The Thrombopoietin Receptor Can Mediate Proliferation without Activation of the Jak-STAT Pathway

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Summary

Cytokine receptors of the hematopoietic receptor superfamily lack intrinsic tyrosine kinase domains for the intracellular transmission of their signals. Instead all members of this family associate with Jak family nonreceptor tyrosine kinases. Upon ligand stimulation of the receptors, Jaks are activated to phosphorylate target substrates. These include STAT (signal transducers and activators of transcription) proteins, which after phosphorylation translocate to the nucleus and modulate gene expression. The exact role of the Jak-STAT pathway in conveying growth and differentiation signals remains unclear. Here we describe a deletion mutant of the thrombopoietin receptor (c-mpl) that has completely lost the capacity to activate Jaks and STATs but retains its ability to induce proliferation. This mutant still mediates TPO-induced phosphorylation of Shc, Vav, mitogen-activated protein kinase (MAPK) and Raf-1 as well as induction of c-fos and c-myc, although at somewhat reduced levels. Furthermore, we show that both wild-type and mutant receptors activate phosphatidylinositol (PI) 3-kinase upon thrombopoietin stimulation and that thrombopoietin-induced proliferation is inhibited in the presence of the PI 3-kinase inhibitor wortmannin. These results demonstrate that the Jak-STAT pathway is dispensable for the generation of mitogenic signals by a cytokine receptor.

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The proto-oncogene c-mpl (1, 2) is the receptor for thrombopoietin (TPO)[1], a cytokine which has been shown to be the major regulator of megakaryopoiesis and platelet formation (3–5). C-mpl was originally isolated as the cellular homologue of the transforming oncogene v-mpl of the myeloproliferative leukemia virus (MPLV) (1). Like many cytokine receptors, c-mpl is a member of the hematopoietic receptor superfamily (6). This family is characterized by conserved cysteine residues and a common amino acid motif -WSXWS- in the extracellular domain, and by the lack of intrinsic tyrosine kinase activity in the intracellular domain (6). Nevertheless, tyrosine phosphorylation plays an important role for the intracellular signaling events initiated by these receptors. It has become apparent that nonreceptor tyrosine kinases, such as Jak and Src family members, are recruited by these receptors and mediate the tyrosine phosphorylation of cellular target proteins (6, 7). The signal transduction of cytokine receptors has been extensively studied over the last several years and numerous proteins have been identified which are involved in the signaling pathways leading from the membrane to the nucleus. The Jak kinases seem to function very early on in this process (6, 7). They bind to the intracellular part of cytokine receptors either constitutively or after ligand stimulation and their kinase activities are upregulated after receptor activation. This is believed to result in tyrosine phosphorylation of the receptor itself and of the STAT proteins, a novel class of SH2 domain-containing transcription factors. The STATs become activated upon phosphorylation and translocate from the cytoplasm to the nucleus where they bind to specific DNA motifs. To date, four Jak kinases, Jak-1, Jak-2, Jak-3 and Tyk-2, and at least six different STAT proteins (STAT 1-6) have been described (6, 7). Different cytokine receptors activate distinct but overlapping sets of Jaks and STATs.

Ligand stimulation of c-mpl has been shown to result in the phosphorylation and activation of Jak-2, Tyk-2 and

[1]*Abbreviations used in this paper:* EMSA, electrophoretic mobility-shift assay; MAPK, mitogen-activated protein kinase; MPLV, myeloproliferative leukemia virus; PI, phosphatidylinositol; STAT, signal transducers and activators of transcription; TPO, thrombopoietin.

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STAT1, STAT3, and STAT5 (8-12). Furthermore, TPO-induced phosphorylation of Shc, MAPK, Raf-1, Cbl, Vav, SHPTP-1, and SHPTP-2 has been described (8–11). It is not clear to what extent the Jak kinases are responsible for phosphorylation of proteins other than STATs.

The intracellular domains of receptors of the hematopoietic receptor superfamily share two membrane-proximal regions of weak homology, designated box1 and box2 (6). Both motifs have been shown to be crucial for ligand-induced cell proliferation and activation of Jaks (13–17). Box1 is required for binding of Jaks (14, 18, 19). Previous studies of c-mpl and various other cytokine receptors with mutations in the box1/box2 region have shown a correlation between Jak activation and cell proliferation (13, 14, 16, 20, 21), suggesting that Jak activation might be essential. Here we describe a deletion mutant of the thrombopoietin receptor c-mpl which reveals that proliferation can be induced without activating Jaks.

Materials and Methods

Antibodies. Polyclonal rabbit antisera against Jak-1, Jak-2, Jak-3, and Shc were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies against Vav, Raf-1, STAT3, STAT5a, STAT5b, and c-myc, and a monoclonal anti-Erk2 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–Tyk-2 antibodies were kindly provided by Dr. John Krolewski (Columbia University, New York). Horseradish peroxidase–conjugated anti-phosphotyrosine mAb RC20 (clone PY20) was purchased from Transduction Laboratories (Lexington, KY). Anti-active MAPK polyclonal antibodies were obtained from Promega (Madison, WI). Polyclonal anti–c-fos antibodies were purchased from Oncogene Sciences. Anti-STAT1 antbodies (29130) were kindly provided by Dr. Christian Schindler (Columbia University).

