Janus Kinases Affect Thrombopoietin Receptor Cell Surface Localization and Stability*

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The thrombopoietin receptor (TpoR) regulates hematopoietic stem cell renewal, megakaryocyte differentiation, and platelet formation. TpoR signals by activating Janus kinases JAK2 and Tyk2. Here we show that, in addition to signaling downstream from the activated TpoR, JAK2 and Tyk2 strongly promote cell surface localization and enhance total protein levels of the TpoR. This effect is caused by stabilization of the mature endoglycosidase H-resistant form of the receptor. Confocal microscopy indicates that TpoR colocalizes partially with recycling transferrin in Ba/F3 cells. The interaction with JAK2 or Tyk2 appears to protect the receptor from proteasome degradation. Sequences encompassing Box1 and Box2 regions of the receptor cytosolic domain and an intact JAK2 or Tyk2 FERM domain are required for these effects. We discuss the relevance of our results to the reported defects of TpoR processing in myeloproliferative diseases and to the mechanisms of Tpo signaling and clearance via the TpoR.

The thrombopoietin receptor (TpoR)1 is a member of the cytokine receptor superfamily that regulates hematopoietic stem cell renewal (1), megakaryocyte differentiation, and platelet formation (2, 3). Downstream signaling mediated by the TpoR is dependent on two cytoplasmic Janus tyrosine kinases, JAK2 and Tyk2 (4–8), with JAK2 being the main JAK required for TpoR effects (9, 10). Ligand binding triggers activation and phosphorylation of JAKs and of the cytoplasmic domain of the TpoR, providing docking sites for the Src homology 2 domains of many signaling proteins, such as the signal transducers and activators of transcription 1, 3, and 5 (STAT1, STAT3, and STAT5, respectively), Shc, SHIP, Grb2, SOS, Vav, Cbl, and phosphatidylinositol 3-kinase (8, 11–16).

Recently it has been found that JAK proteins may play important roles in regulating the cellular localization and traffic of their cognate receptors. In the case of the EpoR and oncostatin M receptor, expression of their cognate JAKs, JAK2 and JAK1, was found important for receptor cell surface localization, with enhanced endoplasmic reticulum to Golgi maturation. The overall cellular levels of these receptors were not changed by the presence or absence of JAKs (17, 18). Sequences encompassing the N-terminal domain JH7–JH6 regions of JAKs were required for these effects. Early on after the discovery of the JAK-STAT pathway, the IFNAR1 subunit of the type 1 IFN receptor complex was shown to require the expression of Tyk2 for stability at the cell surface (19). This effect was mediated by sequences in the JH7–JH6 region of Tyk2 (20). Tyk2 expression leads to enhanced protein levels of IFNAR1 by preventing endosomal targeting (21). JAK3 was found to promote cell surface localization of the common gamma (γc) subunit of the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptor complexes (22). Furthermore, overexpression of JAK1, but not of JAK2, Tyk2, or JAK3, leads to enhanced cell surface localization of IL-9Rα and IL-2Rβ, whereas JAK3 and not the other JAKs promotes enhanced cell surface localization of the γc.2 In these cases, it was the FERM domain of JAKs and not the kinase or pseudokinase domain that was required for mediating enhanced cell surface localization of receptors. Because the endogenous JAK1 was reported to be exclusively localized at the plasma membrane via interactions with cytokine receptors (23), it is now considered that receptor-JAK complexes function as one unit, equivalent with receptor tyrosine kinases. Furthermore, the plasma membrane localization of JAK3 depends on coexpressed γc (22). Taken together these data indicate that JAKs may fulfill important roles other than triggering downstream signaling by cytokine receptors.

The traffic of the TpoR to and from the cell surface is special among cytokine receptors for three reasons. First, TpoR was found to recycle to the membrane in hematopoietic cells after activation and withdrawal of ligand (24). Second, clearance of circulating Tpo may occur via binding, internalization of the ligand-receptor complex, and degradation of Tpo by platelets, which express high affinity receptors for Tpo (25–28). TpoR apparently is not recycled in platelets, but its recycling in myeloid progenitors and hematopoietic cell lines may be rele-

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1 The abbreviations used are: TpoR, thrombopoietin receptor; CIS, cytokine-inducible Src homology 2-containing protein; Endo-H, endoglycosidase H; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorter; γc, common gamma subunit; GFP, green fluorescent protein; HA, hemagglutinin; IFN, interferon; IL, interleukin; IRES, internal ribosome entry site; JAK, Janus kinase; Luc, luciferase; MAP, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; SRE, serum response element; STAT, signal transducers and activators of transcription; TK, thymidine kinase; Tyk, tyrosine kinase; EpoR, erythropoietin receptor.

2 Y. Royer, P. J. Courtoy, and S. N. Constantinescu, unpublished observations.
JAK2 and Tyk2 Enhance TpoR Cell Surface Levels and Stability

For Tpo functions in early hematopoiesis. Third and most interestingly, TpoR traffic, maturation, glycosylation, and stability were found to be altered in myeloproliferative diseases such as polycythemia vera, essential thrombocythemia, and idiopathic myelofibrosis (29, 30). Whether this defective maturation is linked to the pathogenesis of these diseases or is a sign of stress hematopoiesis (31) is not known, but all available evidence indicates that the study of TpoR traffic may reveal novel regulation mechanisms.

