The Membrane-proximal Region of the Thrombopoietin Receptor Confers Its High Surface Expression by JAK2-dependent and -independent Mechanisms*

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Janus tyrosine kinase 2 (JAK2) is essential for signaling by the thrombopoietin (TpoR) and erythropoietin (EpoR) receptors. In the absence of JAK2 most EpoR molecules are retained in the endoplasmic reticulum in an Endo H-sensitive form. In contrast, we show that in the absence of JAK2 a large fraction of the TpoR is processed to the mature Endo H-resistant form and reaches the cell surface. By studying chimeras of the TpoR and EpoR we show that high surface expression of the TpoR is entirely conferred by the membrane-proximal region of the intracellular domain that includes the juxtamembrane, Box 1, and Box 2 regions. The TpoR intracellular domain shows similar effects on receptor endocytosis rate as that of the EpoR, but does stabilize the mature receptor isoform from degradation. Co-expression of JAK2 further stabilizes mature TpoR and thus further increases its surface expression. This JAK2 effect depends on the Box 1 region, the only JAK2 interacting site in the TpoR. By contrast, EpoR requires Box 1 as well as the flanking 20 residues on the C-terminal side for JAK2 interaction and JAK2-dependent surface expression. Our study suggests that whereas cell surface expression of type I cytokine receptors requires their cognate JAKs, the mechanisms governing receptor-JAK interactions differ among receptors interacting with the same JAK protein.

Most cytokine receptors lack intrinsic catalytic activities and rely on the Janus tyrosine kinase (JAK) family for signal transduction. A common theme of signal transduction by these receptors involves ligand-induced changes in receptor oligomerization, which leads to the activation of the intracellular JAKs. This results in the phosphorylation of tyrosine residues in the cytoplasmic domain of the receptors, thereby providing a platform for the recruitment and activation of multiple signaling pathways through SH2 domain-mediated interactions (1). The type I cytokine receptors include the prolactin receptor (PrlR), crucial for mammalian reproduction (2), the erythropoietin receptor (EpoR), essential for erythropoiesis (3), and the thrombopoietin receptor (TpoR), or mpl, important for thrombopoiesis (4, 5).

The TpoR, EpoR, and PrlR all form homodimers, associate with JAK2, and rely on JAK2 for signaling (6–8). Upon ligand stimulation, the membrane-proximal region(s) of these receptors activates JAK2 in the cytosol (8–11). The membrane-proximal region of the EpoR, lacking all of the intracellular tyrosines, is sufficient for the EpoR to generate functional erythropoiesis in knock-in mouse models (12). A truncated TpoR with just the membrane-proximal region in its cytosolic domain generated normal platelet count when replacing the wild-type TpoR locus in mice (13). Therefore, this region of the cytokine receptors represents a minimal functional core for their signal transduction. This core region extends from the juxtamembrane region through Box 2 and includes Box 1 and Box 2, the only two regions in cytokine receptor cytoplasmic domains that share limited sequence homology (Fig. 1) (14). Box 1, represented by “PXXXP,” is crucial for cytokine receptors to bind and activate their cognate JAKs. Box 2, which is loosely defined as a motif with acidic and hydrophobic residues, is required for maximal cell growth in response to cytokines (15, 16).

We previously found that in the absence of JAK2 a large fraction of the EpoR is retained in the endoplasmic reticulum (ER). JAK2 facilitates EpoR exit of the ER and promotes EpoR surface expression (8). We also showed that Box 1 and about 20 residues C-terminal to it are essential for JAK2-binding and JAK2-dependent EpoR surface expression. Furthermore, we identified a juxtamembrane hydrophobic motif in the EpoR that is essential for JAK2 activation upon Epo stimulation (17). This motif is characterized by $\Phi_1$XXX$\Phi_2$, where $\Phi$ represents a hydrophobic residue and X represents any residue. Both the sequence and the conformation of this motif are crucial for EpoR signal transduction (17).

Recently, Royer et al. (18) reported that JAK2 and TYK2 stabilize the mature form of the TpoR to promote its cell surface expression, possibly by protecting it from proteasomal degradation. They also showed that some residues encompassing Box 1 and Box 2 regions of the TpoR are important for TpoR...
mitogenic activity and for JAK2 and TYK2 to increase cell surface TpoR (18).

