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Thrombopoietin-Induced Differentiation of a Human Megakaryoblastic Leukemia Cell Line, CMK, Involves Transcriptional Activation of p21$^{WAF1/Cip1}$ by STAT5

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Although thrombopoietin (TPO) is known to play a fundamental role in both megakaryopoiesis and thrombopoiesis, the molecular mechanism of TPO-induced megakaryocytic differentiation is not known. In a human megakaryoblastic leukemia cell line, CMK, that showed some degree of megakaryocytic differentiation after culture with TPO, the cyclin-dependent kinase (Cdk) inhibitor p21$^{WAF1/Cip1}$, but not p27^{Kip1}, p16^{INK4A}, p15^{INK4B}, or p18^{INK4C}, was found to be upregulated in an immediately early response to TPO. The expression of p21 was found to be sustained over a period of 5 days by treatment with TPO in large polyploid cells that developed in response to TPO, but not in small undifferentiated cells, indicating a close correlation between the ligand-induced differentiation and p21 induction in CMK cells. To examine potential roles of Cdk inhibitors in megakaryocytic differentiation, CMK cells were transfected with the p21, p27, or p16 gene, together with a marker gene, β-galactosidase, and were cultured with medium alone for 5 days. The ectopic expression of p21 or p27 but not of p16 led to induction of megakaryocytic differentiation of CMK cells. Overexpression of the N-terminal domain (amino acids [aa] 1 to 75) of p21 was sufficient to induce megakaryocytic differentiation, whereas that of the C-terminal domain (aa 76 to 164) had little or no effect on morphological features. Furthermore, we found that although TPO induced tyrosine phosphorylation of both STAT3 and STAT5 in CMK cells, only STAT5 showed binding activities to potential STAT-binding sites that locate in the promoter region of p21 gene (p21-SIE sites), thereby leading to transactivation of p21. These results suggested that p21 induction, possibly mediated through activated STAT5, could play an important role in TPO-induced megakaryocytic differentiation.

Thrombopoietin (TPO) is a novel hematopoietic growth factor identified as a ligand for the c-*mpl* proto-oncogene that is a member of the hematopoietin receptor superfamily with high sequence similarity to the receptors for erythropoietin (EPO) and granulocyte-colony-stimulating factor (G-CSF) (for a review, see reference 29). The c-*mpl* proto-oncogene is expressed in hematopoietic tissues, particularly in CD34$^+$ hematopoietic progenitor cells, megakaryocytes, and platelets (59), whereas its ligand TPO is detected primarily in the liver, kidney, and smooth muscle, with lesser amounts present in the spleen and bone marrow (8). It has been shown that daily infusion of TPO into mice or nonhuman primates induces a marked increase in the counts of platelets, megakaryocytes, and megakaryocytic progenitor cells (13, 30). Furthermore, c-Mpl- or TPO-deficient mice generated by gene targeting have been demonstrated to exhibit a striking decrease in the number of platelets and megakaryocytic progenitor cells (9, 19). These findings indicate that the TPO–c-Mpl system is a physiological regulator of platelet and megakaryocyte production.

Like many other hematopoietic growth factor receptors, the TPO receptor c-Mpl does not contain any recognized kinase domain, nucleotide binding site, or enzymatic motif in the cytoplasmic domain (59). Upon ligand binding, however, c-Mpl has been shown to transmit a series of biochemical events, including tyrosine phosphorylation and activation of Janus family of protein tyrosine kinases (JAKs), signal transducers and activators of transcription (STATs), phosphatidylinositol 3-kinase (PI 3-kinase), and Shc (20, 41, 42, 47, 50). Using mutational analysis of the c-Mpl cytoplasmic domain, Gurney et al. have shown that the membrane proximal region including box 1 and box 2 motifs is able to induce both cellular proliferation and activation of the JAK-STAT signal transduction pathway (20). Furthermore, Porteu et al. have recently demonstrated that the membrane proximal region is necessary for both TPO-induced mitogenesis and maturation of a human UT7 megakaryoblastic leukemia cell line and that the distal region (amino acids [aa] 71 to 94) is dispensable for proliferation but required for differentiation (50). However, it is not known yet which signaling molecules are involved in the process of TPO-induced proliferation and differentiation of megakaryocytic progenitor cells.

The growth and differentiation of hematopoietic cells are tightly regulated in a cell cycle-dependent manner. Numerous studies have shown that the cell cycle is controlled by a series of regulatory molecules called cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitors (for a review, see reference 55). Among these molecules, p21, also known as WAF1 (wild-type p53-activated fragment 1) (11), Cip1 (Cdk-interacting protein 1) (23), or Sdi1 (senescent cell-derived inhibitor 1) (46), is a dual inhibitor of Cdks and the replication factor PCNA (proliferating-cell nuclear antigen), thereby leading to

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cell cycle arrest and inhibition of DNA replication, respectively (5, 34, 60). Expression of $p21^{WAF1/Cip1}$ is known to be regulated by the tumor suppressor protein p53; in response to DNA-damaging agents such as irradiation or anticancer agents, the expression level of p53 increases, resulting in induction of p21 (11, 12, 35). However, p53-independent expression of p21 is observed in a number of tissues over the course of murine development (48) and can be induced in vitro by a number of agents, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), retinoic acid, vitamin D_3, okadaic acid, platelet-derived growth factor, and interleukin-2 (IL-2) (39). Recently, several groups of investigators have reported a strong correlation between rising p21 levels and differentiation of muscle cells, keratinocytes, and human HL-60 and U937 leukemia cells (1, 21, 27, 40). The rise of p21 was independent of p53, and, in the case of muscle cells, p21 seemed to be induced by MyoD, a classic muscle cell differentiation protein (21). These findings led us to hypothesize that p21 might be involved in some aspects of TPO-induced megakaryocytic differentiation.

In the present study, therefore, we examined the involvement of p21 and other Cdk inhibitor families, including $p27^{Kip1}$, $p16^{INK4A}$, $p15^{INK4B}$, and $p18^{INK4C}$ (18, 22, 24, 49, 54, 58), in megakaryocytic differentiation of the p53-deficient human megakaryoblastic leukemia cell line CMK (52, 57) and also investigated the regulation of p21 expression by TPO. Here, we show that megakaryocytic differentiation of CMK cells is closely associated with increased expression of p21 and that STAT5 activation appears to be involved in TPO-induced upregulation of p21. Furthermore, we demonstrate that ectopic expression of p21 or p27 is sufficient to induce megakaryocytic differentiation of CMK cells. Thus, our data provide a unique evidence for the role of two downstream targets of c-Mpl, STAT5 and p21, in TPO-induced differentiation of megakaryocytic cells.

MATERIALS AND METHODS

Reagents and antibodies. Highly purified recombinant human TPO (rhTPO), rhIL-3, rhIL-6, and rhGM-CSF were provided by Kirin Brewery Company Ltd. (Tokyo, Japan). Highly purified rh gamma interferon (rhIFN-γ) was provided from Shionogi Pharmaceutical Company Ltd. (Tokyo, Japan). Cytosine arabinoside (Ara-C), TPA, actinomycin D, and cycloheximide (CHX) were purchased from Sigma (St. Louis, Mo.). AP1 (anti-human GP Ib α) and AP2 (anti-human GP IIb-IIIa complex) monoclonal antibodies (MAbs) were generously provided by T. Kunicki (Scripps Research Institute, La Jolla, Calif.). Antiphosphotyrosine, a murine MAb, was supplied from B. Drucker (Oregon Health Science University, Portland, Oreg.). Anti-STAT3, anti-STAT5b Abs, and anti-Cdk2 rabbit antiserum were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.) and Pharmingen (Tokyo, Japan), respectively. Anti-STAT1 and anti-p21 MAbs for immunoblotting were purchased from Transduction Laboratories (Lexington, Ky.). Anti-p21 rabbit polyclonal Ab directed against the C terminus of p21 was purchased from Santa Cruz Biotechnology, Inc., and was used for immunohistochemical staining.

