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Distinct regions of c-Mpl cytoplasmic domain are coupled to the JAK–STAT signal transduction pathway and Shc phosphorylation

(thrombopoietin/cytokine/receptor/box 1)

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ABSTRACT **c-Mpl, a member of the hematopoietic cytokine receptor family, is the receptor for thrombopoietin. To investigate signal transduction by c-Mpl, a chimeric receptor, composed of the extracellular domain of human growth hormone receptor and the intracellular domain of c-Mpl, was introduced into the interleukin 3-dependent cell line Ba/F3. In response to growth hormone, this chimeric receptor induced growth in the absence of interleukin 3. Deletion analysis of the 123-amino acid intracellular domain indicated that the elements responsible for this effect are present within the 63 amino acids proximal to the transmembrane domain. Mutation of the recently described box 1 motif abrogated the proliferative response. Tyrosine phosphorylation of the tyrosine kinase JAK-2 and activation of STAT proteins were dependent on box 1 and sequences within 63 amino acids of the plasma membrane. STAT proteins activated by thrombopoietin in a megakaryocytic cell line were purified and shown to be STAT1 and STAT3. A separate region located at the C terminus of the c-Mpl intracellular domain was found to be required for induction of Shc phosphorylation and c-fos mRNA accumulation, suggesting involvement of the Ras signal transduction pathway. Thus, at least two distinct regions are involved in signal transduction by the c-Mpl.**

c-Mpl, a member of the cytokine receptor superfamily, was originally identified as the cellular homolog of a retroviral oncogene (1). The extracellular domain of c-Mpl contains two repeats of a characteristic domain structure that includes four conserved cysteine residues and a WSXWS motif at its C terminus. Overall, c-Mpl exhibits highest homology to the erythropoietin (Epo) and interleukin 3 (IL-3) receptors (2). c-Mpl expression appears largely limited to tissues that support hematopoiesis: bone marrow, spleen, and fetal liver (3). The receptor is highly expressed in $CD34^+$ cells and cells of the megakaryocyte lineage, suggesting a role in the regulation of platelet production. Involvement in regulating megakaryocytopoiesis has recently been confirmed through the study of mice deficient in c-Mpl (4). These mice, generated by gene targeting, display pronounced thrombocytopenia with an 85% decrease in their numbers of platelets and megakaryocytes, but they have normal levels of other hematopoietic cell types.

Recently, our laboratory and others have purified and cloned the ligand for c-Mpl and shown it to be thrombopoietin (TPO), an activity capable of stimulating the proliferation and maturation of megakaryocytes (reviewed in ref. 5). TPO is a cytokine of 353 amino acids composed of an N-terminal domain homologous to Epo and a glycosylated carboxyl domain of unknown function. Recombinant TPO stimulates megakaryocyte proliferation and maturation *in vitro* from primitive hematopoietic stem cell populations alone or in the presence of other exogenous hematopoietic growth factors (6). TPO induces dramatic increases in platelet production and megakaryocyte numbers in mice, suggesting that it regulates both thrombocytopoiesis and megakaryocytopoiesis *in vivo* (5).

To examine the mechanisms of c-Mpl signal transduction, we have studied a series of chimeric receptors containing deletions within the c-Mpl intracellular domain. Previous studies have demonstrated that chimeric receptors containing the c-Mpl intracellular domain are able to stimulate cellular proliferation (7–9). Our results suggest that at least two distinct signal transduction pathways are induced by receptor activation. Elements within 63 amino acids proximal to the plasma membrane are able to induce both cellular proliferation and activation of the JAK–STAT signal transduction pathway. This region has been shown to be required for the transforming pathogenicity of MPLV (10). A second element present within the carboxyl 20 amino acids of the c-Mpl intracellular domain is required for tyrosine phosphorylation of Shc and induction of c-fos mRNA, suggesting a possible involvement of the Ras signal transduction pathway.

