Studies with Chimeric Mpl/JAK2 Receptors Indicate That Both JAK2 and the Membrane-proximal Domain of Mpl Are Required for Cellular Proliferation*

Received for publication, February 4, 2002, and in revised form, April 17, 2002 Published, JBC Papers in Press, April 29, 2002, DOI 10.1074/jbc.M201120200

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The thrombopoietin (TPO) receptor c-Mpl, like other members of the cytokine receptor superfamily, requires the association and activation of Janus kinases (JAKs) for normal signal transduction. The membrane-proximal portion of the signaling domain, containing conserved box1 and box2 motifs, is sufficient to support the proliferation of cytokine-dependent cell lines and basal megakaryocytogenesis in vivo. We hypothesized that activation of the JAK2 kinase alone might be sufficient for proliferative signaling. To test this premise, we constructed chimeric receptors in which the extracellular and transmembrane portions of Mpl were fused to the pseudokinase and kinase domains of murine JAK2 kinase. When expressed in the interleukin-3-dependent cell line Ba/F3, the chimeric receptors were appropriately expressed on the cell surface and were able to initiate tyrosine kinase activity upon exposure to TPO. However, chimeric receptors lacking an intact box2 domain of Mpl were unable to support proliferation at any concentration of TPO. Only chimeric receptors containing both JAK2 kinase activity and the box2 region initiated proliferative signaling. Within the box2 motif, we determined that the sequence Glu56-Ile57-Leu58 of the Mpl cytoplasmic domain is critical for proliferation of the chimeric receptors. Furthermore, TPO-dependent induction of c-myc transcription is also dependent on this motif. These results indicate that JAK2 activation alone is not sufficient for TPO-induced proliferation and that one or more essential signaling pathways must arise from the cytoplasmic domain of Mpl that includes box2. Although the nature of the signal transduction pathway is not yet known, this second proliferative event is likely to regulate c-myc expression.

The thrombopoietin (TPO) receptor c-Mpl is necessary for normal megakaryocyte and platelet development and promotes the survival of hematopoietic progenitor and stem cells (reviewed in Ref. 1). Like other members of the cytokine receptor superfamily, Mpl does not contain endogenous tyrosine kinase activity, but instead regulates the activity of Janus kinases (JAKs). It has been widely reported that mutations or deletions of the Mpl box1 and box2 motifs abrogate JAK2 phosphorylation and also prevent TPO-induced proliferation (2–6) as well as the transforming potential of the v-mpl oncogene (7). Similarly, an engineered cell line that lacks JAK2 expression cannot support TPO signal transduction (8), and fetal liver cells derived from jak2 nullizygous mouse embryos do not contain megakaryocyte progenitors or TPO-responsive cells (9). Therefore, the bulk of the available data indicate that JAK2 activation is essential for normal TPO signaling.

Our group (6) and others (2) have demonstrated that the membrane-proximal portion of the Mpl signaling domain is sufficient for both JAK2 phosphorylation and proliferation, comparable to that seen with the wild-type receptor. This was confirmed in vivo by the recent report of experiments in which an altered Mpl receptor with only 61 membrane-proximal cytoplasmic residues was targeted to the native mpl locus; such homozygous mice display normal base-line megakaryocyte and platelet production (10).

All of the truncated Mpl receptors utilized in the previously reported studies include the box1 and box2 motifs, believed to be required for JAK docking to the receptor. The core of box1 contains two proline residues, separated by either one or two intervening amino acids, usually within the membrane-proximal 25 residues of the cytoplasmic domain (11). For Mpl, box1 includes the sequence Pro-Ser-Leu-Pro (residues 17–20) (7). Box2 is less precisely conserved, but is usually recognized by its position (within residues 40–65 of the membrane-spanning domain) and by a cluster of acidic residues embedded in a hydrophobic region containing one or two basic residues at the carboxyl-terminal end (11). In the Mpl receptor, box2 may include much of the region between cytoplasmic residues 45 and 60. At present, it is not known whether the membrane-proximal domain of Mpl fulfills any role other than the recruitment and activation of JAKs.

