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Role of a Serine/Threonine Kinase, Mst1, in Megakaryocyte Differentiation

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Abstract Platelets, which play a central role in thrombosis and hemostasis, develop from megakaryocytes. Signal transduction originated from the megakaryocyte growth and development factor, the Mpl ligand, which leads to megakaryocyte differentiation, polyploidization, and maturation, has been gradually characterized. In this study, we report the inducibility of Mst1, a recently described serine/threonine kinase, by Mpl ligand and the effect of its induced expression on megakaryocyte differentiation. The steady-state level of mst1 message and Mst1-associated kinase activity increased in response to Mpl ligand. Ectopic expression of human mst1 in a mouse megakaryocytic cell line resulted in a drastic increase in DNA content per cell. Elevated expression of megakaryocyte differentiation markers, such as acetylcholine esterase, PF4, and GPIIb was also observed in hmst1-expressing cells. Activation of p38 MAPK, a known downstream effector of Mst1, was shown to be required for polyploidization, but not for enhanced expression of differentiation markers. Our study thus designates Mst1 as a Mpl ligand-responsive signaling molecule that promotes induction of lineage-specific cellular programming. J. Cell. Biochem. 76:44–60, 1999.

Key words: megakaryocyte; Mst1 kinase; Mpl ligand; signal transduction

Megakaryocytes, the platelet precursors, undergo endomitotic cell cycles as part of the maturation process [Jackson, 1990]. The endomitotic cell cycle commences at a still uncharacterized point, before which megakaryocytes are believed to proliferate through repeated rounds of normal mitotic cell cycles. Previous studies in this laboratory showed that the megakaryocytic endomitotic cell cycle comprises a prolonged S phase and a short gap phase [Wang et al., 1995]. Recent studies by Nagata et al. [1997] indicated that megakaryocytes do enter mitosis. Polyploidizing megakaryocytes displayed nuclear arrangements characteristic of prophase and metaphase with morphologically intact spindle. However, cells caught at anaphase B, telophase, or cytokinesis were never found [Nagata et al, 1997]. Because of the omission of cytokinesis, the DNA content gradually accumulates and cells become polyploid. In addition to polyploidization, a set of genes such as, platelet factor 4 (PF4), acetylcholine esterase, and glycoprotein IIb (GPIIb) [Lepore et al., 1984; Vinci et al., 1984] are turned on in maturing megakaryocytes.

Megakaryocyte differentiation is promoted by a cytokine, thrombopoietin (TPO), the endogenous Mpl ligand [Kaushansky, 1995]. The effect of Mpl ligand on megakaryocytes is twofold, having the ability to stimulate proliferation of megakaryoblasts and to promote polyploidization and maturation. The mechanisms that lead to megakaryocyte proliferation and maturation are still not entirely clear. It is known, however, that ligand binding leads to tyrosine phosphorylation of Shc [Nagata and Todokoro, 1995], an Src-homolgy 2 (SH2) domain-containing adaptor protein known to participate in signal transduction originated from various ligand-challenged receptor tyrosine kinases. Jak2, a member of the Janus family nonreceptor tyrosine kinase, is also activated upon stimulation by Mpl ligand [Sattler et al., 1995]. Tyrosine phosphorylation of signal transducer and activator of transcription (STATs) by Jak family members aids their nuclear localization, lead-

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ing to transactivation of target genes [reviewed in Darnell et al., 1994]. p44, p42 MAPKs were also reported to be activated by Mpl ligand in cells engineered to express copious amounts of Mpl receptor. These include human UT7 megakaryocytes [Rouyez et al., 1997; Yamada et al., 1995], murine TPO-dependent FDCP cells [Nagata and Todokoro, 1995], and prolymphoid Ba/F3 cells [Rouyez et al., 1997]. Treatment of UT7-mpl cells with an MEK1/2-specific inhibitor, PD 98059 [Dudley et al., 1995], was reported to lift the growth inhibition imposed by Mpl ligand. By contrast, expression of megakaryocyte differentiation markers was diminished, suggesting a positive regulatory role of p44, p42 MAPKs in megakaryocyte differentiation.

Mammalian ste-20 like (Mst1) is a recently characterized serine/threonine kinase that shares significant sequence homology with yeast Ste20 within the kinase catalytic domain [Creasy and Chernoff, 1995a]. Mst1 and its close relative, Mst2, have been assigned to a growing kinase family that also includes p21-activated protein kinase (Pak) [Manser et al., 1994], germinal center (GC) kinase [Katz et al., 1994], and hematopoietic progenitor kinase (HPK1) [Hu et al., 1996]. The activity of Mst1 is negatively regulated by protein phosphorylation, at least in the case of epidermal growth factor (EGF) stimulation [Creasy and Chernoff, 1995a], while the biological substrate for Mst1 was not yet identified. The biological function(s) of Mst1 was first hinted in a study that indicated its activation during stress [Taylor et al., 1996]. As a result, it was alternatively named kinase responsive to stress 2 (Krs2). Mst1 was recently shown to play a role in Fas-induced apoptosis in B lymphocytes [Graves et al., 1998]. Specifically, Mst1 is activated via caspase-mediated removal of its C-terminal negative regulatory domain during Fas-induced apoptosis. Overexpression of Mst1 in B lymphocytes activates p38 MAPK and SAPK/JNK, presumably through activating the respective upstream regulatory kinase [Graves et al., 1998], MKK6 [Han et al., 1996] and MKK7 [Holland et al., 1997].

In this study, we report up-regulation of mst1 by Mpl ligand in cultured bone marrow cells. Induced expression of mst1 in a mouse megakaryocytic cell line, Y10, enhanced the expression of various differentiation markers, and increased polyploidization in response to phorbol 12-myristate 13-acetate (PMA). A known effector downstream of Mst1, p38 MAPK, may be involved in some of these processes.

MATERIALS AND METHODS

Cell Culture

Rat and mouse bone marrow cultures were prepared as previously described [Wang et al., 1995]. Y10 mouse megakaryocytes [Zhang et al., 1998] were maintained in F12 nutrient mixture plus 10% fetal bovine serum (FBS). Cells were induced to differentiate by growing in Iscove's modified Dulbecco's medium (IMDM) (Gibco-BRL, Gaithersburg, MD) containing 10% FBS in the presence of either 50 ng/ml of an Mpl ligand, PEG-rHu-MGDF (generously supplied by Amgen, Thousand Oaks, CA), or 50 nM phorbol 12-myristate 13-actate (PMA) for the indicated times. A p38 MAPK inhibitor, SB 203580 [Lee et al., 1994] (Calbiochem, La Jolla, CA), was added at a final concentration of 25 μM, when indicated.

Degenerate Cloning

Rat bone marrow cells were grown in IMDM medium containing 10% FBS in the presence of 50 ng/ml PEG-rHu-MGDF for 3 days. Total RNA was extracted and 4 μg RNA was used in a reverse transcription coupled polymerase chain reaction (RT-PCR). Sequences of the degenerate primers used in PCR correspond to regions in kinase subdomains V and VIII that are conserved among kinases [Hanks et al., 1988]. A total of 10 μM sense primer, TA(C/T)CT(A/G/C)TA(C/T)ATGGA(A/G)TA(C/T)TG (degeneracy of 48) that represents heptapeptide YLYMEYC, and 10 μM anti-sense primer, AX(A/G)TA(A/G)TA(T/G/A)GG(T/G/A)GT(C/T/G)CC (degeneracy of 432) that represents hexapeptide GTPYY (L/V/M) were mixed with 2.5 mM $MgCl_2$, 200 μM of each of the deoxynucleotides, and 5 U *Taq* polymerase (Promega, Madison, WI) in a final volume of 30 μl. The amplification condition was 1 min at 94°C, 2 min at 37°C, and 3 min at 62°C for the first five cycles, followed by 1 min at 94°C, 2 min at 45°C, and 3 min at 62°C for another 30 cycles, plus a final 30-min extension at 62°C; 10 μl of the PCR product was analyzed on a 2.5% agarose gel, and the band of the expected size (approximately 270 base pairs [bp] in length) was excised and subjected to reamplification. The final amplification products were ligated to a TA cloning vector (Invitro-

gen, Carlsbad, CA) and introduced into *Escherichia coli*, according to the manufacturer's instructions. Plasmid DNA isolated from recombinant clones was analyzed by restriction digest. Clones that contained an insert of the expected size were subjected to sequencing analysis using a dsDNA cycle sequencing kit (Gibco-BRL, Gaithersburg, MD). Sequences obtained were compared with known sequences available from GenBank, using the Blast similarity search.

