Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand

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Physiological platelet synthesis is thought to require the humoral activities of meg-CSF and thrombopoietin, which respectively promote proliferation and maturation of megakaryocytic cells. A meg-CSF/thrombopoietin-like protein that is present in plasma of irradiated pigs has been purified and cloned. This protein binds to and activates the c-*mpl* protein, a member of the cytokine receptor superfamily. The isolated Mpl ligand shares homology with erythropoietin and stimulates both megakaryocytopoiesis and thrombopoiesis.

PLATELET production has long been thought to be regulated by lineage-specific humoral factors. Although several known haematopoietic growth factors and cytokines stimulate megakaryocytopoiesis and platelet production (reviewed in ref. 1), most are pleiotropic and consequently their roles in physiological regulation of thrombocytopoiesis are unclear. However, the plasma, serum and urine of thrombocytopenic animals and humans do contain megakaryocytopoietic and thrombopoietic activities[2,13] that are lineage-specific and distinct from known cytokines[14,17]. These activities are referred to as meg-CSF or thrombopoietin on the basis of their ability to affect either proliferation (meg-CSF) or maturation (thrombopoietin) of megakaryocytes[18,19]. Although these activities have been described since the 1960s, attempts to purify them have been unsuccessful.

Recently, the orphan cytokine receptor encoded by c-*mpl* (c-Mpl)[20,23] has been implicated in the regulation of megakaryocytopoiesis[24]. The expression of c-*mpl* appears to be restricted to primitive stem cells, megakaryocytes and platelets, indicating its downregulation during the differentiation of all haematopoietic lineages except megakaryocytes[24]. Moreover, c-*mpl* antisense oligonucleotides selectively inhibit megakaryocytic colony formation *in vitro* without affecting the growth of erythroid and granulomacrophage colonies[24]. These results suggest that a putative ligand for c-Mpl may be a megakaryocyte lineage-specific growth factor similar to the meg-CSF or thrombopoietin activities present in thrombocytopenic plasma.

Consistent with previous observations[2,13], we have found that aplastic porcine plasma (APP) obtained from irradiated pigs stimulates human megakaryocytopoiesis *in vitro*. Here we report that this stimulatory activity is abrogated by the soluble extracellular domain of c-Mpl, identifying APP as a source of the putative Mpl ligand (ML). We have purified the ML from APP and used the amino-acid sequence information to isolate a human ML complementary DNA. The ML has sequence homology to erythropoietin and has both meg-CSF and thrombopoietin-like activities.

Aplastic porcine plasma contains Mpl ligand

APP obtained from irradiated pigs stimulated human megakaryocytopoiesis *in vitro* (Fig. 1*a*). To determine whether this stimulation was dependent on the putative ML, we examined whether a human Mpl–IgG fusion protein containing the extracellular domain of Mpl neutralized this activity. Mpl–IgG inhibited the megakaryocytopoietic activity of APP, whereas a control (natriuretic peptide receptor–IgG) fusion protein had no effect (Fig. 1*a*). This indicates that APP contains an ML, and that this ligand is necessary for the property of this plasma to stimulate megakaryocytopoiesis *in vitro*.

To facilitate purification of the ML, an Mpl-dependent cell proliferation assay was developed. Stable expression of several different growth factor receptors in factor-dependent cell lines confers responsiveness to the respective growth factor[25,27]. Because the cytoplasmic domain of c-Mpl can transduce a proliferation signal[22,23] and the extracellular domain of c-Mpl appeared to bind ML (Fig. 1*a*), we predicted that c-Mpl might be a single-chain receptor. We therefore expressed c-*mpl* in the murine interleukin-3 (IL-3)-dependent Ba/F3 cell line and tested the ability of APP to stimulate proliferation of the Ba/F3-*mpl* cells. Stable expression of human c-*mpl* indeed conferred APP responsiveness to Ba/F3-*mpl* cells cultured in the absence of IL-3 (Fig. 1*b*). This activity was completely abolished by soluble Mpl–IgG, but not by the control fusion protein (CD4–IgG). Normal porcine plasma did not stimulate the Ba/F3-*mpl* cells. These results indicate that APP contains a factor(s) that can bind the extracellular domain of Mpl and transduce a proliferative signal.

Purification of the Mpl ligand from APP

The ML was purified from APP using hydrophobic interaction, immobilized dye, and Mpl-affinity chromatography (Table 1). The activity present in column fractions was determined using the Ba/F3-*mpl* cell proliferation assay. The overall purification from 5 litres of APP was greater than 4×10^6-fold, with about 10% recovery of activity. We estimated the specific activity of the ligand eluted from the Mpl-affinity column to be $\sim 3 \times 10^6$ units mg^{-1}.