Our previous studies (6) and those of others (5) have shown a direct correlation between the level of JAK2 phosphorylation (activation) and the level to which cellular proliferation is supported. This finding led to the hypothesis that activation of JAK2 was not only necessary, but also sufficient for Mpl-directed proliferation. To test this hypothesis directly and to

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* This work was supported by United States Public Health Service Grants K08 HL03498 and R01 HL65498 (to J. G. D.) and Grant R01 CA31615 (to K. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: TPO, thrombopoietin; JAK, Janus kinase; JH, JAK homology; mIL-3, murine interleukin-3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GFP, green fluorescent protein; STAT, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase.
was generated at the desired position of the c-mpl coding region, immediately before amino acid 526 of the full-length protein, upstream of both the pseudokinase and kinase domains. Repeated published studies using the growth hormone receptor in COS cells demonstrated that this technique is feasible and that the fused JAK2 kinase domain is activated, as predicted, in a growth hormone-dependent manner (12). Using both the kinase (JH1) and pseudokinase (JH2) domains of murine JAK2, there should be less constitutive tyrosine kinase activity than was seen when only the kinase domain was used (12). In this study, we found that a chimeric receptor containing 12 cytoplasmic residues of Mpl fused directly to the JH2 and JH1 domains of JAK2 (T12/JAK) was phosphorylated in a TPO-dependent manner, but was not able to support proliferation of Ba/F3 cells, even at high TPO concentrations. In a series of add-back constructs, we found that JAK2 fused after 28 cytoplasmic residues of Mpl (T28/JAK, including box1) was also incapable of proliferative signaling. By mutating critical residues of the box1 motif, we were able to inactivate forms of Mpl containing 53 and 69 cytoplasmic residues (T53/box1(φ) and T69/box1(φ), respectively). When JAK2 was fused to these truncated Mpl constructs, TPO-dependent proliferation was restored if all (T69/box1(φ) JAK), but not part (T53/box1(φ)JAK2, of box2 was present, despite both chimeric receptors displaying JAK2 activity. Moreover, alanine substitution of only three amino acids in this region eliminated its function. This study establishes that, although JAK2 activation is necessary, it is not sufficient for proliferation of Ba/F3 cells and that one or more additional signaling events arise from the box2 subdomain. Finally, additional studies demonstrate that the same residues of box2 are essential for ligand-dependent c-myc induction. Because the box1/box2 organization of Mpl is similar to that of many other receptors, it is likely that our results reflect signal transduction mechanisms shared by other cytokine receptors.

**Experimental Procedures**

**Plasmid Construction**—A wild-type murine mpl cDNA (13) was cloned into the expression vector pcDNA3 (Invitrogen, Carlsbad, CA) using EcoRI (5′) and XhoI (3′) as cloning sites. Carboxy-terminal truncations (T53 and T69) were described previously (6). The wild-type murine jak2 cDNA was generously provided by James Ihle (St. Jude Children’s Research Hospital, Memphis, TN) and Stuart Frank (University of Alabama at Birmingham, Birmingham, AL). To make the chimeric jak2 constructs, a new XhoI site was created immediately downstream of the jak2 stop codon. Also, a unique ClaI site was generated in the jak2 cDNA immediately before amino acid 526 of the full-length protein, upstream of both the pseudokinase and kinase domains as utilized by previous studies (12). Finally, a new ClaI site was generated at the desired position of the c-mpl expression plasmid, and the ClaI-XhoI fragment of jak2 (1812 bp, encoding 604 residues) was ligated into the corresponding sites of pcDNA3/Newmpl. The ClaI site resulted in the addition of two residues (Ile-Asp) at the fusion site. This technique is feasible and that the fused JAK2 kinase domain is activated, as predicted, in a growth hormone-dependent manner (12). Using both the kinase (JH1) and pseudokinase (JH2) domains of murine JAK2, there should be less constitutive tyrosine kinase activity than was seen when only the kinase domain was used (12). In this study, we found that a chimeric receptor containing 12 cytoplasmic residues of Mpl fused directly to the JH2 and JH1 domains of JAK2 (T12/JAK) was phosphorylated in a TPO-dependent manner, but was not able to support proliferation of Ba/F3 cells, even at high TPO concentrations. In a series of add-back constructs, we found that JAK2 fused after 28 cytoplasmic residues of Mpl (T28/JAK, including box1) was also incapable of proliferative signaling. By mutating critical residues of the box1 motif, we were able to inactivate forms of Mpl containing 53 and 69 cytoplasmic residues (T53/box1(φ) and T69/box1(φ), respective-ly). When JAK2 was fused to these truncated Mpl constructs, TPO-dependent proliferation was restored if all (T69/box1(φ) JAK), but not part (T53/box1(φ)JAK2, of box2 was present, despite both chimeric receptors displaying JAK2 activity. Moreover, alanine substitution of only three amino acids in this region eliminated its function. This study establishes that, although JAK2 activation is necessary, it is not sufficient for proliferation of Ba/F3 cells and that one or more additional signaling events arise from the box2 subdomain. Finally, additional studies demonstrate that the same residues of box2 are essential for ligand-dependent c-myc induction. Because the box1/box2 organization of Mpl is similar to that of many other receptors, it is likely that our results reflect signal transduction mechanisms shared by other cytokine receptors.