Plasmid construct. Human p15 and murine p18 cDNAs were generously supplied from K. Kataoka (Institute of Medical Science, Tokyo University, Tokyo, Japan) and C. Sherr (Howard Hughes Medical Institute, Memphis, Tenn.), respectively. Expression vectors of human p21 (pcDSR α-Sdi1), human p16 (CMV-p16), and murine p27 (pCMV5/kip p27) were kindly provided by A. Noda (Meiji Institute of Health Science, Odawara, Japan), T. Hama (Cancer Research Institute, Tokyo, Japan), and H. Kiyokawa (Memorial Sloan-Kettering Cancer Center, New York, N.Y.), respectively. Truncated mutants of human p21 were generated by PCR. Briefly, the N-terminal domain of p21 (aa 1 to 75) was amplified by PCR with oligonucleotide p21N5 (5′-ATAGCGGCCGCACCGA GGCACTCAGAGGAGGCGC-3′) in combination with p21N3 (5′-TTTGCGG CCGCTTACTTGGGCAGGCCAAGGCCCCGCA; the stop codon is underlined). The C-terminal domain of p21 (aa 76 to 164) was obtained with oligonucleotides p21C5 (5′-ATAGCGGCCGCCCACCATGCTCTACCTTCCC ACGGGGCC-3′; the Kozak consensus sequence and start codon are underlined) and p21C3 (5′-TTTGCGGCCGCTTCCAGGACTGCAGGCTTCC-3′). Expression vectors of the N- and C-terminal domain of p21 were generated from an expression vector, pOPRSVI-CAT (Stratagene, La Jolla, Calif.), by replacing the chloramphenicol acetyltransferase (*CAT*) gene with *Not*I-digested PCR fragments. An expression vector of β-galactosidase (BOS-LacZ) was constructed by subcloning β-galactosidase cDNA into the BSTXI site of an expression vector, pEF-BOS (43).

Cell lines and cultures. CMK, a human megakaryoblastic leukemia cell line (52), was kindly provided from Otsuka Pharmaceutical Company, Ltd. (Tokushima, Japan). CMK cells lack p53 expression owing to gross rearrangement in one allele and deletion in the other (57). CMK cells were cultured in RPMI 1640 (Nakarai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; Flow, North Ryde, Australia) at 37°C.

Morphological analysis. CMK cells were cultured for 5 days in RPMI 1640 supplemented with 10% FCS in the presence or absence of TPA (10 nM), rhTPO (30 ng/ml), or Ara-C (50 nM) or in combination. The morphological characteristics of cultured cells were determined by staining the cytospin preparations (Shandon, Pittsburgh, Pa.) with May-Grunwald-Giemsa stain.

Flow cytometry. The surface phenotypes of cells were examined by the indirect immunofluorescence method on a FACSort cell sorter (Becton Dickinson, Oxnard, Calif.). The DNA contents of cultured cells were quantitated by staining with propidium iodide (PI) and were analyzed on a FACSort cell sorter as previously described (56).

Northern blot analysis. The isolation of total cellular RNA and the method for Northern blotting were described previously (36).

Immunoprecipitation, electrophoresis, and immunoblotting. Immunoprecipitation, gel electrophoresis, and immunoblotting were performed according to methods described previously (37). Briefly, CMK cells cultured with or without rhTPO and/or Ara-C were lysed in lysis buffer, and insoluble material was removed by centrifugation. For immunoprecipitation, the precleared lysates obtained from 10^7 cells were incubated with 1 μg of anti-STAT1, anti-STAT3, or anti-STAT5b Ab followed by the addition of protein G Sepharose beads. The immunoprecipitates or the whole-cell lysates (15 μg per each lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, Mass.). After blocking of residual binding sites on the filter, immunoblotting was performed with an appropriate Ab. Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (DuPont NEN, Boston, Mass.).

Size fractionation of CMK cells by centrifugal elutriation. After 5 days of culture with rhTPO (30 ng/ml), CMK cells (2×10^8 cells) were resuspended in 10 ml of culture medium and were loaded onto a cell elutriator (SRR6Y; Hitachi Industrial, Tokyo, Japan) running at 1,800 rpm. All operations were performed at 4°C in RPMI containing 0.1% bovine serum albumin. Four fractions (each fraction consists of 100 ml) were collected according to cell size by changing the flow rate to 20, 30, 45, and 80 ml/min, successively. Small and large cell fractions that eluted at flow rates of 20 and 80 ml/min, respectively, were subjected to further analyses.

Cdk2-associated histone H1 kinase assay. Cdk2 was immunoprecipitated from equal amounts of cell lysates obtained from size-fractionated small and large CMK cells. In vitro kinase assaying was performed in kinase buffer containing 5 μg of histone H1 (Boehringer, Mannheim, Germany) and 20 μCi of [γ-^{32}P]ATP for 30 min at 30°C (7). After addition of protein loading buffer, samples were boiled and subjected to SDS-PAGE. The gels were stained with Coomassie blue to confirm the amounts of immunoprecipitates and then destained, dried, and subjected to autoradiography.

EMSA. CMK cells were serum starved for 6 h and then stimulated with rhTPO (30 ng/ml) for 15 min. Nuclear extracts were isolated according to the procedures described by Sadowski and Gilman (51). In p21 promoter, three different types of potential STAT-binding sites (the palindromic sequence TTCNNNGAA) are located at nucleotides −640 (p21-SIE1), −2540 (p21-SIE2), and −4183 (p21-SIE3) from the TATA promoter site of p21 (6). These three types of double-stranded oligonucleotides were synthesized, and their sequences are as follows: p21-SIE1, 5′-GATCTCCTTCCCGGAAGCA-3′; p21-SIE2, 5′-GATCCTTTCT GAGAAATGG-3′; p21-SIE3, 5′-GATCCCTCAGTCTTCTTGGAAATTC-3′. These oligonucleotides were end labeled with [γ-^{32}P]ATP by kinase reaction and were used as probes for electrophoretic mobility shift assaying (EMSA). One more double-stranded oligonucleotide with a mutated recognition site, 5′-GAT CCTATCTGAGAGATGG-3′ (p21-SIE2m; mutated nucleotides are underlined), was synthesized and used as a competitor oligonucleotide. EMSA was performed as previously reported (6). Briefly, nuclear extract (15 μg of each sample) was incubated in 30 μl of binding buffer containing 2 μg of poly(dI-dC) (Pharmacia Biotechnology, Uppsala, Sweden) and labeled probe (20,000 cpm) for 20 min at room temperature. The reaction mixture was loaded onto a 5% polyacrylamide gel in 1× Tris-borate-EDTA buffer, electrophoresed, dried, and subjected to autoradiography. In the competition assays, nuclear extracts were preincubated with a 200-fold molar excess of unlabeled competitor oligonucleotides, and a binding reaction with labeled probe was performed. Binding of STAT proteins to the three SIE sites in the p21 promoter was examined by electrophoretic mobility supershift assaying. The nuclear proteins were incubated with 1 μg of anti-STAT1, anti-STAT3, or anti-STAT5b Abs for 30 min at 4°C, and then the binding reaction was performed with labeled probes.

Luciferase assaying. Four types of double-stranded oligonucleotides with potential STAT-binding sequences were subcloned immediately upstream of the murine minimal JunB promoter (−42 to +136) linked to the luciferase gene. They are as follows: three tandem repeats of the fragment from the *GBP* gene which contains overlapping GAS and ISRE, (5′-TCGACACTTTCAGTTTCAT

ATTACTCTAATAC-3′; (recognition sites are underlined) recognized by both STAT1 and STAT2 (32), eight tandem repeats of APRE from the rat macroglobulin gene (5′-GCGCCTTCTGGGAAGATCCTTACGGGAATTCAG-3′; recognition site is underlined) with binding activity to STAT3 (61), four tandem repeats of p21-SIE2, and four tandem repeats of p21-SIE2m. CMK cells (10^7 cells) were electroporated (250 V, 960 μF) (Bio-Rad Laboratories, Richmond, Calif.) with 15 μg of reporter gene together with 15 μg of BOS-LacZ. After 12 h of culture, CMK cells were serum starved for 8 h and then stimulated with rhTPO (30 ng/ml) or rhIFN-γ (1,000 U/ml) for 5 h. The cells were lysed with lysis buffer supplied by the manufacturer (Promega, Madison, Wis.), and β-galactosidase activity was measured to normalize the transfection efficiency by a previously reported method (43). Adjusted amounts of cell lysates containing equal levels of β-galactosidase activity were subjected to luciferase assaying. Luciferase activity was measured according to the instructions of the manufacturer (Promega) on luminometer LB96P (Berthold Japan, Tokyo, Japan).

