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Induction of Disabled-2 Gene during Megakaryocyte Differentiation of K562 Cells

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Megakaryocyte differentiation is often accompanied by the changes of gene expression pattern. Here we reported that the expression of DAB2, a putative adaptor protein in cell signaling, was induced at the protein and mRNA levels upon 12-*O*-tetradecanoylphorbol-13-acetate-mediated megakaryocyte differentiation of human chronic myeloid leukemic K562 cells. On the other hand, the differentiation agents DMSO and retinoic acid had no effect on DAB2 expression. Analysis of promoter activity with the human DAB2 luciferase reporter constructs suggested that the regulation is partially at the transcriptional level. The responsive sequences located within an 80-bp DAB2 promoter region. To determine the involvement of MEK1-p42/p44 MAPK pathway in mediating DAB2 gene expression, we have performed the following experiments and found that (i) there was sustained activation of p42/p44 MAPK, but not p38 MAPK, upon K562 cells differentiation; (ii) application of MEK1 inhibitor U0126 reduced the expression of DAB2 protein, mRNA and promoter activity, as well as cell differentiation; (iii) constitutively active MEK1 increased DAB2 promoter activity; and (iv) dominant negative ERK2 abolished constitutively active MEK1-induced DAB2 promoter activity. Taken together, our results indicate that DAB2 gene is induced upon megakaryocyte differentiation by the MEK1-p42/p44 MAPK pathway and may define a new role of DAB2 in hematopoietic cell differentiation.

Key Words: **disabled-2 gene; K562 cells; megakaryocyte differentiation.**

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; MAPK, mitogen-activated protein kinase; CML, chronic myeloid leukemia.

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Human disabled-2 (DAB2) was first identified as a transcript down-regulated in ovarian carcinoma (1). By stable expression of DAB2 cDNA in cancer cell lines, others and we have showed that DAB2 acts as a growth inhibitory molecule and plays a crucial role in cell proliferation/differentiation (2–4). Recent studies addressing the expression pattern of DAB2 in epithelial cell and rat prostate indicate that DAB2 may function in epithelial cell positioning control and prostate regeneration (2, 5). However, little is known regarding the expression pattern of DAB2 in hematopoietic cell differentiation and the signaling pathway mediating DAB2 expression.

Treatment of K562 cells with differentiation agents, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), DMSO, and retinoic acid, has been commonly used to analyze the lineage-specific signal transduction and gene expression upon megakaryocyte differentiation (6–9). For instances, Tetteroo *et al.* reported the expression of megakaryoblastoid differentiation marker platelet glycoprotein IIIa (GPIIIa) is strongly enhanced upon treatment of K562 cells with TPA (6). Simultaneously with the appearance of megakaryocytic markers, the expression of the erythroid lineage specific glycophorin A decreases at both the mRNA and protein levels (7). In this study, we report the involvement of p42/p44 MAPK pathway in the induction and regulation of DAB2 gene upon megakaryocyte differentiation of K562 cells. The potential role of DAB2 gene in megakaryocyte differentiation is discussed.