Expression Constructs. c-mpl deletion mutants were constructed by sequential PCR using the murine c-mpl cDNA (plasmid pSK-c-mpl, provided by Dr. Philip Leder, Harvard Medical School, Cambridge, MA; reference 2) as a template and cloned into the mammalian expression vector MT21myc (22) in frame with a myc-epitope at the 3′ end of the cloning site. Deletion mutants were generated with the help of overlapping oligonucleotides by standard methods (23). To delete aa 505-514 in c-mplΔ7, internal oligonucleotides were: 5′-ATGCCTCAGTAGCAGCAGTAGGCCCAG-3′ and 5′-CTGCTGCTACTGAGGCATGCTTTTGTGG-3′; to delete aa 515-522 in c-mplΔ8: 5′-GTCTGGAAGTCTCCTGTAGTGCGCAGG-3′ and 5′-TACAGGAGACTTCCAGACCTACACCGG-3′. The deletions were introduced into a 850-bp fragment of c-mpl extending from the BamHI site (bp 1124) to the stop codon; the flanking oligonucleotides used to amplify the region were: 5′-TTTTGGATCCACCAGGCTGTGCTCC-3′ and 5′-GACTGCGTCGACGGCTGCTGCCAATAGCTTAG-3′. The amplified fragments were digested with BamHI and SalI and cloned into SH-mpl-N (plasmid SH2-1 containing the EcoRI-BamHI fragment of c-mpl). The resulting EcoRI-SalI fragment encoding for the full-length c-mpl cDNA or the different deletion mutants was isolated and cloned into the plasmid MT21myc in frame with the myc epitope. In the double mutant c-mpl-Δ7ΔC the COOH-terminal 25 amino acids were deleted using an internal NcoI site (bp 1796). The deletions were confirmed by sequence analysis.

Proliferation Assay. Cells were cultured at a density of 5×10^4 per 200 μl in a 96-well round-bottom microtiter plate with varying concentrations of recombinant TPO in culture medium for 48 h. During the last 6 h of culture, cells were pulse-labeled with 0.5 μCi of [^{3}H]thymidine (specific activity 5 Ci/mmol; Amersham), and [^{3}H]thymidine incorporation was quantified by scintillation counting as described (24).

Cell Culture and Transfections. BAF/3 cells were cultured in RPMI medium supplemented with 10% FCS, 2 mM l-glutamine, antibiotics and 10% WEHI-3 supernatant as a source of IL-3. COS cells were maintained in DMEM containing 10% FCS, 2 mM l-glutamine and antibiotics. BAF/3 cells were cotransfected with pSV2neo (1 μg) and the receptor expression plasmids (10 μg) by electroporation using a Gene-Pulser (Bio-Rad Laboratories, Richmond, CA). 2×10^7 cells in 0.5 ml PBS were pulsed in a 0.4-cm cuvette with 250 V, 960 μF. Stable transfectants were selected sequentially in 2 mg/ml G418 and 25 ng/ml TPO. Receptor expression was confirmed by Western blot analysis with a monoclonal antibody against the myc-epitope (anti-human myc mAb, clone 9E10; Oncogene Science). COS cells were transfected with 5 μg of DNA per 2×10^6 to 4×10^6 cells by the chloroquine DEAE-dextran method (25). Stimulation experiments with COS cells were performed 48 h after transfection.

Growth Factor Stimulation, Western Blot Analysis and Immunoprecipitation. BAF/3 transfectants were growth factor-starved for 8–12 h in RPMI supplemented with 10% FCS. Stimulation was performed at a concentration of 1×10^8 cells/ml with 200 ng/ml recombinant human TPO (generously provided by Amgen, Thousand Oaks, CA) or 50 ng/ml recombinant murine IL-3 (Sigma Chem. Co., St. Louis, MO). Stimulation was stopped and cell extracts were prepared with lysis buffer (20 mM Tris-HCl, pH 8, 138 mM NaCl, 10% glycerol, 1% NP-40, 0.025 mM *p*-nitrophenyl guanidinobenzoate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 2 mM EDTA, 10 mM NaF) at 1×10^7 cells/250 μl as described (8). Proteins were resolved by SDS-PAGE (7.5% gel). Western blot analysis and immunoprecipitations were performed as described (8). Kinase activity of Jak-2 immunoprecipitates was analyzed in an in vitro kinase assay (26). In brief, immunoprecipitates were washed twice in kinase buffer (10 mM Hepes, pH 7.4, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 0.1 mM Na_3VO_4) and resuspended in 40 μl kinase buffer. 20 μCi [^{32}P]ATP (specific activity 3000 Ci/mmol) were added and the kinase reactions were incubated for 30 min at room temperature. Reactions were terminated with 2× Laemmli buffer and analyzed by SDS-PAGE.

Electrophoretic Mobility-Shift Assay. Whole cell extracts and shift reactions were prepared as described previously (26). The probe used was from the IRF-1 GAS element; 5′-gatc-GATTTCCCCGAAAT-3′ (reference 7). For supershift assays, standard shift reactions were incubated with pre-immune antibodies or antibodies to STAT1, STAT3, and STAT5 (1:20 dilution) for 30 min at 4°C.

