A Structure-Function Analysis of Serine/Threonine Phosphorylation of the Thrombopoietin Receptor, c-Mpl*

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Thrombopoietin (TPO), the critical regulator of platelet production, acts by binding to its cell surface receptor, c-Mpl. Numerous studies have shown that TPO binding leads to JAK2 kinase activation and Tyr phosphorylation of c-Mpl and several intracellular signaling intermediates, events vital for the biological activity of the hormone. In contrast, virtually nothing is known of the role of Ser or Thr phosphorylation of c-Mpl. By using phosphoamino acid analysis we found that Ser residues of c-Mpl were constitutively phosphorylated in receptor-bearing cells, levels that were increased following exposure of cells to TPO. To identify which residues were modified, and to determine the functional consequences of their phosphorylation, we generated a series of Ser to Ala mutations of a truncated c-Mpl receptor (T69) capable of supporting TPO-induced cell growth. Of the eight Ser within T69 we found that at least four are phosphorylated in TPO-stimulated cells. The mutation of each of these residues alone had minimal effects on TPO-induced proliferation, but substitution of all of the phosphoserine residues with Ala reduced the capacity of the receptor to support cell growth by over 50%. Additionally, the Ser at cytoplasmic position 18 is not detectably phosphorylated. However, the mutation of Ser-18 to Ala nearly abrogates TPO-induced proliferation and co-precipitation of JAK2 with Mpl. This study provides the first systematic analysis of the role of Ser residues in c-Mpl signaling.

Thrombopoietin is a hematopoietic cytokine regulating megakaryopoiesis and platelet production (1). The receptor for thrombopoietin, c-Mpl, is expressed on hematopoietic stem cells, immature hematopoietic progenitor cells, megakaryocytes, and platelets. The generic elicitation of either TPO or c-Mpl in mice reduces the levels of all of these cell types by 70–90%, establishing that TPO-induced signaling plays a major physiological role in hematopoiesis in general and megakaryopoiesis in particular (2, 3). The intracellular signaling pathways utilized by c-Mpl have been extensively studied, focusing primarily on tyrosine phosphorylation events. After stimulation with TPO, the c-Mpl receptor is believed to alter its conformation resulting in a homodimeric receptor complex capable of supporting the transphosphorylation and activation of JAK2 and TYK2 tyrosine kinases (4–6). Once activated, JAK kinases phosphorylate a number of substrates, including c-Mpl itself, providing docking sites for Src homology 2- and phospho-tyrosine-binding motif-containing proteins, including the latent transcription factors STAT3 and STAT5 and a number of adapter proteins including Shc, Vav, and Cbl (7, 8). Other signaling intermediates have also been reported to be activated by TPO in various cell lines and primary cells including mitogen-activated protein kinase (MAPK), protein kinase C, and phosphatidylinositol 3-kinase (9, 10).

Despite the many published reports on tyrosine phosphorylation of cytokine receptors, it is apparent that other mechanisms for signal transduction must exist. For instance, a mutant receptor of c-Mpl, in which all of the tyrosine residues were eliminated by deletion or Phe substitution, is capable of supporting cellular proliferation in cytokine-responsive cell lines (4). Moreover, it has been reported that elimination of the proximal nine residues of the Mpl receptor eliminates JAK2 activation but is capable of supporting cell growth (11). Candidate sites for supporting alternate signaling pathways are phosphorylation of Ser and/or Thr residues.

