YRRL motifs in the cytoplasmic domain of the thrombopoietin receptor regulate receptor internalization and degradation.

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Abstract
Thrombopoietin (Tpo), acting through the c-Mpl receptor, promotes the survival and proliferation of hematopoietic stem and progenitor cells and drives megakaryocyte differentiation. The pro-proliferation and survival signals activated by Tpo must therefore be tightly regulated to prevent uncontrolled cell growth. In this work we determined the mechanisms that control Tpo-stimulated c-Mpl internalization and defined the processes leading to its degradation. Stimulation of BaF-Mpl cells with Tpo leads to rapid, clathrin-dependent endocytosis of the receptor. Using small interfering (si)RNA we found that inhibition of adaptor protein 2 (AP2), which mediates endocytosis of transmembrane proteins, strongly attenuates Tpo-stimulated c-Mpl internalization. AP2 interacts with YXXΦ motifs and we identified 2 such motifs in c-Mpl (Y8RRL and Y78RRL) and investigated Tpo-stimulated internalization of receptors bearing point mutations at these sites. After Tpo stimulation, internalization was greatly reduced in c-Mpl Y78F and c-Mpl Y8+78F and these cell lines also exhibited increased proliferation and increased strength and duration of Jak2, STAT5, AKT, and ERK1/2 activation in response to Tpo. We also found that the Y8RRL motif regulates Tpo-stimulated lysosomal degradation of c-Mpl. Our data establishes that c-Mpl cytoplasmic YRRL motifs are responsible for both Tpo-mediated internalization via interactions with AP2 and lysosomal targeting following endocytosis.
Introduction

Thrombopoietin (Tpo) is critical for the maintenance of hematopoietic stem and progenitor cells and is also the primary regulator of megakaryocyte development\(^1\,^2\). The binding of Tpo to its receptor, c-Mpl, causes associated Janus kinase 2 (Jak2) activation, which in turn phosphorylates (activates) several downstream effectors including signal transducers and activators of transcription (STAT) 3 and 5, mitogen-activated protein kinase (MAPK), phosphotidylinositol-3-kinase (PI3-K) and protein kinase C (PKC)\(^3\,^7\). Activation of these pathways promotes proliferation and survival in c-Mpl-expressing cell lines and hematopoietic progenitor cells, in addition to megakaryocyte lineage differentiation and maturation\(^8\,^11\). It is critical that Tpo signal transduction is stringently controlled to prevent uncontrolled proliferation. Suppressors of cytokine signaling (SOCS) proteins, phosphatases and negative regulators such FAK, Lnk and Lyn have all been shown to down-modulate Tpo-induced signaling\(^12\,^15\). However, the most effective method of regulating Tpo signaling is by controlling expression of c-Mpl on the plasma membrane. Tpo-mediated c-Mpl endocytosis, recycling and degradation are rapid mechanisms to control signaling longevity and represent a mechanism by which Tpo signaling can be regulated without new protein expression.

The principal mechanism of receptor mediated endocytosis in eukaryotic cells is the clathrin-coated vesicle\(^16\). Soluble clathrin molecules self-assemble and are recruited to the plasma membrane where they form lattice structures and interact with transmembrane receptors via adaptor proteins (AP), such as AP2, to form clathrin-coated pits\(^17\,^18\). These pits then further invaginate before finally budding from the membrane to form clathrin-coated vesicles. AP2 is a heterotetramer composed of \(\alpha\)2, \(\beta\)2, \(\mu\)2 and \(\sigma\)2 subunits. The \(\alpha\)2 and \(\beta\)2 subunits localize AP2 to the membrane, recruit endocytic accessory proteins and bind clathrin heavy chain\(^19\,^21\). Transmembrane proteins are associated with the AP2-clathrin complex via the \(\mu\)2 domain, which binds directly to cytoplasmic YXX\(\Phi\) (where X=any amino acid and \(\Phi\)=bulky hydrophobic residue) and [DE]XXXL[IL] motifs\(^22\,^23\). To ensure that AP2 specifically associates with membrane-bound proteins, phosphorylation of \(^{156}\)Thr in the \(\mu\)2 subunit results in a conformational change in AP2, dramatically increasing the affinity of \(\mu\)2 for YXX\(\Phi\) motifs\(^24\,^25\). \(^{156}\)Thr is phosphorylated by adaptor-associated kinase 1 (AAK1)\(^26\), the activity of which is maximized by its association with clathrin\(^27\,^28\), ensuring that AP2-cargo protein interactions are initiated only at the plasma membrane. In addition to being an endocytic signal, YXX\(\Phi\) motifs
located between 6-9 amino acids from the transmembrane domain mediate targeting of cargo protein to the lysosome and lysosome-like organelles via interactions with AP-3\textsuperscript{29-32}.

