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# Functional Regions of the Mouse Thrombopoietin Receptor Cytoplasmic Domain: Evidence for a Critical Region Which Is Involved in Differentiation and Can Be Complemented by Erythropoietin

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**Thrombopoietin (TPO) is the major regulator of growth and differentiation of megakaryocytes. To identify functionally important regions in the cytoplasmic domain of the TPO receptor, mpl, we introduced wild-type mpl and deletion mutants of murine mpl into the granulocyte-macrophage colony-stimulating factor (GM-CSF)- or erythropoietin (EPO)-dependent human cell line UT7. TPO induced differentiation of UT7-Wtmpl cells, not parental UT7 cells, along the megakaryocytic lineage, as evidenced by decreased proliferation, changes in cell morphology, and increased surface expression and mRNA levels of megakaryocytic markers CD41, CD61, and CD42b. When UT7-mpl cells were cultured long-term in EPO instead of GM-CSF, the TPO effect was dominant over that of EPO. Moreover, the differentiation induced by TPO was more pronounced for cells shifted from EPO to TPO than for cells shifted from GM-CSF to TPO, as shown by the appearance of polyploid cells. Mutational analysis of the cytoplasmic domain of mpl showed that proliferation and maturation functions of mpl can be uncoupled. Two functional regions were identified: (i) the first 69 amino acids comprising the cytokine receptor motifs, box 1 and box 2, which are necessary for both TPO-induced mitogenesis and maturation; and (ii) amino acids 71 to 94, which are dispensable for proliferation but required for differentiation. Surprisingly, however, EPO could complement this latter domain for TPO-induced differentiation, suggesting a close relationship between EPO and TPO signaling.**

The regulatory system governing megakaryocytopoiesis and platelet production is a complex process involving the commitment of multipotential progenitors to megakaryocyte precursors and maturation of committed cells, leading to the formation of platelets. In their development, megakaryoblasts undergo a series of morphological changes, including expression of surface lineage-specific differentiation antigens, such as integrins gpIIb-gpIIIa and gpIb, endomitotic replications, and cytoplasmic partition, leading to platelet shedding (51). The recently cloned cytokine thrombopoietin (TPO) has been shown to be the major regulator of both the proliferation and maturation of megakaryoblasts (4, 11, 30, 34, 56). TPO exhibits significant homology to erythropoietin (EPO), the lineage-specific growth factor for erythrocyte production (4, 11, 16, 34).

TPO was identified as the ligand for the proto-oncogene c-*mpl*. c-mpl is a member of the cytokine receptor superfamily, which includes the receptors for EPO, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), most interleukins (IL), growth hormone, and prolactin (45, 52, 53). mpl was first isolated as an oncogenic truncated form (v-mpl) transduced in the envelope gene of the myeloproliferative leukemia virus (MPLV) (48). MPLV induces an acute myeloproliferative disorder in mice. In vitro, MPLV infection promotes growth factor-independent proliferation and terminal differentiation of cells of various hematopoietic lineages (48, 57). The restricted expression of c-mpl to primitive hematopoietic stem cells, megakaryocytes, and platelets (10, 36) and the blockage of in vitro megakaryocyte colony formation by antisense oligodeoxynucleotides to c-mpl (36) led to a suspected role for c-mpl in megakaryocytopoiesis long before the identification of TPO. In addition, a pronounced thrombocytemia was observed in c-mpl-deficient mice (22).

The recent discovery of TPO has provided an impetus for the study of megakaryocyte biology. However, the study of molecular mechanisms responsible for TPO functions is just at its onset. The intracellular domain of mpl does not encode any known kinase or other enzymatic activity (45, 52, 53). As reported for other members of the cytokine receptor family, TPO induces tyrosine phosphorylation of a certain number of cellular proteins, including the receptor itself, shc, and phosphatidylinositol 3 kinase, and leads to activation of the signal transduction pathway involving Janus tyrosine kinases (JAK) and the signal transducers and activators of transcription (STAT) (13, 23, 41, 49). Deletion analysis of the cytoplasmic domain of mpl within a chimeric growth hormone receptor-mpl receptor construct has shown that the first 63 juxtamembrane amino acids (aa), encompassing box 1 and box 2 motifs conserved in the cytokine receptor family, are necessary for JAK-STAT pathway activation and proliferation in Baf3 cells (23). This region was already reported to be absolutely required for both v-mpl-induced pathogenicity in vivo and growth-factor-independent proliferation of FDCP-1 cells in vitro (6, 9).

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Probably because of the difficulty in finding in vitro models for megakaryocytic differentiation, all of these studies have focused on the proliferative effects of TPO; nothing is known about the TPO-mpl signaling events leading to cell maturation. To address this question, it is important to dissect the functions of the cytoplasmic domains of the receptor in a cellular context in which megakaryocytic maturation can occur. In the present study, we have introduced wild-type (wt) mpl and deletion mutants of mpl into UT7, a human cell line of megakaryoblastic origin (32). Despite their origin, UT7 cells display features of multipotential cells able to differentiate toward eosinophil and basophil promyelocytes in the presence of GM-CSF and IL-3, toward erythroblasts in the presence of EPO, and toward megakaryocytes upon treatment with phorbol esters (24, 32, 33). We show that UT7-mpl can undergo megakaryocytic maturation upon TPO treatment. By deletion analysis of the cytoplasmic domain of mpl, we identified a specific region which is involved in the maturation process but dispensable for proliferation. In addition, we show that EPO, although inducing by itself an erythroid phenotype in UT7 cells, can cooperate with TPO to induce more pronounced megakaryocytic differentiation and to restore the response of the maturation-defective mpl mutant. This provides a so-far unique in vitro model to study the common and divergent signaling pathways leading to megakaryopoiesis and erythropoiesis.

## MATERIALS AND METHODS

**Cytokines and antibodies.** Murine TPO obtained as a serum-free medium from baby hamster kidney cells engineered to stably express TPO (34) was provided by Don Foster (Zymogenetics, Inc., Seattle, Wash.). The dilution that produced half-maximal proliferation of Baf3 cells expressing murine mpl was defined as 10 U/ml. The batch used in the present study contained 145,000 U/ml. Recombinant human EPO was from Boehringer Mannheim.

Purified immunoglobulin G1 (IgG1) monoclonal antibodies specific for the Flag epitope tag sequence (M1 and M2) were kindly donated by D. Cosman (Immunex, Seattle, Wash.). Purified monoclonal anti-human CD41 (gpIIb; clone SZ22), CD61 (gpIIIa; clone SZ.21), and fluorescein isothiocyanate (FITC)-labeled anti-CD42b (gpIb; clone SZ2) were from Immunotech (Marseille, France). Monoclonal antibody against glycophorin A (GpA), FITC-conjugated goat $F(ab')_2$ fragments specific for mouse IgG, and purified goat IgG were purchased from Sigma (St. Louis, Mo.). Goat IgG, used to saturate cell Fc receptors prior to antibody binding (see below), was aggregated by treatment at 56°C for 30 min.

