP50, a component of the AP2 complex known to interact with Tyr-based linear endocytic motifs (4). We observed that in the presence of lysosomal inhibitors preventing IFNAR1 degradation, endogenous AP50 co-immunoprecipitated with endogenous IFNAR1 in 11,1 cells. However, this interaction was noticeably less efficient in 11,1-derived KR cells (Figure 6A) that stably express the catalytically inactive K930R Tyk2 mutant (17). In line with these observations, when Tyk2 and IFNAR1 were co-expressed, increasing concentrations of Tyk2 yielded elevated levels of IFNAR1 protein (Figure 6B, see “before normalization” or in whole cell extracts). When the amounts of IFNAR1 taken into immunoprecipitation were first normalized to load comparable levels of IFNAR1 in each lane, we observed that co-expression of Tyk2 prevented the co-immunoprecipitation of Flag-IFNAR1 and HA-AP50 in a dose-dependent manner (Figure 6B). These data suggest that when Tyk2 associates with IFNAR1, this protein kinase also interferes with the docking of AP50. Indeed, the expression of the interaction-deficient Tyk2\textsuperscript{YFAA} mutant did not prevent co-immunoprecipitation (Figure 6C), indicating that the IFNAR1-Tyk2 interaction is required for such inhibition. Together, these results suggest that Tyk2 physically binds to IFNAR1 and shields the Tyr466-based linear motif within the receptor from interaction with AP2 that would otherwise cause basal ubiquitination-independent endocytosis and subsequent lysosomal degradation.

DISCUSSION

The regulation of clathrin-dependent internalization of signaling receptors is complex and involves recognition of both linear and ubiquitin-derived endocytic signals by components of the endocytic machinery. Recent clues suggest a functional interplay between ligand-induced ubiquitination and recognition of the Tyr466-based linear motif by AP2 in the internalization of IFNAR1 (15). However, in the absence of the ligand, the interaction of Tyr466 with AP2 is weak suggesting that some entity may mask this linear motif and prevent basal endocytosis of IFNAR1.

Here we demonstrate that in the absence of either a ligand or ubiquitination of the receptor, the direct binding of Tyk2 in the proximity of the
Tyr-based linear motif of IFNAR1 physically shields this motif from its recognition by the AP50 subunit of AP2. This masking mechanism plays an important role in preventing an unbalanced basal endocytosis of IFNAR1. These results provide a foundation for delineating a long sought mechanism by which Tyk2 contributes to the maintenance of total levels and the density of cell surface IFNAR1 (16,29). Furthermore, given the importance of the Tyr-based endocytic motif in the protection of human IFNAR1 endowed with Tyk2, our present data provide a clue regarding why murine IFNAR1, which exhibits a clear evolutionary departure from other species and lacks this motif (Figure 1), is largely impervious to Tyk2-dependent regulation ((31-33) and B.V. and S.Y.F., unpublished data).

From a functional point of view, our data provide an important mechanistic insight into the ability of Janus kinases to contribute to the activity of cognate receptors in a kinase-independent manner. Unlike growth factor receptors that harbor inherent catalytic domains to transduce signals, receptors for helical cytokines associate with Jak family members to form a modular receptor-kinase complex (reviewed in (36)). In addition to their catalytic role, Jak proteins appear to function as regulators of cell surface expression levels of their associated receptors in a catalytically-independent manner. A stabilizing effect of Jak proteins on the cell surface levels of cytokine receptors has been shown for all members of the Jak family including Tyk2 (for IFNAR1 (16,29), and TpoR (18)), Jak1 (for the Oncostatin M receptor (37,38), IL-9Rα, and IL-2Rβ (18) and our unpublished data), Jak2 (for TpoR (18), the erythropoietin receptor (39), and GHRr (40)), and Jak3 (for the common γ chain of the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptor complexes (41)).

