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Phosphorylated c-MPL tyrosine 591 regulates thrombopoietin-induced signaling

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Abstract

Thrombopoietin (TPO) is the primary regulator of platelet production, affecting cell survival, proliferation and differentiation through binding to and stimulation of the cell surface receptor c-MPL. Activating mutations in c-MPL constitutively stimulate downstream signaling pathways, leading to aberrant hematopoiesis and contribute to development of myeloproliferative neoplasms. Several studies have mapped the tyrosine residues within the cytoplasmic domain of c-MPL that mediate these cellular signals; however, secondary signaling pathways are incompletely understood. In this study we focused on c-MPL tyrosine 591 (Y₅₉₁). We found Y₅₉₁ of wild-type c-MPL to be phosphorylated in the presence of TPO. Additionally, eliminating Y₅₉₁ phosphorylation by mutation to Phe resulted in decreased total receptor phosphorylation. Using an SH2/PTB domain binding microarray, we identified novel c-MPL binding partners for phosphorylated Y₅₉₁, including Src homology phosphatase-1 (SHP-1), spleen tyrosine kinase (SYK) and Bruton's tyrosine kinase (BTK). The functional significance of binding partners was determined through siRNA treatment of Ba/F3-MPL cells, confirming that the increase in pERK1/2 resulting from removal of Y₅₉₁ may be mediated by SYK. These findings identify a novel negative regulatory pathway that controls TPO-mediated signaling, advancing our understanding of the mechanisms required for successful maintenance of hematopoietic stem cells and megakaryocyte development.

INTRODUCTION

c-MPL is a member of the hematopoietic receptor superfamily and is the receptor for the hematopoietic growth factor thrombopoietin (TPO). TPO is the primary regulator of megakaryopoiesis and of critical importance for hematopoietic stem cell maintenance[1, 2]. c-MPL is a type I cytokine receptor that requires bound Janus kinase 2 (JAK2) for kinase activity and cell surface localization[3, 4]. Upon TPO binding, c-MPL monomers homodimerize and undergo a conformational change, allowing the juxtaposition of JAK2

proteins bound to the conserved box 1 and box 2 motifs of the receptor, resulting in trans-auto-phosphorylation of JAK2[5, 6]. Phosphorylated JAK2 can then, in turn, phosphorylate tyrosine residues within the cytoplasmic domain of c-MPL, in addition to signal transduction proteins including JAK-STAT, the mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol-3-kinase (PI3K) and protein kinase C (PKC)[7–11] to promote cell survival and proliferation.

c-MPL tyrosine 625 (Y₆₂₅) is phosphorylated in response to TPO stimulation[7] and is essential for TPO-mediated proliferation. Phosphorylation of Y₆₂₅ is necessary for activation of downstream signal transduction proteins including GRB2-associated-binding proteins (GAB1/2), phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1), Src homology containing protein (SHC), and signal transducer and activator of transcription 3 (STAT3), resulting in downstream activation of the MAPK pathway and PI3K[7, 8, 12]. Removal of this site via receptor truncation or introduction of a tyrosine to phenylalanine point mutation (Y₆₂₅F) severely impairs signaling and growth in response to TPO[7]. Additionally, Y₆₃₀ is also phosphorylated in response to TPO and like Y₆₂₅, is involved in STAT3 activation[7]. Unregulated positive signaling results in hematological malignancies as observed in patients with gain of function mutations in c-MPL[13–16]. Therefore, a thorough understanding of the regulation of TPO signaling will provide important insights into normal and malignant hematopoiesis.

