Regulation of NF-E2 Activity in Erythroleukemia Cell Differentiation*

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The erythroid transcription factor NF-E2 is an obligate heterodimer composed of two different subunits (p45 and p18), each containing a basic region-leucine zipper DNA binding domain, and it plays a critical role in erythroid differentiation as an enhancer-binding protein for expression of the β-globin gene. We show here that dimethyl sulfoxide treatment of wild-type murine erythroleukemia cells, but not a mutant clone of dimethyl sulfoxide-resistant cells, increases NF-E2 activity significantly, which involves both up-regulation of DNA binding and transactivation activities. Both activities were reduced markedly by treatment of cells with 2-aminopurine but not by genistein. Activation of the Ras-Raf-MAP kinase signaling cascade increased NF-E2 activity significantly, but this was suppressed when MafK was overexpressed. Domain analysis revealed an activation domain in the NH2-terminal region of p45 and a suppression domain in the basic region-leucine zipper of MafK. These findings indicate that induction of NF-E2 activity is essential for erythroid differentiation of murine erythroleukemia cells, and serine/threonine phosphorylation may be involved in this process. In addition, they also suggest that a MafK homodimer can suppress transcription, not only by competition for the DNA binding site, but also by direct inhibition of transcription. Hence, MafK may function as an active transcription repressor.

The erythroid transcription factor NF-E2 is present in extracts of erythroid cell lines (1, 2) and has been shown to be a heterodimer formed between the two basic region-leucine zipper (b-zip)1 proteins, i.e. the p45 and the p18 subunits. p45, which together with the Drosophila cap’n-collar (CNC) protein defines a b-zip subfamily, is expressed in hematopoietic cells of the erythroid, megakaryocytic, and mast cell lineages (1), whereas p18 is one of the small Maf family proteins including MafK2 (1–4), and its expression is not restricted to hematopoietic cells (5). Involvement of NF-E2 in erythroid cell differentiation of mouse erythroleukemia (MEL) cells has been suggested by its role as an enhancer-binding protein for expression of the β-globin gene, by the lack of β-globin mRNA expression in a MEL cell line (CB3) devoid of NF-E2 protein as a result of integration of Friend viral sequences within the p45 NF-E2 gene locus (6), and by the restoration of β-globin mRNA by forced expression of p45 cDNA in these cells (6, 7). It has also been found that NF-E2-DNA binding increases during erythroid differentiation of MEL cells (8–10). These results suggest NF-E2 to be one of the key transcription factors that regulate erythroid differentiation of MEL cells. Unexpectedly, targeted disruption of the p45 gene in mice showed essentially no abnormality in erythropoiesis (11, 12). It is possible in these conditions, however, that p45-related b-zip factors, such as Nrfl (13), or Nrfl2 (14), may compensate for deficient NF-E2 activity.

Previous studies showed that p45 can be phosphorylated by a cAMP-dependent protein kinase in vitro (15) and that both DNA binding of NF-E2 and α-globin locus control region enhancer activity are diminished significantly in a cAMP-dependent protein kinase-deficient clone of MEL cells after hexamethylenedisuccinimide treatment compared with wild-type cells (15). These findings suggest that NF-E2 function may be regulated by a cAMP-dependent protein kinase; however, the exact mechanism of NF-E2 regulation remains unclear. With this view in mind, we examined the possibility that NF-E2 activity may be regulated by a post-translational mechanism in MEL cell differentiation. Our results in this study show that dimethyl sulfoxide (Me2SO) increased NF-E2-DNA complex formation in the wild-type MEL cells, in part by the induction of p45 gene expression, and that both DNA binding and transactivation activity of NF-E2 are regulated by serine/threonine phosphorylation such as a Ras signaling cascade. Our findings also suggest that an MafK homodimer can suppress transcription not only by competition for the DNA binding site, but also by directly inhibiting transcription. Hence, MafK may be viewed as an active transcription repressor.