The murine c-mpl gene encodes a polypeptide predicted to contain a 25-residue secretory leader, a 457-amino acid extracellular domain, a 22-residue transmembrane domain, and a 121-amino acid cytoplasmic domain (12). The cytoplasmic domain of the Mpl receptor contains 5 Tyr, 8 Thr, and 13 Ser residues. However, it is clear from both in vitro and in vivo studies that distal truncation of up to one-half of the receptor cytoplasmic domain maintains its capacity to support cell proliferation and differentiation (4, 13). To facilitate our analysis of the Ser and Thr phosphorylation of c-Mpl, we have utilized a truncated form of the receptor bearing the membrane-proximal 69 residues of the cytoplasmic domain (T69), and site-specific Ser and Thr to Ala mutants of this receptor. T69 includes two Tyr residues that are not phosphorylated in response to TPO stimulation in BaF3/mpl cells and contains four Thr and eight Ser residues. In this study, we demonstrated that at least four of the Ser residues in T69 are phosphorylated and that the Thr residues are not modified. Furthermore, multiple substitution

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† The abbreviations used are: TPO, thrombopoietin; rmTPO, recombinant murine TPO; Mpl, myeloproliferative leukemia virus proto-oncogene product; JAK, Janus kinase; STAT, signal transducer and activator of transcription; MAPK, mitogen activated protein kinase; PKC, protein kinase C; IL, interleukin; FCS, fetal calf serum; MTI, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TNF, tumor necrosis factor; EPO, erythropoietin; GM-CSF, granulocyte-macrophage colony-stimulating factor; CK, casein kinase; PAGE, polyacrylamide gel electrophoresis.
of all Ser(P) residues significantly reduced the capacity of the receptor to support cellular proliferation. Moreover, we found that the Ser at position 18 is critical for JAK2 binding to the receptor and cellular proliferation although it is neither constitutively nor inducibly phosphorylated. These results provide the first systematic structure-function analyses of the Ser and Thr residues of the c-Mpl receptor.

**Experimental Procedures**

**Reagents**—Purified recombinant murine TPO (rmTPO) was the generous gift of Dr. Akihiro Shimosaka (Kurin Pharmaceuticals, Tokyo, Japan). Western blot chemiluminescence reagents were purchased from PerkinElmer Life Sciences, and all other reagents were purchased from Sigma unless otherwise indicated.

**Cell Lines and Site-directed Mutagenesis**—The mammalian expression vector, pcDNA3 (Invitrogen, Carlsbad, CA), containing murine c-mpl, was used as a template to make multiple Ser and Thr to Ala mutations using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA). The PCR products were sequenced to confirm the result of mutagenesis with the BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The murine interleukin (IL)-3-dependent cell line BaF3 was engineered to express the full-length murine Mpl receptor (BaF3/Mpl), the T69 truncation (BaF3/T69), and the site-directed T69 mutants by electroporation and limiting dilution into 1 ml of phosphate-free RPMI 1640 medium (Life Technologies, Inc.) with 0.5% bovine serum albumin for 14 h and labeling with 2.5 mCi of [32P]orthophosphate (PerkinElmer Life Sciences) for 18 h and separated on cellulose coated 20 × 20-cm thin layer chromatography (TLC) glass plates (Merck) in 10% acetic acid, 1% pyridine (v/v) buffer at 1000 V for 2 h at 10 °C. The plate was then used for TLC in the second dimension for 5 h in a buffer of 30% 1-butanol, 30% pyridine, 6% glacial acetic acid (v/v/v). The radioactive spots were visualized and quantitated by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

**Immunoprecipitation and Western Blot Analysis**—BaF3/T69 cells and cells bearing the S1A mutation were incubated in serum- and cytokine-free medium for 14 h, stimulated with or without 25 ng/ml rmTPO for 10 min, and lysed as described previously (4). The protein concentration of the lysate was measured using the Protein DC assay kit (Bio-Rad). Specific proteins were immunoprecipitated with the indicated antibodies and protein A/G-conjugated agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were subjected to SDS-PAGE and Western blot analysis as described previously using anti-JAK2 and anti-phosphotyrosine (4G10) antibodies obtained from Upstate Biotechnology (Lake Placid, NY) (4).