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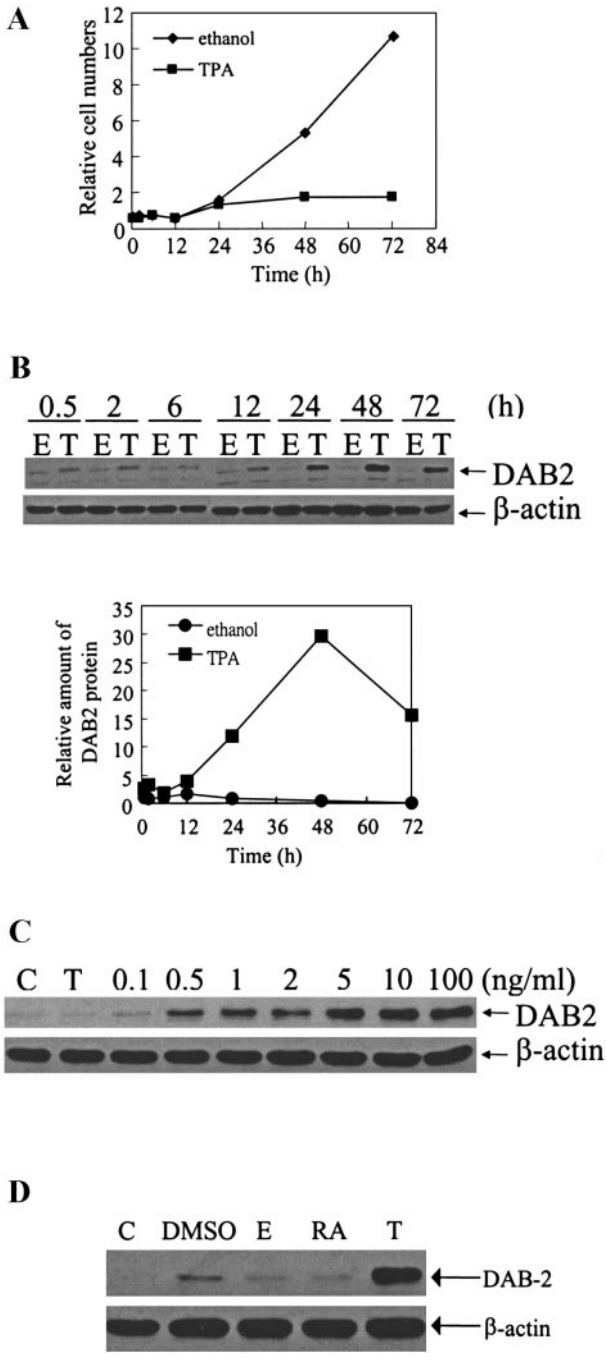

FIG. 1. Induction of DAB2 protein during megakaryocyte differentiation of K562 cells. (A) Growth inhibitory effect of TPA. The K562 cells were plated at a density of 1.5×10^4/ml. At the following day, cells were subjected to the indicated treatment. The cell numbers were determined by counting with the hemocytometer and the relative cell numbers (day 0 was arbitrary set as 1) was plotted versus days of treatment. E, ethanol; T, TPA (10 ng/ml). (B) Time-dependent induction of DAB2 protein. At the indicated time after TPA treatment, cell lysates were collected and Western blot analysis (30 μg protein/lane) was performed using mouse anti-p96 (DAB2) antibody. After quantification by densitometer, the relative expression level of DAB2 protein was plotted against treatment time (lower panel). (C) Dose-dependent induction of DAB2 protein by TPA. The K562 cells were treated with TPA (0.1–100 ng/ml) for 48 h and Western blot analysis was performed as described for B. All the membranes were reprobed with the anti-β-actin antibody for the control of equal loading. (D) Effects of DMSO and RA on the expression of DAB2. The cell lysates from the control K562 cells (C) and cells treated with DMSO (1.4%), ethanol (E, 0.01%), RA (100 μM), or TPA (T, 10 ng/ml) for 48 h were analyzed by Western blot for the expression of DAB2. All the membranes were reprobed with the anti-β-actin antibody for the control of equal loading.