**Cell culture.** Parental (32) or mpl-infected UT7 cells were maintained in α-minimum essential medium supplemented with 10% fetal calf serum and 2.5 ng of recombinant GM-CSF per ml or 2 U of EPO per ml. Before any change of growth factor, cells were washed three times in medium without serum. In some experiments, cells were deprived of growth factors by being washed three times in phosphate-buffered saline (PBS) and replated in Iscove medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 20 μg of transferrin per ml, and 0.4% bovine serum albumin (deprivation medium).

Recombinant ecotropic and amphotropic packaging cell lines GP+E86 and GP+envAm12 were from A. Bank (Columbia University, New York, N.Y.) and were obtained from Genetix Pharmaceuticals, Inc. GP+E86 cells were maintained in Dulbecco's modified minimal medium containing 10% newborn calf serum, 250 μg of xanthine per ml, 15 μg of hypoxanthine per ml, and 25 μg of mycophenolic acid per ml (HMX medium). GP+envAm12 cells were cultured in HMX medium supplemented with 200 μg of hygromycin B per ml.

**Cell growth assays.** DNA synthesis was measured by thymidine incorporation as follows. Briefly, after three washes in PBS, cells were resuspended at $10^5$/ml in deprivation medium and incubated on 96-well culture-treated plates in the presence or absence of serial dilutions of growth factors for 48 h. [$^3$H]thymidine was added, and the amount of radioactivity incorporated in cells was determined 4 h later. To assess cell number and viability, cells were washed three times and cultured in medium containing 10% fetal calf serum and either 2.5 ng of GM-CSF per ml, 200 U of TPO per ml, 1 U of EPO per ml, or a mixture of these factors. At the indicated time intervals, cells were counted and viability was determined by trypan blue exclusion. Cultures were split twice a week to maintain cell concentrations of less than $4 \times 10^5$/ml.

**Plasmid constructs.** The full-length murine mpl cDNA (52), modified by the replacement of the mpl signal peptide by that of murine IL-7 followed by the Flag epitope tag sequence (26, 56) and obtained from D. Cosman (Immunex), was cloned into the *Sal*I site of plasmid pBluescript II KS.

Truncation mutants Δ1 (lacking residues 515 to 541), Δ2 (lacking residues 550 to 575), and Δ3 (lacking residues 576 to 599) within the intracellular domain of mpl were previously generated by site-directed mutagenesis of the mpl sequence of v-mpl (6). Mutations were introduced into the full-length Flag-mpl by exchanging the *Cel*II-*Nco*I fragment of Flag-mpl for the *Cel*II-*Nco*I fragment of v-mpl Δ1, Δ2, or Δ3. All constructs were then subcloned into the *Sal*I site of retrovirus expression vector pBabepuro, carrying the puromycin resistance gene (38).

**Retrovirus production and infection.** pBabepuro vectors encoding wild-type mpl or mutants were transfected into the ecotropic packaging cell line GP+E86 by the calcium phosphate precipitation procedure (19), and individual puromycin-resistant clones were derived in the presence of 1 μg of puromycin (Sigma) per ml. Positive clones were then tested for mpl expression by fluorescence analysis of anti-Flag antibody M1 bound to methanol-permeabilized cells (see below). Supernatants of cells showing high-level staining were used to infect GP+envAm12 amphotropic packaging cells. At 48 h after infection, cells were selected in the presence of 1 μg of puromycin per ml. Supernatants of clones with high levels of expression (tested as described above) were used to infect $3 \times 10^6$ to $4 \times 10^6$ UT7 cells. After 24 h, cells were either stood in liquid culture or plated in a semisolid medium containing 0.8% methylcellulose and selected in medium containing 2.5 ng of GM-CSF per ml and 0.5 μg of puromycin per ml. After about 2 weeks, positive individual clones were picked up and expanded in liquid medium. mpl expression was assessed by Western blotting (immunoblotting) or flow cytometry analysis using anti-Flag antibodies. In some instances, cells were subjected to one round of fluorescence-activated cell sorting using anti-Flag antibodies and an ELITE flow cytometer (Coultronics).

**Flow cytometric analysis of surface antigens.** Cells ($5 \times 10^5$), treated or not treated for various days with TPO, were washed twice in PBA (PBS containing 0.2% sodium azide and 1% serum albumin) and incubated for 30 min at 4°C with 1 mg of normal goat aggregated IgG per ml to saturate Fc receptors. After one wash in PBA, mouse monoclonal primary antibodies were added and the incubation continued for 30 min at 4°C. Then cells were washed three times and incubated with FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG (1:200) for another 30 min. In the case of anti-Flag antibody M1, whose binding is dependent on calcium (26), 1 mM $CaCl_2$ was added in the buffer during incubations with antibodies and during washes. After two washes, cells were fixed with 1% formaldehyde and analyzed for fluorescence on an ELITE flow cytometer. The percentage of positive cells and the mean fluorescence intensity (MFI), a measure of the relative density of the antigen on the surfaces of stained cells, were determined for each sample.

**Morphological studies.** Cells were collected every 2 or 3 days after TPO treatment and concentrated on glass slides by cytocentrifugation. Cell morphology was examined after May-Grunwald-Giemsa staining.

**Northern (RNA) blots.** Cells were washed twice in cold PBS, and total RNA was prepared by the guanidium isothiocyanate-cesium chloride method (8). Fifteen to twenty micrograms of RNA was denatured in glyoxal buffer and electrophoresed as previously described (48). RNAs were transferred on nylon membranes (GeneScreen) and hybridized with either gpIIb, GpA, α-globin, or mpl probes.

## RESULTS

**UT7 cells expressing mpl proliferate and differentiate in response to TPO.** The UT7 cell line is a human cell line derived from a patient with megakaryoblastic leukemia; it is strictly dependent on either GM-CSF or EPO for its survival (32) but does not respond to murine or human TPO (reference 41 and data not shown). Murine wt mpl was introduced into UT7 cells maintained in GM-CSF (UT7.G) by retrovirus infection. Clones expressing high levels of mpl were isolated after one round of fluorescence-activated cell sorting using antibodies to the Flag epitope tag sequence located at the N terminus of mpl. All studies described below were performed on cells which were 70 to 90% positive for anti-Flag antibody staining, as detected by flow cytometry.

