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Murine c-*mpl*: a member of the hematopoietic growth factor receptor superfamily that transduces a proliferative signal

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The murine myeloproliferative leukemia virus has previously been shown to contain a fragment of the coding region of the c-*mpl* gene, a member of the cytokine receptor superfamily. We have isolated cDNA and genomic clones encoding murine c-*mpl* and localized the c-*mpl* gene to mouse chromosome 4. Since some members of this superfamily function by transducing a proliferative signal and since the putative ligand of mpl is unknown, we have generated a chimeric receptor to test the functional potential of mpl. The chimera consists of the extracellular domain of the human interleukin-4 receptor and the cytoplasmic domain of mpl. A mouse hematopoietic cell line transfected with this construct proliferates in response to human interleukin-4, thereby demonstrating that the cytoplasmic domain of mpl contains all elements necessary to transmit a growth stimulatory signal. In addition, we show that 25–40% of *mpl* mRNA found in the spleen corresponds to a novel truncated and potentially soluble isoform of mpl and that both full-length and truncated forms of mpl protein can be immunoprecipitated from lysates of transfected COS cells. Interestingly, however, although the truncated form of the receptor possesses a functional signal sequence and lacks a transmembrane domain, it is not detected in the culture media of transfected cells.

Key words: alternative splicing/chimeric receptor/chromosomal mapping/c-*mpl*/cytokine receptor

Introduction

Mpl is a member of the cytokine receptor superfamily for which no ligand has as yet been identified. A portion of the c-*mpl* gene was originally discovered fused to viral sequences encoding the envelope protein of a mutant Friend murine leukemia virus (Souyri *et al.*, 1990). This mutant strain was called myeloproliferative leukemia virus (MPLV) because it caused a broad spectrum of myeloid leukemias in mice including erythroid, granulocytic, monocytic, megakaryocytic and mast cell leukemias (Wendling *et al.*, 1986, 1989).

The human homolog of v-*mpl* has been shown to have sequence homology to members of the cytokine receptor superfamily (Vigon *et al.*, 1992), which is characterized by a common structural design of the extracellular domain, with four conserved cysteine residues in the N-terminal portion, and a short motif, tryptophan-serine-x-tryptophan-serine (WSXWS), located proximal to the transmembrane domain (Bazan, 1990). All but 40 amino acids of the extracellular domain of mpl, including the conserved cysteines, were deleted in the fusion protein encoded by v-*mpl* and replaced by viral envelope sequences. The WSXWS motif, the transmembrane domain and the entire cytoplasmic domain of mpl were preserved (Souyri *et al.*, 1990). By Northern analysis, mpl was found to be expressed only in hematopoietic tissue, spleen, bone marrow and fetal liver. No expression was detected in thymus.

Members of the cytokine receptor superfamily may be grouped into three functional categories (for review see Nicola and Metcalf, 1991): single chain receptors, such as erythropoietin receptor (EPO-R), granulocyte colony stimulating factor receptor (G-CSF-R) or interleukin-4 receptor (IL4-R), bind ligand with high affinity via the extracellular domain and also generate an intracellular signal. A second class of receptors, so-called α-subunits, includes interleukin-6 receptor (IL6-R), granulocyte-macrophage colony stimulating factor receptor (GM-CSF-R), interleukin-3 receptor (IL3-Rα) and other members of the cytokine receptor superfamily. These α-subunits bind ligand with low affinity but cannot transduce an intracellular signal. A high affinity receptor capable of signalling is generated by a heterodimer between an α-subunit and a member of a third group of cytokine receptors, termed β-subunits, e.g. β_c, the common β-subunit for the three α-subunits IL3-Rα, IL5-Rα and GM-CSF-R. It is not known whether mpl is capable of transducing a signal, or whether mpl can bind a ligand. The hematopoietic lineages in which mpl is physiologically active have not been determined, but the wide range of leukemias caused by v-mpl suggests that this receptor may affect multiple myeloid lineages and/or act at the level of a myeloid progenitor.

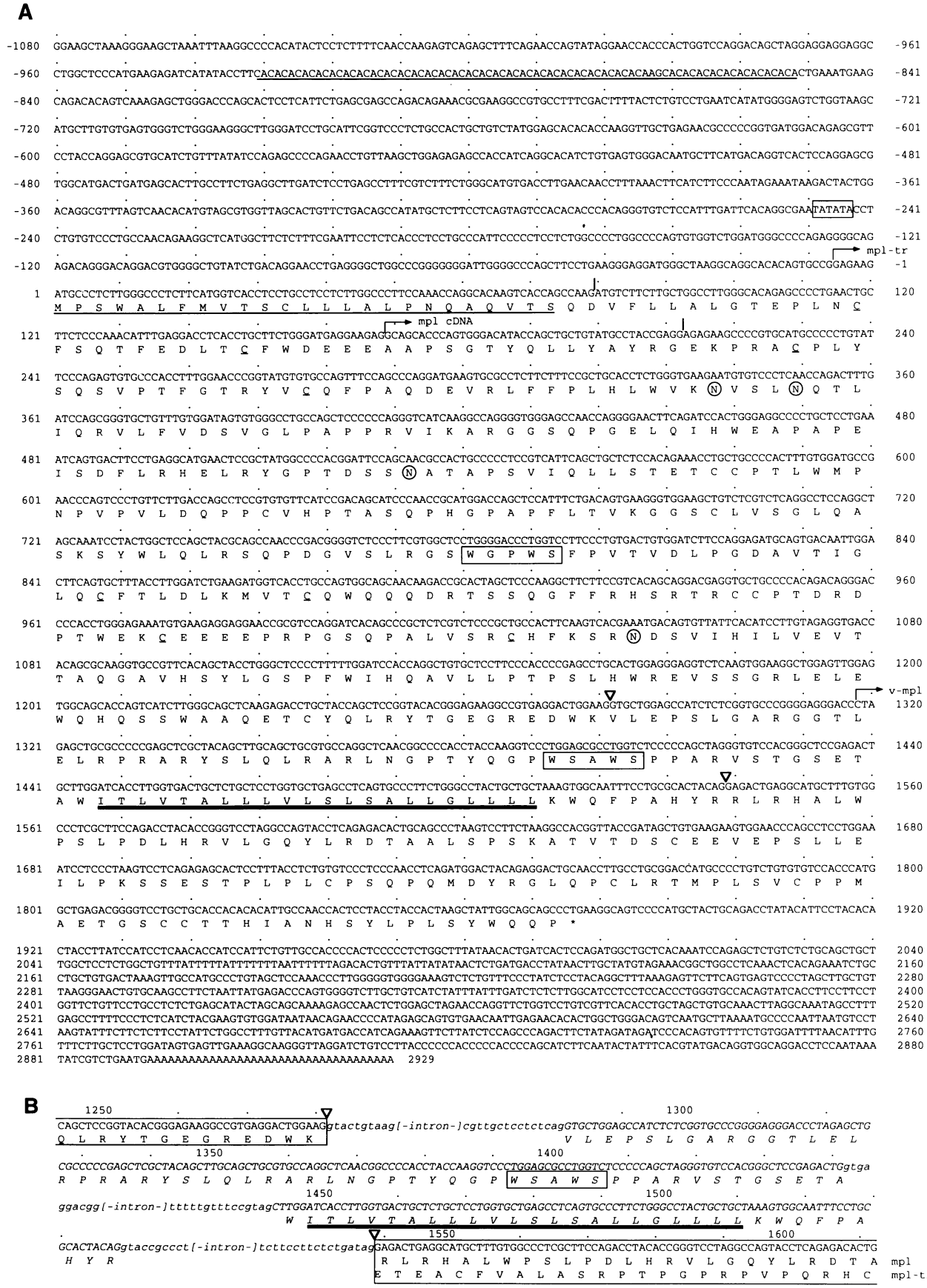
Here we present the full structure of the putative murine mpl transmembrane receptor and a potentially soluble isoform. Further, using a chimeric receptor construct we show that the cytoplasmic portion of mpl contains the elements necessary to transduce a proliferative signal in murine hematopoietic cells. Our results indicate that mpl is either a single chain receptor or a signal transducing β-subunit and suggest that v-mpl may function as a constitutively activated receptor.

