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Short Communication

Promoter motifs required for *c-mpl* gene expression induced by thrombopoietin in CMK cells

Masataka Sunohara[1,2*], Iwao Sato[1], Shigeru Morikawa[3]

[1] Department of Anatomy, School of Life Dentistry at Tokyo, The Nippon Dental University,1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan
[2] Department of Safety Research on Blood Products and Biologics, National Institute of Infectious Diseases, 4-7-1, Gakuen, Musashimurayama, Tokyo 208-0011, Japan
[3] Department of Veterinary Science, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Correspondence to: ma-suno@tky.ndu.ac.jp

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Abstract: Thrombopoietin (TPO) and its receptor, c-Mpl, are the central regulators of megakaryocyte development and platelet production and are also crucial to regulate megakaryocytopoiesis. TPO remarkably elevated *c-mpl* promoter activity, while the protein kinase C (PKC) inhibitors, GF109203, H7 and Calphostin C, clearly reduced the steady level of its promoter activity. In the present study, motifs crucial for *c-mpl* promoter activity induced by TPO treatment have been analyzed using a human megakaryoblastic cell line, CMK. Destruction of the –107Sp1 and the –57Sp1 sites in the *c-mpl* promoter enhancer region resulted in decrease of the promoter activity by 53.1% and 64.4%, respectively, and destruction of –69Ets and –28Ets elements dramatically decreased the promoter activity by 96.4% and 87.8%, respectively, while mutation of –77GATA moderately reduced the activity by 31.4%. The result was in agreement with our previous report that showed the crucial motifs in the *c-mpl* promoter for the promoter activity induced by PMA-treatment. This indicates that TPO-induced activation of the *c-mpl* promoter activity is fully modulated by transcription through a PKC-dependent pathway and the two Sp1 and two Ets motifs are crucial for the activation of the *c-mpl* promoter activity rather than a GATA motif in the *c-mpl* promoter of CMK cells.

Key words: Thrombopoietin; *c-mpl*; Protein kinase C; Megakaryocyte; CMK.

Introduction

Thrombopoietin (TPO) (also referred to as megakaryocyte growth and development factor, MGDF), a ligand for the c-Mpl receptor, plays a major role in the control of megakaryopoiesis (1-3) and is necessary for development of normal megakaryocytes and platelets (4-7). The experiments with knock-out mice, engineered to lack either c-Mpl or TPO, have demonstrated the importance of TPO in the regulation of megakaryopoiesis *in vivo* (8-10). Ligand-induced down-regulation, subsequent to receptor activation, is an important aspect of the normal physiology of cell-surface receptors (11). As reported previously, a variety of ligands and their receptors that activated protein kinase C (PKC) have been shown to induce phosphorylation of the receptors and uncoupling and/or internalization, which resulted in the loss of ligand binding activity (12-15). Previously, we have characterized the expression of c-Mpl on the human megakaryoblastic cell line CMK (16) and we have shown that pretreatment with TPO inhibited the binding of ^{125}I-labeled TPO to c-Mpl on the CMK cells and this inhibition was not caused by alteration of the c-Mpl to TPO but was caused by the down-regulation of c-Mpl on the cell surface. Phorbol 12-myristate 13-acetate (PMA) has also been shown to induce down-regulation of c-Mpl on CMK cells. This down-regulation of c-Mpl was transient (17). On the other hand, TPO has been shown to induce different biological responces in c-Mpl-transduced BaF-3 cells depending on the cell surface density of c-Mpl (18) and TPO has been also shown to activate PKC in CMK cells (19). Recently, we have elucidated that the promoter activity of *c-mpl* induced by TPO was modulated through a PKC-dependent pathway in CMK cells (20). PKC is a member of a family of serine/threonine protein kinases in the cytosol involved in pleiotropic processes such as cell growth, differentiation and cytokine secretion (21, 22). The PKC isoenzyme network plays also a role in the regulation of cell growth and differentiation (15, 23, 24). Previously, we have determined motifs crucial for the *c-mpl* promoter activity induced upon treatment of the CMK cells with PMA (26). Further, we have elucidated the role of some of promoter elements in *c-mpl* gene expression induced by TPO (30). In the present study, we have analyzed the effect of mutations in the *c-mpl* promoter on TPO induced promoter activity to clarify the motifs crucial for *c-mpl* gene expression.