In this work we discovered that in the absence of JAK2 a much larger fraction of the TpoR than the EpoR or PrlR is on the cell surface. We showed that the cytosolic membrane-proximal region of the TpoR stabilizes the mature receptor isoform from degradation, and is both necessary and sufficient for its high level surface expression in the absence of JAK2. We also confirmed results shown by Royer et al. (18) that JAK2 promotes TpoR surface expression by further stabilizing the mature receptor isoform. Additionally, we performed an extended detailed mapping of the entire membrane-proximal region of the TpoR cytoplasmic domain and identified specific residues in the TpoR that are important for its JAK2-independent high level surface expression, and specific residues essential for JAK2 interaction, JAK2 activation, and mitogenic activities. The TpoR is the primary regulator for megakaryocyte and platelet development, and is important for hematopoietic stem cell self-renewal (4, 5, 19–21). Our findings on the mechanism by which the TpoR confers its high surface expression and signaling may shed light on how it controls hematopoietic stem and progenitor cell survival, proliferation, and differentiation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—cDNAs encoding HA-tagged chimera between the EpoR and the TpoR, and between the EpoR and the PrlR were generated by PCR. Alanine scanning mutants of EET were generated using the QuikChange site-directed mutagenesis kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.).

**Generation of Cell Lines**—Ba/F3 cell lines stably expressing HA-tagged EpoR/TpoR chimera—cDNAs encoding HA-tagged chimera between the EpoR and the TpoR, and between the EpoR and the PrlR were generated by PCR. Alanine scanning mutants of EET were generated using the QuikChange site-directed mutagenesis kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.). All constructs were subcloned into the pMX-internal ribosome entry site-GFP bicistronic retroviral expression kit (Stratagene, Inc.).

**Surface Expression of HA-tagged EpoR/TpoR Chimera**—Surface expression of all the constructs in the γ2A cells was measured as described previously (8), except that allophycocyanin (APC)-conjugated anti-mouse IgG secondary antibody was used (BD Pharmingen). Cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences). GFP positive cells, indicating the expression of our constructs, were gated for analysis of APC fluorescence. Median fluorescence intensities of APC were used for the quantification of receptor surface expression. For each sample, half a million cells were collected.

**Glycosidase Digestion of the Receptors**—γ2A cells stably expressing HA-tagged EpoR or EET with JAK2 or vector alone were lysed in lysis buffer (150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris, pH 7.4) with protease inhibitors at 4 °C. Supernatants from these lysates were subjected to digestions with either Endo H (New England Biolabs, 500 units) alone, or with PNGase F (New England Biolabs, 50 units) and neuraminidase (New England Biolabs, 50 units) at 37 °C for 16 h. For digestions with PNGase F and neuraminidase, Nonidet P-40 was added to the final concentration of 1%. The digested products were then separated on SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-HA antibody.

**Metabolic Labeling and Pulse-Chase Analysis**—γ2A cells stably expressing HA-tagged EET with JAK2 or vector alone were washed once in methionine and cysteine-free medium, and placed in methionine and cysteine-free medium containing 10% dialyzed fetal bovine serum for 40 min at 37 °C. 0.5 mCi of [35S]methionine and cysteine mixture was then added, and the culture was incubated for a further 60 min (pulse). The cells were washed once with chase medium, and 10 ml of chase medium (Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum, 100 μg/ml methionine, and 500 μg/ml cysteine) was added. After incubation at 37 °C for the appropriate time (chase), the cells were placed at 0–4 °C, washed once in phosphate-buffered saline (PBS), and lysed in 1% Nonidet P-40 lysis buffer with 0.5% sodium deoxycholate and protease inhibitors. The lysates were then precipitated with HA affinity matrix (Roche). Proteins associated with the matrix after extensive washing were eluted with 0.5% SDS and 1% β-mercaptoethanol and digested with glycosidases. The samples were then run on SDS-PAGE and the gels were dried and examined by autoradiography or on a phosphorimager.

**Endocytosis Assay**—Recombinant human Epo was labeled with iodine 125 (125I-Epo) as described (22). γ2A cells stably expressing HA-tagged EpoR or EET were harvested from plates with PBS (pH 7.3) with 5 mM EDTA, washed and suspended in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics, then preincubated for 10 min at 37 °C.
Membrane-proximal Region of the TpoR

To begin the endocytosis assay an excess of $^{125}$I-Epo (0.5 nM) was added to the medium and at selected time points samples were removed into pre-chilled tubes in an ice-water bath, where endocytosis is stopped due to the low temperature. Cells were washed with medium at 4°C to remove free $^{125}$I-Epo, then surface bound and internal $^{125}$I-Epo were separated by acid stripping the cells as described (22). Non-specific radioactivity in internal and surface-bound fractions was measured in samples that included 100-fold excess unlabeled Epo as competitor. Specific $^{125}$I-Epo was determined as the difference between total and non-specific radioactivity. The endocytic rate constant, $k_{ew}$, was determined by plotting internal Epo versus the integral of surface-bound Epo, where the slope of the straight line fitted through these data points is equal to $k_{ew}$ (23–25). The cumulative integral of the average of specific surface-bound Epo with respect to time was computed by the trapezoidal method, using MATLAB software (The Mathworks, Inc., Natick, MA).

