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Tyrosine-599 of the c-Mpl receptor is required for Shc phosphorylation and the induction of cellular differentiation

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Interaction of thrombopoietin (TPO) with its receptor, c-Mpl, triggers cell growth and differentiation responses controlling primitive haemopoietic cell production and megakaryocytopoiesis. To examine the important receptor domains and signal transduction pathways involved in these cellular responses, c-Mpl cytoplasmic domain truncation and tyrosine substitution mutants were generated. In the myelomonocytic leukaemia cell lines WEHI3B-D$^+$ and M1, ectopic expression of the wild-type c-Mpl receptor induced TPO-dependent cellular differentiation characterized by increased cell migration through agar and acquisition of the morphology and molecular markers of macrophages. Consistent with the concept that proliferative and differentiation signals emanate from distinct receptor domains, the C-terminal 33 amino acids of c-Mpl were dispensable for a proliferative response in Ba/F3 cells but proved critical for WEHI3B-D$^+$ and M1 differentiation. Finer mapping revealed that substitution of Tyr599 by phenylalanine within this c-Mpl domain was sufficient to abolish the normal differentiation response. Moreover, in contrast to the normal c-Mpl receptor, this same *mpl*Y599F mutant was also incapable of stimulating TPO-dependent Shc phosphorylation, the association of Shc with Grb2 or c-Mpl and of inducing c-*fos* expression. Thus activation of components of the Ras signalling cascade, initiated by interaction of Shc with c-Mpl Tyr599, may play a decisive role in specific differentiation signals emanating from the c-Mpl receptor.

Keywords: c-Mpl/differentiation/Shc/signal transduction/thrombopoietin

Introduction

The c-*mpl* gene, first discovered as the cellular homologue of the v-*mpl* retroviral oncogene (Souyri *et al.*, 1990), encodes a member of the haemopoietin receptor superfamily, a group of transmembrane proteins characterized by conserved extracellular domain motifs (Gearing *et al.*, 1989). The c-Mpl ligand, recently identified as thrombopoietin (TPO; Bartley *et al.*, 1994; deSauvage *et al.*, 1994; Lok *et al.*, 1994), plays a prominent role in haemopoiesis, particularly in the regulation of megakaryocyte production. Studies with purified TPO in culture revealed that alone, or in combination with other cytokines, TPO stimulates proliferation of megakaryocyte progenitor cells (Kaushansky *et al.*, 1994; Banu *et al.*, 1995; Broudy *et al.*, 1995; Hunt *et al.*, 1995) as well as their maturation to mature, polyploid megakaryocytes (Kaushansky *et al.*, 1994; Ziegler *et al.*, 1994; Banu *et al.*, 1995; Broudy *et al.*, 1995; Choi *et al.*, 1995; Debili *et al.*, 1995). These properties result in significantly elevated platelet numbers in animals injected with or constitutively overexpressing TPO (Kaushansky *et al.*, 1994; Lok *et al.*, 1994; Farese *et al.*, 1995; Ulich *et al.*, 1995; Yan *et al.*, 1995; Harker *et al.*, 1996) and can ameliorate the thrombocytopenia associated with administration of cytotoxic drugs (Hokom *et al.*, 1995; Ulich *et al.*, 1995). The indispensable role of TPO and c-Mpl in megakaryocytopoiesis *in vivo* was demonstrated in animals genetically manipulated to lack these molecules. Such mice produce dramatically reduced numbers of megakaryocytes and their progenitors, resulting in severe thrombocytopenia (Gurney *et al.*, 1994; Alexander *et al.*, 1996; deSauvage *et al.*, 1996). The role of TPO and c-Mpl is not restricted to megakaryocytopoiesis. Deficiencies in progenitor cells of other haemopoietic lineages in *mpl*$^{-/-}$ mice (Alexander *et al.*, 1996) also suggest a role for TPO in the production of immature haemopoietic cells. Indeed, recent studies *in vitro* support a direct role for TPO in supporting proliferation of primitive haemopoietic progenitors (Ku *et al.*, 1996).

The spectrum of biological activities exhibited by TPO suggests that activation of c-Mpl generates both proliferative and differentiation signals. Studies with the granulocyte colony-stimulating factor (G-CSF) receptor and the gp130 signalling chain suggest that the intracellular domains of these receptors may be functionally sub-divided, with a membrane-proximal region critical for proliferation and a more distal domain providing differentiation-specific signals (Dong *et al.*, 1993; Fukunaga *et al.*, 1993; Yoshikawa *et al.*, 1995; Yamanaka *et al.*, 1996). Similarly, the interleukin (IL)-7 receptor α-chain transmits distinct signals for B-lymphoid growth and differentiation (Corcoran *et al.*, 1996). Emerging evidence suggests that distinct regions of c-Mpl may also contribute to proliferative and differentiation responses (Porteu *et al.*, 1996). Following TPO binding, the biochemical changes within cells expressing c-Mpl appear to conform generally to the pattern observed with activation of other members of the haemopoietin receptor family. Stimulation of c-Mpl by TPO binding induces receptor phosphorylation and activation of a largely common set of signalling molecules including members of the Janus kinase (Jak) and signal transducers and activators of transcription (STAT) families, components of the Ras signalling cascade and protein tyrosine phosphatases (Drachman *et al.*, 1995; Gurney *et al.*, 1995; Miyakawa *et al.*, 1995, 1996; Morella *et al.*, 1995; Mu *et al.*, 1995; Pallard *et al.*, 1995; Sattler *et al.*,

1995; Tortolani *et al.*, 1995). While distinctive regions of the c-Mpl cytoplasmic domain have been linked with Jak/STAT and Shc activation (Gurney *et al.*, 1995; Morella *et al.*, 1995), the precise roles that such molecules may play in particular cellular responses remain unknown.

In order to dissect the important receptor domains and signal transduction pathways involved in mediating proliferative and differentiation responses, we generated c-Mpl cytoplasmic domain mutants, including progressive C-terminal truncations and tyrosine substitutions. Several laboratories, including our own, have manipulated factor-dependent cell lines such as Ba/F3 and FDC-P1 to study proliferative responses mediated by c-Mpl (Skoda *et al.*, 1993; Benit *et al.*, 1994; Lok *et al.*, 1994; Wendling *et al.*, 1994; Alexander *et al.*, 1995; Gurney *et al.*, 1995). Here we show that c-Mpl signals TPO-dependent macrophage differentiation when ectopically expressed in the WEHI3B-D$^+$ or M1 myelomonocytic leukaemia cell lines. While largely dispensable for Ba/F3 proliferation, a C-terminal domain of c-Mpl was critical for this differentiation induction, and finer mapping revealed that a single tyrosine residue within this region was required for the differentiation response. The same tyrosine was also essential for activation of Shc and induction of c-*fos* mRNA following TPO stimulation, suggesting that the Ras signalling cascade may make an important contribution to differentiation responses induced by TPO.