MATERIALS AND METHODS

Construction of Growth Hormone Receptor (GHR)–Mpl Expression Vectors. A chimeric receptor, GHR–Mpl, composed of the extracellular and transmembrane domains of the human GHR (amino acids 1–291) (11) and the intracellular domain of the human c-Mpl receptor (amino acids 514–635) (2) was prepared by sequential PCR as were subsequent deletions and site-directed substitutions. The changes introduced in the box 1 mutation are alanine substitutions of the following c-Mpl amino acids: 529, tryptophan; 531, serine; 533, proline; 534, aspartic acid. Stable cell lines expressing each of the chimeric receptors were obtained and maintained as described (12).

Proliferation Assays. Cells were cultured in the absence of IL-3 for 16 hr [in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS)]. Cells were then washed twice with PBS and plated in 96-well dishes at 50,000 cells per well in 0.2 ml of serum-free RPMI 1640 medium supplemented with the indicated concentrations of human GH. Each concentration was tested in quadruplicate and [^{3}H]thymidine incorporation was determined as described (12).

Electrophoretic Mobility-Shift Assays (EMSAs). Cells cultured in the absence of IL-3 for 16 hr were exposed to GH (10 ng/ml) for 15 min or left unstimulated as controls. Cell extracts were prepared by washing cells twice with cold PBS followed by resuspension of the cells at a concentration of 10^8 cells per ml in lysis buffer (10 mM Tris·HCl, pH 7.5/50 mM NaCl/5 mM EDTA/30 mM sodium pyrophosphate/5 mM sodium fluoride/100 mM sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride/1% Triton X-100). Cells were incubated on ice

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Abbreviations: Epo, erythropoietin; IL-3, interleukin 3; TPO, thrombopoietin; GH, growth hormone; GHR, GH receptor; SIE, sis-inducible element; CSF, colony-stimulating factor; EMSA, electrophoretic mobility-shift assay.

for 1 hr. Precipitate was removed by centrifugation and the supernatant was made 10% with glycerol and frozen at −70°C. A high-affinity sis-inducible element (SIE) (m67) (13) encoded by complementary oligonucleotides (5′-CTAGAGTCGACATTTCCCGTAAATCT-3′ and 5′-CTAGAGATTTACGGGAAATGTCGACT-3′) was labeled with the Klenow fragment of DNA polymerase and [α-^{32}P]dATP. Binding reactions were performed as described (13).

STAT Purification. CMK cells (30 liters at 7×10^5 cells per ml) were grown in high-glucose DMEM supplemented with 10% FCS. Cells were harvested and resuspended in 500 ml of serum-free DMEM containing 10 ng of recombinant TPO per ml. After 15 min of incubation, cells were harvested by centrifugation and lysis extracts were prepared as described above. Supernatant was incubated overnight with a SIE affinity resin composed of biotinylated SIE concatemers bound to Ultralink immobilized NeutrAvidin (Pierce). The affinity matrix was collected by centrifugation, washed twice with 20 ml of lysis buffer, and loaded onto a disposable column. The column was then washed successively with 5 ml of lysis buffer supplemented to 200 mM with NaCl, three times with 5 ml of binding buffer [20.0 mM Hepes, pH 7.6/0.1 mM EDTA/100 mM NaCl/10% (vol/vol) glycerol] supplemented with a mutant SIE (complementary oligonucleotides 5′-CTAGAGTCGAGATATCGCGCAAATCT-3′ and 5′-CTAGAGATTTGCGCGATATCTCGACT-3′) (0.2 mg/ml) and NaCl to concentrations of 100, 200, and 400 mM, respectively, and with 5 ml of binding buffer supplemented with 400 mM NaCl. Proteins were then eluted with 1 ml of binding buffer supplemented with 1 M NaCl. The NaCl concentration of the eluted fraction was reduced to 100 mM by dilution (1:10) in binding buffer lacking KCl or NaCl. Agarose-conjugated anti-phosphotyrosine antibody (50 μl) (Upstate Biotechnology, Lake Placid, NY) was added and proteins were immunoprecipitated overnight at 4°C. Bound proteins were eluted from beads in 50 μl of binding buffer supplemented with 100 mM phenylphosphate (Aldrich).

