BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **247,** 65–69 (1998)
ARTICLE NO. RC988736

Transcription Factor NF-E2 Is Essential for the Polyploidization of a Human Megakaryoblastic Cell Line, Meg-J

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Received April 27, 1998

Transcription factors regulating the process of megakaryocyte development remain largely unclarified. To clarify them further, we used a human megakaryoblastic cell line, Meg-J, which showed prominant polyploidization and augmented platelet glycoprotein (GP) Ib expression after incubation with thrombopoietin (TPO, c-mpl ligand) and K252a (an indolocarbasole derivative). Under these conditions, we analyzed the expression of the transcription factors and observed that the expression of NF-E2 p45, but not those of GATA-1, GATA-2, Tal-1/SCL, Evi-1, and MafK, was increased after TPO and K252a stimulation. Gel-shift assay confirmed the enhanced binding activity to the NF-E2 site. The abolishment of NF-E2 p45 with NF-E2 antisense oligomers inhibited TPO plus K252a-induced polyploidization. These findings suggest that NF-E2 p45 is essential for the polyploidization of megakaryocytic cells.

Megakaryocytopoiesis is initiated with the proliferation of committed megakaryocytic progenitor cells and subsequently followed by terminal differentiation which includes nuclear polyploidization, size growth, and the generation of specific cytoplasmic apparatus, such as demarcation membranes, α-granules, and surface platelet glycoproteins.

A wealth of knowledge about megakaryocytopoiesis has been accumulated since thrombopoietin (TPO) was molecularly cloned(1,2). However little is known about the molecular basis of megakaryocytic development, especially about transcriptional factors crutial for it. This is partially due to the difficulties in obtaining sufficient numbers of normal megakaryocytes for genetic research, and also due to absence of an in vitro model system which could reflect the normal processes of megakaryocytic differentiation.

Therefore, we have tried to establish an in vitro alternative model for studying megakaryocytic development processes using an established megakaryocytic cell line, Meg-J. K252a, an indolocarbasole derivative, was isolated from a culture broth of Nocardiosis SP(3,4). It has been known to cause polyploidization without interventing mitosis in some non-hematological cell lines in vitro(5,6). Although the mechanisms of the action of K252a has not been elucidated, we applied K252a in our study and observed that K252a could induce polyploidization and differentiation of megakaryocytic cell lines. These effects of K252a were further augmented by simultaneous addition of TPO into the culture (paper in preparation).

Using this culture system, we have tried to identify the transcription factors which play critical roles in the process of polyploidization and expression of platelet glycoproteins (GPs) We analyzed the transcriptional factors, GATA-1, GATA-2, NF-E2 (heterodimer of NF-E2 p45 and p18/MafK), Tal-1/SCL, and Evi-1, which have been reported to be expressed in megakaryocytic cells(7-14).

MATERIALS AND METHODS

Reagents. Recombinant human thrombopoietin(rhTPO) was provided by the Kirin Brewery Co.(Takasaki, Japan). K252a was purchased from Funakoshi Co.(Tokyo, Japan), dissolved in dimethyl sulfoxide(DMSO) and stored in the dark at −20°C. Propidium iodide(PI) was purchased from Sigma Co.(St. Louis, MO). Fluorescein isothiocyanate(FITC)-conjugated monoclonal antibodies(MoAbs) against platelet glycoprotein(GP)IIb/IIIa(CD41a) and GP Ib(CD42b) were purchased from Immunotech Co.(Marseille, France).