**Assay for Epo-dependent Proliferation—**Ba/F3 cells stably expressing wild-type or mutant EETs were maintained in interleukin-3 and washed extensively in RPMI medium with 10% fetal bovine serum before 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. We seeded triplicate samples of cells (2 × 10^4/well in 200 μl) in 96-well plates, and cultured them for 3 days with different concentrations of Epo. We then added 15 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Promega, Madison, WI). 48 h later, cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. After permeabilization with 0.1% Triton X-100 in PBS for 2 min, cells were blocked for 1 h in PBS containing 3% bovine serum albumin and 0.2% Tween 20. Coverslips were then incubated with anti-HA monoclonal antibodies (1:1000, Covance) for 1 h at 37°C, washed 3 times with PBS, and incubated with fluorescence isothiocyanate-conjugated donkey anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories). After 3 washes with PBS, the coverslips were mounted with anti-fading agent Vectashield (Vector Laboratories). Fluorescent images were taken using a Zeiss LSM510-Meta laser scanning confocal microscope with Zeiss acquisition software.

**RESULTS**

The Transmembrane and Intracellular Domains of the TpoR Confer Its High Level Cell Surface Expression—We previously found, using a FACS-based assay in the JAK2-deficient cell line γ2A, that in the absence of JAK2 most EpoR proteins are retained in the ER and only a small fraction on the cell surface (8). In this assay, receptors are HA-tagged at the extracellular domains and expressed in a bicistronic vector that also expresses GFP. The presence of the receptors on the cell surface can be detected by staining non-permeabilized cells with anti-HA antibody and APC-conjugated secondary antibodies. For each cell, GFP fluorescence is proportional to the total amount of the receptors present, whereas APC fluorescence indicates receptor surface expression. Surprisingly, we found that the fraction of the TpoR on the cell surface was much greater than that of the EpoR and PrlR (data not shown).

To eliminate potential detection bias due to differences in accessibility of the HA epitope between receptors, we generated chimeric receptors expressing the same HA-tagged extracellular domain of the EpoR. ETT and EPP contain the transmembrane and cytoplasmic domains of the TpoR or PrlR, respectively. In the absence of JAK2, ETT was targeted to the cell surface much more efficiently than the EpoR and EPP, whereas the total protein levels were comparable as judged by anti-HA immunoblot analysis (Fig. 2, A and B) and GFP fluorescence (data not shown). Therefore, a larger fraction of the TpoR was expressed on the plasma membrane compared with the EpoR and the PrlR, and the difference was attributable to the transmembrane and cytoplasmic domains of the TpoR. In addition, JAK2 promoted surface expression of the TpoR, as well as the EpoR and PrlR, consistent with our and the previous findings of Royer et al. (8, 18) (Fig. 2A). The percentage enhancement of each receptor on the cell surface by JAK2 co-expression was similar.
To confirm these results, we examined the subcellular localization of HA-tagged TpoR and EpoR in γ2A cells using confocal microscopy. Consistent with the FACS assay, TpoR was localized at the plasma membrane in the absence of JAK2 and co-expression of JAK2 further increased the TpoR on the cell surface (Fig. 2C). Some TpoR was also observed in the ER, Golgi, and vesicles (data not shown). The TpoR, when expressed in HeLa and 293T cells with endogenous JAK2, also localized mainly on the plasma membrane (data not shown). In contrast, in the absence of JAK2 in γ2A cells, EpoR was localized intracellularly (mainly in the ER) (Fig. 2C). In the presence of JAK2, we observed the EpoR on the plasma membrane in cells that expressed EpoR highly, and in the ER in cells that have lower EpoR expression levels (Fig. 2C). Together these results suggested that at similar expression levels, the TpoR had a much greater fraction expressed on the plasma membrane compared with the EpoR, and that this difference was conferred by its transmembrane and cytoplasmic domains.