Protein Sequence Analysis. Purified proteins were subjected to SDS/PAGE, transferred to poly(vinylidene difluoride) membrane (Millipore), stained for 0.5 min with 0.1% Coomassie blue R-250 in 50% methanol, destained for 2 min with 10% acetic acid/50% methanol, and then washed with water and allowed to dry. The 80- and 87-kDa bands were reduced and alkylated with isopropylacetamide as described (14, 15) and digested in 25 μl of 0.1 M ammonium bicarbonate/10% acetonitrile with 0.2 μg of Lys-C (Wako Biochemicals, Osaka) at 37°C for 17 hr. Peptides generated from the Lys-C digest were separated on a C_{18} capillary column (0.32×100 mm) (LC Services, Woburn, MA) and sequenced on an Applied Biosystems sequencer (model 470A).

Immunoprecipitation and Western Blot Analysis. Cell extracts were prepared as described for SIE binding experiments. Extracts were precleared by incubation with protein A agarose for 4 hr at 4°C and then incubated overnight at 4°C with 10 μl per 10^8 cells of agarose-conjugated anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology). Western blot analyses of tyrosine phosphorylation of JAK-2 (Santa Cruz Biotechnology, Santa Cruz, CA), Shc (Upstate Biotechnology), STAT1 (Upstate Biotechnology), and STAT3 (Santa Cruz Biotechnology) were performed essentially as recommended by the manufacturers.

Northern Blot Analysis. Poly(A)$^+$ RNA was isolated from 10^8 cells per sample. mRNA (2 μg per lane) was resolved on a 1.2% agarose gel containing formaldehyde and transferred to nylon membrane (GeneScreen*Plus*, DuPont). The blot was probed with a ^{32}P-labeled oligonucleotide probe specific for murine c-fos. The blot was washed at 42°C in 0.2× SSC/0.1% SDS and exposed overnight to X-Omat film (Kodak).

RESULTS

To dissect the regions of the c-Mpl intracellular domain involved in signal transduction, a chimeric receptor construct encoding the extracellular domain of the human GHR and the intracellular domain of the human c-Mpl (GHR–Mpl) was stably transfected into IL-3-dependent mouse pre-B-cell line Ba/F3. Fluorescence-activated cell sorter analysis confirmed that pools of GHR–Mpl-transfected cells exhibited cell surface expression of GHR extracellular domain (Fig. 1*B*). To determine whether these cells could respond to GH, cells were deprived of IL-3 and subsequently exposed to a range of GH concentrations. GHR–Mpl induced proliferation in response to GH, whereas no induction was seen with untransfected Ba/F3 cells. Moreover, cells expressing GHR–Mpl were able to be continuously maintained in the absence of IL-3 if provided with GH (data not shown). To define regions of the receptor required for the proliferative response, a series of deletions were introduced into the c-Mpl intracellular domain. This analysis indicated that the carboxyl half of the intracellular domain is dispensable for the proliferative response (Fig. 1). This proliferative effect could be observed in either the presence or absence of serum. Further deletions of carboxyl deletions of 80, 100, or 110 amino acids abrogated the proliferative capacity of GHR–Mpl completely. Comparison of the intracellular domains of members of the hematopoietic receptor family has identified two regions of partial sequence homology termed box 1 and box 2 (10). c-Mpl contains an element located proximal to the membrane that displays particularly strong homology to box 1 of IL-7 and granulocyte colony-stimulating factor (CSF) receptors and a second more distal element that resembles the loosely conserved box 2 element. The box 2 element, located between amino acids 565 and 574 of c-Mpl, is removed in the carboxyl deletions of 80 or more amino acids. Thus, deletion of box 2 correlates with the loss of proliferative ability of c-Mpl. To determine whether the box 1 element is also required for the proliferative response, mutations were introduced to delete box 1. This receptor exhibited complete loss of proliferative response (Fig. 1*C*).