The mechanisms by which different Jak family members appear to increase the cell surface levels of their associated receptor chain are yet to be fully understood. In addition to protecting the receptors from basal internalization by shielding the linear motifs (as Tyk2 does for IFNAR1), additional modes of action proposed to date include a chaperone-like assistance in receptor folding, maturation and delivery to the cell surface, inhibition of post-internalization sorting into the late endosomes/lysosomes, as well as protection from proteasome-mediated degradation (11,16,18,37,39). As for the similarity of the ligand-dependent events, the interplay between the linear and ubiquitin-derived motifs is particularly evident in promoting the efficient internalization of IFNAR1; similar relationships are revealed for regulating the internalization of the prolactin receptor (our unpublished data).

Other masking determinants, that protect various endocytic motifs and hence limit basal internalization of other signaling receptors to preserve a physiological density of surface receptors, should be eventually identified. While in some cases interacting proteins will be involved (similar to the function of Tyk2 towards IFNAR1), masking could also be achieved by intramolecular folding of the receptor itself. Thus, the functional role of a given endocytic determinant should always be investigated in the context of the full length receptor. There might be a need to revise the interpretation of data obtained from studies that relied on deletions of the intracellular domains as a strategy for delineating either ubiquitination-related or linear endocytic motifs of signaling receptors. Such deletions might potentially remove not only important positive regulators of endocytosis (such as recognition signals for a ubiquitin ligase or a ubiquitin-acceptor site) but also docking sequences or surfaces involved in recruitment of masking proteins.

At present, we do not know exactly how the ligand-driven ubiquitination of IFNAR1 that we have recently reported (15) promotes the exposure of the Tyr466-based motif. Ubiquitination-induced dissociation of Tyk2 from IFNAR1 is an attractive hypothesis. However, we did not detect a decrease in Tyk2 binding to IFNAR1 within 15 min of IFN addition, while a substantial fraction of IFNAR1 is internalized at that time (S.K. and S.F., unpublished data). Another possibility is the ubiquitination-induced dissociation of an additional masking component(s), whose recruitment onto IFNAR1 could be mediated by Tyk2. A more plausible scenario is a ubiquitination-dependent change in the conformation of the intracellular domain of IFNAR1 (a change in the overall configuration of the IFNAR1/Tyk2 complex) that would ameliorate a juxtaposition of Tyr466 and Tyk2 and therefore allow for the recruitment of AP2 and subsequent endocytosis. Future studies will address these possibilities.
ABBREVIATIONS: AP2, adaptin protein complex 2; AP50, µ2 subunit of AP2; EGFR, epidermal growth factor receptor; GHR, growth hormone receptor; HA, hemagglutinin tag; IFN, interferon; IFNAR1, interferon alpha/beta receptor 1; IL, interleukin; Jak, Janus kinase; TpoR, thrombopoietin receptor.

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Legends to Figures

Figure 1 Sequence alignment of the proximal portions of the intracellular domain of IFNAR1 receptors from different species.

All currently known sequences of IFNAR1 are depicted starting from the end of the transmembrane domain. Putative endocytic motifs are depicted in bold letters. In human IFNAR1, the position of Y466 is noted by number and the amino acid residues situated within a region shown to participate in the interaction with Tyk2 (28) are underlined.

Figure 2 Knockdown of Tyk2 decreases the levels of endogenous IFNAR1 in 293T cells.

A. Characterization of shRNA constructs against Tyk2. 293T cells were transfected with the indicated constructs and the levels of Tyk2 and α-tubulin were analyzed by immunoblotting using respective antibodies.

B. Effect of either Tyk2 or Jak2 knockdown on the steady state level of endogenous IFNAR1 analyzed by immunoprecipitation with anti-IFNAR1 antibody EA12 followed by immunoblotting with anti-IFNAR1 antibody GB8. Asterisks denote non-specific bands that reflect equal protein loading. Levels of Tyk2 and Jak2 in whole cell extracts analyzed by immunoblotting using appropriate antibodies are also shown.
C. Effect of either Tyk2 (closed squares) or Jak2 (closed circles) knockdown on the internalization of endogenous IFNAR1 in 293T cells that were not treated with the ligand measured using the anti-IFNAR1 AA3 antibody-based fluorescent assay as described in reference (15) and outlined in Experimental Procedures. ShCON represent cells transfected with shRNA against GFP. An average result of four experiments (±S.E.) is shown.