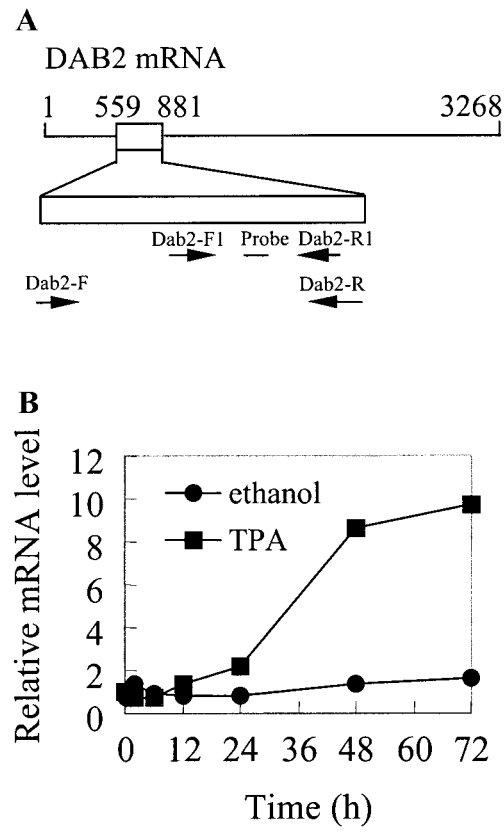

FIG. 2. Induction of DAB2 mRNA during megakaryocyte differentiation of K562 cells. (A) Design of real-time RT-PCR for detection of DAB2 mRNA. The relative positions of the TaqMan probe and the primers for synthesis of cDNA fragment (Dab2-F and Dab2-R) and for real-time RT-PCR (Dab2-F1 and Dab2-R1) were shown. (B) Quantification of hDAB2 mRNA by real-time RT-PCR. The K562 cells total RNA (200 ng) isolated at the indicated time and treatment was subjected to real-time RT-PCR analysis. The data have been normalized by the level of β-actin mRNA as determined by real-time RT-PCR. Similar results were obtained in two independent experiments.

MATERIALS AND METHODS

Materials. TPA was purchased from Sigma (St. Louis, MO). The p96 antibody was purchased from Transduction Laboratories (Lexington, KY). The phospho-MAPK sampler kit, the p38 MAPK antibody and the p42/p44 MAPK antibody were purchased from New England BioLab (Beverly, MA). The MEK1 inhibitor U0126, the pGL3-basic plasmid, and the Steady-Glo luciferase assay system were purchased from Promega (Madison, WI). The constitutively active MEK1 cDNA was purchased from Upstate Biotechnology (Lake Placid, NY).

Preparation of cell lysates and Western blot analysis. The K562 cells were pelleted by centrifugation at 3000 rpm for 10 min. After

A

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  1 ACATCACTCNCAGTGGCAATCCAGGGAACGGATCTGTGAAACGAAGCTCG   50
 51 GTGGATCCCACCCCTTTCCTCAGAGCTCCTTCCTGTCCTTATTTGCACCC  100
101 GCCCCCCGCCCGCGTCGGTCTGGGGCTTTTGACTTCTCCCCGAACACATT  150
151 TCCCCTCCCGTCGCTGGGTCCCTGCGATCGCCCCCAGCTGGTGGGGCTCG  200
201 CGGAGCTCAGGGGAGCGGGGTCCTCTGCGCCGCTGCAGCGCGAGTTAATA  250
251 AACAGTTAAGTTTGGAAGACTCTGCAGACACGTTGAGGGGGAGTTACCAA  300
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351 CAGCATCTGATTAGAACCATATCTCTCGCCGGG                   383
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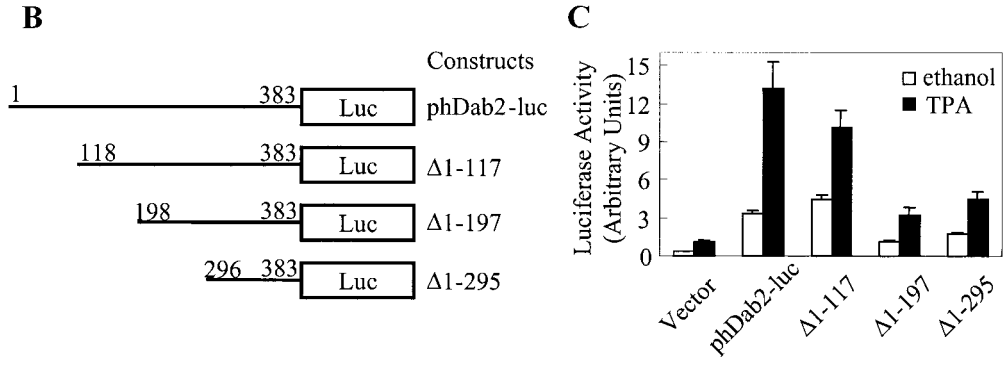

FIG. 3. Activation of DAB2 promoter during TPA-induced megakaryocyte differentiation of K562 cells. (A) Sequence of the human DAB2 promoter. (B) Schematic representation of the human DAB2 luciferase reporter constructs. The 383-bp DAB2 promoter or its deletion mutants were subcloned into pGL3-basic vector to drive the expression of a luciferase reporter gene. (C) Promoter activity of DAB2 gene. The K562 cells were transfected with the indicated plasmids (1 μg) and were treated with ethanol or TPA for 24 h followed by luciferase activity assay. The results represented the mean of triplicate assays ± SE. Similar results were obtained from three independent experiments with two plasmid preparations.

removing the medium, cell pellets were washed twice with phosphate-buffered saline (PBS), lysed in the lysis buffer A (10), and kept on ice for 30 min. After centrifugation, the supernatants were collected as the total cell lysates. The expression of protein was determined by Western blot analysis as described previously (10).