Plasmid Construction

A 400-bp *Sal*I-*Not*I fragment from SPC/SV40 (a gift of Dr. Q. Yu, Pulmonary Center, Boston University School of Medicine), containing the SV40 small t intron and the polyadenylation signal, was ligated to the *Sac*I-*Eco*RI fragment of PF4-myc-hGH [Thompson et al., 1996] via polylinker adaptors containing *Sac*I, *Nco*I, *Kpn*I, *Sal*I, *Not*I, *Nde*I, and *Eco*RI sites. The human growth hormone reporter gene originally included in PF4-myc-hGH was removed by digestion with *Sac*I and *Eco*RI, resulting in plasmid PF4c. The *Bam*H1-*Sma*I fragment of the hmst1 cDNA (a generous gift of Dr. Jonathan Chernoff, Fox Chase Cancer Center, Philadelphia, PA) was then inserted into the *Nco*I site of PF4c by blunt-end ligation. Recombinant plasmid, referred to as PF4-hmst1, with hmst1 inserted in the right orientation, was selected by examination with *Sal*I, *Eco*RV double digestion. All constructs were verified by DNA sequencing.

Transfection

PF4-hmst1, or vehicle plasmid PF4c (100 μg each), were electroporated into Y10 cells along with 20 μg pCDNA3 (Invitrogen, Carlsbad, CA), which encodes neomycin acetyltransferase that confers resistance to neomycin analogue G418, and with 5 μg of cytomegalovirus (CMV)-β-galactosidase, as described elsewhere [Wang et al., 1999]. Cells were selected by 400 μg/ml G418 (Gibco-BRL, Gaithersburg, MD) for four weeks. Resistant cells were used in PMA induction experiments. Individual clones were obtained by limiting dilution.

FACS Analyses and Acetylcholine Esterase Staining

Determination of DNA content per cell by fluorescein-activated cell scanning (FACS) analysis and staining for acetylcholine esterase activity were performed as we previously described [Zhang et al., 1996].

Northern Blot Analysis

Preparation of total RNA and Northern blot analysis were performed as described elsewhere [Sun et al., 1999]. Data were quantitated using the Electrophoresis Documentation Analysis System (Kodak), when indicated.

Preparation of Whole Cell Lysates

Cells were collected by centrifugation at 1,500*g* for 7 min. Pellets were washed once with ice-cold phosphate-buffered saline (PBS) (136 mM NaCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.6 mM KCl, pH 7.4) and lysed on ice with four times pellet-size IP buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% (v/v) Tween 20, 10% glycerol) containing 0.4 M NaCl, protease inhibitors (Complete, Boehringer-Mannheim, Indianapolis, IN), and phosphatase inhibitors (20 mM β-glycerophosphate, 2 mM Na_3VO_4, and 30 mM NaF) for 30 min, followed by centrifugation at 12,000*g* to remove cellular debris. Protein concentration of

Fig. 1. mst1 is induced in primary rat bone marrow cells and in a megakaryocytic cell line stimulated with PEG-rHu-MGDF. **A:** The deduced amino acid sequence of clone 5 is identified as rat mst1. A search in GenBank that human mst1 (hMst1) is the most closely related species. The conserved kinase domains are marked by Roman letters. The position of the antisense degenerate primer used in RT-PCR is underlined. Identical amino acid residues found in both clone 5 and hMst1 are indicated by vertical bars. Analogous amino acids shared by both cDNAs are labeled by +. **B:** For Northern blot analysis, total RNA was extracted from rat bone marrow cells or from a derivative of L8057 megakaryocytic cell line, Y10/PF4c (see under Methods), cultured in the absence (control) or presence of 50 ng/ml PEG-rHu-MGDF (MGDF), as detailed under Methods. 20 μg of total RNA was loaded per lane. Fractionated RNA was transferred to a nylon membrane and probed with ^{32}P-labeled hmst1 cDNA, yielding a single band of approximately 7 kilobase (kb), corresponding to the published mst1 mRNA [Creasy and Chernoff, 1995a]. Equal loading of RNA was confirmed by ethidium bromide staining of the ribosomal RNAs. Similar results were obtained when the blot was probed by clone 5 cDNA (not shown). For Mst1 kinase assay, 200 μg protein from bone marrow cultures or 50 μg protein form platelet-enriched fraction were immunoprecipitated with a hMst1 antiserum and the kinase activities associated with the immunoprecipitates were determined, using mylein basic protein (MBP) as an in vitro substrate, as described under Materials and Methods. The intensity of individual band of interest was measured using the Electrophoresis Documentation and Analysis System (Kodak) and fold of induction was determined. The data shown are of a representative experiment out of three performed.

A

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Clone 5: DIIRLRNKTLTEHEIATILQSTLKGLEYLHFMRKIHRDIKAGNILLNTEGHAKLADFGVA
         |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
hMst1:   DIIRLRNKTLTEDEIATILQSTLKGLEYLHFMRKIHRDIKAGNILLNTEGHAKLADFGVA
                                    VIa         VIb                 VII

Clone 5: GQLTDTMAKRNTVIGTPYY
         |||||||||||||||||++
hMst1:   GQLTDTMAKRNTVIGTPFW
                        VIII
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B

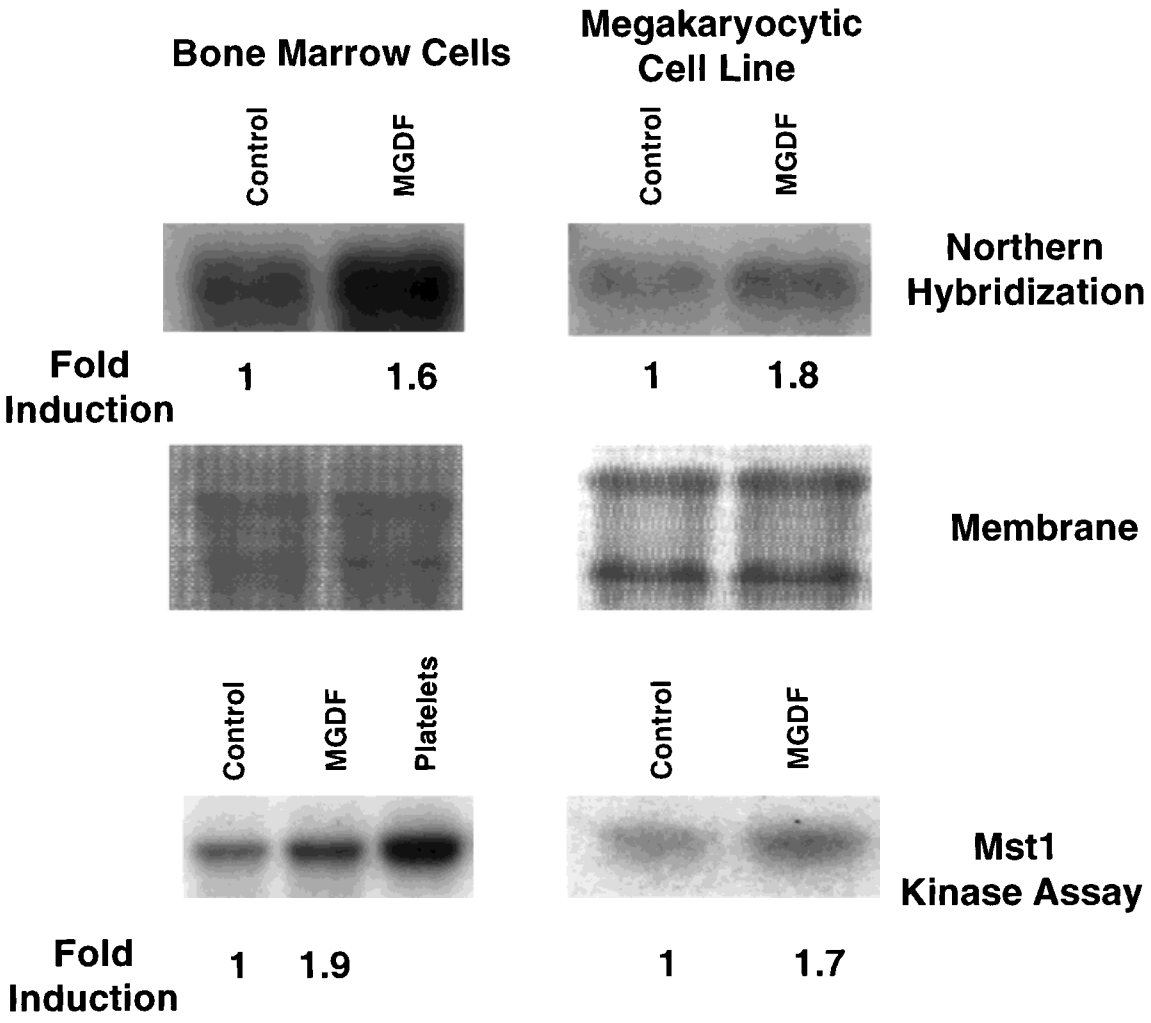


Figure 1.

the lysates was determined by using the Bradford method (Bio-Rad, Hercules, CA).