PI 3-Kinase Assay. PI 3-kinase activity was measured as described (27). Cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies (PY20; Transduction Laboratories) and the immunoprecipitates were washed three times with lysis buffer, twice with LiCl buffer (0.5 M LiCl in 0.1 M Tris, pH 7.5), twice with kinase buffer (20 mM Hepes, pH 7.4, 15 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5 mM PMSF), and subsequently resupended in 45 μl of kinase buffer containing 10 μCi γ-[^{32}P]ATP and 25 μM cold ATP. 5 μl PI (4 mg/ml in DMSO; Avanti Polar Lipids, Alabaster, Alabama) were added and the reaction was incubated for 15 min at RT. The kinase reaction was stopped by adding 40 μl

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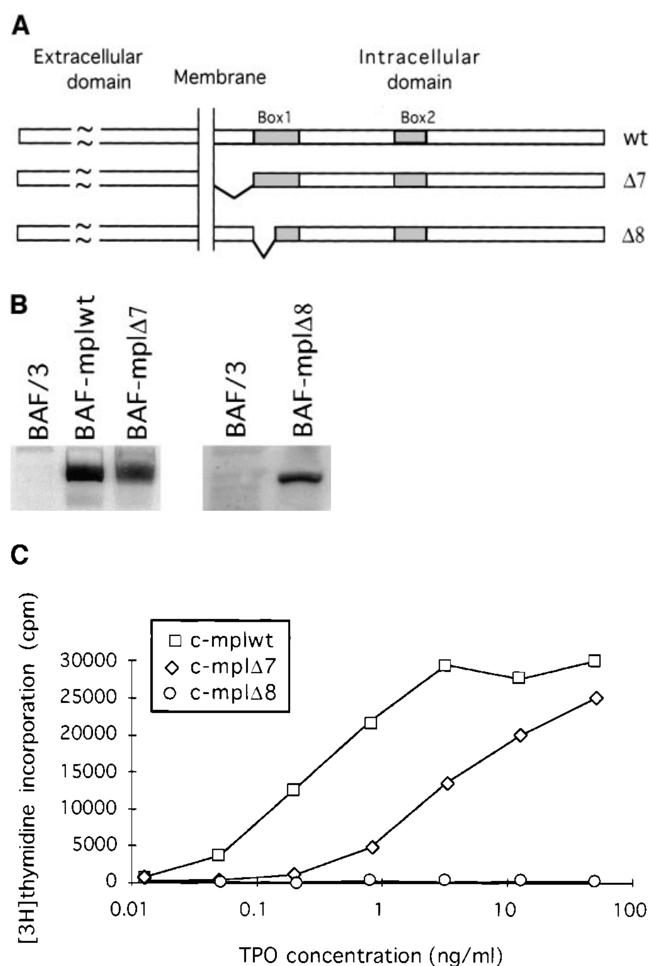

Figure 1. Mitogenic response of BAF/3 cells expressing c-mpl mutants. (*a*) Schematic representation of c-mplwt and deletion mutants c-mplΔ7 and c-mplΔ8. (*b*) Stable expression of c-mplwt, c-mplΔ7 or c-mplΔ8 in BAF/3 transfectants. Cell lysates prepared from 2×10^6 cells were resolved on a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted with a mAb against the myc-epitope. (*c*) TPO-induced proliferation of BAF-mplwt, BAF-mplΔ7, and BAF-mplΔ8 cells. [^{3}H]thymidine incorporation as an indicator of cellular proliferation was measured at different TPO concentrations. The mean of triplicate counts for each data point is shown.

of 1 N HCl and the lipids were extracted with 80 μl of CH3CL/MeOH (1:1) and analyzed by thin layer chromatography. Unlabeled PI3-P (Sigma) detected by iodine staining was used as a standard. Labeled PI3-P was visualized and quantified using a PhosphorImager.

Results and Discussion

Mitogenic Response Mediated by c-mpl Deletion Mutants. A series of deletion mutants of c-mpl was constructed; two selected mutants are depicted in Fig. 1 *a*. Mutant c-mplΔ7 lacks the first 10 amino acids (aa) (KWQFPAHYRR, aa 505-514; reference 2) of the cytoplasmic domain but retains an intact box1, whereas mutant c-mplΔ8 retains the juxtamembrane region but lacks the NH_2-terminal half of box1 (LRHALWPS, aa 515-522). Cell lines stably expressing the wild-type and mutant receptors were established by transfection of the IL-3–dependent cell line BAF/3. Comparable levels of receptor expression were detected in cells expressing c-mplwt (BAF-mplwt), c-mplΔ7 (BAF-mplΔ7), and c-mplΔ8 (BAF-mplΔ8) (Fig. 1 *b*). The transfected cells were then analyzed for their mitogenic response to TPO (Fig. 1 *c*). Expression of c-mplwt conferred responsiveness to TPO as shown previously (4, 5) (Fig. 1 *c*). BAF-mplΔ7 cells also showed a strong proliferative response to TPO, though higher levels of TPO were required when compared to BAF-mplwt. BAF-mplΔ8 cells were completely unresponsive to TPO (Fig. 1 *C*) and parental BAF/3 cells (not shown), demonstrating that TPO-responsiveness required expression of a functional receptor in these cells. These results indicate that the first 10 aa of the c-mpl cytoplasmic domain are dispensable for a mitogenic response, whereas an intact NH_2-terminal half of box1 is absolutely required. BAF-mplΔ7 cells retained their proliferative capacity in TPO for a prolonged period of time (>3 mo, data not shown), suggesting that the mutant receptor provides the signals necessary for long-term survival.