Plasmid construction. The 383-bp human DAB2 promoter (GenBank Accession No. AF218839) was obtained by PCR amplification of K562 genomic DNA with the forward primer hDab2-promoter-F (5′-GAATTCGGTACCACATCACTCCCAGTGGCAAT-3′) and the reverse primer hDab2-promoter-R (5′-GAATTCAGATCTCCCGGCGAGAGATATGGTTC-3′). The *Kpn*I–*Bgl*II fragment of the PCR product was subcloned into the pGL3-basic vector to obtain phDab2-luc. To construct pTOPO-hDab2, the human DAB2 cDNA fragment flanking nt 599 to nt 881 (GenBank Accession No. NM_001343) was obtained by RT-PCR amplification of K562 total RNA with the forward primer Dab2-F (5′-GGCAACAGGCTGAACCATTAGT-3′) and reverse primer Dab2-R (5′-TTGGTGTCGATTTCAGAGTTTAGAT-3′) followed by subcloning the 283-bp PCR product into the pTOPO-PCRII vector. To construct pTOPO-β-actin, we used the primer set actin-F (5′-TCACCCACACTGTGCCCATCTACG-3′), and actin-R (5′-CAGCGGAACCGCTCATTGCCAATG-3′) to perform RT-PCR with the RNA from K562 cells. A 294 bp PCR product was obtained and subcloned into the pCRII-TOPO.

Real time quantitative RT-PCR. The one-step real-time quantitative RT-PCR was performed with the GenAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Briefly, 200 ng of total RNA was added into a reaction mixture containing 25 μl of 2× RT-PCR universal master mix, 2.5 μl of 40× reverse transcriptase mixture, 5 μl of forward primer Dab2-F1 (5 μM, AGTGAGGCCCTAAGGATTCTAGATGACCAAACTA), 5 μl of reverse primer (5 μM, CAGGATATCTTTGCTTTCTGTTGGACTATTTAGGTC), and 5 μl of TaqMan probe (2 μM, CGGGTGTTGACCAGATGGATTTGTTTGGGGACATGTCTAC) in a final volume of 50 μl. The condition for RT-PCR was: 1 cycle of 48°C for 30 min; 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min. The cycle threshold value (C_T) used to assess the quantity of target gene was determined by which the fluorescence exceeds a preset limit.

Transient transfection and luciferase activity assay. The K562 cells were transfected with the DMRIE-C reagent as described by the manufacturer (Life Technologies, Gaithersburg, MD). Twenty-four hours after transfection, cells were treated with TPA for 1 day. Equal amount of cell extracts were incubated with Steady-Glo luciferase reagent and the luciferase activity was determined with the TopCount Microplate Scintillation & Luminescence Counter (Packard Instrument, Meriden, CT).

RESULTS

Induction of DAB2 Protein during Megakaryocyte Differentiation of K562 Cells

The K562 cells underwent megakaryocyte differentiation upon treatment of TPA and resulted in the decrease of cell growth rate (Fig. 1A). Since DAB2 gene has been shown to associate with cell proliferation and differentiation, we determined the expression pattern of DAB2 during megakaryocyte differentiation of K562 cells by Western blot analysis. As shown in Fig. 1B, the major DAB2 protein in K562 cells corresponded to the rat p82 isoform (2). DAB2 expressed at a relatively lower level in the untreated control culture. However,

its expression was markedly elevated to the maximal level at 2 days after TPA treatment. The induction of DAB2 was as high as 29-fold of the control level (Fig. 1B). A TPA dose-dependent experiment (0.1–100 ng/ml) further confirmed the induction of DAB2 with a maximal effect at 10 ng/ml TPA (Fig. 1C). On the other hand, treatment with differentiation agent DMSO that programmed cells toward granulocyte differentiation (11) or RA that did not affect the differentiation of K562 cells (12) has no effect on DAB2 expression (Fig. 1D). These results indicate that DAB2 protein expression is modulated during megakaryocyte differentiation in K562 cells.