c-Mpl contains two YXXΦ motifs in its cytoplasmic domain at Tyr8 (Y\textsubscript{8}RRL; numbering is from the first cytoplasmic domain residue) and Tyr78 (Y\textsubscript{78}RRL). Previous findings suggest that Y\textsubscript{78} acts as a negative regulator in a truncated (T-83) form of c-Mpl\textsuperscript{33}, although the function of this residue in full length receptor remains unclear. A role for Y\textsubscript{8} in c-Mpl signaling has not been identified. Additionally, c-Mpl contains 2 di-leucine/isoleucine repeats at L\textsubscript{54}L\textsubscript{55} and I\textsubscript{57}L\textsubscript{58} in the cytoplasmic box2 region. Using a series of truncated receptors and point mutations, Dahlen and colleagues\textsuperscript{34} reported that c-Mpl requires L\textsubscript{54}L\textsubscript{55} and I\textsubscript{57}L\textsubscript{58} in addition to Y\textsubscript{112}, for Tpo-dependent internalization. In this work, we demonstrate the importance of AP2 in controlling c-Mpl internalization and determine the roles of the two YRRL motifs in the receptor following Tpo stimulation. We found that AP2 is essential for Tpo-stimulated clathrin-mediated c-Mpl internalization by reducing expression of the AP2α2 subunit using siRNA. By generating cells stably expressing c-Mpl with a Y to F point mutation at Y\textsubscript{8} and Y\textsubscript{78}, we found that Y\textsubscript{78}RRL but not the Y\textsubscript{8}RRL motif mediated the AP2-Mpl interactions and is essential for receptor internalization. Additionally, cells expressing the Y\textsubscript{78}F mutation exhibited increased proliferation and Jak2 and AKT phosphorylation in response to Tpo. Interestingly, although c-Mpl\textsubscript{Y8} appears not to be significantly involved in c-Mpl internalization, receptors bearing this mutation are not degraded as rapidly as wildtype forms and appear not to be targeted to the lysosome. Our findings provide novel insights into the regulation of Tpo signaling, and potentially that of other hematopoietic growth factors, via receptor internalization and degradation.

**Materials and Methods**

**Chemicals and Reagents**
Pharmacological inhibitors JakI, LY294002, SU6656 and U0126 where all purchased from Calbiochem (San Diego, CA.). The clathrin inhibitor monodansylcadaverine (MDC), the proteosome inhibitor Z-LLL-al (ZL) and cycloheximide were purchased from Sigma (St. Louis, MO.). Extracellular-specific polyclonal rabbit anti-human c-Mpl was provided by Amgen Pharmaceuticals (Thousand Oaks, CA.) and intracellular-specific polyclonal rabbit anti-human c-Mpl was purchased from Upstate (Lake Placid, NY.). Monoclonal mouse anti-AP2α2 antibody
was purchased from BD Biosciences (La Jolla, CA.). Antibodies specific to phospho-specific and total Jak2, STAT5, AKT and ERK1/2 were purchased from Cell Signaling Technologies (Danvers, MA.) Monoclonal mouse anti-actin was purchased from Sigma. Secondary antibodies, goat anti-rabbit-horseradish peroxidase (HRP) and rabbit anti-mouse-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA.) and fluorescently labeled goat anti-rabbit-488 was purchased from Invitrogen (Carlsbad, CA.). Membrane-impermeable sulfo-
NHS-biotin and Neutravidin-coupled agarose beads were purchased from Pierce (Rockford, IL.). Recombinant human (rh) Tpo was a gift from Don Foster, Zymogenetics (Seattle, WA).

**Plasmid Construction**

Wildtype human c-Mpl (NM_005373.1) was cloned into the retroviral expression vector pMX-
puro using EcoRI and XhoI cloning sites. The c-Mpl intracellular domain point mutations (c-Mpl Y₈F, c-Mpl Y₇₈F and c-Mpl Y₈ + 7₈F) were introduced using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA.) with the following oligonucleotides: Y₈F- forward primer 5’-
CCTGCACACTTCAGGAGACTGAGGCAT-3’; Y₈F reverse primer 5’-
ATGCCTCAGTCTCCTGAAGTGTGCAGG-3’; Y₇₈F  forward primer 5’-
CCAGATGGACTTCCGAAGATTGCAGCAGCTT-3’; Y₇₈F reverse primer 5’-
AAGGCTGCAATCTTCCGGAAGTCCATCTCGG-3’. Mutations were confirmed by sequencing.