Results

Molecular cloning of mouse mpl

We isolated 14 independent clones encoding mpl from an unamplified mouse spleen cDNA library using a 590 bp probe representing the entire v-*mpl* sequence. The clones can be grouped into three classes. The first represents clones coding for mpl, a transmembrane protein. The longest cDNA clone of this class starts 165 bp 3′ of the putative initiator codon ATG (Figure 1A), as defined by the genomic c-*mpl*

clone (see below). The size of the full length composite *mpl* cDNA (2930 bp) is consistent with the reported size of 3 kb for mouse *mpl* mRNA on Northern blots (Souyri *et al.*, 1990). The open reading frame encodes a protein of 625 amino acids with a calculated mol. wt of 69 817, including a putative signal peptide of 25 amino acids. The extracellular



portion of the encoded protein is composed of two subdomains, each with four conserved cysteines and a WGXWS or a WSXWS motif. A hydrophobic transmembrane segment of 22 amino acids is followed by a 120 amino acid cytoplasmic domain. In addition to this clone, five shorter clones with identical sequence were isolated.

Clones of the second class encode a truncated form of mpl, designated mpl-tr. The longest of these cDNA clones contains the putative initiator codon ATG (Figure 1A). *Mpl-tr* is identical in sequence to *mpl* except for a deletion of 257 bp of the cDNA coding for the 54 C-terminal amino acids of the extracellular domain including the conserved WSXWS motif as well as the transmembrane domain and the first eight amino acids of the cytoplasmic domain (Figure 1A). This deletion generates a frame shift, and the reading frame terminates after 30 amino acids (Figure 1B). Mpl-tr cDNA encodes a protein of 457 amino acids including the signal peptide, with a calculated mol. wt of 51 162. A total of four clones were isolated that possess the identical deletion. On Northern blots only a single band was detected. However, this method does not resolve the difference of 257 nucleotides between *mpl* and *mpl-tr* transcripts (see below).

The third class of clones represents unspliced pre-mRNA. Three overlapping clones were sequenced across the 3′ portion of the region deleted in *mpl-tr* (Figure 1B). The sequence at this 3′ boundary agrees with intron/exon consensus sequences (Shapiro and Senapathy, 1987). Thus, the region of 257 bp deleted in *mpl-tr* represents two exons of 161 bp and 96 bp encoding the WSXWS motif and the transmembrane domain, respectively, interrupted by an intron of 160 bp.

The 5′ boundary of the excluded sequence was defined by sequencing a mouse c-*mpl* genomic clone. Using an *Eco*RI−*Stu*I fragment representing the 5′ end of the *mpl* cDNA to screen a mouse genomic DNA library, we isolated a 16 kb genomic fragment that encompasses the excluded region. This genomic sequence is identical to the cDNA sequence up to the 5′ boundary of the deleted region, where it diverges from the cDNA sequence and displays a splice donor consensus (Figure 1B). Thus, the truncated form is generated by excluding two exons.

In order to confirm that the first ATG in *mpl-tr* cDNA is the initiation codon we sequenced the corresponding region of the genomic clone (Figure 1A). Upstream of the putative initiation codon we found stop codons in all three reading frames. The sequence surrounding this ATG agrees with the Kozak consensus (Kozak, 1989).

Chromosomal localization of c-mpl

The presence of a polymorphic CA-dinucleotide repeat just 5′ of the c-*mpl* gene readily permitted us to locate this gene on the mouse genetic map. This was done using two approaches: typing the strain distribution patterns (SDP) of c-*mpl* in recombinant inbred (RI) strains (Taylor, 1989) (Table I) and using an interspecific backcross of (C57BL/6 × *Mus spretus*) × *Mus spretus* (Table II). The CA-dinucleotide repeat is located in the genomic c-*mpl* sequence 931 bp upstream of the putative initiator ATG (Figure 1A). Oligonucleotides flanking this repeat amplify a 205 bp product from C57BL/6J DNA, a 185 bp product from DBA/2J DNA and a 195 bp product from *M.spretus* DNA that can be separated by electrophoresis on 1.4% agarose gels or 8% polyacrylamide gels (not shown). This polymorphism was used to type the strain distribution pattern of c-*mpl* in the C57BL/6J × DBA/2J (BXD) RI strains (Table I). By this approach, c-*mpl* maps to mouse chromosome 4 between the previously mapped markers *Pmv-19* and *D4Mit12*. The CA repeat polymorphism was also used to type the strain distribution pattern of c-*mpl* in an interspecific backcross of (C57BL/6 × *M.spretus*) × *M.spretus* (Table II) and confirmed the location on chromosome 4, between *Pmv-19* and *D4Mit11*. The

Table I. Strain distribution pattern of c-*mpl* polymorphisms among BXD recombinant inbred mice