Thus, we investigated whether the two JAKs can affect TpoR traffic and metabolism. Here we show that JAK2 and Tyk2, but not JAK1 or JAK3, strongly promote cell surface localization of the TpoR by stimulating recycling and enhancing the protein stability of the mature, Golgi-processed form of the TpoR. Expression of JAK2 or Tyk2 did not change the internalization kinetics of the TpoR, whereas it promoted recycling. Under cycloheximide treatment, which blocks protein synthesis, inhibitors of proteasome degradation prolonged the half-life of the mature TpoR band in the absence of overexpressed JAK2 or Tyk2, suggesting that a fraction of the mature TpoR is normally degraded via the proteasome. Confocal microscopy studies suggest that JAK2 and Tyk2 prevent degradation of an intracellular pool of TpoR, which partially colocalizes with recycling transferrin. By site-directed mutagenesis, we show that an intact FERM domain of JAK2 or of Tyk2 is required for this effect and that receptor sequences encompassing Box1 and Box2 are likely to make the first contact with JAK FERM domains. Although a hydrophobic motif preceding Box1 is not required for JAK2-dependent traffic effects, this motif is crucial, as for the homologous EpoR, for switching on JAK kinase activity upon ligand binding to the TpoR extracellular domain. We discuss the significance of our data for the mechanisms of signaling, down-modulation, and recycling of the TpoR.

MATERIALS AND METHODS

cDNA Constructs—Receptors were tagged after the cleavage site of the signal sequence as described (32). The HA-tagged mouse TpoR and the HA-tagged human γc were subcloned into the pMX-IRES-GFP bicistronic retroviral vector upstream of the IRES as described previously (33). The human IL-9Rα and the human JAK3 were subcloned into the pREX-IRES-CD4 vector, and the murine JAK1 and the human Tyk2 were subcloned into the pREX-IRES-CD2 vector. Furthermore, we used the previously described HA-tagged EpoR, wild type JAK2, and kinase-inactive JAK2 cloned in the same vectors (17). cDNAs coding for the mutagenic TpoR inhibitors H7 (Roche Applied Science) were recovered by binding to protein G-agarose beads (Invitrogen). As described previously (40), the antibodies were then preincubated with antibodies against the HA tag (monoclonal HA.11). Immune complexes were recovered by binding to protein G-agarose beads (Invitrogen). Proteins bound to the beads were then eluted with 0.5% SDS and 1% β-mercaptoethanol and digested or not with Endo-H (New England Biolabs) for 1 h at 57 °C. Proteins were separated on 10% SDS-PAGE followed by immunoblotting with anti-HA antibody.

Ba/F3 cells stably transduced were incubated at 37 °C with 20 μg/ml cycloheximide for different periods of time to block protein synthesis. Control cells were kept nontreated to have 100% cell surface localization of the TpoR and γc at the same moment. Analysis of the surface pool of the TpoR or γc was performed by flow cytometry (HA.11) on the living cells. Both receptor bands were tagged with HA. Three independent experiments were performed. An aliquot of each cell line was taken at each time point to verify the receptor expression by Western blot. For measuring the contribution of proteasome or lysosome-mediated degradation of the TpoR, stably transduced Ba/F3 cells were incubated at 37 °C for 3–20 h with 20 μg/ml cycloheximide and with inhibitors of proteasome activity, MG132 (9 μM) or lactacystin (4 μM) (both from Calbiochem), or with 20 μg/ml cycloheximide and lysosome inhibitors chloroquine (200 μM) (42) or leupeptin (200 μM) (43, 44). Cells were then lysed and analyzed by Western blotting with antibodies against HA for the relative levels of the mature and immature TpoR.

Internalization Measurements—Ba/F3 cells expressing the HA-tagged TpoR or γc along with various JAKs were labeled for 2 h at 4 °C with 2 μg/ml 125I-anti-HA in the presence or absence of 200 μg/ml cold anti-HA. After three washes, the cells were incubated at 37 °C for different periods of time. Cell surface radioactivity was removed by acid wash for 1 h at 4 °C (250 mM sodium acetate, 150 mM NaCl, pH 0.5), and acid-resistant radioactivity was intracellular. The mean efficiency of the acid wash was ~75%. Viability of Ba/F3 cells in this acidic buffer was verified at room temperature for 2 h. Separation between the cells and the supernatant was performed by centrifugation through a cushion of sodium. Radioactivity was measured using a gamma counter. Counts that could not be stripped by the acid wash in the absence of internalization were subtracted from the overall counts. All measurements were done in triplicate, and three independent experiments were performed.

Confocal Microscopy—Confocal microscopy was performed as described previously (48) (Molecular Probes) and was acquired with a 60× oil objective. Confocal images were acquired with a Bio-Rad MRC-1024 confocal microscope.

Surface Expression of TpoR by FACS—Flow cytometry was used to measure TpoR expression by Ba/F3 cells. Cells were incubated at 37 °C for 2 h with 2 μg/ml 125I-anti-HA in the presence or absence of 200 μg/ml cold anti-HA. After three washes, the cells were incubated at 37 °C for different periods of time. Cell surface radioactivity was removed by acid wash for 1 h at 4 °C (250 mM sodium acetate, 150 mM NaCl, pH 0.5), and acid-resistant radioactivity was intracellular. The mean efficiency of the acid wash was ~75%. Viability of Ba/F3 cells in this acidic buffer was verified at room temperature for 2 h. Separation between the cells and the supernatant was performed by centrifugation through a cushion of sodium. Radioactivity was measured using a gamma counter. Counts that could not be stripped by the acid wash in the absence of internalization were subtracted from the overall counts. All measurements were done in triplicate, and three independent experiments were performed.