Western blot analysis with anti-phosphotyrosine antibody revealed a band of ≈130 kDa that is induced by GH in cells expressing GHR–Mpl but not in untransfected Ba/F3 cells (data not shown). This molecular mass agrees well with the size of JAK family members. In extracts prepared from GHR–Mpl-expressing cells that were stimulated with GH, tyrosine phosphorylation of JAK-2 was markedly induced (Fig. 2). Tyrosine phosphorylation of JAK-2 is induced by GH in cells expressing the GHR–Mpl Δ60 but not in cells expressing GHR–Mpl Δ80 or GHR–Mpl box 1. Thus, the ability of the GHR–Mpl mutants to induce JAK-2 tyrosine phosphorylation correlates with the ability to induce proliferation in Ba/F3 cells.

The activation of members of the rapidly growing STAT family of transcription factors has been observed recently for a number of cytokine receptor systems (16). To examine whether similar DNA binding activities are activated through GHR–Mpl, EMSAs were conducted with SIE previously shown to bind multiple STAT-related proteins (13). In response to GH, several bands of SIE binding activity were observed in extracts from Ba/F3 cells expressing GHR–Mpl (Fig. 3). These bands could be specifically blocked by competition with unlabeled SIE but not with other unrelated oligonucleotides (data not shown). Analysis of the c-Mpl deletion mutants indicated that the ability to induce these DNA binding activities correlated with the ability to induce tyrosine phosphorylation of JAK-2. DNA binding activity was induced through GHR–Mpl Δ60 but not through GHR–Mpl Δ80 or the GHR–Mpl box 1 mutant. The relative intensity of the three bands of binding activity was significantly altered with the receptors containing carboxyl deletions of 20 or 60 amino acids

