blood 1995 85: 391-401

The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets

N Debili, F Wendling, D Cosman, M Titeux, C Florindo, I Dusanter-Fourt, K Schooley, N Methia, M Charon and R Nador

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

The Mpl Receptor Is Expressed in the Megakaryocytic Lineage From Late Progenitors to Platelets

By Najet Debili, Françoise Wendling, David Cosman, Monique Titeux, Christina Florindo, Isabelle Dusanter-Fourt, Ken Schooley, Nassia Methia, Martine Charon, Roland Nador, Ali Bettaieb, and William Vainchenker

The Mpl receptor (Mpl-R) is a cytokine receptor belonging to the hematopoietin receptor superfamily for which a ligand has been recently characterized. To study the lineage distribution of Mpl-R in normal hematopoietic cells, we developed a monoclonal antibody (designated M1 MoAb) by immunizing mice with a soluble form of the human Mpl-R protein. With few exceptions, Mpl-R was detected by indirect immunofluorescent analysis on all human leukemic hematopoietic cell lines with pluripotential and megakaryocytic phenotypes, but not on other cell lines. By immunoprecipitation and immunoblotting, M1 MoAb recognized a band at 82 to 84 kD corresponding to the expected size of the glycosylated receptor. Among normal hematopoietic cells, M1 MoAb strongly stained megakaryocytes (MK) and Mpl-R was detected on platelets by indirect immunofluorescence staining or immunoblotting. On purified CD34$^+$ cells, less than 2% of the population was stained, but the labeling was weak and just above the threshold of detection. However, dual-labeling with the M1 and antiplatelet glycoprotein MoAbs showed that most Mpl-R$^+$/CD34$^+$ cells coexpressed CD41a, CD61, or CD42a, suggesting that cell surface appearance of Mpl-R and platelet glycoproteins could be coordinated. M1-positive and M1-negative subsets were sorted from purified CD34$^+$ cell populations. Colony assays showed that the absolute number of hematopoietic progenitors was extremely low and no primitive progenitors were present in the CD34$^+$/Mpl-R$^+$ fraction. However, this cell fraction was significantly enriched in low proliferative colony-forming units-MK. When the CD34$^+$/Mpl-R$^+$ fraction was grown in liquid culture containing human aplastic serum and a combination of growth factors, mature MK were seen as early as day 4, whereas the predominant cell population was erythroblasts on day 8. Similar data were also obtained with the CD34$^+$/Mpl-R$^-$ fraction with, however, a delay in the time of appearance of both MK and erythroblasts. In conclusion, Mpl-R is a cytokine receptor restricted to the MK cell lineage. Its expression is low on CD34$^+$ cells and these cells mainly correspond to late MK progenitors and transitional cells. These data indicate that the action of the Mpl-R ligand might predominate during the late stages of human MK differentiation.

PRODUCTION OF blood cells is regulated by specific hematopoietic growth factors acting on survival, proliferation, and differentiation. These cytokines exert their biologic effects through the binding of specific receptors on the cell surface. Most cytokine receptors are members of a large family characterized by a common structural motif within the extracellular domain, containing four conserved cysteine residues and a short tryptophan-serine-X-tryptophan-serine (WSXWS) motif located proximal to the transmembrane region. These receptors do not contain consensus sequences for catalytic activities within the cytoplasmic domain.[1]

The murine myeloproliferative leukemia virus (MPLV) contains a truncated form (*v-mpl*) of the coding region of the *c-mpl* gene that is a member of the hematopoietin receptor superfamily.[2] This mutant virus was isolated from a mouse originally infected at birth with a Friend helper virus.[3] It was shown that the oncogene *v-mpl* transforms, in vivo and in vitro, murine multipotential and committed progenitors leading to the generation of various immortalized hematopoietic cell lines capable of spontaneous differentiation.[2,4,5] cDNAs encoding the human and murine *c-mpl* proto-oncogenes were isolated and sequence analyses showed that *c-mpl* had strong homology to members of the cytokine receptor superfamily.[6-8] Using chimeric receptor constructs, it was shown that the cytoplasmic portion of *c-mpl* contained the elements necessary to transduce a proliferative signal in murine hematopoietic cells.[6,8]

Several lines of evidence strongly suggested that the *c-mpl* product was the receptor of a cytokine specifically involved in the regulation of the megakaryocytic lineage. *c-mpl* transcripts were detected by reverse transcription-polymerase chain reaction (RT-PCR) analyses in human leukemic cell lines with a megakaryocytic phenotype, in megakaryocytes (MK) and platelets, as well as in CD34$^+$ cells.[9] Addition of *c-mpl* synthetic antisense oligodeoxynucleotides significantly decreased *c-mpl* transcrits in CD34$^+$ cells and resulted in a profound reduction of in vitro colony-forming unit-MK (CFU-MK) colony formation, whereas the development of erythroid (burst-forming unit-erythroid [BFU-E]) and granulocyte-macrophage (CFU-GM) colonies was not impaired.[9] Biologic and molecular characterization of the Mpl ligand (Mpl-L) have just been performed.[10-14]

To further clarify the role of Mpl-R in human hematopoiesis, we developed a monoclonal antibody (M1 MoAb) directed to the extracellular portion of the human receptor. Here, we provide data showing that Mpl-R is a growth factor receptor restricted to the MK lineage of differentiation from late MK progenitors to platelets.

MATERIALS AND METHODS

Cell lines culture. UT-7, Mo-7E, and TF-1 (factor-dependent pluripotent cell lines obtained from Dr Komatsu, Tochigi-ken, Japan,

From INSERM U362, Institut Gustave Roussy, Villejuif, France; Immunex Corp, Seattle, WA; INSERM U363, Hôpital Cochin, Paris, France; and the Centre Départmental de transfusion, Hôpital Henri Mondor, Créteil, France.

Submitted July 13, 1994; accepted September 29, 1994.

Supported by the Institut National de la Santé et de la Recherche Médicale, the Ligue Nationale contre le Cancer, the Association de Recherche contre le Cancer (Grants No. 6210 and 6688), the Fondation de France, and the Institut Gustave Roussy.

Address reprints requests to William Vainchenker, MD, PhD, INSERM U362, Institut Gustave Roussy, Pavillon de Recherche 1, 39 Rue Camille Desmoulins, 94805 Villejuif Cedex, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/95/8502-0024$3.00/0

and Dr Drexler, Hannover, Germany)[15-17]; Dami, HEL, LAMA, and AP217 (erythro/megakaryoblastic cell lines[18-20]; AP217 kindly provided by R. Berthier, Grenoble, France); CHRF and ELF 153 (megakaryocytic cell lines)[21,22]; K562 (erythroblastic cell line)[23]; KG1a, HL60, and U 937 (myeloid cell lines); and RPMI and CEM (lymphoid cell lines) were maintained in α-minimum essential medium (α-MEM; GIBCO-BRL, France) and 10% heat-inactivated fetal bovine serum (FBS; Gentech, Les Ulis, France). UT-7, TF-1, and Mo-7E were cultivated with 10 ng/mL recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF). Human recombinant cytokines were gifts from Immunex Corp (Seattle, WA) and Amgen (Thousand Oaks, CA). All media were complemented with L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (250 U/mL).

Generation of a human anti–Mpl-R polyclonal antibody. A recombinant protein was obtained by subcloning a *Sph*I-*Hin*dIII fragment encompassing amino-acids 84-231 of the extracellular domain of the human *c-mpl* cDNA[7] into the pGEX2T vector. The recombinant protein was expressed in bacteria. The purified fusion protein (100 μg) was injected subcutaneously into New Zealand rabbits that were boosted twice intramuscularly at 3-week intervals.

Generation of human anti–Mpl-R MoAb. The N-terminal hematopoietin receptor domain of the human *c-mpl* cDNA, from amino acids 26-284,[7] was amplified by PCR and modified at the N-terminus by the addition of a Flag epitope tag[24] and at the C-terminus by the addition of a termination codon. The mpl domain cDNA was inserted into a yeast expression vector under the control of the ADH-2 promoter as a fusion to the α factor leader for secretion as described.[25] The recombinant Flag-Mpl protein was expressed in yeast and purified from culture supernatants by affinity chromatography using an anti-Flag MoAb as described.[26]

A Balb/c mouse was immunized by six subcutaneous injections of 3 to 10 μg of the purified protein. Hybridomas were generated by standard techniques and supernatants from growth-positive wells were tested for their ability to bind the biotinylated immunogen using an antibody capture assay as previously described.[27] Cells from one positive well were single-cell cloned and used to produce the anti–Mpl-R antibody designated as M1 (anti-Mpl receptor-1). M1 was identified as an IgG1 isotope using a typing kit (Sigma Chemical, St Louis, MO). This antibody was then shown to immunoprecipitate ^{35}S-labeled full-length human *c-mpl*–encoded protein from CV-1/EBNA cells[28] transfected with a human *c-mpl* expression vector (data not shown). M1 MoAb was unable to immunoprecipitate murine *c-mpl*–encoded protein under the same conditions (unpublished data).