Results

To dissect the signal transduction domains of the c-Mpl receptor, a set of truncation mutants bearing progressive C-terminal deletions was constructed (Figure 1). MplIC88 lacks the C-terminal 33 receptor amino acids but retains the membrane-proximal region including the box1 and box2 motifs that are conserved among most haemopoietin receptors. In MplIC36, a further 52 residues were deleted, including box2, and MplTM retains only one intracellular amino acid. To assess the capacity of these receptors to stimulate proliferation, each mutant cDNA was incorporated into the LXSN retroviral vector (Miller and Rosman, 1989) and expressed in the IL-3-dependent Ba/F3 cell line. Three independent clones expressing each receptor were selected by fluorescence-activated cell sorting (FACS; Figure 1) and analysed in microwell proliferation assays. Because Ba/F3 cells lack endogenous c-Mpl receptors, upon withdrawal of IL-3 control Ba/F3-LXSN cells died (Figure 2). However, Ba/F3 cells expressing the wild-type receptor proliferated in response to TPO, exhibiting maximal growth at 0.8 ng/ml with no evidence of morphological alteration. As previously described (Gurney *et al.*, 1995; Porteu *et al.*, 1996), the C-terminal domain of c-Mpl was largely dispensable for proliferation. Dose–response analysis revealed that Ba/F3-*mpl*IC88 cells proliferated in response to TPO concentrations within 3- to 4-fold of those stimulating Ba/F3-*mpl* cells (Figure 2). However, further deletion of 52 amino acids encompassing the conserved box2 motif dramatically impaired the proliferative response. TPO concentrations up to 200-fold higher than normal were required to induce Ba/F3-*mpl*IC36 proliferation, and a maximal response was observed only in one of three clones tested (Figure 2). Further deletion of the box1 motif abolished all proliferative response

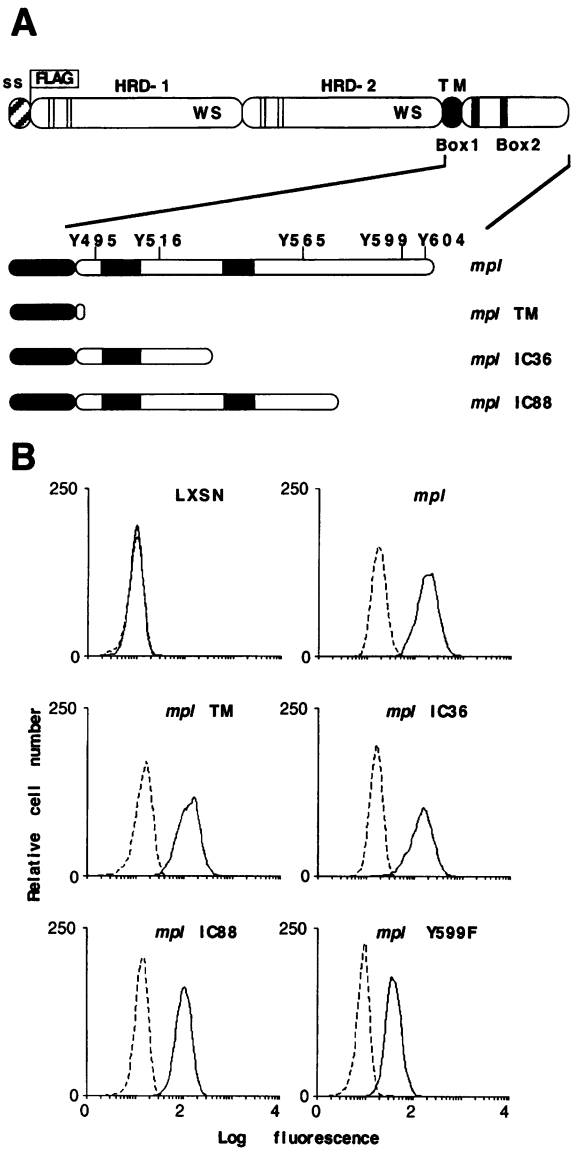

Fig. 1. c-Mpl receptor mutants. (**A**) The structure of the wild-type c-Mpl receptor is represented schematically including the signal sequence (SS), two haemopoietin receptor domains (HRD) with conserved cysteine residues (vertical lines) and WSXWS motif (WS), transmembrane domain (TM) and cytoplasmic tail including the box1 and box2 motifs conserved with other members of the haemopoietin receptor family. All Mpl receptors used had a FLAG octapeptide tag incorporated at the N-terminus. The structures of the progressive C-terminal truncation mutants which retain 88 (*mpl*IC88), 36 (*mpl*IC36) or one (*mpl*TM) intracellular amino acids are shown below. The positions of the five c-Mpl intracellular tyrosine residues, that were mutated to phenylalanine in this study, are also indicated, with numbers representing amino acid positions in the mature c-Mpl receptor. (**B**) Cell surface expression of wild-type (*mpl*), truncation (*mpl*TM, *mpl*IC36 and *mpl*IC88) and *mpl*Y599F mutant receptors in representative clones of Ba/F3 cells. Cells were stained with the M2 anti-FLAG antibody (unbroken line) or isotype control antibody (broken line) and analysed by flow cytometry as described in Materials and methods.

(*mpl*TM, Figure 2). Similar results were also obtained in proliferation assays performed on FDC-P1 cells expressing the wild-type or mutant c-Mpl receptors (data not shown).

c-Mpl signals differentiation in WEHI3B-D$^+$ cells

To study the role of c-Mpl in non-proliferative cellular responses, we examined the capacity of the receptor to

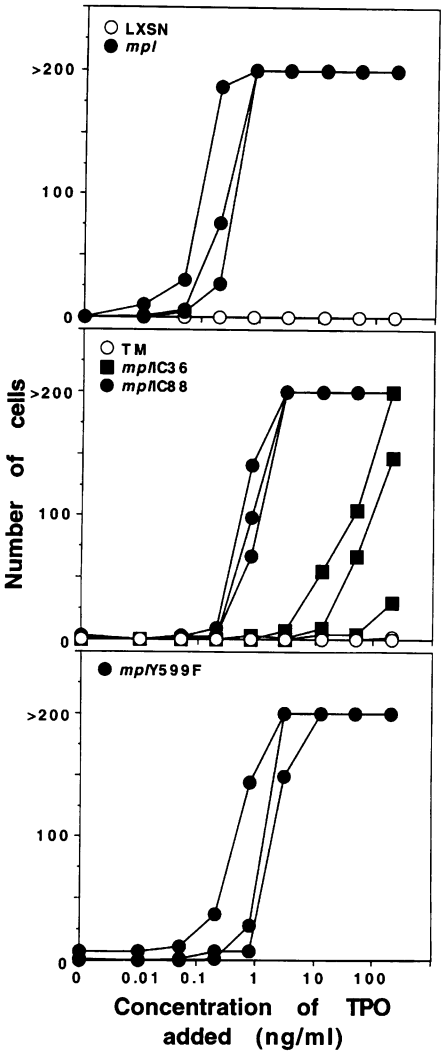

Fig. 2. Proliferative response to TPO of control Ba/F3 cells (LXSN) or Ba/F3 cells expressing the wild-type c-Mpl receptor (*mpl*), truncated (*mpl*TM, *mpl*IC36 and *mpl*IC88) or *mpl*Y599F mutants. A total of 200 cells in 10 μl were added to 5 μl of serially diluted concentrations of TPO and the numbers of viable cells were scored microscopically after 48 h. The mean of duplicate counts for each of three independent Ba/F3 cell clones expressing each mutant are shown.