**Results**

**Phosphoamino Acid Analysis of the Mpl and T69 Receptors**—We previously reported that two tyrosine residues in c-Mpl are phosphorylated after stimulation with TPO (4). The cytoplasmic domain of c-Mpl includes 13 Ser and 8 Thr residues, but little is known about their phosphorylation status and functional role in TPO-induced signaling. To explore whether some of these residues were constitutively or inducibly phosphorylated, BaF3 cells were engineered to express full-length c-Mpl receptor (BaF3/mpl) or a truncation mutant containing the membrane-proximal 69 amino acids of the cytoplasmic domain of c-Mpl (Fig. 1, T69) and then metabolically labeled with [32P]orthophosphate. The phosphorylation status of the receptor was analyzed before and after stimulation with TPO by phosphoamino acid analysis (Fig. 2). In BaF3/mpl cells, Ser residues were constitutively phosphorylated, and their radioactive intensities were enhanced 3-fold following stimulation with TPO (Fig. 2B). Tyrosine phosphorylation was also markedly enhanced upon stimulation by TPO, but Thr phosphorylation was barely detectable in BaF3/mpl cells under any conditions. Like BaF3/mpl cells, constitutive and TPO-inducible phosphorylation of Ser was also observed in T69 (Fig. 1, T69) and then metabolically labeled with [32P]orthophosphate. The phosphorylation status of the receptor was analyzed before and after stimulation with TPO by phosphoamino acid analysis (Fig. 2). In BaF3/mpl cells, Ser residues were constitutively phosphorylated, and their radioactive intensities were enhanced 3-fold following stimulation with TPO (Fig. 2B). Tyrosine phosphorylation was also markedly enhanced upon stimulation by TPO, but Thr phosphorylation was barely detectable in BaF3/mpl cells under any conditions. Like BaF3/mpl cells, constitutive and TPO-inducible phosphorylation of Ser was also observed in T69 cells. As anticipated from the results of our previous studies utilizing Western blotting with a Tyr(P)-specific antibody (4), neither constitutive nor inducible Tyr phosphorylation of the T69 receptor was detected.

**Tryptic Peptide Mapping of the T69 Mpl Receptor**—Since one or more of the Ser residues of c-Mpl and T69 were found to be phosphorylated, we performed additional studies to identify which Ser residues were modified. As shown in Fig. 1, a predicted trypptic peptide map suggests that the complete digestion of T69 with trypsin should generate nine peptides, four of...
which bear one or more potential sites of Ser phosphorylation. To begin to determine the sites of Ser(P), we performed phosphopeptide mapping of BaF3/T69 cells (Fig. 3). We found that seven phosphopeptides were reproducibly generated by tryptic digestion of [32P]metabolically labeled T69. The intensity of all the radioactive spots was enhanced after stimulation with TPO (Fig. 3B). Although it is possible that an extracellular domain peptide was labeled, it was more likely that incomplete digestion of the intracellular domain of Mpl was responsible for our finding more than the four predicted labeled peptides. For example, although trypsin digests polypeptides following lysine (Lys) and arginine (Arg) residues, it cuts poorly if the basic residue is preceded by a charged residue. Since one predicted tryptic fragment (SSESTPLPL) has Ser in the first position (Fig. 1), if phosphorylated it might prevent complete trypsin digestion at that site and lead to an extra peptide. Subsequent digestion at that site and lead to an extra peptide. Subsequent analysis of a mutant Mpl receptor bearing multiple Ser to Ala mutations (see below) confirmed that all of the receptor phosphorylation events can be accounted for by Ser phosphorylation within the cytoplasmic domain of Mpl.