Analysis of eluted fractions from the Mpl-affinity column by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) revealed the presence of several proteins (Fig. 2*a*), the most intensely staining of which migrated at positions corresponding to M_r values of ~66K, 55K, 30K, 28K and 14K. To determine the size of the ML, an aliquot of affinity-purified protein preparation was subjected to SDS–PAGE and resolved

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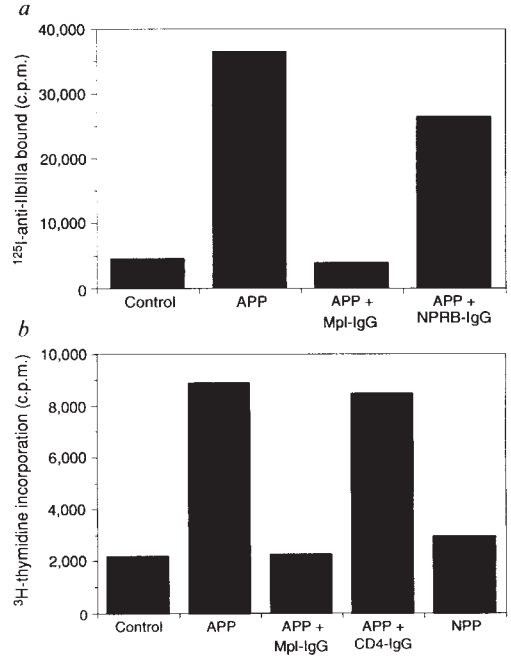

FIG. 1 Stimulation by APP and inhibition by Mpl–IgG of human *in vitro* megakaryocytopoiesis (*a*) and Ba/F3-*mpl* cell proliferation (*b*).
METHODS. Platelet-poor plasma was collected from normal or aplastic anaemic pigs. Pigs were rendered aplastic by irradiation with 900 cGy of total body irradiation using a 4 meV linear accelerator. The irradiated pigs were supported for 6–8 days with intramuscular injections of cefazolin. Subsequently, their total blood volume was removed under general anaesthesia, heparinized, and centrifuged at 1,800*g* for 30 min to make platelet-poor plasma. The megakaryocyte-stimulating activity was found to peak 6 days after irradiation. *a*, Liquid suspension megakaryocytopoiesis assay: the effect of APP on human megakaryocytopoiesis was determined using a modification of the liquid suspension megakaryocytopoiesis assay described in ref. 43. Circulating peripheral blood progenitor cells (PSC) obtained from consenting patients were diluted fivefold with PBS and centrifuged at 800*g* for 15 min at room temperature. The cell pellets were resuspended in Iscove's modified Dulbecco's media (IMDM) and layered onto 60% percoll (density 1.077 gm ml^{-1}, Pharmacia) and centrifuged at 800*g* for 20 min. The light-density mononuclear cells were collected at the interface and washed twice with IMDM and plated out at $1–2 \times 10^6$ cells per ml in IMDM containing 30% FBS (1 ml final volume) in 24-well tissue culture clusters (Costar). The cultures were incubated with or without 10% APP for 12–14 days in a humidified incubator at 37 °C in 5% CO_2 and air; 0.5 μg ml^{-1} Mpl–IgG or natriuretic peptide receptor/IgG (NPRB–IgG[44]) was added at days 0, 2 and 4. After 12–14 days in culture, megakaryocytopoiesis was quantified using a radiolabelled murine IgG monoclonal antibody (HP1-1D) against the megakaryocyte-specific glycoprotein GPIIbIIIa (provided by W. L. Nichols, Mayo Clinic) as described[43]. The average of duplicate determinations is shown. There was less than 10% variation between each duplicate. Mpl–IgG: a cDNA fragment encoding amino acids 1–491 of human Mpl was obtained by PCR from a human megakaryocytic CMK cell cDNA library and fused in-frame to a cDNA encoding the Fc region of human IgG1, as described[44]. The Mpl–IgG construct was subcloned into pRK5-tkneo and transfected into 293 human embryonic kidney cells by the calcium phosphate method[45]. The cells were selected in 0.4 mg ml^{-1} G418 and individual clones were isolated. Mpl–IgG expression from isolated clones was determined using a human Fc-specific ELISA. The recombinant Mpl–IgG was purified on a protein A-Sepharose column (Pharmacia). *b*, Ba/F3-*mpl* cell proliferation assay: a DNA fragment corresponding to the entire coding sequence of *mpl* P (ref. 21) was obtained by PCR using as template cDNA prepared from the human megakaryocytic CMK cell line and was cloned into pRK5-tkneo. After linearization, this construct was introduced in Ba/F3 cells by electroporation (250 volts, 960 μF). Neomycin-resistant cells were selected in 2 mg ml^{-1} G418 and individual clones were obtained by limiting dilutions. Clones expressing *mpl* P were identified by FACS analysis using rabbit polyclonal antiserum raised against human Mpl–IgG. Stimulation of proliferation of Ba/F3-*mpl* cells in response to aplastic pig plasma was measured by the extent of ^{3}H-thymidine incorporation in the DNA. Cells were starved of IL-3 for 16 h then seeded in 96-well plates at a density of 25,000 cells per well in media containing APP at various concentrations in the presence or absence of Mpl–IgG or CD4–IgG[42] at 200 pM. After incubation for 22 h, 1 μCi ^{3}H-thymidine per well was added and the cells incubated for an additional 6 h before being collected. Incorporated radioactivity was determined in the presence of 40 μl of scintillation fluid (μicroscint 20) using a Top Count Counter (Packard Instruments). The average of duplicate determinations is shown. There was less than 10% variation between each data point. One unit of activity produced 50% maximal stimulation.