**The Membrane-proximal Region of the TpoR Intrinsic Domain Controls Its High Cell Surface Expression**—To assess the contribution of the transmembrane and intracellular domains of the TpoR to its high level surface expression, we examined the surface expression of EET and ETE in γ2A cells using the FACS assay (Fig. 3A). EET, consisting of HA-tagged EpoR extracellular and transmembrane domains fused to the TpoR cytoplasmic domain, expressed on the cell surface at a level similar to that of ETT with or without JAK2 co-expression. Conversely, ETE, consisting of the HA-tagged EpoR extracellular domain, the TpoR transmembrane domain, and the EpoR cytoplasmic domain, were expressed on the plasma membrane at a level similar to that of the EpoR. These results indicate that the cytoplasmic, rather than the transmembrane, domain of the TpoR is necessary and sufficient for high level surface expression both with and without the co-expression of JAK2.

The cytosolic domains of cytokine receptors are divergent in sequence, with the exception of the membrane-proximal Box 1 and Box 2 (14). To identify specific regions in the TpoR cytoplasmic domain responsible for its efficient surface expression, we further dissected EET. We divided the intracellular domain of the TpoR into the juxtamembrane region to Box 2, and the region C-terminal to Box 2. As shown in Fig. 3B, in the absence of JAK2, E(juxta to B2)T expressed on the cell surface similar to that of the EpoR. In contrast, T(juxta to B2)E expressed on the cell surface similar to EET (Fig. 3B). These results indicated that TpoR sequences C-terminal to Box 2 are not required for high surface expression. Therefore, the crucial region for high surface expression of EET is the membrane-proximal region from the juxtamembrane region to the Box 2 of the TpoR. JAK2 promoted cell surface expression of all chimeric receptors regardless of the origin of the membrane-proximal portion (Fig. 3B).

**High TpoR Surface Expression through JAK2-independent Mechanisms**—Our results suggested that the membrane-proximal region of the TpoR confers its high level surface expression through JAK2-independent and -dependent mechanisms. We first probed the mechanisms underlying high level TpoR surface expression in the absence of JAK2.

To study the mechanism underlying the abundant TpoR surface expression in the absence of JAK2, we compared receptor maturation from the ER through the Golgi, using glycosylation states as a readout. Populations of γ2A cells stably expressing EET or the EpoR were examined. Three forms of each receptor were observed: a non-glycosylated immature form, an Endo H-sensitive immature form, and an Endo H-resistant Golgi-processed mature form (Fig. 4A). In the absence of JAK2, very little EpoR was expressed as an Endo H-resistant Golgi-processed mature form, whereas a much larger fraction of EET was
Membrane-proximal Region of the TpoR

A

| Extracellular | TMD | Cytoplasmic |
|---------------|-----|-------------|
| EpoR          | ETT | ETE         |
| JAK2          |     |             |

B

- Cytoplasmic domain
- Relative surface expression

TpoR | 100 | 391
EpoR | 20  | 115
E(justa to B2)T | 18  | 224
T(justa to B2)E | 110 | 347

Endo H-resistant, indicating that EET matured more efficiently than the EpoR.

Because differences in internalization kinetics could affect receptor levels on the cell surface, we measured internalization rates of EET and EpoR by measuring $^{125}$I-labeled Epo ($^{125}$I-Epo) internalized via receptor-mediated endocytosis (Fig. 4B). γ2A cells stably expressing EET or the EpoR were incubated with an excess (0.5 nM) of $^{125}$I-Epo at 37°C, and surface-bound and internal $^{125}$I-Epo were measured. Fig. 4B shows that Epo rapidly bound to the surface of both EpoR and EET cells, appeared as internalized ligand within minutes in both cells, and internal $^{125}$I-Epo increased during the 15-min endocytosis assay (Fig. 4B, left and middle panels). We determined each endocytic rate constant of the receptor, $k_{in}$ (Fig. 4B, right panel) (23–25). EET and the EpoR had essentially the same $k_{in}$, 0.019 min$^{-1}$ for EET and 0.020 min$^{-1}$ for the EpoR, thus EET and the EpoR were internalized at the same rates. EET cells internalized more $^{125}$I-Epo than EpoR cells simply because they expressed more surface receptors and bound more Epo. Therefore, the higher fraction of mature EET than mature EpoR could not be attributed to slower internalization from the cell surface.