Uncontrolled activation of several cytokine receptors have been shown to contribute to hematological malignancy. Thus, under normal conditions, mechanisms must exist to turn growth factor induced signaling off. Previous studies have identified a number of negative regulators of TPO signaling, including protein phosphatases, suppressors of cytokine signaling (SOCS) proteins, Lnk, Lyn kinase and focal adhesion kinase (FAK)[17–19]. Additional mechanisms to limit activated receptor signaling include receptor internalization via clathrin-dependent endocytosis and CBL-mediated ubiquitination and degradation through both lysosomal and proteosomal pathways[20, 21]. Soon after its identification, we proposed that Y₅₉₁, also referred to as intracellular tyrosine 78, acts as a negative regulatory residue[7]. We found that the receptor trafficking protein AP2 binds to Y₅₉₁, resulting in endocytosis of the receptor[20]. In the current work we explored additional mechanisms involving c-MPL Y₅₉₁ that might contribute to the negative regulation of TPO signaling.

Receptor tyrosine phosphorylation is a critical step in growth factor induced signal transduction. Previous studies have reported that in wild-type (WT) c-MPL, only Y₆₂₅ and Y₆₃₀ are phosphorylated in response to TPO[7] whereas Y₅₉₁ is also phosphorylated in the mutant MPLW₅₁₅L receptor[22]. In the current study we demonstrate that Y₅₉₁ is also phosphorylated in WT c-MPL in response to TPO. Furthermore, to identify signaling pathways dependent on phosphorylated Y₅₉₁ (pY₅₉₁), we used Ba/F3 cells expressing a Y₅₉₁F mutant c-MPL in order to characterize the signaling effects resulting from elimination of phosphorylation at this site. We identified binding partners for pY₅₉₁ using an SH2/PTB domain binding array. Peptide pulldowns were used to confirm binding partners identified in the arrays, and siRNA used to show that SYK may mediate the increased MAPK pathway signaling in response to TPO in WT cells. Together, these results provide new insights into the molecular mechanisms involved in the regulation of TPO-mediated signaling and

proliferation, and identifies a potential therapeutic target to enhance or block c-MPL signaling for therapeutic benefit.

EXPERIMENTAL PROCEDURES

Reagents

Extracellular-specific polyclonal rabbit anti-human c-MPL was provided by Amgen Pharmaceuticals (Thousand Oaks, CA), intracellular-specific polyclonal rabbit anti-human c-MPL, AP2 and 4G10 anti-phosphotyrosine were purchased from Millipore (Billerica, MA). Phosphospecific and total JAK2 (phospho-JAK2 clone C80C3 and total JAK2 clone D2E12), STAT5 (phospho-STAT5 clone D47E7), Akt (phospho-AKT clone D9E), ERK1/2 (phospho-ERK1/2 clone D13.14.43 and total ERK clone 137F5), SYK (phospho-SYK Y323, phospho-SYK Y352 and phospho-SYK Y525/526 clone C87C1 and total SYK clone D115Q) and SHP-1 (phospho-SHP-1 clone D11G5 and SHP-1 clone C14H6) in addition to total BTK antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Monoclonal mouse anti-actin (clone AC-15) was purchased from Sigma-Aldrich (St. Louis, MO). 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). Recombinant human (rh) TPO was a gift from Don Foster (Zymogenetics, Seattle, WA).

Mass Spectrometry

See supplemental methods.

Plasmid Construction

Wild-type human c-MPL and intracellular domain point mutation c-MPL Y₅₉₁F cloned into the retroviral expression vector pMX-puro using EcoRI and XhoI cloning sites as previously described[20].

Cell Lines and Culture Conditions

Ba/F3-MPL cells were generated by retroviral infection with pMX-puro-c-MPL constructs using Platinum E (PLAT/E) retroviral packaging cells and cultured as previously described[20, 21]. F-36P cells were obtained from the European Collection of Cell Culture and cultured as previously described[23]. The Phoenix-amphotropic packaging cell line (φNX) was cultured in Dulbecco modified Eagle medium (DMEM) containing 10% FBS. To generate stable F-36P-MPL cell lines, subconfluent φNX cells were transfected with pMX-puro-c-MPL constructs using FuGENE 6 transfection reagent (Roche Applied Sciences, Indianapolis, IN) for 48 hours before viral supernatant collection. F-36P parental cells were then incubated in viral supernatant supplemented with 10ng/mL rhIL-3 and 5μg/mL polybrene (Millipore) for 48 hours before selection with puromycin(2μg/mL). Cells lines were cultured and used as pools.

