Role of promoter element in *c-mpl* gene expression induced by TPO

By

Masataka SUNOHARA^{1,2}, Shigeru MORIKAWA³, Akira FUSE², and Iwao SATO¹

¹Department of Anatomy, School of Life Dentistry at Tokyo, The Nippon Dental University,
1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan

²Department of Safety Research on Blood Products and Biologics, National Institute of Infectious Diseases,
4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan

³Department of Veterinary, Science National Institute of Infectious Diseases,
1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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Summary: Thrombopoietin (TPO) and its receptor, c-Mpl, play the crucial role for the development of megakaryocyte and considered to regulate megakaryocytopoiesis. Previously we reported that TPO increased the *c-mpl* promoter activity determined by a transient expression system using a vector containing the luciferase gene as a reporter and the expression of the *c-mpl* gene is modulated by transcription through a protein kinase C (PKC)-dependent pathway in the megakaryoblastic cells. In this research, to elucidate the required elements in *c-mpl* promoter, the promoter activity of the deletion constructs and site-directed mutagenesis were measured by a transient transfection assay system. Destruction of –77GATA in *c-mpl* promoter decreased the activity by 22.8%. Our study elucidated that –77GATA involved in TPO-induced *c-mpl* gene expression in a human megakaryoblastic cell line, CMK.

Introduction

Thrombopoietin (TPO) and its receptor, c-Mpl, are required for megakaryopoiesis and platelet development (Lok *et al.*, 1994¹), De Sauvage *et al.*, 1994²), Wendling *et al.*, 1994³), Kaushansky, 1999⁴). PKC is a member of a family of serine/threonine protein kinases in the cytosol involved in pleiotropic processes such as cytokine secretion, cell growth and differentiation (Ohno *et al.*, 1991⁵), Nishizuka *et al.*, 1992⁶). CMK, the human megakaryoblastic cell line, was established from the peripheral cells obtained from a patient with acute megakaryoblastic leukemia and Down' syndrome (Komatsu *et al.*, 1989⁷), Sato *et al.*, 1989⁸). Previously we characterized an expression of c-Mpl on the CMK cells (Sato *et al.*, 1989⁸) and found that TPO down-regulated the expression of c-Mpl on the cell surface (Sato *et al.*, 1998⁹). TPO has also been shown to activate PKC in the CMK cells (Hong *et al.*, 1998¹⁰). Recently we suggested that TPO-induced *c-mpl* promoter activity was modulated at transcription level through a PKC-dependent pathway in the CMK cells (Sunohara *et al.*, 2003¹¹), 2004¹²). In this research, we

investigated the required promoter elements in *c-mpl* gene expression induced by TPO.

Materials and methods

Cell Culture

The CMK cell line was established from a patient with acute megakaryoblastic leukemia and Down' syndrome (Komatsu *et al.*, 1989⁷), Sato *et al.*, 1989⁸). As described previously (Sunohara *et al.*, 2003¹¹), 2009¹³), 2010¹⁴), 2008¹⁵), the cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% FCS, 2mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B at 37°C in humid air containing 5% CO₂ atmosphere. CMK cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B at 37 °C in humidified air containing 5% CO₂ atmosphere.

Reagents

Protein kinase C modulator, GF 109203 (CALBIO-CHEM Co., La Jolla, CA) was 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.

Thrombopoietin (Recombinant human TPO) was purified as previously described (De Sauvage *et al.*, 1994²⁾).

Construction of a reporter plasmid to measure the *c-mpl*-promoter activity.

The construct[pGL3-*c-mpl* (-310)] has been reported previously (Sunohara *et al.*, 2003¹¹⁾, 2009¹³⁾, 2010¹⁴⁾), the 310 bp upstream promoter region of *c-mpl* was amplified from the chromosomal DNA of the CMK cells by polymerase chain reaction using the following primer set, (forward 5'-CTA TTC TGA TGC TAT TAT ACC ATT C-3', reverse 5'-CTT GGC TGC TGA CTT GGG C-3'). The PCR product was cloned into pGEM-Teasy vector (Promega, Madison, WI) and designated as pGEM-*c-mpl*-prom. For the determination of the promoter activity of *c-mpl*, a *Bgl* II /*Hind* III fragment of pGEM-*c-mpl*-prom was subcloned into a defined *Bgl* II /*Hind* III site of the pGL3-basic vector (Promega) to construct pGL3-*c-mpl* (-310) in which the luciferase gene was driven under control of the *c-mpl* promoter (Fig.1).