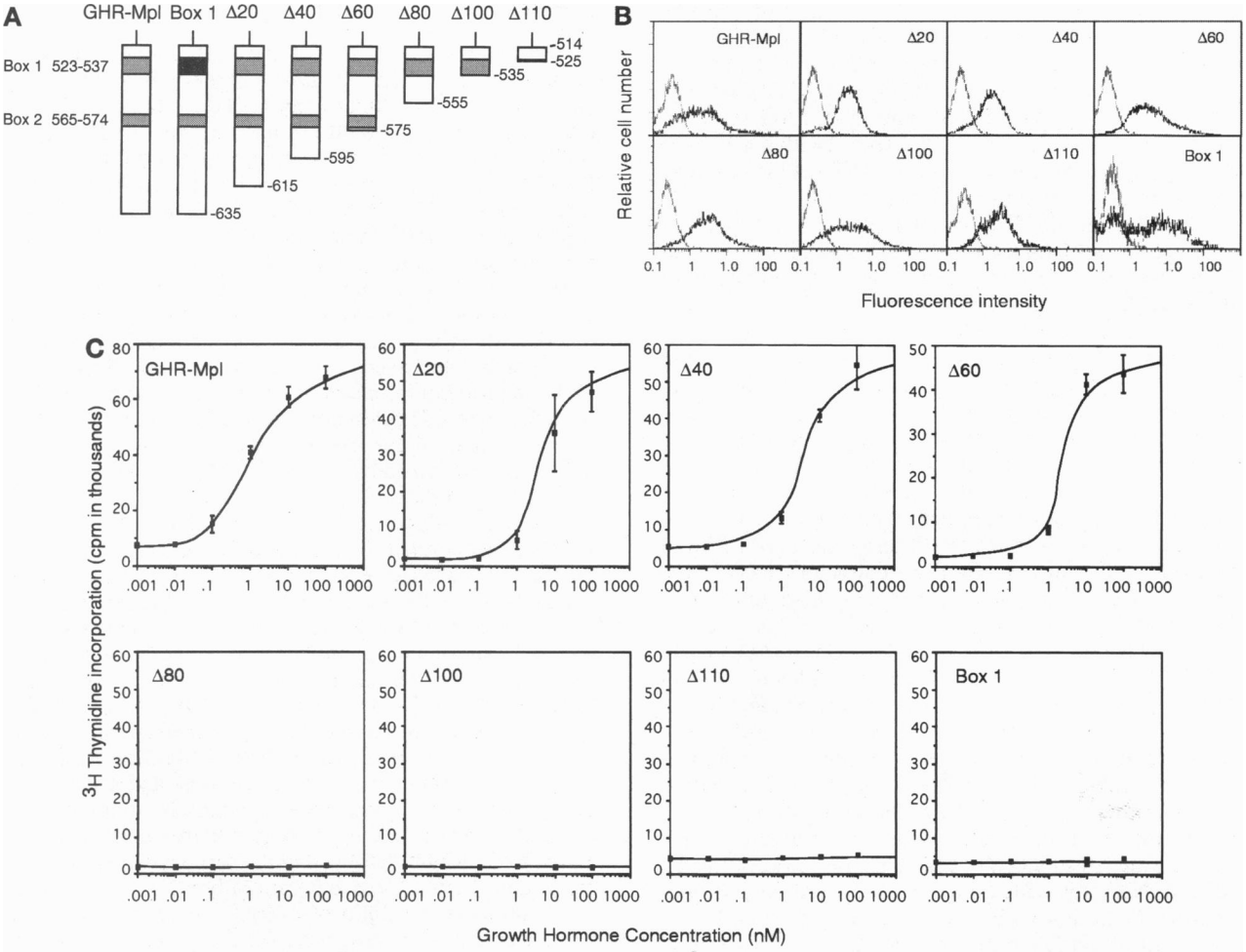

FIG. 1. Deletion analysis of proliferative response. (*A*) Schematic representation of the intracellular domain of the GHR–Mpl chimera introduced into Ba/F3 cells. Deletion numbers refer to the number of amino acids removed from the C terminus. Amino acid residues of c-Mpl contained within each of the GHR–Mpl mutants are indicated on the right. Locations of box 1 and box 2 are depicted by shaded boxes. Mutation of box 1 in the GHR–Mpl box 1 mutant is indicated by a solid box. (*B*) Expression of GHR on Ba/F3 cells transfected with the indicated GHR–Mpl expression vectors was assessed by flow cytometric analysis. Cells were stained with anti-GHR monoclonal antibody 3B7 and R-phycoerythrin-conjugated goat anti-mouse immunoglobulins. Shown are histograms displaying log R-phycoerythrin fluorescence (*x* axis) vs. relative cell number (*y* axis) for transfected cells (dark curve) and untransfected control cells (light curve). (*C*) Proliferation of Ba/F3 cells expressing chimeric GHR–Mpl in response to human GH. The ability of GH to induce proliferation in Ba/F3 cells expressing the indicated GHR–Mpl mutants was measured by the extent of incorporation of [^{3}H]thymidine into DNA.

relative to full-length GHR–Mpl; however, induction of all three bands is clearly observed in longer exposures (data not shown).

To identify the relevant SIE binding activities induced by c-Mpl, we stimulated endogenous c-Mpl present on human megakaryoblastic CMK cells with TPO. TPO induced SIE binding activity in CMK cells (Fig. 4*A*). Four tyrosine phosphorylated bands, with apparent molecular masses of 80, 87, 89, and 91 kDa, were retained on SIE affinity columns from extracts prepared from TPO-treated cells (Fig. 4*B*). The 91-kDa band agrees in size with STAT1 and was recognized in Western blot analysis with monoclonal antibody directed against STAT1 while the 80-, 87-, and 89-kDa bands each interact with polyclonal anti-STAT3 antibody (Fig. 4*C*). The

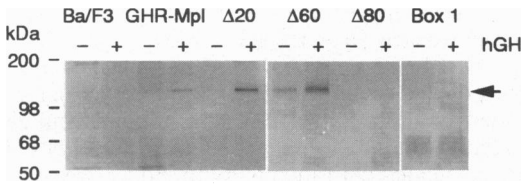

FIG. 2. Induction of JAK-2 tyrosine phosphorylation. Ba/F3 cells expressing the indicated GHR–Mpl mutants were starved of IL-3 for 14 hr and then exposed to human GH (hGH) for 10 min or left unstimulated as a control. Western blot analysis was performed as described.