Monoclonal antibodies—The monoclonal HA.11 antibody was iodinated with Na125I to a specific activity of 15 Curie/g using IODO-GEN-precoated tubes (Pierce) following the recommendations of the manufacturer. 125I-Anti-HA binding was used to measure the cell surface localization of the TpoR. The cells were incubated for 2 h at 4 °C with 2 μg/ml 125I-anti-HA in the presence or absence of 200 μg/ml cold anti-HA and then separated from unbound antibodies by a centrifugation through a cushion of sucrose. Bound and unbound antibody fractions were measured using a gamma counter. All measurements were done in triplicate.

Immunoprecipitation, Endoglycosidase H (Endo-H) Digestion, and Immunoblotting—Ba/F3 cells were lysed in Nonidet P-40 buffer with sodium orthovanadate, sodium fluoride, phenylmethanesulfonyl fluoride, and Complete protease inhibitor mixture (Roche Applied Science), as described previously (40). The antibodies were then preincubated with antibodies against the HA tag (monoclonal HA.11). Immune complexes were recovered by binding to protein G-agarose beads (Invitrogen). Proteins bound to the beads were then eluted with 0.5% SDS and 1% β-mercaptoethanol and digested or not with Endo-H (New England Biolabs) for 1 h at 57 °C. Proteins were separated on 10% SDS-PAGE followed by immunoblotting with anti-HA antibody.

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were stained with monoclonal anti-HA followed by a goat anti-mouse IgG linked to Alexa Fluor 568 (Molecular Probes). The cells were treated with methanol/acetone at 20 °C to remove the GFP and mounted with ProLong Antifade (Molecular Probes). The complete procedure was performed at room temperature. For the LAMP1 staining, the cells were incubated with anti-HA followed by a goat anti-mouse IgG linked to Alexa Fluor 647 (Molecular Probes) and anti-mouse LAMP1 (1D4B, Developmental Studies Hybridoma Bank) followed by a goat anti-rat IgG linked to Alexa Fluor 568.

Image acquisitions were made on a Bio-Rad MRC-1024 confocal laser scanning imaging system associated with the Lasersharp 2000 (Bio-Rad) acquisition software. For the LAMP1 staining, similar experiments were performed with HeLa cells that were transiently transfected using ExGen500 with the cDNA coding for the HA-tagged TpoR or γ. The cells were incubated with rabbit polyclonal HA.11 followed by a goat anti-rabbit IgG linked to Alexa Fluor 568 and anti-human LAMP1 (HA3, Developmental Studies Hybridoma Bank) followed by a goat anti-mouse IgG linked to Alexa Fluor 647.

**Assay for Tpo-dependent Proliferation—Ba/F3 cells expressing wild type TpoR or mutated TpoR were washed three times in RPMI 1640 medium to remove IL-3 completely. Washed cells were plated at 100,000 cells/well in a 24-well plate with 1 ng/ml Tpo in RPMI supplemented with 10% fetal bovine serum. Proliferation was measured after 3, 5, and 7 days using a Coulter Counter Z1. All experiments were done in triplicate.**

**Dual Luciferase Assays—STAT1 and STAT3 transcriptional activation was assessed by measuring luciferase production in γ2A cells transfected with the pGR5-Luc, which contains five copies of a GRR sequence (32). MAP kinase activation was assessed with the pSRE-Luc construct (45). As an internal control, we used the pRL-TK vector (Promega) containing the Renilla luciferase gene under the control of the thymidine kinase promoter. γ2A cells were transfected with Lipofectamine 2000 (Invitrogen) for 8 h and then stimulated or not with 50 ng/ml Tpo. The cells were lysed 16 h after transfection, and luciferase assays were performed in triplicate using the dual luciferase reporter assay kit (Promega).**

**RESULTS**

**Effect of JAK Proteins on Cell Surface Levels of TpoR—**To study the effect of the different Janus kinases on the cell surface expression of the TpoR, we constructed four different Ba/F3 cell lines overexpressing JAK1, JAK2, JAK3, or Tyk2, as we have described previously for JAK2 (40). This was achieved by retroviral transduction using bicistronic retroviral vectors, which code for the gene of interest separated from a marker (GFP, CD4, or CD2) by an IRES (33). The cells were sorted for CD2 or CD4 expression. Western blots showed total protein level as well as a 1/5 dilution using anti-JAK1, anti-JAK2, anti-JAK3, or anti-Tyk2 antibodies. The cells were transduced with the TpoR and other signaling molecules (i.e. SH-PTP2) or mutated TpoR were washed three times in RPMI 1640 medium to remove IL-3 completely. Washed cells were plated at 100,000 cells/well in a 24-well plate with 1 ng/ml Tpo in RPMI supplemented with 10% fetal bovine serum. Proliferation was measured after 3, 5, and 7 days using a Coulter Counter Z1. All experiments were done in triplicate.