MATERIALS AND METHODS

Cell Culture—A clone of dimethyl sulfoxide-sensitive (DS) MEL cells (DS-19) (16) and a clone of dimethyl sulfoxide-resistant (DR) MEL cells (DR-1) (17, 18) were grown in suspension in modified Ham’s F-12 medium containing 10% heat-inactivated bovine calf serum (Hyclone Laboratories, Logan, UT), as described previously (18). DS cells undergo erythroid differentiation after treatment with Me2SO and express erythroid markers such as β-globin (19, 20), heme pathway enzymes (18, 21), and erythropoietin receptor (22, 23); they ultimately cease to grow in culture after a few days by completing differentiation (24). In

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2 MafK is a protein, while mafK represents its gene.

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4 The abbreviations used are: b-zip, basic region-leucine zipper; MEL, murine erythroleukemia; Me2SO, dimethyl sulfoxide; DS, Me2SO-sensitive; DR, Me2SO-resistant; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; 2-AP, 2-aminopurine; ALAS-E, erythroid-specific δ-aminolevulinate synthase; ALAS-N, nonspecific δ-aminolevulinate synthase.
contrast, DR cells fail to show terminal erythroid differentiation when treated with Me₂SO, and they continue to grow as hemoglobin-free cells in culture (17). Both DS and DR cells were split every 3–4 days to maintain a logarithmic growth.

DNA Probes—DNAs for mouse p45 and MafK were generated by polymerase chain reaction using cDNAs reverse transcribed from MEL cell RNA and a set of primers as described previously (3). Human ribosomal DNA was obtained from the Japanese Cancer Research Resources Bank, Tokyo, and used as an internal control.

Northern Blot Analysis—Total RNA from untreated and treated MEL cells was isolated using the acridinum thiocyanate–phenol chloroform method (25). 20 μg of each RNA was loaded onto a 1.2% agarose-formaldehyde gel, electrophoresed, and transferred to a sheet of Zeta-Probe filter (Bio-Rad). The filters were hybridized with appropriate RNA probes at 50 °C in a solution containing 50% formamide, 1.5 × SSPE (270 mmol/liter NaCl, 15 mmol/liter Na₂HPO₄, and 1.5 mmol/liter EDTA), 1% SDS, 0.5% bovine lactotransferase optimizer (BLOTTO), 0.2 mg/ml yeast transfer RNA, and 0.5 mg/ml sonicated salmon sperm DNA. Hybridized filters were washed and exposed to Kodak X-OMat XR-5 films (Eastman Kodak). mRNA concentrations were quantified by LKB Ultrascan XL enhanced laser densitometry (Pharmacia Biotech Inc.). Experiments were performed two or three times using separate preparations of RNA, and representative results are shown in the figures.

Western Blot Analysis—Nuclear extracts were prepared from 1 × 10⁶ cells according to the method described previously (26). 10 μg of nuclear extracts was separated electrophoretically using 10% polyacrylamide gel. Immunoblotting and detection by enhanced chemiluminescence were performed as described previously (18).

DNA Gel Mobility Shift Assays—To evaluate the DNA binding activities of NF-E2, DNA gel mobility shift assays were performed using the oligomer 5′-CTGGTGTCGTCATGATTCGAGG-3′, which contains the 11-base pair NF-E2 consensus and its flanking sequences. 5 μg of nuclear extract was incubated with 5′-end-labeled oligomers in a binding buffer containing 20 mM Hepes buffer (pH 7.8), 60 mM KCl, 0.2 mM EDTA, 6 mM MgCl₂, 0.5 mM dithiothreitol, 10% v/v glycerol, and 1.5 μg of an equimolar mixture of poly(dI-dC) and poly(dA-dT). The reaction mixture was incubated at room temperature for 15 min. In antibody-mediated competition assays, 1 μl of rabbit anti-mouse p45 serum was first incubated with nuclear extracts on ice for 10 min and then incubated with the probes for an additional 10 min. For dephosphorylation, 5 μg of nuclear extracts from Me₂SO-treated DS cells was incubated with 0.001–0.01 unit of calf intestine phosphatase for 15 min before the addition of the ³²P-labeled probe. The mixture was then loaded onto a 4% polyacrylamide gel, and electrophoresis was carried out under room temperature.