Western Blotting and Immunoprecipitation

Western Blotting and immunoprecipitations were performed as previously described[21].

Detection of Active, GTP-bound Ras

Levels of activated Ras were detected using an Active Ras Pull-down and Detection Kit (Thermo Scientific) following the manufacturers' protocol. Ba/F3-MPL cells were treated with or without 1ng/mL rhTPO for 5 minutes and lysed in the kit-provided lysis buffer supplemented with 1% protease inhibitors (Sigma-Aldrich).

SH2-domain Binding Assay

Protein microarrays were fabricated to contain 136 human Src homology 2/phosphotyrosine-binding (SH2/PTB) domains as described previously[24, 25]. Arrays were probed with synthesized phosphopeptide labeled on the N-terminus with 5-(and-6)-carboxytetramethylrhodamine [5(6)-TAMRA]. Binding results were analyzed as described previously. A phospho-tyrosine peptide corresponding to the Y₅₉₁ region of c-MPL was synthesized to include nine residues upstream and seven residues downstream of Y₅₉₁, plus a C-terminal Asp to aid synthesis and solubility (LCSSQAQMDpY₅₉₁RRLQPSCD).

Peptide Binding Assays

Biotinylated peptides corresponding to c-MPL residues 582–601 including an aminohexanoic acid spacer (Biotin-Ahx-LCSSQAQMDY₅₉₁RRLQPSCLT-NH₂ and Biotin-Ahx-LCSSQAQMD(pY₅₉₁) RRLQPSCLT-NH₂) were synthesized by RS Synthesis (Louisville, KY). Lyophilized peptides were reconstituted to 10mM in DPBS and 400μL was conjugated to 400μL High capacity NeutrAvidin Agarose Resin (Thermo Scientific, Rockford, IL) for 4.5 hours at room temperature. F-36P-MPL WT cells were treated with or without 1ng/mL rhTPO for 5 minutes and lysed in NP-40 lysis buffer. Lysates were pre-cleared by incubation with 40μL High capacity NeutrAvidin agarose resin for 1 hour at 4°C. 1mg of pre-cleared lysate was incubated with 20μL peptide conjugated beads overnight at 4°C. The samples were centrifuged and washed 5 times with lysis buffer, resuspended in Lammeli buffer and subjected to immunoblotting.

RNA Interference (siRNA)

Syk siRNAs were purchased from Dharmacon RNAi Technologies as a SMARTpool, and Ptpn6 siRNA and control siRNA were purchased from Invitrogen. All siRNAs were reconstituted according to the manufacturers' instructions. Cells were treated using the Neon® Transfection System (Invitrogen). Ba/F3-Mpl WT and Y₅₉₁F cells were washed in DPBS and resuspended in R buffer at 2.0×10^7 /mL; 2.0×10^6 cells were transfected with siRNA at a final concentration of 1μM. Cells were cultured for 24 hours post-transfection in RPMI 1640 +10% FBS supplemented with murine IL-3, they were then cytokine starved in RPMI 1640 +2% FBS for 16 hours, then stimulated with or without 10ng/mL rhTPO for 15min and lysed in NP-40 lysis buffer. Lysates were then subjected to immunoblotting or Ras pulldown.

RESULTS

Tyrosine residue 591 (Y₅₉₁) of c-MPL is phosphorylated in response to TPO stimulation

To develop a better understanding of c-MPL phosphorylation in response to TPO, tandem mass spectrometry was used to determine sites of TPO-mediated phosphorylation of c-MPL. c-MPL peptides were obtained by immunoprecipitating c-MPL from rhTPO stimulated Ba/F3-MPL cells followed by trypsin digest. Phosphorylated peptides were enriched using IMAC and subjected to tandem mass spectrometry. Generated data was analyzed using SEQUEST® and revealed the mass spectrum of the peptide TPLPLCSSQAQMDYR (Figure 1). The quantity computation with the help of the b- and y-ion series revealed that the mass of tyrosine (Y) 14 is 243Da, instead of the native 163Da. This is indicative of a phosphomodification (+80Da) of this tyrosine residue. Tyrosine 14 of the peptide is equivalent to Y₅₉₁ in the intracellular domain of c-MPL, indicating that this residue is phosphorylated in response to TPO.