Transient transfection and detection of β-galactosidase activity. CMK cells were cotransfected with full-length expression vector (pcDSR α-Sdi1), the N terminus (pOPRSVI-p21N) and C terminus (pOPRSVI-p21C) of human p21, murine p27 (pCMV5/kip p27), human p16 (CMV-p16), or empty expression vector (pcDSR α) in combination with BOS-LacZ by using Lipofectin (Gibco BRL, Gaithersburg, Md.) according to the manufacturer's instructions. Briefly, a mixture of 3 μg of DNA (2.7 μg of each expression vector and 0.3 μg of BOS-LacZ) and 20 μl of Lipofectin was added to CMK cells (10^6) resuspended in 800 μl of Opti-MEM (Gibco BRL) in a 35-mm dish, and the mixtures was incubated for 5 h, followed by the addition of 1 ml of culture medium. The cells were washed 24 h after transfection, resuspended in culture medium, and cultured for 5 days. To detect β-galactosidase activity in the cells, the cytospin preparations were fixed with 0.5% glutaraldehyde for 10 min at room temperature and washed extensively in phosphate-buffered saline (PBS) with 1 mM $MgCl_2$. The cells were then stained with PBS containing $K_3Fe(CN)_6$ (20 mM), $K_4Fe(CN)_6$ (20 mM), $MgCl_2$ (1 mM), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 1 mg/ml) for 3 h at 37°C. The reaction product of β-galactosidase was blue. The morphological characteristics of the cells were examined after counterstaining with Nuclear Fast Red.

Immunohistochemical staining of p21. p21-transfected cells cultured for 5 days were attached to sylane-coated slide glasses (DAKO, Glostrup, Denmark) by cytospinning and were fixed with 4% paraformaldehyde. To detect p21 protein, a three-step immunofluorescence method was performed. The cell preparations were incubated with 0.1% Triton-X in 0.01 M PBS for permeabilization for 10 min, rinsed three times in PBS, and incubated with Block Ace (Dainihon Pharmaceutical Co. Ltd., Suita, Japan) for 30 min. The cell preparations were then incubated with anti-p21 rabbit polyclonal Ab raised against the C terminus of p21 (Santa Cruz Biotechnology, Inc.) diluted 400:1 in PBS for 40 min, biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Inc., Burlingame, Calif.) diluted 200:1 in PBS, and fluorescein avidin D (Vector) diluted 500:1 in PBS for 30 min. Before mounting with aqueous mounting medium (Perma-Fluor; Immunon, Pittsburgh, Pa.), the cells were incubated for 5 min with 1 μg of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes Inc., Eugene, Oreg.) for nuclear staining and were washed with the rinsing solution described above.

RESULTS

Effect of rhTPO, Ara-C, and TPA on megakaryocytic differentiation of CMK cells. The morphological characteristics, surface phenotypes, and DNA contents of CMK cells were examined after culture for 5 days with medium alone, rhTPO, or Ara-C or in combination. Since DNA-specific antitumor agents such as Ara-C are reported to enhance the cytokine-induced differentiation of a human leukemia cell line (2), we used Ara-C in combination with rhTPO to augment the effect of rhTPO. CMK cells were also cultured with TPA, which was shown to be a potent inducer of megakaryocytic differentiation (14, 52). As shown in Fig. 1A, a small but detectable fraction (~4%) of CMK cells showed megakaryocytic differentiation after culture with rhTPO (30 ng/ml), whereas CMK cells cultured with medium alone were composed of small undifferentiated blast cells. Furthermore, although Ara-C alone had little or no effect on megakaryocytic differentiation of CMK cells (data not shown), the treatment of CMK cells with rhTPO in combination with Ara-C (50 nM; at this concentration, growth of CMK cells was reduced by half) resulted in morphological alterations indicative of megakaryocytic maturation in a substantial proportion (~20%) of CMK cells (Fig. 1A). In the case of TPA (10 nM), most CMK cells exhibited megakaryocytic differentiation (Fig. 1A).

Flow cytometry analysis with anti-GP Ib α (AP1) and anti-GP IIb/IIIa (AP2) MAbs also demonstrated that rhTPO treatment led to a slight increase in GPIb and GPIIb/IIIa expression (Fig. 1B). Furthermore, the expression levels of GPIb and GPIIb/IIIa were significantly augmented by treatment with the combination of rhTPO and Ara-C (rhTPO–Ara-C) and by that with TPA (Fig. 1B). DNA content analysis revealed that 54% of CMK cells cultured with medium alone were in G_0/G_1 (4N) and that 8% of the cells were in G_2/M (8N) (Fig. 1B), because CMK cells are originally in tetraploid. rhTPO treatment of CMK cells induced a slight increase in the 8N fraction up to 20%; combination treatment with rhTPO–Ara-C led to a striking increase in the 8N fraction up to 46% in addition to the appearance of the 16N faction (Fig. 1B). TPA treatment yielded the most prominent polyploid formation up to 64N (Fig. 1B).

Changes in expression of Cdk inhibitors during megakaryocytic differentiation of CMK cells. In order to characterize the mechanism underlying megakaryocytic differentiation of CMK cells, we investigated the effects of rhTPO, rhTPO–Ara-C, and TPA on expression of several Cdk inhibitors, including p21, p27, p16, p15, and p18 genes, for up to 120 h (Fig. 2A). Treatment with Ara-C alone did not affect expression of these Cdk inhibitors (data not shown). When CMK cells were cultured with rhTPO, p21 expression was rapidly induced (~2 h), peaked at 4 h, and declined thereafter. In the case of treatment with the combination rhTPO–Ara-C or TPA, induction of p21 was similarly rapid (~2 h) but the increased expression of p21 continued over the 120-h test period. By contrast, expression of p16 and p15 was not detected before or after each treatment. Expression of p18 and p27 was detectable even before treatment, but none of the treatment significantly augmented such expression during the test period.

In addition to p21 mRNA expression, the induction of p21 protein was also examined by immunoblotting with anti-p21 Ab (Fig. 2B). Treatment of CMK cells with rhTPO, rhTPO–Ara-C, or TPA resulted in upregulation of p21, and the levels of p21 induction were almost comparable to those of p21 mRNA induction.

Sustained expression of p21 in mature megakaryocytes developed in response to TPO. In order to further analyze a relationship between TPO-induced megakaryocytic differentiation and p21 expression, we isolated mature megakaryocytes that developed in response to rhTPO. CMK cells were cultured with rhTPO alone for 5 days and were then size fractionated with a cell elutriator. The small cell fraction was exclusively (over 99%) composed of undifferentiated blastic cells, and the large cell fraction was composed of mature polyploid megakaryocytes with a purity about 75% by May-Grunwald-Giemsa staining (Fig. 3A). In agreement with morphological features, large cells exhibited prominent ploidy up to 64N, while small cells remained between 4N and 8N by DNA content analysis (Fig. 3B). GPIb and GPIIb/IIIa were found to be more intensely expressed in large cells than in small cells (Fig. 3B). Furthermore, it was noted that expression of p21 was detected in large cells but not in small cells by Western blot analysis (Fig. 3C). In addition, Cdk2 activity in large cells was found to be significantly lower compared with that in small cells, possibly due to an inhibitory effect of p21 (Fig. 3D). These results suggested that prolonged expression of p21 is closely associated with megakaryocytic differentiation of CMK cells.