**Cell Line and Culture Conditions**—Parental Ba/F3 cells were originally provided by Alan D’Andrea (Dana-Farber Cancer Institute, Boston, MA). These cells and all derived sublines were maintained in RPMI 1640 medium (Bio-Whittaker, Inc., Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, penicillin, and streptomycin. Parental Ba/F3 cells were tested for Mpl expression by immunoprecipitation and Western blotting (see below). If the cells were transfected, they were then washed extensively to remove growth factor and allowed to incubate in serum- and cytokine-free medium supplemented with 0.5% bovine serum albumin for 14–18 h.

**Reverse Transcription-PCR to Detect c-myc Induction**—Ba/F3 cells expressing truncated and mutant Mpl receptors were maintained in RPMI 1640 medium containing 10% fetal calf serum and IL-3 while growing in log phase. Cells were again washed and analyzed on a flow cytometer (FACScalibur, BD Pharmingen) to detect fluorescence intensity. Parental Ba/F3 cells were used as a negative control.

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Recombinant murine TPO was added (10 ng/ml), and aliquots were withdrawn at 0, 1, and 3 h. Total RNA was isolated from cells using the RNeasy minikit (QIAGEN Inc., Valencia, CA); the concentration was determined by spectrophotometry; and the quality was assessed on agarose gel. One microgram of total RNA was subjected to reverse transcription using oligo(dT) (41.7 ng/ml; Invitrogen) and Superscript II (Invitrogen) according to the manufacturer’s directions. One-tenth of the reaction was subsequently removed for PCR with either c-myc oligonucleotides (primers: 5′-CCCACCTCTCCCAACCC-3′ (sense) and 5′-GTCCTCTCAAGTAACTCG-3′ (antisense); predicted product of 607 bp) or glyceraldehyde-3-phosphate dehydrogenase oligonucleotides (primers: 5′-CCATGGAGAAGGCTGGGG-3′ (sense) and 5′-CAAAGTGTGATGAGATGCC-3′ (antisense); predicted product of 194 bp). PCR products were analyzed by electrophoresis on a 1% agarose gel in the presence of ethidium bromide. The gel was photographed with a Polaroid camera on a UV transilluminator.

**RESULTS**

**Truncated and Chimeric Mpl Constructs**—The receptors utilized in this study are illustrated in Fig. 1A. For all constructs, the wild-type extracytoplasmic and transmembrane domains of Mpl were intact. Carboxyl-terminal truncations of the signaling domain are denoted by “T” followed by the number of remaining membrane-proximal amino acids (e.g., in the T69 receptor, cytoplasmic residues 70–121 were deleted, and only amino acids 1–69 remain). Chimeric receptors containing the JH1 and JH2 domains of JAK2 are indicated by the suffix “JAK.” A double mutation in box1 that prevents activation of endogenous JAK2 (i.e., P17G/P20G) is indicated as “box1(Δ),” and a triple alanine substitution in box2 (E56A/I57A/L58A) is indicated as “box2(ΔAA).” Chimeric receptors in which the kinase domain of JAK2 is inactivated by a Tyr-to-Phe substitution (T69/JAK) were starved of serum and growth factor for 6 h and then stimulated with either TPO or IL-3 in the presence of 4-hydroxymethoxifen (Sigma) or an equivalent amount of vehicle (ethanol) for 16 h. Cellular DNA was stained with Hoechst dye 33342 (Sigma), and cells were analyzed by flow cytometry. For each sample, the gate was set to include only cells expressing GFP, and then cell cycle histograms based on DNA content were constructed. The percentage of cells in the G1, S, and G2/M phases of the cell cycle was determined by manual gating.