Next we determined the effect of Y₅₉₁ on total receptor phosphorylation. To assess the phosphorylation state of c-MPL, Ba/F3-MPL cells were stimulated with 10ng/mL rhTPO for 5 minutes, lysed and subjected to immunoprecipitation using an anti-c-MPL antibody. Precipitated protein was then analyzed via western blot using an anti-phospho-tyrosine probe. Consistent with previous studies, we found a marked increase in wild-type (WT) receptor phosphorylation in response to TPO[1] and significantly decreased receptor phosphorylation in the Y₆₂₅F mutant (Figure 2A) [7]. Likewise, the Y₅₉₁F mutant displayed a similar decrease in total c-MPL receptor phosphorylation upon TPO stimulation, whereas the Y₆₃₀F mutant was phosphorylated to a similar extent as the wild-type receptor (Figure 2A). Additional experiments were performed to determine the kinetics of receptor phosphorylation and without Y₅₉₁, c-MPL phosphorylation is less sensitive to TPO (Figure 2B) and duration of phosphorylation is shortened (Figure 2C).

c-MPL Y₅₉₁F exhibits hypersensitive, enhanced and prolonged ERK1/2 activation upon TPO stimulation

TPO binding to c-MPL results in the activation of numerous signaling cascades including the JAK/STAT, PI3K/Akt and MAPK pathways[5]. To determine the effects of the Y₅₉₁F mutation on TPO-mediated signaling, Ba/F3-MPL WT or Y₅₉₁F cells were cytokine starved and stimulated with 10ng/mL rhTPO for up to 2hr. As previously reported, cells expressing the Y₅₉₁F displayed a significant increase in the level and duration of, ERK1/2 phosphorylation compared to WT (Figure 3A)[20]. Cells expressing c-MPL Y₅₉₁F stimulated with increasing concentrations of rhTPO for 5min exhibited a significant hypersensitive response in phosphorylation of ERK1/2 at low doses (0.1ng/mL) of rhTPO and enhanced maximal levels ERK1/2 phosphorylation (Figure 3B). In a previous work, we show Akt phosphorylation is also increased in the Y₅₉₁F mutant; however, activation of the JAK/STAT signaling pathway remained the same in both c-MPL WT and Y₅₉₁F expressing cells [20]. To further understand the mechanism behind the enhanced and prolonged ERK1/2 phosphorylation in c-MPL Y₅₉₁F cells, we assessed the activation state of a stimulator of the MAPK/ERK pathway, the small GTPase, Ras. Ba/F3-MPL WT or Y₅₉₁F cells were cytokine starved for 18hr prior to 10ng/mL rhTPO stimulation. Cells were lysed and subjected to a

pull-down using the Ras binding domain of RAF1 to isolate the active, GTP-bound Ras, and analyzed using western blot to detect levels of active Ras. We observed a significant, approximate two-fold increase in the level of Ras-GTP in cells expressing the c-MPL Y₅₉₁F mutant receptor compared to WT (Figure 3C).