To examine if the higher surface expression level of the TpoR compared with the EpoR could be attributed to differences in protein stability, we compared the half-life of mature EET and EpoR in γ2A cells stably expressing either receptor after blocking protein synthesis with cycloheximide. Consistent with our previous findings that very little EpoR exit ER without JAK2 (8), and possibly because of the heterogeneity in the glycosylation pattern of the mature EpoR protein, we could hardly detect the mature EpoR without JAK2 co-expression in the cycloheximide experiment (data not shown). In contrast, the mature form of the EET was readily detected and had an estimated half-life of 6 h (Fig. 5, EET-JAK2). Therefore, the TpoR intracellular domain stabilized the mature receptor isoform from degradation, which may contribute to its higher surface expression level.

To study if JAK2 promotes TpoR maturation, we examined the effect of JAK2 co-expression on the glycosylation state of EET. JAK2 co-expression further increased the amount of Endo H-resistant EET (Fig. 4A).

To examine if JAK2 facilitates EET biosynthesis and processing, we performed pulse-chase analysis of EET in γ2A cells. As shown in Fig. 4C, in the absence of JAK2 immediately after a 60-min pulse labeling, the majority of labeled EET was Endo H-sensitive, whereas a small amount of mature Endo H-resistant EET was detected. After a 90-min chase, the level of Endo H-sensitive EET decreased (asterisks) in the Endo H plus lanes indicate digested Endo H-sensitive EET, whereas the level of Endo H-resistant EET did not change. After a 200-min chase, only Endo H-resistant EET was detected. These results sug-
FIGURE 4. Processing and endocytosis of the TpoR and EpoR. A, the glycosylation states of HA-tagged EET and EpoR were examined in γ2A cells stably expressing either receptor as well as JAK2 or vector alone. The receptors were first treated either with Endo H or with PNGase F and neuraminidase, resolved by SDS-PAGE, and then immunoblotted with anti-HA antibodies. B, EpoR and EET are internalized at similar rates. Left panel shows endocytosis of Epo by EpoR cells and middle panel shows endocytosis of Epo by EET cells. Equivalent numbers of γ2A cells stably expressing either receptor were incubated with 125I-Epo for the indicated times. The amount of surface-bound (circles) and internal (square) 125I-Epo was determined as described under “Experimental Procedures.” The data are the mean of triplicate samples with standard deviations shown by error bars. Data from the left and middle panels were transformed and plotted in an internalization plot (right panel), which plots the amount of internal Epo versus the integral of the amount of surface-bound Epo. In this graph the endocytic rate constant, k_{in}, is equal to the slope of the line fitted through the plotted points (23–25). C, maturation of EET is not affected by JAK2 co-expression. γ2A cells stably expressing HA-tagged EET with JAK2 or vector alone were pulse-labeled with 500 μCi of a [35S]methionine and [35S]cysteine mixture for 60 min and chased with cold methionine and cysteine for the indicated times. The cells were lysed and immunoprecipitated with HA affinity matrix. The immunoprecipitates were resolved by SDS-PAGE and detected by autoradiography.
Membrane-proximal Region of the TpoR

FIGURE 5. Mature TpoR is more stable than mature EpoR. γ2A cells stably expressing HA-tagged EET or the EpoR were treated with cycloheximide for the indicated duration. Cells were lysed in 1% Nonidet P-40 lysis buffer and soluble fractions were subjected to SDS-PAGE and immunoblot analysis with anti-HA antibodies. The level of β-actin was shown as loading controls.

suggested that a small amount of Endo H-resistant mature EET was formed quickly and independently of JAK2, and that the bulk of the Endo H-sensitive EET was degraded. Importantly, these kinetics were not affected by JAK2, indicating that JAK2 did not significantly promote EET maturation to the Golgi (Fig. 4C). Shorter pulse and chase times showed similar results (data not shown).

We next compared the half-life of mature EET in the absence and presence of JAK2. In γ2A cells stably expressing EET, the EET had a half-life of ~6 h (Fig. 5). Co-expression of JAK2 resulted in a larger fraction of EET being in the Endo H-resistant form (EET-JAK2 versus EET + JAK2, short exposure, time 0), which is consistent with the results shown in Fig. 4A. More importantly, JAK2 co-expression prolonged the estimated half-life of mature EET to 9 h (Fig. 5), indicating that JAK2 stabilizes mature EET and protects it from degradation. In contrast, mature EpoR, even with JAK2 co-expression, had a half-life of ~130 min (Fig. 5). This is in agreement with the findings in TpoR-containing Ba/F3 cells described by Royer et al. (18).