**Chimeric Mpl/JAK2 Receptors with 12 or 28 Cytoplasmic Residues of Mpl Do Not Support Proliferation**—Based on our previous observation that the level of JAK2 phosphorylation and proliferation appears to be directly correlated (6), we hypothesized that phosphorylation (i.e., activation) of JAK2 alone would be sufficient for proliferative signaling. Previous studies with the erythropoietin and epidermal growth factor receptors fused to the JAK2 kinase domain suggested that this might also hold for Mpl (16, 17). Therefore, we initially designed a chimeric Mpl/JAK2 receptor that included only 12 cytoplasmic residues from Mpl plus the JAK2 JH1 (kinase) and JH2 (pseudokinase) domains (T12/JAK). After verifying that the receptor was adequately expressed on the cell surface of Ba/F3 cells (Fig. 1B), distinct clones were tested for TPO-induced proliferation by MTT assay (Fig. 4A). Contrary to expectation, we found that the chimeric receptor was unable to support proliferation, even at high TPO concentrations. We then constructed a receptor containing 28 membrane-proximal cytoplasmic residues of Mpl including the entire box1 motif (T28/JAK). Again, despite high level receptor expression (Fig. 1B), the chimeric receptor failed to support Ba/F3 cellular proliferation in TPO (Fig. 4A). These experiments suggest that, in addition to phosphorylation of the fused JAK2 domain, a portion of the Mpl cytoplasmic domain distal to residue 28 may be required for cellular proliferation.

**Box1 of Mpl Is Essential for JAK2 Activation and Proliferation**—The importance of box1 for cytokine receptor signaling has been well documented, especially the characteristically spaced proline residues within the first 15–25 cytoplasmic residues (7, 11). To confirm the role of box1 in the truncated Mpl receptor containing 69 cytoplasmic residues, we mutated the two proline residues in the Mpl box1 motif to glycine (P17G/P20G). As predicted, the resulting T69/box1(Δ) receptor was completely deficient in both TPO-dependent proliferation (Fig. 2A) and phosphorylation of endogenous JAK2 (Fig. 2B). The same results were obtained using a T53/box1(Δ) receptor (data not shown). These data confirm that box1 provides a critical signal for cellular proliferation and is necessary for JAK2 tyrosine phosphorylation in the T69 truncated Mpl receptor.

The Box2 Core Motif Glu56-Ile57-Leu69 Plays a Major Role in Signaling by the T69 Receptor—From our studies of truncated Mpl receptors, we know that there is a major decrease in TPO-dependent proliferation when residues 53–69 are deleted from the cytoplasmic domain (6). Further truncation of residues 28–53 eliminates the residual proliferative response. By mutating amino acids between cytoplasmic positions 53 and 69 to alanine, we were able to measure the effect on receptor function. The greatest effect on proliferation of Ba/F3 cells was observed when three residues (Glu56-Ile57-Leu69) were substituted with alanines, creating a box2 mutant designated T69/box2(ΔAA) (Fig. 3A). By itself, this mutation diminished (but did not eliminate) tyrosine phosphorylation of the native JAK2 kinase (Fig. 3B).
**Fig. 1.** Receptor constructs and expression in Ba/F3 cells. A, the cytoplasmic domains of wild-type (wt) Mpl, truncation mutants (T53 and T69), and chimeric receptors (T12/JAK, T28/JAK, T53/JAK, and T69/JAK) are depicted. The cell membrane is shown on the left, and the carboxyl terminus is to the right. Mpl is in dark gray with the box1 and box2 motifs indicated. For receptors containing all or part of box2, critical proline residues within box1 were mutated to glycine (P17G/P20G) to prevent activation of endogenous JAK2 (denoted box1(φ)). The pseudokinase and kinase domains of JAK2 (light gray) represent the same 604-amino acid fragment in all chimeric constructs (indicated by the suffix JAK). Mutation of critical box2 residues to alanine is indicated by box2(AAA), and substitution of a critical tyrosine residue in the JAK2 kinase domain is represented by JAK(Y1007F). B, representative clones were analyzed by fluorescent immunostaining for the surface expression of the Mpl extracellular domain. The control cells (Ba/F3, unshaded curve) are shown for reference.
JAK2 kinase domain, it is theoretically possible that T69/box1(H9278)/JAK supports proliferation because of a favorable orientation of the fused JAK2 kinase compared with its orientation in T53/box1(H9278)/JAK, allowing interaction with signaling molecules docked on the Mpl cytoplasmic residues. Because the box2 core sequence Glu56-Ile57-Leu58 is critical for proliferation (Fig. 3A), we engineered the triple alanine substitution in T69/box1(H9278)/JAK, creating the T69/box1(AAA)/JAK receptor with no change in the number of residues acting as a spacer between Mpl and the JAK kinase domain. This mutation completely eliminated TPO responsiveness of the chimeric receptor (Fig. 4C), verifying the importance of the box2 motif for proliferation.