	BXD																								
	1	2	5	6	8	9	11	12	13	14	15	16	18	19	20	21	22	23	24	25	27	28	29	30	31	32
Pmv-19	D	B	D	B	B	D	B	D	B	D	D	B	D	D	B	D	D	D	B	D	D	D	D	D	B	D
Mtv-13	D	B	D	B	B	D	B	D	B	D	D	D	D	D	B	D	D	D	B	D	D	D	D	D	B	D
Cyp-4a	D	B	D	B	B	D	B	D	B	D	D	D	D	D	B	D	D	D	B	D	D	D	D	D	B	D
Glut-1	D	B	D	B	B	D	B	D	B	D	D	D	D	D	B	D	D	D	B	D	D	D	D	D	B	D
Ms15-1	D	B	D	B	B	D	B	D	B	D	D	D	D	D	B	D	D	D	B	D	D	D	D	D	B	D
c-*mpl*	D	B	D	B	B	D	B	D	B	D	D	D	D	D	B	D	D	D	B	D	D	D	D	D	B	D
D4Mit12	D	B	D	B	D	D	B	D	D	D	D	D	D	D	B	D	B	D	B	D	D	D	D	D	B	D

The SDPs of c-*mpl* and flanking markers on chromosome 4 are shown. Strains carrying the C57BL/6J allele are denoted by a B, and those carrying the DBA/2J allele are denoted by a D. Recombination events are indicated by a line. There are no recombinants between *Mtv-13*, *Cyp-4a*, *Glut-1*, *Ms15-1* and c-*mpl*.

Fig. 1. (**A**) Composite genomic and cDNA sequence for c-*mpl*. The sequence shown is composed of genomic sequence (position −1080 to +216) and of cDNA sequence (position −6 to +2929) for c-*mpl*. The sequence of the overlapping region from −6 to +216 was identical. The position of two introns is indicated by vertical lines. The 5′ end of the longest cDNA clone for mpl-tr and mpl and the beginning of the sequence captured in v-*mpl* are indicated by arrows. The cDNA for mpl-tr contains the putative initiator ATG. The cDNA for mpl is missing 165 bp at the 5′ end. The boundaries of the deletion in the mpl-tr cDNA are marked by triangles. Thin lines demarcate the CA-dinucleotide repeat and the putative signal peptide; the thick line indicates the transmembrane domain. The putative TATA-box and the conserved WSXWS and WGXWS motifs are boxed, and the conserved cysteines are underlined. The four potential N-glycosylation sites are circled. (**B**) Intron/exon structure of the region deleted in *mpl-tr*. The numbers refer to the nucleotide position as in (A). The 5′ and 3′ boundaries of the deleted region are boxed. The region deleted in mpl-tr is italicized. The exon/intron junction at the 5′ boundary was defined by sequencing a genomic clone for c-*mpl*. The 3′ boundary and the two exons coding for the region deleted in mpl-tr are derived from sequencing of unspliced cDNA clones. The two reading frames utilized in mpl and mpl-tr are shown for the 3′ boundary.

Table II. Recombination frequencies of c-*mpl* with flanking markers in an interspecific backcross

Interval	*R*	*N*	Recombination % ± SE	Confidence 95% limits
D4Mit9–c-*mpl*	12	91	13.2 ± 3.55	7.0–21.9
c-*mpl*–*D4Mit11*	3	92	3.26 ± 1.85	0.7–9.2

Percentage recombination between markers, standard error and confidence limits were calculated on RI manager version 2.3 (Manly *et al.*, 1991) from the number of recombinants (*R*) in a sample size (*N*).

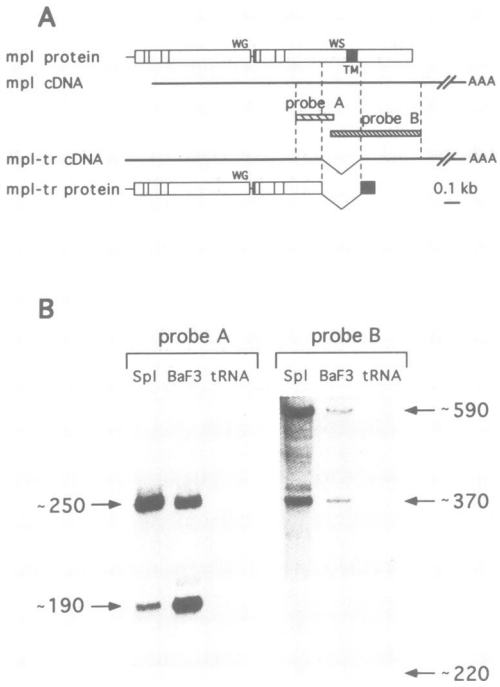


Fig. 2. (**A**) The position and length of the two RNase protection probes A and B, as used in (B), are shown. The two longest cDNA clones for mpl and mpl-tr are drawn to scale and aligned with their deduced protein products. Boxes represent the domains of the mpl protein. The position of each of the four conserved cysteines is indicated by a vertical line, 'WG' and 'WS' show the position of the conserved WGXWS and WSXWS motifs respectively. The deletion in the central portion of the *mpl-tr* cDNA and in the corresponding mpl-tr protein region is indicated by the thin line. This causes a frame shift and the mpl-tr protein terminates prematurely after 30 amino acids as indicated by the stippled box (see also Figure 1B). (**B**) Ribonuclease protection assay with *mpl* antisense RNA probes. Numbers indicate the approximate length of protected fragments in nucleotides. The expected length (220 nt) of a protected fragment for a mouse analogue of the truncated human MPLK is marked but not observed. Sixty and 20 μg of total RNA from spleen (Spl) or BaF3 cells or yeast tRNA were used for each hybridization with probe A and probe B respectively. No *mpl* mRNA was detected in MEL cells or in FDC-P1 cells (not shown).

polymorphic markers *D4Mit12* and *D4Mit11* are in close proximity to each other on mouse chromosome 4 and to date have not been separated in crosses.

Analysis of alternative splicing

To assess whether the cDNA encoding mpl-tr represents a physiologically transcribed mRNA in mouse tissues we used a ribonuclease (RNase) protection assay (Krieg and Melton, 1987) with probes that distinguish between the full length and the truncated forms. Probe A spans the region deleted in the *mpl-tr* cDNA from the 5′ end (Figure 2A) and protects a 250 nucleotide (nt) fragment from mRNA corresponding to mpl and a 190 nt fragment corresponding to mRNA coding for mpl-tr. Probe B is identical to the part of mpl transduced by the myeloproliferative leukemia virus. This probe spans the breakpoint of the deleted region from the 3′ end (Figure 2A). A protected fragment of 590 nt was expected for mpl and a 370 nt fragment for mpl-tr. By densitometry of the protected fragments shown in Figure 2B *mpl-tr* accounts for 25% and 40% of the total spleen *mpl* mRNA with probe A and probe B, respectively.