Regulation of p21 gene expression. It has been shown that expression of p21 mRNA is controlled at both transcriptional and posttranscriptional levels (1, 35, 53). Therefore, we examined regulation of p21 expression by using actinomycin D and

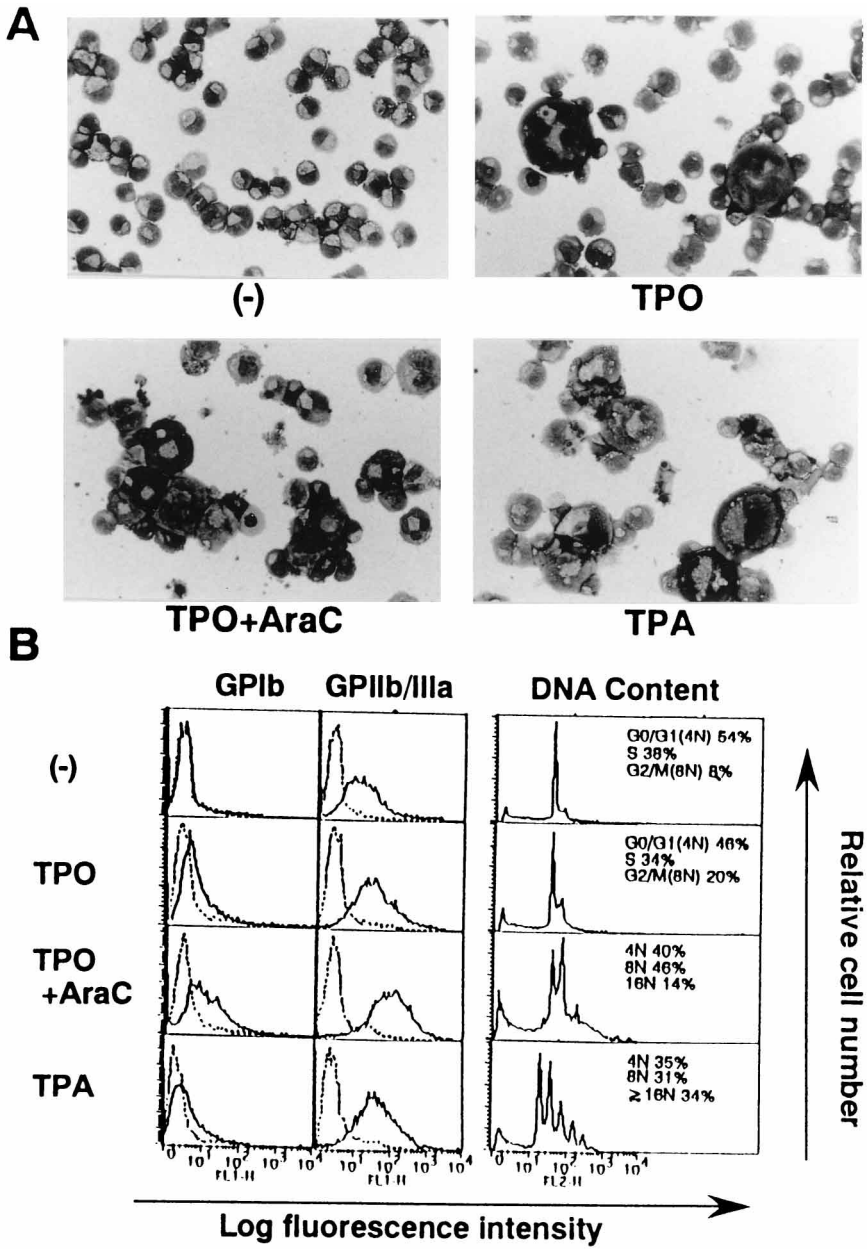

FIG. 1. Effects of rhTPO, rhTPO–Ara-C, or TPA on megakaryocytic differentiation of CMK cells at morphological, surface phenotyptic, and DNA content levels. The analyses were performed after culture for 5 days under the condition indicated. (A) Light micrograph of CMK cells. Cytocentrifugation preparation from each culture was stained with May-Grunwald-Giemsa stain (magnification, ×100). (B) Flow cytometric analyses of CMK cells. The expression levels of GPIb and GPIIb/IIIa were examined by staining with AP1 and AP2 MAbs (———) or control Ab of the same isotype (– – –). The DNA contents of CMK cells were examined by staining with PI solution and analyzed on a FACSort cell sorter.

CHX, which inhibit RNA synthesis and protein synthesis, respectively. CMK cells were preincubated with actinomycin D (10 μg/ml) or CHX (40 μg/ml) for 2 h and then treated with rhTPO, TPO–Ara-C, or TPA for 4 h. Pretreatment with actinomycin D was found to completely block rhTPO-, rhTPO–Ara-C- or TPA-induced p21 mRNA expression by Northern blot analysis (Fig. 4A), suggesting that induction of p21 mRNA was transcriptionally regulated by each treatment. By contrast, p21 mRNA expression by rhTPO, rhTPO–Ara-C, or TPA was significantly increased by pretreatment with CHX (Fig. 4A). These results suggested that p21 is an immediate-early response gene and that its induction does not require protein synthesis in CMK cells, although the levels of p21 mRNA expression may be posttranscriptionally regulated by labile proteins. Furthermore, because of p53 deficiency in CMK cells (57), it was suggested that p21 expression was transcriptionally regulated in a p53-independent manner.

In addition to TPO, CMK cells express receptors for IL-3, IL-6, granulocyte-macrophage (GM)-CSF, and IFN-γ, although these cytokines are not capable of inducing megakaryocytic differentiation of CMK cells. In order to determine if other cytokine stimuli could induce p21 expression, p21 mRNA expression was examined by Northern blot analysis after 4 h of treatment of CMK cells with rhTPO (30 ng/ml),

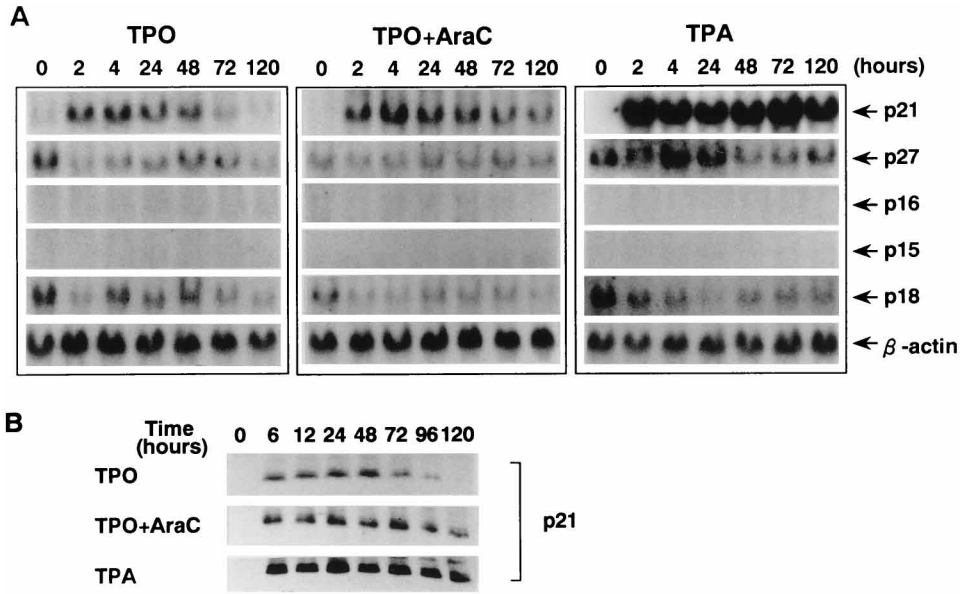

FIG. 2. (A) Northern blot analysis of Cdk inhibitor expression during cultures with rhTPO-, rhTPO–Ara-C, and TPA. Total cellular RNA was isolated at the times indicated, and 15 μg of each sample was electrophoresed on formaldehyde agarose gels. The filters were hybridized with ^{32}P-labeled probes for Cdk inhibitors and β-actin. (B) Induction of p21 proteins by treatment with rhTPO, rhTPO–Ara-C, or TPA. Fifteen micrograms of each whole lysate was subjected to SDS-PAGE and probed with anti-p21 Ab.

rhIL-3 (10 ng/ml), rhIL-6 (50 ng/ml), rhGM-CSF (10 ng/ml), or IFN-γ (1,000 U/ml). As shown in Fig. 4B, p21 expression was also induced by rhIL-3 and rhGM-CSF, but not by rhIL-6 or IFN-γ (Fig. 4B). However, rhTPO revealed a more durable effect on p21 expression than rhIL-3 by time course analysis (Fig. 4C). As in the case of rhIL-3, only a transient effect on p21 expression by rhGM-CSF was observed (data not shown).