Immunoprecipitation and immunoblot analysis. Immunoprecipitations and immunoblotting were performed as described.[29] Briefly, 2×10^7 cells were solubilized in a mild lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP40, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL pepstatin A, 10 μg/mL leupeptin, 10 μg/mL aprotinin). All reagents were purchased from Sigma. Lysates were incubated with the polyclonal rabbit anti–Mpl-R serum (J2) and immune complexes were collected by incubation with protein A-sepharose beads (Pharmacia Biotech, Saint Quentin Yvelines, France). Samples were subjected to 8% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) under reducing conditions and, when indicated, transferred to nitrocellulose membranes for immunoblotting. Blots were incubated with the M1 MoAb, washed, and incubated with a horseradish peroxydase-conjugated antimouse antibody (Amersham, Paris, France). Immune complexes were detected by electrochemoluminescence (ECL; Amersham).

For biosynthetic labeling, cells were preincubated for 1 hour at 37°C in culture medium without methionine and cysteine, and further incubated for 3 hours in the same medium supplemented with a mixture of L^{35}S-methionine and L^{35}S-cysteine (250 μCi/mL; cell labeling mix; Amersham). After washing, cells were solubilized and immunoprecipitated as described above.

Northern analysis. Total cellular RNAs were prepared by the acid guanidium thiocyanate/phenol/chloroform method.[30] Total RNAs (10 μg) were electrophoresed on 1% agarose gels followed by blotting onto nitrocellulose filters (Hybond C; Amersham). The probe was a *Pvu*II-*Pvu*II fragment, covering the third and fourth exons of the *c-mpl* c-DNA,[31] which was (α-^{32}P)dCTP-labeled using a random priming labeling kit (Rediprime; Amersham) to a specific activity of about 5×10^8 cpm/μg. Hybridization was performed overnight at 42°C. Filters were washed with a solution containing 1× SSPE and 0.1% SDS (1× SSPE: 0.15 mol/L NaCl, 0.01 mol/L NaH_2PO_4, and 0.001 mol/L EDTA) and exposed for 10 days at −80°C with intensifier screens.

RT-PCR analysis. Total cellular RNAs were extracted from a pellet of 5×10^6 cells following the procedure described by Gough.[32] RT-PCR was performed as previously described without modification,[9] except that 1 μg of total RNA was used. 5′ and 3′ oligodeoxynucleotides used to amplify c-mpl and β2-microglobulin transcripts and internal oligonucleotide probes were described.[9]

Isolation of bone marrow cell populations. Bone marrow or blood mononuclear cells (PBL) from normal informed individuals were separated through a Ficoll-Hypaque metrizoate gradient (1.077 g/L; Eurobio, Paris, France). CD34$^+$ cells were recovered from marrow light-density cells (usually 2×10^8 cells) by either the immunomagnetic bead or immunopanning techniques. For the immunomagnetic bead technique, cells were first incubated at 4°C for 30 minutes with the QBEND 10 MoAb (CD34; Immunotech, Lumigny, France) at a dilution of 1/2,500 (3 μg/mL) and then with paramagnetic beads coupled to a goat antibody to mouse IgG (Dynabeads M-450; Dynal, Oslo, Norway) at a bead to target cell ratio of 5 to 1. CD34$^+$ cells were isolated by magnetic separation and detached from the beads by chymopapain treatment (Sigma; 130 U/mL for 10 minutes).[33] The CD34 epitope recognized by QBEND 10 is cleaved by chymopapain. This procedure allows the collection of CD34$^+$ cells free from beads and an immediate relabeling of the cells with MoAbs recognizing chymopapain-resistant epitopes on the CD34 molecule. A commercial immunopanning technique (AIS; Techgen, Les Ulis, France) was also used according to the manufacturer's instructions. It includes two steps of purification, i.e., isolation of soybean agglutinin (SBA)-negative cells followed by the isolation of CD34$^+$ cells from the SBA-negative population.

Platelets were isolated from the blood by differential centrifugation and contaminating leukocytes were eliminated by filtration.

To obtain a highly enriched population of human megakaryocytes, total bone marrow cells were cultured in liquid suspension cultures containing Iscove's Modified Dulbecco's Medium (IMDM; GIBCO BRL), 10% serum from human aplastic patients (AP), and 1% deionized bovine serum albumin (BSA; fraction Cohn V; Sigma), as previously described.[34] Cells were washed and usually serum-depleted before being analyzed.

In addition, in a patient with an M7 leukemia, blood samples containing more than 75% CD41$^+$ blast cells were collected and deep frozen for subsequent biochemical analysis.

Antibodies and flow cytometric analysis. Several MoAbs were used for flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated HPCA 2 (8G12, anti-CD34), Beb 1 (anti-CD42a), IOA71 (YDJ1.2.2., anti-CD71), IOB6 (T16, anti-CD38), Dako IIIA (Y2-51, anti-CD61), and anti-CD15 were purchased from Becton Dickinson Monoclonal Center (Mountain View, CA), Immunotech (Lumigny, France), and Dako (Glostrup, Denmark), respectively. Phycoerythrin (R-PE)-conjugated anti-Leu-12 (4G7, anti-CD19) and anti–Leu-M3

(anti-CD14) were purchased from Becton Dickinson. Control isotype antibodies (IgG1, IgG2a, FITC-IgG1, and PE-IgG1) were obtained from Dako. FITC- or PE-labeled sheep antimouse antiserum or IgG F(ab)$'_2$ fragments were obtained from Silenius (Hawthorn, Australia) and Dako, respectively.

Briefly, cells were incubated with M1 MoAb (purified IgG1, 236 μg/mL, 1/100 dilution) in phosphate-buffered saline (PBS; pH 7.4) supplemented with 0.1% BSA for 30 minutes at 4°C. Cells were washed in PBS and then incubated with 1/100 dilution of FITC- or PE-conjugated sheep antimouse IgG F(ab)$'_2$ fragments for 30 minutes at 4°C. The negative control for M1 MoAb was an unrelated mouse IgG1. Cells were subsequently incubated for 10 minutes at 4°C with mouse serum and washed. Cells were then incubated with a directly conjugated MoAb for 30 minutes at 4°C and washed. 7 Amino-actinomycin-D (1 μg/mL; Sigma) was added just before analysis with a FACsort (Becton Dickinson) to eliminate dead cells.[35]

In some experiments, cells were cytospun onto slides, fixed with methanol, and doubly stained with a polyclonal rabbit antiserum against the von Willebrand factor (vWF; Dako) and M1 MoAb. Antibody binding was revealed by a rhodamin isothiocyanate-conjugated sheep antiserum against rabbit immunoglobulins or FITC-conjugated antimouse IgG F(ab)$'_2$ fragments, respectively.

Cell sorting. Purified CD34$^+$ cells were either stained with M1 MoAb alone or doubly labeled with M1 and the R-PE HPCA 2 (anti-CD34) MoAbs. After one washing, cells were suspended in IMDM at a concentration of 5×10^5 cells/mL and separated by cell sorting. Cells were sorted on an ODAM, ATC 3000 cell sorter (ODAM/Bruker, Wissembourg, France) equipped with an INNOVA 70-4 argon ion laser (Coherent Radiation, Palo Alto, CA) tuned at 488 nm and operating at 500 mW or on a FACsort. For both cytometers, a "morphologic" gate that included 80% of the events and all the CD34$^+$ cells was determined on two-parameter histograms by side scatter (SSC) versus either electric measurement of the cell volume or forward scatter (FSC). Compensation for two-color labeled samples was set up with singly labeled samples. Among the CD34$^+$ population, M1 MoAb positivity or negativity were determined by using cells labeled with the R-PE HPCA 2 and an FITC-labeled irrelevant IgG1 MoAb. Because the expression of Mpl-R was quite low in comparison to the IgG1 control, the M1-positive (Mpl-R$^+$) fraction was contaminated by about 15% of negative cells.