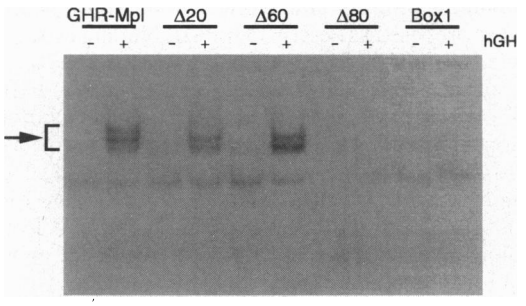

FIG. 3. Induction of STAT activity in Ba/F3 cells through GHR–Mpl. Ba/F3 cells expressing the indicated GHR–Mpl mutants were starved of IL-3 for 14 hr and then exposed to human GH (hGH) for 15 min or left unstimulated as a control. Cells were harvested and lysed as described. Shown is an analysis of SIE binding activity by EMSA. Positions of retarded complexes of STAT binding activity are indicated by arrow.

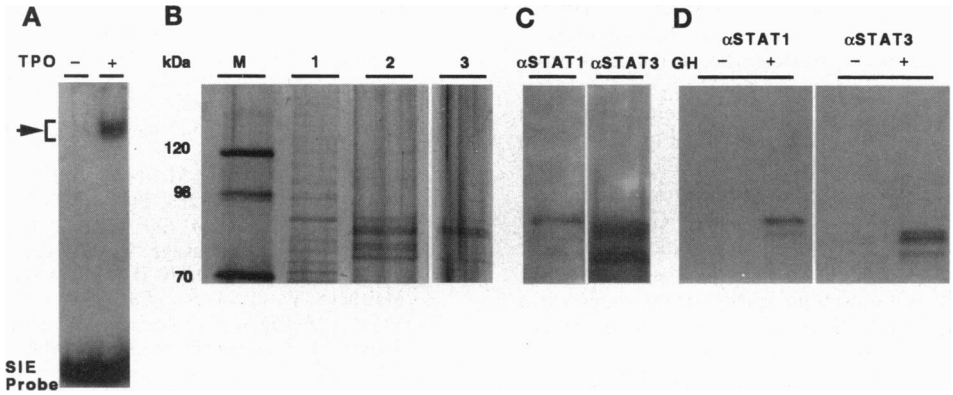

FIG. 4. Identification of STAT1 and STAT3 activation by TPO. (*A*) Induction of STAT activity in CMK cells by TPO. CMK cells were exposed to recombinant human TPO (10 ng/ml) for 15 min and harvested as described. EMSA was performed in the same manner as with Ba/F3 cell extracts. (*B*) Purification of SIE binding activity. STAT proteins activated by TPO in CMK cells were purified as described. Proteins were resolved by SDS/PAGE on a 6% gel in the presence of 2-mercaptoethanol and were visualized by silver staining. Lanes: M, molecular size markers; 1, CMK lysate starting material; 2, proteins eluted from SIE affinity column; 3, proteins eluted from SIE affinity column and then immunoprecipitated with agarose-conjugated anti-phosphotyrosine antibody resin. (*C*) STAT1 and STAT3 Western blot analysis of CMK STAT proteins. Purified proteins were resolved by SDS/PAGE on a 6% gel as in *B* and transferred to nitrocellulose. Western blot analysis was conducted with monoclonal antibody specific for STAT1 or polyclonal antibody directed against STAT3 as indicated. Bands were visualized with alkaline phosphatase-conjugated secondary antibodies. (*D*) Western blot analysis of STAT proteins induced in GHR–Mpl-expressing Ba/F3 cells by GH. Proteins retained on SIE affinity resin from extracts of unstimulated or stimulated cells were resolved by SDS/PAGE, transferred to nitrocellulose, and subjected to Western blot analysis as in *C*. Cells were harvested and lysed as described.