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**Figure 1. JAK2 and Tyk2 promote cell surface localization of the TpoR in Ba/F3 cells.** A. Ba/F3 cells were transduced with the four different wild type Janus kinases as well as with FERM domain mutants of JAK2 and Tyk2. Cells were sorted for CD2 or CD4 expression. Western blots showed total protein level as well as a 1/5 dilution using anti-JAK1, anti-JAK2, anti-JAK3, or anti-Tyk2 antibodies. B. TpoR was stably transduced in these different Ba/F3 cells and sorted for equal GFP levels. Cell surface localization was assessed by a monoclonal HA.11 antibody and FACS analysis. Values are normalized to the wild type receptor surface level in parental Ba/F3 cells (100%). C. TpoR or EpoR was stably transduced in IL-9R-Ba/F3 cells proliferating in the presence of IL-9 and overexpressing the wild type JAK2 or the kinase-inactive JAK2 K882D. FACS analysis using HA.11 antibody was performed for equivalent GFP expression. D. JAK1 Tyr107 and JAK3 Tyr102 are involved in the interaction with gp130 (JAK1) and γ (JAK3). These tyrosines are localized in a conserved sequence shared by all Jaks. Mutations of JAK2 Tyr114 and Tyk2 Tyr102-Phe103 into alanines destroyed interactions with the TpoR (B).
phenylalanine residue to alanine (Tyr → Ala/Phe → Ala for Tyk2) (Fig. 1D). We chose these mutations because it has been shown that a tyrosine at this position is necessary for the interaction between JAK1 and gp130 (48) and between JAK3 and Tyk2. C, TpoR Ba/F3 cells overexpressing each JAK were lysed in Laemmli buffer. On a 10% Tris-glycine SDS-polyacrylamide gel, the TpoR migrates into two bands at 95 and 80 kDa. The intensity of the 95 kDa band was increased by JAK2 and Tyk2. D, the TpoR was immunoprecipitated from stably transduced Ba/F3 cells using monoclonal HA.11 antibody and protein G-agarose. The eluted receptor was then digested with Endo-H. Only the 80 kDa band was sensitive to digestion.

Detection of the Enhancement by JAK2 and Tyk2 of Cell Surface TpoR Levels by $^{125}$I-labeled Anti-HA Antibody and by Confocal Microscopy—To prove that the enhancement of TpoR cell surface localization was not an artifact of the multistep labeling with a phycoerythrin-labeled secondary antibody or the result of a redistribution effect, we performed binding assays with the same monoclonal HA.11 antibody directly iodinated with $^{125}$I. Again, the TpoR cell surface localization was increased more than 3-fold in the presence of JAK2 and Tyk2, confirming the FACS results (Fig. 2A).

Immunofluorescence staining on saponin-permeabilized Ba/F3 cells was performed to verify the distribution of the TpoR in the presence or absence of overexpressed JAK2 and Tyk2. By confocal imaging, the TpoR distribution was comparable between the cells, with essentially an image of a rim on the
plasma membrane (Fig. 2B). The staining intensity was largely increased in the presence of overexpressed JAK2 or Tyk2.

In summary, JAK2 and Tyk2 strongly promoted increased surface levels of the TpoR. These observations initially documented by FACS were confirmed by radioactive labeling and by confocal microscopy. The effects of JAK2 and Tyk2 required an intact N-terminal FERM domain. Although JAK1 weakly increased TpoR cell surface levels also, JAK3 had no effect. Because TpoR is known to activate primarily JAK2 and Tyk2, and not JAK1 or JAK3, these results suggest that cognate JAKs affect receptor cellular localization as a function of their ability to interact and be utilized by the receptor. Our results suggest that for receptors that may utilize several JAKs, an additional layer of regulation may be represented by different extents to which those JAKs affect receptor traffic.

Effect of Janus Kinases on TpoR Protein Levels—The next question was whether the TpoR cell surface localization correlated with the total receptor protein levels. TpoR was immunoprecipitated from stably transduced Ba/F3 cells overexpressing each of the four JAKs or control mock-infected cells. By Western blot and SDS-PAGE on 10% Tris-glycine gels, the receptor migrated as two different bands of 95 and 80 kDa (Fig. 2C). The intensity of the 95-kDa complex was increased strongly in the presence of JAK2 or Tyk2. Again, JAK1 had a marginal effect, whereas JAK3 did not change the basal receptor expression levels. By densitometry, JAK2 and Tyk2 enhanced by 3.5–4-fold the levels of the 95 kDa band, which was in total agreement with the results obtained by FACS and by binding assays. The intensity of the 80 kDa band did not vary among the different samples. To test whether the 95 kDa band was in fact the TpoR mature Golgi-processed form that includes the cell surface pool, we performed an Endo-H digestion on the immunoprecipitated receptor (Fig. 2D). The 80 kDa band was sensitive to Endo-H digestion, as shown by its shift in size after digestion. This band thus corresponds to the immature form of the receptor. The 95 kDa band was resistant to Endo-H digestion, demonstrating that this band corresponds to the Golgi-processed TpoR. This band reflects therefore both the cell surface and mature intracellular pool of TpoR. Furthermore, only this upper TpoR band can become tyrosine phosphorylated after the addition of ligand (not shown). Thus, the pool of mature TpoR is likely to contain the cell surface TpoR, the intracellular (Golgi) pool as well as the recycling TpoR.

For the EpoR and the oncostatin M receptor, expression of the cognate Janus kinases promotes maturation to the Endo-H-resistant band, but the total receptor protein levels remain constant (17, 18), whereas expression of Tyk2 promotes cell surface levels of IFNAR1 and is required for protein stability at the cell surface (19, 21). In the case of the TpoR, the intensity of the 80 kDa band was constant (and not decreased as for the homologous EpoR Endo-H-sensitive band), whereas the Endo-H-resistant band was increased strongly by JAK2 or Tyk2 overexpression. These data suggest that JAK2 and Tyk2 may reduce TpoR internalization, increase the recycling rate, or stabilize the mature intracellular pool.