Induction of DAB2 mRNA Expression during Megakaryocyte Differentiation of K562 Cells

To determine whether the induction of DAB2 protein was mediated by the up-regulation of its mRNA level, we developed a real-time quantitative RT-PCR to quantify DAB2 mRNA. At first, a 10-fold serial dilution of pTOPO-hDab2 (from 100 pg to 1 fg) containing 283-bp hDAB2 cDNA fragment specific to the p82 isoform (Fig. 2A) was subjected to the one-step real-time PCR. Then the [log (DNA concentration)] was plotted versus the threshold cycle value C_T of each reaction to establish the standard curve for quantification. Our results indicate this quantification method had a linear range across at least five logs of DAB2 DNA concentrations with a correlation coefficient = 0.998 (data not shown). Similarly, we have established a real-time RT-PCR assay for quantification of β-actin mRNA that was used for internal control of RNA quality and quantity (data not shown).

To quantify DAB2 mRNA expression during megakaryocyte differentiation of K562, TPA was applied to the cells and the total RNA was isolated at the indicated time for real-time RT-PCR analysis. We found that DAB2 mRNA began to elevate at 24 h after TPA treatment. The induction of DAB2 mRNA was at a time-dependent manner with a maximal effect (approximately 9-fold) persisted from day 2 to day 3 (Fig. 2B). These results indicate that DAB2 mRNA was up-regulated during megakaryocyte differentiation of K562 cells.

Regulation of DAB2 Promoter Activity during Megakaryocyte Differentiation of K562 Cells

To determine the DAB2 promoter activity during megakaryocyte differentiation of K562 cells, we isolated 383-bp human DAB2 promoter (13) by PCR and generated a luciferase reporter construct phDab2-luc under the control of human DAB2 promoter (Figs. 3A and 3B). Similar to the previous report, phDab2-luc increased the luciferase activity approximately 10-fold vs pGL3 basic vector transfected cells, suggesting this 383 bp DNA fragment contained the functional promoter activity. Transfection of phDab2-luc into K562 cells followed by TPA treatment increased the DAB2 promoter activity (Fig. 3C). The TPA effect on DAB2 promoter was not reduced significantly when we deleted the distal 117 bp promoter regions (i.e., Δ1–117). However, deletion of the distal 198-bp (i.e., Δ1–197) or 295-bp (i.e., Δ1–295) promoter region dramatically reduced the TPA effects to approximately 1/4 of the phDab2-luc (Fig. 3C). These results suggested that DAB2 promoter is activated upon TPA-induced megakaryocyte differentiation and the putative TPA responsive sequence is located between 118 and 198 bp upstream of DAB2 gene.