Materials and Methods

Tissue culture

The CMK cell line was established from a patient with acute megakaryoblastic leukemia and Down' syndrome (16, 25). As reported previously (20, 26), the cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% FCS, 2mM L-glutamine, 100 units/ml penicillin, 100µg/ml streptomycin,

and 0.25µg/ml amphotericin B at 37 °C in humid air containing 5% CO_2 atmosphere.

Reagents

Recombinant human TPO was purified as previously described (4). PKC modulators, GF 109203 (CALBIOCHEM Co., La Jolla, CA), Calphostin C and (Sigma Chem.Co.,St.Louis, MO) were dissolved in DMSO as a stock solution and kept at -80 °C until use. Control medium included DMSO at the highest concentration used in stock solutions did not change *c-mpl* gene expression. H-7 (SEIKAGAKU AMERICA INC, Md, USA) was dissolved in distilled water as a stock solution and kept at -80 °C until use.

A reporter plasmid to analyze *c-mpl* promoter activity

As described previously (20, 26), a reporter plasmid, pGL3-*c-mpl*(-310), in which the luciferase gene was driven under control of the *c-mpl* promoter was used.

Reporter plasmids carrying a variety of truncation in *c-mpl* promoter

A series of reporter plasmids carrying various truncations in the pGL3-*c-mpl* (-310), pGL3-*c-mpl* (-214), pGL3-*c-mpl* (-121), pGL3-*c-mpl* (-83), pGL3-*c-mpl* (-73), pGL3-*c-mpl* (-60) and pGL3-*c-mpl* (-47), respectively, were used (Fig. 1A) (26).

Reporter plasmids carrying destruction in various motifs in *c-mpl* promoter

As previously described (26), a variety of mutations were introduced into pGL3-*c-mpl*(-121) to destroy motifs, Sp1, GATA, Ets at respective positions, respectively. The resulting plasmids, pGL3-107Sp1(-), pGL3-77GATA(-), pGL3-69Ets(-), pGL3-57Sp1(-) and pGL3-28Ets(-), respectively, were used in the present study (Fig. 2A) (26).

Transient transfection assay

As reported previously (26), three constructs, the pGL3-Control Vector, in which the luciferase gene is driven under SV40 promoter (as a positive control, Promega), the pGL3-Basic Vector without any promoter upstream of the luciferase gene (as a negative control) and the constructs described above were used for transfection assay. CMK cells were pretreated with TPO (100 ng/ml for 3 h), then the cells were washed twice in PBS. The plasmids (1 µg) were co-transfected with a plasmid, pRL-CMV, in which a wild-type Renilla luciferase gene is driven under CMV promoter (TOYO INC MFG CO., LTD., Tokyo), into CMK cells for 5 h by a lipofection method using the DMRIE-C reagent (GIBCO, Grand Island, NY) according to the manufacturer's instructions. Then, the cells were cultured in growth medium (RPMI1640, 10%FCS) for 19 h, washed with PBS twice, lyzed in a cell culture lysis reagent, LCß (TOYO INC MFG CO., LTD., Tokyo) and stored at -80 °C until luciferase assay. The luciferase activity was quantified by using a PicaGene-Dual-SeaPansy Luminescence Kit (TOYO INC MFG CO., LTD., Tokyo). For luciferase assay, 20 µl of the cell lysates were added to 100 µl of luciferase reaction mixture supplied in the kit, and luciferase activity was determined with a luminometer in accordance with the manufacturer's instructions. The lysates of the cells transfected with a promoterless vector, pGL3-Basic Vector, were used as a negative control and the Renilla luciferase activity expressed from pRL-CMV was used to evaluate transfection efficiency and to normalize the luciferase activity in all the lysates. Results are expressed as the percent of the control values. All the experiments were performed at least three times, and results represent means ± SD of at least 3 measurements.