Expression of mpl protein

To study the expression pattern and distribution of mpl protein, we generated rabbit polyclonal anti-mpl antibodies directed against the N-terminal half of the extracellular domain of mpl. Although BaF3 cells express endogenous mRNA corresponding to both forms of mpl, the levels of mpl protein expression are too low for detection by immunoprecipitation or by Western blot (not shown). Therefore, we chose to overexpress mpl in transfected cell lines. To create a full length cDNA for mpl, the 5′ end of mpl-tr cDNA was added to the cDNA for mpl. This composite cDNA and the cDNA for mpl-tr were subcloned into the expression vector pcDNA1. Transiently transfected COS cells were metabolically labeled with [^{35}S]methionine, lysed and unique proteins of 78 and 55 kDa were immunoprecipitated from the mpl and mpl-tr transfectants, respectively (Figure 3A). In addition, proteins of 81 and 43 kDa were detected in both mpl and mpl-tr transfectants. The identity of these proteins is at present unknown.

Surprisingly, given the presence of a signal sequence and the absence of the transmembrane and cytoplasmic domains, no mpl-tr protein was found in the tissue culture supernatant (Figure 3A). This might be due to a defective leader peptide, attachment of the mpl-tr protein to the cell membrane through a lipid anchor, intracellular retention of mpl-tr possibly due to the loss of the WSXWS motif, or instability of secreted mpl-tr protein. To show that the mpl signal sequence is functional, we expressed a fusion protein consisting of the entire extracellular domain of mpl fused via the C-terminus to human secreted placental alkaline phosphatase (Figure 4) (Flanagan and Leder, 1990) in transfected COS cells. As shown in Figure 3A, a protein of the expected 135 kDa was readily immunoprecipitated from both cell lysates and supernatant (Figure 3A). As expected, alkaline phosphatase activity was also detected in the supernatant. Again, a protein of 81 kDa appeared in the immunoprecipitate.

To determine whether truncated mpl is associated with the outer cell membrane through a lipid anchor, as has been described for ciliary neurotrophic factor receptor, another member of the cytokine receptor family (Davis *et al.*, 1991), we labeled transiently transfected COS cells with ^{125}I using lactoperoxidase (LPO) (Marchalonis, 1969). Only proteins located on the outside of the cell membrane are labeled by this procedure. We were able to label and immunoprecipitate the full length form of mpl from COS cells transfected with the composite mpl cDNA (Figure 3B), indicating that all elements necessary for correct sorting and transport to the cell surface are present. Mpl-tr, however, was not detected on the surface of COS cells transfected with *mpl-tr* cDNA and appears to be an exclusively intracellular protein. Interestingly, the bands of 81 and 43 kDa observed in the

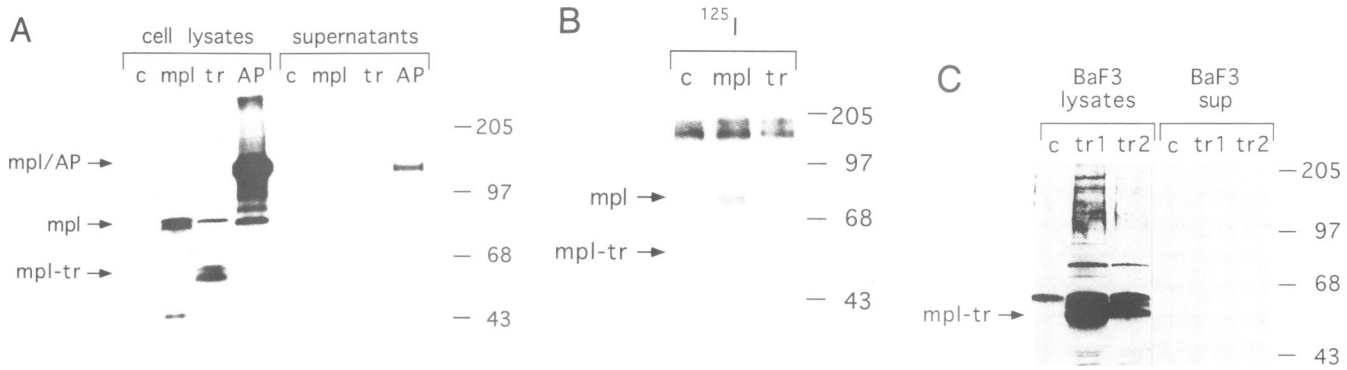


Fig. 3. Immunoprecipitation of mpl and mpl-tr with anti-mpl antibodies. 'c', indicates mock transfected controls; 'mpl', cells transfected with full length *mpl* cDNA; 'tr', cells transfected with *mpl-tr* cDNA; 'AP', cells transfected with mpl/AP fusion construct. Numbers indicate mol. wt in kDa. (**A**) Transiently transfected COS cells were metabolically labeled with [^{35}S]methionine. As indicated, cell lysates or culture supernatants were immunoprecipitated with anti-mpl antibodies. (**B**) ^{125}I/lactoperoxidase cell surface labeling and immunoprecipitation of transiently transfected COS cells. (**C**) Stably transfected BaF3 cell lines were metabolically labeled with [^{35}S]methionine and cell lysates or culture supernatants were immunoprecipitated with anti-mpl antibodies. The identity of the 63 kDa band present in the cell lysates of transfected BaF3 cells as well as in the control is at present unknown. A similar band was also observed in MEL cells, which do not express *mpl* mRNA (not shown).

[^{35}S]methionine immunoprecipitates were not detected with ^{125}I labeling. Thus, these proteins may be intracellular or they may lack tyrosine residues necessary for LPO labeling.