To identify which of the 8 Ser residues within T69 are phosphorylated, multiple Ser to Ala T69 receptor mutants were generated, and phosphopeptide maps of each were determined (Fig. 4). As the phosphopeptide map of T69/S18A was identical to T69, we established that Ser-18 was not constitutively or inducibly phosphorylated (compare Figs. 4A and 3B). The remaining predicted tryptic phosphopeptides all contained more than a single Ser residue, requiring the generation of multiple Ser to Ala mutants. A phosphopeptide map of T69/S37A/S39A lost two radioactive spots compared with that of T69 (Fig. 4B), indicating that either one or both of Ser-37 and Ser-39 were phosphorylated in response to TPO. As this mutant retained full proliferative activity (see below), we did not further analyze these sites. Like T69/S37A/S39A, the T69/S46A/S53A double mutant also lost two [32P]-radiolabeled spots in the phosphopeptide map (Fig. 4C). A single T69/S46A mutant also lost the same spots as T69/S46A/S53A (Fig. 4D), indicating that Ser-46 is phosphorylated. This conclusion could be extended; our finding an identical phosphopeptide map of T69/S53A and T69 indicated that only Ser-46 was phosphorylated within this peptide (compare Figs. 4E and 3B). There are three Ser residues and one Thr residue in the last predicted tryptic peptide, SSESTPLPL (Fig. 1). Analysis of the T69/S61A/S62A/S64A/T65A mutant revealed the loss of two radiolabeled peptides (Fig. 4F), consistent with at least one of the three Ser sites being phos-
phorylated, with partial tryptic digestion prior to Ser-61 accounting for the loss of two phosphopeptides. Additional T69 mutants were analyzed to dissect the phosphorylation status of this region. Interestingly, compared with T69, T69/S61A/S62A (Fig. 4G) and T69/S64A/T65A (Fig. 4H) both lost the same spots as did the T69/S61A/S62A/S64A/T65A mutants (Fig. 4F). It is possible that like the p55 tumor necrosis factor (TNF) receptor, heat shock factor-1, and rhodopsin proteins (15–17), phosphorylation of one Ser residue might be responsible for phosphorylation of subsequent Ser residues, helping to explain why the same spots were lost in different mutations. Nevertheless, the results in Fig. 4, G and H, indicate that Ser-61 (due to the partial digestion at Lys-60/Ser-61) and Ser-64 (since Thr-65 is not phosphorylated), and possibly Ser-62, are phosphorylated in TPO-stimulated BaF3 cells bearing these mutant receptors. Finally, to be certain that all of the sites of Ser phosphorylation were accounted for by Ser-37, Ser-39, Ser-46, Ser-61, Ser-62 and Ser-64, a mutant Thr-69 receptor bearing Ser to Ala changes at all these sites (and at Thr-65; referred as T69/S37A/S39A/S46A/S61A/S62A/S64A/T65A) was tested and found to be devoid of Ser(P) in 32P-labeled, TPO-stimulated BaF3 cells (Fig. 5). In these experiments the same number of 32P-labeled T69 cells were used as a positive labeling control. This result confirmed that these residues contain the sole sites for Ser phosphorylation of the proximal 69 residues of the c-Mpl receptor.

Mutant T69 Receptor Cell Proliferation Assays—To determine if any of the Ser residues of T69 play a functional role in TPO-induced BaF3 cells, the cell lines used in our Ser(P) analyses were evaluated in cell proliferation assays. By using only clones that express the same level of the mutant receptors as seen in BaF3/T69 cells, we found that BaF3 cells bearing the S37A/S39A, S46A, S53A, S61A/S62A, and S64A/T65A mutant T69 receptors displayed a nearly identical dose-response proliferation curve as BaF3/T69 cells (Fig. 6, A and C–G). To exclude the possibility that the function of these Ser residues is redundant, we also tested T69/S37A/S39A/S46A/S61A/S62A/S64A/T65A cells; we found >50% reduction of proliferation in response to TPO stimulation (Fig. 6H). These results suggest that although any single Ser(P) may not be crucial, the presence of some Ser(P) within the first 69 cytoplasmic domain of Mpl contribute to the capacity of the Mpl receptor to support the signals responsible for cell proliferation.