Colony assays and liquid cultures. For colony assays, cells were plated in the fibrin clot culture system as previously described.[36] Culture medium was composed of IMDM containing 10% preselected serum from an aplastic patient, 1% deionized BSA, L-asparagine (20 μg/mL), $CaCl_2$ (28 μg/mL), 10% bovine citrated plasma (BCP; GIBCO BRL), recombinant human interleukin-3 (r-hu IL-3; 100 U/mL), r-hu GM-CSF (2.5 ng/mL), r-hu G-CSF (20 ng/mL), and recombinant human erythropoietin (r-hu Epo; 1 U/mL). Colonies were counted under an inverted microscope after 12 days of incubation. In addition, to accurately identify MK colonies, clots were dried in situ, fixed with methanol, and reacted with the Y2-51 anti-GpIIIa MoAb for 30 minutes. Fixation of Y2-51 MoAb was then shown using mouse 1/100 diluted IgG + IgM F(ab)$'_2$ coupled to alkaline phosphatase (Caltag Lab, San Francisco, CA). Enzymatic activity was detected as described.[9] Clots were counterstained with hematoxylin.

For liquid cultures, CD34$^+$ cells positive for Mpl-R surface expression were sorted and cultured in 24-well plates (Costar, Dutscher, Brumath, France) at a cell concentration of 500 cells/mL of IMDM medium containing 10% preselected serum from an aplastic patient, a combination of growth factors (stem cell factor, Epo, and IL-3) and 1% deionized BSA as described.[37] Analysis of the cell population growing in each well was performed by flow cytometry after labeling with directly conjugated MoAbs against CD61, GPA, CD14, and CD15.

RESULTS

Surface expression of Mpl-R on human leukemic cell lines. A number of human hematopoietic cell lines were examined for Mpl-R display by indirect immunofluorescence using the M1 MoAb and the fluorescent profiles of these cell lines are shown in Fig 1. Binding of M1 was clearly detected in the HEL, Mo-7E, and Dami cell lines. A weaker labeling was observed on UT-7, AP217, and TF-1, whereas CHRF, KU 812, K 562, and KG 1a showed binding that was barely detectable. In contrast, HL 60, U 937, or CEM and Jurkatt (not shown) were consistently negative. In general, these results confirmed the data obtained by RT-PCR (data not shown). However, some differences must be pointed out. HEL[7] and Dami cells, which both expressed high amounts of c-mpl mRNA as detected by Northern analysis, showed the highest Mpl-R expression on their cell surface. In contrast, binding of M1 MoAb to CHRF was variable ranging from the threshold of detection to a clear labeling, whereas mRNA expression was at a similar level to Dami (Fig 2). The intensity of staining on Mo-7E, UT-7, AP217, and TF-1 was also variable from one experiment to another. This variability was not dependent on the culture conditions (plateau or exponentially growing cells) or on the presence of serum or growth factors (data not shown). In K 562 cells, a labeling just over the control IgG1 could be detected in some experiments, whereas no transcript could ever be detected by RT-PCR.

Immunobiochemical characterization of Mpl-R from human leukemic cell lines. Expression of Mpl-R on human leukemic cell lines was further examined by immunoprecipitation from metabolically labeled Dami cells or by immunoprecipitation followed by immunoblotting from unlabeled cells. A rabbit polyclonal antiserum (J2) raised against a portion of the Mpl-R extracellular domain was used for immunoprecipitation and M1 MoAb for immunoblotting. As shown in Fig 3, Mpl-R appeared as a protein of approximately 82 to 84 kD that was recognized by both the polyclonal and monoclonal anti-Mpl-R antibodies in lysates from metabolically labeled (lanes 1 and 2) or unlabeled cells (lanes 3 and 4). When analyzing various cell lines, we observed that Dami cells expressed high amounts of Mpl-R protein. The amount of Mpl-R protein was weak in HEL, Mo-7E, and CHRF, low in UT-7, and undetectable in TF-1 or AP 217 (data not shown). These results are in partial agreement with the data obtained from indirect immunofluorescence as exemplified by AP 217 or TF-1 cells, which showed weak surface staining (Fig 1). It is noteworthy that, from cell lines to cell lines, the size of the Mpl-R protein identified by the M1 MoAb varied from 84 kD (Mo-7E and UT-7) to 70 kD (HEL) or appeared as a doublet at 84 kD and 74 kD in the CHRF cell line. These bands could correspond to multiple forms of Mpl-R such as those detected by the cloning data.[6-8]

Mpl-R expression on normal hematopoietic cells. To examine the distribution of Mpl-R among bone marrow cells, light-density mononuclear cells were labeled with M1

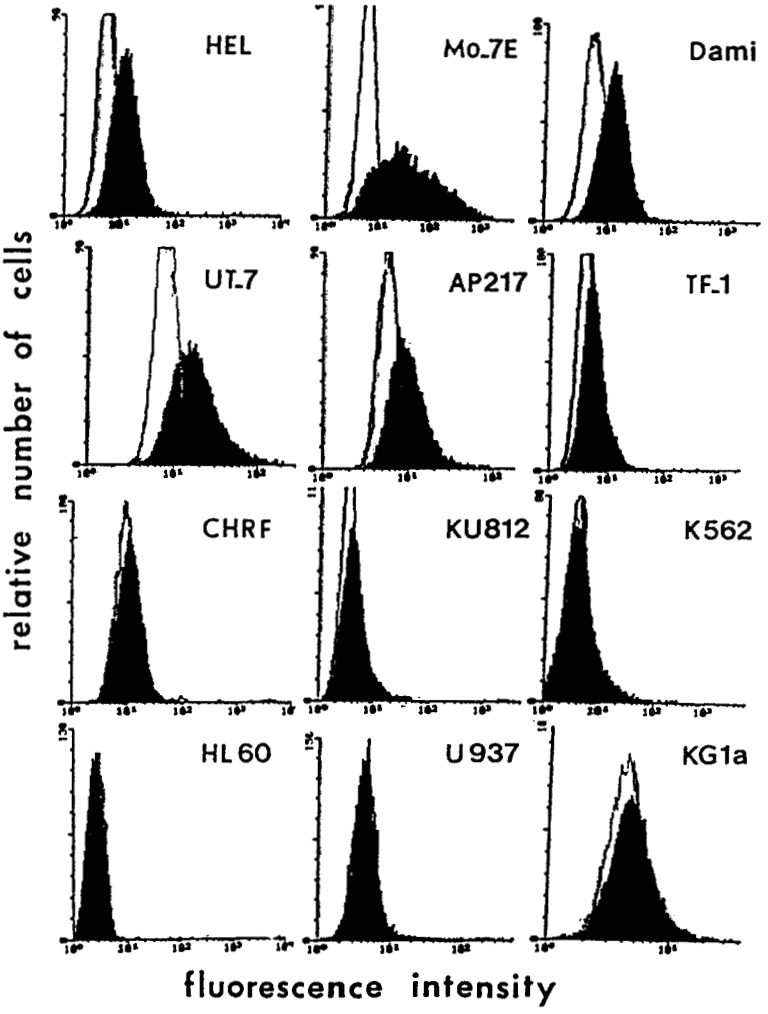

Fig 1. Mpl receptor expression on human leukemic cell lines. Fluorescent profile of representative cell lines. The cells were labeled with M1 MoAb (black profile) or with a control IgG1 (thin line) and indirectly stained with FITC-conjugated sheep anti-mouse IgG F(ab)$_2$ fragments. Cytofluorographs were obtained on a FACsort.

MoAb. In the different scatter gates for granulocytic cells, blast cells, and lymphocytes, no staining distinguishable from the background level of the nonrelevant IgG1 control was observed, with the exception of very rare cells (<0.1%). Mpl-R staining of cells with high scatter properties, which could correspond to polyploid MK, was not investigated because these cells were too rare in marrow samples. No significant labeling was detectable on mononuclear cells from peripheral blood.

Because we have previously shown that c-mpl transcripts were detected by RT-PCR in CD34$^+$ cell populations, MK, and platelets,[9] we focused our studies on these three populations. To determine whether the Mpl-R protein could be seen at the cell surface of MK, bone marrow cells were cultured in liquid in the presence of human aplastic serum to obtain a culture highly enriched in MK (up to 10%). Cells were dually labeled with a PE-conjugated anti-CD61 MoAb and M1 MoAb indirectly labeled with FITC. Staining was analyzed by flow cytometry. The majority of CD61$^+$ cells were stained by M1 (Fig 4). However, some CD61$^-$ cells also appeared to be weakly stained with M1. This was caused by a nonspecific reactivity of M1 MoAb on dead cells, as shown by the addition of 7-amino-actinomycin D to the medium. Among MK, those with large forward and side scatters properties (which corresponded to MK with a high ploidy level) exhibited the strongest staining. Cells from these liquid cultures were also cytospun onto slides and smears were dually labeled with M1 MoAb and a polyclonal anti-vWF antibody. All MK were strongly stained with both antibodies, whereas no significant M1 labeling was seen on cells unlabeled with the anti-vWF antibody (data not shown).