Since COS cells are monkey cells of non-hematopoietic origin, we hypothesized that co-factors necessary for secretion of mpl-tr might be missing. To test the possibility that mpl-tr might be secreted by hematopoietic cells, we subcloned *mpl-tr* cDNA into a suitable vector (Daley *et al.*, 1990) and tested mpl-tr expression in stably transfected BaF3 cells. Two independent BaF3 clones express mpl-tr in the cell lysate, but did not secrete any detectable mpl protein into the superantant (Figure 3C). Thus, there is no apparent difference between COS cells and BaF3 cells with respect to trafficking of mpl-tr.

hIL4-R/mpl chimera

As is the case for other members of the cytokine receptor superfamily, the cytoplasmic domain of mpl lacks any recognizable enzymatic motif that would hint at a signalling capability. Therefore, it was important to test directly whether mpl is a functional receptor that can transduce a proliferative signal. For this purpose, we designed a chimeric receptor consisting of the cytoplasmic domain of mpl fused to the extracellular and transmembrane domains of the human interleukin-4 receptor (hIL4-R) (Figure 4). This chimera, designated hIL4-R/mpl, was tested for its ability to bind human IL4 and signal when stably transfected into BaF3 cells (Palacios and Steinmetz, 1985). BaF3 is a murine interleukin-3 (IL3) dependent cell line that stops proliferating and undergoes rapid apoptosis when IL3 is withdrawn from the media (Rodriguez-Tarduchy *et al.*, 1990). BaF3 cells stably transfected with the wild type human IL4-R grow normally in human IL4 (D.C.Seldin, in preparation). Thus, the human IL4-R activated by human IL4 can substitute for the IL3-R-mediated proliferative signal. The cytoplasmic domain of the IL4-R is required for signal transduction (Mosley *et al.*, 1989; D.C.Seldin, unpublished results). Therefore, replacing the cytoplasmic domain with an exogenous cytoplasmic domain is a suitable test for activity. In addition, the interaction of human IL4 with its receptor is species specific (Lowenthal *et al.*, 1988); therefore the results are not

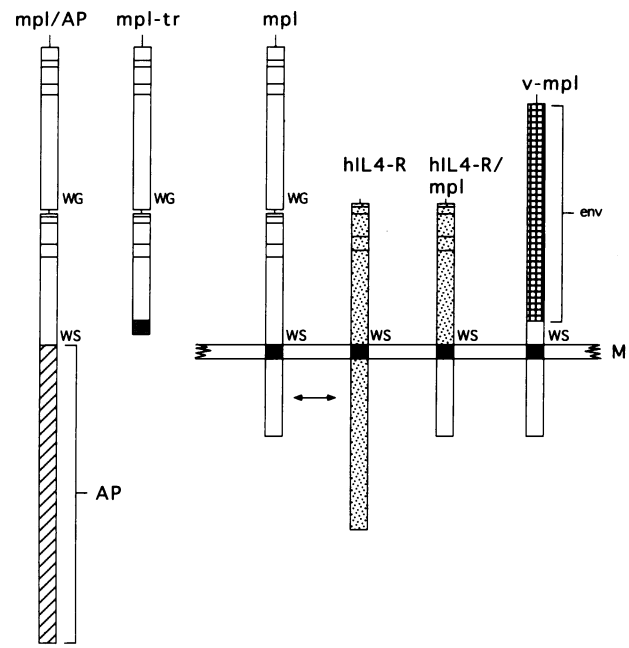


Fig. 4. Schematic representation of mpl and mpl variants used in this study in relation to the oncogenic v-mpl protein. Open boxes represent mpl sequences. Horizontal lines indicate the position of the conserved cysteines. 'WG' and 'WS' indicate the positions of the WGXWS and WSXWS motifs respectively. Mpl/AP is a 135 kDa fusion protein between the extracellular domain of mpl and human placental secreted alkaline phosphatase (hatched). In the hIL4-R/mpl chimera the cytoplasmic domain of the hIL4-R (stippled) was replaced by the corresponding mpl sequence (open box). The transmembrane domain of the chimera is derived from hIL4-R.

complicated by activation of endogenous mouse IL4-R.

The chimeric human IL4-R/mpl was transfected into BaF3 cells and six G418-resistant clones were selected and assayed for expression of the chimeric receptor. Two clones were found to express detectable levels of cell surface binding sites for human IL4, as assayed by binding of a human IL4/AP fusion protein (Figure 5A) (Morrison and Leder, 1992). When IL3 was removed from the culture medium, both cell lines were able to proliferate in the presence of human IL4

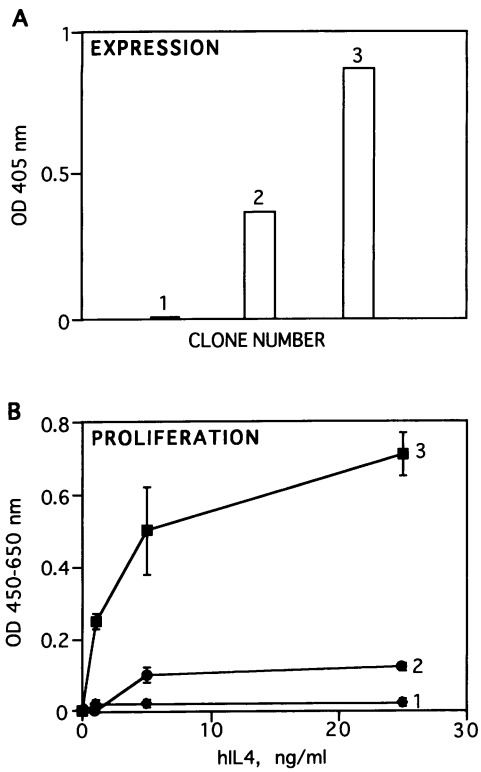


Fig. 5. (**A**) Expression of the hIL4-R/mpl chimeric receptor. Examples of three G418-resistant clones are shown. The relative expression was assessed by enzymatic assay of bound hIL4/AP fusion protein. The product of *p*-nitrophenyl phosphate cleavage was quantitated by measurement of absorbance at 405 nm. Clone 1 was a non-expressor, and clones 2 and 3 expressed intermediate and higher levels of binding sites for human IL4, as shown. (**B**) Signalling via the hIL4-R/mpl chimeric receptor. The individual clones were incubated in the presence of increasing doses of human IL4. Proliferation was measured by reduction of XTT, with quantitation of the orange product by absorbance at 450 nm and background subtraction at 650 nm. Error bars indicate the mean and standard deviation for three wells assayed at each concentration.

in a dose-dependent manner (Figure 5B). The maximal response observed with clone 3 is the same as that seen with transfected wild type human IL4-R (not shown). Thus, these results indicate that the cytoplasmic domain of mpl contains all elements necessary to transduce a proliferative signal.

Discussion

Comparison of human and mouse mpl genes

We have isolated cDNAs and a genomic clone for mouse c-*mpl*. The deduced mouse mpl protein is 86% similar and 82% identical to the longer form of human MPL, termed MPLP. This remarkably high degree of conservation extends throughout the entire sequence including the cytoplasmic portion. The extracellular portion can be divided into two subdomains, each having the characteristic four conserved cysteines and a WSXWS or WGXWS motif. A similar duplication is also found in two other members of the cytokine receptor family, namely, the common β-subunit (human β_c; mouse AIC2A and AIC2B) for IL3, IL5 and GM-CSF and the low affinity LIF receptor (LIF-R). This structural feature *per se* does not offer a clue to whether mpl can bind a ligand. AIC2A and LIF-R bind ligand with low affinity, whereas AIC2B and human β_c do not bind ligand at all.