TPO-mediated STAT phosphorylation and binding of STAT5 to the SIE sites in the p21 promoter. STAT proteins have been shown to be phosphorylated by a variety of cytokines such as TPO, and a number of immediate-response genes are considered to be regulated by activated STAT proteins (25, 26). Since p21 expression was induced in an immediate-early response to either rhTPO or rhTPO–Ara-C, we examined the effect of rhTPO on tyrosine phosphorylation of STAT proteins and also on their binding to the SIE sites in the p21 promoter.

Although TPO was reported to induce tyrosine phosphorylation of STAT1 and STAT3 proteins in CMK cells (20), we found constitutive tyrosine phosphorylation of STAT1 and a significantly increased amount of protein tyrosine phosphorylation of STAT3 and STAT5 from stimulation with rhTPO (Fig. 5), suggesting that STAT3 and STAT5 were activated in response to rhTPO in our CMK cells. In order to determine if the rhTPO-activated STAT proteins were involved in p21 induction, we performed EMSA using three types of probes, named p21-SIE1, -SIE2, and -SIE3, that are potential STAT-binding sites in the promoter region of p21 (6). As shown in Fig. 6, nuclear extracts from rhTPO-treated CMK cells did not show any change in DNA binding activity with p21-SIE1 (lanes 1 and 2). In contrast, the rhTPO-treated nuclear extracts came to possess DNA binding activity with p21-SIE2 (lane 4) and p21-SIE3 (lane 11). The TPO-induced complexes bound to p21-SIE2 or p21-SIE3 were competed effectively by the p21-SIE2 and p21-SIE3 oligonucleotides (lanes 5 and 12), respectively, but not by the SIE2m oligonucleotides containing a mutated recognition site (lanes 6 and 13), suggesting that the rhTPO-induced DNA binding complexes were formed in a sequence-specific manner. Furthermore, in accord with the findings on p21 gene expression (Fig. 2A), time course study of EMSA with a p21-SIE2 probe showed that the combination of rhTPO and Ara-C had a more-durable effect on DNA binding activity than rhTPO alone (Fig. 7).

In order to further determine which STAT protein was involved in the formation of DNA-binding complexes, supershift assaying was performed by preincubation of the rhTPO-stimulated nuclear extracts with anti-STAT1, anti-STAT3, or anti-STAT5b Ab (Fig. 6). Both p21-SIE2 and p21-SIE3 binding complexes were found to be supershifted by preincubation with anti-STAT5b Ab (Fig. 7, lanes 9 and 16), whereas no effect was observed with anti-STAT1 or anti-STAT3 Ab (lanes 7, 8, 14, and 15). These results suggested that rhTPO-activated STAT5, but not STAT3, may bind to both p21-SIE2 and p21-SIE3 sites, resulting in induction of p21. By contrast, nuclear extracts from TPA-treated CMK cells did not show any change in DNA binding activity to p21-SIE1, -SIE2, or -SIE3 sites (data not shown). Therefore, it was supposed that TPA induced p21 expression independently of STATs, possibly through the TPA response element in the promoter of the p21 gene that was previously identified in TPA-treated U937 cells (1).

Transactivation of the p21-SIE2 site by rhTPO. To examine changes in transactivating activities of STAT proteins by treatment with rhTPO, a reporter gene containing GAS/ISRE, APRE, p21-SIE2, or p21-SIE2m was transduced into CMK cells. Luciferase activity driven by GAS/ISRE remained at low levels before and after treatment with rhTPO, while treatment with IFN-γ augmented luciferase activity 15-fold (Fig. 8), suggesting that neither STAT1 nor STAT2 is activated by rhTPO. Although constitutive tyrosine phosphorylation of STAT1 was observed in CMK cells (Fig. 5), STAT1 appeared to be inactive until some modulations were added. In contrast, rhTPO transactivated APRE 26-fold, possibly through activation of STAT3. In addition, the p21-SIE2 element but not p21-SIE2m was found to be transactivated 19-fold in response to rhTPO. These results were largely consistent with the results from Western blot and

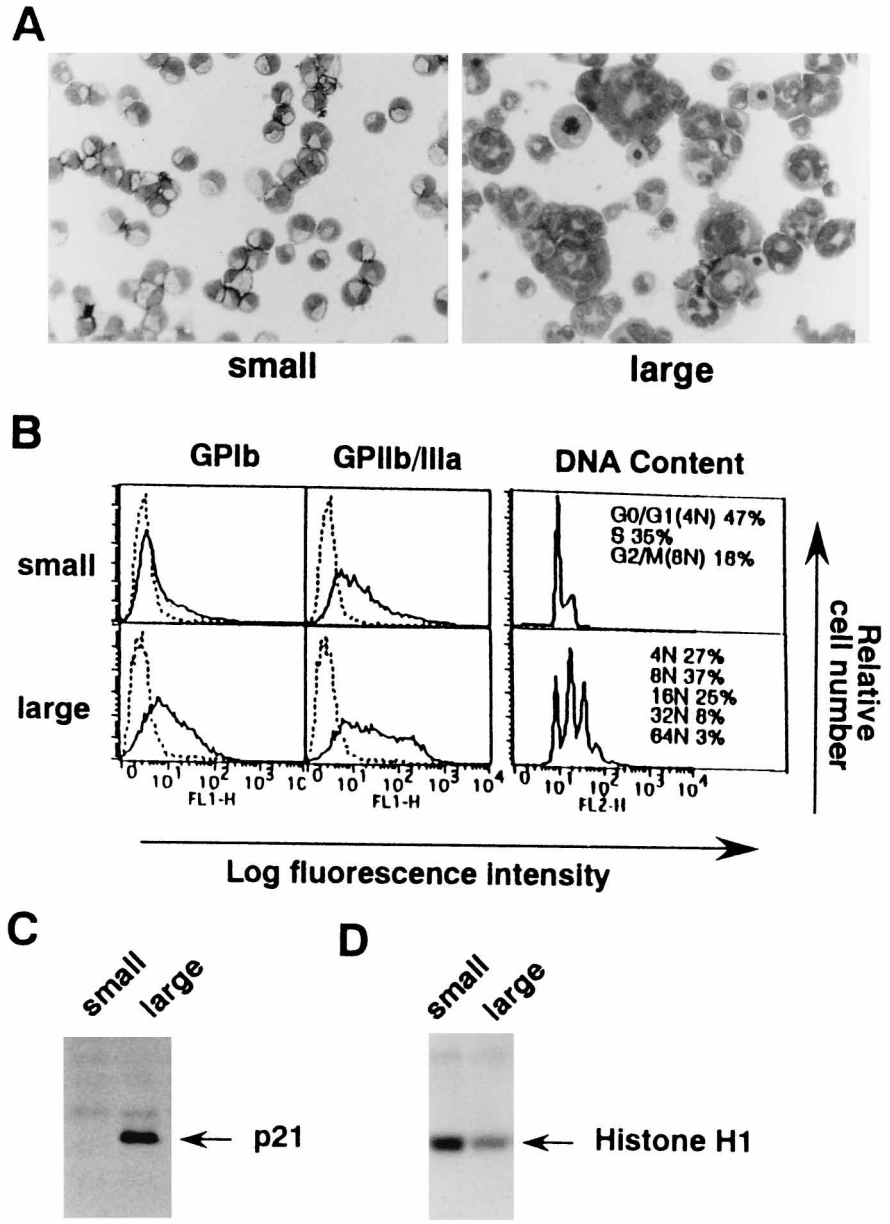

FIG. 3. Morphological, immunological, and biochemical analyses of size-fractionated CMK cells after culture with rhTPO. CMK cells were cultured with rhTPO (30 ng/ml) for 5 days and then subjected to size fractionation with a cell elutriator. (A) Light micrograph of the cells collected in small (eluted at a flow rate of 20 ml/min) and large (eluted at a flow rate of 80 ml/min) cell fractions. Cytocentrifugation preparation from each fraction was stained with May-Grunwald-Giemsa stain (magnification, ×100). (B) Flow cytometric analysis of CMK cells in each fraction. The expression levels of GPIb and GPIIb/IIIa were examined by staining with AP1 and AP2 MAbs (———) or control Ab of the same isotype (– – –). The DNA contents were examined by staining with PI solution and analyzed on FACSort cell sorter. (C) Expression of p21 in each fraction by Western blot analysis. Fifteen micrograms of each whole lysate was subjected to SDS-PAGE and probed with anti-p21 Ab. (D) Inhibition of Cdk2-associated histone H1 kinase activity by p21. Cdk2 was immunoprecipitated from equal amounts of cell lysates prepared from each fraction. In vitro kinase assaying was performed with kinase buffer containing 5 μg of histone H1 and 20 μCi of [γ-^{32}P]ATP for 30 min at 30°C.