Next, Mpl-R expression was investigated on freshly isolated platelets by both indirect immunofluorescence and immunoblotting. Figure 5 shows that M1 MoAb binds to the Mpl-R expressed on the platelet surface. Fluorescence was much weaker than that observed with an anti-CD61 MoAb, but clearly higher than the background level (Fig 5A). By immunoblotting, M1 MoAb recognized a protein of expected size (82 kD) in a platelet lysate (Fig 5B). The same band but much fainter was also found in lysate from M7 leukemia cells (Fig 5B, lane 3) but not from normal PBL (Fig 5B, lane 2). In addition, in some but not all experiments, a 150-

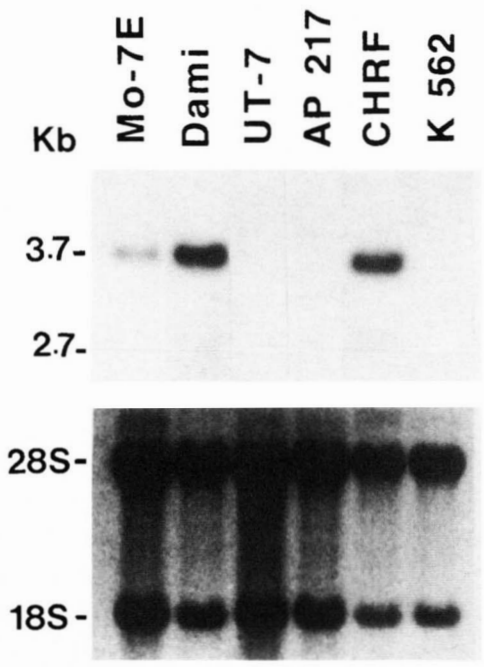

Fig 2. Northern analysis of c-mpl in leukemic cell lines. Total cellular RNA (10 μg) was size separated by formaldehyde/agarose gel and stained with ethidium bromide to visualize 28S and 18S ribosomal RNA. Nitrocellulose filter was hybridized with a human Mpl-R probe. Bands at 3.7 kb are the major c-mpl mRNA. A faint band at 2.7 kb is also seen in Dami and CHRF.[7] Autoradiogram corresponds to a 10-day exposure.

to 160-kD band was also detected in platelet lysates that may correspond to a dimer. Together, these data indicate that both MK and platelets display a detectable number of Mpl receptor molecules on their cell surface.

Mpl-R expression on CD34⁺ cells. Mpl receptor display was then studied on $CD34^+$ cells that are recovered with a purity of about 90% (Fig 6) when either immunomagnetic bead or immunopanning is used. However, the percentage of $CD34^+$ cells stained with M1 MoAb averaged 0.5%.

Mpl-R expression was next analyzed on subsets of $CD34^+$ cells purified by immunomagnetic beads, because chymopapain treatment helps to eliminate most platelets or platelet fragments attached to $CD34^+$ cells. To determine whether $CD34^+/Mpl\text{-}R^+$ cells coexpressed platelet glycoproteins, two-color flow cytometry analyses were performed using $CD34^+$ cells labeled with either FITC-conjugated CD41a (anti-GpIIb), CD42a (anti-GpIX), or CD61 (anti-GpIIIa) and R-PE–labeled M1. Results showed that the vast majority (>80%) of the $CD34^+/M1^+$ cells coexpressed the platelet glycoproteins CD41a (Fig 6) or CD61 (not shown). Nevertheless, a significant fraction of $CD41a^+$ or $CD61^+$ cells were not labeled with M1 MoAb. In contrast, all $CD42a^+$ cells were $M1^+$. However, because the percentage of $CD42a^+$ cells in the $CD34^+$ population was very low, a large proportion of the $M1^+$ cells were in the $CD42a^-$ fraction. These data indicate that the expression of Mpl-R on $CD34^+$ cells appears to be coordinated with the appearance of GpIIb/IIIa. No labeling with M1 was observed on the $CD34^+/CD19^+$ and $CD34^+/CD13^+$ cell populations (data not shown).

Cell sorting. Several cell sorting experiments (n = 14) were performed using $CD34^+$ cell populations. Clonogenic assays and liquid cultures were used to determine the number and nature of progenitors contained in the $CD34^+/M1^+$ or $CD34^+/M1^-$ subpopulations. In the $CD34^+/M1^+$ cell fraction, the absolute number of day-12 clonogenic progenitors (BFU-E, CFU-GM, and CFU-MK) was extremely low and reproducibly less than 2% of the total number of hematopoietic progenitors contained in the initial unseparated $CD34^+$ cell populations (Fig 7A). No primitive progenitors giving rise to colonies within 20 days in culture were present in this cellular fraction. When analyzed in detail, the number of CFU-MK–derived colonies containing 3 or more MK was twofold to threefold increased in the $CD34^+/M1^+$ fraction as compared with the $CD34^+/M1^-$ fraction (Fig 7B). However, the majority of these MK colonies was of small size (range, 3 to 10 MK/colony). In addition, in all experiments, we observed a marked enrichment in individual MK and clusters composed of only 2 or 3 MK. Notably, the ratio between BFU-E and CFU-GM appeared to be slightly inverted in the $CD34^+/M1^+$ fraction when compared with the $CD34^+/M1^-$ or unfractionated $CD34^+$ cells (Fig 7B). Because the $CD34^+/M1^+$ fraction could be contaminated by $M1^-$ cells, the rare cells (<0.1% of the $CD34^+$) that were stained by the control nonrelevant IgG1 were sorted and plated in similar semisolid culture conditions. No isolated MK, MK clusters, or MK colonies were observed. The only contaminant progenitors were rare CFU-GM. As controls, cell sorting was also performed in parallel using $CD34^+$ cells labeled either with anti-CD41a or anti-CD61 MoAbs. A positive staining was clearly seen on a subset (about 3%) of the cells. These positive cells were sorted and grown in culture. Within 4 to 8 days, they gave rise to a nearly pure population of MK clusters (2 to 3 MK) and MK colonies composed of up to 8 cells.

$CD34^+/M1^+$ cell fractions were also cultured for 12 days in liquid culture containing aplastic plasma and a combination of growth factors (r-hu IL-3, 100 U/mL; r-hu GM-CSF, 2.5 ng/mL; r-hu SCF, 20 ng/mL, and r-hu Epo, 1 U/mL). At various times, cells were phenotyped by flow cytometry after been labeled with anti-CD41a, anti-glycophorin A, anti-CD14, and anti-CD15 MoAbs. Mature MK were detected as soon as day 4 in these cultures. At later times, the number of erythroblasts increased greatly and became the predominant population (>90%) on day 8. Similar data were obtained with the $CD34^+/M1^-$ fraction with, however, two main differences: MK appeared later (day 8) and erythroblasts were also the main population at the end of the cultures, but glycophorin A was detectable only after day 10. These results suggest that the $CD34^+/M1^+$ cell fraction may have been slightly enriched in late erythroid progenitors.

DISCUSSION

In this study, we have used a novel MoAb called M1 directed to the extracellular domain of the human Mpl recep-