proteins were eluted from gel slices and assayed (Fig. 2*b*). Most of the activity was found in the 28K–32K region of the gel, with a smaller amount of activity eluting in the 18K–20K region. The only proteins visible in these regions had M_r values of 30K, 28K and 18K. To obtain sequence information on these proteins, the Mpl-affinity-purified preparation was separated by SDS–PAGE and electroblotted onto polyvinylidenefluoride (PVDF)[28]. Amino-terminal sequence analysis[29] of these three proteins gave the identical sequence, SPAPPACDPRLLNKLLRDDHVLHGRL (single-letter amino-acid code), indicating that these proteins are derived from a common precursor. Computer-assisted analysis showed this amino-acid sequence to be unique.

TABLE 1 Purification of the Mpl ligand from APP

Sample	Volume (ml)	Protein (mg ml^{-1})	Units ml^{-1}	Units	SA (Unit mg^{-1})	Yield (%)	Fold purification
APP	5,000	50	40	200,000	0.8	—	1
Phenyl	4,700	0.8	40	188,000	50	94	62
Blue-S	640	0.93	400	256,000	430	128	538
Mpl-UL	12	0.0005	1,666	20,000	3.3×10^6	10	4.1×10^6

Aplastic porcine plasma obtained from irradiated pigs was made 4 M in NaCl and stirred for 30 min at room temp. The resultant precipitate was removed by centrifugation at 3,800 r.p.m. in a Sorvall RC3B and the supernatant was loaded onto a phenyl-Toyopearl column (220 ml) equilibrated in 10 mM sodium phosphate containing 4 M NaCl. The column was washed with this buffer until the absorbance at 280 nm was <0.05 and eluted with H_2O. The eluted protein peak was diluted with H_2O to a conductivity of 15 mS and loaded onto a blue-Sepharose column (240 ml) equilibrated in PBS. Subsequently, this column was washed with 5 column volumes each of PBS and 10 mM sodium phosphate containing 2 M urea. The column was eluted with 10 mM sodium phosphate containing 2 M urea and 1 M NaCl. The eluted protein peak was made 0.01% in octyl glucoside (*n*-octyl β-D-glucopyranoside) and 1 mM each in EDTA and Pefabloc (Boehringer Mannheim) and loaded directly onto tandemly linked CD4–IgG[42] and Mpl–IgG ultralink (Pierce) columns. The CD4–IgG (2 ml) column was removed after the sample was loaded and the Mpl–IgG (4 ml) column washed with 10 column volumes each of PBS and PBS containing 2 M NaCl, then eluted with 0.1 M glycine-HCl, pH 2.25. Fractions were collected into 0.1 volume of 1 M Tris, pH 8.0. The CD4–IgG and Mpl–IgG columns were made by coupling 10–20 mg of each to 0.5 g of Ultralink (UL) resin according to the manufacturer's instructions. A summary of the purification of the ML from 5 litres of APP is shown.