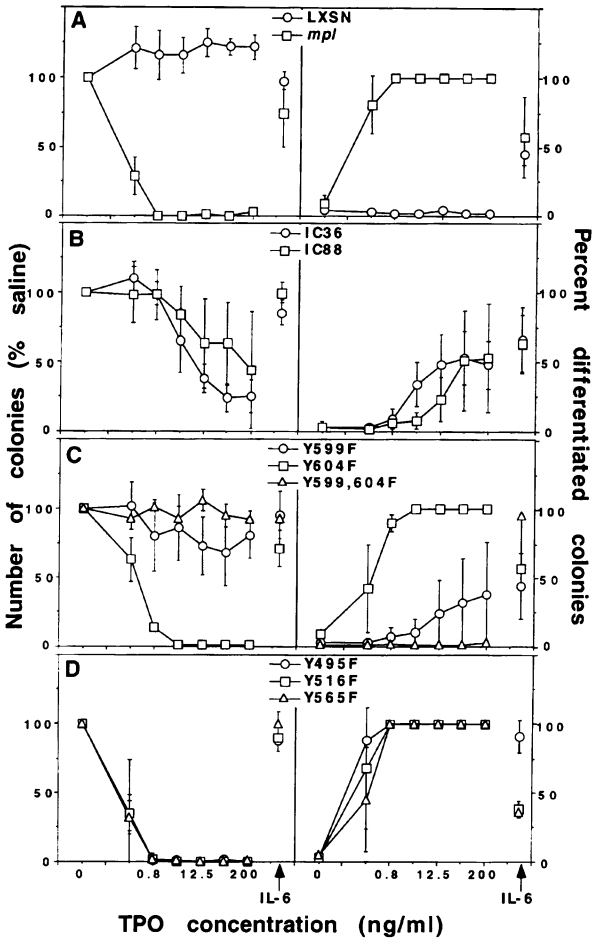

Fig. 3. TPO-dependent differentiation of WEHI3B-D$^+$ cells expressing c-Mpl receptor mutants. Cells expressing the control vector or wild-type c-Mpl receptor (LXSN and *mpl*, **A**), truncated receptors (*mpl*IC36 and *mpl*IC88, **B**) or specific tyrosine→phenylalanine mutants (*mpl*Y599F and *mpl*Y604F, **C**; *mpl*Y495F, *mpl*Y516F and *mpl*Y565F, **D**) were cultured in agar with increasing concentrations of TPO, or with saline or IL-6 (20 ng/ml). Colonies were enumerated (left panels) and scored for differentiation (right panels) after 7 days. The mean and standard error of duplicate assays for several independent WEHI3B-D$^+$ clones expressing each receptor are graphed (LXSN, *mpl*, *mpl*Y495F, *mpl*Y516F, *mpl*Y565F: n = 3; *mpl*IC36, *mpl*IC88, *mpl*Y599F: n = 5; *mpl*Y604F, *mpl*Y599,604F: n = 2).

induce differentiation in WEHI3B-D$^+$ cells. This cell line, established from a murine myelomonocytic leukaemia, proliferates autonomously *in vitro*, but can be induced to undergo granulocyte–macrophage differentiation in response to external stimuli such as G-CSF or IL-6 (Metcalf, 1989). As WEHI3B-D$^+$ cells lack endogenous c-Mpl receptors, in the presence of TPO, control cells (D$^+$-LXSN) remained undifferentiated, forming large compact colonies in agar cultures (Figure 3). However, in WEHI3B-D$^+$ cells expressing the wild-type c-Mpl receptor, dose-dependent differentiation was observed, characterized by a marked reduction in colony numbers and increased cell migration producing dispersed colony morphology (Figure 3). In liquid cultures containing TPO, the clonal suppression observed in agar was reflected by inhibition of D$^+$-*mpl* cell proliferation (Figure 4). Moreover, unlike D$^+$-LXSN cells, within 24 h the D$^+$-*mpl* cultures became adherent and developed a differentiated morphology, with increased cell size, a smaller nuclear to cytoplasmic ratio and prominent vacuolation. Their macrophage identity was confirmed in Northern blot RNA analysis (Figure 5) which revealed induction of expression of the macrophage markers lysozyme (Bonifer *et al.*, 1991) and Mac-1α (Rosmarin *et al.*, 1989) with kinetics consistent with the morphological changes. Expression of the myeloperoxidase gene, which is associated with less mature myeloid cells (Rosmarin *et al.*, 1989), was diminished. Changes in expression of these genes were not observed in D$^+$-LXSN cells.

The C-terminal domain of c-Mpl is required for induction of differentiation

To determine the receptor domains required to mediate differentiation, the c-Mpl truncation mutants were transfected into WEHI3B-D$^+$ cells, and lines expressing each receptor were selected by FACS (see Materials and methods). The response to TPO of five clones expressing each mutant was then analysed in agar cultures. While

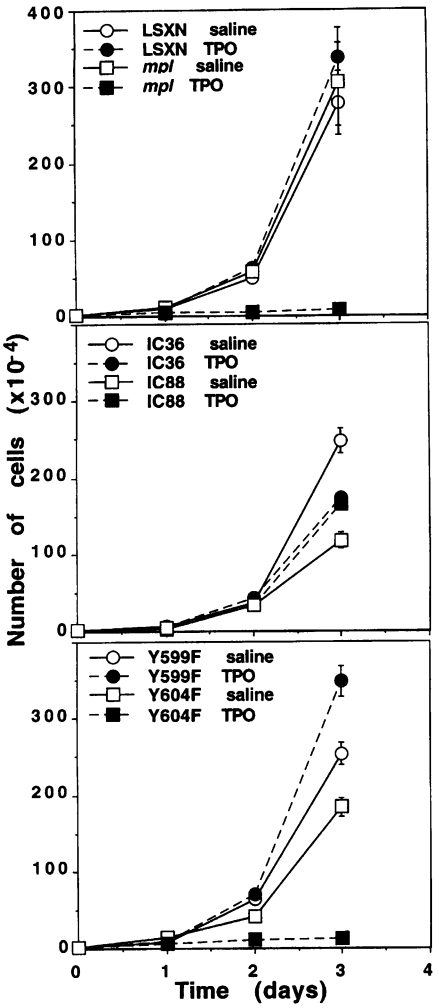

Fig. 4. Growth of WEHI3B-D$^+$ cells in liquid cultures. Control cells (LXSN) or cells expressing the wild-type c-Mpl receptor (*mpl*), truncated (*mpl*IC36 and *mpl*IC88) or *mpl*Y599F or *mpl*Y604F mutants were plated at 2×10^4 cells/ml in 20 ng/ml TPO (closed symbols) or saline (open symbols) and the total cell number was determined daily. The mean and range of duplicate counts are graphed.

complete clonal suppression and differentiation of D$^+$-*mpl* cells occurred at 0.8 ng/ml TPO, no response was observed in D$^+$-*mpl*IC88 cells at similar ligand concentrations. Some D$^+$-*mpl*IC88 differentiation occurred at higher TPO concentrations, but even at cytokine levels of 200 ng/ml remained sub-maximal (Figure 3). As expected, the differentiation response of WEHI3B-D$^+$ cells expressing the more extensively truncated MplIC36 receptors was similarly deficient (Figure 3). The cells had not simply become generally refractory to differentiation because a normal differentiation response to IL-6 was observed (Figure 3). The incapacity of the truncated c-Mpl receptor mutants to signal growth arrest and differentiation efficiently was also evident in liquid cultures. While proliferation of D$^+$-*mpl* cells was inhibited by TPO, the addition of cytokine to cells expressing *mpl*IC36 or *mpl*IC88 did not prevent cell growth (Figure 4).