Western Blot Analysis

A total of 20 µg of protein was loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel and electrophoresed. Separated polypeptides were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), using a semidry blotter (Trans-Blot SD, Bio-Rad, Hercules, CA). The membrane was blocked in TBST (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.05% (v/v) Tween 20) plus 5% (w/v) nonfat dry milk, and was allowed to react with the primary antibody for desired time. After washing in blocking buffer three times, 10 min each, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit serum (diluted 1:2,500 in blocking buffer) (Promega). Immunoreactive polypeptide was detected by using the ECL system (Amersham, Arlington Heights, IL). Anti-p38 MAPK, anti-phospho-p38 MAPK, anti-phospho-MAPK ($p44^{MAPK1}$ and $p42^{MAPK2}$), and anti-phospho-SAPK/JNK were purchased from New England Biolabs (Beverly, MA). Incubation with the anti-MAPK antibody was performed according to the manufacturer's instructions. Rabbit anti-hMst1 antiserum (a generous gift of Dr. Jonathan Chernoff) was diluted 1:2,500 in blocking buffer when used in Western blot analysis.

Immune Complex Kinase Assay

A total of 50 µg protein was incubated with hMst1 antiserum in IP buffer containing 140 mM NaCl (IP/0.14), protease inhibitors, and phosphatase inhibitors for 2 h at 4°C, while rocking. A total of 40 µl protein G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the reaction was allowed to continue for another 2 h. Immunoprecipitates collected on protein G beads were washed three times with ice-cold IP/0.14, twice with kinase buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 10% glycerol), and finally resuspended in 12 µl kinase buffer containing 1mM DTT, protease inhibitors and phosphatase inhibitors. Kinase reaction was carried out in the presence of 10 mM $MgCl_2$, 20 µM ATP, 2–4 µCi γ-^{32}P-ATP, and 4 µg mylein basic protein (MBP) (Gibco-BRL) in a volume of 20 µl. Reactions were started by adding $MgCl_2$ and ATP and were stopped by adding 5µl of 5× SDS-gel sample buffer after incubation at 37°C for 30 min. The samples were analyzed on a 17.5% SDS gel, which was then dried and exposed to an X-ray film. Data were quantitated using the Electrophoresis Documentation Analysis System (Kodak), when indicated.

RESULTS

Elevated Expression of mst1 in Mpl Ligand-Treated Megakaryocytes

A degenerate cloning strategy was employed to identify kinase(s) that might perform important function(s) during megakaryopoiesis. RNA derived from rat bone treated with PEG-rHu-MGDF, a recombinant Mpl ligand, for 3 days was used for RT-PCR, in which degenerate primers corresponding to conserved regions found in kinases were included (see under Methods). As reported previously [Zhang et al., 1998], a 3-day exposure of bone marrow cells to PEG-rHu-MGDF resulted in frequent detection of large mature polyploid megakaryocytes as a result of the maturation-promoting effect of this differentiation activator. A DNA (clone 5) with a sequence that bears 96% identity with a human cDNA, identified as hmst1 [Creasy and Chernoff, 1995a], at the amino acid level and 90% at the nucleotide level was isolated through degenerate cloning (Fig.1A). Clone 5 also shared significant homology to hmst2 [Creasy and Chernoff, 1995b], a close relative of hmst1, with a 93% identity at the amino acid level. However, homology to hmst2 at the nucleotide level dropped to 77%. On the basis of this information, we believe that clone 5 represents the rat homologue of hmst1, rather than the closely related hmst2. We were also able to isolate other serine/threonine kinases from PEG-rHu-MGDF-treated rat bone marrow culture by this procedure. Interestingly, the clonal frequency of mst1 (26.9%) in PEG-rHu-MGDF-treated rat bone marrow cells was relatively higher than that of nek2 (15.4%) [Schultz et al., 1994], IKKβ [Manning and Rao, 1997; Woronicz et al., 1997; Zandi et al., 1997] (7.7%), hmst2 (3.8%) and plk (3.8%) [Clay et al., 1993]. The significance of the appearance of some of these kinases in the maturing megakaryocytes will be addressed elsewhere.

Consistent with its presumed relative abundance in response to PEG-rHu-MGDF, a two-fold induction of mst1 mRNA and Mst1 kinase activity by Mpl ligand were observed in primary bone marrow cells. Interestingly, platelets have a high Mst1 kinase activity as well. As

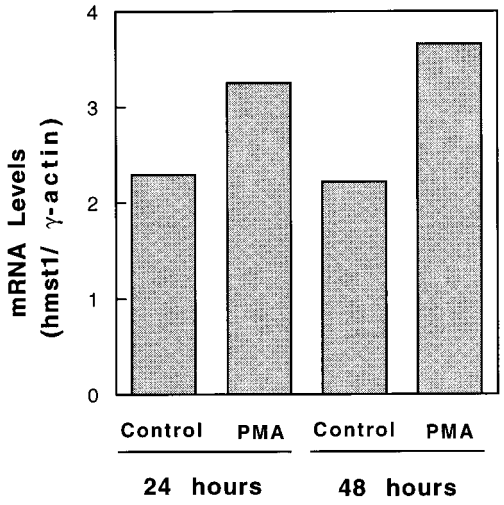
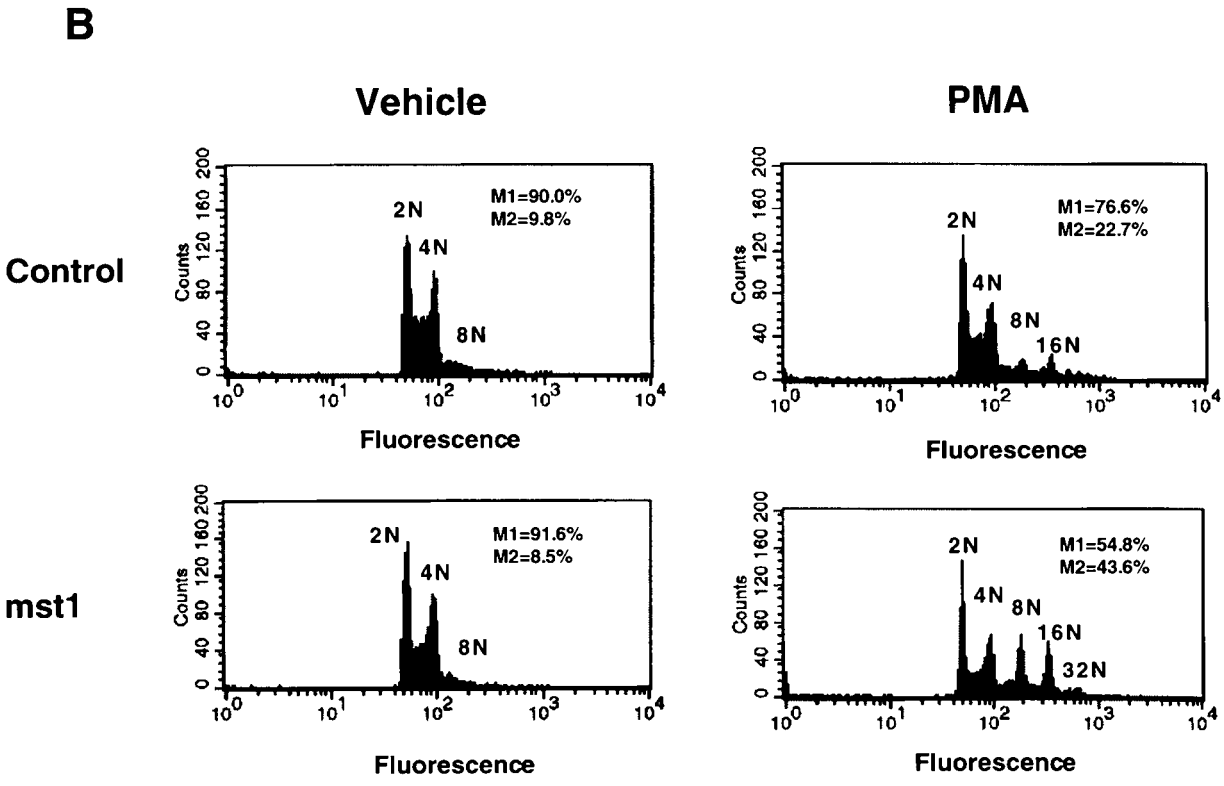