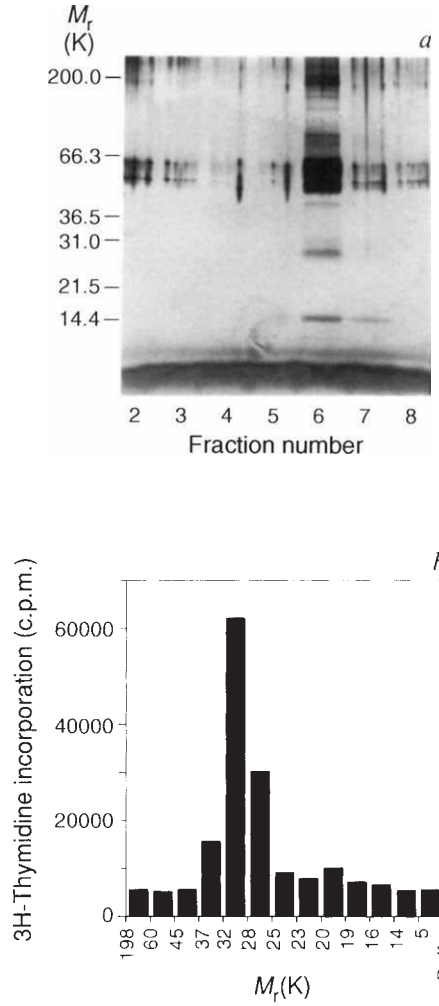

FIG. 2 Identification of ML by SDS–PAGE (*a*) and gel elution (*b*). METHODS. *a*, SDS–PAGE of purified ML. 200 μl each of the eluted fractions 2 to 8 from the Mpl-affinity column were precipitated with acetone and solubilized in Laemmli-SDS-sample buffer. The sample was resolved on a 4–20% SDS–polyacrylamide gel (Novex) and proteins were visualized by silver staining. *b*, Elution of ML activity from an SDS gel. The affinity-purified ML (50 μl) was resolved on a Novex 14% SDS–polyacrylamide gel run under non-reducing conditions. The sample was not heated. As a control, sample buffer without ligand was applied to a separate gel under identical conditions. Following electrophoresis the gel was cut into 12 slices, noting their position in relation to M_r markers. The 12 gel slices were placed into the cells in two Biorad model 422 electro-eluters and proteins were electro-eluted as previously described[46]. The eluted proteins from the 12 gel slices were dialysed overnight at 4 °C against PBS. Samples were then incubated on ice for 1 h. Precipitated SDS was removed by centrifugation in a microfuge. The supernatants were then again placed on ice for 1 h and microfuged. The final supernatants were diluted in PBS and assayed for Ba/F3-*mpl* cell proliferation activity.

Furthermore, this protein(s) is probably the ML because it co-elutes with activity from an SDS gel at two different regions along the gel.

Molecular cloning of the Mpl ligand

On the basis of the N-terminal sequence obtained from the purified ligand, two degenerate oligonucleotide primer pools were used to amplify porcine genomic DNA by polymerase chain reaction (PCR). Assuming that the N terminus of the ML is encoded by a single exon, the correct amplification product should be 69 base pairs (bp) long. A fragment of the expected size was obtained and sequenced. The amino-acid sequence (PRLLNKLLR) encoded between the PCR primers was identical to that obtained by N-terminal protein sequencing.

A synthetic oligonucleotide based on the sequence of the PCR fragment was used to screen a human genomic DNA library. Four clones with a similar restriction pattern were isolated and one of them was characterized further. A 390-bp *Eco*RI–*Xba*I fragment was found to contain a putative exon encoding 42 amino acids (Fig. 3*a*). The deduced amino-acid sequence varies by only five amino acids when compared to the first 26 amino acids of the porcine ML. The predicted amino-acid sequence immediately upstream of the sequence determined by direct protein sequencing is highly suggestive of a signal peptide. However, this sequence does not contain an initiation codon, suggesting the existence of an additional upstream exon.

To identify a source of ML messenger RNA for cDNA cloning reverse-transcribed PCR (RT-PCR) analysis was done on mRNA prepared from various human fetal and adult tissues and cell lines, using as primers oligonucleotides corresponding to the ends of the exon sequence. A DNA fragment of the expected size (140 bp) was detected in adult kidney and fetal liver. A human fetal liver cDNA library (7×10^6 clones) in λDR2 was screened with the oligonucleotide probe and four positive clones were isolated. The sequence of a clone with a 1.8-kilobase (kb) insert was determined.

Structure of the Mpl ligand

The human ML cDNA clone consists of 1,774 nucleotides followed by a poly(A)$^+$ tail (Fig. 3*a*). The presumed initiation codon at nucleotide position 216–218 is within a consensus sequence favourable for eukaryotic translation initiation[30]. It defines an open reading frame of 1,059 nucleotides, which predicts a primary translation product of 353 amino acids. Flanking this open reading frame are 215 nucleotides of 5′ and 498 nucleotides of 3′ untranslated sequence. The N terminus of the predicted amino-acid sequence is highly hydrophobic and probably represents a signal peptide. Computer analysis[31] indicates a potential cleavage site for signal peptidase between residues 21 and 22. Cleavage here would generate a mature polypeptide of 332 amino acids (38K) beginning with the N-terminal sequence obtained from protein sequencing of the porcine ML.

Comparison of the ML sequence with the Genbank sequence database revealed 23% identity between the N-terminal 153 residues of the ML and erythropoietin (Fig. 3*b*). When conservative substitutions are taken into account, this region of ML shows