Plasmids—p45 and MafK expression vectors were described previously (3, 5). FLAG epitope tags were introduced into the binding domain fusions were constructed using pGBT9 (CLONTECH). The NcoI/HindIII fragment of pIKIII (3) was cloned in the EcoRI site of pGBT9 after blunting of the end. Portions of p45 cDNAs were also inserted into pGBT9 using conventional restriction enzyme sites, resulting in G4-p45 fusion plasmids. Various portions of murine mafK cDNA (5) were isolated by polymerase chain reaction and cloned also in pGBT9, resulting in G4-MaK fusion plasmids. These fusion cDNAs were transfectioned into the BstIII site of pEFBssIII by isolating the HindIII fragment, resulting in G4-p45(1–272), (1–83), (1–36), (39–83), (86–272), G4-MF, G4M3AF, and G4MPAC. An expression plasmid for H-RasVT12 was kindly provided by Dr. M. Nakafuku. A plasmid expressing dominant positive MAPKK (SESE-MAPKK) (27) was kindly provided by Drs. Y. Gotoh and E. Nishida. pRBGP10 reporter plasmid was generated by inserting a single copy of the NF-E2 site derived from the chicken β-globin 3′ enhancer into pRBGP3 (3).

Transfection and Luciferase Assays—pRBGP2 that contains three copies of NF-E2 site (3), pRBGP10, or GAL4 × 5 luciferase plasmid was used as a reporter. Transfection of DNA into DS or DR cells was carried out using the transfection reagent DOTAP (Boehringer Mannheim). Briefly, a total of 1 × 10⁶ cells was incubated with 2 μg of reporter and effecter plasmids and 3 μg of pSV-β-galactosidase (as an internal control) in 1 ml of medium culture without serum for 7 h at 37 °C. Then 2 ml of fresh medium containing 10% bovine calf serum was added, and the cells were incubated in the absence or presence of 1.5% Me₂SO or kinase inhibitors for another 8–72 h. Cell lysates were prepared using reporter lysis buffer (Promega, Madison, WI). Luciferase and β-galactosidase activities were determined using the luciferase assay system and β-galactosidase enzyme assay system (Promega), respectively. Luciferase activity was normalized on the basis of β-galactosidase activity. NIH3T3 and QT6 cells were transfected by the calcium-phosphate precipitation procedure as described previously. To examine the effect of H-RasVT12 and MAPKK, cells were incubated in the presence of 0.25% fetal bovine serum for 36 h after transfection.

RESULTS

Effect of Me₂SO on NF-E2-DNA Complex Formation—To define the role of NF-E2 in erythroid differentiation, we first examined the effect of Me₂SO on NF-E2-DNA complex formation in DS and DR cells using a gel mobility shift assay. In DS cells a band was detected which increased its intensity significantly after treatment of cells with Me₂SO for 72 h (Fig. 1A). This band was suppressed completely by the addition of anti-p45 antiserum (Ab) on the formation of NF-E2-DNA complex. 1 μl of rabbit anti-mouse p45 serum was first incubated with nuclear extracts on ice for 10 min and then incubated with the probes for additional 10 min.

Effect of Me₂SO on NF-E2-DNA Complex Formation—To define the role of NF-E2 in erythroid differentiation, we first examined the effect of Me₂SO on NF-E2-DNA complex formation in DS and DR cells using a gel mobility shift assay. In DS cells a band was detected which increased its intensity significantly after treatment of cells with Me₂SO for 72 h (Fig. 1A). This band was suppressed completely by the addition of anti-p45 antiserum, but not by preimmune serum, indicating that it is an NF-E2-DNA complex (Fig. 1B). In contrast to DS cells, the corresponding band in DR cells did not increase after Me₂SO treatment (Fig. 1A). A band corresponding to an AP-1-DNA complex that migrated slightly slower than the NF-E2-DNA complex was not influenced by Me₂SO treatment in either DS or DR cells (Fig. 1A).