Fig. 2. mst1-transfected Y10 cells exhibit elevated DNA content per cell in the presence of phorbol myristate acetate (PMA). **A:** Total RNA extracted from a PF4-mst1 co-transfected population in the absence (control) or presence of 50 nM PMA was fractionated on a formaldehyde gel. Fractionated RNAs were transferred to a nylon membrane and hybridized with a ^{32}P-labeled hmst1 cDNA or with γ-actin cDNA to confirm equal loading of RNA per lane. The level of ectopically expressed hmst1 mRNA was measured using the Electrophoresis Documentation and Analysis System (Kodak) and was normalized to γ-actin mRNA. **B:** FACS analysis was performed on the mock-treated (treated with the PMA vehicle, dimethylsulfoxide [DMSO], diluted 20,000-fold) control population and on the PF4-mst1 population 48 h after PMA treatment. The percentage of cells that have either a mitotic DNA content (2N plus 4N, M1) or a DNA content greater than 4N (M2) was determined using the CELLQuest application program.



the bone marrow contains a mixture of cells of various lineages, of which megakaryocytes represent a small fraction, we presume that the level of activation of this kinase in megakaryocytes by PEG-rHu-MGDF is greater than the one monitored. Because of the rarity of megakaryocytes in the marrow, we resorted to a megakaryocytic cell line, Y10 [Zhang et al., 1998], to further explore inducibility of Mst1 by Mpl ligand and its relevance to megakaryopoiesis. Y10, a derivative of the megakaryocytic cell line L8057 [Ishida et al., 1993], differentiates in response to Mpl ligand. Mst1 mRNA and kinase activity were similarly induced by Mpl

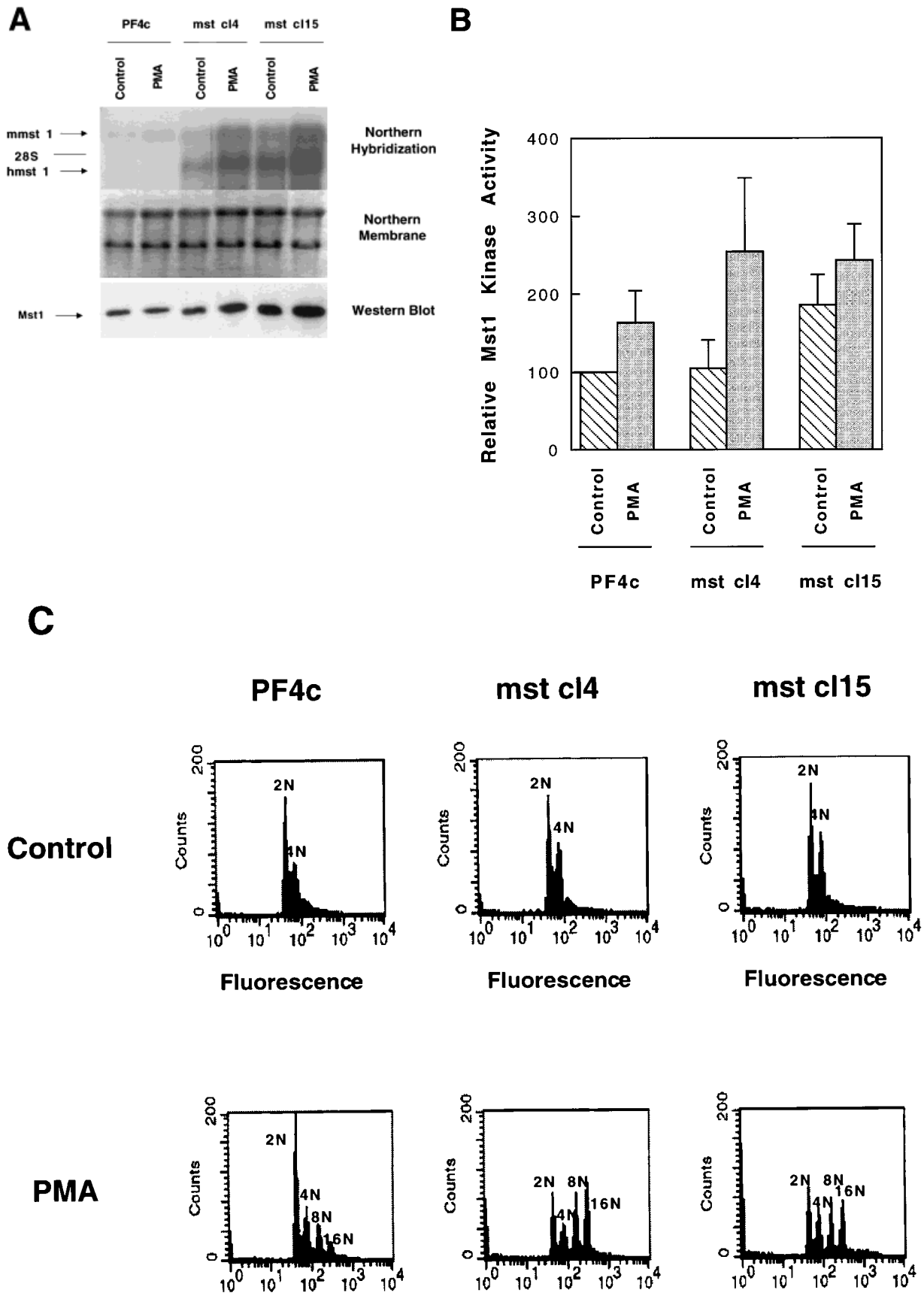

A
PF4c
mst cl4
mst cl15
Control
PMA
Control
PMA
Control
PMA
mmst 1
28S
hmst 1
Northern Hybridization
Northern Membrane
Mst1
Western Blot
B
Relative Mst1 Kinase Activity
400
300
200
100
0
Control
PMA
Control
PMA
Control
PMA
PF4c
mst cl4
mst cl15
C
PF4c
mst cl4
mst cl15
Control
PMA
Counts
Fluorescence
2N
4N
8N
16N

Figure 3.

ligand in Y10 cells (Fig. 1B), suggesting a specific activation in megakaryocytes. On the basis of these observations, we concluded that mst1 expression and activity are increased in response to Mpl ligand. Activation of this kinase in other cell types in the marrow is not ruled out, however.

Induced Expression of mst1-Promoted Polyploidization

Having established the PEG-rHu-MGDF-induced expression of mst1, we proceeded to investigate the potential involvement of mst1 expression during megakaryopoiesis. Repeated attempts to express hmst1 constitutively under the control of the CMV promoter in Y10/L8057 [Zhang et al., 1998] megakaryocytes by introducing a recombinant plasmid that bears a neomycin-resistant marker yielded neomycin-resistant transfectants whose hmst1 expression was undetectable. On the basis of this observation, we speculated a probable negative effect of CMV-driven high-level expression of Mst1 on survival of Y10 cells. As an alternative to examine the effect of mst1 on megakaryopoiesis, expression of hmst1 was placed under the control of the rat platelet factor 4 (PF4) promoter so that the selection of the hmst1-transfectants would more likely be achievable under noninducing conditions. Induced expression of a target gene from the PF4 promoter by PMA has been demonstrated to be mild, within a two- to threefold range [Sun et al., 1999] (Fig. 2A). Co-transfection of Y10 cells with PF4-hmst1 and pCDNA3 or with PF4 control vector (PF4c) and pCDNA3 generated G418-resistant populations with comparable co-transfection efficiency. Both populations were subjected to examination of DNA content in the absence or presence of PMA. Ploidy analyses showed that PMA treatment resulted in an apparent increase in 8N and 16 N cells in the control population. When the expression of hmst1 was induced, PMA treatment induced a significantly larger increase (twofold compared with the control population) in the number of cells that bears a DNA content greater than 4N (Fig. 2B). The fact that ectopic expression of hmst1 is induced in the nonselected PMA-treated hmst1-transfected population (Fig. 2A) is consistent with the notion that induced expression of hmst1 caused the observed increase in ploidy, and leads us to believe that the observation is not a result of a clonal artifact.

