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PREP1, MEIS1 homolog protein, regulates PF4 gene expression

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Abstract

We have previously demonstrated that homeodomain proteins, MEIS1 and PBXs, transactivate the PF4 gene through the novel regulatory element termed TME. This study focuses on Pbx regulating protein 1 (PREP1), a MEIS1 homolog protein, for its transcriptional activity in the PF4 promoter. PREP1 binds to the TME in HEL cells. PREP1 was expressed in human megakaryocytes that differentiated from $CD34^+$ cells. EMSA shows that either PREP1 by itself or PREP1/PBX complexes bind to the two TGACAG motifs in the TME and activate the PF4 promoter. Furthermore, PREP1 and PREP1/PBX complexes synergistically activate the PF4 promoter with GATA-1 and ETS-1. These data demonstrate that PREP1 is also an important transcription factor that regulates PF4 gene expression such as MEIS1. Additionally, these data imply functional similarities and differences between PREP1 and MEIS1 in the regulation of PF4 gene expression.

Keywords: PF4; Homeodomain; PREP1; MEIS1; PBX; Megakaryocyte; TGACAG; GATA-1; ETS-1; Transcription

Megakaryocytes are the hematopoietic precursors of platelets. Platelets play an essential role in thrombosis and hemostasis. Platelet factor 4 (PF4) is expressed exclusively in megakaryocytes and platelets, and serves as a lineage specific marker of megakaryocyte development [1]. To investigate megakaryocytic gene expression, the PF4 promoter has been studied [2–11]. We previously demonstrated that GATA-1 and ETS-1 are important transcription factors for the transactivation of the PF4 gene [12]. Furthermore, in our recent study, we identified the novel regulatory element termed TME in the PF4 promoter and demonstrated that homeodomain proteins, MEIS1 and PBXs (PBX1B and PBX2), bound to the TGACAG motifs in the TME by forming MEIS1/PBX complexes (Y. Okada et al., Blood, in press). It has also been demonstrated that these complexes strongly activate the PF4 promoter synergistically with GATA-1 and ETS-1. Because the GATA-1 and ETS family proteins were essential transcription factors for megakaryocytopoiesis, this synergism indicated the importance of homeodomain proteins for the megakaryocytic gene expression.

Pbx regulating protein 1 (PREP1) has been identified as the transcription factor that binds to the enhancer region in the urokinase plasminogen activator gene [13,14]. PREP1 forms complexes with PBX1B or PBX2 and binds to the TGACAG motif in this enhancer region. Analysis of the amino acid sequence revealed that the PREP1 homeodomain belongs to the TALE class of homeodomains that includes other homeodomain proteins such as MEIS1, PBXs, and TGIF. These reports suggest the structural and functional similarity between PREP1 and MEIS1. On the other hand, previous studies have indicated that MEIS1, not PREP1, complicates leukemia induced by the overexpression of HOXA9 and suggest the functional differences between them [15,16]. The details of the similarities and differences of these proteins are not well understood.

This study demonstrated that PREP1 and PREP1/PBX complexes bound to the TME and both of them activated the PF4 promoter synergistically with GATA-1

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and ETS-1. Furthermore, PREP1 expression was detected in human megakaryocytes. These results indicate the important role of PREP1 in the regulation of PF4 gene expression.

Materials and methods

Plasmid construction. cDNA of PREP1 was obtained from cDNAs prepared from HEL cells by PCR amplification. This PCR product was cloned into the pcDNA3 vector (Invitrogen). The sequence of the inserted PREP1 fragment was confirmed by DNA sequencing. The preparations of two expression plasmids, pcDNA3-hEts-1 and pXM-mGATA-1, and a reporter plasmid, PF4luc, were described previously [12].

Cell culture and isolation of nuclear extract. HEL and K562 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin, and 100 μg/ml streptomycin (P/S). HeLa and HepG2 cells were maintained under the same conditions except that Dulbecco's modified Eagle's medium (DMEM) was used. The isolation method for nuclear extracts was described previously [12].

Transient transfection assay. Transient transfection assays were performed using expression plasmids and PF4luc. 0.5 μg of PF4luc was transfected into HepG2 cells with 0.5 μg of pβactin-lacZ using Lipofectamine2000 reagent (Gibco-BRL). For the overexpression of the transcription factors, 1 μg of each expression plasmid was used. In all assays, cells were harvested approximately 48 h after transfection. Each assay was performed more than three times.