A c-mpl Mutant That Mediates Proliferation without Jak-STAT Activation. Stimulation of c-mpl by its ligand results in tyrosine phosphorylation and activation of Jak-2 (references 8–11, 13). As expected, tyrosine phosphorylation of Jak-2 was observed as early as 5 min after stimulation and was still visible after 30 min in cells expressing the wild-type receptor (Fig. 2 *a*). Jak-2 phosphorylation was not induced in TPO-stimulated BAF-mplΔ8 cells (not shown). Surprisingly, stimulation of BAF-mplΔ7 with TPO also failed to induce tyrosine phosphorylation of Jak-2 (Fig. 2 *a*), even after increasing the TPO concentration ten-fold (data not shown). IL-3 was able to induce tyrosine phosphorylation of Jak-2 in both BAF-mplwt and BAF-mplΔ7 cells at comparable levels, demonstrating that Jak-2 is intact in BAF-mplΔ7 cells (Fig. 2 *a*). Stable expression of c-mplΔ7 in the IL-3–dependent myeloid cell line 32D further confirmed the ability of this mutant to induce a mitogenic response (not shown) in the absence of Jak-2 phosphorylation (Fig. 2 *b*).

The unexpected inability of c-mplΔ7 to mediate phosphorylation of Jak-2 was also confirmed in COS cells transiently transfected with the deletion mutants (Fig. 2 *c*). COS cells transfected with c-mplwt or c-mplΔ7 were stimulated with TPO and tyrosine phosphorylation of Jak-2 was analyzed (Fig. 2 *c*). Tyrosine phosphorylation of Jak-2 was detected in cells transfected with c-mplwt but not with c-mplΔ7, although similar amounts of receptor were expressed in both transfectants (Fig. 2 *c*).

Tyrosine phosphorylation of Jaks leads to activation of their kinase function (6). To monitor Jak-2 activation, the kinase activity of Jak-2 immunoprecipitates from TPO-stimulated BAF-mplwt and BAF-mplΔ7 cells was measured in an in vitro kinase assay (26). Jak-2 kinase activity was strongly activated in stimulated BAF-mplwt but not in BAF-mplΔ7 cells (Fig. 2 *d*).

Tyk-2, another member of the Jak family, has recently been reported to be tyrosine phosphorylated after TPO receptor stimulation (11). To determine whether Tyk-2 is

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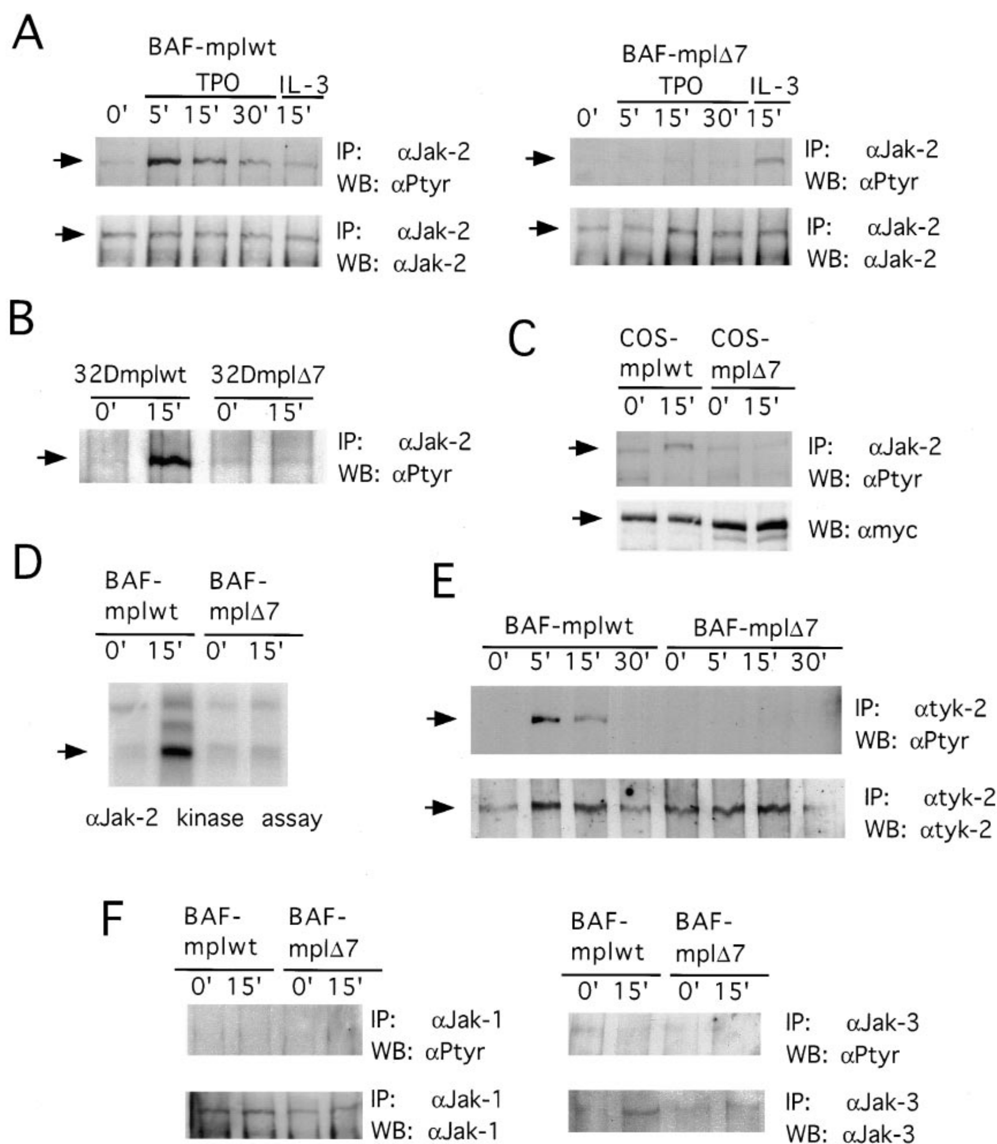

