GATA-dependent regulation of TPO-induced *c-mpl* Gene Expression during Megakaryopoiesis

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Summary: Thrombopoietin (TPO) and its receptor, c-Mpl, play the crucial role during megakaryocytopoiesis. Previously, we have shown that the promoter activity of *c-mpl* induced by TPO is modulated by transcription through a PKC-dependent pathway and that GATA(-77) is involved as a positive regulatory element in TPO-induced *c-mpl* gene expression in the megakaryoblastic CMK cells. In this research, to examine participating possibility of GATA promoter element in TPO-induced *c-mpl* gene expression through a PKC-independent pathway, the promoter activity of site-directed mutagenesis and the effect of protein kinase C modulator were measured by a transient transfection assay system. Together with our previous results on the TPO-induced *c-mpl* promoter, this study indicates destruction of -77GATA in *c-mpl* promoter decreased the activity by 47.3% under existence of GF109203. These results suggest that GATA promoter element plays significant role in TPO-induced *c-mpl* gene expression through a PKC-independent pathway.

Introduction

c-Mpl and its ligand, Thrombopoietin (TPO), are required for megakaryopoiesis and platelet development (Lok *et al.*, 1994¹, De Sauvage *et al.*, 1994², Wendling *et al.*, 1994³, Kaushansky, 1994⁴). A protein kinase C (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). The human megakaryoblastic cell line, CMK, 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). We have 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). In the CMK cells, TPO has also been shown to activate PKC. (Hong *et al.*, 1998¹⁰). Recently we suggested that TPO-induced *c-mpl* promoter activity was modulated at

transcription level through a PKC-dependent pathway (Sunohara *et al.*, 2003¹¹, 2004¹²) and that GATA(-77) is involved as a positive regulatory element in TPO-induced *c-mpl* gene expression in the megakaryoblastic CMK cells (Sunohara *et al.*, 2013¹³). In this research, we investigated role of GATA promoter element in TPO-induced *c-mpl* gene expression through a PKC-independent pathway by using a transient transfection assay system.

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, 2013¹³, 2009¹⁴, 2010¹⁵, 2008¹⁶), the cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% FCS, 2mM L-glutamine, 100 units/ml penicillin, 100µg/

Fig. 1A. 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. 1A.

ml streptomycin, and 0.25 μ g/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 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B at 37 °C in humidified air containing 5% CO₂ atmosphere.

Reagents

Protein kinase C modulators, GF 109203 (CALBIOCHEM 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⁾, 2013¹³⁾, 2009¹⁴⁾, 2010¹⁵⁾), 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.*, 2008¹⁶⁾), 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. 1A). 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.*, 2008¹⁶⁾), 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. 2A). 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 residues are underlined).

Transient transfection assay

As described previously (Sunohara *et al.*, 2003¹¹⁾, 2013¹³⁾, 2009¹⁴⁾, 2010¹⁵⁾), 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,

Fig. 1B. 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. 1B). 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.

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. TPO (100 ng/ml) was added to the CMK cells and then the cells were washed twice in PBS. The reporter plasmids (1 μ g) 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, LC β (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). 20 μ l aliquot of cell lysates were added to 100 μ l 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 $\pm$ 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 site-directed mutagenesis and PKC inhibitor (GF109303X) on TPO-induced *c-mpl* promoter activity.

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, 2008¹⁶), we confirmed again that PKC plays the key role in maintaining *c-mpl* transcription in CMK cells (data not

Fig. 2A. 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 mutation within the positive regulatory motif on the promoter activity. The mutation within the positive regulatory motif (-77GATA) is represented in Fig. 2A. The crosses (X) designate the mutated regions.

shown).

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

As previously described (Sunohara *et al.*, 2013¹³), 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. 1A, 1B).

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

We already reported the effect of the mutation within the positive regulatory motifs on the promoter activity 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. Destruction of -77GATA in *c-mpl* promoter decreased the activity by 22.8% (Sunohara *et al.*, 2013¹³). In this study, destruction of -77GATA motif in *c-mpl* promoter decreased its activity by 21.66% and we confirmed again GATA(-77) is involved as a positive regulatory element in TPO-induced *c-mpl* gene expression in CMK cells..

Effect of GF109203 and mutation within the positive regulatory motif on TPO-induced c-mpl promoter activity

In this research, to examine participating possibility of GATA promoter element in TPO-induced *c-mpl* gene expression through a PKC-independent pathway, CMK cells were cultured with GF109203 and then the promoter activity of site-directed mutagenesis and the effect of protein kinase C modulator were measured by a transient transfection assay system.

As shown in Fig. 2B, destruction of -77GATA in *c-mpl* promoter decreased the activity by 47.3% under existence of GF109203. Take this results into consideration,

GATA promoter element play significant roles in TPO-induced *c-mpl* gene expression through a PKC-independent pathway.

Discussion

It was recently reported 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 role of GATA promoter element in TPO-induced *c-mpl* gene expression through a PKC-independent pathway by using a transient transfection assay system.

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).

The glycoprotein IIb promoter contains a specific erythromegakaryocytic enhancer that may interact with GATA-1 in megakaryocytic cells (Prandini *et al.*, 1992¹⁷) and also GATA and Ets recognition sequences are an important determinant of megakaryocytic gene expression (Lemarchandel *et al.*, 1993¹⁸). On the other hand, GATA-1-induced upregulation of *c-mpl* is involved in erythroid and megakaryocytic differentiation in the murine myeloid cell line (Yamaguchi *et al.*, 1998¹⁹) and GATA-1 serves multiple functions during megakaryocyte development

Fig. 2B. Effect of GF109203 and mutation within the positive regulatory motif on TPO-induced *c-mpl* promoter activity. The promoter activity of the mutated construct [pGL3- 77GATA(-)] was measured by transient transfection assay in CMK cells (Fig. 2B). CMK cells were pretreated with TPO (100 ng/ml for 3 hr), washed twice and transfected with *c-mpl*-prom-luc for 5 h and then incubated in the complete medium for 19 h. Protein kinase C inhibitor, GF109203 (10 μ M) was added to CMK cells from 1 h before TPO pretreatment. Then the cells were washed twice and transfected with *c-mpl*-luc for 5 h. After incubation in complete medium for 19 h, the *c-mpl* promoter activity was measured and 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.

(Vyas *et al.*, 1999²⁰).

As reported before, our research clearly defines that -77GATA was a positive regulatory element and involved in *c-mpl* gene expression induced by TPO.

Together with our previous results, this study suggests clearly that destruction of -77GATA in *c-mpl* promoter decreased the activity by 47.3% under existence of GF109203. These results are shown that GATA promoter element plays significant role in TPO-induced *c-mpl* gene expression through a PKC-independent pathway.

Further studies are needed to elucidate the PKC-independent modulation mechanism of *c-mpl* gene expression induced by TPO in human megakaryocytic CMK cells.

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