PKC plays a crucial roles in c-mpl gene expression in megakaryoblastic cells

By

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Summary: Thrombopoietin is the cytokine involved in megakaryopoiesis and its receptor (c-Mpl) is considered to regulate development of megakaryocyte. In this research, to elucidate the underlying mechanisms of c-mpl gene expression in megakaryoblastic cells, we investigated the effect of a protein kinase C (PKC) on c-mpl promoter activity in a time-dependent manner. PKC is a member of a family of serine/threonine protein kinases in the cytosol involved in cell growth and differentiation. Phorbol 12-myristate 13-acetate (PMA) is known as PKC activator, significantly enhanced the c-mpl promoter activity and PKC inhibitor, 2-methylpiperazine dihydrochloride (H-7) suppressed the up-regulation of PMA-induced promoter activity and this effect decreased in a time-dependent manner. These results clearly suggest that in megakaryoblastic cells, PKC plays the crucial role in the initiation of up-regulation of PMA-induced c-mpl promoter activity.

1. Introduction

Thrombopoietin (TPO), a ligand for the C-Mpl receptor is required for megakaryopoiesis and platelet development (Lok et al., 1994); De Sauvage et al., 1994; Wendling et al., 1994; Kaushansky, 1999). 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., 1991; Nishizuka et al., 1992). 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., 1989; Sato et al., 1989). Previously we characterized an expression of c-Mpl on the CMK cells (Sato et al., 1989) and found that TPO down-regulated the expression of c-Mpl on the cell surface (Sato et al., 1989). TPO has also been shown to activate PKC in the CMK cells (Hong et al., 1998). 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., 2003). In this research, we investigated the effect of PKC on c-mpl promoter activity in a time-dependent manner.

2. Materials and methods

2.1. Cell Culture

The CMK cell line was established from a patient with acute megakaryoblastic leukemia and Down’s syndrome (Sato et al., 1989). The cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2 mM 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.

2.2. Reagents

PKC inhibitor, 2-methylpiperazine dihydrochloride (H-7) (SEIKAGAKU AMERICA INC, Md, USA), was dissolved in distilled water as a stock solution and kept at −80°C until use. Phorbol 12-myristate 13-acetate (PMA) was dissolved in DMSO as a stock solution and kept at −80°C until use. Control medium including DMSO at
the highest concentration used in the stock solution was confirmed to have no effect on c-mpl gene expression.

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

The following constructs have been described previously (Sunohara et al., 2003\(^1\), 2009\(^2\)), 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.

2.4. Transient transfection assay

As reported previously(Sunohara et al., 2003\(^1\), 2009\(^2\)), 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 (20 nM) was added to 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, lyzed in a cell 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). 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 promoterless 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.

3. Results

3.1 Dose-dependent effect of H7 on the activation of the c-mpl promoter

H7 is well known as an inhibitor of PKC. To examine whether the promoter activity of c-mpl is modulated by transcription through a PKC-dependent pathway or not, CMK cells were cultured with or without different concentrations (1.0–100 µM) of H7. As shown in Fig. 1, the steady level of the c-mpl promoter activity was found to decrease in a dose-dependent manner with H7 and 100 µM of H7 was chosen for all subsequent experiments.

3.2 Effect of PMA treatment on the activation of the c-mpl promoter

PMA (20 nM) was added to CMK for 3 h and then the c-mpl promoter activity was measured. As shown in Fig. 2, PMA significantly elevated the c-mpl promoter activity.

3.3 Effect of H7 on the PMA-induced c-mpl promoter activity

To test the role of PKC in PMA-induced c-mpl promoter activity, PKC inhibitor was added to CMK cells 1 h before PMA pretreatment (20 nM for 3 h) and c-mpl promoter activity was measured. As shown in Fig. 3, H7 completely inhibited the PMA-induced increase in c-mpl promoter activity.
3.4 Effect of H7 on the activation of the c-mpl promoter activity

Protein kinase C inhibitor, H7 (100 µM) was added to CMK cells at different times of addition (−1 h, 3 h, 6 h, 12 h, 18 h) with PMA pretreatment (20 nM for 3 h) and then, the c-mpl promoter activity was measured. As shown in Fig. 4, H-7 suppressed the up-regulation of PMA-induced promoter activity and this effect decreased in a time-dependent manner.

4. Discussion

We have already confirmed that TPO pretreatment induced the up-regulation of c-mpl promoter activity and also this TPO induced up-regulation was completely abolished by treatment with PKC inhibitors (Sunohara et al., 2003).

In this research, to further explore the role of PKC in c-mpl gene expression at transcription level, we examined the effect of the PKC activator, phorbol 12-myristate 13-acetate (PMA) and the PKC inhibitor, 2-methylpiperazine dihydrochloride (H-7) on c-mpl promoter activity in the CMK cells. As shown in Fig. 2, PMA significantly elevated the c-mpl promoter activity, while H7, known as PKC inhibitor, suppressed the up-regulation of PMA-induced promoter activity (Fig. 3) and reduced the steady level of its activity (Fig. 1).

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of PMA-induced promoter activity and this effect decreased in a time-dependent manner.

These results clearly suggest that in megakaryoblastic cells, PKC plays a crucial role in the initiation of upregulation of PMA-induced \textit{c-mpl} promoter activity.

The results described here reveal the first insight into the timing of activation of a PKC-dependent pathway in \textit{c-mpl} gene expression at transcription level.

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**References**