amino acid sequences of peptide fragments generated by Lys-C digestion of the 80-kDa band (TQIQSVEPYTK) and the 87-kDa band (TQIQSVEPYTK and FNILGTNTK) are found in STAT3 (residues 384–392 and 632–642). To determine whether STAT1 and STAT3 were also activated through GHR–Mpl in Ba/F3 cells, Western blot analysis was conducted with proteins retained on SIE affinity columns from extracts of Ba/F3 cells expressing GHR–Mpl. GH stimulation resulted in the activation of both STAT1 and STAT3 (Fig. 4*D*).

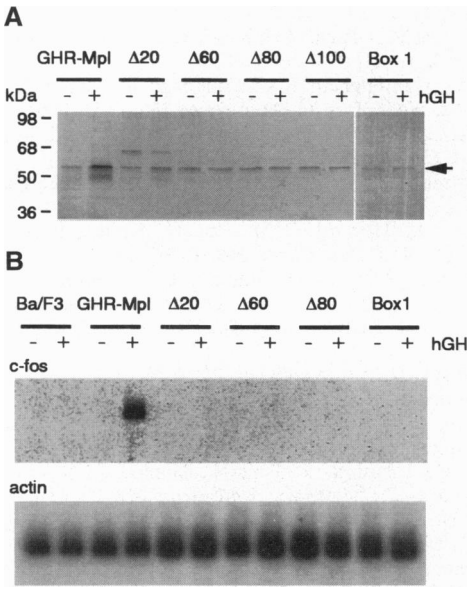

FIG. 5. Identification of a carboxyl element within c-Mpl involved in Shc phosphorylation and c-fos induction. (*A*) Induction of Shc tyrosine phosphorylation. Ba/F3 cells expressing the indicated GHR–Mpl mutations were exposed to human GH (hGH) for 10 min or left unstimulated as a control. Western blot analysis was performed as described. (*B*) Northern blot analysis of c-fos expression. Poly(A)$^+$ RNA (2 μg per lane) isolated from Ba/F3 cells expressing the indicated GHR–Mpl mutants stimulated 10 min with 10 nM hGH or left unstimulated as a control was resolved on a 1.2% denaturing agarose gel, transferred to nylon, and hybridized sequentially with ^{32}P-labeled oligonucleotides specific for c-fos and actin.

Previous studies have implicated involvement of the Ras signal transduction pathway in mediating cytokine action (17). To probe the involvement of this pathway, the level of tyrosine phosphorylation of Shc, a protein previously shown to be involved in the activation of p21^{ras}, was examined. In Ba/F3/GHR–Mpl, the level of tyrosine phosphorylation of Shc was rapidly increased by GH stimulation (Fig. 5*A*). However, induction was not observed with the Δ20, Δ60, or box 1 mutations. Because the Δ20 and Δ60 mutant receptors are able to mediate a proliferative signal in response to GH, this result suggests the existence of at least two distinct functional domains within the c-Mpl intracellular domain.

c-Fos mRNA level in Ba/F3 cells expressing full-length GHR–Mpl was greatly increased in response to GH (Fig. 5*B*). Induction of c-fos was not observed in Ba/F3 cells expressing the Δ20, Δ60, or box 1 mutations. This pattern correlates with Shc tyrosine phosphorylation and further supports the existence of a separable functional element at the C terminus of c-Mpl that is dispensable for proliferation. c-Fos induction was abrogated by the box 1 mutation within the full-length GHR–Mpl receptor. Thus, the element(s) present within the carboxyl 20 amino acids that is required for c-fos induction is not sufficient and requires a functional box 1 element.