EMSA studies and suggested that TPO-activated STAT5 could transactivate the p21-SIE2 site.

Induction of megakaryocytic differentiation of CMK cells by ectopic expression of p21. In order to assess whether increased expression of p21 was directly associated with megakaryocytic differentiation of CMK cells, the cells were transiently cotransfected with the expression vectors of p21 and β-galactosidase by lipofection and were cultured with medium alone for 5 days. The cultured cells were then stained for β-galactosidase, and their morphology was visualized by counterstaining with Nuclear Fast Red. As shown in Fig. 9, most (~95%) of the p21-transfected cells indicated by blue staining were large and had polyploid nuclei with morphological features of mature megakaryocytes (Fig. 9B), while cells transfected with an empty expression vector revealed small undifferentiated features (Fig. 9A). In addition, enforced expression of the p21 protein in large differentiated cells was confirmed by immunohistochemical staining with the anti-p21 antibody (data not shown). These results suggested that ectopic expression of p21 for 5 days was sufficient to induce megakaryocytic differentia-

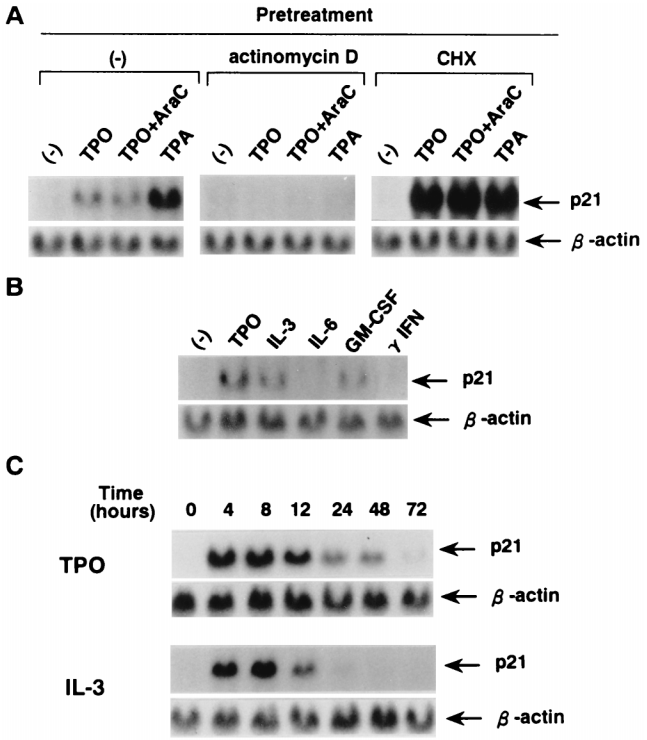

FIG. 4. Regulation of p21 mRNA expression. (A) CMK cells were preincubated in the presence or absence of actinomycin D (10 μg/ml) or CHX (40 μg/ml) for 2 h and monitored by treatment with rhTPO, rhTPO–Ara-C, or TPA for 4 h. Northern blot analysis was performed with ^{32}P-labeled probes for p21 and β-actin. (B) CMK cells were treated with rhTPO, rhIL-3, rhIL-6, rhGM-CSF, or IFN-γ, and p21 expression was examined by Northern blot analysis. (C) Changes in expression levels of p21 were investigated during culture with rhTPO or rhIL-3 by Northern blot analysis.

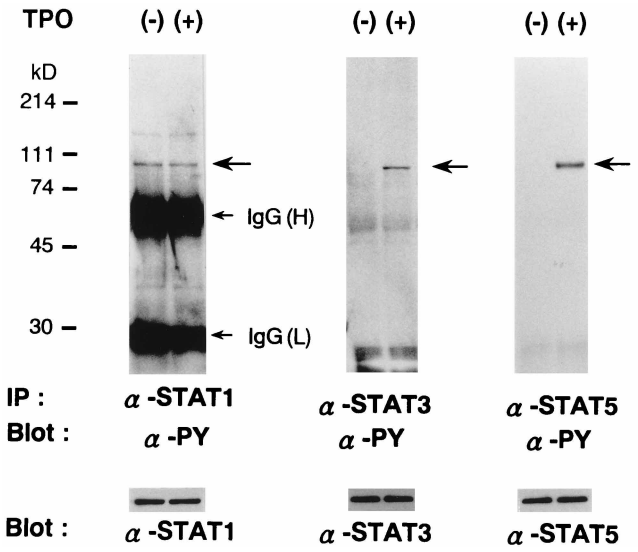

FIG. 5. TPO-induced tyrosine phosphorylation of STAT proteins in CMK cells. CMK cells were serum starved for 3 h and then either not stimulated or stimulated with rhTPO for 15 min. Total cell lysates were immunoprecipitated with anti-STAT1, anti-STAT3, or anti-STAT5b Ab. The blots were probed with antiphosphotyrosine MAb. Then, the filters were stripped and reprobed with anti-STAT1, anti-STAT3, and anti-STAT5b Abs, respectively.

tion of CMK cells. To further determine the functional domain of p21 required for inducing megakaryocytic differentiation, truncated mutant forms of p21 were transfected into CMK cells. Overexpression of the N-terminal domain of p21 revealed an effect comparable with that of full-length p21 (Fig. 9E), whereas that of the C-terminal domain failed to induce megakaryocytic differentiation (Fig. 9F). Furthermore, when CMK cells were transfected with other Cdk inhibitors, p16 and p27, overexpression of p27 led to megakaryocytic differentiation of the cells as well as that of p21 (Fig. 9C), whereas ectopic expression of p16 was not capable of affecting CMK differentiation (Fig. 9D).

DISCUSSION

By using a series of acute myeloblastic leukemia (AML) cells, we have previously shown that the proliferation of AML cells can be stimulated by rhTPO in approximately 70% of c-Mpl-positive cases (36). Furthermore, we have recently shown that rhTPO is capable of inducing megakaryocytic differentiation of AML cells, albeit in a limited number of cases (37). However, it remains to be determined how rhTPO promotes megakaryocytic differentiation of AML cells.

Since cellular differentiation has been reported to be coupled with cell cycle arrest (28), we initially investigated changes in expression levels of several Cdk inhibitors during induced differentiation of the human megakaryoblastic leukemia cell line CMK. Following culture with rhTPO, rhTPO–Ara-C, or TPA for 5 days, CMK cells showed some degree of megakaryocytic differentiation in response to rhTPO and a more prominent degree of megakaryocytic differentiation in response to rhTPO–Ara-C or TPA. Northern blot analysis demonstrated that the treatment of CMK cells with each of the agents resulted in an immediate-early induction of p21; and p21 levels remained elevated over a period of 5 days with rhTPO–Ara-C or TPA and for 2 to 3 days with rhTPO, while each of the treatments did not significantly induce or augment expression of p27, p16, p15, or p18. Furthermore, when CMK cells were size fractionated after culture with rhTPO for 5 days, p21 expression was observed in large differentiated cells but not in small undifferentiated cells. Thus, there seemed to be a close correlation between the levels of ligand-induced megakaryocytic differentiation and p21 induction in CMK cells. To examine a potential role of p21 in megakaryocytic differentiation, CMK cells were transfected with a p21 gene together with a marker gene (β-galactosidase) and were cultured with medium alone for 5 days. Morphological analysis of the cultured cells revealed that p21-transfected cells exhibited the morphology of differentiated megakaryocytes. These results suggested that enforced expression of p21 for 5 days is sufficient for inducing megakaryocytic differentiation of CMK cells and also suggest that p21 is involved in ligand-modulated megakaryocytic differentiation of the cells.