Figure 2. C-mplΔ7 does not activate Jaks. (*a*) TPO stimulates tyrosine phosphorylation of Jak-2 in BAF-mplwt but not in BAF-mplΔ7 cells. Growth factor-deprived cells were left untreated or were stimulated with TPO or IL-3 for the indicated times and lysates were prepared. Jak-2 was immunoprecipitated with anti–Jak-2 antiserum and subsequently immunoblotted with anti-phosphotyrosine antibodies. Membranes were stripped and reprobed with anti–Jak-2 antiserum to confirm equal loading of protein in all lanes. (*b*) TPO stimulates tyrosine phosphorylation of Jak-2 in 32Dmplwt but not 32DmplΔ7 cells. (*c*) Jak-2 is tyrosine phosphorylated in TPO-stimulated (15 min) COS cells transiently expressing c-mplwt but not in cells expressing c-mplΔ7. Lysates were prepared and probed with anti-myc antibodies to confirm equal levels of receptor expression in all samples. An antiphosphotyrosine immunoblot of Jak-2 immunoprecipitates was performed. (*d*) Activation of Jak-2 kinase in TPO-stimulated BAF-mplwt but not in BAF-mplΔ7 cells. Kinase activity of Jak-2 immunoprecipitates was measured as autophosphorylation in an in vitro kinase assay. (*e*) TPO-stimulated tyrosine phosphorylation of Tyk-2 in BAF-mplwt but not in BAF-mplΔ7 cells. Tyk-2 was immunoprecipitated with anti–Tyk-2 antibodies and subsequently blotted with antiphosphotyrosine antibodies. Membranes were stripped and reprobed with anti–Tyk-2 antibodies to confirm equal protein loading. (*f*) Antiphosphotyrosine blot of Jak-1 and Jak-3 immunoprecipitates. *IP*, immunoprecipitation; *WB*, Western Blot.

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activated and might compensate for the lack of Jak-2 activation in BAF-mplΔ7 cells, we analyzed tyrosine phosphorylation of Tyk-2 in TPO-stimulated BAF-mplwt and BAF-mplΔ7 cells. Phosphorylation of Tyk-2 was detected at 5 min and 15 min after stimulation of the wild-type receptor but not after stimulation of c-mplΔ7 (Fig. 2 *e*). Furthermore, neither Jak-1 nor Jak-3 were tyrosine phosphorylated in TPO-stimulated BAF-mplwt and BAF-mplΔ7 cells (Fig. 2 *f*). Thus, c-mplΔ7 mediates a mitogenic response without detectable phosphorylation of any of the known Jaks.

We next analyzed whether the failure of c-mplΔ7 to activate Jaks was also reflected in a lack of activation of their major targets, the STAT proteins. Tyrosine phosphorylation of STATs by Jaks leads to activation of their DNA-binding activity (6, 7). Stimulation of the TPO receptor has been described to activate STAT1, 3, and 5 (10–12). Using a DNA probe (GAS-element) (7) which can detect several activated STATs (including STAT1, 3, and 5), we measured STAT DNA-binding activity in lysates prepared from TPO-stimulated BAF-mplwt and BAF-mplΔ7 cells (Fig. 3 *a*), and also 32D-mplwt and 32D-mplΔ7 cells (data not shown) in an electrophoretic mobility-shift assay (EMSA). Complex formation was detected in cells expressing the wild-type receptor but not in cells expressing the mutant receptor. The GAS-binding activity was seen as early as 5 min after TPO stimulation and was still present after 1 h of stimulation (Fig. 3 *a*) whereas no GAS-binding activity was detected in BAF-mplΔ7 cells at any of the time points analyzed. Increasing the concentration of TPO up to 500 or 1,000 ng/ml did not enhance the GAS-binding activity in BAF-mplwt cells and did not result in any detectable activ-