**Cell Lines and Culture Conditions**

The interleukin-3 (IL-3)-dependent prolymphoid cell line BaF3 and BaF3-Mpl clones were maintained in RPMI1640 medium containing 10% fetal bovine serum (FBS) supplemented with IL-3 (2µg/ml conditioned medium from IL-3-producing baby hamster kidney cells). The retroviral packaging cell line Platinum-E (Plat-E) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. To generate BaF3-Mpl clones, sub-
confluent Plat-E cells were transfected with pMX-puro-c-Mpl constructs using FuGENE 6 transfection reagent (Roche Applied Sciences, Indianapolis, IN.) for 48hr, prior to viral supernatant collection. BaF3 parental cells were then incubated in viral supernatant supplemented with IL-3 for 48hr before selection with puromycin (2µg/ml). Clonal populations were generated by limiting dilution. Total c-Mpl protein expression was determined by western blot and cell surface expression by flow cytometry.
RNA Interference

The previously described AP2α2 subunit siRNA target sequence AAGAGCAUGUGCACGCUGGCCA$^{35}$ and the DY547-labeled negative control were purchased from Dharmacon (Chicago, IL.). BaF3-Mpl cells were transfected using an Amaxa nucleofector (Gaithersburg, MD.) according to the manufacturer’s protocol. Briefly, 4x10$^6$ BaF3-Mpl cells were resuspended in solution V (Amaxa) containing 2µg of either negative control or AP2α2 siRNA and electroporated using the program X-001. SiRNA efficiency was determined by western blot analysis for AP2α2 subunit after 24, 48 and 72 hr. Maximal knockdown occurred 48hr post transfection.

Western Blotting

Following chemical treatment or Tpo-stimulation BaF3-Mpl cell lines were washed 3 times with cold PBS and lysed in NP-40 buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1mM EDTA, 1mM Na$_3$VO$_4$, 1mM NaF) containing 1% protease inhibitors (Sigma). Denatured proteins were fractionated by NaDodSO$_4$-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Protein expression was detected by incubating with specific antibodies and visualized by chemiluminescent detection reagent (ECL-plus; GE Healthcare, Little Chalfont, UK).

Internalization Assays

BaF3-Mpl clones were starved of IL-3 for 2 hr before stimulation with 50ng/ml rhTpo for 0, 10, 30 and 60 min. Cells were washed 3 times in cold phosphate buffered saline (PBS) before biotinylation of the extracellular domains of membrane-bound proteins with 5mg/ml sulfo-NHS-biotin for 30 min at 4°C, a cell impermeable biotinylation reagent. Further protein biotinylation was prevented by washing the cells 3 times with cold PBS containing 100mM glycine. Cells were lysed in NP-40 buffer and protein concentrations determined using the BCA protein assay kit (Pierce). Biotinylated proteins were pulled down using Neutravidin-conjugated agarose beads, proteins denatured by adding NaDodSO$_4$-loading dye and boiling for 5 min and c-Mpl expression analyzed by western blotting.
MTT Proliferation Assay

BaF3-Mpl clones in log-phase growth were washed 3 times with PBS to remove IL-3, resuspended in RPMI 1640 supplemented with 2% FBS and plated into 96-well plates at a concentration of 1000 cells / well before rhTpo was added at concentrations ranging from 1pg/ml to 10ng/ml. Cells were incubated for 48 hr before treatment with 1mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma) and incubation at 37°C for 3 hr. Cells were lysed and formazen crystals dissolved in 100µl MTT lysis solution (dH2O, 20% NaDodSO4, 40% N,N-dimethyl formamide (DMF), 2% acetic acid, 0.2% HCl) for 6 hr at 37°C and absorbance read on a colorimetric plate reader at 570nm. Each data point is expressed as a percentage of proliferation stimulated by a maximal dose of murine IL-3 (4µl/ml murine IL-3 supernatant). Each experiment was performed in triplicate with 3 different clones for each wild type or mutant Mpl construct.

Flow Cytometry

Tpo-stimulated BaF3-Mpl cells were washed in cold labeling buffer (PBS with 0.5% bovine serum albumin) and fixed in cold 2% paraformaldehyde for 5 min. Cells were then labeled with primary antibody specific to the extracellular domain of c-Mpl for 30 min at 4°C. Unbound antibody was removed by washing and cells were incubated for 30 min at 4°C with goat anti-rabbit-488 fluorescent secondary antibody. Cells were then washed and analyzed using a FACSCalibur flow cytometer and CellQuest Pro analysis software (BD Biosciences).