To characterize further the effect of mst1 on megakaryocyte polyploidization, we isolated individual clones of hmst1 stable transfectants. Two stable transfectants (clones 4 and 15) displayed high levels of hmst1 transcript, particularly in the presence of PMA (Fig. 3A). Similarly, elevated levels of Mst1 protein were detected in the hmst1-expressing cells, although the increase was less striking compared with what was observed at the message level. Expression of mst1 in clone 15 was high, possibly as a result of integration of a high copy number of the PF4-hmst1. Further induction of hmst1 transcription by PMA in this clone was, nevertheless, evident. It is interesting to note that the hmst1-expressing clones also had higher levels of endogenous mmst1, as compared with control, perhaps due to an autoregulatory mechanism. As the original Y10 cells responded to both PMA and Mpl ligand by increasing the level of ploidy [Zhang et al., 1998], the hmst1 clones retained the ability to respond only to PMA. Responsiveness to Mpl

Fig. 3. mst1-expressing clones develop higher ploidy in response to phorbol myristate acetate (PMA). **A:** Control cells, PF4-mst1 cl4, or PF4-mst1 cl15 cells were seeded at a density of 2×10^5 cells/ml. Cells were cultured in the absence (control, treated with dimethylsulfoxide [DMSO] diluted 20,000-fold) or presence of 50 nM PMA for 2 days before RNA extraction. For Northern blot, 20 μg total RNA was loaded onto each lane. The membrane was hybridized with the ^{32}P-labeled hmst1 probe, yielding a 7-kb band (mmst1) corresponding to published mst1 mRNA [Creasy and Chernoff, 1995a] and a 3.5-kb band corresponding to the one expressed from the PF4-hmst1 plasmid, with a shorter 3′ tail. Equal loading of RNA was confirmed by ethidium bromide staining of the ribosomal RNAs. For Western blot analysis, lysates were prepared, and 20 μg of protein was loaded and electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Separated polypeptides were transferred onto a nitrocellulose membrane and probed with anti-Mst1, as described under Methods. Equal loading of protein per lane was confirmed by staining the blot with Ponceau S (not shown). Data are representative of two experiments. **B:** For kinase assay, 50 μg protein was immunoprecipitated by anti-hMst1 and kinase activity associated with the immunoprecipitates was measured using MBP as an in vitro substrate. MPB phosphorylation was quantitated using the Electrophoresis Documentation and Analysis System (Kodak). The results shown are averages ±SD for three experiments. The data were subjected to a one-tailed Student's *t*-test, which showed that when PF4-mst cl15 cells are compared with control cells, $P < 0.05$ in the presence of PMA and $P = 0.05$ in nontreated cells. **C:** Cells were collected after a 48-h exposure to the indicated stimulator and DNA content per cell was determined by flow cytometry, as described under Methods. The data shown are representative of three experiments.

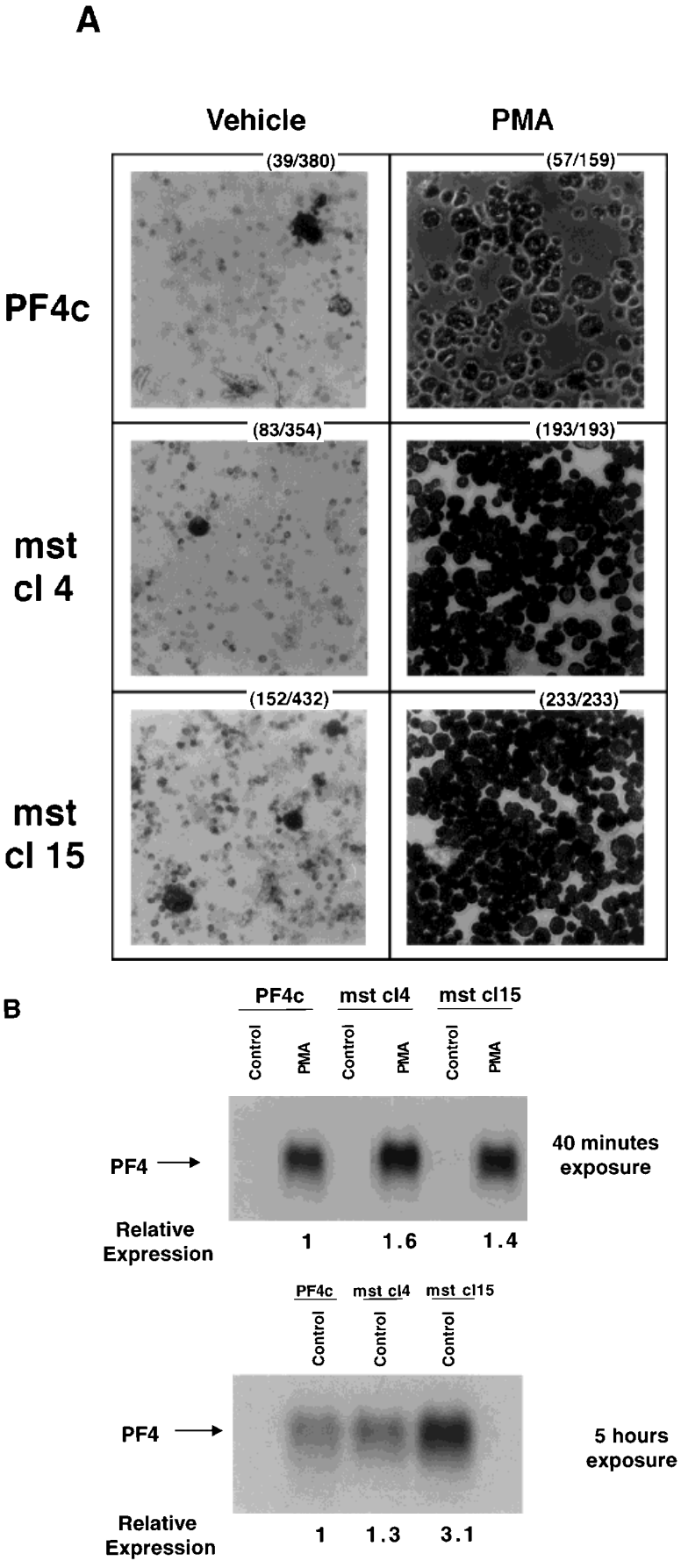


Fig. 4. Mst1 potentiates the expression of various megakaryocyte differentiation markers. **A:** Control PF4c cells and PF4-mst1 clones were grown in the absence (3 days) or in the presence of 50 nM phorbol myristate acetate (PMA) (2 days). 2×10^5 cells were cytospun onto a slide. Duplicate slides were stained for acetylcholine esterase activity. ×200. Fractions in the parentheses represent the number of positively stained cells over total cell number per field. **B:** Northern blotting. Cells were treated without (control) or with PMA for 48 h, and total RNA was extracted. 20 μg RNA was fractionated on a 1% formaldehyde gel, followed by Northern blot transfer onto a nylon membrane. The membrane was hybridized with a mouse PF4 cDNA probe (B) or with a mouse GPIIb cDNA probe **(C)** and exposed to an X-ray film for indicated times, yielding a 900-bp band of PF4 mRNA and a 2.2-kb band of GPIIb mRNA. The relative abundance of PF4 mRNA and GPIIb mRNA was determined by using the Electrophoresis Documentation and Analysis System (Kodak).

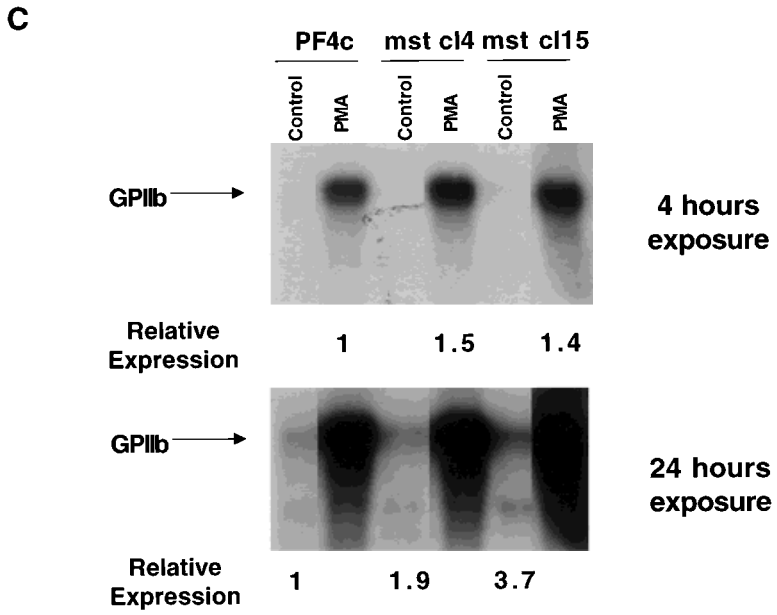


Figure 4. (Continued.)

was lost in the hmst1 cells during the selection process, as was the control PF4c cells. Similar observation was obtained in the original Y10 cells as well after prolonged culturing. Since repeated cloning is likely to produce clones with reduced sensitivity to Mpl ligand as well, we focused our studies with the above cloned cells on PMA-induced polyploidization.