SHP-1 and SYK associate with phosphorylated c-MPL Y₅₉₁ in vitro

An SH2/PTB domain microarray was used to identify potential binding partners for pY₅₉₁. A 5,(6)-TAMRA labeled pY₅₉₁ sequence peptide was diluted to 8 separate concentrations and used to probe a protein array comprised of 136 SH2 or PTB domains (Supplemental Table 1). Peptides bound at <2μM, with R² = 0.89 were considered viable targets (Supplemental Figure 1). mRNA expression of targets was confirmed in bone marrow, Ba/F3, CD41⁺ cells and megakaryocytes by reverse transcriptase PCR (Supplemental Figure 2). The pY₅₉₁ peptide bound with high affinity in a manner consistent with saturation binding to domains from BTK, SHP-1 and SYK (Figure 4A). To validate the protein interaction data obtained with the SH2 domain binding array, peptide pull-down assays were performed using Y₅₉₁ and pY₅₉₁ peptides. To mimic cellular conditions, Y₅₉₁ peptides were incubated with lysate from the human cell line, F-36P without TPO stimulation and pY₅₉₁ peptide was incubated with lysate from F-36P cells stimulated with 1ng/mL rhTPO. We observed SHP-1 preferential binding to phosphorylated Y₅₉₁, SYK binding to only phosphorylated Y₅₉₁ and BTK binding equally to phosphorylated and unphosphorylated Y₅₉₁ (Figure 4B). Therefore, we continued to study both SHP-1 and SYK. The clathrin-mediated endocytosis adaptor protein, AP2 was used as a control [26].

SYK binds c-MPL pY₅₉₁ to negatively regulate TPO-mediated ERK1/2 signaling

After identification of SHP-1 and SYK as potential binding partners for Y₅₉₁, we sought to determine if they were responsible for the increased ERK1/2 and Akt signaling observed in Ba/F3-MPL Y₅₉₁F cells. To examine the role of SHP-1 and SYK in TPO-induced ERK1/2 and Akt signaling, we used specific siRNAs to either *Ptpn6* (the gene encoding SHP-1) or *Syk* to reduce SHP-1 and SYK protein expression, respectively, in Ba/F3-c-MPL WT and Y₅₉₁F cells. In c-MPL Y₅₉₁F cells, the siRNA-induced reduction of SHP-1 protein resulted in no significant change in TPO mediated ERK1/2 phosphorylation (Figure 5A) or Akt phosphorylation (Supplemental Figure 3A), relative to the siRNA control. However, specific siRNA-induced reduced SYK protein expression in WT c-MPL cells resulted in a significant increase in the level of pERK1/2 after TPO stimulation compared to the control (Figure 5B). The increase in pERK1/2 levels in WT cells mediated by SYK siRNA treatment is not to the same extent as observed when tyrosine 591 is mutated to phenylalanine; however, this is likely the result of incomplete knockdown of SYK (Figure 5B, top panel). We observed no difference in Akt phosphorylation as a result of SYK knockdown (Supplemental Figure 3B). To determine if SYK plays an active role in the observed regulation of ERK1/2 phosphorylation, we looked at the phosphorylation state of SYK in response to TPO. We assessed the phosphorylation state of multiple sites of SYK including Y₃₁₇, Y₃₄₆ and Y_{519/520} (Y₃₂₃, Y₃₅₂ and Y_{525/526} in human SYK, respectively). Phosphorylation of Syk Y₃₁₇, a negative regulatory residue [27], was unaltered between c-MPL WT and c-MPL Y₅₉₁F in response to TPO (Figure 6A). However, baseline phosphorylation of Syk Y₃₄₆ was higher in WT c-MPL compared to Y₅₉₁F (Figure 6B). We were unable to detect

phosphorylation of SYK Y_{519/520} (data not shown). We found no difference in the phosphorylation state of SHP-1 (Figure 6C).

DISCUSSION

c-MPL is critical for the maintenance of hematopoietic stem cells, in addition to being the key regulator of megakaryopoiesis[1, 2, 28]. Therefore, its regulation is essential to prevent uncontrolled activation of the receptor, which can result in the development of myeloproliferative neoplasms (MPNs)[14, 16]. In this study, we demonstrate for the first time that Y₅₉₁ in wild-type c-MPL is phosphorylated in response to TPO and that removal of Y₅₉₁ phosphorylation results in decreased total receptor phosphorylation. Y₅₉₁ regulates the level active Ras within the cell following TPO stimulation thus, its removal contributes to the higher levels of pERK1/2 observed in Y₅₉₁F. Additionally, we identify SYK and SHP-1 as potential binding partners for pY₅₉₁, and moreover, that knockdown of SYK mimics the Y₅₉₁F phenotype, suggesting that SYK contributes to the negative regulatory effects of Y₅₉₁ in wild-type cells. These findings suggest a significantly more important role than previously thought for Y₅₉₁ in TPO signaling and receptor function.