The putative translational start site was present in only one of our cDNAs (*mpl-tr*). However, we were able to deduce the position of the putative initiator ATG from the sequence of a genomic clone for mouse c-*mpl* and constructed a composite full length cDNA by fusing the two longest cDNA clones. This cDNA was expressed in COS cells and anti-mpl antibodies were used to immunoprecipitate a protein of 78 kDa from these cells, slightly larger than the predicted mol. wt of 69 817. Since there are four potential N-glycosylation sites present in the mpl sequence, these may account for the slower mobility on SDS–PAGE.

Chromosomal localization

We have localized c-*mpl* to mouse chromosome 4. On the consensus map of chromosome 4 (Abbot *et al.*, 1992) c-*mpl* is in proximity to the genes for lymphocyte tyrosine kinase (*lck*), L-*myc* and stem cell leukemia (*Scl*). The CA-dinucleotide repeat we have identified and used in the mapping of c-*mpl* should facilitate the inclusion of c-*mpl* as a typable marker in other genetic crosses. Two phenotypic mutants, *Hairpatches* (*Hpt*) (Schultz *et al.*, 1991) and *Repeated epilation* (*Er*) (Guenet *et al.*, 1979), map to this region of chromosome 4. Both exhibit pleiotropic defects; however, a molecular link with c-*mpl* is not obvious from the phenotypes. This region of mouse chromosome 4 is syntenic to human chromosome 1p34 (Nadeau *et al.*, 1992) to which human *MPL* has previously been mapped by *in situ* hybridization (Le Coniat *et al.*, 1989).

Truncated forms of mpl

We identified transcripts encoding a truncated form of mouse mpl (mpl-tr) both by cDNA cloning and by RNase protection. Comparison of sequences of unspliced pre-mRNA and the genomic clone revealed that mpl-tr mRNA is generated by failing to incorporate two exons, resulting in the deletion of the WSXWS motif and the transmembrane domain (Figure 1). We therefore expected this protein to be secreted. However, mpl-tr protein was not detectable in the culture supernatants of transfected COS cells, although an appropriately sized protein was present in the cell lysate.

We tested some of the possible reasons for this discrepancy. The failure of the mpl-tr protein to be exported was not due to a defective signal peptide since the mpl/AP fusion protein was secreted (Figure 3A) and the full length mpl was appropriately directed to the cell membrane (Figure 3B). Mpl-tr was not found on the cell surface by ^{125}I/lactoperoxidase labeling (Figure 3B) and therefore does not appear to be attached to the outside of the cell. BaF3, a hematopoietic cell that normally expresses both forms of mpl at low levels also failed to secrete detectable levels of the transfected truncated form.

Transcripts lacking the transmembrane domain and encoding a potentially soluble form of receptor have been described for most members of the cytokine receptor superfamily (reviewed in Fernandez-Botran, 1991), including the receptors for IL3-Rα, IL4-R, IL5-Rα, IL6-R, IL7-R, IL9-R, G-CSF-Rα, GM-CSF-Rα, LIF-R, GH-R and EPO-R. Alternative splicing was found to be the mechanism in most cases examined. The soluble forms of the IL4-R (Mosley *et al.*, 1989), hIL5-Rα (Tavernier *et al.*, 1991), hIL7-Rα (Goodwin *et al.*, 1990), GM-CSF-R (Raines *et al.*, 1991) and mouse EPO-R (Kuramochi *et al.*, 1990) have been shown to be secreted into the culture supernatant when

overexpressed in COS cells. These soluble receptors were able to bind ligand and had an inhibitory effect by competing for ligand in *in vitro* assays.

The deletion in mpl-tr removes the transmembrane domain as well as the conserved WSXWS motif. The soluble forms of all other members of the cytokine receptor family retain the conserved WSXWS element, with the exception of the soluble human EPO-R (Todokoro *et al.*, 1991). Mutations in the WSXWS motif of the mouse EPO-R resulted in retention of the mutant protein in the endoplasmic reticulum in BaF3 cells (Yoshimura *et al.*, 1992). In contrast, EPO-R mutated in the WSXWS motif was expressed on the cell surface in FDC-P1 cells, but had decreased affinity for ligand and in some cases had lost signalling ability (Quelle *et al.*, 1992). Similarly, deletion of 108 amino acids from the extracellular domain including the WSXWS motif in the G-CSF-R (Fukunaga *et al.*, 1991) and mutations in the WSXWS motif of the IL-2Rβ (Miyazaki *et al.*, 1991) did not interfere with cell surface expression, but in some cases abolished signal transduction. Although the importance of the WSXWS motif in protein trafficking of cytokine receptors remains to be clarified, it is conceivable that the deletion of the WSXWS motif in mpl-tr is responsible for the apparent lack of secretion that we have observed. We cannot exclude that mpl-tr in an appropriate cell type *in vivo* might be efficiently secreted. The secretion process may require additional signals, and a formal possibility is that mpl-tr is secreted, but very unstable and therefore not detectable unless in complex with a soluble cofactor. Alternatively, the truncated form of mpl might have a role within the cell.

Interestingly, a rather different truncated form of MPL has been described in the human (Vigon *et al.*, 1992). This form retains the transmembrane domain, diverges from the full length MPL after nine amino acids of the cytoplasmic domain, and terminates prematurely after 60 amino acids. Three out of a total of eight human *MPL* cDNA clones analyzed were of the truncated type (Vigon *et al.*, 1992), suggesting that this transcript is abundant in humans. We did not find an analogous form in the mouse. RNase protection probe B would have protected a fragment of ~220 nucleotides for an analogous mouse mRNA, but no signal was detected for this form in RNA from spleen or BaF3 cells (Figure 2B). Conversely no analogue corresponding to murine *mpl-tr* has been described in humans (Vigon *et al.*, 1992). Interspecific differences in the expression pattern of alternative forms have been found for other members of the cytokine receptor superfamily, e.g. G-CSF-R (Fukunaga *et al.*, 1990a,b), GM-CSF-Rα (Crossier *et al.*, 1991; Raines *et al.*, 1991) and IL5-Rα (Takaki *et al.*, 1990; Tavernier *et al.*, 1991, 1992). These discrepancies may reflect species differences in hematopoiesis or, alternatively, may simply reflect frequent aberrant splicing events.