EMSA and supershift assay. The double-stranded DNA fragments for the TME and the TME mutant (TMEmut) probe were constructed from each of the two oligonucleotides, 5′-TCCTGCTGACAGCTGCT GACAGCTGGCCTCAGCTGC-3′ and 5′-CGCAGCTGAGGCCAG CTGTCAGCAGCTGTCAGCAGGA-3′, and 5′-TCCTGC**AGCGAT** CTGC**AGCGAT**CTGGCCTCAGCTGC-3′ and 5′-CGCAGCTGAG GCCAG**ATCGCT**GCAG**ATCGCT**GCAGGA-3′, respectively. These double-stranded oligonucleotides were labeled with Klenow polymerase and used as the probes. EMSA was performed according to the method described previously [12]. The binding reaction was conducted at 4 °C for 45 min. In the supershift assay, 6 μg of the nuclear extract was incubated with antibodies at room temperature for 20 min before the probe was added. Antibodies (PREP1 (N-15), PBX1 (P-20), PBX2 (G-20), Meis1 (N-17), and TFIID (SI-1) as a control IgG) were obtained from Santa Cruz Biotechnology.

In vitro transcription/translation. PREP1, PBX1B, and PBX2 for the EMSA experiments were prepared in vitro using the "TNT coupled transcription–translation reticulocyte lysate (T7 polymerase version)" (Promega) according to the manufacturer's instructions. The obtained proteins were analyzed by Western blotting and 1/25 of the total product of each protein was used for the EMSA.

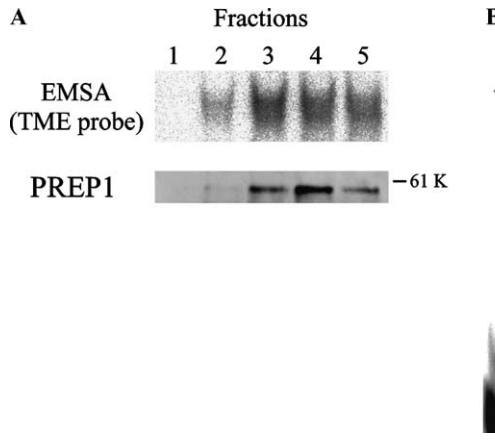

Fig. 1. Binding of PREP1 to the TME in megakaryocytic HEL cells. (A) TME binding proteins were prepared from the nuclear extracts of HEL cells by DNA affinity chromatography. The obtained fractions were analyzed by EMSA (upper panel) and Western blotting (lower panel). (B) Supershift assay was performed using 6 μg of nuclear extract from HEL cells and antibodies (anti-PREP1 Ab and control IgG).