Cells and culture. A human megakaryoblastic cell line, Meg-J was established in our laboratory from a patient with chronic myeloid leukemia of blastic crisis. Meg-J cells were maintained in RPMI 1640 medium(GIBCO, USA) containing 10% heat-inactivated fetal calf serum(FCS) at 37°C in a humidified atmosphere of 5% CO_2. In experi-

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TABLE 1

GATA-1	Sense	5′-CAGTAAACGAGCAGGTACTC-3′
	Antisense	5′-CATAAAGCCACCAGCTGGTC-3′
GATA-2	Sense	5′-AGCCGGCACCTGTTGTGCAA-3′
	Antisense	5′-TGACTTCTCCTGCATGCACT-3′
SCL/Tal-1	Sense	5′-TTGGGGAGCCGGATGCCTTC-3′
	Antisense	5′-CTCCCGGCTGTTGGTGAA-3′
Evi-1	Sense	5′-AGCAACGTCGAATCAAGACCTGCTTCAGAT-3′
	Antisense	5′-TCAGACTGTAAGAGCTCACTGGCCTCAGGT-3′
NF-E2 p45	Sense	5′-ACAGTACCATGGCCCCGTGTCCTC-3′
	Antisense	5′-AGACCAGCTCAATCTGTAGCCTCC-3′
MafK	Sense	5′-ATGACGACTAATCCCAAACCG-3′
	Antisense	5′-ACGGAACTGGATGAGATTTC-3′

ments, cells were cultured with K252a(0.3μM) and TPO(10 ng/mL) in 10 mL of culture medium, containing 10% FCS for 5 days for morphological and marker studies. For RT-PCR and gel-shift assay, cells were incubated for 1 hour.

NF-E2 p45 sense and antisense oligonucleotides. 15-base antisense oligomers toward NF-E2 p45 (5′-AGGTCTCCACAAGCACAAAGGATT-3′) were prepared (Cyclone, Milligen, Tokyo, Japan). Control sense oligonucleotides were used in each experiment. Cells were incubated with 5 μM NF-E2 sense or antisense oligos in RPMI 1640 medium containing 2.5% FCS for 5 days.

Reverse transcription-PCR. Cellular RNA was obtained from untreated and TPO plus K252a treated cells using the method of RNAzol (BIOTEX Laboratories, USA) according to the manufacturer's instructions. The first strand of cDNA synthesis and PCR was performed with the primers specific for the GATA-1, GATA-2, Tal-1/SCL, Evi-1, NF-E2 p45, and MafK gene (Table 1). The mixture was then subjected to PCR amplification, using a Perkin-Elmer Cetus thermal cycler. Cycling conditions were as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes, and extension at 72°C for 2 minutes for 30 cycles.

Ethidium bromide-stained 2% agarose gels were used to separate PCR fragments.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared and double-stranded oligonucleotide DNA was radiolabeled with γ-32P ATP and used as probes for analysis. Gel shift assay was performed by previously described methods (15). The following oligonucleotides were used for EGMSA; NF-E2 p45; 5′-TGGGGAACCTGTGCTGAGTCACTGGAG-3′ GATA-1; 5′-GGTAAAGAGATAAGGCCCAT-3′.

DNA quantitation. To analyze the ploidy distribution of cells, cells were harvested and stained with 0.1% sodium citrate solution containing 50 μg/mL propidium iodine (Sigma), 100 mg/ml RNase (Sigma) and 0.1% Triton X-100 (Sigma) at 4°C for 60 minutes. Then the DNA content was measured by a flow cytometer (EPICS Profile II, Coulter Corporation, Miami, FL).

Detection of surface platelet glycoproteins. To investigate the surface expression of markers, cells were washed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Sigma) incubated with each monoclonal antibody at 4°C for 30 min. Cells were washed again and analyzed by flow cytometry.