The p21 protein is known to have two functional domains; one is the N-terminal domain that shows homology to the other Cdk inhibitors with a Cdk2-binding motif (aa 53 to 58), and the other is the C-terminal domain with a PCNA-binding motif (aa 141 to 155) (5, 34, 45). The p21 protein binds directly to a Cdk in complexes formed with a cyclin and can act as an inhibitor of Cdks, resulting in cell cycle arrest (16, 23). The p21 protein also binds to a PCNA and inhibits the ability of DNA polymerase δ to extend new DNA chains but does not affect DNA repair (62). It has been shown that the G_1-S transition block is executed by a truncated mutant of p21 containing only the N-terminal domain that inhibits Cdks but not PCNA, while a truncated mutant of p21 containing the C-terminal domain

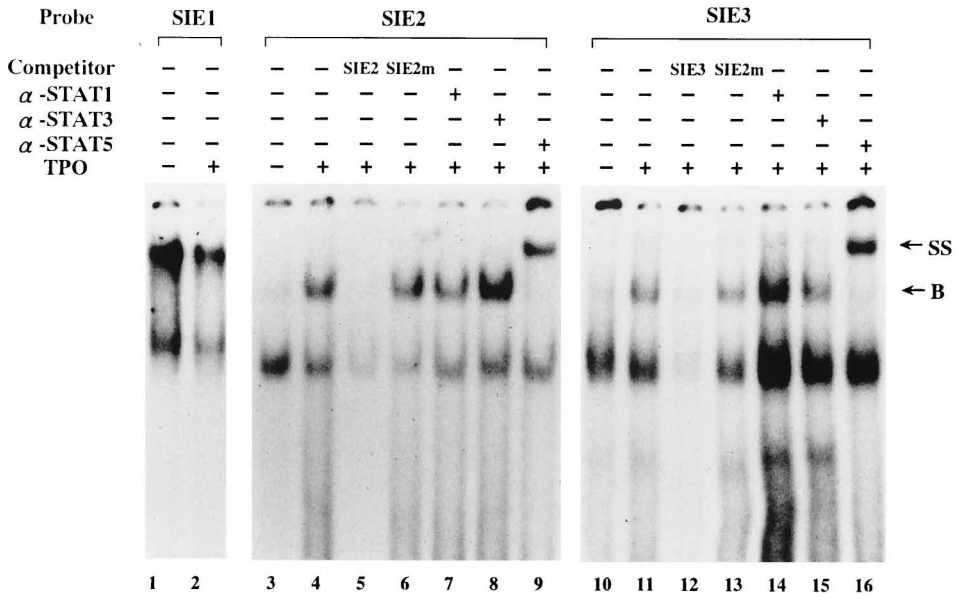

FIG. 6. Identification of p21-SIE sites binding proteins by EMSA. CMK cells were serum starved for 6 h and then either not stimulated or stimulated with rhTPO for 15 min. The nuclear lysates were incubated with the end-labeled probes (p21-SIE1, -2, or -3) for 20 min at room temperature, and then the reaction mixtures were loaded onto a 5% polyacrylamide gel. In the competition assays, nuclear extracts were preincubated with a 200-fold molar excess of unlabeled competitor oligonucleotide, and binding reaction to the labeled probe was performed. In the supershift assay, nuclear extracts were preincubated with 1 μg each of anti-STAT1, anti-STAT3, or anti-STAT5b Ab, followed by binding reactions to the labeled probes. B, TPO-induced DNA-binding complex; SS, supershifted DNA-binding complex.

showed little or no inhibitory effect on cell growth (5, 34). To determine a functional domain of p21 required for inducing megakaryocytic differentiation, in the present study we introduced expression vectors of a truncated mutant of p21 into CMK cells. Overexpression of the N-terminal domain (aa 1 to 75) of p21 was found to be capable of inducing megakaryocytic differentiation, whereas that of the C-terminal domain (aa 76 to 164) was not. These results suggested that the N-terminal domain, which was reported to induce cell cycle arrest, was also required for p21-modulated megakaryocytic differentiation of CMK cells.

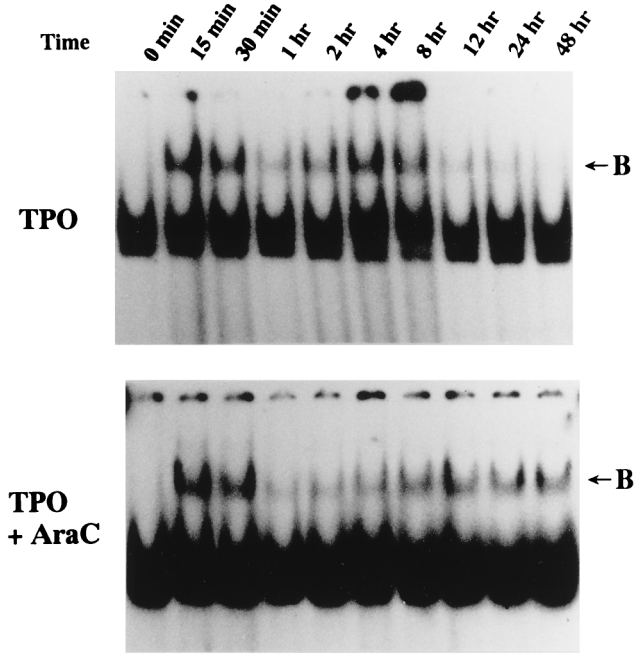

FIG. 7. Time course study of EMSA during culture with rhTPO or rhTPO–Ara-C. CMK cells were serum starved for 6 h and cultured with rhTPO or rhTPO–Ara-C under serum-free conditions for the times indicated. The nuclear lysates were isolated and incubated with the end-labeled p21-SIE2 probe for 20 min at room temperature, and then the reaction mixture was loaded onto a 5% polyacrylamide gel. B, TPO-induced DNA-binding complex.

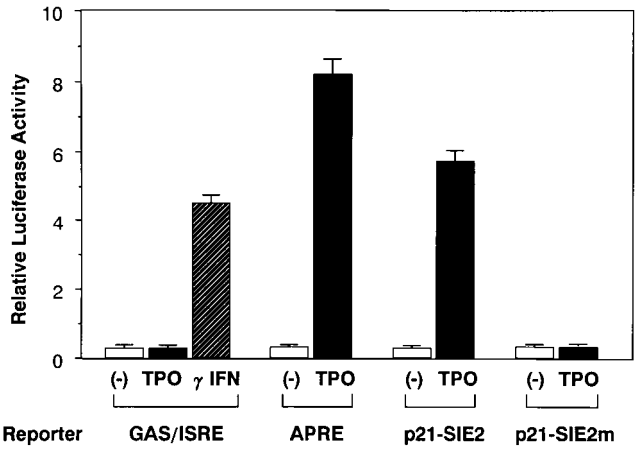

FIG. 8. Changes in transactivating activities of STAT proteins after treatment with rhTPO or IFN-γ by luciferase assaying. Various types of STAT-responsive elements (4× GAS/ISRE [with binding potential to STAT1 and STAT2], 8× APRE [with binding potential to STAT3], 4× p21-SIE2, and 4× p21-SIE2m) were subcloned immediately upstream of the murine minimal JunB promoter (−42 to +136) linked to the luciferase gene and were used as reporter genes. CMK cells were electroporated (250 V, 960 μf) with 15 μg of reporter gene together with 15 μg of BOS-LacZ. After 12 h of culture, CMK cells were serum starved for 8 h and then stimulated with rhTPO or rhIFN-γ for 5 h. Adjusted amounts of cell lysates containing equal levels of β-galactosidase activity were subjected to luciferase assays. The results are means ± standard deviations of experiments done in triplicate.