TpoR Mutants Define Specific Residues Important for JAK2 Activation and Mitogenic Capability in Response to Cytokines—To examine the biological functions of the membrane-proximal region of the TpoR, we analyzed the ability of all the EET mutants in supporting cytokine-dependent JAK2 activation and cell proliferation in a hematopoietic cell line, Ba/F3. Populations of Ba/F3 cells that stably express wild-type or mutant EET receptors were isolated by flow cytometry and maintained in the presence of interleukin-3. These cells were then depleted of interleukin-3 by extensive washing, and their ability to grow in Epo was analyzed. The Box 1 mutants, EET(PSLP) and EET(DLH), did not confer Epo-dependent growth in Ba/F3 cells (Fig. 7A), as expected from their inability to interact with JAK2 (Fig. 6B). We further assayed the ability of these mutant receptors to activate JAK2 by measuring tyrosine phosphorylation of JAK2 upon Epo stimulation in Ba/F3. Consistent with the lack of mitogenic activities, these Box 1 mutants did not activate JAK2 (Fig. 7B). Therefore, the Box 1 mutants, EET(PSLP) and EET(DLH), failed to interact with or activate JAK2, and could not support Epo-dependent growth.

We next screened our mutant EETs for those that interacted with JAK2 normally and showed a JAK2-dependent increase in surface expression but were defective in supporting mitogenic activity in Ba/F3 cells. These mutants fall into two categories. One category, represented by EET(517HALW -> AAAA), was totally inactive in mitogenic signaling in Ba/F3 cells. This mutant could not activate JAK2 upon ligand stimulation (Fig. 7B). We confirmed that JAK2 bound to EET(517HALW -> AAAA) by co-immunoprecipitation assays (Fig. 7C). The Leu519 and Trp520 residues mutated in HALW are two highly conserved hydrophobic residues in the juxtamembrane region (17). Thus, as in the EpoR, these residues in TpoR are not required for JAK2 binding and JAK2-dependent surface expression, but for turning on JAK2 kinase activity upon cytokine stimulation.

The second category was represented by mutants in the Box 2 motif, EET(559LEI -> AAA) or EET(LEI), and in the region between Box 1 and Box 2, EET(525RVL -> AAAA) and EET(554LRD -> AAA), abbreviated as EET(RVL) and EET(LRD), respectively. These mutants interacted normally with JAK2 and conferred ligand-dependent growth in Ba/F3
cells; however, their mitogenic activity was dramatically decreased compared with wild-type EET (Fig. 8A). They showed decreased sensitivity to Epo and required higher concentrations of Epo to support maximal cell growth (Fig. 8A).

Consistent with these results, these mutants were markedly impaired in activating JAK2 (Fig. 8B). This was in contrast to the first category of mutants (Fig. 7, EET(HALW) or the Box 1 mutants (Figs. 7 and 8, EET(PSLP) and EET(DLH)), which did not activate JAK2 or support cell growth at all, even at high Epo concentrations.

In summary, the Box 1 mutants, EET(PSLP) and EET(DLH), failed to interact with JAK2, and could not activate JAK2 or support factor-dependent growth. Mutation of two of the three residues in the conserved hydrophobic motif, as in EET(HALW), allowed normal interaction with JAK2 as judged by JAK2-induced TpoR surface expression and co-immunoprecipitation, but completely lost the ability to activate JAK2 or confer mitogenic activity. The Box 2 mutant EET(LEI) and mutants EET(RVL) and EET(LRD), interacted with JAK2, but required higher Epo concentrations to activate JAK2 or support cell growth. The ability of all mutants to activate JAK2 correlated perfectly with their ability to sustain factor-dependent growth, reinforcing the notion that JAK2 is essential in TpoR signaling.

**DISCUSSION**

This study demonstrated that the membrane-proximal region of the TpoR cytoplasmic domain confers its high surface expression through JAK2-dependent and -independent mechanisms. In the absence of JAK2, a much larger fraction of the TpoR than the EpoR is in the mature Endo H-resistant form and is present on the cell surface. The membrane-proximal portion of the TpoR, which includes the juxtamembrane, Box 1 and Box 2 regions, is both necessary and sufficient for high level surface expression. Although the TpoR and EpoR intracellular domains confer similar rates of ligand-induced endocytosis, the TpoR membrane-proximal region stabilizes the mature receptor isoform from degradation. We also showed that JAK2 co-expression further increases TpoR surface expression, not by affecting receptor biosynthesis, but by stabilizing the mature receptor isoform. This JAK2 and TpoR interaction relies solely on the conserved Box 1 region, in contrast to the EpoR that requires Box 1 and an additional 20 residues on the C-terminal side for functional interaction with JAK2. Our study suggests that although cytokine receptors are all regulated by their cognate JAKs, their surface levels and receptor-JAK interactions are unique.