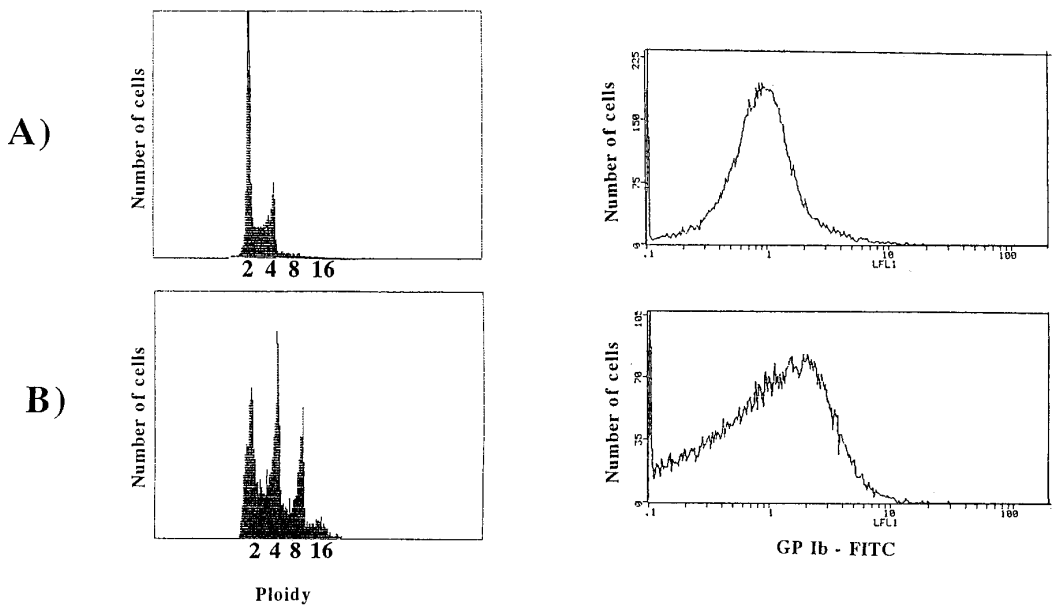

FIG. 1. Left, Histogram of the DNA content of Meg-J cells; right, expression of glycoprotein Ib (GP Ib) of cells. (A) Untreated, (B) cells cultured with K252a plus TPO for 5 days.

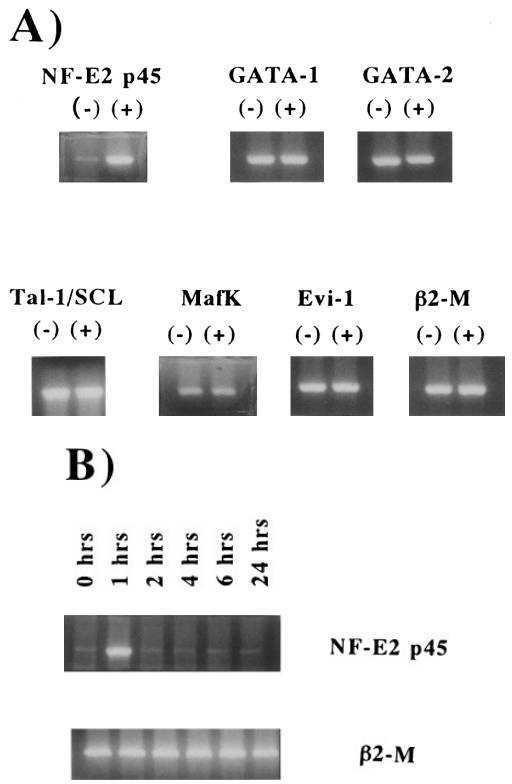

FIG. 2. (A) RT-PCR ananlysis of expression of transcription factors; NF-E2 p45, GATA-1, GATA-2, Tal-1/SCL, MafK, Evi-1, β2-Microglobulin (β2-M), respectively, of untreated control cells (−) and of cells after stimulation with K252a and TPO for 1 hour (+). (B) Sequential expression of NF-E2 p45 of cells after stimulation with K252a and TPO.