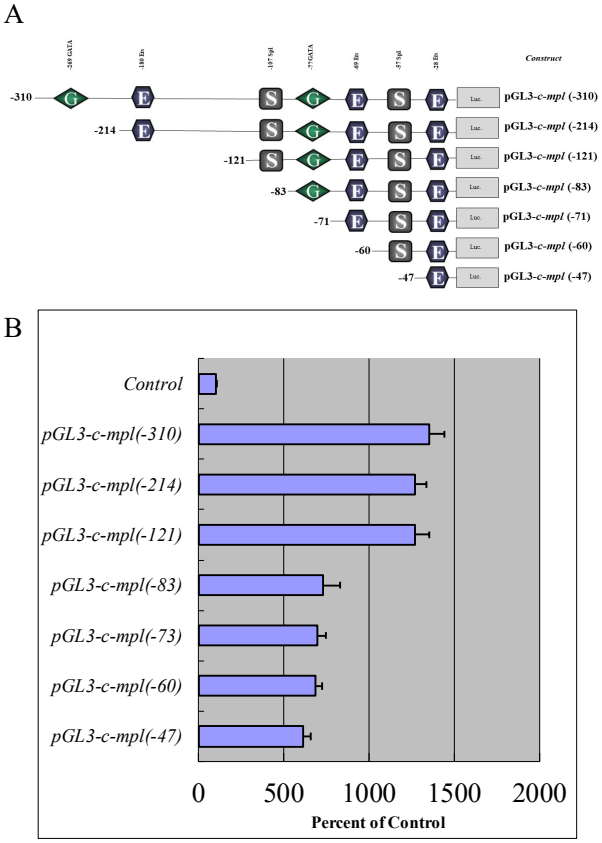

Figure 1. A. Schematic representation of putative regulatory elements in the *c-mpl* promoter and the organization of these sites in the deletion constructs. B. Effect of deletions of various regulatory elements on the *c-mpl* promoter activity. After pretreatment with TPO (100 ng/ml for 3 h), CMK cells were cultured and transfected with various reporter plasmids. Then the *c-mpl* promoter activity was measured in the same manner. Results are expressed as the percent of the control values. All the experiments were performed at least three times, and results represent means ± SD of at least 3 measurements.

Results

Effect of TPO and protein kinase C inhibitors on the activation of the *c-mpl* promoter

To elucidate the regulation mechanism of TPO in *c-mpl* gene expression during megakaryopoiesis, we have previously investigated the effect of TPO on the activation of *c-mpl* promoter activity and at the same time, we have examined the effect of PKC inhibitors on its promoter activity (20). In the present study, we first conducted similar experiments to confirm the previous observation. As shown in the previous report (20), TPO treatment significantly elevated the *c-mpl* promoter activity in CMK cells and this is markedly inhibited by PKC inhibitors, GF109203, H7, and Calphostin C (data

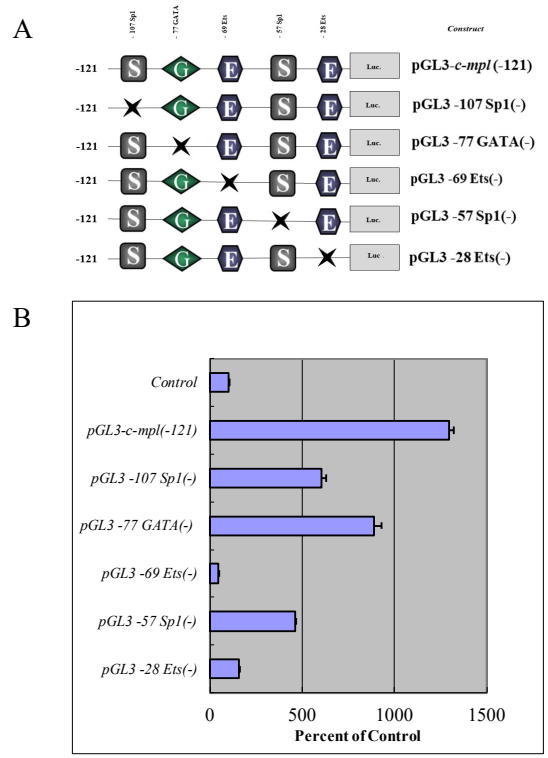

Figure 2. A. Schematic representation of putative regulatory elements in pGL3-*c-mpl*(-121) and the organization of these sites in the mutated constructs. B. Effect of a variety of mutations within the positive regulatory motifs on the *c-mpl* promoter activity. After pretreatment with TPO (100 ng/ml for 3 h), CMK cells were cultured and transfected with various reporter plasmids. Then the *c-mpl* promoter activity was measured in the same manner. Results are expressed as the percent of the control values. All the experiments were performed at least three times, and results represent means ± SD of at least 3 measurements.

not shown).