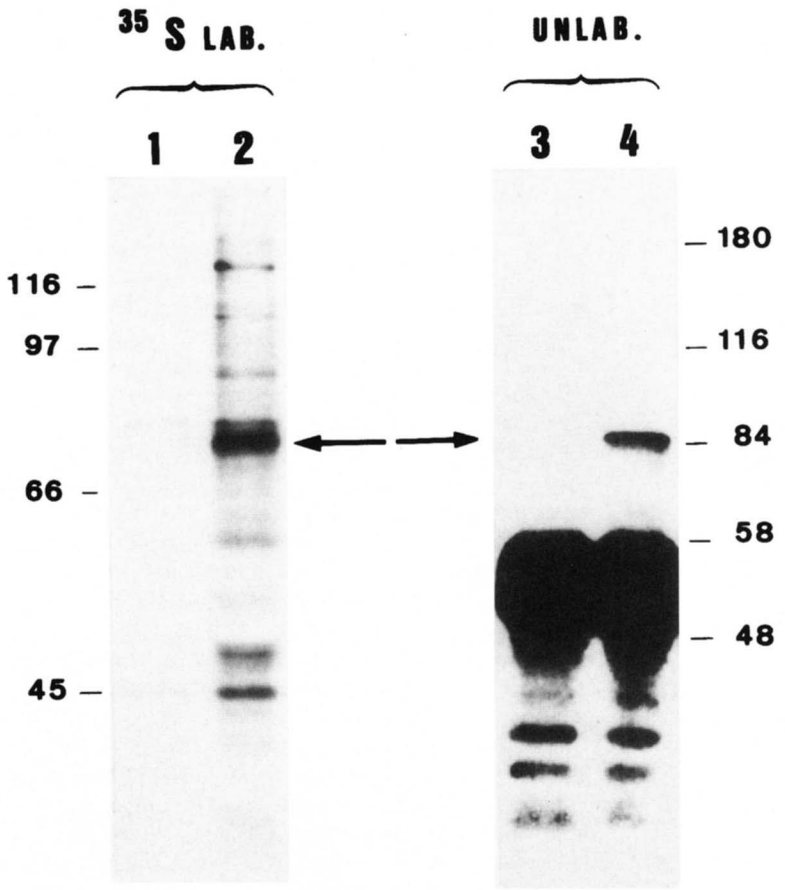

Fig 3. Immunoprecipitation of Mpl-R from a human leukemic cell line. Dami cells (2×10^7) were incubated in the presence (lanes 1 and 2) or the absence (lanes 3 and 4) of ^{35}S-methionine/^{35}S-cysteine for a few hours and lysed. Extracts were immunoprecipitated with a preimmune (lanes 1 and 3) or immune (lanes 2 and 4) anti–Mpl-R rabbit polyclonal antiserum. Immunoprecipitated Mpl-R was detected by autoradiography (lanes 1 and 2) or immunoblotting using the M1 MoAb (lanes 3 and 4) and shown by chemoluminescence.

tor (Mpl-R), the c-mpl product, to define the distribution of this growth factor receptor among hematopoietic cells. M1 MoAb recognizes the Mpl-R on leukemic and normal human hematopoietic cells committed to the MK lineage.

With a few exceptions, all studied human leukemic cell lines with an erythroid/megakaryocytic phenotype display the Mpl-R at their cell surface. In contrast, cell lines with erythroid, myeloid, or lymphoid phenotypes were negative with the exception of a KG1a clone, which, in some experiments, showed a low staining with M1 MoAb. It is noteworthy that this KG1a clone expresses a low level of GpIIb/IIIa.[38] The Mpl-R protein was also detected on three pluripotential factor-dependent cell lines (UT-7, TF-1, and Mo-7E). These cell lines might be useful models to characterize the

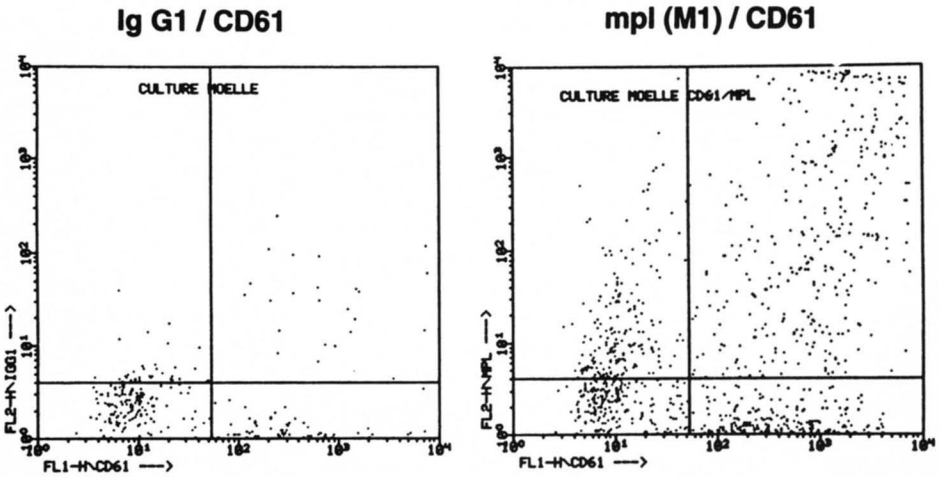

Fig 4. Coexpression of GpIIIa (CD61) and Mpl-R on cultured human MK. Marrow cells were cultured for 12 days in liquid cultures stimulated with a preselected human aplastic serum. Nonadherent cells were labeled simultaneously with a PE-conjugated anti-CD61 MoAb (Y2-51) and either a nonrelevant control IgG1 (left panel) or the specific anti–Mpl-R M1 MoAb (right panel), followed by sheep anti-mouse FITC-conjugated IgG F(ab)$_2$ fragments. The upper right panel indicates cells positive for both GpIIIa and Mpl-R surface expression.

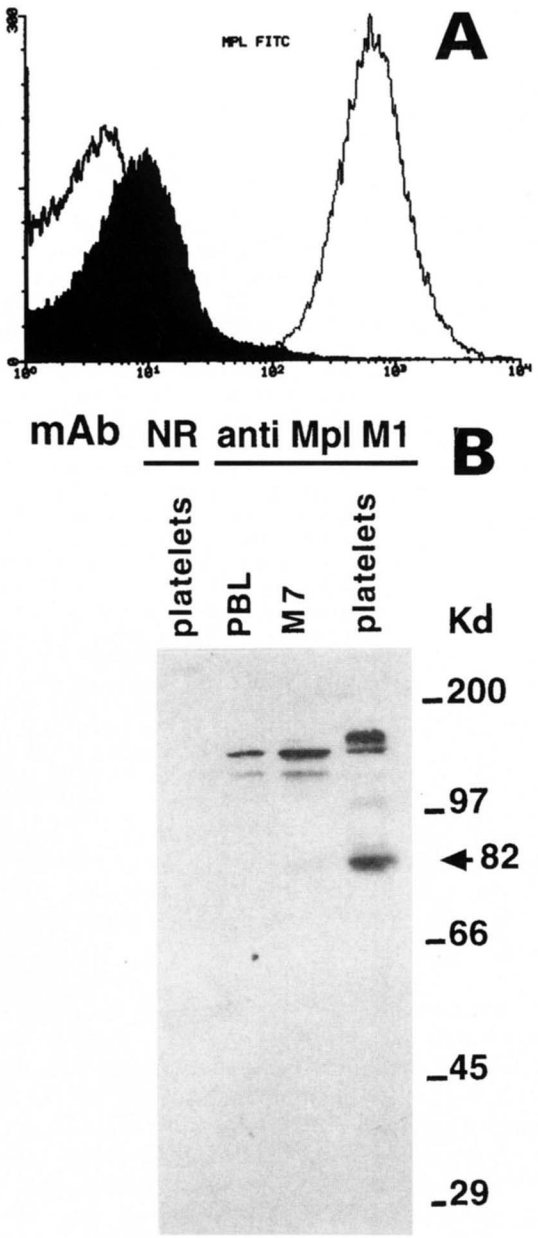

Fig 5. Immunofluorescence staining and immunoblotting of Mpl-R on peripheral blood platelets. (A) Platelets, depleted in leukocytes, were labeled with the anti–Mpl-R M1 MoAb and indirectly stained with FITC-conjugated sheep antimouse IgG F(ab)$_2$ fragments (black profile). Labeling with the control IgG1 is shown by the thick line. The thin line shows the staining with an anti-GpIIIa MoAb. (B) Immunoblotting of Mpl-R in a total platelet lysate shown with M1 MoAb and chemoluminescence. The positions of prestained molecular mass markers are shown on the right. The arrow indicates the molecular mass of the Mpl-R. NR is a nonrelevant IgG1.

effects of the Mpl ligand and to study the transduction signals generated through activation of Mpl-R. Our data are in agreement with prior evaluation using RT-PCR.[9] They indicate that distribution of this growth factor receptor is restricted within the hematopoietic hierarchy. However, we did not find a clear correlation between the cell surface expression of the Mpl-R protein and the amount of mRNA by Northern analysis. For example, CHRF and Dami cells had similar levels of c-mpl transcripts by Northern blot analysis. However, in contrast to Dami cells, binding of M1 MoAb to CHRF was low. Several hypotheses could explain this observation. The human Mpl-R molecule might be heterogeneous, possibly due to different alternate splicing as already described[7,31] and observed for other hematopoietic receptors such as the Epo-R.[39] Thus, the epitope recognized by M1 MoAb may not be present on all Mpl-R molecules. Alternatively, only a fraction of the Mpl-R molecules might be transported to the cell surface, with the remainder retained in the cytoplasm. Further experiments are required to determine the exact processing of Mpl-R and its intracellular trafficking.