Thus, distinct regions of the c-Mpl receptor cytoplasmic domain are required to mediate efficient cellular growth and differentiation. While the membrane-proximal region of c-Mpl is sufficient for proliferative signals in Ba/F3

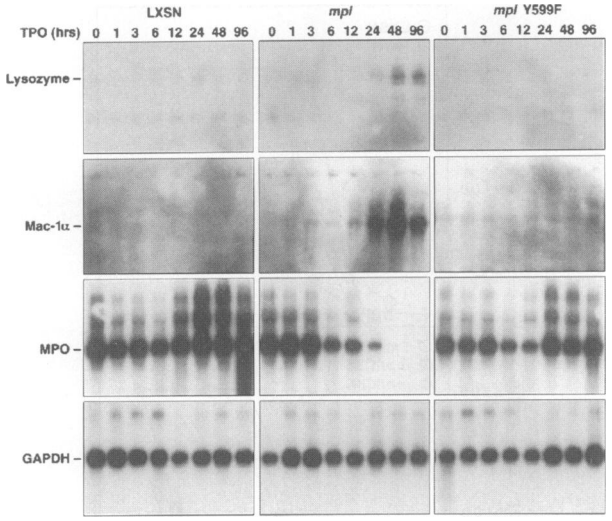

Fig. 5. Myeloid-specific gene expression in WEHI3B-D$^+$ cells. D$^+$-LXSN, D$^+$-*mpl* or D$^+$-*mpl*Y599F cells were stimulated with TPO (20 ng/ml) for the times indicated and then poly(A)$^+$ RNA was extracted, separated in denaturing agarose gels (1 μg), transferred to nylon membranes and hybridized sequentially with cDNA probes to lysozyme, Mac-1α, myeloperoxidase (MPO) and GAPDH.

cells, macrophage differentiation of WEHI3B-D$^+$ cells depends critically on the C-terminal 33 amino acids of the receptor.

c-Mpl Tyr599 is required for induction of differentiation

Phosphorylation of tyrosine residues is considered a crucial process in the initiation of signal transduction from haemopoietin receptors following ligand binding (Ihle, 1996). The c-Mpl intracellular domain contains five tyrosine residues, two of which, Y599 and Y604, are located within the C-terminal 33 amino acids (Figure 1). To determine if phosphorylation at these sites is important in differentiation signals, receptors in which one or both of these residues were mutated to phenylalanine were constructed. When expressed in WEHI3B-D$^+$ cells, MplY604F mediated TPO-dependent clonal suppression and differentiation with a similar dose–response to the wild-type receptor (Figure 3). In contrast, TPO stimulation of D$^+$-*mpl*Y599F cells or cells expressing the *mpl*Y599,604F double mutant did not induce clonal suppression and stimulated at elevated cytokine concentrations only sub-maximal differentiation (Figure 3). As expected, clonal suppression and differentiation with a TPO dose–response similar to that of D$^+$-*mpl* cells was observed in cells expressing c-Mpl receptors substituted at Y495, Y516 or Y565, which lie outside the C-terminal domain (Figure 3). A normal differentiation response to IL-6 was observed in agar cultures of WEHI3B-D$^+$ cell lines expressing each of the various tyrosine substitution mutants (Figure 3). Unlike D$^+$-*mpl* cells or cells expressing other tyrosine→phenylalanine mutants, proliferation of D$^+$-*mpl*Y599F cells was not inhibited by TPO in liquid cultures (Figure 4) and the cells grew with an undifferentiated morphology. In keeping with their inability to respond to TPO with morphological differentiation, induction of the macrophage markers lysozyme and Mac-1α and the suppression of MPO expression were also not observed in TPO-treated D$^+$-*mpl*Y599F

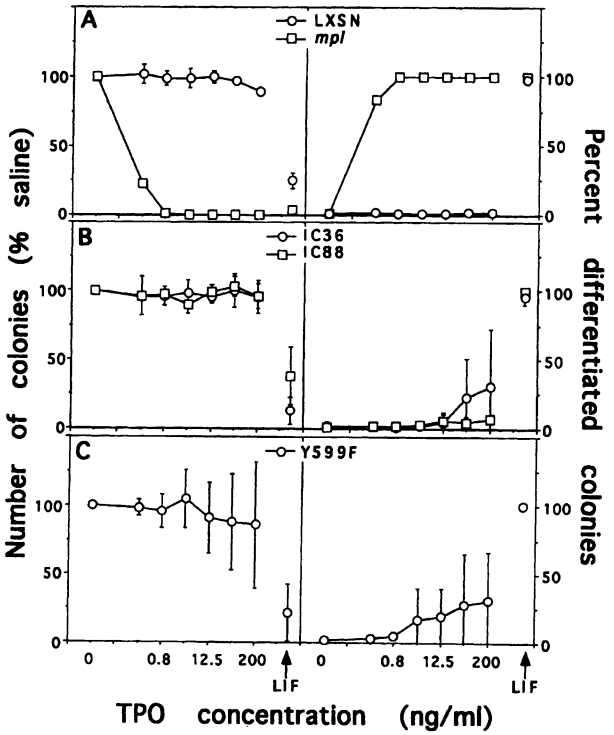

Fig. 6. TPO-dependent differentiation of M1 cells expressing c-Mpl receptor mutants. Cells expressing the control vector or wild-type c-Mpl receptor (LXSN and *mpl*, **A**), truncated (*mpl*IC36 and *mpl*IC88, **B**) or *mpl*Y599F (**C**) mutants were cultured in agar with increasing concentrations of TPO, in saline or in LIF (10 ng/ml). Colonies were enumerated (left panels) and scored for differentiation (right panels) after 7 days. The means and standard errors of duplicate assays for three independent WEHI3B-D$^+$ clones expressing each receptor (except *mpl*Y599F, n = 5) are graphed.

cells (Figure 5). Finally, consistent with the C-terminal 33 c-Mpl amino acids being largely dispensable for Ba/F3 proliferation, Ba/F3 cells expressing *mpl*Y599F proliferated maximally at TPO concentrations within 3- to 4-fold of those required by Ba/F3-*mpl* cells (Figure 2).

M1 is a myelomonocytic leukaemia cell line that can be induced to undergo macrophage differentiation by external stimuli, most notably leukaemia inhibitory factor (LIF) or IL-6 (Metcalf, 1989). To confirm our observations in an additional cell line, the wild-type c-Mpl receptor and the *mpl*IC36, *mpl*IC88 and *mpl*Y599F mutants were expressed in M1 cells and their response to TPO was compared with control M1-LXSN cells. As in WEHI3B-D$^+$ cells, expression of the wild-type receptor resulted in complete clonal suppression and morphological differentiation of M1 cells in agar cultures containing TPO at concentrations down to 0.8 ng/ml (Figure 6). However, while they responded normally to LIF, M1-*mpl*IC36, M1-*mpl*IC88 and M1-*mpl*Y599F cells exhibited essentially no clonal supression and only residual morphological differentiation, even at 200 ng/ml TPO (Figure 6). Thus Tyr599 within the C-terminal domain of c-Mpl is essential for the receptor to signal TPO-dependent differentiation efficiently in myelomonocytic cells.

c-Mpl Tyr599 is required for phosphorylation of Shc and induction of c-fos mRNA

The amino acid sequence upstream of Y599 in c-Mpl, Asn–His–Ser–Tyr, conforms to the consensus for inter-