As previously described (41) and as shown in Fig. 1, UT7.G-Wtmpl cells exhibited a proliferative response to TPO, whereas parental cells did not. DNA synthesis, as measured by [$^3$H]thymidine incorporation during the first 48-h treatment, was half maximal with 100 U of TPO per ml. Maximal stimulation was obtained with 300 U/ml and was comparable in intensity to that induced by GM-CSF (Fig. 1A). However, when cell number was assessed over longer periods, growth was considerably reduced in the presence of TPO, reaching only about 5 to 10% of GM-CSF levels after 10 days (Fig. 1B). This slow growth rate was not due to ongoing cell death, since cells remained 90% viable over this period. Rather, changes in cell morphology (adherence and size increase) seen in cultures with TPO

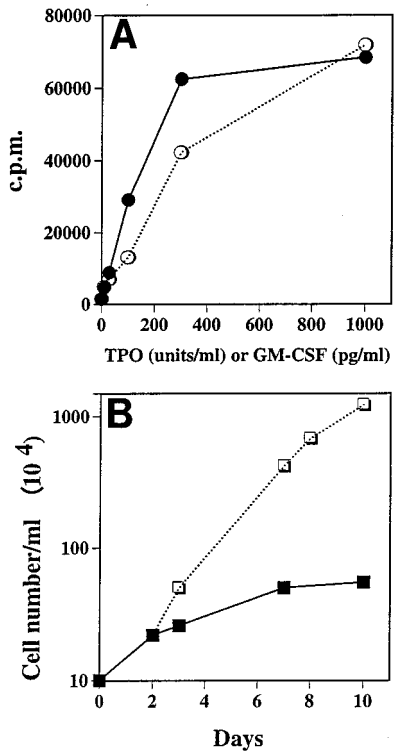


FIG. 1. Proliferation of UT7.G-Wtmpl cells in response to GM-CSF and TPO. (A) DNA synthesis was measured by [$^3$H]thymidine incorporation after 48 h of treatment with the indicated doses of GM-CSF (○) and TPO (●). Data are the means of triplicate determinations. (B) Long-term growth in the presence of GM-CSF or TPO. Cells were washed and incubated with saturating doses of either GM-CSF (2.5 ng/ml; □) or TPO (400 U/ml; ■), and the numbers of viable cells were determined at the indicated times.

(see below) seemed to indicate that TPO induced UT7.G-Wtmpl cells to maturate.

We therefore examined the expression of megakaryocyte-specific differentiation antigens CD41, CD61 (gpIIb-gpIIIa complex), and CD42b (gpIb) in cells stimulated with a dose of TPO inducing maximal proliferation (200 to 400 U/ml). These three antigens showed basal expression on parental UT7.G and UT7.G-Wtmpl cells maintained in GM-CSF (Fig. 2A and Table 1). As shown in Fig. 2A, exposure to TPO for 7 days led to a three- to fourfold increase in expression of these markers, as detected by flow cytometry and Northern blot analysis. This increase was not due to cell enlargement or nonspecific binding, since the surface expression level of the erythroid marker glycophorin A or the second antibody alone remained very low after TPO treatment. By contrast, treatment of UT7.G-Wtmpl cells with EPO instead of TPO led to decreases for the three megakaryocytic antigens and an increase for GpA (Fig. 2B and C).

Kinetic analysis showed that surface levels of the megakaryocyte-specific antigens CD41 and CD42b were induced within 4 days of culture in the presence of TPO and reached maximal expression after 7 days (Fig. 3). High-level expression was maintained for at least 1 month (data not shown). Both the number of positive cells and the relative density of the antigens on cells (as measured by the MFI) were increased. The changes observed after TPO treatment were similar to those induced by phorbol esters (24, 32) (data not shown).

**TPO acts dominantly over GM-CSF and EPO to induce megakaryocytic maturation.** TPO-induced maturation was dominant over GM-CSF, as shown by the increase in megakaryocyte-specific antigens, changes in cell morphology, and reduced growth rate (albeit less than in the presence of TPO alone) induced on UT7-Wtmpl cells by a combination of TPO and GM-CSF (Table 1).

UT7 cells express high levels of receptors for EPO and can be maintained in long-term culture in the presence of EPO. In the presence of this growth factor, they acquire features of erythroid lineages (24, 33). By contrast with the dominant effect of TPO over GM-CSF observed here, previous studies have shown that EPO could not increase expression of erythroid markers on UT7 cells in the presence of GM-CSF (24). To study the relationships between EPO and TPO with respect

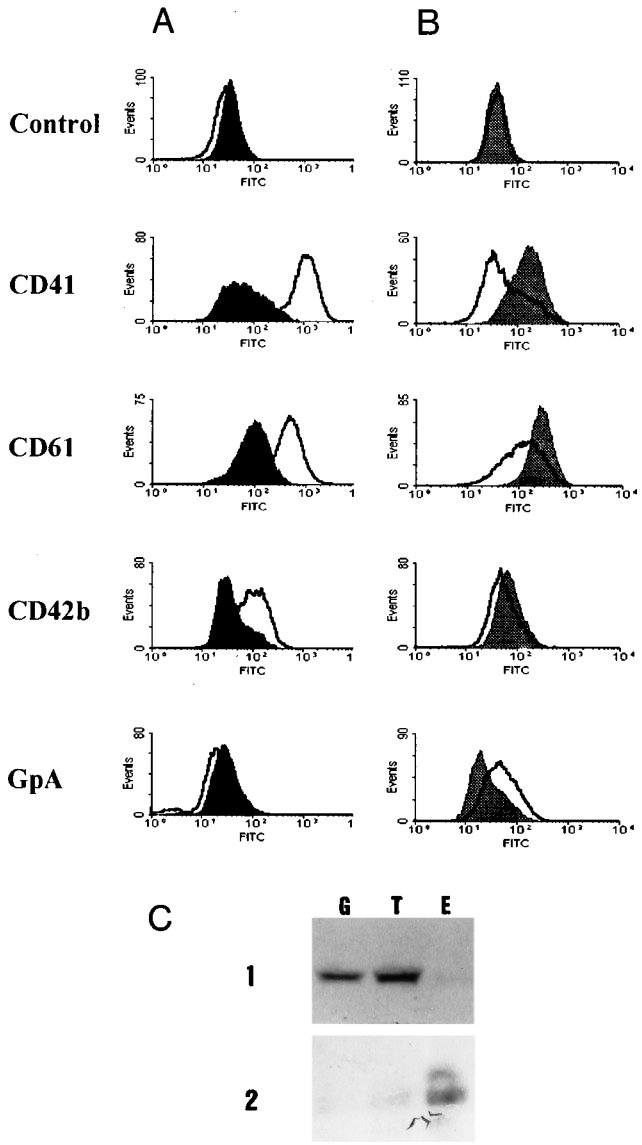


FIG. 2. TPO increases and EPO decreases expression of megakaryocytic markers in UT7.G-Wtmpl cells. UT7.G-Wtmpl cells were washed three times and incubated in the presence of 400 U of TPO per ml, 2 U of EPO per ml, or 2.5 ng of GM-CSF per ml. The expression of megakaryocytic (CD41, CD61, and CD42b) or erythroid (GpA) differentiation antigens was measured after 7 days. (A and B) Flow cytometry analysis. (A) Filled areas, GM-CSF; open areas, TPO. (B) Grey areas, GM-CSF; open areas, EPO. (C) Northern blot analysis of CD41 (1) and GpA (2) mRNA expression. Lane G, cells in GM-CSF; lane T, TPO-treated cells; lane E, EPO-treated cells.