It is also possible that the T69/box1(φ)/JAK receptor acts by recruiting endogenous JAK2 to the signaling complex, despite the fact that T69/box1(φ) can neither proliferate nor support JAK2 phosphorylation (Fig. 2). To prove that chimeric JAK2, rather than endogenous JAK2, is necessary for proliferation, we introduced a mutation in the chimeric JAK2 kinase domain that has previously been shown to eliminate essentially all auto-phosphorylation and kinase activity (Y1007F) (14). When expressed in Ba/F3 cells, the resulting T69/box1(φ)/JAK(Y1007F) receptor supported no TPO-dependent proliferative capacity (Fig. 4C), confirming the essential role of the chimeric JAK2 kinase for proliferation induced by T69/box1(φ)/JAK.

Chimeric Receptors Undergo Tyrosine Phosphorylation in Response to TPO Stimulation—We have previously demonstrated that full-length Mpl receptors are tyrosine-phosphorylated in response to TPO binding at the carboxyl terminus (Tyr112 and Tyr117), but that the truncated T69 receptor is not tyrosine-phosphorylated despite the presence of two residual cytoplasmic tyrosine residues, Tyr6 and Tyr28 (6). Thus, it is likely that TPO-dependent phosphorylation of a chimeric receptor represents tyrosine phosphorylation of the covalently attached JAK2 kinase domain, an essential step during JAK2 activation (14). We tested Ba/F3 cells expressing each of the chimeric receptors
Furthermore, the T53/JAK receptor underwent TPO-dependent tyrosine phosphorylation of the expressed receptor (Fig. 5). Immunoprecipitation of Mpl and Western blot analysis to detect tyrosine phosphorylation showed that the wild-type Mpl receptor was inducibly phosphorylated (Fig. 5, lanes 3 and 4), whereas the T69/JAK receptor, lacking the distal tyrosine residues, was not (lanes 5 and 6). The chimeric receptors T28/JAK (lanes 7 and 8), T69/box1(ϕ)/JAK (lanes 9 and 10), and T69/box1(ϕ)/box2(AAA)/JAK (lanes 11 and 12) were each phosphorylated in response to TPO stimulation, but the T69/box1(ϕ)/JAK (Y1007F) construct (lanes 13 and 14) was not, reflecting mutation of a residue critical for JAK2 kinase activity (14). It should be noted that the chimeric Mpl/JAK receptor (~140 kDa) is significantly larger than the wild-type Mpl receptor (95 kDa) and that the major sites of tyrosine phosphorylation are different for the native receptor (Mpl Tyr112) and the chimeric receptors (JAK2 Tyr1007), perhaps making it difficult to compare the intensity of these phosphotyrosine bands. Despite variation between clones, the extent of tyrosine phosphorylation for each chimeric receptor was similar (lanes 8, 10, and 12). We also confirmed that the chimeric receptors T12/JAK, T53/box1(ϕ)/JAK, T53/JAK, and T69/JAK underwent TPO-dependent tyrosine phosphorylation (data not shown). Furthermore, the T53, T53/box1(ϕ), and T69/box1(ϕ) receptors were not phosphorylated, as they contain neither the Mpl Tyr112 nor the JAK2 Tyr1007 site (compare with T69; lane 6) (data not shown). From these data, we conclude that steric constraints within the chimeric receptors do not preclude activation of the chimeric JAK2 kinase and that each of the chimeric receptors is phosphorylated to a comparable extent. Thus, quantitative differences in activation of chimeric JAK2 are unlikely to have affected our proliferation results. Of note, after SDS-PAGE, all Mpl receptors appeared as doublets that differed in size by ~8 kDa regardless of TPO stimulation (Figs. 5 and 6, lower panel). Although the post-translational modification responsible for this is not known, only the upper bands were tyrosine-phosphorylated.