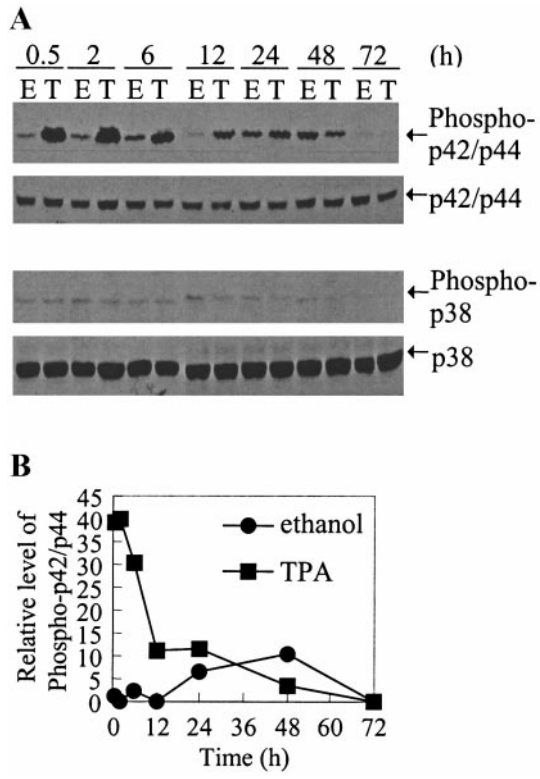

FIG. 4. Time course of p42/p44 MAPK activation in TPA-induced megakaryocyte differentiation of K562 cells. (A) Effect of TPA on the activation of p42/p44 and p38 MAPK. At 24 h after plating, the K562 cells were subjected to the indicated treatment. Thirty micrograms total cellular proteins was used for Western blot analyses with anti-phospho-p42/p44 MAPK, p42/p44 MAPK (reprobed), phospho-p38 MAPK, and p38 MAPK (reprobed) antibodies, respectively. E, ethanol; T, TPA. (B) Relative levels of phospho-p42/p44 MAPK. The film from A probed with anti-phospho-p42/p44 MAPK antibody was quantified by densitometer and was plotted against the treatment time. The expression level of phospho-p42-p44 MAPK at 30 min ethanol treatment was arbitrary set as 1.

Involvement of MEK1-p42/p44 MAPK Pathway in Megakaryocyte Differentiation and Up-Regulation of DAB2

Activation/phosphorylation of p42/p44 MAPK (ERK1/2) by MEK1 is a major event in transducing the signals