The Mst1-associated kinase activity was higher in PMA-treated hmst1-expressing cells, as compared with PMA-treated control cells (Fig. 3B). Ploidy analysis of the hmst1 transfectants demonstrated a minute shift toward the high ploidy classes in the absence of PMA. By contrast, an increase in DNA content per cell was robust in the presence of PMA, as compared with that of the control (Fig. 3C). We conclude that an increase in Mst1 kinase can potentiate megakaryocyte polyploidization, provided that other PMA-induced signaling pathways are activated.

Induced Expression of hmst1 Enhanced Acetylcholine Esterase Activity and Increased the Level of PF4 and GPIIb mRNA

Because polyploidization is only one of several markers of megakaryocyte differentiation, we were interested in determining the impact of high-level mst1 on the expression of other lineage-specific markers. Similar to what was observed for ploidy, a substantial increase in acetylcholine esterase activity was observed in hmst1-expressing cells when induced by PMA (Fig. 4A). When treated with PMA, there was a four- to fivefold increase in the number of positively stained cells in both mst1 clones, as compared with control. The intensity of the staining was substantially higher in mst1 clones as well, indicating a higher level expression of the acetylcholine esterase in individual cells. The levels of PF4 and GPIIb mRNAs were also examined. PMA significantly increased the levels of PF4 and GPIIb mRNA in all cell types. A further elevation of the steady-state levels of PF4 and GPIIb messages was detected in hmst1-expressing cells in the presence of PMA (Fig. 4B,C). Moreover, a three- to fourfold increase in the basal levels of PF4 and GPIIb mRNA was detected in cells that express high levels of hmst1 (clone 15) under noninduced conditions, as compared with control cells (Fig. 4B,C). We conclude that high-level expression of mst1 could potentiate the expression of megakaryocyte differentiation markers, such as acetylcholine esterase, PF4, and GPIIb.

Activation of p38 MAPK Is Involved in Megakaryocyte Polyploidization

In order to understand the underlying mechanism(s) by which megakaryocyte differentiation was promoted by induced expression of mst1, we examined signal transduction pathways that may be involved. Graves et al. [1998] recently showed that the p38 MAPK signaling

pathway is activated in B lymphocytic cells that overexpress Mst1. Specifically, overexpression of Mst1 activated a co-expressed p38 MAPK, presumably by activating the upstream signal transducer MKK6. Also, the kinase activity of the overexpressed Mst1 was enhanced due, at least in part, to caspase-mediated removal of a negative regulatory domain of the kinase. Accordingly, possible activation of p38 MAPK in hmst1-expressing cells was investigated.

To characterize the involvement of p38 MAPK in megakaryopoiesis, we took advantage of a p38 MAPK-specific inhibitor, SB 203580 [Lee et al., 1994]. In the Y10/PF4c and mst1-overexpressing clones, an induction of p38 MAPK by PMA was not observed (Fig. 5A). Nor did we detect a significant increase in the level of activated p38 MAPK in mst1-expressing cells, as compared with that of control cells. This may reflect an already high basal level of activated p38 MAPK in Y10 megakaryocytes. Upon treatment with SB 203580, the level of the activated p38 MAPK was reduced, but not completely abolished. Notably, in cells that constitutively express hmst1, activation of p38 MAPK became less sensitive to SB 203580 treatment, implying a role for Mst1 in p38 MAPK activation in PMA-stimulated megakaryocytes. Attenuated activation of p38 MAPK in the presence of SB 203580 can be correlated with a 28% reduction in the number of cells that bears a DNA content greater than 4N (Fig. 5C). These observations suggest a role for the activated p38 MAPK in polyploidization and imply that p38 MAPK mediates, at least in part, some of the effect on ploidy induced by mst1. Interestingly, the p38 MAPK inhibitor caused a significant increase in the accumulation of 4N cells (Fig. 5), perhaps as a result of inhibition of repeated rounds of DNA replication.

Involvement of p38 MAPK in the Expression of Megakaryocyte Differentiation Markers

As activation of p38 MAPK plays a role in polyploidization, we were interested in determining its potential role in the expression of differentiation markers. Incubation of PF4c or hmst1-expressing cells with SB 203580 failed to impair PMA-induced increases of PF4 and GPIIb mRNAs (Fig. 6A), or of acetylcholine esterase activity (Fig. 6B). In both PF4c and clone 15 cells treated with SB 203580, the frequency of small cells observed per field was greater (in accordance with the ploidy data), and so was the intensity of staining for acetylcholine esterase in each small cell. These results indicated that p38 MAPK does not play a central role in promoting expression of megakaryocyte markers and that the effect of mst1 on megakaryocyte differentiation is not via p38 MAPK activation.

DISCUSSION

Degenerate cloning identified mst1 as a Mpl ligand-inducible serine/threonine kinase in rat bone marrow cells. In addition, Mst1-associated kinase activity was induced both in bone marrow cells and in a megakaryocytic cell line by Mpl ligand. Ectopic expression of hmst1 greatly potentiates polyploidization and expression of megakaryocyte differentiation markers in mouse Y10 megakaryocytes. We conclude that Mst1 participates in the Mpl ligand-induced signaling cascades and functions to potentiate polyploidization and differentiation during megakaryopoiesis. Examination of possible role(s) played by Mst2, a closely related kinase to Mst1, would aid our understanding to the functional significance of the Mst family

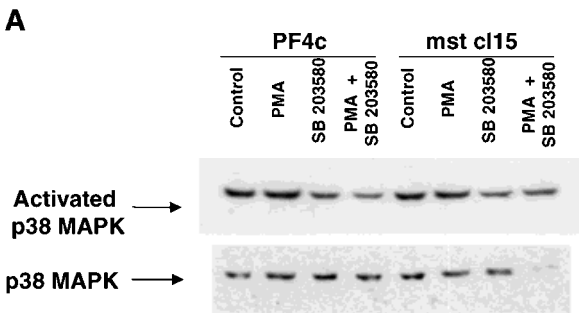


Fig. 5. Involvement of p38 MAPK in polyploidization. **A:** PF4c control Y10 cells or mst1 cl15 cells were cultured in the absence (control) or presence of the indicated stimulators. A total of 25 μM of the p38 MAPK inhibitor, SB 203580, was included at the beginning of the culturing, when indicated. Cells were harvested and detergent extracts were prepared; 20 μg protein was fractionated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. The Western blot was probed with anti-p38 MAPK or with anti-phospho-p38 MAPK. **B:** PF4c control cells or mst cl15 cells were cultured in the absence (control) or in the presence of 50 nM PMA. SB 203580 (25 μM) was added at the beginning of the culturing, when indicated. DNA content per cell was determined after 2 days, via flow cytometry analysis. The percentage of cells that have either a mitotic DNA content (2N plus 4N, M1) or a DNA content of greater than 4N (M2) was determined using the CELLQuest application program.