JAK2 and Tyk2 Promote Stabilization of a Mature Intracellular Pool of TpoR—To assess the internalization rate of the TpoR, the monoclonal HA.11 antibody was first iodinated with 125I, then used to label the TpoR on the cell surface and to follow TpoR internalization. Cells were washed and allowed to warm up to 37 °C for different periods of time. Internalization occurred, and the antibodies remaining bound to the cell surface were removed by a strong acid wash (pH 0.5) at 4 °C for 1 h. The average results from three different experiments showed that TpoR internalization was not blocked or reduced in the presence of JAK2 or Tyk2 (Fig. 3A).

The half-lives of the two TpoR forms (immature and mature) were then tested by incubating the cells at 37 °C in the presence of cycloheximide to block new protein synthesis. At the indicated times (hours), we measured the disappearance of the TpoR from the cell surface of living cells by FACS analysis. Values were normalized to the signal obtained in control samples assayed in parallel at each time point. Fig. 3B shows that JAK2 or Tyk2 increased the TpoR abundance on the cell surface after 15 h of cycloheximide treatment. Even for the TpoR in parental Ba/F3 cells, the stability at the cell surface was extremely long compared with that of a known internalized and degraded cytokine receptor as the γc (Fig. 3B). That in the presence of JAK2/Tyk2 the cell surface TpoR levels increased after 10 h cycloheximide was surprising, as we expected a delayed decrease, but the result was reproducible. Cycloheximide treatment may deplete cells of a short lived protein that contributes in a JAK-independent manner to receptor down-modulation. Thus, in the presence of both JAK2/Tyk2 and cycloheximide, the cell surface levels of TpoR would increase after a certain interval of cycloheximide treatment.

To dissect the consequences of cycloheximide treatment on the TpoR total protein pool, Western blot experiments were performed on Ba/F3 cell lysates treated for the same periods of time (Fig. 3C). In the presence of JAK2 or Tyk2, the stability of the 95-kDa complex was prolonged for more than 28 h compared with parental Ba/F3 cells expressing the TpoR. In contrast, the 80 kDa band had nearly completely disappeared after 3 h of treatment in all cases, showing that the two forms of the TpoR exhibit different half-lives. Interestingly, at late time points, such as 20–25 h, the levels detected at the cell surface were more stable than the total pool of mature TpoR, which contains the cell surface but also the Golgi and recycling pool. We do not have definitive proof, but the most likely explanation would be that that the TpoR recycles at higher rates at these late times. In summary, JAK2 and Tyk2 did not affect the TpoR internalization rate but strongly increased the stability of the cell surface TpoR.

Fig. 3D shows that in the presence of cycloheximide, the decrease in the levels of mature TpoR at 20 h (similar to Fig. 3C) is significantly prevented by two different proteasome inhibitors, MG132 and lactacystin (left panel). In contrast, two lysosome inhibitors, chloroquine and leupeptin, failed to increase the levels of the mature TpoR band after 20 h of cycloheximide. Even at high concentrations (200 μM) compared with the usual 50–100 μM concentrations, the lysosome inhibitors failed to enhance the stability of the TpoR. For the proteasome inhibitors, the stabilization effects were noticed starting with 3 h of treatment. In the presence of JAK2, which itself results in stabilization of the mature TpoR band, the proteasome inhibitors exert a modest effect, suggesting that in fact the presence of JAK2 may protect the TpoR from proteasome degradation, and this in turn may result in enhanced recycling. With respect to lysosomes, it is still possible that they play a role in TpoR degradation, but under cycloheximide treatment, we were not able to observe any stabilization of the mature form of the receptor in the presence of lysosome inhibitors.

To study traffic of the TpoR to and from the cell surface further, confocal imaging experiments were performed on Ba/F3 cells permeabilized with saponin and fixed with paraformaldehyde. Adherence was achieved by spinning the cells on poly-L-lysine-coated coverslips. First, transferrin was used as a marker for rapid recycling to the cell surface through the early endosome compartment. Living cells were first incubated at 37 °C for 25 min with transferrin to allow internalization and distribution in recycling endosomes and then fixed.
prior to receptor labeling. Colocalization was observed between transferrin and TpoR essentially in small vesicles just under the cell membrane (Fig. 4A). Ba/F3 cells expressing HA-tagged γc were used as a negative control.

Second, LAMP1 was used as a lysosomal compartment marker. Colocalization was not detected between LAMP1 and TpoR (Fig. 4B), whereas colocalization between γc and LAMP1 could be detected (Fig. 4B), as described previously (22). As expected, transferrin staining did not colocalize with the LAMP1 signal in Ba/F3 cells (not shown). However, because it was difficult to establish clearly a lack of colocalization between LAMP1 and TpoR in Ba/F3 cells, which have a small cytoplasm, we also transfected HeLa cells with vectors coding for the HA-tagged forms of the TpoR or γc and stained for HA and LAMP1. We chose to use HeLa Tet-Off cells because they were highly transfectable by ExGen500 and because they can be also used for tetracycline-dependent gene expression. These cells were adherent and had well spread cytoplasmic structures.

Their morphology is a bit different from that of parental HeLa cells, and upon transfection with ExGen500 they adopted a rounded appearance atypical for adherent cells. However, this cationic polymer transfection agent is nontoxic and allows them to grow at the normal, fast rate. The resolution of cytoplasmic structures in the transfected cells was very good. As shown in Fig. 4C, TpoR did not colocalize with LAMP1, irrespective or not of JAK2 overexpression. In contrast, γc colocalized with LAMP1, as described previously (22).