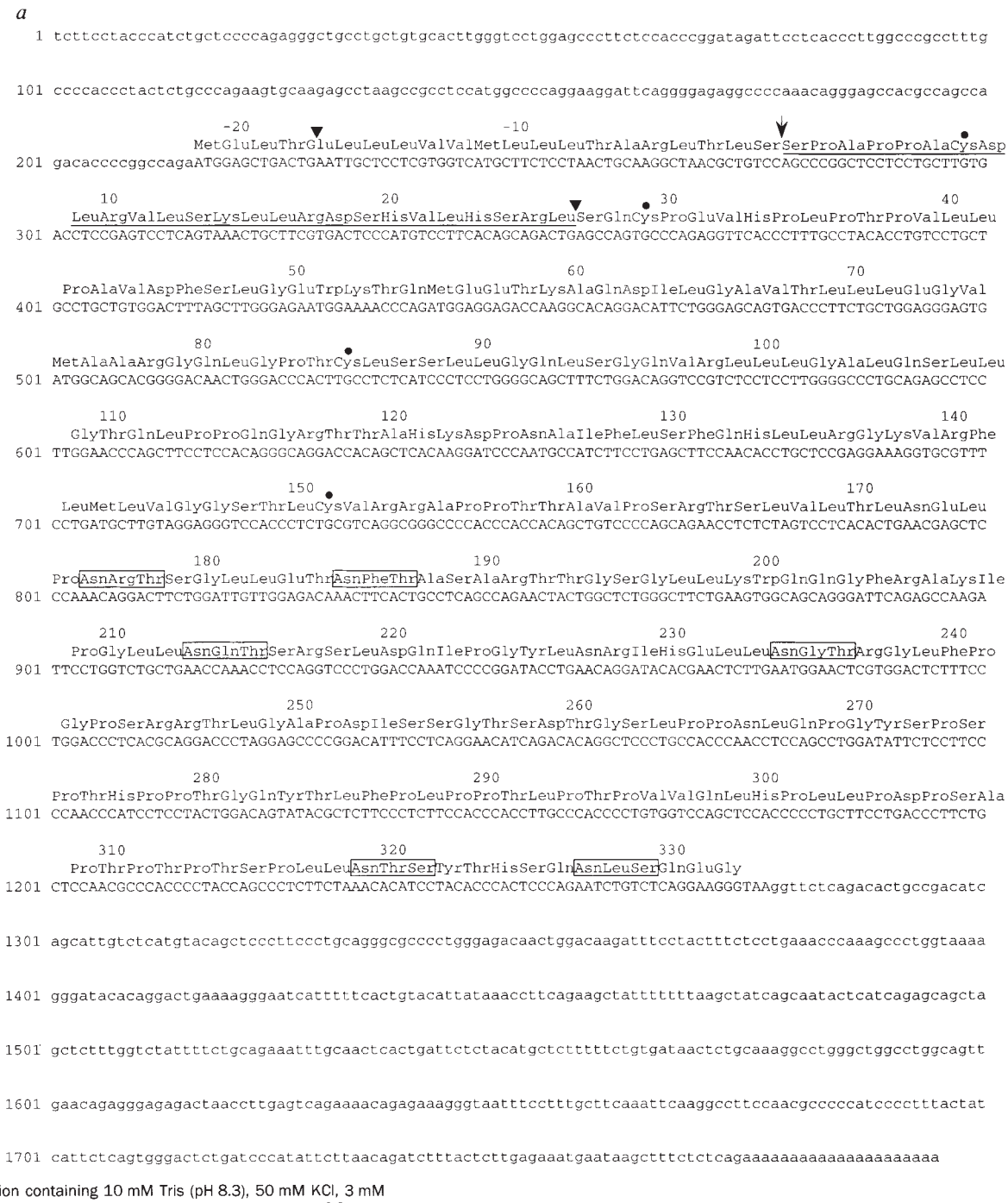

FIG. 3 *a*, Nucleotide sequence and deduced amino-acid sequence of human ML cDNA. Nucleotides are numbered at the beginning of each line. Amino acids are numbered above the sequence starting at Ser 1 of the mature ML protein sequence. The 5′ and 3′ untranslated regions are indicated in lower case letters. The boundaries of the exon contained in the 390-bp *Eco*RI–*Xba*I fragment are indicated by arrows and the potential *N*-glycosylation sites are boxed. Cysteine residues are indicated by a dot above the sequence. The underlined sequence corresponds to the N-terminal sequence determined by protein sequencing of the pig ligand. *b*, Comparison of the ML and erythropoietin sequences. The predicted amino-acid sequence for the human ML is aligned with the human erythropoietin sequence[34]. Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes. Potential *N*-glycosylation sites are underlined with a plain line for the ML and with a broken line for erythropoietin. The two cysteines important for erythropoietin activity are indicated by a dot.