To clarify whether the induction of NF-E2-DNA complex formation by Me₂SO is caused by increased gene expression, we examined the mRNA levels for p45 and MafK by RNA blot analysis and their protein levels by immunoblot analysis. In DS cells, Me₂SO treatment increased p45 mRNA levels significantly at 72 h (Fig. 2A), consistent with the observed increase in NF-E2-DNA complex formation (Fig. 1A). In contrast, DR cells did not show any increase in p45 mRNA after Me₂SO treatment. MafK mRNA levels were not influenced by Me₂SO treatment in either DS or DR cells (Fig. 2A). Consistent with the induction of p45 mRNA in DS cells, p45 protein levels were also increased significantly after Me₂SO treatment in DS cells but not in DR cells (Fig. 2B). In contrast to p45 protein levels, which were readily detectable, MafK protein levels were below the detection level in both DS and DR cells (data not shown). These findings indicate that increased NF-E2-DNA complex

![FIG. 1. Effect of Me₂SO on NF-E2-DNA complex formation in DS and DR cells.](#)
formation by MeSO treatment occurs only in DS cells, which subsequently undergo erythroid differentiation, and that this process involves p45 gene up-regulation.

MeSO Treatment of DS Cells Increases the Enhancer Activity of the NF-E2 Site—To examine whether the MeSO-induced NF-E2 complex is active in transcription, we determined the activity of an NF-E2 binding site-luciferase fusion construct that was transfected into DS or DR cells. The results of these experiments are summarized in Fig. 3. As shown in the figure, luciferase activity was increased by 4-fold in transfected DS cells when they were treated with MeSO for 72 h compared with the level in untreated transfected cells (Fig. 3A). In contrast to DS cells, MeSO treatment did not increase luciferase activity in DR cells (Fig. 3A). These results indicate that NF-E2-DNA complex formation led to the up-regulation of NF-E2 site-dependent promoter activity during erythroid differentiation in DS cells by MeSO treatment. They also suggest that the failure of DR cells in erythroid differentiation may be related to the lack of an increase in NF-E2-DNA complex formation and therefore to the lack of NF-E2-mediated enhancer activity. It should also be noted that induction of luciferase activity in DS cells was already observed at 12 h (7-fold) after MeSO treatment (Fig. 3B). It was earlier than an increase in the amount of NF-E2-DNA complex which was not yet detectable at 12 h (data not shown). Thus it is possible that MeSO treatment may induce not only an increase in the amount of NF-E2-DNA complex but also transformation of the NF-E2 molecules to become functionally active.

The Role of Phosphorylation on NF-E2 Function—Because phosphorylation plays an important role in various signal cascades (28) and because NF-E2 has been shown to be phosphorylated in vitro (15), we examined the effect of protein kinase inhibitors on NF-E2 DNA binding activity and cis-regulatory activity of the NF-E2 site. Combined treatment of DS cells with MeSO and 2-aminopurine (2-AP), a broad specificity serine/threonine kinase inhibitor, resulted in a marked decrease in MeSO-mediated induction of DNA binding activity of NF-E2 (Fig. 4A). In contrast to 2-AP treatment, treatment of DS cells with genistein, a tyrosine kinase inhibitor, had no effect on MeSO-mediated induction of NF-E2 binding activity (Fig. 4A), suggesting that tyrosine phosphorylation is not required for MeSO-mediated induction of NF-E2 activity. Consistent with this finding, preincubation of a nuclear extract of MeSO-treated DS cells with calf intestine phosphatase decreased NF-E2 binding activity in a dose-dependent fashion (Fig. 4B). This inhibition occurred in the absence of any change in the level of p45 protein in both cells and nuclear extracts (Fig. 4C). However, this finding is in contrast to a previous report (15) which found no significant effect by phosphatase treatment. The reason for the observed discrepancy is unclear, and it is not possible to trace it because no detail was provided in the previous report (15).