Taken together these results show that in the absence of ligand, the TpoR and not the γc is recycled to the cell surface through the same pathway as transferrin. Only the γc and not the TpoR colocalized with LAMP1. Overexpression of JAK2 and Tyk2 enhanced the cell surface levels of TpoR (Fig. 2B) by protecting against proteasome degradation. It was highly reproducible that the stability of the TpoR at the cell surface was higher than the stability of the pool of mature TpoR in the cell (Fig. 3, B and C).

**Fig. 3. Effect of JAK2 and Tyk2 on internalization and recycling of wild type TpoR.** A, after 125I-anti-HA labeling of TpoR at 4 °C, internalization was allowed to take place at 37 °C for the indicated times (minutes). Anti-HA antibodies that remained bound to the cell surface were stripped by an acid wash. The efficiency of the acid wash was ~75%. Cell-associated counts were measured using a gamma counter. Counts that could not be stripped by the acid wash in the absence of internalization were subtracted from the overall counts. B, TpoR surface levels were assessed by FACS analysis (anti-HA labeling) after incubation at 37 °C in the presence of 20 μg/ml cycloheximide in IL-3-containing medium for the indicated times (hours). Values are normalized to the signal of nontreated cells incubated in parallel. HA-γc Ba/F3 cells were used as controls. C, for each indicated time, cycloheximide-treated cells were lysed in Laemmli buffer and subjected to SDS-PAGE and Western blot analysis with anti-HA antibodies. Anti-β-actin antibodies were used as loading controls. D, cells were treated for 20 h with 20 μg/ml cycloheximide and proteasome inhibitors MG132 (10 μM) or lactacystin (10 μM) or with 20 μg/ml cycloheximide and lysosome inhibitors chloroquine (200 μM) or leupeptin (200 μM). The detection of the levels of TpoR was performed by Western blot as in C.
Determination of the TpoR Cytosolic Sequences Required for the JAK-dependent Increase in Cell Surface Expression and Protein Levels—TpoR is able to bind and activate both JAK2 and Tyk2 (4–8), but it is not clear whether the sequence requirement on the receptor is the same for both of them. To determine exactly which receptor sequences are important for the effects of JAK proteins on receptor protein and cell surface levels, we mutagenized the juxtamembrane sequence of the TpoR. Thus, groups of two or three amino acids were replaced by alanines (Fig. 5A). Mutations covered the 11 residues preceding Box1. Within this region, there are three hydrophobic residues (H9278–X–X–X–H9278) that were suggested to adopt in gp130 and EpoR an α-helically oriented hydrophobic motif (39, 50). In the TpoR the homologous residues are Leu515 and Leu519–Trp520. We also mutagenized the Box1 region, the Box2 region, and a tyrosine located between Box1 and Box2 which aligns with a conserved tryptophan residue, which in many cytokine receptors is crucial for signaling (51). We reasoned that this aromatic Tyr533 residue in the TpoR would have the same function as, for example Trp282 in the EpoR region between Box1 and Box2.

First, we determined whether these mutant receptors are functional. Tpo-induced proliferation was assayed 3, 5, and 7 days after switching the cells to 1 ng/ml Tpo (Fig. 5B). The proliferation was totally abolished when the two last residues of the hydrophobic motif preceding Box1 (residues) Leu519–Trp520 were mutated, consistent with results obtained for the equivalent mutations in EpoR. However, mutation of Leu515 to Ala had no effect. This is in opposition with the EpoR where the equivalent residue Leu253 was required for Epo-induced proliferation (39). This difference could be the result of a different orientation/structure of the TpoR and EpoR juxtamembrane domains. In fact, this is the case because of a five-amino acid insertion at the end of the transmembrane domain of the TpoR.3 However, mutation or deletion of this transmembrane motif did not change in any way the effects of JAK2 on increasing the cell surface levels of TpoR.

Mutations of Box1, Box2, or of the Gln532–Tyr533–Leu534 sequence between Box1 and Box2 abrogated completely the proliferation. To determine the sequence requirements for the JAKs to bind to the TpoR, cell surface levels of the TpoR mutants were assessed by FACS in parental Ba/F3 cells and in Ba/F3-JAK2 or Ba/F3-Tyk2 cells (Fig. 5C). Values were normalized to those of the wild type TpoR surface level in parental Ba/F3 cells (considered as 100%). Mutation of the hydrophobic motif Leu515–Trp520 decreased cell surface levels in control parental Ba/F3 cells, whereas cell surface levels of mutants Arg514 → Ala514, Arg513 → Ala513, Leu515 → Ala, Box1Dead, and Box2Dead were slightly reduced. However, our major question was to test whether JAK2 or Tyk2 could still increase the cell surface levels of these mutants.

3 J. Staerk, C. Lacout, G. Reubins, S. O. Smith, W. Vainchenker, and S. N. Constantinescu, manuscript in preparation.

488 was adsorbed, internalized, and recycled by the cells for 25 min at 37 °C and then washed. After paraformaldehyde fixation, staining for the receptor (monoclonal antibody HA.11) was achieved. HA-Ba/F3 cells were used as negative controls. B, double immunostaining was realized on the same Ba/F3 permeabilized cells with an anti-mouse LAMP1 antibody and the monoclonal anti-HA antibody for the receptor. By confocal imaging, no colocalization was observed between TpoR and LAMP1. HA-γc Ba/F3 cells were used as positive controls. C, HeLa cells were transiently transfected with cDNAs coding for the HA-tagged TpoR or γc and were immunostained after permeabilization with an anti-human LAMP1 antibody and a polyclonal rabbit HA.11 antibody for the receptor. Confocal imaging showed no colocalization between TpoR and LAMP1, whereas γc colocalized with LAMP1.
Fig. 5. Mutations of the TpoR hydrophobic motif and Box1-Box2 region show different requirements for proliferation and Janus kinase binding.