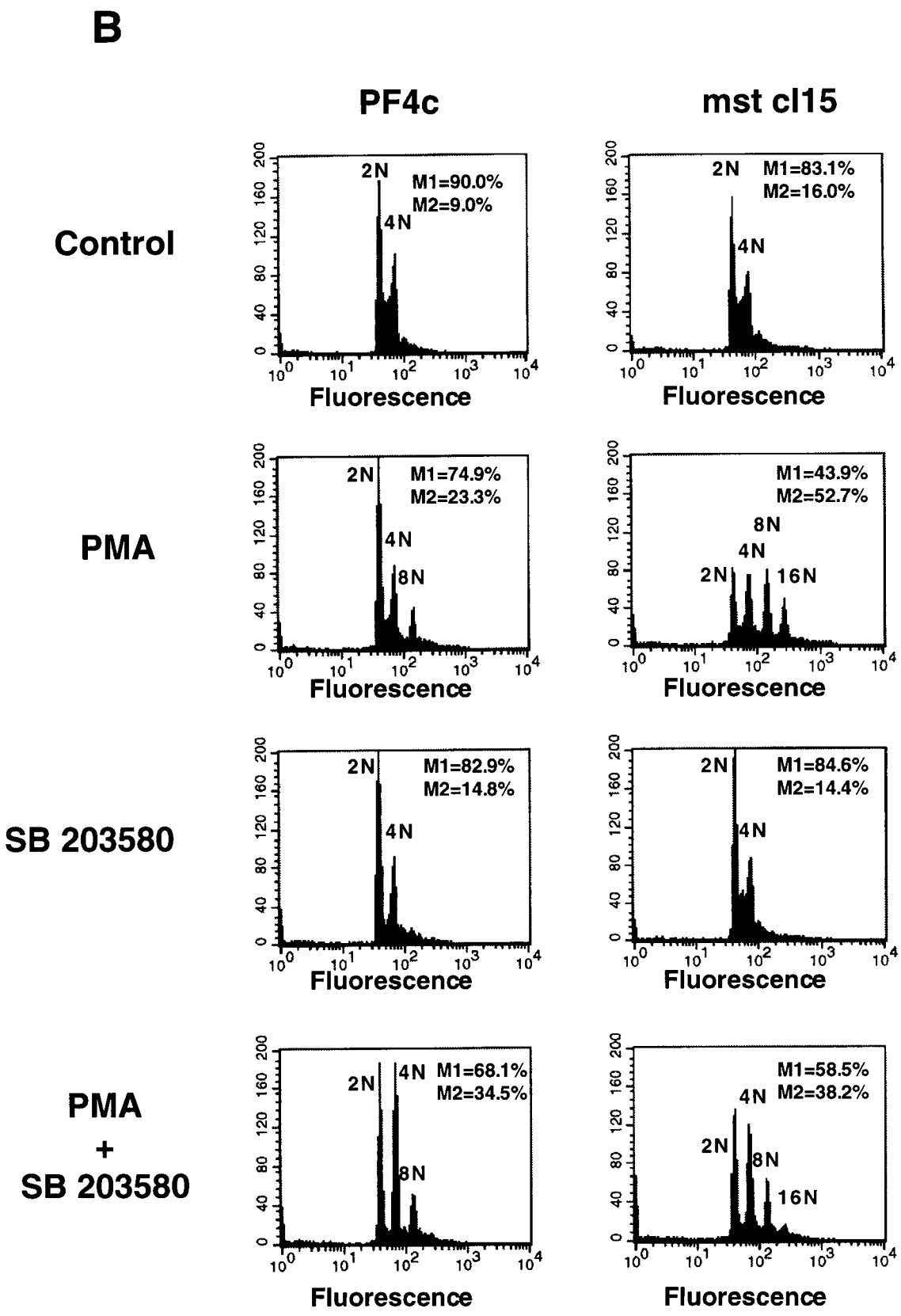


Figure 5. (Continued.)

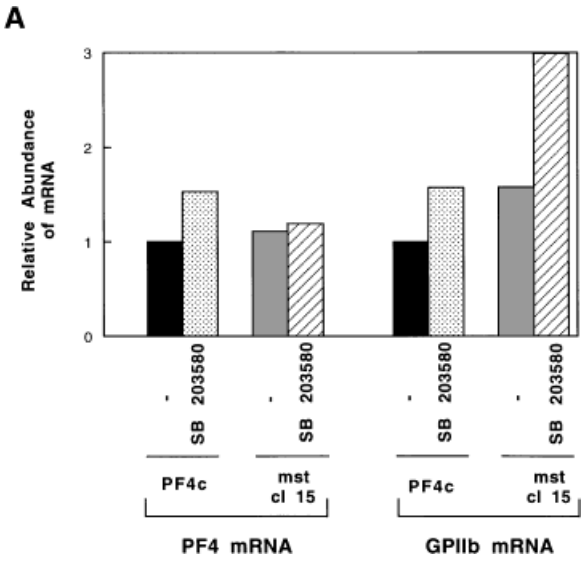
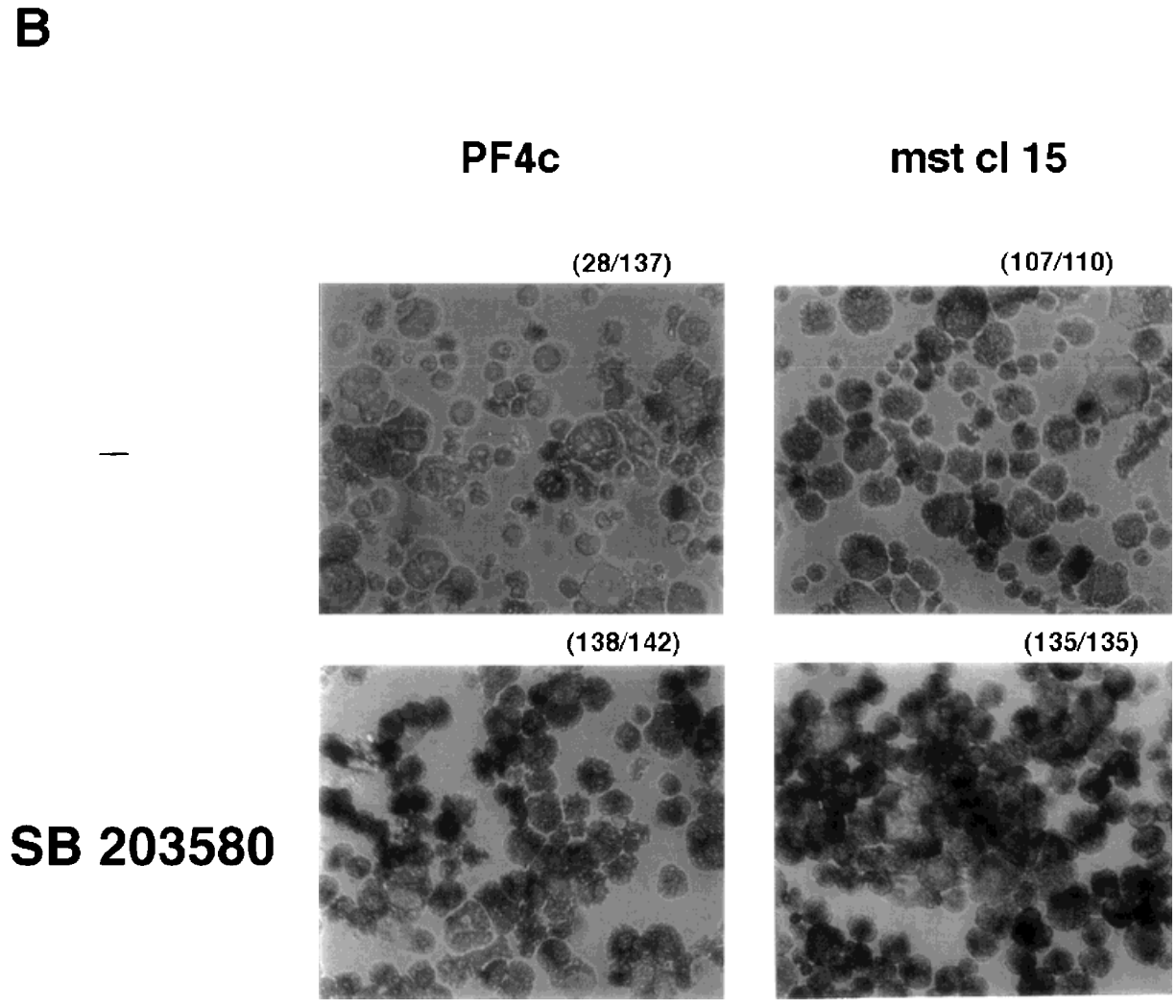


Fig. 6. Involvement of p38 MAPK in the expression of megakaryocyte differentiation markers. **A:** PF4c control cells or mst cl15 cells were stimulated with 50 nM PMA in the absence (−) or presence of the 25 μM p38 MAPK inhibitor SB 203580. Total RNA was extracted, and 20 μg RNA from each sample was loaded onto a formaldehyde gel and electrophoresed for Northern blot analysis. Fractionated RNA was transferred to a nylon membrane. The membrane was blocked, probed either with a labeled PF4 cDNA or with a labeled GPIIb cDNA, and exposed to an X-ray film. The levels of PF4 or GPIIb mRNA were measured using the Electrophoresis Documentation and Analysis System (Kodak) and was normalized to 18S RNA. Because the reference points for PF4 message and GPIIb message were different, comparison should only be made within each group. **B:** PF4c control Y10 cells or mst cl15 cells were PMA-stimulated and cultured in the absence (−) or presence of the indicated p38 MAPK inhibitor. 2×10^5 cells were cytospun onto a slide. Duplicate slides were fixed and stained for acetylcholine esterase activity. ×200. Fractions in the parentheses represent the number of positively stained cells over total cell number per field.



members during megakaryopoiesis, pending on the availability of reagents such as, an antibody to Mst2.