Figure 3 Knockdown of Tyk2 promotes basal internalization of IFNAR1 in an ubiquitination-independent manner
A. Internalization of endogenous IFNAR1 in 293T cells transfected with indicated shRNA constructs (control shRNA, circles, and shRNA against Tyk2, squares) and treated (closed symbols) or not (open symbols) with IFNα was analyzed using the anti-IFNAR1 AA3 antibody-based fluorescent assay as described in reference (15) and outlined in Experimental Procedures;
B. In vivo ubiquitination of Flag-tagged IFNAR1 proteins (wild type or SA mutant, as indicated) expressed in 293T cells was assessed by denaturing immunoprecipitation using anti-Flag (M2) antibody followed by immunoblotting using either anti-ubiquitin (upper panel) or anti-Flag (lower panel) antibodies.
C. Internalization of Flag-tagged IFNAR1 proteins (wild type, open symbols, or ubiquitination-deficient SA mutant, closed symbols) in 293T cells transfected with control shRNA (circles) or shRNA against Tyk2 (squares) in the absence of IFN.

Figure 4 Tyk2 prevents basal internalization of IFNAR1 in a manner that depends on Tyk2-IFNAR1 interaction and on integrity of the Tyr-based endocytic motif.
A. Interaction of wild type Tyk2 and the Y103A/F104A double mutant (Tyk2YFAA) with Flag-IFNAR1 co-expressed in 293T cells analyzed by immunoprecipitation and immunoblotting with the indicated antibodies. Loading of IFNAR1 into the final reaction was normalized to achieve comparable levels of this Flag-tagged protein in all lanes;
B. Effect of co-expression of wild type Tyk2 or the Y103A/F104A double mutant (Tyk2YFAA) on surface (S) and total (T) levels of IFNAR1 in Tyk2-null 11,1 cells. Cell surface IFNAR1 was collected by streptavidin bead-based pull-down from the lysates of cells that underwent surface biotinylation (as described in Experimental Procedures). Both surface and total levels were analyzed by immunoblotting using anti-Flag M2 antibody. Levels of Tyk2 and β-actin in whole cell lysates (WCE) were also analyzed by immunoblotting.
C. Internalization of Flag-tagged IFNAR1 co-expressed with either empty vector (Vec, closed squares), wild type Tyk2 (open squares), or the Y103A/F104A double mutant (Tyk2YFAA) (closed triangles) in 11,1 cells was determined as in Figure 3C.
D. Internalization of the IFNAR1 mutants (IFNAR1S535A - squares; IFNAR1 S535A/Y466F – triangles) co-expressed with (open symbols) or without (closed symbols) Tyk2 was analyzed as in panel C.

Figure 5 Proximity of the Tyk2-binding site to the Tyr-based linear endocytic motif within IFNAR1 is required for Tyk2-mediated inhibition of basal endocytosis of IFNAR1.
A. Graphic depiction of IFNAR1 mutants. The Tyr-based endocytic motif is denoted. BD: binding domain; SP: spacer.
B. Interaction of IFNAR1 mutants with Tyk2 was analyzed by immunoprecipitation and immunoblotting as described in Figure 4A.
C. Internalization of wild type IFNAR1 (closed squares), spacer mutant (SP, closed triangles), and spacer + Y466F mutant (SPYF, open diamonds) in 293T cells in the absence of IFN was analyzed as described in Figure 3C.