Results

Tpo-induced internalization of c-Mpl is mediated by Jak and Src Family Kinase (SFK) signaling.

Using BaF3 cells expressing full-length human c-Mpl (BaF3-Mpl) we studied the kinetics of receptor internalization following stimulation with Tpo. Flow cytometric analyses demonstrated that c-Mpl internalization was near maximal (reduction to 46% ± 1.2% of normal cell surface expression) 10 min after Tpo stimulation and maximal after 60 min (to 34.3% ± 3.2% of baseline; Fig. 1A). These kinetics are comparable to those previously published34. To determine whether c-Mpl internalization was dependent on clathrin-mediated endocytosis cells were pre-treated with an inhibitor of clathrin-coated pit formation, MDC36. MDC addition inhibited c-Mpl internalization in a concentration-dependent manner with maximal attenuation
achieved at 500μM (Fig. 1B), indicating that Tpo-induced receptor internalization is dependent on the formation of clathrin-coated pits. To further determine the intracellular signaling mechanisms responsible for c-Mpl internalization, we used pharmacological inhibitors to several kinases activated by Tpo, including Jak2, SFKs, phosphoinositol 3-kinase (PI3K) and extracellular stimulus response kinase (ERK)1/2. Inhibitors to Jak2 and SFKs (JAK I and SU6656 respectively) greatly reduced Tpo-stimulated c-Mpl internalization, whilst inhibitors to PI3K and ERK1/2 had no effect (Fig. 1C). A concentration-dependent inhibition of c-Mpl internalization by pre-incubation with JAK I and SU6656 was further demonstrated (Fig. 1D and E).

**AP2 is essential for Mpl internalization**

To determine whether AP2 is required for Tpo-induced c-Mpl internalization, BaF3-Mpl cells were transfected with siRNA specific to the α2 subunit of AP2. Western blot analysis of whole cell lysates after treatment with siRNA demonstrated an approximate 4-fold decrease in AP2α2 protein expression after 48 hr (Fig. 2A). For internalization studies, BaF3-Mpl cells were transfected with AP2α2 siRNA for 48 hr prior to stimulation with 50ng/ml Tpo, before c-Mpl expression was analyzed by flow cytometry. AP2α2-depleted cells exhibited greatly reduced c-Mpl internalization in response to Tpo; c-Mpl expression was reduced to 55.3% [± 9.6] of baseline expression in control cultures whereas AP2α2 siRNA-treated cell c-Mpl expression was reduced only to 91.2% (±1.9) of baseline maximal expression (Fig. 2B).

**c-Mpl internalization is mediated via an AP2-Y78RRL motif interaction**

The AP2µ2 domain has previously been shown to interact with YXXΦ motifs on transmembrane receptors and drive clathrin-mediated receptor endocytosis. To determine whether the two YRRL motifs in the cytoplasmic domain of c-Mpl are required for AP2-mediated internalization we generated four expression-matched BaF3-Mpl cell lines, stably expressing either wild-type, or Y to F point mutations at cytoplasmic Y8, Y78 and both Y8 and Y78 (Fig. 3A). In the absence of Tpo, there was no significant difference in cell surface expression of c-Mpl between clones when analyzed by flow cytometry (data not shown).

Using a membrane impermeable biotinylation reagent and a streptavidin pull-down strategy we were able to accurately determine expression of c-Mpl on the cell surface following
Tpo stimulation in the BaF3-Mpl wildtype and mutant clones. Previous findings suggest that post-translational modifications of c-Mpl give rise to a more “mature” form of the receptor, which is expressed at the cell membrane. In accordance with this finding western blot analyses of human c-Mpl –bearing cell lines display two specific protein bands, one migrating at 80kDa and a second at 85kDa, the latter of which represents the mature cell surface form of the c-Mpl receptor. We measured the intensity of the 85kDa band to evaluate Tpo-induced Mpl internalization in the BaF3-Mpl clones. Cells were stimulated with 50ng/ml rhTpo for 10, 30 and 60 min prior to membrane biotinylation, lysis and streptavidin pull-down. Levels of internalization were quantified by densitometry of the 85kDa membrane-localized c-Mpl band in Tpo-stimulated cells compared to unstimulated controls. Wildtype c-Mpl was rapidly internalized in response to Tpo, with cell surface expression reduced to approximately 33% of baseline after 10 min and to 14% after 60 min (Fig 3C). c-Mpl Y5F expressing cells exhibited slightly reduced internalization in response to Tpo compared to wildtype receptor-bearing cells (surface expression reduced to 64% of baseline after 10 min and to 38% after 60 min). In contrast, Y78F c-Mpl bearing cells exhibited greatly blunted internalization in response to Tpo, with cell surface expression remaining at 90% of baseline after 10 min and 68% after 60 min. c-Mpl internalization kinetics of Mpl Y8+78F cells were similar to those of Mpl Y78F cells.