2-AP treatment of cells also suppressed MeSO-mediated induction of NF-E2 site-dependent promoter activity (Fig. 4D), suggesting that serine/threonine phosphorylation may be involved in both the induction of NF-E2-DNA binding and the cis-regulatory activity of the NF-E2 site. Treatment of cells with genistein even increased NF-E2 site-dependent promoter activity (2-fold compared with MeSO-treated cells), although the reason for this increase is not yet understood (Fig. 4D).

Inhibition of Serine/Threonine Phosphorylation Also Inhibits the MeSO-mediated Increase in β-Globin and ALAS-E mRNA Levels and Heme Content—To examine the effect of inhibition of serine/threonine phosphorylation on erythroid differentiation, the effect of 2-AP on MeSO-mediated induction of β-globin and ALAS-E mRNAs and heme content was examined. Combined treatment of cells with 2-AP and MeSO for 48 h reduced the level of β-globin and ALAS-E mRNA levels to 13, and 75%, respectively, compared with MeSO treatment (Fig. 5). Addition of 2-AP to cells that had been incubated with MeSO for 48 h also decreased significantly an increase of these indices in the subsequent 6 h (Fig. 5). Heme contents in cells treated with MeSO for 48 h were 21.36 ± 0.83 pmol of heme/106 cells (mean ± S.D., n = 6), whereas they were decreased to

![FIG. 2. Effect of MeSO on p45 and mafK mRNA and p45 protein levels.](Image)

![FIG. 3. MeSO increased an enhancer activity of the NF-E2 site in DS cells.](Image)
Cells were then incubated with 10 mM with or without NF-E2 binding sites. with a luciferase (LUC) reporter that did not contain NF-E2 sites, or 100 μM 2-AP or 100 μM genistein together with MeSO for another 5 h. Gel mobility shift assays using an oligonucleotide containing three NF-E2 sites were performed as described under “Materials and Methods.” Panel B, 5 μg of nuclear extract from MeSO-treated DS cells were incubated calf intestine phosphatase (CIP) for 15 min before the addition of 32P-labeled probe. Panel C, DS cells were treated as described above, then immunoblot analysis was performed with anti-p45 anti-serum. Panel D, DS cells were transfected with a luciferase activity was performed with anti-p45 anti-serum. Panel D, DS cells were transfected with a luciferase reporter construct containing three NF-E2 sites in front of a minimal promoter. For cells in lanes 1–3, chemicals were added at zero time, and cells were incubated for 48 h. For cells in lanes 3 and 4, cells were first treated with 1.5% MeSO for 48 h, and then 10 mM 2-AP was added. Incubation was continued for another 6 h. Northern blot analysis was performed as described under “Materials and Methods.” Lower left panel, ALAS-E mRNA. Lane 1, 1.5% MeSO; lane 2, 1.5% MeSO + 10 mM 2-AP; lane 3, 1.5% MeSO; lane 4, 1.5% MeSO +10 mM 2-AP. For cells in lanes 1–3, chemicals were added at time zero, and cells were incubated for 48 h. For cells in lanes 3 and 4, cells were first treated with 1.5% MeSO for 48 h, and then 10 mM 2-AP was added. Incubation was continued for another 3 h. Northern blot analysis was performed as described under “Materials and Methods.” Lower left panel, ALAS-E mRNA. Lane 1, 1.5% MeSO; lane 2, 1.5% MeSO + 10 mM 2-AP; lane 3, 1.5% MeSO; lane 4, 1.5% MeSO + 10 mM 2-AP. Upper right panel, β-globin mRNA levels normalized on the basis of 28S rRNA. Lower right panel, ALAS-E mRNA levels normalized on the basis of 28S rRNA. Conditions of incubation were identical to those in the upper left panel. Experiments were repeated twice using a nitrate salt of 2-AP and once using a free base of 2-AP. Similar results were obtained in all of these experiments.