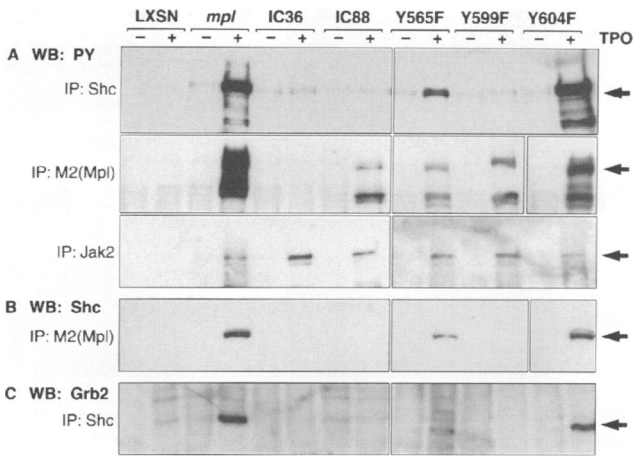

Fig. 7. Shc phosphorylation and complex formation in WEHI3B-D$^+$ cells expressing c-Mpl receptor mutants. (**A**) Cells expressing the control vector (LXSN), the wild-type c-Mpl receptor (*mpl*), truncated (*mpl*IC36 and *mpl*IC88) or specific *mpl*Y565F, *mpl*Y599F or *mpl*Y604F mutants were stimulated with TPO (100 ng/ml) for 7 min then lysed and immunoprecipitates prepared with anti-Shc, M2 (directed against the FLAG epitope tag at the N-terminus of all the receptors) or anti-Jak2 antibodies. Following SDS–PAGE, proteins were Western blotted with an anti-phosphotyrosine antibody (see Materials and methods). The anti-receptor (**B**) and anti-Shc (**C**) immunoprecipitates were also analysed in Western blots with antibodies to Shc and Grb2 respectively to assess formation of Shc–c-Mpl and Shc–Grb2 complexes. In each experiment, membranes were also blotted with the immunoprecipitation antibody to confirm precipitation from all cell extracts (data not shown).

action with the phosphotyrosine binding domain (PTB) of Shc, an adaptor molecule thought to link activation of a wide variety of receptors to the Ras signalling cascade (Laminet *et al.*, 1996). To examine the possibility that this pathway is disrupted by deletion or mutation of Y599 within Mpl and therefore potentially involved in differentiation signals, Shc activation was assessed in WEHI3B-D$^+$ cells expressing the normal or mutant c-Mpl receptors. Consistent with studies in other cells (Drachman, 1995; Gurney *et al.*, 1995; Miyakawa *et al.*, 1995; Mu *et al.*, 1995), rapid phosphorylation of Shc was observed following stimulation of D$^+$-*mpl* cells with TPO (Figure 7A). Moreover, Y599 was required specifically because cells expressing MplY599F or the truncated MplIC36 and MplIC88 receptors, which lack Y599, failed to induce Shc phosphorylation, while D$^+$-*mpl*Y565F and D$^+$-*mpl*Y604F cells responded normally (Figure 7A). The failure of c-Mpl receptors lacking this tyrosine to activate Shc was not simply the result of non-specific receptor inactivation in WEHI3B-D$^+$ cells. Upon stimulation with TPO, the capacity of the D$^+$-*mpl*IC88 and D$^+$-*mpl*Y599F mutants to induce phosphorylation of the Jak2 kinase, as well as the receptors themselves, was still intact (Figure 7A). Co-precipitation experiments also revealed that in D$^+$-*mpl* cells, TPO-induced Shc phosphorylation was accompanied by the association of Shc with the c-Mpl receptor (Figure 7B). Consistent with this interaction occurring directly between Y599 of c-Mpl and the PTB domain of Shc, no association was observed in TPO-stimulated D$^+$-*mpl*Y599F cells. Moreover, unlike wild-type receptors or tyrosine substitution mutants that retain the capacity to induce WEHI3B-D$^+$ differentiation, the truncated receptors and MplY599F were unable to stimulate assembly of Shc–Grb2 complexes (Figure 7C).

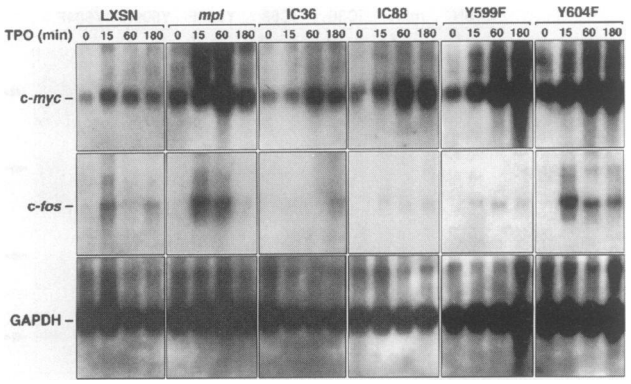

Fig. 8. Immediate-early gene induction in Ba/F3 cells expressing Mpl receptor mutants. Ba/F3 cells expressing the control vector (LXSN), wild-type c-Mpl receptor (*mpl*), truncated (*mpl*IC36 and *mpl*IC88) or specific *mpl*Y599F or *mpl*Y604F mutants were stimulated with TPO (20 ng/ml) for the times indicated and then poly(A)$^+$ RNA was extracted, separated in denaturing agarose gels (1 μg), transferred to nylon membranes and hybridized sequentially with cDNA probes to c-*myc*, c-*fos* and GAPDH.

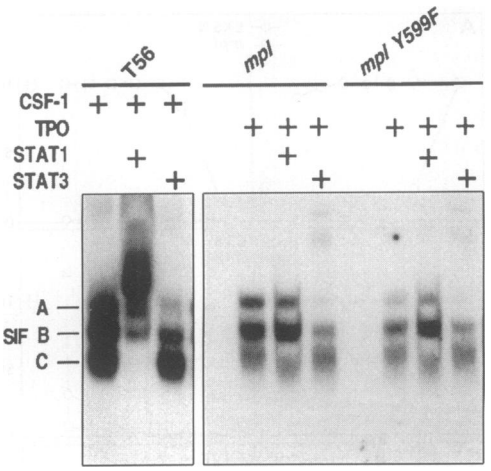

Fig. 9. Activation of STAT3 DNA binding by TPO in WEHI3B-D$^+$ cells expressing the wild-type c-Mpl receptor (*mpl*) or *mpl*Y599F mutant. Total protein extracts were prepared from unstimulated cells (unlabelled lanes) or cells incubated for 5 min with 100 ng/ml TPO (+) and incubated with radiolabelled double-stranded oligonucleotides containing the m67 SIE binding site in the presence (+) or absence of specific antibodies to STAT1 or STAT3. Complexes were resolved by electrophoresis and visualized by autoradiography (see Materials and methods). For reference, the SIF-A, -B and -C complexes induced by CSF-1 in T56 fibroblasts are shown in the left panel.