The data show that, in normal unseparated hematopoietic cells, Mpl-R was detected by flow cytometric analyses on a very small number of cells (<0.1% of total bone marrow cells) that nearly all belonged to the MK lineage. Noteworthy, cells showing the highest fluorescence intensity in total bone marrow cells were polyploid MK, which were excluded from the gates. The ability to detect Mpl-R on MK was further shown by analysis on marrow cultures enriched in MK where M1 MoAb binding clearly increased in parallel to MK maturation. In agreement with this observation, Mpl-R was also detected on platelets both by immunolabeling and Western blotting. These results on Mpl-R protein expression extend our previous observations obtained at the mRNA level.

To further delineate the developmental and lineage expression of Mpl-R in normal hematopoietic progenitors, subsets of CD34$^+$ cells were analyzed because c-mpl transcripts were previously detected in this cell population. Only a small fraction of CD34$^+$ cells (average, 0.5%) reacted positively and unambiguously with M1 MoAb and their great majority coexpressed the platelet glycoproteins GpIIb/IIIa. No labeling was seen on other CD34$^+$ subsets. However, because both the staining intensity obtained with M1 MoAb and the number of positive cells were low, it remains difficult to exclude that a weak staining might also exist on other CD34$^+$ subsets. The weak labeling observed with M1 MoAb is likely due to the low number of receptor molecules expressed on the surface of CD34$^+$ cells as reported for other cytokine receptors.[40,41]

To sort the CD34$^+$ subsets expressing Mpl-R, we cautiously outlined the windows that gave the clearest separation between CD34$^+$/Mpl-R$^+$ and CD34$^+$/Mpl-R$^-$ cells. This did not avoid a cross-contamination of about 15%, in comparison to the control IgG1. Nevertheless, colony assays showed that the CD34$^+$/Mpl-R$^-$ fraction contained a number of BFU-E, CFU-GM, and CFU-MK progenitors similar to that found in unseparated CD34$^+$ populations. In contrast, the CD34$^+$/Mpl-R$^+$ fraction was almost totally depleted in primitive progenitors (<2%) and clearly enriched in late MK progenitors and transitional cells. These MK progenitors had limited proliferative capabilities because they gave rise to MK clusters comprising 2 to 3 MK or individual MK within 5 to 7

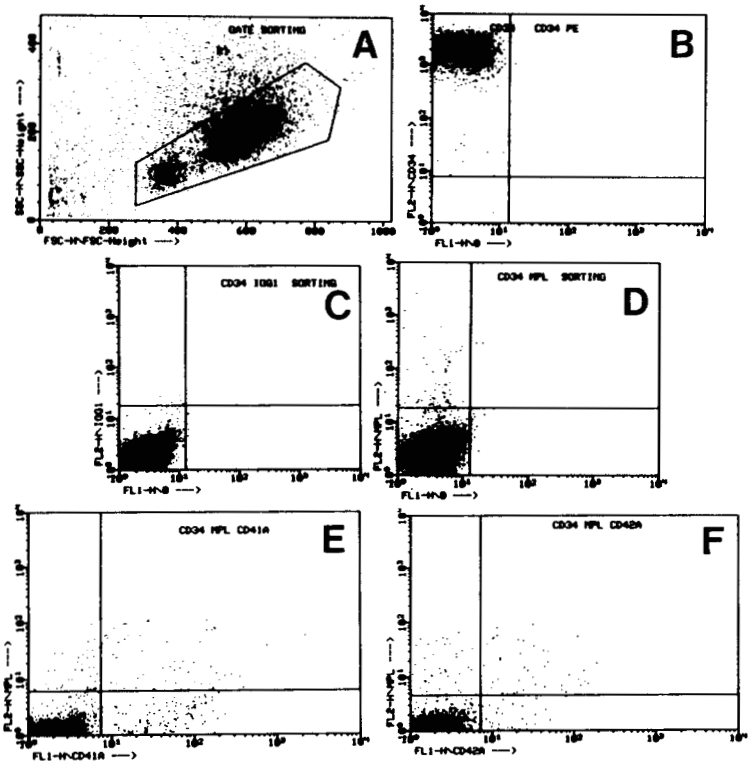

Fig 6. FACS analysis of surface expression of Mpl-R and its coexpression with platelet glycoproteins on purified CD34$^+$ cells. CD34$^+$ cells were purified by the immunomagnetic beads technique using the QBEND 10 MoAb, detached from the beads by chymopapain, immediately labeled with an R-PE–conjugated anti-CD34 MoAb (8G12), and analyzed on a FACsort. (A) The side scatter (SSC) versus forward scatter (FSC) of the cell population. (B) shows that more than 90% of the gated cells expressed the CD34 antigen. (C) and (D) show the fraction of CD34$^+$ cells expressing Mpl-R. Cells were labeled with either a nonrelevant IgG1 (C) or the anti–Mpl-R M1 MoAb (D) and indirectly stained with R-PE–conjugated sheep antimouse F(ab)$_2$ fragments. Cells positively stained with M1 MoAb are shown in the upper left rectangle in (D). (E) and (F) illustrate the results of two-color analyses of CD34$^+$ cells dually stained with R-PE–labeled M1 MoAb (y axis) and FITC-conjugated anti-GpIIb (CD41a) or anti-GpIX (CD42a) MoAbs (X axis). Cells appearing in the upper right panels are those that coexpressed Mpl-R and platelet glycoproteins.

days in culture. Some true day-12 CFU-MK were also present and about twofold to threefold enriched in some experiments. As reported previously, although controversial,[42,43] CFU-MK progenitors coexpress CD34 and GpIIb/IIIa. We thus examined the CFU-MK progenitor content in the CD34$^+$/GpIIb/IIIa$^+$ sorted fraction in comparison to that in the CD34$^+$/Mpl-R$^+$ subpopulation. A much higher proportion of day-12 CFU-MK was found in the CD34$^+$/GpIIb/IIIa$^+$ cell fraction. This difference may only be caused by the low level of cell surface Mpl-R expression as compared with GpIIb/IIIa, which likely introduces a skew in the cell sorting. Previously, we have reported that c-mpl transcripts were present in the CD34$^+$/CD38$^{-/low}$ fraction that has been described to be a cellular fraction highly enriched in primitive progenitors.[44,45] In more recent studies, we found that this fraction is also enriched in MK progenitors, including late CFU-MK.[46] Thus, the presence of c-mpl mRNA in the CD34$^+$/CD38$^{-/low}$ fraction might be more the reflection of an enrichment in MK progenitors than an expression in primitive hematopoietic cells.

It is noteworthy that, in all experiments, we observed an increase in the relative ratio between mature BFU-E and CFU-GM in the CD34$^+$/Mpl-R$^+$ fraction. This enrichment was obvious in liquid cultures because erythroblasts were nearly the only population found after 8 days. Presently, it is difficult to ascertain whether this finding reflects a specific labeling of mature BFU-E by M1 MoAb because only 2% of the total BFU-E number was present in this cellular fraction. Still, when the rare cells (<0.1%) labeled with the nonrelevant IgG1 were sorted and grown in semisolid cultures, almost no BFU-E–derived colonies were observed. Therefore, the data cannot exclude that a subset of BFU-E does express Mpl-R.

Together, our results indicate that expression of Mpl-R seems to follow the same sequence as GpIIb/IIIa during MK differentiation. Interestingly, recent studies on the human c-mpl gene have shown that the structure of its proximal promoter resembles that of GpIIb and other MK-specific genes with both the presence of GATA and ets binding sequences.[31] This finding may explain the restricted expression of the Mpl-R protein to the MK lineage and its coexpression with the platelet glycoproteins IIb/IIIa.