METHODS. *a*, On the basis of the N-terminal sequence of the porcine ML, two primer pools were synthesized: *mpl*.1 5′-CCNGCNCCNCCNCC-NGCNTGY-GA-3′ (2,048-fold degenerate) and *mpl*.2 5′-NCCRTGNARNA-CRTGRTCRTC-3′ (2,048-fold degenerate). Porcine genomic DNA isolated from porcine peripheral blood lymphocytes was used as the template for PCR[47]. DNA (0.8 μg) was amplified in a 50 μl reaction containing 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 100 μg ml^{-1} BSA, 400 μM dNTPs, 1 μM of each primer pool and 2.5 units of *Taq* polymerase. Initial template denaturation was at 94 °C for 8 min. followed by 35 cycles of 45 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The final cycle was allowed to extend for 10 min at 72 °C. A 69-bp product was obtained and subcloned in pGEMt and three clones were sequenced. On the basis of the sequence obtained, a 45-mer deoxyoligonucleotide called pR45 was designed and synthesized: 5′-GCCGTGAAGGACGTGGTCGTCACGAAGCAGTTTATTTAGGAG-TCG-3′. The oligonucleotide was labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and used to screen a human genomic library in λGem12 (provided by U. Schindler) under low-stringency hybridization conditions[48]. Positive clones were isolated and a 390-bp *Eco*RI–*Xba*I fragment hybridizing to the probe was cloned in pBluescript SK- and sequenced. On the basis of this sequence, two oligonucleotides were synthesized corresponding to the ends of the predicted exon sequence: forward primer: 5′-AGAAATTGCTCCTCGTGGTCATGCTTCT-3′ and reverse primer: 5′-CAGTCTGCCGTGAAGGACATGG-3′. These oligonucleotides were used to prime a PCR reaction using the conditions described above. cDNAs from various human tissues and cell lines were used as a template. The expected 140-bp product was identified in fetal liver cDNA. A fetal liver cDNA library in λDR2 was screened with the pR45 oligonucleotide as described above. Positive clones were isolated and characterized. The 1.8-kb insert of clone FL2b was subcloned in M13 and both strands were sequenced by standard fluorescent dye terminator and dye primer methods on an ABI373 automated sequencer.

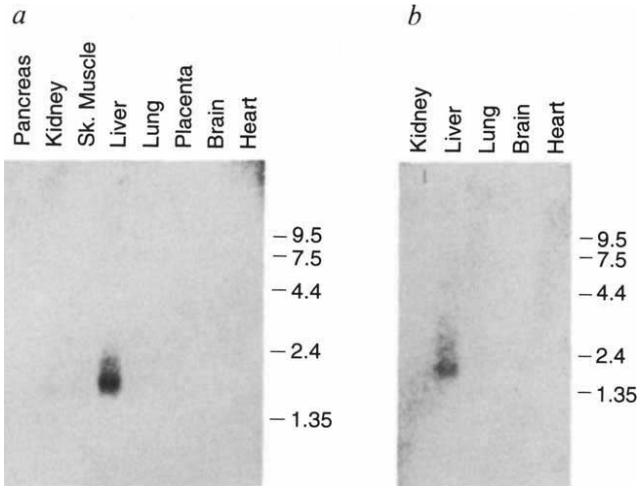

FIG. 4 Northern blot analysis of various human adult (*a*) or fetal tissues (*b*). Northern blots of ~2 μg poly(A)$^+$ RNA from various human tissues were purchased from Clontech. The membranes were hybridized overnight with a single-stranded DNA probe corresponding to position 219–470 of the human ML. The probe was generated by asymmetric PCR in the presence of ^{32}P-dATP. The blot was washed at 42 °C in 0.2 × SSC, 0.1% SDS and then exposed for 3 days to a storage phosphor imaging plate.

50% similarity to erythropoietin. Both erythropoietin and the ML contain four cysteines, three of which are conserved. Site-directed mutagenesis experiments have shown that the first and last cysteines of erythropoietin form a disulphide bond essential for function[32]. By analogy, the first and last cysteines of ML may also form a critical disulphide bond. None of the three glycosylation sites within erythropoietin are conserved in the ML. The six potential *N*-linked glycosylation sites predicted by ML sequence are all located in the C-terminal half of the protein.

Like erythropoietin, the ML mRNA does not contain the consensus polyadenylation sequence AAUAAA, nor the regulatory element AUUUA that is present in 3′ untranslated regions of many cytokines and is thought to influence mRNA stability[33]. Northern blot analysis reveals low levels of a single 1.8-kb ML RNA transcript in both fetal and adult liver (Fig. 4). After longer exposure, a weaker band of the same size could be detected in adult kidney (not shown). By comparison, erythropoietin is expressed in fetal liver and, in response to hypoxia, the adult kidney and liver[34,35].

The importance of the C-terminal region of the ML remains to be elucidated. On the basis of the presence of the six potential sites for *N*-linked glycosylation and the ability of the ligand to bind lectin-affinity columns (data not shown), this region of the ML is probably glycosylated. In some gel-elution experiments we observed activity resolving with M_r ~60K (data not shown), which may represent the full-length, glycosylated molecule. The C-terminal region may therefore act to stabilize and increase the half-life of circulating ML. In the case of erythropoietin, the non-glycosylated form has full *in vitro* biological activity, but has a significantly reduced plasma half-life relative to glycosylated erythropoietin[36–38]. The C-terminal domain of ML contains two di-basic amino-acid sequences (Arg-Arg motifs at positions 153–154 and 245–246; Fig. 3*a*) that could serve as potential processing sites. Cleavage at these sites may be responsible for generating the 30K, 28K and 18K forms of the ML isolated from APP. Importantly, the Arg_{153}-Arg_{154} sequence occurs immediately following the erythropoietin-like domain of the ML.