TABLE 1. TPO dominance over GM-CSF and EPO for inducing megakaryocyte-specific antigens[a]

| Antigen | % Positive cells | | | | | | MFI | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | GM-CSF[b] | GM-CSF/TPO[c] | GM-CSF + TPO[d] | EPO[e] | EPO/TPO[f] | EPO + TPO[g] | GM-CSF[b] | GM-CSF/TPO[c] | GM-CSF + TPO[d] | EPO[e] | EPO/TPO[f] | EPO + TPO[g] |
| CD41 | 61 ± 17 | 90 ± 5 | 78 ± 2 | 38 ± 5 | 93 ± 5 | 90 ± 4 | 100 ± 40 | 430 ± 210 | 300 ± 70 | 50 ± 17 | 585 ± 132 | 638 ± 127 |
| CD42b | 28 ± 10 | 57 ± 17 | 38 ± 11 | 14 ± 4 | 75 ± 5.5 | 68 ± 13 | 55 ± 8 | 101 ± 35 | 74 ± 16 | 40 ± 17 | 161 ± 34 | 179 ± 58 |
| GpA | 18 ± 9 | 12 ± 5 | ND[h] | 46 ± 14 | 16 ± 14 | 23 ± 10 | 41 ± 12 | 31 ± 10 | ND | 75 ± 37 | 31 ± 14 | 38 ± 15 |
| Flag-mpl | 83 ± 6 | 77 ± 10 | ND | 96 ± 2 | ND | 85 ± 14 | 261 ± 17 | 228 ± 58 | ND | 267 ± 108 | ND | 342 ± 105 |

[a] Megakaryocyte marker or Flag-mpl expression was analyzed by flow cytometry after 7 days of treatment. Both the percentage of positive cells and the MFI were measured for each antibody staining. Data are the means ± standard errors of five independent experiments performed on two different clones of UT7-mpl cells.
[b] Cells were maintained in GM-CSF alone (1 ng/ml).
[c] Cells were cultured in GM-CSF, washed three times, and resuspended in medium containing TPO alone (400 U/ml).
[d] Cells were cultured in GM-CSF, washed three times, and resuspended in medium containing TPO (400 U/ml) and GM-CSF (1 ng/ml).
[e] Cells were cultured in EPO (1 U/ml).
[f] Cells were cultured in EPO, washed three times, and resuspended in medium containing TPO alone (400 U/ml).
[g] Cells were cultured in EPO, washed three times, and resuspended in medium containing TPO (400 U/ml) and EPO (1 U/ml).
[h] ND, not determined.




FIG. 3. Kinetic effect of TPO on surface megakaryocytic-marker expression on UT7.G-Wtmpl and UT7.G-Δ3mpl cells. UT.G cells expressing either wt mpl (solid symbols) or mpl mutant Δ3 (open symbols) were washed three times and treated with 400 U of TPO per ml. On the indicated days, cells were harvested and stained with antibodies to CD41 or CD42b antigens and analyzed by flow cytometry. Both the percentage of cells positive for binding and MFI were determined. Results represent the means of at least five experiments.

to differentiation, UT7.G-Wtmpl cells were washed and grown in the presence of EPO instead of GM-CSF for 2 weeks (referred to as UT7.E-Wtmpl cells) before being switched to a medium containing TPO or TPO plus EPO. In agreement with previous studies (24, 33), growth in EPO increased expression of erythroid-cell-specific antigens GpA and α-globin (Fig. 4) but reduced expression of megakaryocytic markers CD41, CD61, and CD42b (Table 1 and Fig. 2B and C). The reverse was seen




FIG. 4. Northern blot analysis of erythroid antigens upon treatment of UT7.G-Wtmpl cells with EPO and/or TPO. UT7.G-Wtmpl cells cultured with 2.5 ng of GM-CSF per ml (lane 1) were shifted to a medium containing either 2 U of EPO per ml (lane 2) or a mixture of EPO and 200 U of TPO per ml (lane 3). Total RNA was isolated after 7 days of treatment, as described in Materials and Methods. Ten micrograms of RNA was transferred and hybridized sequentially with the indicated probes. The relative intensities and positions of 18S (18) and 28S (28) RNAs are shown.

FIG. 5. Morphological changes of UT7-Wtmpl cells grown in either GM-CSF, EPO, or TPO. UT7.G-Wtmpl cells maintained in GM-CSF (A) were washed three times and transferred to a medium containing 400 U of TPO per ml (B). Likewise, UT7.E-Wtmpl cells maintained in EPO (C) were washed and shifted to a TPO-containing medium (D). After 5 to 7 days, cells were cytocentrifuged and stained with May-Grunwald-Giemsa stain. Magnification, ×26.

when cells were cultured in the presence of TPO. The megakaryocytic phenotype triggered by TPO was dominant over the erythroid phenotype induced by EPO, as shown by concomitant increases for CD41 and CD42b and decreases for GpA and α-globin, at the levels of both surface expression (Table 1) and mRNA (Fig. 4), observed on UT7.E-Wtmpl cells treated with a mixture of EPO plus TPO.

**EPO synergizes with TPO to induce megakaryocytic maturation.** As can be seen in Table 1, the increase in expression of markers of megakaryocytic differentiation was far greater when UT7-Wtmpl cells were shifted from EPO to TPO (mean for CD41, 7.5-fold increase; range, 4- to 11-fold) than when they were transferred from GM-CSF to TPO (mean, 3.5-fold increase; range, 2- to 5-fold). The growth rate was even more reduced, with almost complete arrest by the second day of treatment with TPO. After 11 days in TPO, UT7.E-Wtmpl cell cultures contained 100-fold-fewer cells than did cultures maintained in EPO. These cells could not be maintained in TPO for more than 2 to 3 weeks.