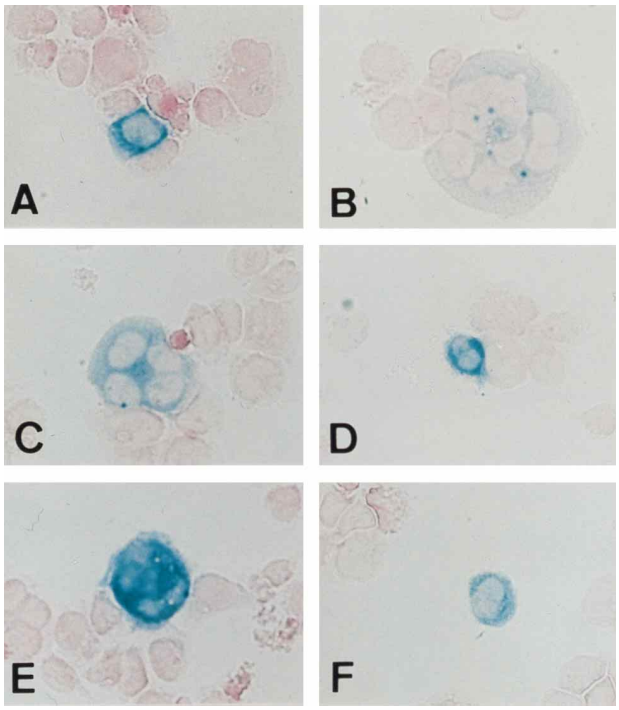

FIG. 9. Effect of the ectopic expression of Cdk inhibitors on megakaryocytic differentiation. CMK cells were cotransfected with each expression vector of Cdk inhibitors and BOS-LacZ by using lipofection. After 5 days of culture in the absence of reagent, aliquots of the cultured cells were subjected to cytospin preparations and β-galactosidase activities in the cells were visualized. The morphological characteristics of the blue cells showing β-galactosidase activity were further examined by counterstaining with Nuclear Fast Red. The ectopically overexpressed Cdk inhibitors are as follows: empty expression vector (A), full-length p21 (B), p27 (C), p16 (D), N-terminal domain of p21 (aa 1 to 75) (E), and the C-terminal domain of p21 (aa 76 to 164) (F).

On the basis of structural characteristics and functions of Cdk inhibitors, they can be grouped into two subfamilies. One class of inhibitors, including p21, p27, and $p57^{Kip2}$ (11, 23, 31, 38, 46, 49, 58), shares a Cdk2-binding motif in the N terminus and acts on a wide range of Cdks. The other class of inhibitors, also known as INK4s (including p16, p15, p18, and p19 [4, 18, 22, 24, 54]), contains fourfold ankyrin repeats and specifically inhibits Cdk4 and Cdk6. In the present study, enforced expression of p21 or p27 resulted in induced differentiation of CMK cells, while that of p16 was unable to affect morphological features. Since megakaryocytic differentiation is exceptionally accompanied by a wave of nuclear endoreplication in a nonproliferating cell, it would be of interest to determine how DNA replication occurs under overexpression of p21 or p27. In the case of TPA-induced megakaryocytic differentiation of HEL and Meg-01 cells, Garcia and Cales recently reported that the expression level of cdc25C was reduced during endoreplication, leading to a lack of cdc2 activation, which is essential for entry into the M phase (14). Since the kinase activity of the cdc2-cyclin B complex is also positively regulated by Cdk2 (17), overexpression of p21 or p27 might result in downregulation of cdc2 activity through inhibition of Cdk2. It was also speculated that because of its inability to inhibit Cdk2, p16 might not be capable of inducing megakaryocytic differentiation. Recently, it was demonstrated that p21 knock-out mice undergo normal development without apparent hematopoietic abnormalities (3, 10). This observation appears to be inconsistent with our findings suggesting the involvement of p21 induction in the megakaryocytic differentiation process. However, the lack of an abnormal phenotype in hematopoiesis may be attributable to functional redundancy of Cdk inhibitors in the case of $p21^{-/-}$ mice.

Our data suggested that the induction of p21 may be transcriptionally regulated in CMK cells, independently of p53. p53-independent induction of p21 in a human HL-60 promyelocytic leukemia cell line and a human U937 monoblastic leukemia cell line undergoing differentiation has also been observed (1, 27, 53). Jiang et al. have reported that p21 is an immediate-early response gene induced during differentiation of HL-60 cells along the granulocytic or macrophage-monocyte pathway (27). Furthermore, Lie et al. have recently reported that p21 is upregulated in a p53-independent manner during the 1,25-dihydroxyvitamin D_3-induced differentiation of U937 cells into monocytes-macrophages and that transient overexpression of p21 in U937 cells results in cell surface expression of monocyte-macrophage-specific markers even in the absence of 1,25-dihydroxyvitamin D_3 (33). These findings, including our own, suggest that the p53-independent induction of p21 may not be restricted to a specific lineage but may be functional in a variety of hematopoietic cell lineages for facilitating terminal differentiation. However, the role of p21 in the growth and differentiation of normal hematopoietic progenitor (committed) or stem (uncommitted) cells is not known. Additional information about the effect of constitutive p21 expression in normal hematopoietic stem or progenitor cells would be particularly helpful in understanding the biological significance of p21 in early- as well as late-stage differentiation.

The present results also demonstrated that p21 is upregulated in an immediately-early response to TPO in CMK cells. After binding of TPO to its receptor c-Mpl, JAKs are known to be rapidly phosphorylated on tyrosine and activated as tyrosine kinases (41, 42). The activated JAKs, in turn, induce phosphorylation of latent cytoplasmic proteins called STATs (20, 41, 47). Upon phosphorylation, STATs dimerize, translocate to the nucleus, and participate in transcriptional regulation by binding to specific DNA sites (reviewed in references 25 and 26). It has previously been reported that STATs are activated by TPO in various combinations as follows: STAT3 in MO7E cells (41), STAT5 in UT7/Mpl cells (47), STAT1 and STAT3 in BaF3/Mpl cells (20), STAT3 and STAT5 in 32D/Mpl cells (42). In the case of our CMK cells, TPO was found to induce tyrosine phosphorylation of STAT3 and STAT5. EMSA and supershift assaying demonstrated that STAT5 but not STAT3 exhibited a binding activity to both p21-SIE2 and p21-SIE3 sites in response to rhTPO. In addition, the p21-SIE2 element was transactivated by the treatment with rhTPO. These results raised the possibility that STAT5 is involved in transcriptional activation of p21 by TPO. This possibility may be supported by the recent finding that epidermal growth factor- or IFN-γ-activated STAT1 protein mediated induction of p21 through binding to the conserved STAT-responsive element in the promoter of the p21 gene (6).

In addition to TPO, STAT5 was reported to be activated by various cytokines and humoral factors such as IL-2, IL-3, GM-CSF, erythropoietin, growth hormone, and prolactin (15, 62). Among these factors, furthermore, IL-3 and GM-CSF as well as TPO were found to induce p21 expression. However, IL-3 and GM-CSF had only a transient effect on p21 expression and were unable to facilitate megakaryocytic differentiation of CMK cells, suggesting that the prolonged expression of p21 by TPO may be important for induction of CMK differentiation. Although the precise role of STAT proteins in cell growth and differentiation remains elusive, our data thus provide unique evidence that, at least in a megakaryocytic lineage, STAT5 may

participate in the regulation of cell differentiation through induction of p21. Recently, Nakajima et al. have shown that although a target gene(s) has not been identified, STAT3 may be indispensable for IL-6-induced macrophage differentiation of the murine leukemia cell line M1, supporting the idea that STAT proteins could be involved in the process of cellular differentiation (44, 63). Additional studies to elucidate the biological functions and biochemical consequences of STAT5 and p21 proteins will undoubtedly lead to a better understanding of the molecular mechanisms underlying megakaryopoiesis.

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