Induced expression of mst1 in a megakaryocytic cell line was associated with an increased kinase activity. A moderate increase in this kinase activity led to a robust elevation of the DNA content per cell when cells were stimulated with PMA (Fig. 3C). It should be emphasized that a noticeable induction of mst1 message was accompanied by a more moderate induction of associated kinase activity. These observations may imply that the steady-state level of Mst1 protein or its kinase activity is tightly controlled. Overexpression of mst1 alone could induce increased expression of megakaryocyte differentiation markers, such as acetylcholine esterase, PF4, and GPIIb (Fig. 4). The fact that induced expression of exogenous mst1 in Y10 megakaryocytes resulted in both elevation of the DNA content per cell and expression of various differentiation markers indicate that increased expression of mst1 constitutes one of the primary signal transduction pathways that lead to a more differentiated state. Our results indicate that mst1 is only one of the signaling components mediating effects of a ploidizing agent on ploidy, and that cellular programming associated with differentiation can be enhanced by mst1 expression. Ectopic expression of a different kinase, known to be involved in transition to M phase of the cell cycle (nimA kinase) in the same Y10 megakaryocytes did not induce the expression of lineage specific markers [Sun et al., 1999]. The specificity of the effect of Mst1 as a kinase, rather than a binding protein, on induction of megakaryocyte differentiation will await investigations involving overexpression of Mst1 mutated in the kinase domain.

Activation of MAPKs ($p44^{MAPK1}$ and $p42^{MAPK2}$) by Mpl ligand has been described in megakaryocytic cell lines [Rouyez et al., 1997; Yamada et al., 1995] or in a promyeloid cell line [Rouyez et al., 1997] that have been engineered to be Mpl ligand-responsive. Overexpression of either MAPK itself or the upstream activating kinase, MEK1, led to an elevated expression of megakaryocyte differentiation markers [Rouyez et al., 1997]. However, it has been shown that, at least in COS cells, activation of $p44^{MAPK1}$ and $p42^{MAPK2}$ remains unaltered upon overexpression of Mst1 [Creasy and Chernoff, 1995a]. Although $p44^{MAPK1}$ and $p42^{MAPK2}$ appeared to be required for megakaryocyte differentiation, our study confirmed that whereas PMA activated $p44^{MAPK1}$ and $p42^{MAPK2}$ in the Y10 cell system, induced expression of mst1 had no effect on their activation (data not shown). This may explain why high levels of mst1 exerted maximal effects on ploidy only in the presence of PMA.

p38 MAPK and SAPK/JNK are known to be activated in hematopoietic cells in response to inflammatory cytokines [Freshney et al., 1994; Lee et al., 1994; Raingeaud et al., 1995; Sluss et al., 1994] and hemopoietic growth factors are known to activate the p38 MAPK pathway [Foltz et al., 1997]. p38 and SAPK/JNK were recently shown to be activated by erythropoietin [Nagata et al., 1998]. Furthermore, these kinases are likely downstream effectors of Mst1, at least in B lymphocytes [Graves et al., 1998]. As a result, we investigated their potential involvement in mediating the Mst1-induced phenotype. In the Y10 system, we did not observe a distinguishable difference in p38 MAPK activation between control PF4c cells and hmst1-expressing cells, although basal level expression of the activated p38 MAPK was high in both cells. This result suggests that p38 MAPK is not the sole mediator of Mst1-induced polyploidization. Likewise, p38 MAPK activation does not seem to play a role in Mst1-induced expression of megakaryocyte differentiation markers (Fig. 6). In cells stimulated to differentiate, inhibition of p38 MAPK did not abrogate expression of differentiation markers. Activation of p38 MAPK and SAPK/JNK in apoptosing B cells involves caspase-mediated cleavage and subsequent activation of Mst1. This does not seem to be the case in hmst1-expressing Y10 cells, as no cleavage product was detected on Western under both noninducing and inducing conditions (not shown). This observation indicates that cleavage-mediated activation of Mst1 does not apply to megakaryocyte differentiation, as would be expected if apoptosis does not take part in early stages of megakaryocyte differentiation.

Although p38 MAPK does not seem to mediate Mst1-induced differentiation, its requirement for megakaryocytes to reach a high ploidy state is, nevertheless, suggested by our results . Incubation of a p38 MAPK inhibitor with PMA-stimulated Y10 cells reduced polyploidization

(Fig. 5B,C). This observation also demonstrated that polyploidization and differentiation could be uncoupled, a conclusion that has been previously deduced by others [Kikuchi et al., 1997]. Further extension of this observation gives rise to the hypothesis that signal transduction pathway leading to polyploidization and differentiation must diverge. Consequently, a regulatory molecule, such as Mst1, whose expression affects both processes, must exert its function early in the signal transduction pathway. In immortalized human B cells, overexpressing Mst1 led to activation of co-expressed p38 MAPK or SAPK/JNK. This was due to activation of their respective upstream activating kinases, MKK6 and MKK7 [Graves et al., 1998]. Accordingly, the position of Mst1 in the signal transduction pathway is, at least, equivalent to Ask1 [Ichijo et al., 1997] or MAPKKKs. Bearing this in mind, dysfunction of a signal transducer further downstream may suppress either polyploidization or differentiation, or both. Whereas high levels of activated p38 were observed in PF4c and clone 15 cells, this was not true for SAPK/JNK. Western blot analysis indicated that activated SAPK/JNK was barely detectable in either control PF4c or hmst1-overexpressing cells under inducing or noninducing conditions. As a result, we could not evaluate the effect of SAPK/JNK in megakaryopoiesis. We have detected, however, a 60 KDa polypeptide that was immunoreactive with an anti-phospho-SAPK/JNK antiserum. The level of this 60 KDa polypeptide appeared to be higher in mst1 cl15 cells than in control PF4c cells. The significance of this 60 KDa polypeptide awaits further analysis.

Our current studies and those reported by others suggest that activation and integration of multiple signal transduction pathways, including that of $p44^{MAPK1}$ and $p42^{MAPK2}$, p38 MAPK, and Mst1, may be required to achieve a fully differentiated state in megakaryocytes. Our data support the hypothesis, as diagrammatically represented in Figure 7, that signal originated from ligand-challenged Mpl receptor leads to activation of p38 MAPK along with an increase in Mst1 kinase activity. Whereas both p38 MAPK activation and increase in Mst1 kinase activity are important for polyploidization, a high level of Mst1 kinase activity is sufficient to enhance differentiation, even in the absence of activated p38 MAPK. The identification of the molecule(s) mediating signaling from the Mpl receptor to Mst1, and the specific targets (e.g., downstream kinase(s) and/or transcription factors) of Mst1 kinase would be of particular interest.

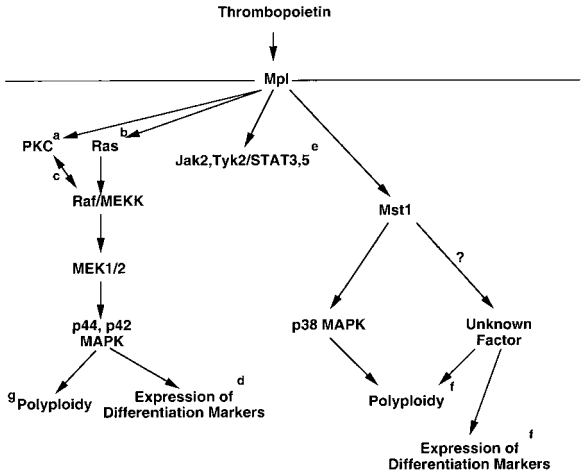


Fig. 7. Proposed role of Mst1 in Mpl ligand-induced megakaryocyte differentiation. **a:** Kunitama et al., Hong et al. **b:** Matsumura et al. **c:** Kolch et al. **d:** Rouyez et al., 1997; Fichelson et al., 1999. **e:** Drachman et al. **f:** This study. **g:** Rojnuckarin et al., 1999.

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