15.74 ± 0.62 pmol of heme/10^6 cells in cells treated with both MeSO and 2-AP for 48 h. These findings indicate that inhibition of serine/threonine phosphorylation plays a critical role in erythroid differentiation of MEL cells.

**Fig. 4. Effect of protein kinase inhibitors on the enhancer activity of the NF-E2 site and NF-E2 binding activity.** Panel A, DS cells were incubated in the presence of 1.5% MeSO (DMSO) for 67 h. Cells were then incubated with 10 mM 2-AP or 100 μM genistein together with MeSO for another 5 h. Gel mobility shift assays using an oligonucleotide containing three NF-E2 sites were performed as described under “Materials and Methods.” Panel B, 5 μg of nuclear extract from MeSO-treated DS cells were incubated calf intestine phosphatase (CIP) for 15 min before the addition of 32P-labeled probe. Panel C, DS cells were treated as described above, then immunoblot analysis was performed with anti-p45 anti-serum. Panel D, DS cells were transfected with a luciferase reporter plasmid with or without NF-E2 binding sites. Cells were then incubated with 10 mM 2-AP or 100 μM genistein together with 1.5% MeSO for 12 h. The results are expressed as the ratio of the luciferase activities of cells to that obtained with a reporter that did not contain NF-E2 sites, based on the mean of two determinations.

**Fig. 5. Effect of inhibition of serine/threonine phosphorylation on erythroid differentiation.** Upper left panel, β-globin mRNA. Lane 1, 1.5% MeSO; lane 2, 1.5% MeSO + 10 mM 2-AP; lane 3, 1.5% MeSO; lane 4, 1.5% MeSO + 10 mM 2-AP. For cells in lanes 1–3, chemicals were added at time zero, and cells were incubated for 48 h. For cells in lanes 3 and 4, cells were first treated with 1.5% MeSO for 48 h, and then 10 mM 2-AP was added. Incubation was continued for another 3 h. Northern blot analysis was performed as described under “Materials and Methods.” Lower left panel, ALAS-E mRNA, Lane 1, 1.5% MeSO; lane 2, 1.5% MeSO + 10 mM 2-AP; lane 3, 1.5% MeSO; lane 4, 1.5% MeSO + 10 mM 2-AP. Upper right panel, β-globin mRNA levels normalized on the basis of 28S rRNA. Lower right panel, ALAS-E mRNA levels normalized on the basis of 28S rRNA. Conditions of incubation were identical to those in the upper left panel. Experiments were repeated twice using a nitrate salt of 2-AP and once using a free base of 2-AP. Similar results were obtained in all of these experiments.

**NF-E2 Function Is Regulated by the Ras Signaling Cascade.**—Various cell surface receptors transmit proliferation and differentiation signals into cells through activation of signaling transduction cascades. The Ras-Raf-MAPKK cascade represents such an important signal transduction cascade which ultimately phosphorylates serine/threonine residues of effector molecules, including various transcription factors (28). Thus we examined whether this cascade is involved in the regulation of NF-E2 function. Coexpression of p45 and MafK in NIH3T3 cells resulted in a small increase of the reporter gene activity (−3-fold) which contained three copies of NF-E2 sites. When a plasmid expressing the oncogenic form of H-ras (H-RasV12) was cotransfected into NIH3T3 cells with p45 and MafK expression plasmids, however, there was a marked increase in the reporter gene activity (−25-fold) (Fig. 6A). In contrast, when a deletion construct of p45 (p45ΔN), which lacked the amino-terminal region thus defective in transcription (5), was cotransfected with a MafK expression plasmid, the reporter gene activity remained suppressed, even in the presence of the H-RasV12 expression plasmid (Fig. 6A). Hence it can be concluded that the observed effect of H-RasV12 involved the functional NF-E2.