c-Mpl Y78F exhibits enhanced Tpo-mediated proliferation and signaling

Our previous studies identified Y78 in the cytoplasmic domain of c-Mpl as a potential negative regulator of Tpo proliferation, although these findings were made using a truncated receptor, missing the distal two tyrosine residues. In the current study we sought to determine the role of Y78 in the context of the full length receptor and assess whether our findings regarding c-Mpl internalization had any effects on Tpo-mediated proliferation and signaling. MTT assays were performed using three clones from each receptor type, wildtype, Y8F, Y78F and Y8+78F. All of the clones analyzed displayed comparable levels of c-Mpl expression, as determined by western blot analysis (data not shown). rhTpo induced a concentration-dependent increase in cell proliferation in all clones, although cells bearing c-Mpl with the Y78F mutation displayed significantly greater maximal proliferation. Using 1ng/ml rhTpo, BaF3/Mpl cells proliferated at 104% (±3.9) of IL-3 stimulated controls, whereas c-Mpl Y78F cells proliferated to 133% (±4.4) of IL-3 stimulated controls (Fig 4A). c-Mpl Y8F was not significantly different from c-Mpl
wildtype receptor bearing cells, and c-Mpl Y8+78F was not significantly different from c-Mpl Y78F. To determine the effects of the Y8F and Y78F mutations on Tpo-induced signaling, cells expressing the different forms of the receptor were starved for 18 hr prior to stimulation with rhTpo (50ng/ml) for 0, 10 and 30 min. Cells bearing the Y78F mutation showed moderately increased phosphorylation of Jak2 and STAT5 and increased longevity of signal and greatly increased phosphorylation of AKT and ERK1/2 (Fig. 4B). Signaling in the c-Mpl Y8F mutation was similar to wildtype c-Mpl control cells. To determine whether the increased Tpo-stimulated signaling was as a result of reduced internalization of c-Mpl observed with the Y78F mutation, we treated wildtype c-Mpl expressing cells with AP2α2 siRNA to inhibit endocytosis. Interestingly, we found that signaling was not affected by knocking down AP2α2 protein expression (Fig. 4C), even to levels that blunted c-Mpl Y78F receptor internalization, suggesting that attenuating receptor internalization may not significantly affect Tpo signaling in these cells.

c-Mpl lysosome targeting is mediated by the Y8RRL motif

It has previously been demonstrated that YXXΦ motifs localized between 6-9 residues from the transmembrane domain mediate lysosomal targeting of internalized receptors31;32. Consequently, we assessed whether the c-Mpl Y8RRL motif was involved in targeting the internalized receptor for lysosomal degradation. To accurately determine the effects on receptor degradation we treated BaF-Mpl cells with the protein synthesis inhibitor cycloheximide (1µg/ml) for a range of time periods from 0 to 120 min; c-Mpl expression was then analyzed by western blot and the ratio of 80kDa (“immature”) to 85kDa (“mature”) Mpl determined by densitometry (Fig. 5A). In the absence of cycloheximide, the majority (~80%) of Mpl exists as an 80kDa form. In the presence of cycloheximide, the mature form (85kDa) becomes increasingly predominant with time. In the subsequent experiments cells were treated with cycloheximide for 60 min, having determined that after this period of treatment synthesis of new protein had been prevented without significant cell death (data not shown). Cycloheximide-treated cells were stimulated with rhTpo (50ng/ml) for 0 to 120 min before the level of c-Mpl degradation was determined by western blot analysis and densitometry. In wildtype c-Mpl-expressing cells Tpo-stimulation resulted in a steady degradation of the receptor (Fig. 5B), which was maximal after 60 min (reduction to ~20% of normal c-Mpl expression). However, in Y8F c-Mpl-expressing cells, receptor degradation only occurred after 30 min of Tpo treatment and
after 60 min the level of degradation was significantly lower than for wildtype c-Mpl (reduced to only ~65% of normal c-Mpl expression). These findings strongly suggest a role for Y8RRL in mediating c-Mpl degradation.