The cooperative effect of EPO on TPO-induced megakaryocytic differentiation could be seen upon morphological examination of cells cytospun on glass slides and stained with May-Grunwald-Giemsa stain. Indeed, when UT7.G-Wtmpl cells were shifted from GM-CSF to TPO, cell enlargement was observed but only a few cells (about 5%) showed a small increase in polyploidy (Fig. 5A and B). Within 4 days of TPO treatment of UT7.E-Wtmpl cells maintained in EPO (Fig. 5D), the increase in the size of cells was more pronounced and about 30% of cells had multilobed nuclei or were multinucleated and showed basophilic staining of the cytoplasm. As can be seen in Fig. 5C, in the presence of EPO alone, most cells resembled proerythroblasts (24, 33) with large nucleoli.

EPO had only a slight effect on surface expression of transfected Flag-mpl (Table 1). Parental UT7 cells maintained in EPO could not respond to TPO for gene induction (data not shown) or STAT5 activation (41). In addition, no endogenous mpl transcripts were detected in UT7-Wtmpl cells cultured in GM-CSF, EPO, or TPO (data not shown). This demonstrates that the differentiation observed with UT7.E-Wtmpl cells was not due to induction of endogenous human mpl by EPO (data not shown). Thus, although EPO increased expression of erythroid-cell-specific genes and decreased expression of megakaryocytic markers in UT7 cells, it synergized with TPO by priming cells to respond to TPO by a greater increase in megakaryocytic size and expression of differentiation markers.

**Proliferation and maturation signals are mediated by distinct regions of the mpl cytoplasmic domain.** In order to study the molecular mechanisms leading to megakaryocytic differentiation upon mpl stimulation, Flag-mpl mutants containing various deletions of the intracellular domain were prepared (Fig. 6A) and stably expressed in UT7 cells maintained in GM-CSF by retrovirus infection. Mutants Δ1 (lacking aa 11 to 36) and Δ2 (lacking aa 45 to 70) have deletions of the box 1 and box 2 homology domains, respectively, conserved in the cytokine receptor superfamily. Mutant Δ3, lacking aa 71 to 94, does not contain any known specific motif. Infected cells were selected in puromycin in the presence of GM-CSF, and clones exhibiting high-level fluorescence staining with anti-Flag antibody (60 to 100% positive cells [Fig. 6B]) were kept for further studies.

The abilities of different forms of mpl to transduce a prolif-

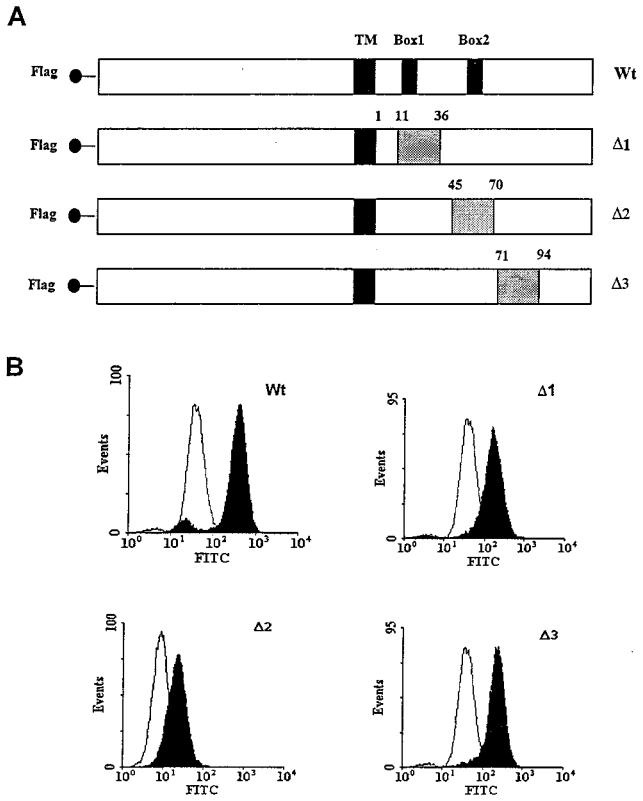


FIG. 6. Deletion mutants of the cytoplasmic domain of mpl. (A) Schematic representation of mpl deletion mutants. 1, the first amino acid of the intracytoplasmic domain, which consists of 120 aa. Scale is not respected for the extracellular domain. TM, transmembrane domain. (B) Flow cytometry analysis of the expression of mpl mutants in representative clones of UT7, using Flag antibody M1. Various Flag-mpl constructions were introduced into UT7.G cells. M1 binding was assessed on cells selected in puromycin and cultured in the presence of GM-CSF. Each open peak corresponds to staining with anti-mouse FITC-conjugated second antibody alone. Several clones with high levels of expression (more than 60% positive cells) were obtained for each construction and kept for further studies.

erative signal were first examined in [$^3$H]thymidine uptake assays. As shown in Fig. 7A, UT7.G cells expressing mutants Δ1 and Δ2 failed to respond to TPO over the range of concentrations tested. In agreement with previous results (23), this shows that both box 1 and box 2 domains are indispensable for TPO-induced mitogenesis. By contrast, mutant Δ3 could transduce an efficient growth signal in UT7 cells, which was comparable to that induced by wild-type mpl and reached the maximal level induced by optimal concentrations of GM-CSF. All the mutants tested responded equally well to GM-CSF (data not shown).

When we next examined the ability of TPO to sustain the survival and long-term proliferation of UT7 transformants, striking differences were observed between Δ3 and full-length mpl. UT7.G-Δ3mpl cells proliferated more rapidly than did UT7.G-Wtmpl cells in response to TPO, and no time-dependent decrease in growth rate was observed with mutant Δ3 (compare Fig. 1B with Fig. 7B). Cells expressing Δ3 mpl could be maintained in the presence of TPO for months, and during that time, they proliferated at the same rate as when they were grown in the presence of GM-CSF.

Since the reduced growth rate of UT7.G-Wtmpl cells cultured in the presence of TPO was accompanied by cell maturation, we tested the capacity of mutant Δ3 to increase expression of megakaryocyte-specific antigens upon TPO stimulation. In contrast to the results with UT7.G-Wtmpl cells, no changes in the surface expression levels of CD41 and CD42b could be observed after the treatment of UT7.G-Δ3mpl cells with TPO for up to 10 days (Fig. 3). No changes in cell morphology or CD41 expression were induced even after more than 1 month of treatment with TPO, indicating that UT7.G-Δ3mpl cells were not delayed in their maturation process. In addition, these cells could acquire a megakaryocytic phenotype upon phorbol myristate acetate induction (data not shown). These results show that aa 71 to 94 of the intracellular domain of mpl are dispensable for proliferation but necessary for megakaryocytic differentiation. Therefore, proliferation and maturation signaling functions of mpl can be uncoupled.