Stimulation of the Ras pathway is associated with the induction of the c-*fos* immediate-early gene (Barone and Courtneidge, 1995). Consistent with a lesion in this pathway, in Ba/F3 cells expressing *mpl*IC36, *mpl*IC88 or *mpl*Y599F, induction of c-*fos* RNA was not observed after growth factor withdrawal and then subsequent stimulation with TPO (Figure 8). In contrast, the wild-type receptor and MplY604F, which stimulate normal Shc phosphorylation, induced c-*fos* expression within 15 min of TPO addition (Figure 8). The induction of c-*myc* expression did not require Y599 or the C-terminal region of c-Mpl and was induced normally by TPO in Ba/F3-*mpl*IC88 and Ba/F3-*mpl*Y599F cells. Indeed, c-*myc* RNA induction correlated with the capacity of c-Mpl to induce proliferation; delayed *myc* expression was observed in Ba/F3-*mpl*IC36 cells (Figure 8).

Together, these data strongly imply that TPO-dependent phosphorylation of Y599 within the cytoplasmic domain of c-Mpl, which conforms to the Shc PTB binding consensus, recruits and activates Shc. This process appears to stimulate the Ras signalling cascade and correlates precisely with the capacity of the receptor to mediate cellular differentiation in WEHI3B-D$^+$ and M1 cells (see Discussion).

MplY599F stimulates STAT3 DNA binding complexes

Recent studies suggest an important role for STAT3 in gp130-mediated differentiation of M1 cells (Minami *et al.*, 1996; Yamanaka *et al.*, 1996). As STAT3 is known to be activated by c-Mpl stimulation (Gurney *et al.*, 1995; Mu *et al.*, 1995; Sattler *et al.*, 1995; Miyakawa *et al.*, 1996), TPO-dependent STAT3 DNA binding in WEHI3B-D$^+$ cells expressing the wild-type or MplY599F receptors was assessed in electrophoretic mobility shift assays (EMSAs). The high affinity m67 SIE DNA sequence forms complexes with activated STAT3 and STAT1 (SIF-A, B and C) derived from cells stimulated by a variety of factors including CSF-1 (Figure 9 and Novak *et al.*, 1995). TPO also induced the formation of three m67 binding complexes in D$^+$-*mpl* cells, and supershifting with anti-STAT3 antibodies suggested that two of these contained STAT3 (Figure 9). Unlike the SIF complexes, the faster migrating TPO-activated proteins were not supershifted by anti-STAT1 antibodies (Figure 9) and their precise composition is unknown. Nevertheless, the MplY599F receptor clearly retained the capacity to activate STAT3, inducing formation of the same complexes as the wild-type receptor upon TPO stimulation (Figure 9). Thus, while these studies do not exclude a role for STAT3 in c-Mpl-mediated WEHI3B-D$^+$ differentiation, activation of STAT3 DNA binding appears insufficient for this response.

Discussion

The manipulation of factor-dependent cell lines such as Ba/F3 provides effective models for the study of proliferative responses mediated by haemopoietin receptors, including c-Mpl. As the biological role of TPO in megakaryocytes and haemopoietic progenitor cells includes stimulation of both proliferation and differentiation, we sought to establish models to also allow the analysis of c-Mpl-induced differentiation signals. The WEHI3B-D$^+$ and M1 myelomonocytic leukaemia cell lines proliferate autonomously in the absence of exogenous growth factors but differentiate in response to stimuli such as G-CSF, IL-6 or LIF (Metcalf, 1989). We show here that when expressed ectopically in these cell lines, the normal c-Mpl receptor can stimulate cellular differentiation. In agar cultures, unlike parental cells, TPO stimulation of D$^+$-*mpl* cells suppressed clonal growth, and the residual colonies displayed a dispersed morphology characteristic of differentiated cells (Figure 3). Indeed, analysis of cells in liquid cultures revealed that growth inhibition was accompanied by differentiation from immature myelomonocytic cells to macrophages, which acquired expression of lysozyme and Mac-1α (Figures 4 and 5). The TPO-induced differentiation of D$^+$-*mpl* cells contrasts

with that induced with G-CSF or IL-6 through endogenous receptors, which do not suppress clonal growth significantly (Metcalf, 1989) and may reflect higher expression levels of the transfected receptors.

To establish the c-Mpl receptor domains and signal transduction pathways that mediate distinct cellular responses, we assessed the capacity of c-Mpl mutants to promote cell growth and differentiation. Consistent with previous studies (Gurney *et al.*, 1995; Porteu *et al.*, 1996), we confirmed with receptor truncations that a membrane-proximal region of the c-Mpl intracellular domain, including the conserved box1 and box2 motifs, is sufficient for Ba/F3 or FDC-P1 proliferation in response to TPO. Deletion of the C-terminal domain or mutation of Tyr599 did slightly increase the concentration of TPO required for maximal growth (Figure 2), raising the possibility that, although not essential, this region and signals from it (see below) may make some contribution to the proliferative stimulus. Only a residual proliferative signal, requiring very high TPO concentrations, remained upon deletion of box2, and further removal of box1 abolished all response (Figure 2). The correlation between retention of box1 and box2 and an efficient mitogenic response is a common feature of the haemopoietin receptor family (Ihle *et al.*, 1996). Despite delivering only a very poor proliferative stimulus, the *mpl*IC36 mutant retained the capacity to induce phosphorylation of Jak2 in WEHI3B-D$^+$ and Ba/F3 cells (Figure 7 and data not shown). Thus, additional signals may be required for proliferation although, at the TPO concentration used to stimulate Jak2 phosphorylation in these studies, Ba/F3-*mpl*IC36 cells did display some residual growth. The capacity of c-Mpl mutant receptors to stimulate proliferation correlated well with their capacity to induce c-*myc* expression efficiently (Figure 8).

Although largely dispensable for proliferative signals, the C-terminal 33 amino acids of the c-Mpl intracellular domain proved essential for effective induction of differentiation. When expressed in WEHI3B-D$^+$ or M1 cells, receptor mutants truncated to exclude this domain were unable to signal growth arrest or morphological differentiation at TPO concentrations sufficient for maximal responses through the wild-type receptor. While some differentiation was evident at very high levels of TPO, a complete response could not be achieved in these cells (Figures 3 and 6). Receptor tyrosine phosphorylation is observed universally among haemopoietin receptors following ligand binding and is considered critical for receptor function by creating sites for interaction with phosphotyrosine-binding motifs present in a diverse range of downstream signalling molecules (Ihle, 1996). Mutational analysis of tyrosine residues in the c-Mpl domain required for differentiation induction revealed that substitution of a single amino acid, Y599, abolished this response. Unlike cells expressing the wild-type receptor, D$^+$-*mpl*Y599F cell growth was not inhibited by TPO, nor did the cells acquire the macrophage morphology or express the molecular markers typical of differentiation (Figures 3, 4 and 5). The differentiation response depended specifically on c-Mpl Y599, because WEHI3B-D$^+$ cells expressing receptors with substitution of Y604, or indeed of each of the three tyrosines located outside the C-terminal domain, differentiated normally in response to TPO.