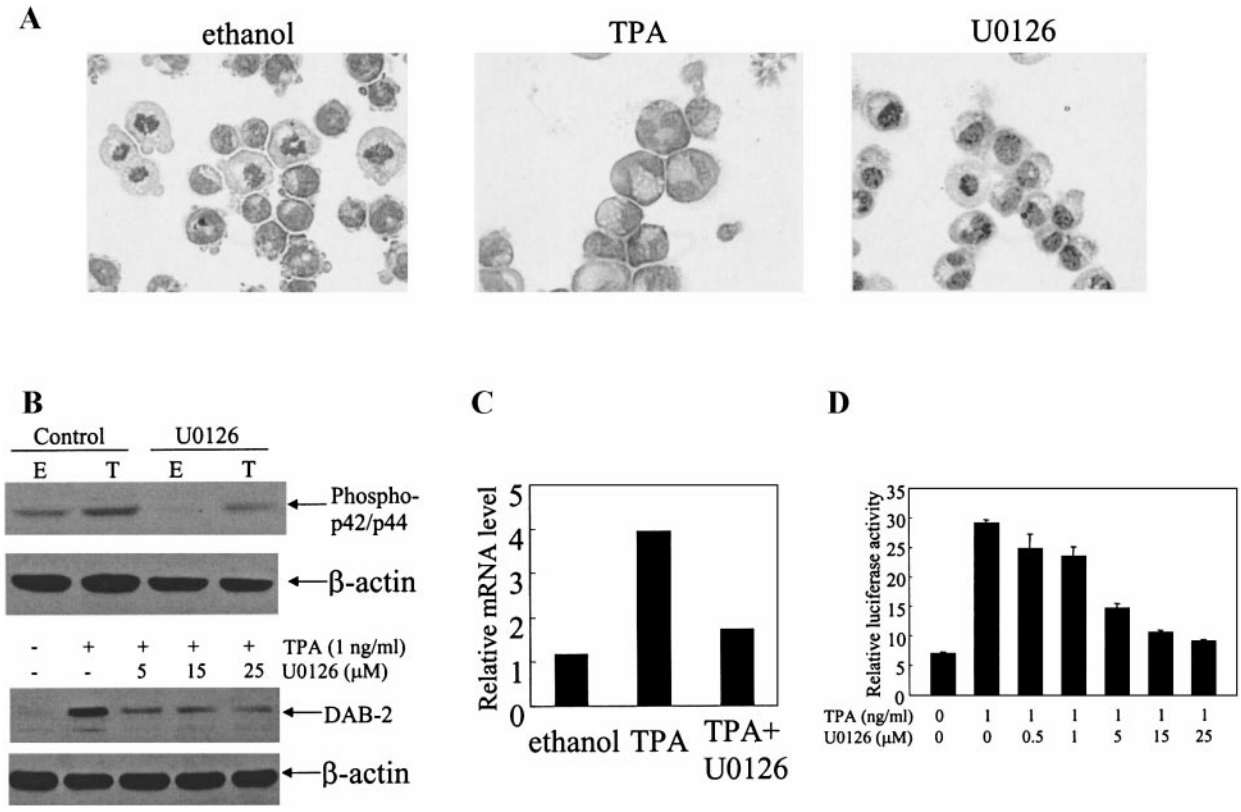

FIG. 5. Effect of MEK1 inhibitor U0126 on megakaryocyte differentiation and DAB2 gene expression. (A) U0126 inhibits megakaryocyte differentiation of K562 cells. The K562 cells were pretreated with U0126 (10 μM) for 1 h followed by 2 days TPA treatment. Aliquots of the cell suspension were cytospin, stained with Liu stain reagents, and observed by light microscopy. A typical field was shown for K562 cells treated with ethanol (control), TPA, and TPA + U0126, respectively. (B) Inhibition of p42/p44-MAPK phosphorylation and DAB2 protein expression by MEK1 inhibitor U0126. The cell lysates from 30 min (for MAPK) and 2 days (for DAB2) treatment with ethanol, TPA, or TPA plus U0126 were collected and subjected to Western blot analyses with anti-phospho-p42/p44 MAPK (upper panel), or anti-DAB2 antibody (lower panel). Both membranes were reprobed with anti-β-actin antibody for the control of equal loading. (C) Inhibition of TPA-induced DAB2 mRNA expression by U0126. The K562 cells were plated as in A and were treated with vehicle ethanol, TPA, or TPA + U0126 (5 μM) for 24 h. The expression of DAB2 mRNA was determined by real-time RT-PCR using 200 ng total RNA and was normalized by the levels of β-actin mRNA. (D) Inhibition of the phDab2-luc activity by U0126. The K562 cells transfected with phDab2-luc were subjected to the indicated treatment for 24 h followed by luciferase activity assay. The data represented the mean of triplicate assays ± SE. Essentially similar results were obtained from three independent experiments.

responsive to cell proliferation/differentiation. We determined the involvement of MAPK activity in the up-regulation of DAB2 gene. With the phosphor-specific antibody, we found that p42/p44 MAPK, but not p38 MAPK, was activated as early as 30 min after TPA treatment (Figs. 4A and 4B). Two days later, K562 cells showed the typical megakaryocyte differentiation phenotypes with the enlargement of the majority of cells, decrease of N/C ratio, and the presence of nucleus fragmentation. By contrast, U0126 strikingly reversed the morphological changes and blocked the megakaryocyte differentiation induced by TPA (Fig. 5A), while the phosphorylation of p42/p44 MAPK and the expression of DAB2 protein were both inhibited (Fig. 5B). Analysis of the DAB2 mRNA expression by real-time RT-PCR further indicated the reduction of DAB2 protein was due to the decrease of DAB2 mRNA (Fig. 5C). U0126 also affected the DAB2 promoter activity as shown by the dose-dependent inhibition on the phDab2-luc activity (Fig. 5D). Therefore, our results indicate the involvement of MEK1-p42/p44 pathway in the regulation of DAB2 gene expression and megakaryocyte differentiation.

To further elucidate the role of MEK1-p42/p44 MAPK in modulating DAB2 gene expression, we determined the effect on DAB2 promoter activity by co-transfecting pUSE-active MEK1, an expression construct with S218D/S222D mutation that demonstrated constitutive MEK1 activity (14), with phDab2-luc. As shown in Fig. 6A, an approximately 6-fold increase of luciferase activity relative to wild type MEK1 or control plasmid pUSEamp$^+$-transfected cells was observed 24 h post transfection. In addition, co-transfection of the dominant negative ERK2 (dnERK2, Ref. 15) with phDab2-luc reduced 40% of the MEK1-induced luciferase activity (Fig. 6B) and approximately 30% inhibi-