Phosphorylation of Endogenous (Native) JAK2 Is Not Required for Proliferation—Next, we studied tyrosine phosphorylation of the chimeric and endogenous JAK2 proteins by immunoprecipitation and Western blot analysis (Fig. 6). As previously demonstrated (6), both the wild-type and truncated (T69) Mpl receptors supported tyrosine phosphorylation of the native JAK2 kinase (125 kDa) in response to TPO (Fig. 6, lanes 1, 2, 5, and 6). The chimeric receptors T28/JAK (lanes 3 and 4), T69/box1(ϕ)/JAK (lanes 9 and 10), and T69/box1(ϕ)/box2(AAA)/JAK (lanes 11 and 12) each demonstrated tyrosine phosphorylation of the larger chimeric JAK2 protein (~140 kDa), but not endogenous JAK2. Cells expressing the T69/JAK receptor (with and intact box1 motif) supported phosphorylation of both the endogenous and chimeric JAK2 molecules (lanes 7 and 8), whereas the T69/box1(ϕ)/JAK (Y1007F) receptor could not phosphorylate either form of JAK2 (lanes 13 and 14).

Proliferation Does Not Correlate Directly with the Quantity of JAK2 Phosphorylation—One possible concern is that subtle differences in the cumulative JAK2 activity, whether endogenous or chimeric, might affect the proliferative capacity of the receptors under analysis. There may be a threshold of JAK2 activity below which no proliferation is possible. To test these hypotheses, we compared Ba/F3 cells expressing the T53 versus T53/JAK receptors (Fig. 7A). Despite the addition of covalently linked JAK2, the chimeric receptor did not proliferate better than its corresponding truncated receptor. Similar results were obtained when the T69 and T69/JAK constructs were compared.

FIG. 5. Tyrosine phosphorylation of chimeric receptors in response to TPO stimulation. Whole cell lysates from Ba/F3 cells expressing the indicated receptors were generated before (~) and after (+) stimulation of cells with TPO (6.7 ng/ml) for 10 min. Anti-Mpl antiserum was used for immunoprecipitation (IP). Samples were analyzed by Western blots and probed for the presence of phosphotyrosine (pY). The blot was then stripped and reprobed to demonstrate equal loading of protein in each lane (lower panel).
are there any signaling pathways, other than those initiated by JAK2, that are required for hematopoietic growth factor-induced cellular proliferation? The major findings of our studies include the following. 1) In our system, JAK2 kinase activity is necessary, but not sufficient, to support TPO-induced hematopoietic cellular proliferation. 2) A second proliferative signal, arising from the box2 subdomain of Mpl, is also necessary (but not sufficient) for proliferative signaling and can complement JAK2 kinase activity. 3) TPO-induced c-myc induction is dependent on an intact box2 motif and does not require JAK2 activation.

A number of previous studies have made use of the chimeric cytokine receptor/JAK model to study the ability of a Janus tyrosine kinase to initiate signal transduction and to support normal cellular physiology. This approach was first tested using a chimeric growth hormone receptor/JAK2 protein (growth hormone receptor/JAK) in COS-7 cells. We hypothesized that the box2 domain of Mpl might promote the formation of JAK2 dimerization in greater cumulative JAK2 phosphorylation (compare lane 2 and 4). However, these studies were done in an autonomous cell line, making it difficult to assess the proliferative potential of the chimeric receptor.

In addition to the data presented above, results from several published studies support the hypothesis that at least one other signaling pathway, in addition to JAK2, is involved in cytokine-mediated proliferation. First, cross-linking of a CD16/CD7/JAK2 fusion molecule resulted in tyrosine phosphorylation and activation of many cytokine pathways, but was unable to induce proliferation of Ba/F3 cells using either full-length JAK2 or the pseudokinase and kinase domains. As appears to be the case for our chimeric Mpl/JAK2 receptors, failure to up-regulate c-myc expression may be responsible for the lack of growth signaling in these other cell systems.

Second, a chimeric receptor consisting of interleukin-2 receptor-γc fused to JAK3 failed to induce proliferation of CTLL-2 cells without a membrane-proximal domain of the γc subunit. Third, expression of a constitutively active form of JAK2 in cytokine-dependent cells did not result in autonomous growth. Finally, a mutant form of Mpl has been described that supports TPO-dependent proliferation without detectable JAK or STAT activation. These data all support a role for at least one distinct non-JAK signaling pathway in cellular proliferation.