In the absence of JAK2, we showed significant TpoR expression on the plasma membrane, in contrast to the EpoR that was mainly retained in the ER. Using chimeric receptors that differ only in the cytoplasmic domain (EpoR versus EET), we found that the TpoR membrane-proximal region is necessary and sufficient for this function. "Knock-in" mice in which a truncated TpoR cytoplasmic domain lacking the distal 60 amino acids replaces the wild-type endogenous receptor, have normal steady-state platelet counts with normal megakaryocyte numbers and ploidy (12, 13). This TpoR mutant retains the intact...
membrane-proximal Box 1 and Box 2 domains that are required for JAK2 activation and TpoR cell surface expression, as shown herein and previously (18). Therefore, the membrane-proximal region of the TpoR is sufficient for its surface expression and biological functions in vivo (12, 13).

To further elucidate the molecular mechanisms underlying the high surface expression level of TpoR in the absence of JAK2, we demonstrated that a much larger fraction of EET was expressed in the Endo H-resistant, Golgi-processed mature form compared with the EpoR. Because we and others have suggested that the EpoR cytosolic domain is not folded well without JAK2 (8), EET may be better folded and processed than the EpoR, enabling a larger percentage of the total EET protein to exit the ER and reach the cell surface independent of JAK2. Furthermore, although the ligand-induced internalization rates are similar between the TpoR and EpoR, the TpoR cytoplasmic domain stabilizes the mature receptor from degradation. Thus, our data suggest that TpoR is intrinsically better folded and more stable than the EpoR, which results in its better glycosylation, maturation, and higher surface expression.

Within the TpoR membrane-proximal region, residues in the juxtamembrane region and between Box 1 and Box 2 are important for its high surface expression in the absence of JAK2 (Fig. 1); however, none is essential. Interestingly, the structure of the juxtamembrane region of the TpoR, the EpoR, and another cytokine receptor gp130 was shown to affect the conformation of its cytosolic domain and downstream signaling (17, 27–29). Therefore, the TpoR juxtamembrane region may also govern the conformation of the cytosolic domain that affects its folding and trafficking. Mutant EET(PSL) between Box 1 and Box 2 was defective in surface expression. The two Ser residues in this mutant were postulated to be phosphorylated (26). The possibility that an unidentified kinase affects the surface expression of the TpoR awaits further study. Mutant EET(KAT) exhibited higher than wild-type surface expression without JAK2. Because the TpoR can recycle to the cell surface in the absence of ligand (18), residues KAT may affect TpoR recycling and cause higher steady-state TpoR levels on the cell surface.

In the presence of JAK2, the TpoR also had a much higher surface expression compared with the EpoR. We found that the mature TpoR was much more stable than the mature EpoR. Moreover, similar to the EpoR, JAK2 co-expression greatly enhanced TpoR surface expression. In contrast to the EpoR that requires JAK2 for ER exit and maturation (8), JAK2 does not affect TpoR biosynthesis and maturation kinetics but stabilizes mature TpoR. This is consistent with results by Royer et al. (18) showing that JAK2 and TYK2 stabilize mature TpoR, possibly by protecting the TpoR from proteasomal degradation.

The requirement of JAK kinases for membrane localization of their cognate receptors has been demonstrated in several systems; however, the molecular mechanisms governing these functions appear to be receptor specific. For example, JAK2 enhances EpoR ER to Golgi processing and surface expression (8). Similarly, JAK1 expression shifted the localization of the Oncostatin M receptor from the ER to the plasma membrane (30). On the other hand, TYK2 affects the surface expression of IFNAR1 by impeding its constitutive endocytosis and degrada-

**FIGURE 7.** The Box 1 and residues HALW of the TpoR are essential for activation of JAK2 and TpoR mitogenic activity. A, Box 1 and residues HALW are critical for TpoR mitogenic activity. The mitogenic activity of EpoR, EET, and EET mutants were measured in Ba/F3 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. B, Box 1 and residues HALW are important for JAK2 activation. EpoR, EET, and EET mutants were analyzed for their ability to activate JAK2 in Ba/F3 cells. Ba/F3 cells stably expressing the corresponding wild-type or mutant receptors were stimulated with various concentrations of Epo as indicated and examined for JAK2 tyrosine phosphorylation using 4G10 antibodies. C, EET(HALW) interacts with JAK2 in co-immunoprecipitation assay. HEK293T cells co-transfected with vector, HA-EET, or HA-EET(HALW) and JAK2 were immunoprecipitated (IP) with antibodies to HA and probed with anti-JAK2 antibodies. Receptor expression levels are shown by immunoblotting (IB) lysates with anti-HA.
Membrane-proximal Region of the TpoR