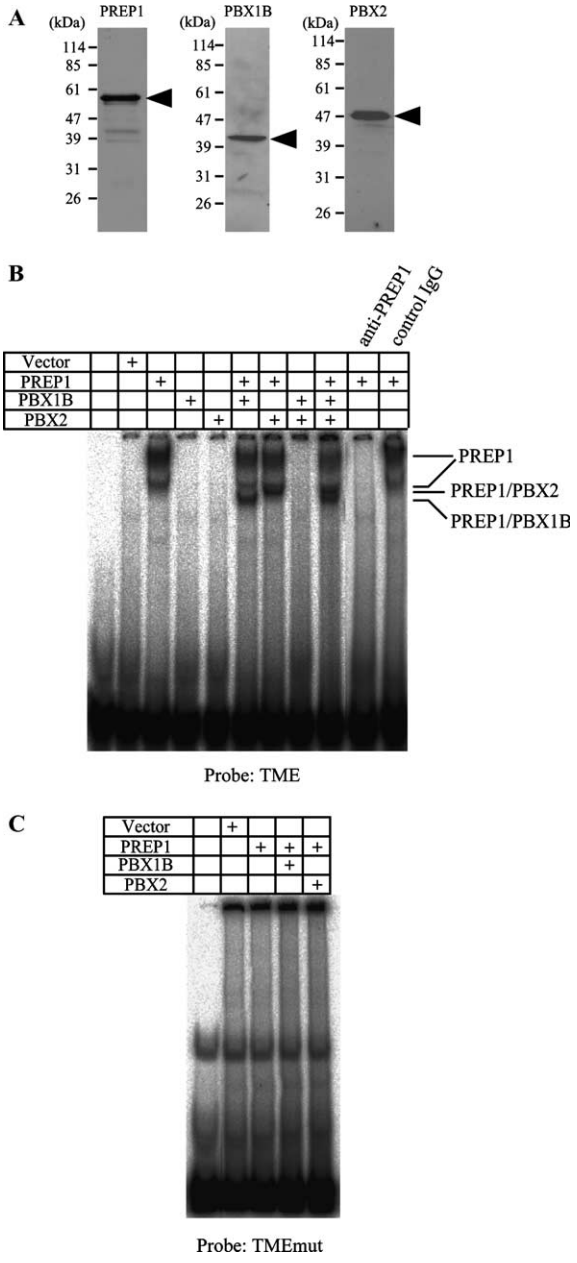

Fig. 2. Binding of PREP1 to the TGACAG motifs in the TME. (A) The productions of in vitro translated PREP1, PBX1B, and PBX2 were confirmed by Western blotting. (B) EMSA was performed using the prepared proteins, PERP1, PBX1B, and PBX2, and the TME probe. Supershift assay was performed with the indicated antibodies. (C) EMSA was performed using the TMEmut probe. This probe is a mutant TME probe in which two TGACAG motifs were disrupted.

Purification of DNA binding proteins. Purification of the TME binding proteins by the DNA affinity column was conducted according to the method described previously (Y. Okada et al., Blood, in press). 6.25 and 20 μl of each collected fraction were used in EMSA and Western blotting, respectively.

Western blotting. Twenty microliter of each purified fraction (Fig. 1A), 5 μl of each in vitro translated protein (Fig. 2A), or 20 μg of each nuclear extract (Fig. 5B) were electrophoresed on 10% SDS–PAGE and the proteins were blotted onto nitrocellulose membranes. They were blocked in TBS containing 3% nonfat milk and 0.1% Tween 20 for 1 h at room temperature and then incubated in the same solution containing the primary antibody (PREP1 (N-15), PBX1 (P-20), or PBX2 (G-20) (Santa Cruz Biotechnology)) at room temperature for 1 h. The membranes were washed and incubated in blocking solution containing the secondary antibody at room temperature for 1 h. The immunoblots were visualized with the ECL Western blotting detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RT-PCR analysis using human megakaryocytes. Umbilical cord blood samples from normal full-term newborn infants were obtained from Tokyo Metropolitan Bokutou Hospital, after obtaining the informed consent of the mothers. The isolation of $CD34^+$ progenitor cells from cord blood and subsequent cell culture for megakaryocytopoiesis was performed as described previously [17]. Total RNA samples were isolated using Trizol reagent (Gibco-BRL). Five micrograms of total RNA was reverse-transcribed by the SUPER SCRIPT First-Strand Synthesis System for RT-PCR (Gibco-BRL), according to the manufacturer's instructions. After the RT reaction, 1/20 volume of the cDNA template and a set of primers (PF4, forward 5′-AG CATGAGCTCCGCAGCCGGGTTCT-3′, reverse 5′-GTAGGCAGC TAGTAGCTAACTCTCC-3′; HPRT (hypoxanthine phosphoribosyltransferase), forward 5′-GGCGTCGTGATT AGTGATGATGAAC C-3′, reverse 5′-CTTGCGACCTTGACCATC TTTGGA-3′; PREP1, forward 5′-CCATGATGGCTACACAGACAT TAAG-3′, reverse 5′-CTACTGCAGGGAGTCACTGTTCGCC-3′) were mixed and used for PCR amplification. The following describes the temperature and time of each step of the PCR (denaturing, annealing, and extension), the number of PCR cycles and the PCR product length obtained: PF4, 95 °C 1 min, 52 °C 1 min, 72 °C 2 min, 30 cycles, 323 bp; HPRT, 95 °C 1 min, 52 °C 1 min, 72 °C 2 min, 30 cycles, 467 bp; Prep1, 94 °C 30 s, 65 °C 30 s, 72 °C 1 min, 35 cycles, 1313 bp.

Results and discussion

PREP1 or PREP1/PBX complexes bind to the two TGACAG motifs in the TME

To investigate the binding of PREP1 to the TME, DNA affinity chromatography was performed using the TME and nuclear extracts from HEL cells. Fractions with the TME binding activity were determined by EMSA. Using these fractions and the anti-PREP1 antibody, Western blotting was performed (Fig. 1A). It was shown that PREP1 was included in the fractions containing the TME binding activity. Furthermore, the supershift assay certified that the shifted band in EMSA was derived from PREP1 (Fig. 1B).