RESULTS

The effects of K252a and TPO on Meg-J cells. Meg-J cells were originally tetraploid cells. When the cells were incubated with K252a and TPO for 5 days, the ploidy of cells was increased toward higher values. The expression of GP Ib was also augmented(Fig. 1). Furthermore, morphologically, cells became larger in size and revealed multilobulated nuclei, which is characteristic of matured megakaryocytes (data not shown).

mRNA expression of transcription factors in Meg-J cells. To determine which transcription factors were crucial for the TPO plus K252a-induced polyploidization and maturation of Meg-J cells, we investigated the changes of expression of GATA-1, GATA-2, Tal-1/SCL, Evi-1 and NF-E2 before and after stimulation with TPO plus K252a for 1 hour, using RT-PCR amplification. As shown in Fig. 2A, the expression of NF-E2 p45 mRNA was increased after the culture. Sequential analysis showed that the expression of NF-E2 p45 mRNA reached a peak at 1 hour after the stimulation (Fig. 2B). No increase of expression of GATA-1, GATA-2, Tal-1/SCL, Evi-1, or MafK was observed after stimulation (Fig. 2A).

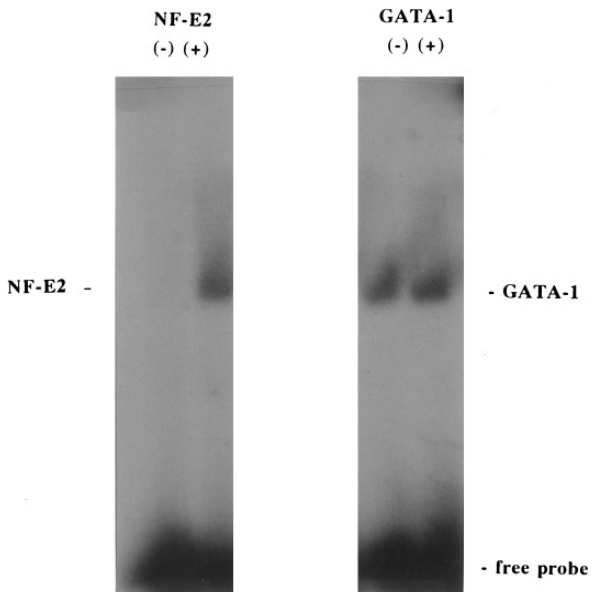

FIG. 3. Electrophoretic mobitity shift assay of NF-E2 and GATA-1 of untreated cells (−), and cells cultured with K252a plus TPO for 1 hour (+).

Electrophoretic mobility shift assay. As shown in Fig. 3, no binding activity was detected when the radiolabeled NF-E2 probe was incubated with untreated Meg-J nuclear extracts. However, after incubation for 1 hour, pronounced binding activity to the NF-E2 site was observed. In contrast, the binding of the GATA-1 probe did not differ before and after the incubation.

The effects of NF-E2 p45 antisense oligos on Meg-J cells. To determine the role of NF-E2 in the process of polyploidization, antisense oligos specific for NF-E2 p45 was added to the culture. When Meg-J cells were incubated with antisense oligos, the NF-E2 p45 transcript became undetectable, whereas the expression of β2-microglobulin(β2-M) was unchanged in the antisense-treated cells(Fig. 4). The expression of both NF-E2 p45 and β2-M was not altered by sense oligos.

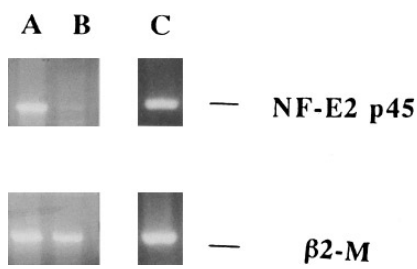

FIG. 4. RT-PCR ananlysis of expression of NF-E2 p45 and β2-Microglobulin, which was assayed after 1 hour-incubation. A, K252a plus TPO, in addition of NF-E2 p45 sense oligos; B, K252a plus TPO, in addition of NF-E2 p45 antisense oligos; C, Meg-J cells cultured with K252a plus TPO.