Our previous studies, using receptor truncations, identified Y₅₉₁ as a potential negative regulator of TPO-mediated proliferation[7]. This has been further supported by our work showing that the Y₅₉₁F point-mutation in c-MPL confers a proliferative advantage to cells in response to TPO[7, 20]. To determine whether the observed phenotype is the consequence of the removal of a phosphorylation site at Y₅₉₁, within the intracellular domain of c-MPL, we determined the phosphorylation state of Y₅₉₁ in wild-type c-MPL after TPO stimulation. Phosphorylation of c-MPL Y₅₉₁ has been previously reported in the disease state associated with a W₅₁₅A activating mutation of c-MPL[22] but here we report that c-MPL Y₅₉₁ is also phosphorylated in response to TPO in the wild-type receptor. As such, we tested whether phosphorylation of Y₅₉₁ provides a docking site for SH2 domain containing proteins to bind and act as direct downstream effectors or as a scaffold for additional effector proteins in TPO mediated signaling.

It has been known for many years that multiple residues of c-MPL are phosphorylated upon TPO binding. Previous studies have focused on Y₆₂₅ and Y₆₃₀[7]; our demonstration in this study that c-MPL Y₅₉₁ is also phosphorylated provides an additional potential point of regulation of receptor activity. Moreover, based on our experiments using single tyrosine to phenylalanine mutations of c-MPL, the decrease in total receptor phosphorylation in the Y₅₉₁F cells is greater than would be predicted based on loss of a single tyrosine site. This result also suggests that c-MPL Y₆₂₅ and Y₆₃₀ phosphorylation levels are, in part, dependent on the phosphorylation state of Y₅₉₁ and that pY₅₉₁ is required for maximal phosphorylation at Y₆₂₅ and Y₆₃₀. If so, our results suggest that Y₅₉₁ might also serve as a docking site of a kinase responsible of phosphorylation of Y₆₂₅ and Y₆₃₀. To better explore this hypothesis the temporal phosphorylation profile of c-MPL would need to be explored.

Since TPO is the primary megakaryocyte growth factor[29], much has been discovered about the positive signals involved in its regulation. However, the mechanisms that negatively regulate its signaling are less well understood. The YRRL motif appears twice in

the cytoplasmic domain of c-MPL. These cytoplasmic c-MPL YRRL motifs are important in negative regulation of TPO signaling. Y₅₂₉RRL is required for lysosomal targeting and subsequent degradation and Y₅₉₁RRL is important for MAPK pathway activation and suppression of proliferation[20]. Our data suggest that Y₅₉₁ can regulate MAPK pathway activation through modulation of active Ras levels within the cells. Active Ras is necessary for megakaryocyte differentiation[28], thus it is possible that a role of Y₅₉₁ is to suppress megakaryocytic differentiation through downregulation of active Ras. The mechanism by which Y₅₉₁ suppresses Ras activation is an important area for future studies as aberrant Ras signaling has been observed in a number of human cancers, including hematological malignancies[30].