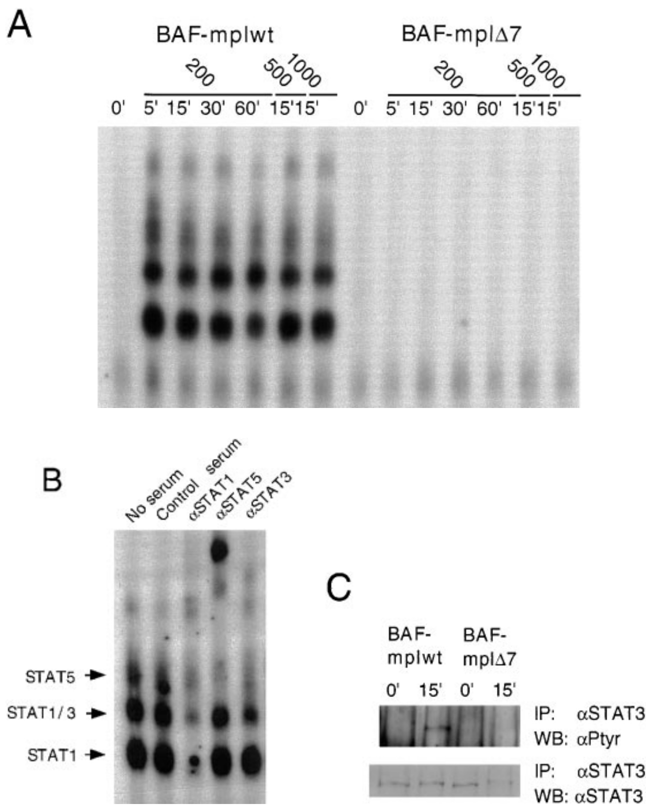

Figure 3. Induction of GAS-binding activity in BAF-mplwt but not BAF-mplΔ7 cells. (*a*) Growth factor–deprived cells were left untreated or were stimulated with TPO. The time points and the TPO concentrations analyzed are indicated. Cell extracts were prepared and analysed by EMSA using the IRF-1 GAS probe. GAS-binding activity was detected in BAF-mplwt but not BAF-mplΔ7 cells. (*b*) The identity of the GAS-binding complexes in BAF-mplwt cells (5′ stimulation) was examined in supershift assays with antibodies specific for STAT1, 3, and 5 (STAT5a and STAT5b antibodies were pooled). (*c*) Antiphosphotyrosine blot of STAT3 immunoprecipitates shows that STAT3 is tyrosine phosphorylated after TPO-stimulation of the wild-type but not the mutant receptor. Membrane was stripped and reprobed with anti-STAT3 antibodies to confirm equal protein loading.

ity in BAF-mplΔ7 cells (Fig. 3 *a*). The identity of the STATs present in the different complexes detected in TPO-stimulated BAF-mplwt cells was analyzed by supershift assays with antibodies to STAT1, 3, and 5 (Fig. 3 *b*). The complex with the lowest mobility was supershifted with anti-STAT5 antibodies. Antibodies to STAT1 supershifted the complex with the highest mobility. The complex with intermediate mobility was diminished by anti-STAT1 and anti-STAT3 antibodies indicating that the complex probably consists of STAT1/STAT3 heterodimers. To confirm the activation of STAT3, an anti-phosphotyrosine immunoblot of STAT3 immunoprecipitates was performed showing phosphorylation of STAT3 by the wild-type receptor but not by the mutant receptor (Fig. 3 *c*). The inability of c-mplΔ7 to induce a STAT-DNA complex is consistent with the observed lack of Jak activation in TPO-stimulated BAF-mplΔ7 cells and 32D-mplΔ7 cells. Moreover, this result excludes the possibility that another, as yet unidentified Jak kinase is activated by the mutant receptor to induce STAT DNA-binding activity.

Effects of TPO Stimulation on Shc, Vav, Raf-1, MAPK and PI 3-Kinase. Our results demonstrate that c-mplΔ7 is able to mediate TPO-stimulated proliferation without activation of the Jak-STAT pathway. We therefore asked if other signaling pathways previously described for c-mpl (8–11) were activated in TPO-stimulated BAF-mplΔ7 or BAF-mplΔ8 cells. As shown in Fig. 4, stimulation of both c-mplwt and c-mplΔ7 induced tyrosine phosphorylation of Shc (a), Vav (b) and c-mpl (c). In constrast, c-mplΔ8 was completely inactive (data not shown). Phosphorylation of Shc and Vav was slightly reduced and phosphorylation of the receptor itself was markedly reduced in BAF-mplΔ7 cells as compared to BAF-mplwt cells. A phosphotyrosine blot of total cell lysates after TPO stimulation (Fig. 4 *d*) was in agreement with the above observations: protein tyrosine phosphorylation was still detectable in BAF-mplΔ7 cells but the number of proteins phosphorylated and the degree of phosphorylation was reduced compared to BAF-mplwt cells. No tyrosine phosphorylated proteins were detected in lysates from TPO-stimulated BAF-mplΔ8 cells (data not shown). These results suggest that c-mplΔ7 mediates activation of tyrosine kinase(s) other than Jaks. The mutation in box1 in c-mplΔ8 appears to disrupt activation of not only the Jaks but also the additional or alternative tyrosine kinase(s) active in BAF-mplΔ7 cells.

c-mplΔ7 also retained the ability of the wild-type receptor (28, 29) to induce phosphorylation of the serine-threonine kinases Raf-1 (Fig. 4 *e*) and MAPK (Fig. 4 *f*), and upregulation of c-fos and c-myc expression (Fig. 5). While the c-mplΔ7-mediated effect on Raf-1 was comparable to the wild-type receptor, the phosphorylation of MAPK induced by the mutant receptor was reduced in its intensity and duration (Fig. 4 *f*). Induction of c-fos and c-myc protein synthesis was reduced approximatively threefold in BAF-mplΔ7 cells as compared to BAF-mplwt cells. In an effort to further investigate the importance of these signals for Jak-independent proliferation, we generated the mutant c-mplΔ7ΔC by introducing an additional COOH-terminal truncation (aa 601-625) in the c-mplΔ7 mutant; it has been previously shown that this region is required for both Shc activation and receptor phosphorylation (13, 21). This double mutant failed to induce tyrosine phosphorylation of Jak and Shc and phosphorylation of Raf-1 but nevertheless was sufficient to mediate proliferation in BAF/3 cells although maximal proliferation was reduced about twofold when compared with the c-mplΔ7 mutant (data not shown). These data suggest that the mitogenic signal required neither Jak activation nor Shc or Raf-1 phosphorylation.