The effect of a constitutively active form of MAPKK was also examined using a transactivation assay (Fig. 6B). In this experiment, we utilized a reporter plasmid pRBGP10, which contained a single NF-E2 site in front of a minimal promoter. Expression of activated MAPKK itself had little effect on the reporter gene activity, indicating that under these experimental conditions, this reporter construct is not a suitable target for endogenous effectors such as AP-1. However, MAPKK increased the reporter gene activity in the presence of both p45 and MafK (−50-fold). As with the activated Ras, MAPKK also required the amino-terminal region of p45 to achieve a high level of reporter gene expression (data not shown).

The effect of H-RasV12 on the DNA binding activity of NF-E2 was also examined using a gel mobility shift assay. Although expression of p45 and MafK by themselves in NIH3T3 cells resulted in only weak DNA binding activity, coexpression with H-RasV12 resulted in a marked increase in p45-MafK het-
erodimer formation (Fig. 7). These results clearly show that the Ras cascade has a significant effect on the DNA binding activity of NF-E2. Because activation of this cascade is known to result in serine/threonine phosphorylation of an effector molecule, these findings are consistent with the results that serine/threonine phosphorylation may play an important role in the regulation of NF-E2 function.

To examine whether these results were caused by changes in the level of expression of NF-E2 subunits, we determined the accumulation of NF-E2 subunit proteins in transfected cells, using FLAG epitope-tagged p45 or tagged MafK expression plasmids. These epitope-tagged proteins behaved in a manner similar to that of the wild-type proteins in transfection assays (data not shown). The levels of p45 were similar for p45 expression by itself (Fig. 8, fourth lane from left), and expressed together with MafK (Fig. 8, sixth lane). Similar results were also observed when exogenous H-RasV12 was expressed additionally (Fig. 8, fifth and seventh lanes). In contrast, the amount of MafK increased slightly in response to exogenous H-RasV12 in both the absence of p45 (Fig. 8, second and third lanes) and the presence of p45 expression plasmid (Fig. 8, sixth and seventh lanes). This change in MafK protein level, however, did not influence NF-E2 function (discussed below). These findings indicate that an effect of the Ras cascade on NF-E2 function is not caused by a change in the level of NF-E2 subunit expression.

The NH2-terminal 83-Amino Acid Region of p45 Contains an Activation Domain That Is Responsible for Ras-mediated Induction of Transactivation—To understand the molecular mechanism of regulation of NF-E2 function, it is essential to define the function of each subunit with respect to its trans-regulation. To examine trans-regulatory function of p45 in independent of DNA binding, we fused various regions of p45 to the GAL4 DNA binding domain, and transfected their expression plasmids into cells together with a reporter gene plasmid. The results demonstrated that the NH2-terminal region of p45 contains a transcription activation domain (Fig. 9A). When fused to the GAL4 DNA binding domain, 1–272 amino acids, or 1–83 amino acids of p45, showed marked transactivation of the reporter gene plasmid. G4-p45 (86–272 amino acids) increased luciferase activity moderately, whereas G4-p45 (1–36 amino acids) had little effect (Fig. 9A). These findings indicate that an effect of the Ras cascade on NF-E2 function is not caused by a change in the level of NF-E2 subunit expression.

The NH2-terminal 83-Amino Acid Region of p45 Contains an Activation Domain That Is Responsible for Ras-mediated Induction of Transactivation—To understand the molecular mechanism of regulation of NF-E2 function, it is essential to define the function of each subunit with respect to its trans-regulation. To examine trans-regulatory function of p45 independent of DNA binding, we fused various regions of p45 to the GAL4 DNA binding domain, and transfected their expression plasmids into cells together with a reporter gene plasmid. The results demonstrated that the NH2-terminal region of p45 contains a transcription activation domain (Fig. 9A). When fused to the GAL4 DNA binding domain, 1–272 amino acids, or 1–83 amino acids of p45, showed marked transactivation of the reporter gene plasmid. G4-p45 (86–272 amino acids) increased luciferase activity moderately, whereas G4-p45 (1–36 amino acids) had little effect (Fig. 9A). These
results indicate that the NH₂-terminal 83-amino acid region is required for a full transactivation activity.