Signal transduction by mpl

Since mpl is a member of the cytokine growth factor receptor superfamily, it was important to determine whether it could deliver a growth stimulatory signal. Using a chimeric receptor, we provide evidence that mpl can transduce a proliferative signal. Because of the species specificity of human IL4, the observed effect cannot be due to stimulation of endogenous mouse IL4-R. The maximal response observed with clone 3 is the same as that seen with transfected wild type human IL4-R. The cytoplasmic domain is highly conserved between mouse and human MPL. The conservation (93.3% similarity, 92.5% identity) is even slightly higher than for the extracellular domain (84% similarity, 79.3% identity). Other chimeric constructs will allow us to define the essential elements in the cytoplasmic domain for signalling and to study post-receptor signalling events. At present we cannot predict whether mpl is a single chain receptor capable of binding a ligand or a signalling β-subunit.

The v-*mpl* oncogene is a fusion of the Friend envelope protein to mpl beginning at leucine 448 (Figure 1A). The coding sequences of v-*mpl* and mouse c-*mpl* are identical, but v-*mpl* terminates prematurely omitting the two C-terminal amino acids. The truncated Friend envelope protein gp55 has been shown to associate with the EPO-R on erythroid precursor cells and to constitutively activate the EPO-R (Li *et al.*, 1990). The region required for this activation has been mapped to the transmembrane domain of gp55 (Chung *et al.*, 1989; Showers *et al.*, 1993). Since in v-*mpl* the region encoding the viral transmembrane domain has been replaced by c-*mpl* sequences encoding the mpl transmembrane domain (Figure 4), the transforming mechanisms of gp55 and v-*mpl* cannot be the same. Our data show that the cytoplasmic domain of mpl is competent for signalling and perhaps it is constitutively activated and responsible for cell transformation in the v-*mpl* fusion. The broad range of hematopoietic lineages transformed by v-*mpl* contrasts with the erythroid specificity of gp55. This may reflect the ability of mpl to transmit a proliferative signal in a broad range of hematopoietic lineages or to act at an early myeloid progenitor stage.

Materials and methods

Cloning of mpl

Oligonucleotides starting at position 301 of the published v-*mpl* sequence (Souyri *et al.*, 1990) (5′-CTAGAGCTGCGCCCCCGAGC-3′) and antisense oligonucleotides at position 890 of v-*mpl* (5′-ATAGGTCTGCAGTAG-CATGG-3′) were used to amplify a 590 bp *mpl* fragment from 1st strand spleen cDNA by PCR. The PCR reactions were carried out in a volume of 100 μl containing 1 μg of each oligonucleotide at an annealing temperature of 55°C. This cDNA was subcloned into pBluescript II (Stratagene) and sequenced by the dideoxy termination method (Sanger *et al.*, 1977) with a modified T7 polymerase (USB) using oligonucleotides covering the sequence. A directional cDNA library was constructed from spleen mRNA of FVB/N mice in Lambda-Zap vector (Stratagene). After adapter ligation the cDNA was size selected by agarose gel electrophoresis and cDNA 2 kb or larger was ligated to the λ-arms. The unamplified library was screened with the 590 bp v-*mpl* fragment labeled with [^{32}P]dCTP. Positive clones were plaque purified, excised with helper phage and sequenced.

A genomic library from the PCC4 teratocarcinoma cell line in Lambda FIX II vector (Stratagene) was screened with a [^{32}P]dCTP labeled *Eco*RI−*Stu*I fragment representing the 5′ end of *mpl* clone 8. Positive clones were subcloned into pBluescript II.

Chromosomal localization

An oligonucleotide at position −1047 (5′-CATACTCCTCTTTTCAAC-CA-3′) and an antisense oligonucleotide at position −832 (5′-CTGGTT-GTCTGCTTCATTTC-3′) flanking a CA-dinucleotide repeat 1 kb upstream of the mpl translational start site amplify a 205 bp product from C57BL/6J DNA, a 185 bp product from DBA/2J DNA and a 195 bp product from *M.spretus* DNA. These polymorphisms were used to type the strain distribution pattern (SDP) of c-*mpl* in the BXD RI strains and in an interspecific backcross of (C57BL/6 × *M.spretus*) × *M.spretus* using DNA obtained from the Jackson Laboratory. PCR reactions were carried out in a volume of 40 μl containing 100 ng of each oligonucleotide primer and 100 ng of genomic template DNA, at an annealing temperature of 55°C with 1.5 mM $MgCl_2$. The polymorphic products were resolved by electrophoresis using both a 1.4% Tris-borate agarose gel and 8% PAGE. Analysis of the SDP was carried out using the RI manager version 2.3 computer program (Manly

and Elliott, 1991), with the following addendum: we have typed BXD strains 1, 22, 30, 31 and 32, which were previously undefined for the simple sequence length repeat polymorphic marker D4Mit12 (Dietrich *et al.*, 1992).

RNase protection assay

RNase protection probe A was generated by PCR using an oligonucleotide (5′-AAGGTGCCGTTCACAGCTAC-3′) starting at position 1088 and an antisense oligonucleotide (5′-GCTCGGGGGCGCAGCTCTAG-3′) starting at position 1337 from *mpl 8* cDNA as a template and subcloned into pBluescript. The full length v-*mpl* sequence (590 bp) in pBluescript was used as probe B. RNA from mouse tissues or cell lines was prepared by the guanidium thiocyanate/CsCl method (Chirgwin *et al.*, 1979). RNase protection was performed as described (Krieg and Melton, 1987). Sixty and 20 μg of total RNA were used for each sample with probe A and probe B, respectively. Quantitation of bands observed on autoradiograms was performed on a Molecular Dynamics Computing Densitometer.

Construction of a full length mpl cDNA, mpl/AP and hIL4-R/mpl chimera

To generate a full length cDNA for mpl the 5′ end of clone *mpl 8* was replaced with the 5′ end of the longer clone *mpl 4* coding for mpl-tr, taking advantage of a unique *Stu*I site at position 710. *Mpl 8* in pBluescript II was cut with *Eco*RI and *Stu*I and ligated to an *Eco*RI−*Stu*I fragment from *mpl 4*. Clones with the correct replacement were selected by restriction endonuclease analysis.

A cDNA coding for an mpl/AP fusion protein was constructed in two steps. First a *Hin*dIII−*Xho*I fragment from the APtag vector (Flanagan and Leder, 1990) containing the alkaline phosphatase region was subcloned into a pcDNA1 vector (InVitrogen) cut with *Hin*dIII−*Xho*I. The entire extracellular domain of mpl was amplified by PCR from the full length *mpl* cDNA with a sense primer (5′-GAAATTAACCCTCACTAAG-3′), corresponding to the T_3 recognition sequence in pBluescript II and an antisense primer (5′-TTAGATCTCCAAGCAGTCTCGGAGCCCG-3′), starting at position 1446, that introduces a unique *Bgl*II site. The PCR product was purified on agarose gel, cut with *Hin*dIII and *Bgl*II and ligated into the modified pcDNA1 vector that had been cut with *Hin*dIII and *Bgl*II.