Although attempts to co-immunoprecipitate c-MPL and SYK have been unsuccessful, which is a likely consequence of the transient nature of SH2 interactions, we found using an SH2-domain binding array followed by a peptide pulldown, that SYK may potentially bind to Y₅₉₁ of c-MPL. SYK typically binds immunoreceptor tyrosinebased activation motifs (ITAMs), characterized by two YXXL/I sequences 6–9 amino acids apart[31]. The region of c-MPL around Y₅₉₁RRL does not contain additional YXXL/I motifs but c-MPL signals as a dimer; therefore, pY₅₉₁RRL could serve as a hemi-ITAM for SYK binding. The role of SYK in immunoreceptor signaling has been well characterized and typically results in receptor activation and positive signaling (reviewed in [31]). However, our preliminary data shows that the elimination of this SYK binding site yields an increase in ERK1/2 signaling, suggesting that in wild-type cells, SYK may potentially downregulate the phosphorylation of ERK1/2 in response to TPO. SYK phosphorylation decreases following TPO stimulation of both WT and Y₅₉₁F c-MPL supporting its role as a negative regulator of TPO signaling. SYK Y₃₄₆ has been linked to ERK activation in immune cells[27] and the decrease in SYK pY₃₄₆ in c-MPL Y₅₉₁F supports a link between the two proteins albeit a negative rather than the typical positive association. SYK function has been reported to interfere with epidermal growth factor (EGF)-mediated cellular responses through epidermal growth factor receptor (EGFR), thus in some cases, SYK functions as a negative regulator[32]. However, the exact mechanisms by which c-Mpl and SYK may interact and how this potential interaction affects TPO signaling requires further investigation. It is possible that there are a number of signaling intermediates between SYK and ERK including other negative regulatory proteins functioning as effectors facilitating negative regulation through Y₅₉₁. However, the mechanisms through which SYK negatively regulates ERK signaling and its role in megakaryopoiesis have yet to be elucidated.

In addition to identifying SYK as a potential binding partner for pY₅₉₁, peptide pulldowns identified two additional novel binding partners for Y₅₉₁, SHP-1 and BTK. SHP-1 is a phosphatase that preferentially binds pY₅₉₁, yet is not responsible for the increased ERK1/2 activation resulting from the Y₅₉₁F mutation; however, SHP-1 could modulate other aspects of TPO signaling outside of ERK1/2 activation. SHP-1 negatively regulates signaling of type I cytokine receptors through binding of phosphorylated tyrosine residues on activated receptors and subsequent dephosphorylation of JAK2[33, 34]. We observed a slight increase in the level of phosphorylated JAK2 in the Y₅₉₁F mutant; therefore, it is possible that in c-MPL, SHP-1 binding to pY₅₉₁ mediates JAK2 activation. Additional experiments are required to further understand the role of Y₅₉₁ in SHP-1 regulation.

In this report we show that c-MPL Y₅₉₁ is phosphorylated in response to TPO, providing a novel docking site for SH2-domain containing proteins SHP-1 and SYK. Once bound to pY₅₉₁, SYK negatively regulates ERK1/2 phosphorylation. Tight regulation of the cellular response to TPO is essential, as loss of this control seen in clinical mutations of c-MPL (*c-MPL* W₅₁₅L) results in development of hematological malignancies. A novel c-MPL Y₅₉₁D mutation was identified in a patient with *JAK2*V₆₁₇F-positive polycythemia vera[35]. At the time of discovery, the function of the mutation was unknown; however, our data suggests that loss of Y₅₉₁ in this patient may be facilitating the disease phenotype due to loss of a negative regulator. Thus, an understanding of the molecular details of TPO signaling is important for identification of areas for therapeutic intervention. A negative regulatory site such as Y₅₉₁ and its associated effector proteins provide potential therapeutic targets for dysregulated cellular responses to thrombopoietin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Phospho-tyrosine peptide from the intracellular domain of c-MPL identified by LC-MS/MS following tandem IP¹

Tandem mass spectrometry was performed using an LTQ tandem mass spectrometer and data analysis was performed using SEQUEST and InsPecT. Tandem mass spectra of the phosphopeptide TPLPLCSSQAQMDpYR were identified. The y and b series of the ions is indicated. The sequence resulting from these spectra is indicated above the panel. The mass of tyrosine (Y) 14 is 243 Da, instead of the native 163 Da. This indicates a phosphomodification (+80 Da) of this tyrosine residue. Tyrosine 14 of the peptide equals tyrosine 591 in the intracellular domain of c-MPL.