Previous studies have implicated PI 3-kinase in the mitogenic response induced by a number of cytokines (6, 30, 31). To study this pathway we analyzed PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates from BAF-mplwt, BAF-mplΔ7 cells and BAF-mplΔ8 cells before and after TPO stimulation. c-mplΔ7 mediated an increase in PI 3-kinase activity comparable to the wild-type receptor

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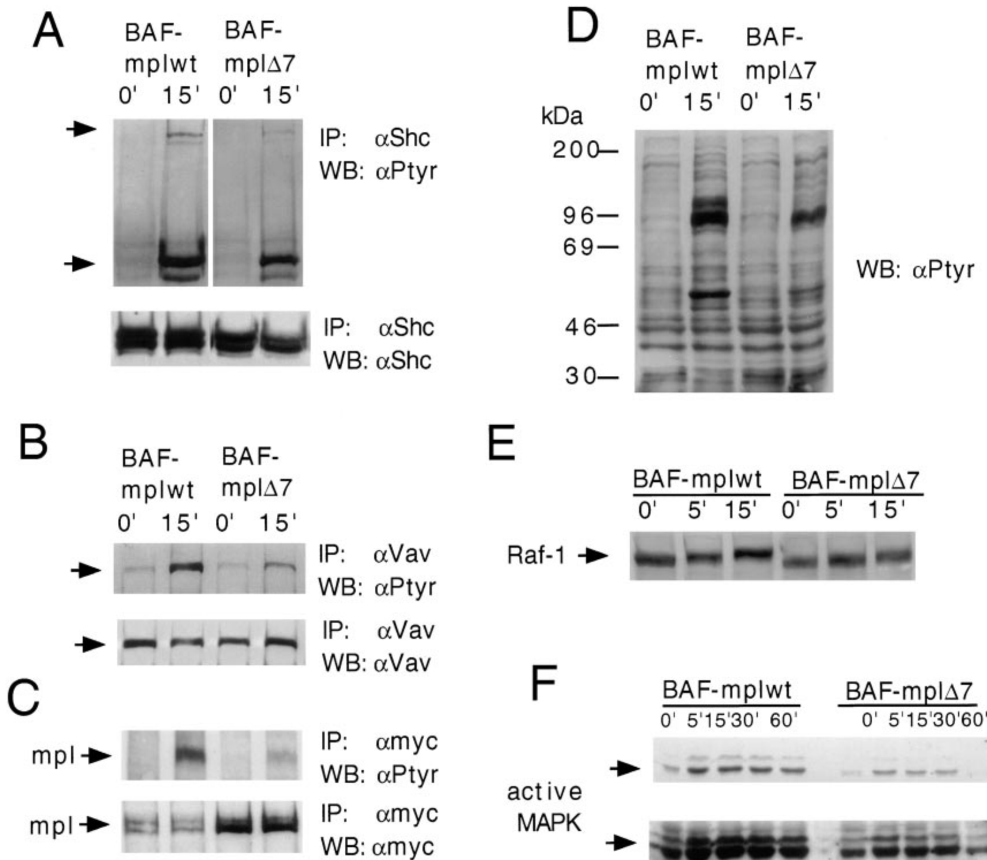

Figure 4. Effect of TPO stimulation on Shc, Vav, the receptor itself, Raf-1, and MAPK. Growth factor-deprived BAF-mplwt and BAF-mplΔ7 cells were either left untreated or stimulated with TPO for the indicated times and cell extracts were prepared. Immunoprecipitations were performed with antibodies to Shc (*a*), Vav (*b*), and myc (*c*) and the immunoprecipitates were blotted with antiphosphotyrosine antibodies (*a–c*). To confirm equal loading of protein, membranes were stripped and reprobed with the antibodies used for immunoprecipitations (lower panel of *a–c*). In (*c*) a higher amount of c-mplΔ7 protein was immunoprecipitated. (*d*) Antiphosphotyrosine immunoblot of total cell lysates. (*e*) Cell lysates were immunoblotted with an antibody to Raf-1. The lower mobility of Raf-1 seen after stimulation with TPO in BAF-mplwt and BAF-mplΔ7 reflects the increased phosphorylation of Raf-1 on serine. (*f*) Cell lysates were immunoblotted with anti-active MAPK antibodies which recognize the active forms of Erk-1 and Erk-2 (different exposures of the same membrane are shown in the upper and middle panel). Membranes were stripped and reprobed with anti-Erk2 antibodies to confirm equal protein loading.

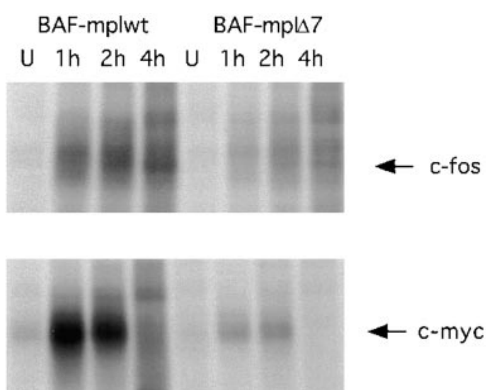

Figure 5. TPO stimulates c-fos and c-myc synthesis in BAF-mplwt and BAF-mplΔ7 cells. Growth factor–deprived cells were washed twice and incubated for 30 min at a density of 10^7 per ml in RPMI 1640 deficient in methionine and cysteine (ICN). Cells were metabolically labeled as described (36) by adding 0.5 mCi of [^{35}S]methionine (Translabel; ICN) per ml to the cell suspension. TPO (200 ng/ml) was added simultaneously and cells were incubated for the indicated times. Unstimulated (U) cells were incubated with [^{35}S]methionine in the absence of TPO for 1 h. Cell extracts were prepared and c-fos and c-myc were immunoprecipitated with antibodies to c-fos (*top*) or c-myc (*bottom*). Immunoprecipitates were resolved by SDS-PAGE (7.5% gel) and analyzed by fluorography. Signals were quantified with a PhosphorImager.