Recombinant PCR was used to generate a chimeric receptor containing the extracellular and transmembrane domains of the human IL4 receptor (hIL4-R) and the cytoplasmic domain of mpl. A 21 nucleotide sense primer (oligo 1: 5′-CTGACCTGGAGCAACCCGTAT-3′) beginning at nucleotide 596 of the hIL4-R, 5′ to a unique *Hin*cII site and a 30 nucleotide antisense primer (oligo 2: 5′-AGGAAATTGCCACTTGGTGATGCTGACATA-3′) which encoded the last five amino acids of the hIL4-R transmembrane domain and the first five amino acids of the mpl cytoplasmic domain were used in 30 cycles of PCR with a plasmid containing the full length hIL4-R cDNA as a template. The complementary 30 nucleotide sense primer (oligo 3: 5′-TATGTCAGCATCACCAAGTGGCAATTTCCTGC-3′) and an antisense primer to the last 15 nucleotides of the v-*mpl* cDNA followed by a stop codon, *Bam*HI and *Eco*RI recognition sequences, and a trinucleotide spacer (oligo 4: 5′-CTTGAATTCGGATCCCTGCTACCAATAGCTTAG-TGGT-3′) were used in a parallel PCR with the *mpl* cDNA as template. 0.5% of each reaction product was then mixed, denatured, annealed and extended with Taq polymerase to form the chimeric receptor. This chimeric molecule was amplified for 30 cycles of PCR using oligos 1 and 4 as primers. The product was gel purified using GeneClean (Bio 101), cut with *Hin*cII and *Eco*RI, and cloned into a PGEM-7zf+ plasmid (Promega) containing the wild type hIL4-R sequence 5′ to the *Hin*cII site. The fidelity of this construct was confirmed by sequencing. From there, the chimeric receptor was subcloned into the expression vector pGD containing TK-neo and a Moloney LTR promoter (Daley *et al.*, 1990) and termed pGD-hIL4-R/mpl.

Generation of anti-mpl antibodies

An oligonucleotide (5′-CGTAGATCTGGCACAGAGCCCCTGAAC-TGC-3′) at position 100 and an antisense oligonucleotide (5′-CACAGA-TCTTCCTGGAAGATCCACAGTCAC-3′) at position 822 amplify a 722 bp fragment from mpl clone 8 as a template. Both primers contain a unique *Bgl*II site. This fragment was gel isolated, cut with *Bgl*II and subcloned preserving the reading frame into the *Bam*HI site of the expression vector pQE-8 (Qiagen) which introduces a C-terminal six histidine tag resulting in the C-terminal amino acid sequence: GSETAWRSHHHHHH. Clones positive for expression of mpl after induction with IPTG were identified by SDS−PAGE. The 29 kDa mpl/6His fusion protein was affinity purified on a Ni^{2+} column and further purified by preparative SDS−PAGE. The protein band was cut out from the gel and 100 μg was mixed with complete Freund's adjuvant and injected subcutaneously into rabbits. The boost was performed after 4 weeks with 50 μg of the same immunogen in incomplete Freund's adjuvant. Serum was collected 10 days after the boost and used at a 1:200 dilution for immunoprecipitations and Western blots.

Cell transfections and immunoprecipitations

COS cell transfections were performed by the DEAE−Dextran method (Seed and Aruffo, 1987). Transfected COS cells were radioactively labeled 48−72 h after transfection. Metabolic labeling was carried out in methionine-free DMEM media supplemented with 10% dialyzed serum and 0.25 mCi/ml of [^{35}S]methionine for 6 h at 37°C. Cell surface labeling with ^{125}I and lactoperoxidase (Marchalonis, 1969) was carried out *in situ* on COS cells growing attached to the Petri dish in PBS with 2 mCi/ml ^{125}I. Labeled cells were washed twice in PBS and lysed for 10 min on ice in 1 × TBS (150 mM NaCl, 50 mM Tris pH 8) with 1% Triton X-100 and protease inhibitors. The lysates were spun for 30 min at 10 000 *g*. Supernatants were adjusted to 0.2% SDS, 0.5% NP40 and 0.5% deoxycholate final concentration and incubated with antibodies at 1:200 dilution for 12 h at 4°C. Protein A−Sepharose beads (Pharmacia) were added, incubated for 2 h and washed four times in RIPA buffer (0.1% SDS, 0.5% NP40 and 0.5% deoxycholate in TBS) and twice with TBS. The immune complexes were eluted with 0.1 M glycine pH 3 for 1 min and neutralized with 1 M Tris bsae and 4 × SDS−PAGE loading buffer.

BaF3 cells were electroporated with 40 μg of pGD-mpl-tr DNA or pGD-hIL4-R/mpl DNA at 250 V/960 μF in PBS and plated in serial dilutions. Clones were selected in 0.6 mg/ml G418 beginning at 24 h. Metabolic labeling of BaF3 clones transfected with pGD-mpl-tr and immunoprecipitations were performed as described above.

Expression and analysis of the chimeric hIL4-R/mpl receptor in BaF3 cells

Stable BaF3 clones transfected with pGD-hIL4-R/mpl were selected and assayed for expression of the chimeric receptor by binding of a human IL4/human placental alkaline phosphatase fusion protein (Morrison and Leder, 1992). The binding was quantitated by measuring alkaline phosphatase activity (Flanagan and Leder, 1990). The function of the hIL4-R/mpl receptors was determined by washing the cells out of IL3-containing medium and plating them in 96 well plates at 10^4 cells per well in 100 μl of medium containing increasing concentrations of conditioned medium from transiently transfected COS cells secreting human IL4. The human IL4 concentration was determined by comparison with a sample from the National Institute for Biological Standards and Control. After 3 days of stimulation, 50 μl of a 1 mg/ml stock solution of XTT, a colorimetric dye {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide} with 5 mM PMS (phenazine methosulfonate), an electron coupling agent, was added to each well (Scudiero *et al.*, 1988). The product of XTT reduction by viable cells, reflecting the number of cells per well, was measured at 4 h on an automated plate reader (Molecular Devices).

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The cDNA sequence and the genomic sequence data reported here have been deposited in the EMBL and GenBank Nucleotide Sequence databases under the accession numbers Z22649 and Z22657 respectively.