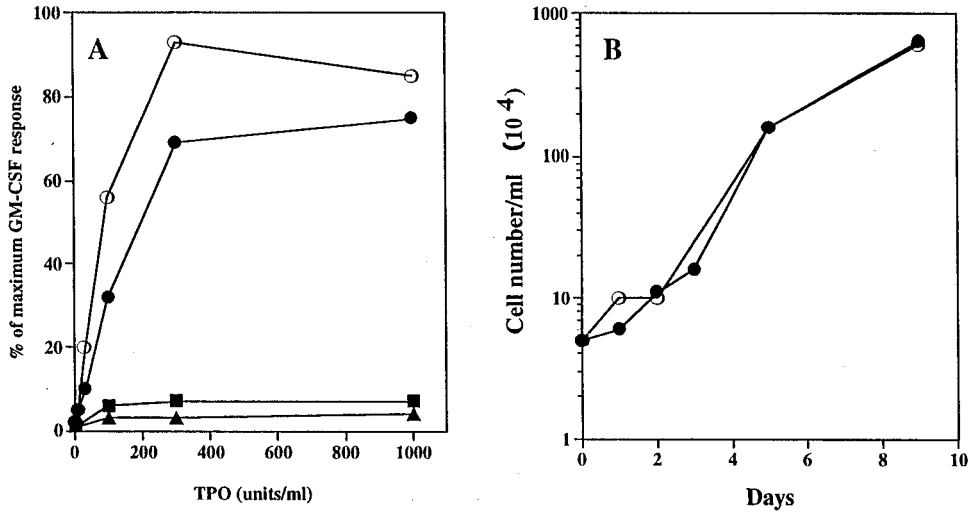


FIG. 7. Proliferation of UT7.G cells expressing various mpl constructs. (A) TPO-dependent DNA synthesis of various mpl mutants. UT7.G transformants expressing wt mpl (●), Δ3 (○), Δ1 (■), or Δ2 (▲) were washed extensively to eliminate GM-CSF and incubated for 48 h with the indicated concentrations of TPO in a deprivation buffer containing no serum. [$^3$H]thymidine incorporation was measured during the last 4 h. Data are plotted as percentages of the maximal response obtained with 2.5 ng of GM-CSF per ml, an average of 95,000 cpm for all types of cells. (B) Long-term growth of UT7.G-Δ3mpl cells in the presence of GM-CSF or TPO. Cells were treated with the optimal concentration of GM-CSF (2.5 ng/ml; closed circles) or TPO (400 U/ml; open circles), and the numbers of viable cells were determined at the indicated times.

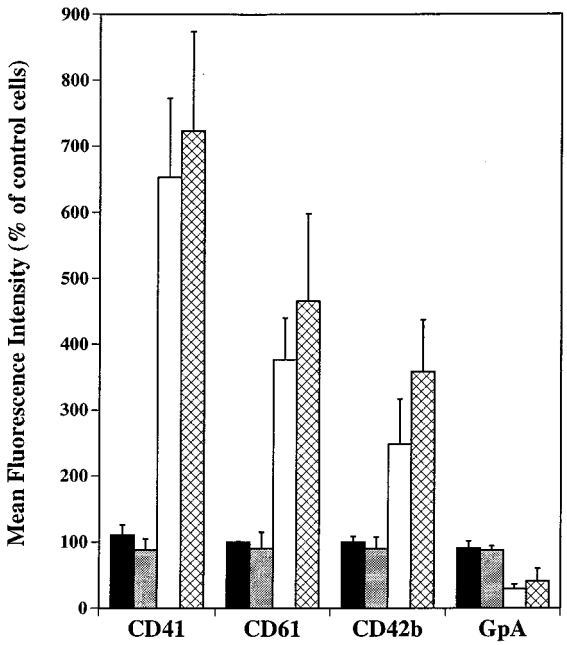


FIG. 8. EPO can restore TPO-dependent megakaryocytic differentiation of the defective Δ3 mutant. UT7.G cells expressing Δ1 (block bars), Δ2 (grey bars), Δ3 (open bars), and wt (hatched bars) mpl were cultured in EPO for about 15 days (UT7.E) and then transferred to a medium containing a mixture of EPO and TPO. After 7 days of treatment, surface megakaryocytic (CD41, CD61, and CD42b) and erythroid (GpA) antigen expression was tested by flow cytometry. Data are expressed as percentages of the MFI of each antigen on cells kept in EPO alone and are means ± standard errors of three or four independent experiments performed with different clones for each mpl construct.

To examine the involvement of the nonproliferative mutants Δ1 and Δ2 in the differentiation process, we took advantage of the dominant differentiating effect of TPO over GM-CSF. UT7.G cells containing full-length mpl or various mpl mutants were shifted to a medium containing a mixture of GM-CSF and TPO, and expression of megakaryocytic markers was assessed by flow cytometry at different time intervals during 1 month of treatment. No change in the expression of CD41, CD61, or CD42b was observed on cells containing any of the mutant forms, Δ1, Δ2, or Δ3, of mpl (data not shown), while transformants expressing wt mpl responded to TPO by inducing expression of all three antigens. Thus, the first 69 aa of the cytoplasmic domain of mpl are involved in the transduction of both proliferation and differentiation signals.

**EPO can restore the TPO-induced differentiation response of cells expressing the Δ3 mutant.** The potentiating effect of EPO on TPO-dependent megakaryocytic differentiation was examined with UT7 transformants expressing mpl mutants by shifting cells to a medium containing EPO instead of GM-CSF for about 2 weeks (UT7.E) and subsequently to a medium with TPO plus EPO. Flow cytometric analysis of erythroid-cell- and megakaryocyte-specific differentiation antigens was performed after 7 days of treatment. Surprisingly, the Δ3 mutant was able to respond to TPO by transducing a megakaryocytic differentiation signal, as shown by increases in CD41, CD61, and CD42b expression and downregulation of GpA expression (Fig. 8). For all markers tested, the magnitude of the response of the Δ3 mutant was similar to that of full-length mpl under the same culture conditions (Fig. 8). In addition, cell growth was reduced and large cells appearing in cultures of UT7.E-Δ3mpl cells stimulated by TPO resembled those seen with UT7.E-Wtmpl (Fig. 5D). These results show that EPO can complement a signal transduced by the Δ3 domain of mpl, leading to megakaryocytic maturation. In contrast, cells expressing the Δ1 and Δ2 mpl mutants did not differentiate in the presence of TPO plus EPO (Fig. 8). The EPO effect was not related to increased surface levels of Δ3 mpl receptors, since the binding of anti-Flag antibodies remained unchanged when cells were shifted from GM-CSF to EPO (data not shown). The continuous presence of EPO in cultures was not necessary for TPO-induced differentiation, since differentiation was also observed when UT7.E-Δ3mpl cells were washed three times and then transferred to a medium containing TPO alone instead of a mixture of TPO and EPO. These results show that EPO can complement a signal transduced by the Δ3 domain of mpl, leading to megakaryocytic maturation.