To determine if the Y8RRL motif is involved in lysosomal-targeting and degradation rather than proteasomal degradation, cycloheximide-treated wildtype and Y8F c-Mpl expressing cells were pretreated with lysosome (NHCl4) and proteasome (Z-LLL-al) inhibitors before Tpo stimulation. Both NHCl4 and Z-LLL-al inhibited receptor degradation following Tpo stimulation in wildtype c-Mpl-expressing cells (Fig. 6A). However, in Y8F Mpl-expressing cells, NHCl4 had no effect on degradation, whilst Z-LLL-al treatment alone almost completely aborted c-Mpl degradation, indicating that Y8RRL is involved in lysosome targeting but not in proteasomal degradation. If the mutation in Y8F c-Mpl-bearing cells destroys the lysosome targeting motif, the receptor would be expected to build up in the early endosome, and be available for rapid recycling. To determine whether this hypothesis was correct, Y8F c-Mpl cells were stimulated with Tpo for a limited period to induce receptor internalization, before Tpo was removed to promote recycling of the receptor back to the membrane. Y8F c-Mpl recycled back to the membrane significantly more rapidly than WT (Fig. 6B), further indicating a failure of Y8F-Mpl to target to lysosomes.

Discussion

In this study we demonstrate a role for AP2 in mediating Tpo-stimulated c-Mpl internalization and determined that Y78RRL is an AP2µ2- cytoplasmic binding motif in the receptor. Additionally, we have shown that an identical sequence located close to the transmembrane domain (Y8RRL) appears to target the receptor for lysosomal degradation following Tpo stimulation. Consistent with previous studies, we have shown that the Y78F mutation results in increased Tpo-induced proliferation and further demonstrate that this mutation also leads to increased activity of Jak2 and AKT, which indicate a possible mechanism for the increase in cell numbers. These findings suggest a critical role for Y8 and Y78 in the cytoplasmic domain of c-Mpl in controlling surface expression and receptor degradation following Tpo stimulation, which is highly significant considering the importance of tightly regulating the response to Tpo.
Although we have been able to show the importance of AP2 and the predicted AP2-binding motif in controlling c-Mpl internalization, we have been unable to demonstrate direct protein-protein interactions between the receptor and the AP2-µ2 subunit by co-immunoprecipitation (data not shown). Interestingly, this finding is similar to previous studies with the transferrin receptor (TfR). Both AP2 and the YTRF motif in the cytoplasmic domain of the TfR are critical for its internalization, but no direct interaction between AP2 and TfR could be detected. Although we were unable to show a direct interaction, having demonstrated that AP2 and Y₇₈RRL are both vital for c-Mpl internalization we hypothesize that the receptor interacts with AP2 at this site to mediate Tpo-induced endocytosis. It is possible that the AP2-c-Mpl interaction is too weak and/or transient to be detected by co-immunoprecipitation and it may be feasible to identify these interactions by other methods, for example, fluorescence resonance energy transfer (FRET) with confocal microscopy.

The position of the YXXΦ motif in the cytoplasmic domain is thought to be of great importance to its function. Purely endocytic YXXΦ sequences are commonly considered to lie within 10-40 residues from the transmembrane domain. The c-Mpl endocytic motif at Y₇₈RRL is therefore considerably further away than in other transmembrane receptors. Whether this is exclusive to c-Mpl or whether it also occurs in other hematopoietic growth factor receptors is currently unknown.

Regulation of AP2 localization and binding is mediated by phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and AAK-1 kinase activity. PI(4,5)P₂ interacts via two binding sites on AP2, one on the α-subunit to dock the complex at the plasma membrane and the other on the µ-subunit. AAK-1 increases the affinity of the µ-subunit to bind its target cargo by phosphorylation of Thr. We have shown that Tpo is able to directly cause the internalization of its receptor via activation of Jak2 and SFKs signaling cascades, although the downstream effectors remain unclear. Interestingly, a previous finding that c-Mpl Y₁₁₂ mediates receptor internalization could also support a potential role for SFKs in regulating c-Mpl endocytosis as this residue has previously been shown to regulate Tpo-stimulated Lyn kinase activity. The role of SFKs in regulating receptor internalization has been studied previously with differing results. For example, the SFK inhibitor SU6656 fails to attenuate internalization of epidermal growth factor receptor (EGFR), but previous studies on the stem cell factor receptor c-Kit, suggest that SFKs are vital for internalization and degradation of the receptor. We have
demonstrated that similar to c-Kit, SFKs are important in regulating c-Mpl internalization. Additionally, we cannot rule out the possibility that c-Mpl Y78 is involved in the activation of another signaling pathway that mediates receptor internalization, in addition to interacting with AP2. Whether the Tpo-mediated stimulation of certain signaling pathways causes changes in AP2 structure and/or phosphorylation, for example by increasing AAK-1 kinase activity, is currently being investigated.