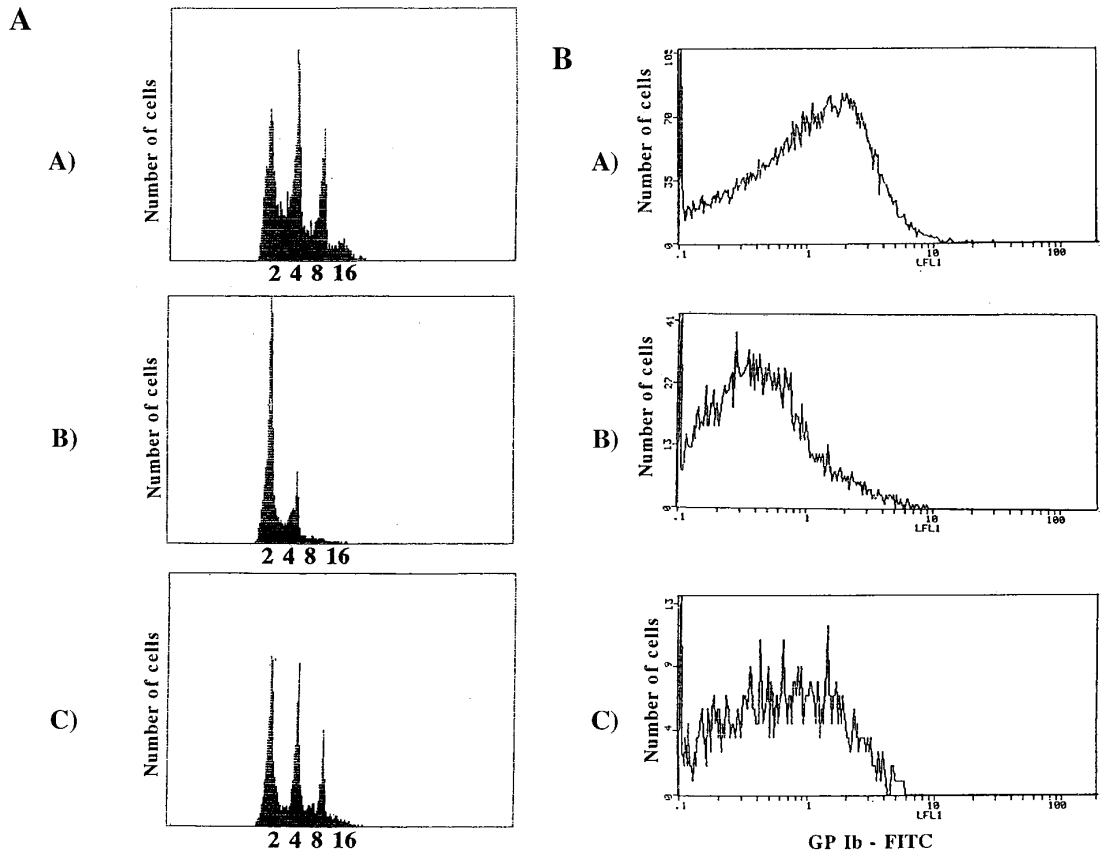

FIG. 5. A, histogram of the DNA content of cells with each culture conditions, described below. B, expression of GP Ib of each condition, described below. (A) Meg-J cells cultured with K252a plus TPO for 5 days; (B) K252a plus TPO, in addition of NF-E2 p45 antisense oligos; (C) K252a plus TPO, in addition of NF-E2 p45 sense oligos.

As shown in Fig. 5A, NF-E2 p45 antisense oligos inhibited the polyplodization, but not sense oligos did not. The inhibitory effect was significant at oligo concentrations as low as 5 μM. The expression of GP Ib was augmented after culture with TPO and K252a, whereas the expression of GP Ib was decreased by incubation with NF-E2 antisense oligos(Fig. 5B). The GP Ib expression was decreased by incubation with NF-E2 antisense oligos in whole cultured cells, whereas when the 4N cell group was gated, the augmented Ib expression was unchanged. Addition of antisense or sense oligos had no effect on untreated Meg-J cells (data not shown). Similar results were observed in another megakaryocytic cell line, CMK (data not shown).