In an effort to link specific biochemical signalling cascades with biological responses in cells following TPO stimulation, we examined the capacity of Mpl receptor mutants to activate molecules implicated in haemopoietin receptor signal transduction. Previous studies from other laboratories (Drachman *et al.*, 1995; Gurney *et al.*, 1995; Miyakawa *et al.*, 1995; Mu *et al.*, 1995) and our own data (Figure 7) revealed that Shc phosphorylation on tyrosine residues following the binding of TPO to c-Mpl required the C-terminal receptor region. We have extended these observations to show that Y599 within this c-Mpl domain is essential for TPO-dependent stimulation of Shc phosphorylation. Mutation of Y599 to phenylalanine, or its deletion in the *mpl*IC36 and *mpl*IC88 truncation mutants, not only prevented TPO-dependent Shc phosphorylation but also the association of Shc with c-Mpl and the assembly of Shc–Grb2 complexes (Figure 7). The inability to activate Shc was not the result of non-specific receptor dysfunction because, in response to TPO, cells expressing these c-Mpl mutants exhibited normal Jak2 phosphorylation, which requires only the membrane-proximal cytoplasmic region (Gurney *et al.*, 1995). Studies of tyrosine kinase and haemopoietin receptor systems (Ihle, 1996) have established that activation of the Ras signalling pathway can be initiated by the association of Shc with the activated receptor, followed by Shc phosphorylation and association with Grb2. The induction of c-*fos* expression is considered an important downstream component of the Ras pathway (Barone and Courtneidge, 1995). Consistent with a lesion in the Ras signalling cascade, the c-Mpl receptor mutants unable to activate Shc also failed to induce c-*fos* expression upon TPO stimulation (Figure 8). The amino acid sequence around c-Mpl Y599, Asn–His–Ser–Tyr, conforms to the Asn–Xaa–Xaa–Tyr consensus motif bound by the PTB domain of Shc (Laminet *et al.*, 1996). Our data, therefore, provide strong evidence that, upon TPO binding, the association of Shc with c-Mpl, which results in Shc phosphorylation and Grb2 association, occurs directly between c-Mpl Y599 and the PTB domain of Shc and is likely to lead to activation of the Ras pathway.

Thus, in addition to defining distinct regions of c-Mpl required for different biological responses, we have established a direct correlation between the ability of mutant Mpl receptors to phosphorylate Shc and their capacity to induce macrophage differentiation of WEHI3B-D$^+$ and M1 cells. Although Ba/F3-*mpl*Y599F cells also fail to phosphorylate Shc upon TPO stimulation (data not shown), their proliferative response to the cytokine was not impaired dramatically. Thus, the contribution of Shc, and therefore probably the Ras cascade, to signal transduction from c-Mpl may be more decisive in differentiation rather than the proliferative responses under study.

Emerging studies suggest that different biological responses mediated by a number of haemopoietin receptors depend on distinct regions of their cytoplasmic domains. In B-lymphopoiesis, the signal mediating proliferative responses through the IL-7 receptor α-chain is distinct from that inducing differentiation (Corcoran *et al.*, 1996). Within the gp130 signalling chain, a membrane-distal receptor region distinct from those sufficient for promoting mitosis is required for the induction of growth arrest and differentiation in M1 cells (Murakami *et al.*, 1991; Yamanaka *et al.*, 1996). Similarly, a C-terminal domain

of the G-CSF receptor is dispensable for cellular proliferation but required for neutrophilic differentiation (Dong *et al.*, 1993; Fukunaga *et al.*, 1993; Yoshikawa *et al.*, 1995). Recent studies by Porteu *et al.* (1996), which defined distinct c-Mpl receptor domains involved in proliferation and differentiation of a megakaryoblastic leukaemia cell line, suggested that a similar pattern characterizes c-Mpl signalling. Our data clearly reinforce the model that distinct regions of c-Mpl signal proliferation and differentiation. However, Y599, identified here as critical for Mpl-induced differentiation, does not fall within the region identified by Porteu *et al.* (1996). In that study, an internal c-Mpl cytoplasmic deletion of 24 amino acids, C-terminal to the box1 and box2 motifs but not including Y599, specifically abolished TPO-dependent differentiation. The capacity of this mutant to phosphorylate or recruit Shc to the receptor was not assessed, and it remains possible that conformational changes wrought by internal deletion might disrupt these events. Studies with the IL-2 receptor suggest that proliferative responses require simultaneous activation of more than one signalling cascade (Miyazaki *et al.*, 1995). Together, these data also raise the alternative possibility that differentiation induction by c-Mpl involves the concerted contribution of multiple signalling pathways. The capacity of c-Mpl mutants to stimulate residual differentiation at high TPO concentrations, despite the lack of Shc activation, is consistent with this model. Our data suggest that although induction of STAT3 DNA binding is not sufficient for c-Mpl-induced differentiation, it is activated following TPO binding by a mechanism distinct from that of Shc (Figure 9). As gp130-induced M1 cell differentiation has been shown recently to require STAT3 activation (Minami *et al.*, 1996; Yamanaka *et al.*, 1996), it will be intriguing, therefore, to determine any role for STAT3 in c-Mpl-mediated differentiation, particularly in the residual response of cells expressing MplY599F. Finally, as the present study was conducted using myelomonocytic cells, it is also feasible that the signals required for differentiation might vary in different cell types. It is important to note that while these studies of macrophage differentiation have defined critical c-Mpl domains required for activation of specific signalling pathways and cellular responses, it remains to be determined whether Tyr599 and Shc phosphorylation are essential for the physiological response of megakaryocytes to TPO. Thus, in future studies, it will be important to assess the relevance of the key c-Mpl receptor domains and signal transduction pathways identified in cell line models, to the responses of primary haemopoietic cells, particularly megakaryocytes.

Materials and methods

Construction of mutant Mpl receptors

Mutant Mpl receptors bearing progressive C-terminal truncations or cytoplasmic domain tyrosine→phenylalanine substitutions were constructed (Figure 1). A c-*mpl2* cDNA (Alexander and Dunn, 1995) encoding the full-length c-Mpl receptor with a FLAG epitope tag at the N-terminus (Alexander *et al.*, 1995) was subcloned into the *Eco*RI site of the LXSN retroviral vector (Miller and Rosman, 1989) to generate pLX*mpl*F. The 0.8 kb fragment extending 3′ from the single *Bam*HI site in c-*mpl* to the *Bam*HI site in the LXSN polylinker was excised and replaced with *Bam*HI-digested fragments bearing specific mutations within the intracellular receptor domain, generated as described below. C-terminal truncations were generated by the polymerase chain reaction (PCR) using pLX*mpl*F as the template, a 5′ oligonucleotide (*mpl*wt-A: 5′-AAGGTGCCGTTCACAGCTAC-3′) located 18 bp upstream of the unique *Bam*HI site in c-*mpl* and 3′ oligonucleotides introducing termination codons and *Bam*HI sites following codons 1 (*mpl*TM: 5′-CGGGATCCCTACTTTAGCAGCAGTAGGCCCA-3′), 36 (*mpl*IC36: 5′-CGGGATCCCTATAGGGCTGCAGTGTCTCTGA-3′) and 88 (*mpl*IC88: 5′-CGGGATCCCTACATGGTCCGCAGGCAAGGTT-3′) of the cytoplasmic domain. Receptor fragments bearing single tyrosine→phenylalanine substitutions were generated by recombinant PCR (Higuchi, 1990) using *mpl*wt-A and *mpl*wt-B (5′-CGGGATCCCTACTGCCAATAGCTTAGTGGTA-3′, which incorporates a *Bam*HI site immediately after the normal *mpl* termination codon) as external primers and oligonucleotides incorporating tyrosine→phenylalanine codon changes as internal primers: *mpl*Y495F, 5′-CCTGCGCACTTCAGGAGACTG-3′; *mpl*Y516F, 5′-CTAGGCCAGTTCCTCAGAGAC-3′; *mpl*Y565F, 5′-CAGATGGACTTCAGAGGACTG-3′; and *mpl*Y599F, 5′-AACCACTCCTTCCTACCACTA-3′. (Sense oligonucleotides are shown with alterations from wild-type sequence underlined.) The *mpl*Y604F fragment was generated in a single PCR reaction using *mpl*wt-A and an oligonucleotide incorporating the mutation and the normal *mpl* termination codon followed by a *Bam*HI site: 5′-CCGGATCCTCAGGGCTGCTGCCAAAAGCTTAGTGG-3′. Residue numbers refer to amino acid positions in the mature Mpl protein encoded by *mpl2*. Each mutant fragment was sequenced to confirm incorporation of the desired mutations and to exclude additional alterations.