Effect of deletions in regulatory elements on *c-mpl* promoter activity

TPO induced *c-mpl* promoter activity in CMK cells was markedly inhibited by PKC treatment indicating PKC is crucial for the activity. Actually we have shown that PMA significantly elevated *c-mpl* promoter activity in CMK cells (26). Moreover, we have shown that PMA responsible element in the *c-mpl* promoter located at position less than -121 in the *c-mpl* promoter (26). Furhter, we have confirmed that the -69Ets and -28Ets elements were critical and that -107Sp1 and -57Sp1 were moderately important (26), while one of promoter elements, -77GATA, in the *c-mpl* promoter has been shown to be involved in TPO-induced promoter activation (30). In the present study, we have analyzed the other elements, Sp1 and Ets, in the *c-mpl* promoter upon TPO treatment to clarify if TPO induced *c-mpl* promoter activity in CMK cells is fully regulated by PKC.

We first analyze the TPO-induced promoter activity of the each deletion construct (pGL3-*c-mpl* (-310), pGL3-*c-mpl* (-214), pGL3-*c-mpl* (-121), pGL3-*c-mpl* (-83), pGL3-*c-mpl* (-73), pGL3-*c-mpl* (-60) and pGL3-*c-mpl* (-47)) in CMK cells (Fig. 1A). The data for the deletion constructs suggested that important positive regulatory elements upon TPO treatment appeared to be at positions within the region [-121, +1] in the *c-mpl* promoter, because the activity was maintained among pGL3-*c-mpl* (-310), pGL3-*c-mpl* (-214) and pGL3-*c-mpl* (-121) , but clearly reduced in pGL3-*c-mpl* (-83) (Fig. 1B), as has been reported previously for PMA induced promoter activation (26). This indicated that -107Sp1 is important for the TPO-induced the *c-mpl* promoter activity. We have also analyzed the effect of further deletion using the deletion constructs, pGL3-*c-mpl* (-73), pGL3-*c-mpl* (-60), and pGL3-*c-mpl* (-47), and found that deletion of -77GATA, -69Ets, and -57Sp1 regions moderately affected the promoter activity, indicating -28Ets is also important for the TPO-induced the *c-mpl* promoter activity (Fig. 1B).

Effect of point mutations in pGL3-*c-mpl* (-121) on the *c-mpl* promoter activity

The above results strongly indicated that the required elements in the *c-mpl* promoter upon TPO treatment was fully regulated by PKC and -107Sp1 and -28Ets were important. Thus, we analyzed the effect of mutations destructing each motif in the pGL3-*c-mpl* (-121) on the promoter activity induced by TPO-treatment. The promoter activity of the mutated constructs (pGL3-107Sp1 (-), pGL3-77GATA (-), pGL3-69Ets (-), pGL3-57Sp1 (-) and pGL3-28Ets (-)) were measured after transient transfection assay in CMK cells (Fig. 2A). The mutations destroyed the GATA sequence (GATA→G<u>C</u>TA) in pGL3-77GATA (-), Ets sequence (AGGAA→A<u>CC</u>AA) in pGL3-69Ets (-) and pGL3-28Ets (-), respectively, and Sp1 sequence (GGGGCAGGG→GG<u>AAA</u>AGGG) in pGL3-107Sp1 (-) and pGL3-57Sp1 (-), respectively (mutated residues are underlined). As shown in Fig. 2B, destruction of –107Sp1 and –57Sp1 in the *c-mpl* promoter resulted in a significant decrease in the promoter activity by 53.1% and 64.4%, respectively. Destruction of –69Ets and –28Ets in the promoter dramatically decreased the activity by 96.4% and 87.8%, respectively, while that of –77GATA moderately reduced the activity by 31.4%. Thees results were in agreement with our previous report that showed the effects of destruction of motifs in the promoter region on decrease of the *c-mpl* promoter activity induced by PMA-treatment.