DISCUSSION

In this report, we present evidence that the c-Mpl intracellular domain is able to activate two distinct signal transduction pathways. A region of the c-Mpl intracellular domain proximal to the cell membrane is necessary and sufficient to transmit a proliferative signal. This membrane proximal region also mediates the tyrosine phosphorylation of JAK-2 and induction of STATs. A second region of the c-Mpl intracellular domain located at the C terminus is required for tyrosine phosphorylation of Shc and induction of c-fos mRNA. These responses are abrogated by removal of as little as 20 amino acids from the C terminus and thus can be clearly dissociated from the membrane proximal element shown to be sufficient for induction of the JAK–STAT pathway. However, all of the responses noted upon activation of GHR–Mpl are lost upon mutation of an element that resembles a previously noted box 1 motif present in several other members of the hematopoietic receptor superfamily. As the box 1 element has been implicated as

a binding site for members of the JAK family of protein kinases (17, 18), it is possible that JAK kinases may be involved in multiple pathways that lead to proliferation, STAT activation, and fos induction.

The deletion analysis of the c-Mpl intracellular domain utilized the chimera between GHR and c-Mpl transfected in the IL-3-dependent mouse pre-B-cell line Ba/F3, demonstrating that c-Mpl can activate multiple signal transduction pathways in cells not of the megakaryocyte lineage. GHR is one of the best characterized members of the cytokine receptor family. Both functional studies and x-ray crystal structure indicate that GH induces homodimerization of the receptor to signal (19, 20). We have recently observed that Shc phosphorylation, JAK-2 phosphorylation, STAT activation, and induction of a proliferative response are also observed in Ba/F3 cells transfected with full-length c-Mpl and stimulated with TPO, suggesting that, like GHR, homodimerization of c-Mpl is sufficient for receptor activation (data not shown).

Despite limited sequence identity, many members of the cytokine receptor superfamily now appear to possess similar functional architecture. Studies of the Epo, IL-2, prolactin, and GH-Rs have shown that the membrane proximal region including box 1 is required for mediating a proliferative response (21–24). Thus, the growth signal induced by c-Mpl is likely to involve a mechanism similar to that used by other members of the cytokine receptor family. The distal element within the c-Mpl intracellular domain is not required for proliferation. It may be functionally related to carboxyl domains identified in granulocyte CSF receptor (25); IL-2 receptor β subunit (26); IL-2 receptor γ subunit; the shared signaling component of IL-2, IL-4, IL-7, IL-9, and IL-13 (27); and AIC2b/KH97, the shared signaling component of IL-3, IL-5, and granulocyte–macrophage CSF (28). Although there is no obvious amino acid similarity between these regions, in each case carboxyl deletions result in a loss of c-fos induction. In the case of granulocyte CSF receptor, this region has been shown to transduce a differentiation signal. It will be of interest to identify proteins that interact with this region of c-Mpl in megakaryocytes.

TPO induces activation of STAT1 and STAT3 through endogenous c-Mpl in CMK cells. Both STAT1 and STAT3 are also activated in response to IL-6 by the IL-6 receptor β-subunit gp130 (16, 29, 30). This signaling subunit is utilized by several cytokines including IL-6, IL-11, OSM, LIF, and CNTF (17). Interestingly, IL-6, IL-11, and LIF have effects on megakaryocytopoiesis (31–33). Perhaps the ability to activate STAT1 and STAT3 underlies the effects common to TPO and these other cytokines. The possibility that TPO may also activate additional proteins with DNA binding specificity distinct from the SIE used in this study cannot be discounted. Identification of specific genes regulated by TPO should facilitate addressing this question. It is clear, however, that signal specificity of individual cytokines is not mediated solely through the action of specific STAT proteins. Other considerations such as the pattern of receptor expression and the competence of individual genes to be transcribed likely play major roles in determining the response of different cells to a particular cytokine. c-Mpl expression is highly restricted to primitive hematopoietic cells and cells of the megakaryocyte lineage. This pattern of expression may provide a key component of TPO signal specificity.

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