Signaling Analysis of Hypoproliferative T69 Mutants—Fig. 6B displays the proliferation response of BaF3/T69/S18A cells; in essence, this single mutation nearly abrogated the capacity of this receptor to support BaF3 cell growth. As Ser-18 resides within box 1 of Mpl, the site of JAK2 binding, we examined the hypothesis that loss of the JAK2 signal was responsible for extremely poor response of BaF3/T69/S18A cells to TPO. JAK2 activation in BaF3/T69/S18A cells was analyzed by Western blotting. In contrast to BaF3/T69 cells, where JAK2 kinase was inducibly tyrosine-phosphorylated within minutes of exposure to TPO, its phosphorylation was markedly reduced in T69/S18A cells (Fig. 7A) despite equal protein loading (Fig. 7B). Although JAK2 binds to T69 constitutively, and their association is increased by TPO stimulation (Fig. 7C), we could not detect JAK2 binding to the T69/S18A receptor before or after stimulation with TPO (Fig. 7C). These results indicate that Ser-18 contributes to JAK2 binding and is thereby responsible for cellular proliferation in BaF3 cells. A similar analysis was conducted using T69/S37A/S39A/S46A/S61A/S62A/S64A/T65A cells, to test whether their impaired response to TPO is due to altered JAK2 activation. As shown in Fig. 7, E and F, we found that JAK2 activation was reduced in the mutant cells in proportion to the level of TPO-induced proliferation.

DISCUSSION

The molecular basis for cytokine responsiveness and cellular proliferation is an intensely studied topic. Several groups have worked out many of the details of the signals that emanate from-c-Mpl tyrosine phosphorylation, but little is known of the Ser/Thr phosphorylation of the receptor. In this study we demonstrate that both full-length c-Mpl and T69 are constitutively phosphorylated on Ser, the levels of which are enhanced following stimulation with TPO. The most important findings in the studies presented here are as follows. 1) We have mapped the Ser and Thr phosphorylation pattern of the region between the transmembrane domain and Leu-69 of the murine Mpl receptor (Ser-37 and/or Ser-39, Ser-46, Ser-61, Ser-64, and possibly Ser-62 are phosphorylated, and Ser-18 and Ser-53 are definitely not phosphorylated), a region sufficient to support cellular proliferation. 2) Phosphorylation of Ser residues of Mpl in BaF3 cells contributes substantially to the capacity of the Mpl receptor to support cellular proliferation (Figs. 6H). 3) Ser-18 is critical for the interaction of Mpl and JAK2 and thus is vital for TPO-induced signal transduction.

The erythropoietin (EPO) receptor is also known to be phosphorylated on Ser and Tyr but not on Thr residues (18). Since TPO and EPO and their receptors display sequence similarities and are almost certainly related on an evolutionary basis, our finding of a conserved phosphorylation pattern is interesting. In contrast, the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor β subunit and the interleukin (IL)-6 receptor β subunit are phosphorylated on Tyr, Ser, and Thr residues (19, 20). However, the functional relevance of Ser and Thr phosphorylation of cytokine receptors is poorly understood. To explore this aspect we used Ser to Ala receptor mutants and assessed their phosphorylation and capacity to support cell proliferation. We found that although alteration of any single Ser residue (except Ser-18) failed to significantly impact the capacity of Mpl to support cellular proliferation, alteration of all sites of Ser(P) from the receptor substantially blunted BaF3 cell growth at all concentrations of TPO tested (Fig. 6H). Although we cannot rule out the possibility that the tertiary structure of the cytoplasmic domain of Mpl might be subtly altered, leading to a global reduction in function, it is also possible that only specific signals are affected. Furthermore, we found that the capacity of the mutant receptor to stimulate JAK2 activation was reduced proportionately to the diminished TPO-induced proliferation of these cells. Interestingly, an observation reported by Sawyer and Penta (18) may relate to our findings. These investigators found that only a highly modified form of the EPO receptor, which was glycosylated and phosphorylated on Ser residues, associated with JAK2.