The observations indicate that full-length ML may represent a precursor protein that undergoes limited proteolysis to generate the mature ligand. Interestingly, a comparison of human and porcine ML sequences shows 83% identity between the erythropoietin-like domains, but only 67% between the C-terminal

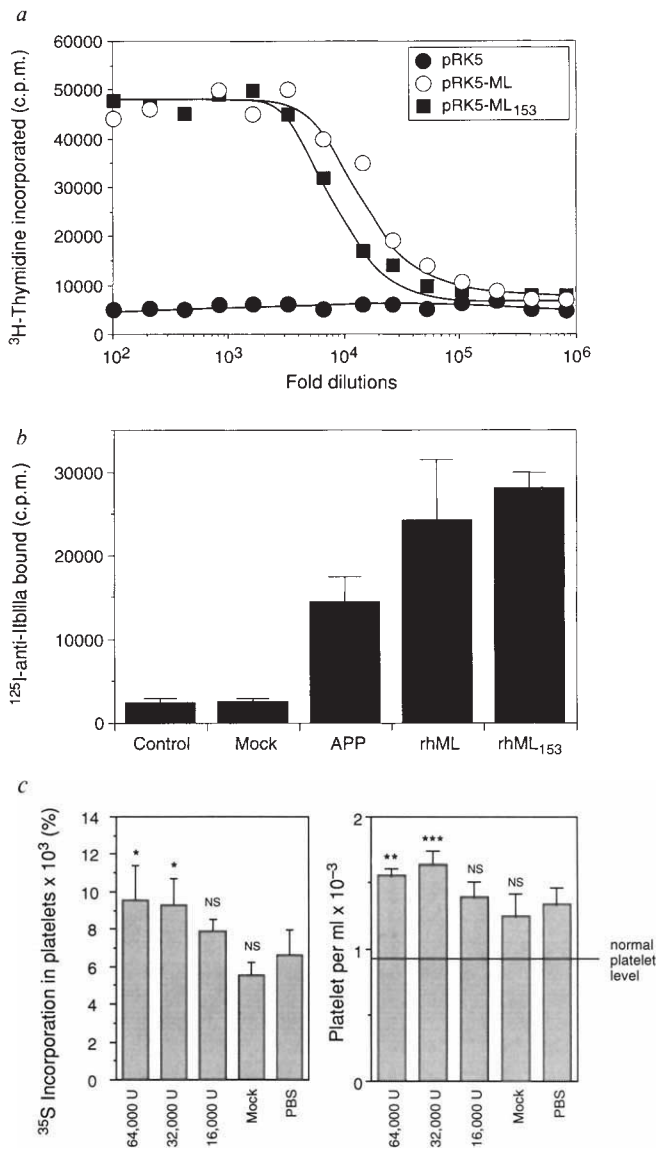

FIG. 5 Effect of rhML on Ba/F3-*mpl* cell proliferation (*a*), *in vitro* human megakaryocytopoiesis (*b*), and murine thrombopoiesis (*c*).
METHODS. pRK5-ML_{153} was generated by introducing a stop codon after residue 153 by PCR. 293 cells were transfected with either pRK5-hML or pRK5-ML_{153} by the calcium phosphate method[45]. Media were then conditioned for 36 h and tested for activity in the Ba/F3-*mpl* cell proliferation (*a*) and *in vitro* megakaryocytopoiesis (*b*) assays described in Fig. 1. The effect of partially purified rhML on platelet production *in vivo* (c) was determined using the rebound thrombocytosis assay described in ref. 39. Briefly, mice were given a single injection of goat anti-mouse platelet serum (day 0) and on day 5 and 6, during rebound thrombocytosis, the mice were injected with test samples or excipient. On day seven, 30 μCi $Na_2{}^{35}SO_4$ was injected and 24 h later ^{35}S incorporation into circulating platelets and platelet counts were determined. Partially purified rhML was prepared from 200 ml conditioned media obtained from 293 cells transfected with pRK5-hML. The media were passed through a 2 ml blue-Sepharose column equilibrated in PBS and the column was washed with PBS and eluted with PBS containing 2 M each of urea and NaCl. The active fraction was dialysed into PBS and made 1 mg ml^{-1} with endotoxin-free BSA. Medium from mock-transfected cells was treated similarly. The sample contained less than one unit of endotoxin per ml (Limulus Amebocyte Assay, Bio Whittaker). Mice were injected with either 64,000, 32,000 or 16,000 units of rhML, a mock preparation, or excipient alone. Each group consisted of six mice. The mean and standard deviation of each group is shown. *P* values were determined by a 2-tailed *t*-test comparing medians; * $P = 0.003$; ** $P = 0.0005$; *** $P = 0.0001$; NS, not significant.

domains. The dibasic site present at position 153–154 in the human ML is conserved in porcine ML (unpublished results), consistent with the possibility that the erythropoietin-like domain of the ML represents the mature ligand.