A, sequence of the juxtamembrane region showing the different alanine mutations corresponding to each mutated TpoR. B, cells stably expressing TpoR mutants were washed three times in RPMI 1640 medium, then plated in 1 ng/ml Tpo. Cells were counted after 7 days using a Coulter Counter Z1. Values are the means ± S.D. of triplicates. C, cell surface localization of the wild type and mutated TpoR in parental and in Ba/F3 cells overexpressing JAK2 or Tyk2. FACS analysis was performed with anti-HA antibodies. Values are normalized to the wild type receptor surface level in parental Ba/F3 cells (100%). D, sequence alignment of the TpoR and IFNAR1 juxtamembrane domains from mouse and human species.

Fig. 5C shows that JAK2 was inefficient to promote TpoR cell surface levels when Box1 residues were mutated and was less efficient when the Gln532-Tyr533-Leu534 sequence between Box1 and Box2 was mutated. Mutation of the hydrophobic motif, especially of the two hydrophobic residues preceding Box1, Leu519-Trp520, did not modify the effect of JAK2. In contrast, the ability of Tyk2 to promote TpoR traffic was decreased by these mutations (Leu519→Ala/Trp520→Ala) and, as expected, by mutating the Box1 sequence. Overall, for JAK2 it is clear that Box1 and the sequences between Box1 and Box2 are required for traffic effects, whereas for Tyk2 a more diffuse region, which includes the hydrophobic motif upstream from Box1, and Box1 may be required. Interestingly, the juxtamembrane domain of the IFNAR1 subunit of the IFNα/β receptor shows similarities to the juxtamembrane domain of the TpoR (Fig. 5D). For IFNAR1, binding to Tyk2 also occurs via the proximal cytosolic 46 amino acid residues (52). Type II cytokine receptors, which include IFN receptors, do not contain precise Box1 and Box2 sequences, although homology was detected between the juxtamembrane sequences of type I and II cytokine receptors (53). Thus, it is possible that Tyk2 binding to the TpoR juxtamembrane domain may occur in a way that is similar to binding of Tyk2 to IFNAR1.

JAK2 and Tyk2 Affect TpoR Traffic Similarly but Function to Different Extents in TpoR Signaling—JAK2 and Tyk2 promote the stabilization of mature TpoR and enhance cell surface receptor levels to apparently similar extents. However, JAK2 is the main JAK utilized by TpoR (9, 10), and it is unclear whether Tyk2 plays a role in TpoR signaling.

First, we tested whether Tyk2 can, in the absence of JAK2, mediate TpoR signaling represented by STAT and MAP kinase activation in the human fibrosarcoma cell line γ2A, which is deficient in JAK2 (35) and in STAT5 (54). This was accomplished by dual luciferase experiments with the pGRR5 luciferase reporter constructs controlled by a promoter containing five GRR (IFN-responsive region of the high affinity receptor for IgG1) binding sites for STATs (32), detecting in γ2A cells STAT1 and STAT3 activation or by a promoter containing a serum response element for the MEK/ERK MAP kinase pathway, pSRE (45), as described previously (40). After 16 h of Tpo...
stimulation, expression in these deficient cells of JAK2 was able to induce a strong luciferase transcription for both the STATs reporter (Fig. 6A) and for the MAP kinases reporter (not shown). Tyk2 had a much smaller effect but was able to induce transcription in the absence of JAK2 (Fig. 6A). JAK1 and JAK3 had no significant effects. By increasing the amount of transfa...
fected Tyk2 cDNA in these cells, we could detect an increase in Tpo-dependent signaling in the absence of JAK2 (Fig. 6B), but it was clear that the ability of Tyk2 to mediate TpoR signaling is much weaker than the one of JAK2.

We also tested whether coexpressing both JAKs, JAK2 and Tyk2, leads to a synergic transcriptional effect. We did not detect a significant increase in signaling by JAK2 when Tyk2 was overexpressed (Fig. 6B). In Ba/F3 cells overexpressing JAK2 or Tyk2 and the TpoR, phosphorylation of STAT1, 3, and 5 by Western blot was nearly identical between the two cell types after 15 min of Tpo stimulation (Fig. 6C). Interestingly, the activation of STATs was prolonged for more than 3 h in the presence of JAK2. Also, the levels of CIS were increased at late time points (2–3 h) mainly in JAK2-overexpressing cells, demonstrating a more sustained activation of the signaling pathway in the presence of JAK2.

It remains to be determined why Tyk2 is much less effective in mediating TpoR signaling and whether its equivalent effects on traffic and on the initial signaling events induced by Tpo are relevant for megakaryocyte and myeloid differentiation or for the pathologic response induced by excessive Tpo levels in certain thrombocytopenia cases.

DISCUSSION

Our main observation is that JAK2 and Tyk2, the two Janus kinases reported to function in signal transduction of the TpoR (4–10), also profoundly affect the levels of the receptor on the cell surface. The interaction between JAK2 or Tyk2 and the TpoR enhance the stability of the mature Endo-H-resistant form of the receptor. Although JAK2 and Tyk2 appear equivalent in their ability to promote TpoR stability, JAK2 was much stronger in transducing ligand-dependent signaling than Tyk2.