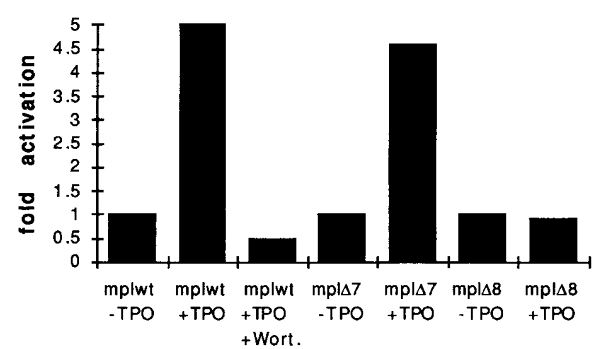

Figure 6. Activation of PI 3-kinase in TPO-stimulated BAF-mplwt and BAF-mplΔ7 cells. Growth factor–deprived cells were left untreated or were stimulated with TPO for 5 min and cell extracts were prepared. Immunoprecipitations were performed with anti-phosphotyrosine antibodies and the immunoprecipitates were analyzed for PI 3-kinase activity. Formation of PI 3-P was inhibited by inclusion of 100 nM wortmannin (+ *Wort.*) in the kinase reaction. The ratios of labeled PI 3-P in stimulated samples/unstimulated samples are shown as fold activation. The results shown represent one out of three experiments with similar outcomes.

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(Fig. 6), indicating that Jak activation is not a prerequisite for PI 3-kinase activation. Mutant c-mplΔ8 showed no increase in PI 3-kinase activity. Incubation of BAF-mplwt and BAF-mplD7 cells with increasing concentrations of the PI 3-kinase inhibitor wortmannin (1, 10, 100, and 1,000 nM) resulted in a concentration-dependent decrease in TPO-dependent proliferation as monitored by [^{3}H]thymidine incorporation after 48 h. Approximatively 50% inhibition of maximal proliferation was observed at a concentration of 100 nM wortmannin, similar to results obtained by others for erythropoietin- or IL-7–induced proliferation (30, 31); proliferation was completely abolished at 1 μM (data not shown). These results suggest that PI 3-kinase may be an essential player in the generation of the mitogenic response by TPO. In this context it is of interest that the proliferation-defective mutant c-mplΔ8 does not activate PI 3-kinase (Fig. 6) but that the proliferation-competent C-terminal truncation mutant c-mplΔ7ΔC still mediates PI 3-kinase activation (M. Dorsch, and S.P. Goff, unpublished observation).

c-mplΔ7 is the first cytokine receptor mutation that disrupts Jak activation while preserving other cytokine-stimulated events. Our results indicate that neither proliferation nor phosphorylation of Shc, Vav, Raf-1, and c-mpl, nor induction of PI 3-kinase activity requires the activation of Jaks. However, the reduction of some of these responses for c-mplΔ7 relative to c-mplwt suggests that the full induction of these events depends upon the cooperation of an intact Jak-STAT pathway with other signaling pathways.

Previous analyses of various cytokine receptors with mutations in the box1/box2 region suggested that the inability to activate Jaks always correlates with the complete loss of the mitogenic response (13, 14, 16, 20, 21) and all major downstream signaling events (13, 20, 21, 32). Unlike our findings, these results suggested an absolute requirement of Jak activation for receptor activity. However, none of these mutants included an internal deletion of the region membrane-proximal to box1 that left box1 intact. The discrepancy between the rather specific effect of the deletion proximal to box1 and the obliterative effects of previous deletions in the box1/box2 region may be explained by the presence of binding or activation domains in box1/box2 for PI 3-kinase and other as yet undefined kinases or signaling molecules. Alternatively, this region may be structurally important for the proper positioning of remaining domains. Nevertheless, our results virtually rule out the possibility that the drastic effects of deletions in the box1/box2 region are solely due to the absence of Jak activation.

Another approach to disrupting the Jak-STAT pathway has been the use of kinase-deficient forms of Jaks as dominant-negative inhibitors of endogenous Jak activity (33, 34). Expression in factor-dependent cells of kinase-deficient Jak-2 decreased IL-3– or GM-CSF–induced cell proliferation and abrogated erythropoietin-induced proliferation (33, 34). The mechanism of inhibition, however, is uncertain. Notably, in one case the Jak-2 mutant suppressed IL-2 signals that do not involve Jak-2 (33), suggesting that the effects of overexpression of such mutants are not restricted to the inhibition of Jak-2 but may also interfere with other signaling events.

The molecular mechanism of the phosphorylation of Shc, Vav and the receptor itself in the absence of Jak activation remains to be elucidated. Src family kinases as well as c-fes, btk, tec, syk (6), and c-kit (35) have all been shown to be activated by various cytokine receptors. However, activation of these kinases is not as universal as activation of the Jaks. To date none of these kinases has been linked to the TPO receptor. In our hands, TPO does not activate lyn, fyn, fes, tec, or syk in BAF-mplwt and BAF-mplΔ7 cells (M. Dorsch and S.P. Goff, unpublished observations).

Our results demonstrate that the Jak-STAT pathway is not essential to all cytokine receptor systems for stimulation of a mitogenic response. Thus, other signaling pathways must be sufficient to mediate this response and PI 3-kinase appears to be at least one essential part of that signal. Nevertheless, we emphasize that our results do not rule out that under physiological conditions the Jak-STAT pathway may contribute to proliferation. The selective effect of the described mutation on Jak-STAT signaling should prove useful in defining the role of this pathway in TPO-mediated differentiation. Finally, it will be important to test whether analogous mutations in other cytokine receptors may have similarly selective effects.

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