## DISCUSSION

Little is known of the mechanisms by which hematopoietic growth factors control cell differentiation. In particular, whether activation of cytokine receptors truly drives maturation or simply supports the proliferation and survival of cells already programmed to mature along a certain lineage remains a matter of debate. In this study we have shown that distinct regions of the intracytoplasmic domain of murine mpl are involved in differentiation and proliferation signals, supporting an active role of mpl in megakaryocytic differentiation.

Murine mpl introduced into the human cell line UT7 (32) allows transduction of both TPO-induced proliferation and maturation signals. Upon TPO treatment, UT7 cells engineered to express murine mpl could develop into megakaryocytes with increased cell sizes and levels of specific megakaryocytic differentiation antigens and reduced levels of erythroid markers. These changes are reminiscent of those obtained upon UT7 treatment with phorbol esters (24, 32). TPO has been shown to induce full megakaryocyte development and production of mature platelets from hematopoietic progenitors (28). Such advanced maturation was not observed here with UT7 cells. However, to our knowledge, this is the first in vitro model allowing comparison of the domains of mpl involved in proliferation and differentiation. Indeed, other cell lines of megakaryoblastic origin, such as MO7e and DAMI, can be induced to maturate into megakaryocytes in the presence of phorbol myristate acetate (3, 20), but these cells were shown to proliferate only in response to TPO (42, 49). In human CMK cells and murine FDCP-2 cells, TPO increased CD61, GATA-1, and NF-E2 expression, but no morphologic changes or polyploidization was observed (30, 40). In addition, these cells express high levels of endogenous mpl (10), precluding the introduction of mutant forms of the receptor. Although in UT7 cells mpl transcripts are seen by PCR and some mpl proteins were immunodetected (10, 53), parental cells were unable to respond to either murine or human TPO even at very high doses.

Mutational analysis of the mpl intracellular domain indicated the presence of two functional regions: (i) the first 69 aa, which are involved in both proliferation and maturation signals; (ii) aa 71 to 94, which are necessary for TPO-induced megakaryocytic differentiation but dispensable for proliferation. In the last few years many studies have examined signal transduction by mutants of several cytokine receptors in the context of the regulation of cell proliferation. This led to the identification of two motifs, box 1 and box 2, which are conserved in the cytoplasmic domains of cytokine receptors and involved in mitogenesis. Box 1 alone or in combination with the box 2 motif is required for the binding of JAK and activa-

tion of the JAK-STAT pathway (reviewed in reference 27). In mpl, in agreement with the recent results of Gurney et al. (23), we found that the first 69 aa of the cytoplasmic domain, encompassing both box 1 and box 2 motifs, are involved in TPO-induced cell growth (Fig. 7A), JAK2 phosphorylation (42), and STAT activation (14, 37). Both motifs have been shown to be necessary to provide a proliferative signal in Baf3 cells (23) and for v-mpl-induced pathogenicity in vivo (6).

In a few examples the receptor elements or domains that confer regulation of gene induction and proliferation have been compared. A role for the C-terminal region of the cytoplasmic domain in transcriptional activity has been reported for several receptors, including those for gamma interferon (21), IL-10 (25), growth hormone (46), G-CSF (59), IL-6, and leukemia inhibitory factor (5). In the unique case of the G-CSF receptor, this region has also been shown to be critical for specific neutrophil gene expression in FDCP-1 cells (18) and for terminal neutrophilic maturation in L-GM cells (12). In addition to this C-terminal domain, the box 1 motif is necessary for G-CSF induction of neutrophilic antigens, while the box 2 motif is dispensable (18). By analogy with the G-CSF receptor, we found that several regions of mpl are required for TPO-induced differentiation. However, in this case, in addition to aa 71 to 94 delineated in our Δ3 mutant, both box 1 and box 2 motifs seemed to be necessary for gene induction, as shown by the absence of maturation of UT7 cells expressing the Δ1 or Δ2 mutant in a medium containing TPO plus GM-CSF or TPO plus EPO, which promoted differentiation of UT7-Wtmpl cells (Fig. 8 and Table 1). This suggests that JAK activation, which is probably one of the first events triggered by TPO, is required for all subsequent downstream signaling. The Δ3 mutant activates JAK2 (42) and STAT1, STAT3, and STAT5 in various cell types (14, 23, 37). Recent studies have shown that the transcriptional activities of STAT1 and STAT3 are modulated by phosphorylation on serine residues (55). In the case of STAT1, hyperphosphorylation on serine residues and a subsequent increase in transcriptional activity are generated during macrophage differentiation upon treatment with gamma interferon (15). We are currently studying whether various combinations of STAT and/or modifications of their activities are involved in TPO-induced differentiation of UT7 cells. On the other hand, Gurney et al. (23) have recently shown that the C-terminal region of mpl (including our Δ3 deletion), which is dispensable for proliferation, is responsible for shc phosphorylation and induction of c-fos mRNA, suggesting the involvement of the Ras–mitogen-activated protein (MAP) kinase signaling pathway in a function of mpl distinct from mitogenesis.

The involvement of the region deleted from mpl in our Δ3 mutant in TPO-induced differentiation in UT7 cells was unexpected in view of the identical pathologies induced by wild-type MPLV and Δ3-MPLV, in which the v-mpl sequence contains the Δ3 deletion (6). However, since v-mpl leads to growth factor-independent proliferation of cells of several hematopoietic lineages (48, 57), the transforming effect of v-mpl might be due primarily to its capacity to induce constitutive activation of a pathway leading to mitogenesis and/or inhibition of apoptosis independently of a differentiation function. In this respect, the normal proliferating function of Δ3 mpl would confer its pathogenicity. On the other hand, since EPO is able to restore the maturation function of the Δ3 mpl mutant, its presence in vivo in the microenvironment of infected cells would allow the development of a normal disease in mice inoculated with Δ3-MPLV.