Our data provide support to the emerging notion that JAKs are integral parts of cytokine receptor complexes, with important effects on receptor availability and fate. A chaperone role has been suggested for JAKs with respect to several cytokine receptors (17, 18), a role that has been revisited for γc, where JAK3 promotes receptor cell surface localization but is not absolutely required for it (22). For the IFNAR1 subunit of the type I interferon receptor, it has been shown that expression of its cognate JAK, Tyk2, prevents internalization in endosomes and further degradation (21), thus Tyk2 enhanced the total levels of IFNAR1 protein expression (19). TpoR appears to be more similar to IFNAR1 where JAKs strongly increase total receptor protein levels than to EpoR, oncostatin M receptor, or the γc. However, TpoR is much more efficient than IFNAR1 in traveling to the cell surface and has the ability to recycle. Taken together these data suggest that JAKs are integral parts of receptors and that their binding to receptor cytosolic domains will significantly affect receptor fate, which can be receptor-specific.

We introduced mutations in the FERM domains of JAK2 and Tyk2 and found that an intact FERM domain is required for the effects of JAKs on TpoR stability and cell surface expression. This result is consistent with the role of FERM domains in JAK-promoted traffic of other cytokine receptors (17–20). However, the kinase-inactive JAK2, which promotes traffic of EpoR to the cell surface to the same or a higher extent than the wild type JAK2 (17), is less able to increase TpoR cell surface levels compared with the wild type JAK2. This is unusual because for the EpoR, JAK2 activity is required to target internalized receptors to lysosomes for degradation (47). These results suggest that the JAK2 domain may have an additional role in promoting TpoR stability and that the recycling function of TpoR (24), which is not present for other receptors, may require different interactions with JAK2. Our results showing that proteasome and not lysosome inhibitors enhance the stability of the mature form of the TpoR after 20 h of cycloheximide treatment (Fig. 3D) suggest that a fraction of the cell surface TpoR is degraded via the proteasome. The combined action of JAK2 and proteasome inhibitors was modest compared with their individual effects, suggesting that JAK2 binding to the TpoR protects against proteasome degradation. Current experiments are examining the possibilities that TpoR may couple to the ubiquitinylation machinery via SOCS/CIS proteins or directly via ubiquitinylation of cytoplasmic lysines. Because the TpoR recycles (24), a higher stability promoted by JAK2 is likely to enhance the total amount of TpoR that recycles.

We have investigated the TpoR sequence requirements for the JAK-dependent effects on traffic. For JAK2, TpoR sequences located in Box1 and between Box1 and Box2 were required. The residues located before Box1 could be mutated to alanine without affecting JAK2-dependent traffic. When receptor function was assayed, we could see that mutations in Box1 and in the region between Box1 and Box2 decreased or abolished signaling. Also, mutation of the two hydrophobic residues Leu519-Trp520 preceding Box1 affected function.

Such a separation between juxtamembrane residues that are involved in traffic/binding and in switching on JAK2 activity after receptor activation has been described for the EpoR (17). Particularly, the hydrophobic residues preceding Box1 (Leu254, Ile257, Trp258) were among such switch residues (17, 39). In the case of the TpoR, only the last two hydrophobic residues, Leu519 and Trp520 (which align with EpoR Ile257 and Trp258), appear to be important for function, in contrast to EpoR where all three residues are important. Unexpectedly, mutation of these two switch residues, Leu519 and Trp520, to Ala, although it did not affect JAK2-dependent traffic, decreased traffic induced by Tyk2. In Fig. 5B, it becomes apparent that the sequences in TpoR necessary for Tyk2 effects are broader and start earlier than those necessary for JAK2 binding. Our assay for receptor-JAK binding is quite sensitive, as shown by the read-out by FACS of an increased receptor level on the cell surface. Thus, binding of JAK2 and Tyk2 to the TpoR juxtamembrane domain may involve different residues, and this is interesting because TpoR appears to be close in sequence and behavior with IFNAR1, a type II cytokine receptor.

Expression of JAK2 and Tyk2 leads to similar increases in cell surface receptor levels and protein levels, and these increases involved the same Endo-H-resistant population of receptors. However, when we tested the ability of each JAK to transduce a signal, we found that Tyk2 was much weaker than JAK2. Although initial signaling events were triggered by Tyk2 with similar efficiency, only JAK2 led to sustained signaling, which is required for the biologic effects of TpoR (55). Thus, JAK2 and Tyk2 may induce similar stabilization of mature receptor, but their signal transduction abilities differ. Although the Tyk2 knock-out mouse has not been reported to exhibit defects in TpoR signaling (e.g. megakaryocyte formation or stem cell renewal) (56), it is possible that the relative expression levels of JAK2 and Tyk2 may be relevant for differentiation. Furthermore, in pathologic situations where very high levels of Tpo are produced, it is conceivable that activation of both JAK2 and Tyk2 will take place and that excessive signaling via these two kinases may occur. Thus, it will be important in the future to determine whether signaling via multiple JAKs is just a redundancy or whether it may have precise roles. This problem is shared with other cytokine receptors, such as those belonging to the gp130 class of receptors, which are also known to activate several JAKs (57).

The effects of JAK2 and Tyk2 on stabilizing the mature form of the TpoR and promoting higher cell surface expression may be relevant for ligand clearance by myeloid progenitors and for
the pathogeny of myeloproliferative diseases. Interestingly, in polycythemia vera and idiopathic myelofibrosis, defects in Tyk2, or an associated protein may be involved in this decrease of the mature Tyk2 in myeloproliferative diseases. Indeed, we and others have just observed that most polycythemia vera patients harbor a unique mutation in the pseudo-JAK2, Tyk2, or an associated protein may be involved in this decrease of the mature Tyk2 in myeloproliferative diseases.

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