Figure 6 Interaction of Tyk2 with IFNAR1 physically shields the receptor from binding AP50.
A. Interaction of endogenous IFNAR1 with endogenous AP50 (μ2) was measured in 11,1 Tyk2-null cells or in their KR derivatives (stably expressing catalytically inactive Tyk2). Cells were pre-treated with
methylamine (40mM, 2hr), an inhibitor of the lysosomal pathway, to prevent IFNAR1 degradation. The interaction was assessed by immunoprecipitation using anti-IFNAR1 (R1) antibodies followed by immunoblotting using the indicated antibodies. Levels of the AP50 subunit and Tyk2 in whole cell lysates (WCE) are also shown. Ig – immunoglobulin heavy chain.

**B.** Transfection of increasing doses of HA-tagged Tyk2 inhibits the interaction between Flag-tagged IFNAR1 and HA-tagged AP50 in 293T cells. This interaction was assessed by immunoprecipitation using anti-Flag antibody followed by immunoblotting using indicated antibodies. The amount of immunoprecipitated IFNAR1 was normalized (upper panel) to achieve comparable levels of loading. Levels of Flag-IFNAR1, HA-Tyk2 and HA-AP50 in whole cell lysates are also shown.

**C.** Effects of expression of either wild type Tyk2 or the Y103A/F104A double mutant (Tyk2^YFAA^) on the IFNAR1-AP50 interaction assessed as described in panel **B**.
H. sapiens  FLRCIN-YVFFPSLKPSSIDEYFSEQPLKN-LLLSTSEEQIEKCFIIENISTIATVETNQ 519
P. troglodyte FLRCIN-YVFFPSLKPSNIDEYFSEQQPLKN-LLLSTSEEQIEKCFIIENISTIATVETNQ 520
P. anubis LLRCIN-YVFFPSLKPSNIDEYFSEQQSLKN-LLLSTSEEQIEKCLIIENISTIATVETNQ 451
M. fascicularis LLRCIN-YVFFPSLKPSNIDEYFSEQQSLKN-LLLSTSEEQIEKCLIIENISTIATVETNQ 439
B. taurus FLRCVK-YVFFPSKPPSSVDEYFSDQPLRN-LLLSTSEEQTERCFIIENASITEIEETDE 520
O. aries FLRCVK-YVFFPSKPPSSVDEYFSDQPLRN-LLLSTSEEQTERCFIIENASITEIEETNE 520
S. scrofa VRSCIN-YVFFPSKPPSTIDEYFAEQPLKN-LLLSTSEEQTERCFIIENASITEIEETTDQ 520
M. musculus VWKYLCHWCFPPLKPRSIDESSEPPSKNLVLLTAEEHTCF-IENITDTVA--VEVKH 509
G. gallus YVNIKIK-YMFFFSCQTPLNIEG-FGAQLFSS-PFVTVEEPVEICYIIIESR----ITEEVNQ 529

YXXφ

LL
A

Tyk2
α-Tub

B

shRNA

IFNAR1

Jak2

Tyk2

Mock
shCON
shTyk2-1
shTyk2-2

C

% internalized

Min

0 5 10

shCON
shTyk2
shJak2

Kumar et al, Figure 2
Kumar et al, Figure 3

A

B

C

IB: Flag

IB: Ub

IB: Flag
Kumar et al, Figure 6

A

IFNAR1

Ig

AP50

Tyk2

Tyk2

AP50

IP: IFNAR1

WCE

B

Tyk2

IFNAR1

AP50

pCDNA3

IFNAR1

AP50

IFNAR1 Before normalization

After normalization

WCE

C

Mock

Tyk2

Tyk2YFA

IFNAR1

AP50

AP50

Tyk2

WCE

IP: M2

IB: M2

IP: M2

IB: M2

IP: M2

IB: M2

IP: M2

IB: M2

IP: M2

IB: M2

IP: M2

IB: M2

IP: M2

IB: M2

IB: M2
Basal ubiquitin-independent internalization of interferon alpha receptor is prevented by Tyk2-mediated masking of a linear endocytic motif

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