To investigate the mechanism of PREP1 binding to the TME, in vitro translated PREP1 and PBXs (PBX1B and PBX2) were prepared. Productions of the proteins were confirmed by Western blotting (Fig. 2A). Using these proteins and the TME probe, EMSA and supershift assays were performed (Fig. 2B). Two shifted bands were detected when PREP1 was used. Because the bands disappeared in the supershift assay using the anti-PREP1 antibody, it was confirmed that both of these bands were derived from PREP1. On the other hand, when both PREP1 and PBXs were added to the probe, extra shifted bands derived from PREP1/PBX complexes were detected. These results suggest that PREP1 or PREP1/PBX complexes bind to the TME. Furthermore, EMSA was performed using the TMEmut probe in which the TGACAG motifs in the TME were disrupted (Fig. 2C). The result showed that PREP1 or PREP1/PBX complexes did not bind to the TMEmut. These results suggest that PREP1 or PREP1/PBX complexes recognized the TGACAG sequence in the TME.

PREP1 or PREP1/PBX complexes activate the PF4 promoter

We demonstrated the bindings of PREP1 or PREP1/PBX complexes to the TME in the PF4 promoter. To investigate the effects of these proteins on the activity of the PF4 promoter, transient transfection assays were performed. The expression plasmids and the PF4 promoter–reporter plasmid (PF4luc) were co-transfected to HepG2 cells (Fig. 3). The overexpression of PREP1 slightly activated the PF4 promoter. The additional

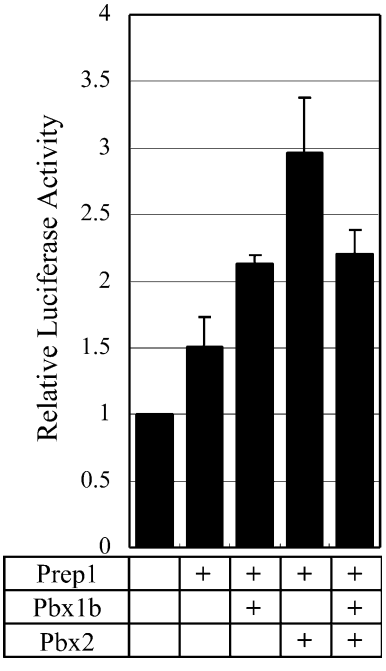

Fig. 3. PREP1 and PBXs synergistically activate the PF4 promoter. Transient transfection assays were performed using the PREP1, PBX1B, and PBX2 expression plasmids. These plasmids and the PF4luc reporter plasmid that contains 1.1 kb of PF4 promoter were transfected into HepG2 cells. Luciferase activities were evaluated and indicated as the relative activities.

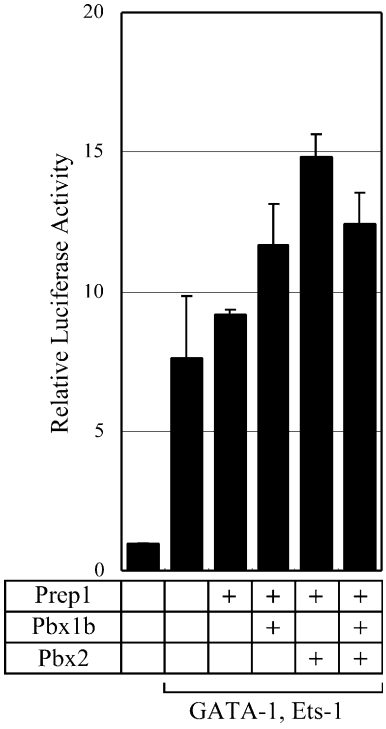

Fig. 4. The synergistic activation of the PF4 promoter by GATA-1, ETS-1, PREP1, and PBXs. The plasmids for expressions of ETS-1 and GATA-1 were transfected into HepG2 cells with the plasmids for expressions of MEIS1, PBX1B, and PBX2. Transcriptional activities were evaluated with the PF4luc plasmid.

overexpression of PBXs enhanced the activation of PREP1. This result indicates that both PREP1 and PBXs are necessary for the effective activation of the PF4 promoter.

To investigate how PREP1 and PBX affect the activation of the PF4 promoter by GATA-1 and ETS-1, transient transfection assays were performed (Fig. 4). The expression of both GATA-1 and ETS-1 activated the PF4 promoter, and the additional expression of PREP1 and PBXs strongly enhanced the activation of GATA-1 and ETS-1. This result indicates that PREP1 and PBXs synergistically activate the PF4 promoter with GATA-1 and ETS-1.