Promoter deletion constructs

As described previously (Sunohara *et al.*, 2008¹⁵⁾), the truncated forms of the 310 bp upstream promoter region of *c-mpl* were amplified from the pGEM-*c-mpl*-prom by polymerase chain reactions using the following primer sets: *c-mpl*-prom (-121) (forward: 50-GGA TGG GCC CCA GA-30, reverse: 50-CTT GGC TGC TGA CTT GGG C-30), *c-mpl* prom (-83) (forward: 50-GGG GCT GTATCT GAC-30, reverse: 50-CTT GGC TGC TGA CTT GGG C-30). The PCR products were cloned into pGEM-Teasy vector and their sequences were confirmed. Then their *Bgl* II/*Hind* III fragments were subcloned into the *Bgl* II/*Hind* III site of the pGL3-basic vector to construct pGL3-*c-mpl* (-121), pGL3-*c-mpl* (-83), respectively (Fig. 2A). In these constructs, the luciferase gene is driven under control of the truncated forms of *c-mpl* promoter.

Site-directed mutagenesis

As reported previously (Sunohara *et al.*, 2008¹⁵⁾), to examine the positive regulatory elements in the *c-mpl* promoter, we examined the effect of a mutation within the positive regulatory motifs on the promoter activity (Fig. 3A). The promoter activity of the mutated construct (pGL3-77GATA (-)) was measured after transient transfection assay in CMK cells. The mutation destroyed the GATA sequence (GATA→GCTA) in pGL3-77GATA (-) (mutated residue is underlined).

Transient transfection assay

As described previously (Sunohara *et al.*, 2003¹¹⁾, 2009¹³⁾, 2010¹⁴⁾), three reporter plasmids, pGL3-basic vector (as a negative control), pGL3-control vector in which the luciferase gene is driven under SV40 promoter (as a positive control, Promega) and the pGL3-*c-mpl* (-310) were used for transfection assay. A plasmid, pRL-CMV (TOYO INC MFG CO., LTD., Tokyo), which expresses Renilla (SeaPansy) luciferase under control of CMV early promoter, was used to normalize the transfection efficiency. PMA (20nM) was added to the CMK cells and then the cells were washed twice in PBS. The reporter plasmids (1 µg) were co-transfected with the pRL-CMV into the 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 the growth medium (RPMI1640, 10% FCS) for 19 h, washed twice in PBS, lysed in a cell lysis reagent, LCB (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). Twenty µl aliquot of cell lysates were added to 100 µl of luciferase reaction mixture supplied in the kit, and luciferase activity was measured with a luminometer in accordance with the manufacturer's instructions. The cell lysate of the cells transfected with a promoter-less control vector, pGL3-Basic Vector, was used as a negative control and the SeaPansy luciferase activity expressed from pRL-CMV was used to evaluate transfection efficiency and to normalize luciferase activity in all lysates. Results are expressed as the percentage of the control values. All the experiments were performed at least three times, and results represent means ± SD of at least 3 measurements.

Results

We examined the effect of TPO on *c-mpl* promoter activity by using transient transfection assay system. Besides pGL3-*c-mpl* (-310), we also examined the effect of the deletion constructs and site-directed mutagenesis on TPO-induced *c-mpl* promoter activity at the same time.

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

After pretreatment with TPO (100 ng/ml for 3 h.), TPO increased the *c-mpl* promoter activity and PKC inhibitor (GF109303X) reduced the basal level of the *c-mpl* promoter activity. As reported previously (Sunohara *et al.*, 2003¹¹⁾, 2008¹⁵⁾), we confirmed again that PKC plays the key role in maintaining *c-mpl* transcription in CMK cells (data not shown).

Fig. 1. Schematic representation of pGL3-*c-mpl* (-310) pGL3-*c-mpl* (-310) is represented in Fig. 1.