In contrast, a number of published reports indicate that direct JAK2 dimerization can be sufficient to support cellular proliferation of hematopoietic cells. For example, fusion of the erythropoietin receptor to the JAK2 kinase and pseudokinase domains (erythropoietin receptor/JAK) produced a chimeric receptor capable of supporting erythropoietin-dependent proliferation in FDCWEHI-1 cells. Similarly, experiments performed with a chimeric epidermal growth factor receptor/JAK2 protein demonstrated epidermal growth factor-dependent proliferation and inhibition of apoptosis in 32D cells as well as erythroid colony formation in mouse fetal liver cells. Furthermore, coumarrycin-induced dimerization of TYK2, a member of the JAK family, supported proliferation of Ba/F3 cells, albeit with absolute dependence on Ras signaling. Finally, other chimeric proteins that affect constitutive dimerization of JAK2 resulted in factor-independent growth of cytokine-dependent cell lines and, in the case of a TEL/JAK translocation, may play a role in leukemogenesis (i.e. TEL/JAK chimera). Clearly, our results differ from the conclusions of these latter studies. Several potential explanations for the discrepant findings are discussed below.
between cell lines utilized, inherent differences in the receptors studied, level of kinase activation, and the steric constraints on JAK2 as a chimeric protein).

As with any signaling studies done in transformed cell lines, there is concern that the specific cell line utilized may affect results. Nearly all of the studies described above were done in cytokine-dependent cell lines (FDCP, 32D, CTLL, and Ba/F3). It is possible that, in some of these cell lines, there was constitutive activation of additional signaling pathways. Although these signaling mechanisms may be unable to support proliferation by themselves, they may complement the incomplete proliferative signal of JAK kinase, making it appear that JAK activation was sufficient for proliferation. To be certain that our Ba/F3 cells did not influence our results, several of the chimeric receptors were expressed in a different cytokine-dependent cell line, FDCP-2. The same results were obtained; TPO-dependent proliferation was observed with cells expressing the chimeric T69/box1(φ)/JAK molecule, but not with the T12/JAK and T28/JAK receptors (data not shown). The confirmation of our findings in a second cell line excludes the possibility of an atypical physiology restricted to our particular strain of Ba/F3 cells.

A second potential explanation for the differences between our conclusions and those of others could lie in the nature of the Mpl receptor. It is possible that the ligand-binding domains of the erythropoietin and epidermal growth factor receptors (but not Mpl) may recruit additional signaling subunits, which can provide a second signal for proliferation. The ability of the extracytoplasmic domain of receptors to influence signaling specificity was previously demonstrated for the erythropoietin and interleukin-2 receptors (32).

FIG. 7. T53 versus T53/JAK. A, MTT assays were done to compare the proliferative potential of cells expressing either the T53 or T53/JAK Mpl construct. Cells expressing T69 are shown to indicate the level of full TPO-dependent proliferation. Each data point represents the mean of triplicate readings. Two additional T53/JAK clones gave similar results. B, cell lysates were generated before (−) and after (+) stimulation of cells with TPO (6.7 ng/ml) for 10 min. Anti-JAK2 antiserum was used for immunoprecipitation (IP). Samples were analyzed by Western blotting and probed for the presence of phosphotyrosine (pY). The JAK2 blot was stripped and reprobed to demonstrate equal loading of protein in each lane (lower panel).

FIG. 8. c-myc up-regulation is dependent on the box2 domain of Mpl. Ba/F3 cells expressing the truncated form of Mpl with 69 cytoplasmic amino acids (T69), the same receptor with a mutation in box1 (T69/box1(φ)), the chimeric Mpl/JAK2 receptor (T69/box1(φ)/JAK), and the chimeric receptor with a mutation of the box2 sequence Glu56-Leu57-Leu58 to alanines (T69/box1(φ)/box2(AAA)/JAK) were exposed to TPO for 0, 1, or 3 h. Total RNA was extracted and subjected to reverse transcription-PCR with oligonucleotide primers specific for c-myc (upper panel; 28 cycles) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; lower panel; 21 cycles). The data are representative of three separate experiments.

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spacer region provided by the pseudokinase domain (JH2), the chimeric kinase domains of adjacent receptors may not be spatially oriented for optimal cross-phosphorylation. Finally, it is possible that excluding the amino-terminal portion of the molecule (JH3–JH7) might affect physiologic function, as this portion of Jak2 contains protein-interactive domains that help target the kinase to sites of action (33, 34) and may also be required for interaction with members of the Src kinase family (35). Published reports on this issue provide conflicting answers. On one hand, direct dimerization of Jak2 using a coumermycin/glycine B system requires the full-length molecule for short-term proliferation (36). On the other hand, the previously described erythropoietin receptor/Jak and epidermal growth factor receptor/Jak receptors, which lack the amino terminus of Jak2, are capable of supporting cellular proliferation (16, 17).