¹Ectopically expressed human c-MPL protein in Ba/F3 cells recovered by tandem IP (anti-c-MPL→trypsin→IMAC). Peptide sequence is indicated along with the position of pY sites in c-MPL, the indicated peptide scores, charge state (z), and observed mass-to-charge ratios (m/z).

Figure 2. c-MPL receptor tyrosine phosphorylation

Ba/F3 cells stably expressing human WT or mutant c-MPL were stimulated with rhTPO, lysed and subjected to immunoprecipitation with an anti-c-MPL antibody. Immunoprecipitates were analyzed via western blot using a phospho-tyrosine probe to compare total receptor phosphorylation of the mutants to the wild-type receptor in response to TPO stimulation, blots were stripped and re-probed with an anti-c-MPL antibody to assess the level of immunoprecipitated protein. Data are representative of 3 independent experiments. (A) Ba/F3 cells expressing different tyrosine to phenylalanine mutants of c-MPL were stimulated with 10ng/mL rhTPO for 5min. (B) Ba/F3 c-MPL WT and Y₅₉₁F

cells stimulated at 0, 0.1, 1 or 10ng/mL rhTPO for 5min. (C) Ba/F3 c-MPL WT and Y₅₉₁F cells were stimulated with 10ng/mL rhTPO for 0, 15, 60 or 120min.

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Figure 3. The Y₅₉₁F mutation leads to increased TPO-mediated ERK signaling via Ras

(A) Top: Western blot analysis of phosphorylation levels of JAK2, STAT5, Akt and ERK1/2 in Ba/F3-MPL wild-type and Y₅₉₁F cells stimulated with 10ng/mL rhTPO for 0, 15, 60 or 120 minutes. Bottom: densitometry of phospho-ERK1/2 blot (*P < 0.05; ***P < 0.001). Error bars represent $\pm$ SEM. (n=3). (B) Top: Western blot analysis of phosphorylation levels of JAK2, STAT5, Akt and ERK1/2 in Ba/F3-MPL wild-type and Y₅₉₁F cells stimulated for 5 minutes with 0, 0.1, 1 or 10ng/mL rhTPO. Bottom: densitometry of phospho-ERK1/2 blot. Error bars represent $\pm$ SEM. (n=3). (C) Top: Detection of active, GTP-bound Ras in Ba/F3-

MPL wild-type and Y₅₉₁F cells. Bottom: Densitometry of GTP-bound Ras blot (*P < 0.05). Error bars represent $\pm$ SEM. (n=3). Densitometry was performed using ImageJ

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Figure 4. SHP-1, SYK and BTK SH2 domain binding affinity for pY₅₉₁

(A) Peptide binding data was gathered by probing an SH2/PTB domain microarray with varying concentrations pY₅₉₁ (CSSQAQMDpY₅₉₁RRLQP) peptide and measuring fluorescence of bound 5,6-TAMARA-labeled peptide. Saturation binding curves were generated and equilibrium dissociation constants (K_D) and R^2 were calculated using GraphPad Prism 5 software. Plots show normalized fluorescence as a function of peptide concentration. (B) To analyze protein binding to tyrosine 591, biotinylated peptides corresponding to the amino acid sequence surrounding Y₅₉₁ were synthesized (LCSSQAQMD(Y₅₉₁/pY₅₉₁)RRLQPSCLT). Biotinylated peptides were conjugated to NeutrAvidin agarose resin and incubated with F-36P-MPL wild-type whole cell lysate. Proteins interacting with peptides were eluted and separated on SDS-PAGE and analyzed via immunoblotting. SHP-1, SYK and BTK antibodies were used to confirm potential partners and AP2 was used as a control.

Figure 6. Phosphorylation of SYK and SHP-1

Western blot analysis of the phosphorylation state of (A,B) SYK and (C) SHP-1 from Ba/F3-MPL wild-type and Y₅₉₁F cells stimulated with 10ng/mL rhTPO for indicated times.