Although we demonstrate significant Tpo signaling alterations in the cells expressing the c-Mpl Y78F mutation, our finding that signaling was not affected following AP2α2 siRNA treatment, strongly suggests that the differences in signaling intensity and duration between wild type c-Mpl and c-Mpl Y78F are due to effects not directly linked with receptor endocytosis. Relatively little is known about the role of the Y78 residue in mediating Tpo signaling, although our data strongly support the previous findings that identified a potential negative regulatory role33. Considering the increase in the extent of protein phosphorylation and longevity of activation, which was particularly evident in AKT and ERK1/2, it is possible that the c-MplY78F mutation affects the activity of one or more phosphatases, for example the protein tyrosine phosphatases SHP1 or 2, which is activated in response to Tpo6. Future experiments aimed at identifying Y78 interacting proteins and the role of Y78 in the activation of specific protein phosphates will elucidate the exact nature of this negative regulation.

As well as identifying a novel c-Mpl internalization motif, we have also described a potential lysosome-targeting motif at Y₈RRL. The location of this motif is consistent with previous findings that di-leucine or YXXΦ motifs located 6-9 residues from the transmembrane domain can target proteins to the lysosome for degradation22;29. Studies of lysosome-associated membrane proteins (LAMPS) and lysosome integral membrane proteins (LIMPs) have highlighted AP3 as the prime candidate for association with lysosome targeting motifs to mediate the transport of protein to the lysosomes32;46. Therefore, internalized c-Mpl is likely routed to the lysosomes via interactions between AP3 and Y₈RRL and this interaction may be responsible for the rapid degradation of c-Mpl following Tpo stimulation. Although recent co-localization studies suggested that c-Mpl is not present in lysosomes47, we have found that the Tpo-stimulated rapid degradation of the receptor can be significantly inhibited by NHCl4, strongly suggesting that there is in fact a role for lysosomal degradation in controlling c-Mpl expression.
This work is the first to describe the YXXΦ motif for receptor internalization and lysosome-targeting in the hematopoietic growth factor receptor (HGFR) family. Importantly, we have recently identified that potential AP2-interaction motifs are also present in a number of other receptors. For example, the erythropoietin receptor (EpoR), which shares significant homology with c-Mpl, contains one [DE]XXXL[IL] motif (D78EGPLL) and several YXXΦ motifs (starting at Y93, Y153, Y181 and/or Y183). Interestingly, granulocyte-colony stimulating factor receptor (G-CSFR) contains two YXXΦ motifs, one of which is deleted by a common truncation in severe congenital neutropenia48. Future study of these and other potential HGFR internalization motifs may well prove important in understanding receptor clearance and degradation.

Our studies provide a novel insight into the mechanisms that regulate Tpo signaling. Interest surrounding the function of c-Mpl in controlling hematopoietic progenitor cell expansion and megakaryopoiesis has recently intensified following the discovery of the Jak2V617F mutation and its role in the myeloproliferative diseases polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (reviewed in49). Given the emerging link between uncontrolled receptor signaling and disease states it has become even more important to determine the precise mechanisms controlling the clearance and degradation of c-Mpl and other HGFRs.
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Authorship
Contributions:
All authors have substantially contributed to the content of the paper and have agreed to the submission in its current format. I.S.H. designed and performed research, analyzed data and wrote the manuscript; J.R.K. and M.M.C. performed research and analyzed data; K.K. designed experiments, interpreted data and wrote the manuscript.

Conflict-of-interest disclosure:
The authors declare no competing financial interests.

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Figure Legends

Figure 1
Tpo-mediated internalization of c-Mpl is dependent on clathrin, Jak2 and SFKs. (A) BaF-Mpl cells were stimulated with 50ng/ml rhTpo for increasing periods of time and cell surface expression of c-Mpl analyzed by flow cytometry. Receptor internalization was determined in the presence of inhibitors to clathrin (B) and a panel of protein kinases (C). (D and E) The effects of JAK1 and SU6656 were further determined by concentration-response. % baseline c-Mpl cell surface expression is determined by comparing Tpo treated cells with unstimulated cells. All data is representative of at least 3 independent experiments using 2 BaF-Mpl clones.

Figure 2
c-Mpl internalization is reduced in AP2α2-depleted cells. BaF-Mpl cells were transfected with siRNA specific to the AP2α2 subunit, which is essential for the function of AP2. (A) AP2α2 depletion was analyzed by western blot and was maximal 48 hr post-transfection (~75% depletion). Tpo-stimulated c-Mpl internalization was then determined by flow cytometry in AP2α2-depleted BaF-Mpl cells 48 hr post-transfection (** = p<0.01; B).