Perhaps the most surprising result to come from our studies is that alteration of Ser-18 nearly eliminated association of Mpl
and JAK2 and caused a profound reduction in the capacity of this receptor to support cell proliferation. We do not feel this conclusion is due to trivial technical problems, as the conclusion is based on the following: 1) performing multiple proliferation assays employing three separate clones of BaF3/T69/S18A cells; 2) these same cell clones grew well in murine IL-3; 3) surface expression in each line was equivalent to that of the truncated but otherwise wild type T69 receptor; and 4) the $M_r$ of the immunoprecipitated receptor matched that of T69 (Fig. 7). Thus, there is ample evidence that technical reasons do not account for our failure to observe a proliferative response to TPO in these cells.

Murakami and colleagues (21) were the first to identify the "box 1" PXXPo rP motif conserved in the intracytoplasmic, membrane-proximal region of all members of the hematopoietic cytokine receptor family and recognized its importance in cytokine-induced activation of cytoplasmic tyrosine kinases. However, alignment of these sequences from multiple cytokine receptors that employ JAK2 (EPO receptor, GM-CSF receptor, Mpl, and gp130) reveals that the internal positions between the two Pro residues are poorly conserved, suggesting they play little role in JAK recruitment or activation. Although cytokine receptors were initially thought to recruit cytoplasmic JAK kinases to the receptor upon ligand-induced dimerization, more recent studies suggest that the Mpl receptor, like most hematopoietic cytokine receptors, displays a basal level of receptor-JAK interaction. The results shown in Fig. 7 indicate that mutation of Ser to Ala at position 18 eliminates all or most of the constitutive association of Mpl with JAK2, as well as that induced by TPO binding. Thus, JAK2 is very minimally phosphorylated in response to TPO stimulation of BaF3/T69/S18A cells. The loss of TPO-induced proliferation in these cells is thus consistent with the majority of studies of Mpl signaling indicating that JAK2 activation is critical for TPO-induced cell signaling (4, 22). This result contradicts that of Dorsch and colleagues (11) in which a membrane-proximal deletion mutant of Mpl, which retained the box 1 and all distal sequence motifs but lost JAK2 activation, retained its capacity to support cel-
cular proliferation in a TPO-dependent manner. The explanation for the discrepancy of the findings is not immediately clear, but our results add to the accumulating evidence that JAK2 activation is vital for hematopoiesis. Moreover, the structural basis for the loss of JAK2 binding and TPO-induced activation in the S18A Mpl mutant is not clear; whether the potential for hydrogen bonding through Ser exceeds that of Ala at position 18 or whether a subtle tertiary structural change was introduced by Ser to Ala substitution at this position are uncertain.

The results of our Ser(P) mapping experiments might also shed some light on the process of Ser phosphorylation in cytokine receptors. For example, T69/S61A/S62A and T69/S64A/T65A cells lost the same radiolabeled phosphopeptides in mapping experiments, suggesting that phosphorylation of residues Ser-61, Ser-62, or Ser-64 is dependent on one or two of the other Ser(P) residues. This result is similar to that with Ser/Thr phosphorylation of the p55 TNF receptor, where phosphorylation of Thr-236 and Ser-270 enable subsequent phosphorylation of Ser-240 and Ser-244 (14). Similar hierarchical and synergistic phosphorylation events were also reported for rho-protein signal transduction (15) and for the TNF receptor (25). In addition, if Ser-37 is phosphorylated, it is immediately followed by Pro and hence represents a site for a Pro-directed Ser/Thr kinase, such as c-Jun NH2-terminal kinase, p38 MAPK, and p44/42 MAPK. Although it is clear that p44/p42 MAPK is activated by TPO stimulation (9), the activity of CKI and CKII following exposure to TPO has not yet been studied. Further work will be necessary to establish the responsible Ser kinases for c-Mpl phosphorylation.

In conclusion, we have identified a critical role for the Ser residue at position 18 of Mpl in TPO-induced cell proliferation, and we have mapped several sites of receptor Ser phosphorylation. The functional role of these individual sites appears at least partially redundant for cellular proliferation, and it remains possible that other physiologic functions of Mpl, such as receptor internalization or cellular differentiation, are also dependent on the modification of these sites. Only further studies of the Mpl receptor will provide a better understanding of the molecular basis of platelet production.

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