Multiple lines of evidence suggest that megakaryocytic and erythroid lineages are closely related in ontogeny and that both cell types seem to be derived from a common progenitor (35). TPO and EPO, as well as their receptors, are structurally related (4, 11, 16, 34). Moreover, surface markers, notably the EPO receptor (17), and several transcription factors, such as GATA-1, NF-E2, and SCL (2, 39, 43), are coexpressed within these two lineages, raising the possibility of common mechanisms of gene activation. The cooperative action of EPO and TPO in UT7 cells shown in the present study supports this hypothesis. Although EPO induced UT7-mpl cells to maturate toward the erythroid lineage and downregulated megakaryocytic markers, it primed cells to respond more efficiently to TPO. In particular, it was only after being cultured in the presence of EPO (in place of GM-CSF) that UT7-mpl cells could undergo some endomitosis (Fig. 5). In addition, EPO can restore the differentiation function of the Δ3 defective-mpl mutant (Fig. 8).

Several in vitro and in vivo studies have previously demonstrated that in addition to being the physiological regulator of erythropoiesis, EPO exerts a positive influence on megakaryocyte formation. Stimulation of murine or human bone marrow cells by EPO can generate megakaryocyte colonies in both serum-deprived and serum-containing cultures (1, 44, 50). More recently, by using recombinant EPO and TPO, a clear synergy was observed in the induction of megakaryocyte colony formation (7). Conversely, TPO has been shown to enhance the proliferation of erythroid progenitors both in vitro (31) and in vivo (29). Overall, these studies suggest that the TPO-mediated differentiation mechanism is, at least in part, common to that of EPO and that erythroid and megakaryocytic progenitors must respond to overlapping signals. Such common pathways could operate at various levels of the ligand-receptor signaling cascade. For example, at the membrane level, EPO and TPO receptors could associate directly or with a common membrane component. Most of the receptors of the hematopoietin family act as multimeric complexes containing two or three different polypeptides (27). Recently, a direct physical association between EPO receptor and c-kit has been demonstrated and suggested to be part of the mechanism by which the kit ligand (SCF) induces proliferation and maturation of erythroid progenitors (58). On UT7-mpl cells, TPO was found to downregulate the numbers of EPO receptors present at the cell surface (47). EPO does not have a profound effect on the expression of transfected Flag-mpl (Table 1), but whether it modulates mpl affinity for TPO and/or its association with another membrane component awaits binding studies with iodinated TPO. On the other hand, EPO and TPO might share a certain number of downstream signaling pathways. In particular, EPO might modulate TPO-dependent megakaryocytic differentiation by modulating the availability and/or activity of transcription factors, such as GATA-1 and NF-E2, which are known to regulate the activities of promoters of genes specific for both lineages (2, 39, 43). Our UT7-mpl cells and Δ3 mpl mutant provide powerful tools for identifying the membrane, cytoplasmic, and/or nuclear signaling factors activated in response to TPO or EPO and involved in specific or common pathways of erythropoiesis or megakaryopoiesis.

Are the capacities to enhance megakaryocytic differentiation and/or to complement the function of the Δ3 mpl mutant in UT7-mpl cells unique to EPO? The GM-CSF receptor, which belongs to the cytokine receptor family and shares signal transduction pathways with EPO and TPO, does not have these properties. IL-6, IL-11, leukemia inhibitory factor, oncostatin M, and SCF have been shown have thrombopoietic activities and/or to cooperate with TPO for megakaryocyte maturation (1, 7, 28, 54). UT7 cells express receptors for IL-6 and SCF. Although none of these factors can maintain long-term cultures, they induce a proliferative burst in UT7 cells (32, 42).

The differentiation of UT7 cells expressing wild-type mpl or mutants of mpl upon treatment with TPO in the presence of SCF, IL-6, or IL-11 is currently under investigation.

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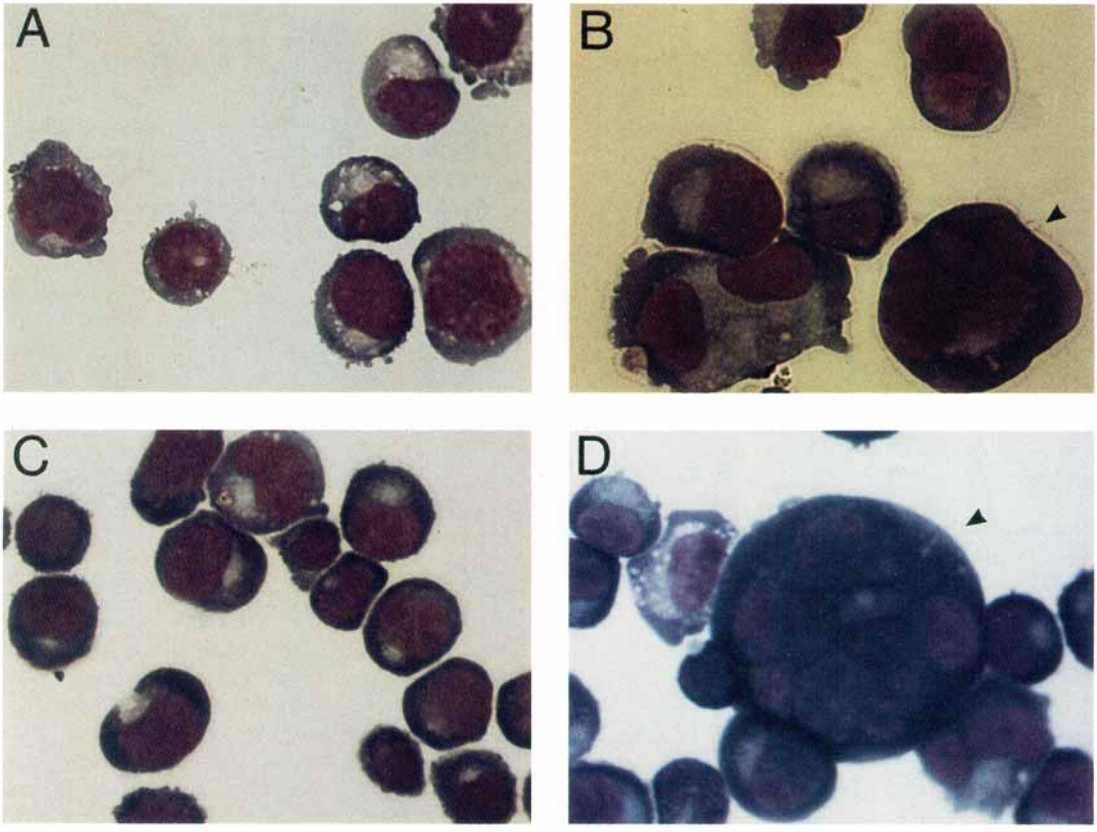
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