PREP1 is expressed in megakaryocytes

Our in vitro studies have documented the binding activities of PREP1 and PREP1/PBX complexes to the

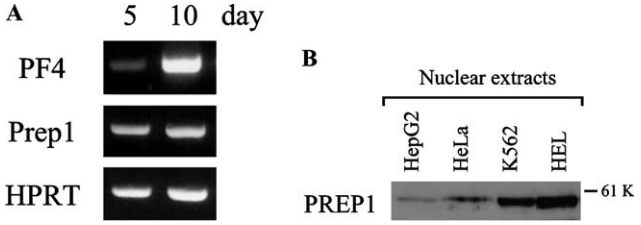

Fig. 5. Expression of PREP1 in megakaryocytes and lined cells. (A) Expression of PREP1 in human megakaryocytes was evaluated by RT-PCR. $CD34^+$ cells in human cord blood were isolated and cultured under the conditions promoting megakaryocytopoiesis. The cultured cells were harvested and used for RT-PCR. (B) Endogenous expression of PREP1 was detected by Western blotting using 20 μg of nuclear extracts from each cell line.

TME and their transcriptional activities have been demonstrated. To confirm their functions in megakaryocytes, the expression levels of Prep1 were evaluated in cultured megakaryocytes. According to our previous report, megakaryocytes were prepared from $CD34^+$ cells in human cord blood. $CD34^+$ cells were cultured in medium containing c-kit ligand and thrombopoietin. About 80% of the cultured cells were differentiated into megakaryocytes. In this condition, erythrocytes were scarcely detected as described previously. After 5 or 10 days incubation, cultured cells were harvested and their total RNAs were used for RT-PCR analysis. The expressions of both PREP1 and PF4 were detected in these differentiated cells (Fig. 5A). This result indicates that PREP1 is expressed in megakaryocytes and may contribute to megakaryocytic gene expression.

Are there any functional differences between PREP1 and MEIS1?

In the experiments described above, it was demonstrated that PREP1 bound to the TGACAG motifs in the TME and strongly activated the PF4 promoter with GATA-1 and ETS-1. Furthermore, PREP1 was expressed in megakaryocytes. These results suggest that PREP1 plays an important role in the regulation of the gene expression in megakaryocytes.

In our previous report, we demonstrated that MEIS1 or MEIS1/PBX complexes also bind to the TGACAG motifs in the TME and strongly activate the PF4 promoter with GATA-1 and ETS-1. Therefore, the tran-

Table 1
Activation of the PF4 promoter by PREP1 and MEIS1 in the transient transfection assays

GATA-1, ETS-1	PREP1	MEIS1	PREP1	MEIS1
	–	–	+	+
	1.5 ± 0.2	**0.9** ± 0.1	**9.2** ± 0.1	**10.4** ± 1.4
+PBX1B	**2.1** ± 0.1	**1.6** ± 0.2	**11.7** ± 1.5	**13.1** ± 1.6
+PBX2	**3.0** ± 0.4	**2.0** ± 0.5	**14.8** ± 0.8	**19.9** ± 1.9

Results of MEIS1 have been described in our previous report (Y. Okada et al., Blood, in press).

scriptional activities of PREP1 were compared to those of MEIS1 using the transient transfection assays (Table 1). PREP1 activates the PF4 promoter with or without GATA-1 and ETS-1 as MEIS1 does. Furthermore, Western blotting using nuclear extracts from various cell lines indicated that PREP1 was strongly detected in megakaryocytic HEL cells (Fig. 5B). These data suggest the functional similarity between PREP1 and MEIS1 for the regulation of PF4 gene expression. However, their physiological functions may not be the same. It has been reported that MEIS1 and HOXA9 cooperatively induce leukemia in mice, but PREP1 and HOXA9 do not [16]. Interestingly, our results show the slight differences in the functions of both proteins. In the EMSA, two shifted bands were detected when only PREP1 was used (Fig. 2B). PREP1 was assumed to form the homomultimer and bind to the TME. This phenomenon was not observed in the EMSA using MEIS1. Furthermore, although the overexpression of PREP1 by itself slightly activated the PF4 promoter, MEIS1 did not (Table 1). This difference between PREP1 and MEIS1 is probably derived from the formation activity of the multimer on the TME. These may explain the functional difference between PREP1 and MEIS1.

Our results on the regulation of PF4 gene expression by PREP1 and MEIS1 suggest the fascinating idea that there are some functional differences between PREP1 and MEIS1. In megakaryocytes, both PREP1 and MEIS1 are expressed. We believe that their synergism or compensation under the physiological state are important for the regulation of PF4 gene expression, but it is not well understood at the present time. Further investigation of this point is required to understand megakaryocytic gene expression and the leukemogenesis.

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