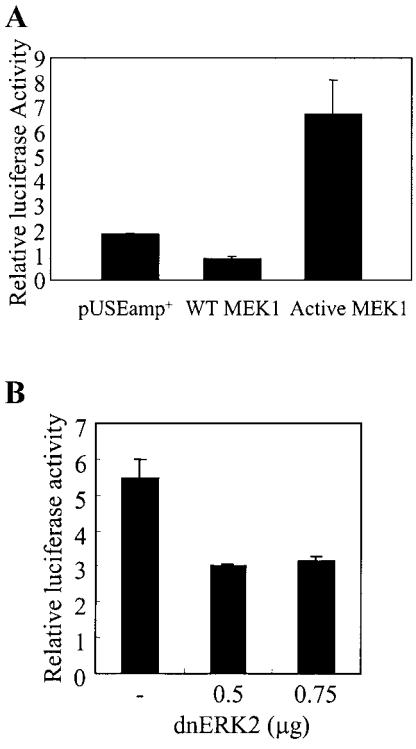

FIG. 6. Modulation of DAB2 promoter activity by constitutively active MEK1 and dominant negative ERK2. (A) Effect of constitutively active MEK1. The K562 cells were transfected with 0.5 μg vector control pUSEamp$^+$, wild type or constitutively active MEK1 expression plasmid and 0.5 μg phDab2-luc. The luciferase activity was analyzed 48 h after transfection. (B) Effect of dominant negative ERK2 on constitutively active MEK1-induced phDab2-luc luciferase activity. The K562 cells were cotransfected with constitutively active MEK1, phDab2-luc and indicated amount of dominant negative ERK2. Two days later, cell lysates were analyzed for luciferase activity. The data represented the mean of triplicate assays ± SE.

tion of the TPA-induced phDab2-luc activity (data not shown). Therefore, our results indicated the involvement of MEK1-p42/p44 MAPK in the regulation of DAB2 gene.

DISCUSSION

In this study, we use K562 cells to investigate DAB2 gene expression during hematopoietic cell differentiation. Our results indicated that the up-regulation of DAB2 is mediated through MEK1-p42/p44 MAPK pathway upon TPA-induced megakaryocytic differentiation of K562 cells. This notion is strongly substantiated by the following observations: (i) TPA causes sustained activation of p42/p44 MAPK but not p38 MAPK; (ii) pretreatment with MEK1 inhibitor U0126 abolishes TPA activation of p42/p44 MAPK as well as DAB2 expression and its promoter activity in a dose-dependent manner; (iii) the DAB2 promoter activity is significantly increased by transfection of constitutively active MEK1, and is down-regulated upon transfection with a dominant negative ERK2. Therefore, our data represent the first important clue to the signal pathway regulating DAB2 gene expression.

By luciferase activity analysis, we have revealed an 80-bp DAB2 promoter sequence contributes to the TPA-responsive activity. Sequence analysis of this promoter region indicated no consensus GATA (TGATTAG) or SP1 binding site (CCGCCC) that have been shown to play a pivotal role in the regulation of megakaryocyte differentiation (16). Instead, a CCTCCC motif that has been shown in the 5′-flanking region of hepatocyte growth factor gene acting as a binding site and enhancer element for Sp family transcription factor (17) was found in this region of DAB2 promoter. A detailed analysis of the involvement of Sp family transcription factors in the regulation of DAB2 promoter activity is under investigation.

The association of DAB2 expression and megakaryocyte differentiation appears to be specific. This was supported by the notion that no induction of DAB2 when cells undergo granulocyte differentiation by DMSO. Since differentiation is a process involved cell growth arrest followed by genotypic and phenotypic changes, the known growth inhibitory activity of DAB2 as demonstrated in several tumor cell lines (2–5) suggests that DAB2 may cause cell cycle arrest and elicit its function at the early stage of differentiation. Alternatively, DAB2 may serve as a differentiation marker, such as CD41, that is up-regulated in the development of megakaryocyte. Although the definite function of DAB2 in megakaryocyte differentiation need to be addressed further, our findings suggest that DAB2 expression is strongly associated with the differentiation status of K562 cells. In summary, our results indicate that induction of DAB2 gene is mediated by p42/p44 MAPK and is associated with megakaryocyte differentiation. Such information can lead to define the role and to expand our understanding of DAB2 in hematopoietic cell differentiation.

ACKNOWLEDGMENTS

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