For one or more of the reasons discussed above, the chimeric Jak2 kinase does not appear to be as efficient in supporting proliferation as endogenous Jak2 activated through an intact box1 motif. This is most clearly shown by the fact that mutation of the box2 core motif partially abrogates proliferation in the context of the intact T69 receptor (Fig. 3A), but completely eliminates proliferation when Jak2 is activated only as part of the fusion protein (T69/box1(Δ)box2/AAA/Jak) (Fig. 4C). Nonetheless, our results demonstrate that, in the context of chimeric Mpl/Jak2 proteins, the covalent kinase domain is active and, in combination with an intact box2 subdomain, can support moderate proliferation of the cells expressing T69/box1(Δ)/jak. As such, our experimental system allows us to map a subdomain of Mpl that is required to complement the chimeric Jak2 kinase signal.

We cannot exclude the possibility that a very small amount of endogenous Jak2, below our limits of detection by immunoprecipitation and Western blotting, is acting to recruit additional signaling molecules through its amino terminus. However, this possibility would require that endogenous Jak2 molecules be recruited to the T69/box1(Δ)/jak molecule, but not to T69/box1(Δ), which contains the identical Mpl domain, nor to T69/box1(Δ)/jak Y100/F, which differs only by a point mutation within the Jak2 kinase domain. From these experiments, it seems likely that a second proliferative signal arises from the membrane-proximal portion of the Mpl cytoplasmic domain, specifically including the box2 region between residues 53 and 69. Although signaling molecules that directly interact with this region have not yet been identified, the induction of c-myc expression appears to be a downstream event that is dependent on the box2 core sequence Glu56/Ile57/Leu58. Moreover, increases in c-myc RNA occur in response to Tpo even when box1 is mutated such that endogenous Jak2 is not phosphorylated and the receptor is incapable of supporting proliferation. In contrast, we were unable to detect tyrosine phosphorylation of STAT5 and ERK1/2 signaling molecules in the absence of endogenous or chimeric Jak2 activity (data not shown). In previous studies, Myc has been identified as a transcription factor that promotes proliferation and inhibits differentiation for multiple cytokine receptors (21, 37–39).

It is interesting that the same box2 motif has also been shown to play a role in ligand-dependent Mpl receptor internalization (40). Our preliminary results studying truncated and mutant Mpl constructs demonstrate that the residual internalization of T69 receptors (30–40% that of full-length molecules) is regulated by the Glu56/Ile57/Leu58 motif and may not require Jak2 phosphorylation (40). It was recently demonstrated that internalization of the erythropoietin and gp130-containing receptors also occurs in a Jak kinase-independent manner (41, 42). Currently, we hypothesize that intracellular trafficking of Mpl and associated molecules serves an important function by targeting or transporting the signaling complex to intracellular compartments, as has been shown for the interferon and several interleukin receptors (43–45). Future studies will focus on the mechanisms linking Mpl internalization with cellular proliferation and an increase in c-myc expression.

In conclusion, our data suggest that Tpo-stimulated proliferation of Ba/F3 cells requires two distinct signaling events. One is the activation of Jak2 kinase, normally mediated through association with the receptor box1 and box2 motifs and ligand-induced dimerization. The second results in the up-regulation of c-myc RNA and requires an intact box2 motif, especially the Glu56/Ile57/Leu58 sequence. In the context of a chimeric Mpl/Jak2 receptor, neither of these signals alone is sufficient for cellular proliferation, but together they promote growth and survival of Ba/F3 cells. Because of the homology between Mpl and the membrane-proximal domains of other cytokine receptors, it should be possible to extrapolate these findings to signaling by other members of the cytokine receptor superfamily.

Acknowledgments—We thank James Ihle and Stuart Frank for murine c-myc cDNA, Don Fostie, Emogene Genetics, Seattle, WA, and Pamela Hunt (Amgen Corp., Thousand Oaks, CA) for murine mpl cDNA and Anti-mpl antisera, Garry Nolan for GFP cDNA, and Ronald Cheung for MycER-GFP. M. Sara Lowe and Andrea Ogston provided assistance with manuscript preparation.

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