Expression of recombinant *mpl* ligand

To confirm that the cloned cDNA encoded a ligand for Mpl, it was expressed in mammalian cells under the control of the cytomegalovirus immediate-early promoter using the expression vector pRK5-hML. Supernatants from transiently transfected human embryonic kidney 293 cells were found to stimulate ^{3}H-thymidine incorporation in Ba/F3-*mpl* cells, but not in parental Ba/F3 cells (Fig. 5*a*). Media from 293 cells transfected with the pRK vector alone did not contain this activity. Addition of Mpl–IgG to the media abolished the stimulation (data not shown). These results show that the cloned cDNA encodes a functional human ML (hML).

To determine whether the erythropoietin-like domain alone can bind and activate Mpl, a truncated form of hML consisting of residues 1–153 ($rhML_{153}$) was expressed in 293 cells. Supernatants from transfected cells have activity similar to that in supernatants from cells expressing the full-length hML (Fig. 5*a*), indicating that the C-terminal domain of ML is not required for binding and activation of c-Mpl.

Stimulation by the *mpl* ligand

Both the full-length (rhML) and the truncated ($rhML_{153}$) forms of recombinant hML stimulated human megakaryocytopoiesis *in vitro* (Fig. 5*b*). Whether this stimulation is caused by direct action of ML or requires a serum or induced cellular factor(s) cannot be ruled out. However, this effect was observed in the absence of other exogenously added haematopoietic growth factors. With the exception of IL-3, the ML is the only haematopoietic growth factor that exhibited this activity. IL-11, IL-6, IL-1, erythropoietin, G-CSF, IL-9, LIF, Kit ligand, m-CSF, OSM and GM-CSF had no effect on megakaryocytopoiesis when tested separately in our assay (data not shown). This result demonstrates that the ML has megakaryocyte-stimulating activity, and indicates a role for ML in regulating megakaryocytopoiesis.

Thrombopoietic activities present in plasma of thrombocytopenic animals stimulate platelet production in a mouse rebound thrombocytosis assay[39,40]. In this model, mice are made acutely thrombocytopenic using specific antiplatelet serum, resulting in a predictable rebound thrombocytosis. Such immunothrombocythaemic mice are more responsive to exogenous thrombopoietin-like activities than are normal mice[39], just as exhypoxic mice are more sensitive to erythropoietin than are normal mice[41]. To determine whether the rML stimulates platelet production *in vivo*, mice in rebound thrombocytosis were injected with partially purified rhML. Platelet counts and incorporation of ^{35}S into platelets were then quantified. Injection of mice with 64,000 or 32,000 units of rML significantly increased platelet production, as shown by a ~20% increase in platelet counts ($P = 0.0005$ and 0.0001, respectively) and a ~40% increase in ^{35}S incorporation into platelets ($P = 0.003$) in the treated mice versus control mice injected with excipient alone (Fig. 5*c*). This level of stimulation is comparable to that observed with IL-6 in this model (data not shown). Treatment with 16,000 units of rML or a preparation from mock-transfected cells did not significantly stimulate platelet production. These results indicate that ML stimulates platelet production in a dose-dependent manner and therefore possesses thrombopoietin-like activity.

Megakaryocytopoiesis and the *mpl*-ligand

It has been proposed that megakaryocytopoiesis is regulated at multiple cellular levels[18,19]. This is based largely on the observation that certain haematopoietic growth factors stimulate proliferation of megakaryocyte progenitors, whereas others appear primarily to affect maturation[1]. Our results, together with previous findings, suggest that the ML acts both as an proliferative and maturation factor. Although the *in vitro* megakaryocytopoiesis assay described here does not differentiate between activities that affect either proliferation or maturation, several lines of evidence suggest that ML stimulates proliferation of megakaryocyte progenitors. APP stimulates both proliferation and maturation of human megakaryocytes *in vitro* (L.A.S., unpublished data), and this stimulation is completely inhibited by Mpl–IgG (Fig. 1*a*). Furthermore, the inhibition of megakaryocyte colony formation by c-*mpl* antisense oligonucleotides[24], and the finding that c-*mpl* can transduce a proliferative signal in cells into which it is transfected[22,23], also indicate that ML stimulates proliferation. The apparent expression of c-*mpl* during all stages of megakaryocyte differentiation[24] and the ability of recombinant ML to stimulate platelet production rapidly *in vivo* indicate that ML also affects maturation. The availability of recombinant ML makes possible a careful evaluation of its role in regulating megakaryocytopoiesis and thrombopoiesis, as well as its potential to influence other haematopoietic lineages. □

Received 30 March; accepted 10 May 1994.

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ACKNOWLEDGEMENTS. We thank M. L. Stewart for APP, M. Shuman for human peripheral stem cells, the Genentech DNA synthesis group, A. Goddard for DNA sequence analysis, R. Thomas for assistance in the platelet rebound assay, J. Baker for criticism of the manuscript, L. Tamayo for preparing figures, and A. Levinson for support and encouragement.