Fig. 2A. Schematic representation of putative regulatory elements in the *c-mpl* promoter and the organization of these sites in the deletion constructs. To determine the suitable construct for high-level specific expression, we examined the three deletion constructs. Each deletion construct is represented in Fig. 2A.

Fig. 2B. Analysis of the *c-mpl* promoter activity by the deletion constructs. The activity of the each deletion construct (pGL3-*c-mpl*(-310), pGL3-*c-mpl*(-121), pGL3-*c-mpl*(-83)) was measured by transient transfection assay in CMK cells (Fig. 2B). Results are expressed as the percent of the control values. All experiments were performed at least three times and results represent means $\pm$ SD of at least 3 measurements.

Fig. 3A. Schematic representation of the organization of putative regulatory elements in the mutated construct. Schematic representation of putative regulatory elements in pGL3-*c-mpl*(121) and the organization of these sites in the mutated constructs. To examine the positive regulatory elements in the *c-mpl* promoter, we examined the effect of a mutation within the positive regulatory motifs on the promoter activity. The mutation within the positive regulatory motif(-77GATA) is represented in Fig. 3A. The crosses (X) designate the mutated region.

Fig. 3B. Effect of mutation within the positive regulatory motif on the promoter activity. The promoter activity of the mutated construct [pGL3-77GATA(-)] was measured by transient transfection assay in CMK cells (Fig. 3B). Results are expressed as the percent of the control values. All the experiments were performed at least three times and results represent means $\pm$ SD of at least 3 measurements.

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

As to the deletion constructs, the activity was clearly reduced from pGL3-*c-mpl* (-83) and we confirmed that pGL3-*c-mpl* (-121) is the most suitable construct for the following our experiments (Fig. 2B).

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

To examine the positive regulatory elements in the *c-mpl* promoter, we examined the effect of the mutation within the positive regulatory motifs on the promoter

activity. The promoter activity of the mutated construct (pGL3-77GATA) was measured after transient transfection assay in CMK cells. As shown in Fig. 3B, destruction of -77GATA in *c-mpl* promoter decreased the activity by 22.8 %.

Discussion

We have already reported that pretreatment with TPO inhibits the binding activity of TPO to its receptor and led to downregulation of the number of c-Mpl on the

cell surface. PMA has also induced downregulation of c-Mpl on CMK cells. These downregulations of c-Mpl were transient (Sato *et al.*, 1998⁹). Recently we clarified that the promoter activity of *c-mpl* was upregulated by TPO pretreatment and TPO-induced *c-mpl* promoter activity was modulated at transcription level through a PKC-dependent pathway in the CMK cells (Sunohara *et al.*, 2003¹¹, 2004¹²). In this research, we investigated the required promoter element in *c-mpl* gene expression induced by TPO. The data of the promoter activity of the deletion constructs upon TPO treatment clearly showed that the important positive regulatory elements appeared to be at position less than -121 in *c-mpl* promoter. So we confirmed that pGL3-*c-mpl* (-121) is the most suitable construct for the following our experiments (Fig. 2B).

To examine the positive regulatory elements in the *c-mpl* promoter, the promoter activity of the mutated construct (pGL3-77GATA) was measured after transient transfection assay in CMK cells and destruction of -77GATA in *c-mpl* promoter decreased the activity by 22.8% (Fig. 3B).

It has been reported that the glycoprotein IIb promoter contains a specific erythromegakaryocytic enhancer that may interact with GATA-1 in megakaryocytic cells (Prandini *et al.*, 1992¹⁶). And also GATA and Ets recognition sequences are an important determinant of megakaryocytic gene expression (Lemarchandel *et al.*, 1993¹⁷). On the other hands, GATA-1-induced upregulation of *c-mpl* is involved in erythroid and megakaryocytic differentiation in the murine myeloid cell line (Yamaguchi *et al.*, 1998¹⁸) and GATA-1 serves multiple functions during megakaryocyte development (Vyas *et al.*, 1999¹⁹).

Our study clearly defines that -77GATA was important as a positive regulatory element and involved in *c-mpl* gene expression induced by TPO.

Further studies are needed to elucidate the required motifs for *c-mpl* gene expression induced by TPO in human megakaryocytic cells.

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