Discussion

TPO is known to play a crucial role in both megakaryopoiesis and thrombopoiesis, and variety of signal transduction cascades from TPO receptor upon TPO treatment were reported such as JAK-STAT pathway [Cell,93(3),385-395, 1998; Exp Hematol. 2000; 28(3):294-304], MAPK-Ras signal cascade [Oncogene. 2002;21(21):3359-67], activation of PI3-K [J Exp Med. 1997;186(12):1947-55.], and PKC activation pathway (19). Recently, we investigated the effect of TPO on *c-mpl* gene expression at the promoter level and found that the promoter activity of *c-mpl* was up-regulated by TPO pretreatment. We confirmed that this up-regulation was completely abolished by PKC inhibitors. A PKC dependent pathway is thought to be involved not only in the process for the up-regulation of *c-mpl* promoter activity but also in a feed back regulatory mechanism for *c-mpl* gene expression itself in megakaryocytic CMK cells (20). These indicated that central of TPO-induced *c-mpl* promoter activation was a PKC-dependent

pathway. We have also reported the required motifs for *c-mpl* gene expression induced by PMA in CMK cells (26).

In megakaryocytic cells, the glycoprotein IIb promoter contains a specific erythromegakaryocytic enhancer that may interact with GATA1 (27). GATA-1-induced up-regulation of *c-mpl* in the murine myeloid cell line is involved in erythroid and megakaryocytic differentiation (28) and during megakaryocyte development, GATA-1 serves multiple functions (29). We have recently confirmed that -77GATA motif in the *c-mpl* promoter in the CMK cells was a positive regulatory element, however, the motif was not extremely important for *c-mpl* gene expression induced by TPO in CMK cells (30).

In the present study, to further explore the effect of TPO on *c-mpl* promoter activity more in detail, we investigated the other required motifs for *c-mpl* gene expression induced by TPO in megakaryoblastic cells using the constructs as described previously (26).

The data of the promoter activity of the deletion constructs upon TPO treatment, as shown in Fig. 1B, clearly showed that the important positive regulatory elements appeared to be within 121 base pairs upstream of the transcription start site in the *c-mpl* promoter. Ets recognition sequences are an important determinant of megakaryocytic gene expression (35). In liver cells, TPO is regulated by transcription factors of the Ets family. Furthermore in the liver, transcription factors of the Ets family are required for a high level expression of the TPO gene (36, 37). On the other hand, modulation of Sp1 levels controls thromboxane receptor expression during megakaryocytic differentiation (38). In the case of *c-mpl,* its promoter also contains Ets and Sp1 as well as GATA-1 binding motifs, which are directly involved in megakaryocyte-specific gene expression (27, 28) and GATA and Ets were shown to be critical for *c-mpl* promoter activity in HEL cells (39).

In this study, it is concluded that the expression of the *c-mpl* gene is modulated by transcription through a PKC-dependent pathway and the Ets motifs at 69 and 28 base pairs upstream of the transcription start site are crucial for the *c-mpl* promoter activity in the CMK cells. Sp1(-107) and Sp1(-57) are also important and GATA(-77) is less involved as a positive regulatory element in *c-mpl* gene expression induced by TPO in the CMK cells. On the other hand, it is reported that a PKC-Sp1 signaling pathway induces early differentiation of human keratinocytes (40). Our observation further indicates Sp1 motif is also involved as a positive regulatory element in *c-mpl* gene expression induced by TPO in megakaryocytic CMK cells. Collectively, in conclusion, these elements reciprocally regulate the expression of the *c-mpl* gene through a PKC-dependent pathway and also, the PKC isoenzyme network might play a role in regulation of this event during megakaryopoiesis. Cytokine-signaling network is complicated so that chimeric receptor system (41) is considered to be useful to analyze TPO signaling pathway in the human megakaryocytic CMK cells, especially to elucidate PKC-dependent modulation mechanism of TPO-induced *c-mpl* gene expression more in detail.

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Interest conflict

The authors have declared no conflicts of interest.

Author's contribution

We would like to type each author's name next to the appropriate category. Conception and design of the study: Masataka Sunohara, Shigeru Morikawa. Acquisition of data: Masataka Sunohara, Shigeru Morikawa. Analysis and interpretation of data: Masataka Sunohara, Shigeru Morikawa, Iwao Sato. Drafting of manuscript: Masataka Sunohara, Shigeru Morikawa, Iwao Sato. All authors read and approved the final manuscript.

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