Finally, it is tempting to compare the expression of the Mpl-R to that of the Epo receptor (Epo-R). Both are lineage-restricted receptors, but a promiscuity between the erythroid and megakaryocytic lineages of differentiation is supported by numerous observations. First, the existence of a bipotential E/MK progenitor has been reported.[46-48] Second, the Epo-R is expressed on cells from the MK lineage[49] and we cannot formally exclude the possibility that the Mpl-R is transiently expressed on erythroid progenitors (this report). Third, production of red blood cells and platelets appears to be inversely related.[50-52] Expression of both the Epo-R and Mpl-R predominates during the late stages of differentiation. In particular, it has been shown using fluorescent-labeled Epo that binding was only detected on glycophorin A$^+$ cells but not on CD34$^+$ cells.[40] Using an MoAb recognizing the Epo-R, we were able to sort immature proerythroblasts and CFU-

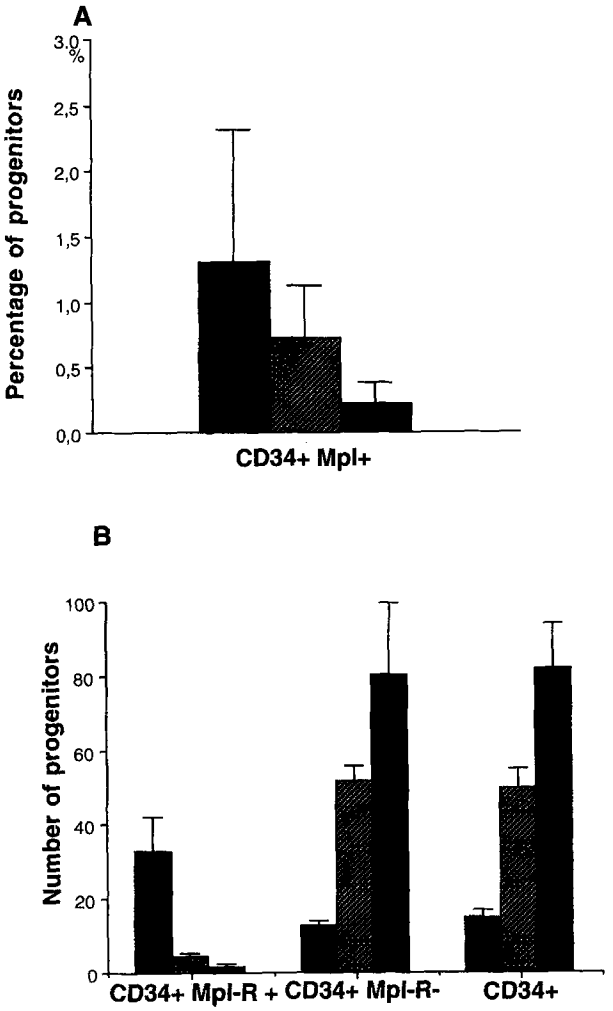

Fig 7. Histograms representing the numbers of progenitors determined by clonogenic semisolid culture assays of $CD34^+/Mpl\text{-}R^+$ or $CD34^+/Mpl\text{-}R^-$ sorted subpopulations. The percentage of (■) CFU-MK, (▨) BFU-E, and (▩) CFU-GM progenitors recovered in the $CD34^+/Mpl\text{-}R^+$ fraction is represented in (A). The number of CFU-MK–, BFU-E–, and CFU-GM–derived colonies in the $CD34^+/Mpl\text{-}R^+$, $CD34^+/Mpl\text{-}R^-$, or unseparated $CD34^+$ fractions are compared in (B). Results represent the mean ± SD of 10 replicate experiments where sorted cells were plated at a concentration of 1,000 cells/mL/dish, with each culture being performed in duplicate.

E to near purity but no BFU-E from normal marrow (personal unpublished data). Similarly, the fluorescence intensity with M1 MoAb is maximal on MK that are already engaged in the endomitotic process of maturation (Fig 4). However, it must be emphasized that the immunolabeling technique has a low-detection sensitivity to investigate hematopoietic growth factor receptor expression.[41] Therefore, the absence of high proliferative CFU-MK and BFU-E in the $Mpl\text{-}R^+$ or $Epo\text{-}R^+$ cell fractions, respectively, does not exclude the presence of these receptors at low levels on primitive progenitors. In favor of this hypothesis, it has been shown that the GM-CSF-R could not be detected on normal CFU-GM by cell sorting.

From the recent experiments that have functionally and molecularly characterized Mpl-L,[10-14] it is clear that Mpl-L is a humoral growth factor restricted to the MK cell lineage that has both the properties of thrombopoietin and MK-CSF. Its biologic activity on the megakaryocytic lineage mimics that of Epo in the erythroid lineage. The present study further strengthens the assumption that the Mpl-R is restricted to the MK lineage and strongly suggests that the action of its ligand will predominate during late stages of MK differentiation.

ACKNOWLEDGMENT

We thank André Katz for expert assistance in cell sorting experiments and Laura Periman and Ginny Price for yeast expression expertise. We are grateful to Dr D. Mason (Oxford, UK) and M. Greave (London, UK) for their gifts of anti-GpIIIa (Y2-51) and anti-CD34 (QBEND 10) MoAbs, respectively; Steven Gillis (Immunex) and Joan Egrié (Amgen) for providing us with human recombinant cytokines; the Bioservices Group at Immunex for transfection of CV-1/EBNA cells; and Dr D. Williams for helpful discussions. We thank the Medical Staff of the Hematology Department of the Institut Gustave Roussy and Hôpital Henri Mondor for providing us with plasma samples from their thrombocytopenic patients.

REFERENCES

1. Bazan JF: Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 87:6934, 1990

2. Souyri M, Vigon I, Penciolelli JF, Heard JM, Tambourin P, Wendling F: A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. Cell 63:1137, 1990

3. Wendling F, Varlet P, Charon P, Tambourin P: A retrovirus complex inducing an acute myeloproliferative leukemic disorder in adult mice. Virology 149:242, 1986

4. Wendling F, Penciolelli JP, Charon M, Tambourin P: Factor-independent erythropoietic progenitor cells in leukemia induced by the myeloproliferative leukemia virus. Blood 73:1161, 1989

5. Wendling F, Vigon I, Souyri M, Tambourin P: Myeloid progenitor cells transformed by the myeloproliferative leukemia virus proliferate and differentiate in vitro without addition of growth factors. Leukemia 3:475, 1989

6. Skoda RC, Seldin DC, Chiang M, Peichel CL, Vogt TF, Leder P: Murine *c-mpl:* A member of the hematopoietic growth factor receptor superfamily that transduces a proliferative signal. EMBO J 12:2645, 1993

7. Vigon I, Mornon JP, Cocault L, Mitjavila M, Tambourin P, Gisselbrecht S, Souyri M: Molecular cloning and characterization of *MPL,* the human homolog of the v-*mpl* oncogene: Identification of a member of the hematopoietic growth factor receptor superfamily. Proc Natl Acad Sci USA 89:5640, 1992

8. Vigon I, Florindo C, Fichelson S, Guenet J, Mattei M, Souyri M, Cosman D, Gisselbrecht S: Characterization of the murine Mpl proto-oncogene, a member of the hematopoietic cytokine receptor family: Molecular cloning, chromosomal location and evidence for a function in cell growth. Oncogene 8:2607, 1993

9. Methia N, Louache F, Vainchenker W, Wendling F: Oligodeoxynucleotides antisense to the protooncogene c-mpl specifically inhibit in vitro megakaryocytopoiesis. Blood 82:1395, 1993

10. Bartley TD, Bogenberger J, Hunt P, Li YS, Lu HS, Martin F, Chang MS, Samal B, Nichol JL, Swift S, Johnson MJ, Hsu RY, Parker VP, Suggs S, Skrine JD, Merewether LA, Clogston C, Hsu E, Hokom MM, Hornkohl, Choi E, Pangelinan M, Sun Y, Mar V,

McNinch J, Simonet L, Jacobsen F, Xie C, Shutter J, Chute H, Basu R, Selander L, Trollinger D, Sieu L, Padilla D, Trail G, Elliott G, Izumi R, Covey T, Crouse J, Garcia A, Xu W, Del Castillo J, Biron J, Cole S, Hu MCT, Pacifici R, Ponting I, Saris C, Wen D, Yung YP, Lin H, Bosselman RA: Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. Cell 77:1117, 1994

11. de Sauvage FJ, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA, Darbonne WC, Henzel WJ, Wong SC, Kuang WJ, Oles KJ, Hultgren B, Solbert LA, Goeddel DV, Eaton DL: Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. Nature 369:533, 1994

12. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, Forstrom JW, Buddle MM, Oort PJ, Hagen FS, Roth GR, Papayannopolou T, Foster DC: Murine thrombopoietin: The ligand for c-mpl expands megakaryocyte progenitors, induces their differentiation and stimulates platelet production. Nature 369:568, 1994

13. Lok S, Kaushansky K, Holly RD, Kuijper JL, Lofton-Day CE, Oort PJ, Grant FJ, Heipel MD, Burhead SK, Kramer JM, Bell LA, Sprecher CA, Blumberg H, Johnson R, Prunkard D, Ching AFT, Mathewes SL, Bailey MC, Forstrom JW, Buddle MM, Osborn SG, Evans SJ, Sheppard PO, Presnell SR, O'Hara PJ, Hagen FS, Roth GJ, Foster DC: Murine thrombopoietin: Expression cloning, cDNA sequence and stimulation of platelet production *in vivo*. Nature 369:565, 1994