DISCUSSION

A human megakaryoblastic cell line, Meg-J, is able to increase the ploidy and platelet membrane GP expression when cultured with K252a plus TPO. During this process, the expression of NF-E2 p45 was increased, although the expression of GATA-1, GATA-2, Tal-1/SCL, and MafK was unchanged. Abolishment of NF-E2 p45 expression with antisense oligos inhibited K252a plus TPO-induced increment of ploidy. The GP Ib expression was found decreased by incubation with NF-E2 antisense oligos when whole cultured cells were analyzed, whereas when cells with tetraploidy were gated and analyzed, GP Ib expression was not decreased. It is apparent that polyploidization is accompanied by an increase in cell size, which subsequently causes a shift of GP Ib expression toward higher values in a flow cytometer. Therefore the reduction of GP Ib expression of K252a plus TPO-treated cells by NF-E2 antisense oligos might be a secondary event accompanying the abrogation of polyploidization by NF-E2 antisense oligos. These findings suggest that NF-E2 is essential for the megakaryocytic polyploidization of Meg-J cells.

NF-E2 is a heterodimer of two components of the basic leucine zipper proteins, NF-E2 p45 and NF-E2 p18/MafK(12-14). The expression of NF-E2 p45 is hematopoietically restricted, whereas p18 is widely expressed. NF-E2 had been reported to be essential for human globin synthesis in in vitro studies(16). Orkin et al. generated mice lacking NF-E2 p45 and NF-E2 p18, respectively(17-19). In NF-E2 p45 −/− mice, anemia was mild, but thrombocytopenia was severe, and

subsequently the mice died of hemorrhage. In the mutant megakaryocytes, polyploidization and expression of GPs were not impaired, but the formation of demarcation membranes and granules was poor. Subsequently, they generated NF-E2 p18 −/− mice, which showed normal hematopoiesis(19). Our results that the abolishment of NF-E2 p45 suppressed polyploidization differ from those obtained in the study of knockout mice. It is unlikely that the effects of NF-E2 p45 obtained here are special events restricted to Meg-J cells, because effects similar to NF-E2 p45 were observed in another megakaryoblastic cell line, CMK (data not shown). It is possible that in the knockout mice, alternative mechanisms which compensated for the dysfunction of NF-E2 p45 could have occurred, resulting in the normalization of polyploidization.

It has been reported that transfection of the GATA-1 gene to the early myeloid cell line 416B induced the differentiation toward the megakaryocytic lineage, but not toward the erythroid lineage(20). Furthermore, Orkin et al. recently generated the lineage-selective GATA-1 knockout mouse and showed that GATA-1 was critical in megakaryocytopoiesis(21). The mutant mice had markedly reduced platelet numbers, deregulated megakaryocyte proliferation and impaired cytoplasmic maturation. However, in our experiments, GATA-1 expression and the binding capacity of GATA-1 were not changed before and after stimulation. The reason for the difference between knockout mice study and our results is unclear, however, might be due to the difference of experimental design used. Other transcription factors including GATA-2, Tal-1/SCL, and Evi-1 showed no change in the expression after treatment with TPO and K252a.

In conclusion, our study has demonstrated that NF-E2 p45 is essential for megakaryocytic polyploidization in some megakaryocytic cell lines.

ACKNOWLEDGMENTS

We thank Professor Norio Hayashi of the Department of Biochemistry, Tohoku University, and Dr. Naoko Minegishi of the Institute of Basic Medical Science, Tsukuba University, for teaching. We also thank Kirin-Brewery Company Limited for supplying TPO. This work is supported in part by a grant-in-aid from the Ministry of Education, Science, and Culture, Japan, and by a grant from the Sankyo Foundation of Life Science.

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