Cell lines and cytokines

The factor-dependent Ba/F3 (Palacios and Steinmetz, 1985) and FDC-P1 (Dexter *et al.*, 1980) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) and 10% WEHI3B-D$^-$-conditioned medium as a source of IL-3 (Metcalf, 1984). The WEHI3B-D$^+$ and M1 myelomonocytic leukaemia cell lines (Metcalf, 1989) were maintained in DMEM supplemented with 10% fetal calf serum (FCS). Recombinant murine TPO was expressed in Chinese hamster ovary cells and purified as described (Alexander *et al.*, 1996). Recombinant murine LIF was obtained from AMRAD (Melbourne, Australia) and recombinant murine IL-6 was a gift from Dr Richard Simpson, JPSL, Melbourne.

Transfections

Cells were washed and then resuspended at 2.5×10^6 cells/ml in phosphate-buffered saline (PBS). A 0.8 ml aliquot was mixed with 10 μg of linearized plasmid DNA encoding the normal or mutant c-Mpl receptors and electroporated with a Gene Pulser (Bio-Rad, Hercules, CA) at 270 V and 960 μFD. The cells were centrifuged through FCS to remove debris, resuspended in growth medium and distributed over four 48-well tissue culture plates (Costar, Cambridge, MA). After 48 h, transfected cells were selected in G418 (GibcoBRL, Gaithersburg, MD) at 1500 μg/ml (WEHI3B-D$^+$) or 800 μg/ml (Ba/F3, FDC-P1 and M1). Wells containing live cells after 10 days were assayed for cell surface receptor expression. Cells were incubated with saturating amounts of the M2 monoclonal antibody (Eastman Kodak, New Haven, CT) directed against the FLAG epitope incorporated into the N-terminus of each receptor. Cells binding M2 were then labelled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin. Analyses were performed on a FACSCAN cell sorter (Becton Dickinson) excluding dead cells by propidium iodide (1 μg/ml) staining.

Proliferation and differentiation assays

The proliferative response to TPO of Ba/F3 cells expressing normal or mutant c-Mpl receptors was measured in microwell assays of 200 cells stimulated with serially diluted quantities of TPO as described (Alexander *et al.*, 1995). Cell numbers were scored after incubation for 2 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air. The capacity of parental or transfected WEHI3B-D$^+$ or M1 cells to differentiate in response to TPO was assessed in agar cultures. One hundred or 200 cells in DMEM containing 20% FCS and 0.3% agar were plated in 1 ml cultures in 35 mm Petri dishes (Metcalf, 1984) stimulated with various concentrations of TPO, IL-6 (20 ng/ml, WEHI3B-D$^+$) or LIF (10 ng/ml, M1) as a positive inducer of differentiation, or with saline. Colony numbers and morphology were scored after 7 days incubation at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Undifferentiated colonies were large and compact while colonies composed of dispersed cells, or which had a halo of migrating cells around a central core, were scored as differentiated. For differentiation of WEHI3B-D$^+$ or M1 cells in liquid culture, 2×10^4 cells were plated in 2 ml cultures of DMEM

supplemented with 10% FCS and 20 ng/ml TPO and monitored over the subsequent 4 days.

RNA analyses

For analysis of immediate-early gene induction in Ba/F3 cells, the cells were withdrawn from IL-3 for 8 h then stimulated with TPO (20 ng/ml) for various times at 37°C. Myeloid gene expression in WEHI3B-D^+ cells was also assessed after stimulation with 20 ng/ml TPO. Polyadenylated RNA was extracted and analysed in Northern blots as described (Alexander *et al.*, 1995). Membranes were hybridized with the following α-^{32}P-labelled cDNA probes: a 780 bp *Eco*RI murine lysozyme fragment; a 1.2 kb *Eco*RI murine Mac-1α fragment; a 1.9 kb *Pst*I murine myeloperoxidase fragment; a 1.4 kb *Xho*I murine c-*myc* fragment; a 1.0 kb *Pst*I v-*fos* fragment; and a 1.2 kb *Pst*I chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment.

Immunoprecipitations and Western blot analysis

WEHI3B-D^+ cells expressing normal or mutant Mpl receptors were incubated with or without TPO (100 ng/ml) for 7 min at 37°C. Cells were washed once with ice-cold PBS, lysed at 2×10^7 cells/ml in lysis buffer A containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 2 mM EDTA, 10 μg/ml leupeptin, 50 μg/ml aprotinin, 1 mM iodoacetic acid, 0.5 mM EGTA, 50 μg/ml soya bean trypsin inhibitor, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM Na_3VO_4. Lysates were centrifuged for 5 min at 4°C at 13 000 r.p.m. and supernatants were incubated with antibodies to Jak2 (Santa Cruz Biotechnology, Santa Cruz), Shc (Transduction Laboratories, Lexington, KY) or the FLAG epitope M2 (Eastman Kodak, New Haven, CT) for 2 h at 4°C. After 1 h, protein A–Sepharose (Pharmacia Biotech, Uppsala, Sweden) was added. The antibody–protein complexes were washed three times with lysis buffer A and then boiled for 5 min in sample buffer containing 30% glycerol, 7% SDS, 0.125 M Tris–HCl (pH 6.8), 10% 2-mercaptoethanol and 0.005% bromophenol blue. Following SDS–PAGE using 4–15% gradient polyacrylamide gels (Bio-Rad, Hercules, CA), proteins were electrophoretically transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, MA). Blots were blocked with PBS containing 1% bovine serum albumin, then incubated with horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20 (Transduction Laboratories, Lexington, KY) before adding ECL substrate solution (Amersham Corp., Arlington Heights, IL) for visualization by autoradiography. In some experiments, blots were stripped with 0.2% glycine (pH 3), reblocked, washed and reprobed with antibodies to either Jak2, Shc, FLAG (M2) or Grb2 (kindly provided by Dr Francesca Walker, Ludwig Institute, Melbourne).

Electrophoretic mobility shift assays

Labelling of oligonucleotides containing the high affinity SIF binding site m67, extraction of protein from cells and electrophoresis of protein–DNA complexes were performed as described (Novak *et al.*, 1995). Briefly, 8 μg of total protein extracted from unstimulated or TPO (100 ng/ml)-treated WEHI3B-D^+ cells was mixed with 1 ng of labelled DNA and 600 ng of sonicated salmon sperm carrier DNA in the presence or absence of antibodies to STAT1 (Transduction Laboratories, Lexington, KY) or STAT3 (Santa Cruz Biotechnology, Santa Cruz). After 15 min at room temperature, complexes were loaded onto a non-denaturing 5% polyacrylamide gel, separated by electrophoresis and visualized by autoradiography.

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