14. Wendling F, Maraskovsky E, Debili N, Florindo C, Teepe M, Titeux M, Methia N, Breton-Gorius J, Cosman D, Vainchenker W: The Mpl ligand is a humoral regulator of megakaryocytopoiesis. Nature 369:571, 1994

15. Komatsu N, Nakauchi H, Miwa A, Ishihara T, Eguchi M, Moroi M, Okada M, Sato Y, Wada H, Yawata Y, Suda T, Miura Y: Establishment and characterization of a human leukemic cell line with megakaryocytic features: Dependency on granulocyte-macrophage colony-stimulating factor, interleukin-3, or erythropoietin for growth and survival. Cancer Res 51:341, 1991

16. Avanzi G, Lista P, Giouinazzo B, Minlero R, Saglio G, Benetton G, Coda R, Cattoretti G, Pegoaro L: Selective growth response to IL-3 of a human cell line with megakaryoblastic features. Br J Haematol 69:359, 1988

17. Kitamura T, Tange T, Terasawa T, Chiba T, Kuwaki T, Miyagawa K, Piao Y, Miyazono K, Urabe A, Takaku F: Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. J Cell Physiol 140:323, 1989

18. Greenberg SM, Rosenthal DS, Greeley TA, Tantravahi R, Handin RI: Characterization of a new megakaryocyte cell line: The Dami cell. Blood 72:1968, 1988

19. Martin P, Papayannopoulou T: HEL cells: A new erythroleukemia cell line with spontaneous and induced globin expression. Science 216:1233, 1982

20. Seigneurin D, Champelovier P, Mouchiroud G, Berthier R, Leroux D, Prenant M, McGregor J, Stack J, Morle F, Micouin C, Pietrantuono A, Kolodie L: Human chronic myeloid leukemic cell line with positive Philadelphia chromosome exhibits megakaryocytic and erythroid characteristics. Exp Hematol 15:822, 1987

21. Fugman DA, Witte DP, Jones CLA, Aronow BJ, Lieberman MA: In vitro establishment and characterization of a human megakaryoblastic cell line. Blood 75:1252, 1990

22. Mouthon M, Freund M, Titeux M, Katz A, Guichard J, Breton-Gorius J, Vainchenker W: Growth and differentiation of the human megakaryoblastic cell line (ELF-153): A model for early stages of megakaryocytopoiesis. Blood 84:1085, 1994

23. Lozzio CB, Lozzio CB: Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. Blood 45:321, 1975

24. Hopp TP, Prickett KS, Price VL, Libby RT, March CJ, Cerretti DP, Urdal DL, Conlon PJ: A short polypeptide marker useful for recombinant protein identification and purification. Biotechnology 6:1204, 1988

25. Price V, Mochizuki D, March CJ, Cosman D, Deeley MC, Klinke R, Clevenger W, Gillis S, Baker D, Urdal D: Expression, purification and characterization of recombinant murine granulocyte-macrophage colony-stimulating factor and bovine interleukin-2 from yeast. Gene 55:287, 1987

26. Prickett KS, Amberg DC, Hopp TP: A calcium-dependent antibody for identification and purification of recombinant proteins. Biotechniques 7:580, 1989

27. Beckmann MP, Schooley KA, Gallis B, Vanden Bos T, Friend D, Alpert AR, Raunio R, Prickett KS, Baker PE, Park LS: Monoclonal antibodies block murine IL-4 receptor function. J Immunol 144:4212, 1990

28. McMahan CJ, Slack JL, Mosley B, Cosman D, Lupton SD, Bruton LL, Grubin CE, Wignall JM, Jenkins NA, Brannan CI: A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. EMBO J 10:2821, 1991

29. Dusanter-Fourt I, Muller O, Ziemiecki A, Mayeux P, Drucker B, Djiane J, Wilks A, Harpur AG, Fischer S, Gisselbrecht S: Identification of JAK protein tyrosine kinases as signaling molecules for prolactin. Functional analysis of prolactin receptor and prolactin-erythropoietin receptor chimera expressed in lymphoid cells. EMBO J 13:2583, 1994

30. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156, 1987

31. Mignotte V, Vigon I, Boucher de Crèvecoeur E, Roméo PH, Lemarchandel V, Chrétien S: Structure and transcription of the human c-mpl gene (MPL). Genomics 20:5, 1994

32. Gough N: Rapid and quantitative preparations of cytoplasmic RNA from small numbers of cells. Anal Biochem 173:93, 1988

33. Sawada K, Dai CH, Koury ST, Horn ST, Glick AD, Civin CI: Purification of human blood burst-forming units erythroid and demonstration of the evolution of erythropoietin receptors. J Cell Physiol 142:219, 1990

34. Debili N, Hegyi E, Navarro S, Katz A, Mouthon M, Breton-Gorius J, Vainchenker W: In vitro effects of hematopoietic growth factors on the proliferation, endoreplication, and maturation of human megakaryocytes. Blood 77:2326, 1991

35. Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV: Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry 13:204, 1992

36. Mitjavila MT, Vinci G, Villeval JL, Kieffer N, Henri A, Testa U, Breton-Gorius J, Vainchenker W: Human platelet alpha granules contain a non specific inhibitor of megakaryocyte colony formation: Its relationship to type β transforming growth factor (TGF-β). J Cell Physiol 134:93, 1988

37. Debili N, Massé J, Katz A, Guichard J, Breton-Gorius J, Vainchenker W: Effects of recombinant hematopoietic growth factors (IL-3, IL6, SCF, LIF) on the megakaryocyte differention of CD34 positive cells. Blood 82:84, 1993

38. Berridge MV, Ralph SJ, Tan AS: Cell lineage antigens of the stem cell-megakaryocyte platelet lineage are associated with the platelet IIb-IIIa glycoprotein complex. Blood 66:76, 1985

39. Nakamura Y, Komatsu N, Nakauchi H: A truncated erythropoietin receptor that fails to prevent programmed cell death for erythroid cells. Science 257:1138, 1992

40. Wognum W, Krystal G, Eaves CJ, Eaves AC, Lansdorp PM:

Increased erythropoietin-receptor expression on CD34-positive bone marrow cells from patients with chronic myeloid leukemia. Blood 79:642, 1992

41. Wognum AB, van Gils FCJM, Wagemaker G: Flow cytometric detection of receptors for interleukin-6 on bone marrow and peripheral blood cells of humans and rhesus monkeys. Blood 81:2036, 1993

42. Debili N, Issaad C, Massé JM, Guichard J, Katz A, Breton-Gorius J, Vainchenker W: Expression of CD34 and platelet glycoproteins during human megakaryocytic differentiation. Blood 80:3022, 1992

43. Thoma SJ, Lamping CP, Ziegler BL: Phenotype analysis of hematopoietic $CD34^+$ cell populations derived from human umbilical cord blood using flow cytometry and c-DNA-polymerase chain reaction. Blood 83:2103, 1994

44. Issaad C, Croisille L, Katz A, Vainchenker W, Coulombel L: A murine stromal cell line allows the proliferation of very primitive human $CD34^{++}/CD38^-$ progenitor cells in long-term cultures and semi-solid assays. Blood 81:2916, 1993

45. Terstappen LWMM, Huang S, Safford M, Lansdorp PM, Loken M: Sequential generations of hematopoietic colonies derived from single nonlineage-committed $CD34^+CD38^-$ progenitor cells. Blood 77:1218, 1991

46. Debili N, Croisille L, Coulombel L, Breton-Gorius J, Vainchenker W: Identification of a human bipotent erythroid-megakaryocytic (MK) progenitor. Blood 82:603a, 1992 (abstr, suppl 1)

47. McLeod DL, Shreeve MM, Axelrad AA: Induction of megakaryocyte colonies with platelet formation in vitro. Nature 261:492, 1976

48. Vainchenker W, Guichard J, Breton-Gorius J: Growth of human megakaryocyte colonies in culture from fetal, neonatal and adult peripheral blood cells. Ultrastructural analysis. Blood Cells 5:25, 1979

49. Fraser JK, Tan AS, Lin F, Berridge MV: Expression of specific high-affinity binding sites for erythropoietin on rat and mouse megakaryocytes. Exp Hematol 17:10, 1989

50. McDonald TP, Clift RE, Cottrell MB: Large, chronic doses of erythropoietin cause thrombocytopenia in mice. Blood 80:352, 1992

51. McDonald TP, Sullivan PS: Megakaryocytic and erythrocytic cell lines share a common precursor cell. Exp Hematol 21:1316, 1993

52. Villeval J, Metcalf D, Johnson GR: Fatal polycythemia induced in mice by dysregulated erythropoietin production by hematopoietic cells. Leukemia 6:107, 1992