FIGURE 8. The Box 2 motif and residues between the Box 1 and Box 2 of the TpoR are important for JAK2 activation and TpoR mitogenic activity. A, Box 2 and residues between Box 1 and Box 2 are important for TpoR mitogenic activity. The mitogenic activity of EET and EET mutants were measured in Ba/F3 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. B, Box 2 and residues between Box 1 and Box 2 are important for JAK2 activation. EET and EET mutants were analyzed for their ability to activate JAK2 in Ba/F3 cells as described in the legend to Fig. 7B.

The common γ chain localizes to the cell surface in the absence of JAK3, however, overexpression of JAK3 up-regulates its membrane accumulation (32). The TpoR is expressed on the cell surface in the absence of JAK2, and JAK2 and TYK2 further increases its surface expression (our data and Ref. 18). In contrast to the γ chain, which requires the entire JAK3 for optimal surface expression, we found that the N-terminal domain alone of JAK2 was sufficient for TpoR surface expression (data not shown), similarly to that of the EpoR (8). These results highlight the distinctive relations between individual JAKs and their receptors.

The 30 or more known cytokine receptors depend on only four JAKs for signal transduction. It is generally believed that Box 1 is essential for receptors to bind to their cognate JAKs; however, how each receptor-JAK pair achieve specificity remains elusive. We used a JAK2-dependent increase of surface expression as a readout of receptor-JAK2 interactions. Using a set of Ala scanning mutants, we showed that Box 1 is the only TpoR cytosolic sequence required for JAK2 interaction, consistent with results by Miyakawa et al. (26) showing that mutation of the Ser residue in Box 1 dramatically decreases JAK2 binding and Tpo-induced proliferation. This is in contrast to the EpoR, whose JAK2 interacting surface spans about 26 amino acids including Box 1 and residues immediately C-terminal to it (Fig. 1 and Ref. 8). Thus, the modes and characteristitcs of cytokine receptor-JAK interactions differ among receptors signaling through the same JAK protein.

Different from our results, Royer et al. (18) showed that residues 532QYL between Box 1 and Box 2, in addition to Box 1, are also important for JAK2-dependent TpoR surface expression in Ba/F3 cells. This difference could be due to differences in the combination of residues mutated or differences in the cell lines used.

JAK2 activation is a pre-requisite for TpoR mitogenic activity; mutant TpoRs that failed to activate JAK2 also failed to support cytokine-dependent Ba/F3 cell growth. However, interaction with JAK2, although required, is not sufficient for JAK2 kinase activation. Mutant EET(HALW) interacted with JAK2 as judged by a JAK2-dependent increase in surface expression in γ2A cells and by co-immunoprecipitation, yet this mutant failed to turn on JAK2 kinase activity upon ligand stimulation or to confer mitogenic activity in Ba/F3 cells. The Leu and Trp residues of this mutant correspond to φ₂ and φ₃ of the predicted hydrophobic motif characterized by φ₁XXXφ₂φ₃, where φ represents a hydrophobic residue and X represents any residue. This hydrophobic motif is conserved among many cytokine receptors (17). Mutating individual φ residues in the EpoR abolished JAK2 activation, whereas mutation of φ₃ abrogated gp130 signaling (8, 33). For the TpoR, a Leu residue is at φ₁, but this residue was not required for TpoR to activate JAK2. Therefore, the requirement of φ₁ in the hydrophobic motif may not be as stringent as φ₂ or φ₃.

We found that the Box 2 region of the TpoR was important for maximal TpoR mitogenic activity and maximal JAK2 activation, although not for JAK2 interaction. We found similar results in the EpoR. In addition, we showed that the TpoR required residues between Box 1 and Box 2, namely 528RVL and 533LRD, for optimal JAK2 activation and signaling upon ligand stimulation. Asp536 in 533LRD aligns with Asp287 in the EpoR that is also essential for EpoR signaling (8). In summary, we propose that the highly conserved Box 1 is the main JAK-interacting site on cytokine receptors, whereas the whole membrane-proximal region spanning the juxtamembrane region to Box 2 fine-tunes the specificity for each receptor-JAK pair. Our characterization of the mechanisms governing TpoR surface

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expression and its signal transduction may help understand how it governs megakaryocyte and platelet development and hematopoietic stem cell self-renewal.

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