Figure 3
Cytoplasmic Y_{78} is essential for c-Mpl internalization. (A)Human c-Mpl cytoplasmic amino acid sequence is shown with the 2 potential AP2 interaction motifs highlighted in bold. Three constructs were made with Y→F point mutations at Y_{8}, Y_{78} and both Y_{8} and Y_{78}. (B) Biotinylation of membrane proteins demonstrates expression of the larger (~85 kDa) mature form of c-Mpl at the at the cell surface (WCL = whole cell lysate). (C) The effects of Tpo stimulation on receptor surface expression in each of the clones was determined by membrane biotinylation and western blot analysis. Internalization was quantified by densitometry; comparing expression of membrane localized c-Mpl (85kDa) to Tpo starved cells (0 min). The data is representative of 3 independent experiments.

Figure 4
The Y78F mutation leads to increased Tpo-mediated proliferation and signaling. (A) MTT proliferation assays were performed on c-Mpl wildtype, Y8F, Y78F and Y8+78F–expressing cells. Data points represent the mean of 3 independent experiments using 3 different c-Mpl-expressing clones (** = p<0.01). (B) Levels of phosphorylated Jak2, STAT5, AKT and ERK1/2 in response to 50ng/ml Tpo were determined by western blot analyses. (C) Tpo-stimulated activation of Jak2, STAT5, AKT and ERK1/2 in wildtype c-Mpl-expressing cells treated with control or AP2α2 siRNA. Data is representative of 3 independent experiments.

Figure 5
Cytoplasmic Y8 is important in Tpo-induced c-Mpl degradation. (A) Wildtype c-Mpl expressing cells were treated for increasing periods of time with 1µg/ml cycloheximide to inhibit the synthesis of new protein. The lower panel represents densitometry of the 2 different forms of c-Mpl compared to actin. The data is representative of 2 independent experiments. (B) Wildtype and c-Mpl Y8F– expressing cells were pretreated with 1µg/ml cycloheximide for 60 min before stimulation with Tpo (50ng/ml) for increasing periods of time. Degradation of the mature 85kDa form of c-Mpl was determined by western blot and density compared to unstimulated cells. The data is representative of 4 independent experiments.

Figure 6
The Y8F mutation inhibits lysosome targeting of c-Mpl. (A) Cells expressing wildtype or c-Mpl Y8F were pretreated with cycloheximide for 30mins with or without lysosome or proteasome inhibitors for an additional 30mins prior to Tpo stimulation for 60mins. Receptor expression was determined by western blot and degradation quantified by comparing the density of cells with or without Tpo. The data represents 3 independent experiments. (B) Flow cytometry for surface c-Mpl expression of wildtype and of c-Mpl Y8F expressing cell lines following Tpo stimulation followed by Tpo removal. The data represents mean of 3 independent experiments.
References


Figure 2

(A) Western blot analysis of AP2α2 and Actin expression over time post transfection. AP2α2 (112kDa) and Actin (45kDa) expression levels were measured at 0, 24, 48, and 72 hours.

(B) Mpl surface expression following TPO treatment. The graph shows a comparison between Control and AP2α2 siRNA groups. The difference is statistically significant (***).
Figure 3

A

<table>
<thead>
<tr>
<th>Variant</th>
<th>TM</th>
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<tbody>
<tr>
<td>Mpl</td>
<td>RWQFPAHYRLLRHALWPSL-/-MDYRRLQPSCLGTM-</td>
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<td>RWQFPAHFRRLRHALWPSL-/-MDFRRRLQPSCLGTM-</td>
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</tbody>
</table>

B

1 = BaF3-Mpl WCL
2 = BaF3-Mpl Membrane biotinylation Streptavidin pulldown

C

Membrane c-Mpl

BaF-Mpl

BaF-Mpl Y8F

BaF-Mpl Y78F

BaF-Mpl Y8+78F

% cell surface expression

Time (mins; Tpo 50ng/ml)
Figure 6

A

- = - = + + = NH$_4$Cl (20mM)
- = - - + + Z-LLL-al (10μM)
- = + + + + Tpo (50ng/ml; 60min)

Mpl (85kD)

Actin (50kDa)

BaF-Mpl

Mpl (85kD)

Actin (50kDa)

BaF-Mpl Y8F

% expression 85kDa

Cont +TPO  NH$_4$Cl +TPO  Z-LLL-al +TPO

B

Mpl cell surface expression (%)

0 TPO  TPO +30  TPO -30  TPO -60

BaF-Mpl

BaF-Mpl Y8F
YRRL motifs in the cytoplasmic domain of the thrombopoietin receptor regulate receptor internalization and degradation

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