Using transactivation assays of p45 fusion constructs with the GAL4 DNA binding domain, we investigated next whether this region contains the Ras-responsive activation domain. As shown in Fig. 9B, expression of H-RasV12 augmented reporter gene activity markedly in the presence of p45 that contained the 83-amino acid NH₂-terminal region, whereas it had little effect on reporter gene activity when a p45 fragment lacking the amino-terminal region was used (Fig. 9B). These results indicate that the activity of the activation domain of p45 can be regulated by the Ras cascade. Hence, it can be concluded that the Ras cascade also has a direct stimulatory effect on p45 transactivation potential.

Our previous studies demonstrated that MafK repressed the NF-E2 site-dependent promoter activity in the absence of p45 and that the presence of p45 reversed MafK transcription repression activity (5). Therefore we examined in this study the effect of MafK on NF-E2 activity stimulated by the Ras cascade. First, we titrated the effect of the MafK expression plasmid on reporter gene activity in the absence or the presence of MAPKK. As shown in Fig. 10A, the reporter gene activity showed a biphasic response to an increasing amount of the MafK plasmid in the presence of a fixed amount of p45 expression plasmid. For example, MafK plasmid transfection strongly augmented reporter gene expression at low levels, whereas it suppressed at levels higher than 0.2 μg. This suppression can be accounted for by the fact that an excess amount of MafK homodimer is known to compete against p45 heterodimers for binding at the NF-E2 site (3, 5). These results thus indicate that in the Ras-mediated regulation of NF-E2 activity, MafK exhibits in the presence of p45 a positive transactivation effect at low levels of expression, whereas it represses transcription when expressed in excess. Because 0.2 μg of MafK expression plasmid was used in all of these experiments, it can be concluded that the induction of NF-E2 activity by the Ras cascade (Fig. 6) is not caused by an increase in the amount of MafK protein. Thus, the Ras signaling cascade stimulates NF-E2-dependent transcription by eliciting a qualitative, rather than a quantitative, change in the NF-E2 subunits.

Domain analysis of MafK using fusion constructs with GAL4DBD revealed an additional mechanism for the repression of reporter activity by a excess amount of MafK. Namely, the GAL4DBD-MafK fusion protein repressed the reporter activity in QT6 fibroblasts (Fig. 10B). Because such repression was observed with a GAL4 fusion product but not with wild-type MafK or with GAL4DBD, the results indicate that MafK possesses direct trans-repression activity, which does not involve competition for a binding site. MafK derivatives that lacked an
NH₂-terminal or COOH-terminal region also suppressed the reporter gene activity potently (Fig. 10B), indicating that the trans-repression activity of MafK is confined to the b-zip domain. A similar experiment in MEL cells was not possible, however, because a basal reporter activity in these cells was too low. Nonetheless, these results taken together indicate that MafK exerts transcription repression not only by competition for its binding site but also by direct trans-repression.

**DISCUSSION**

Previous studies using MEL cells suggested that NF-E2 may provide the major enhancer function for globin gene expression (6, 7). For example, the DNA binding activity of NF-E2 was shown to be increased during erythroid differentiation of MEL cells (8–10); however, the exact mechanism of regulation of NF-E2 activity remained unclear. The level of p45 mRNA itself was reported either to increase (29) or not to change (15) during erythroid differentiation of MEL cells. Our results in this study demonstrated that the levels of p45 mRNA, p45 protein, and the NF-E2-DNA complex were all clearly increased in DS cells during erythroid differentiation (Figs. 1 and 2). The reason for this difference is unclear, but it may be related to different clones of MEL cells used in these studies. It should be noted that the clone of DS (DS-19) cells used in this study is one of the most efficiently differentiating MEL cell clones (16). It is also known that there is a significant increase in p45 protein levels in erythroid differentiation of normal hematopoietic progenitor cells which precedes the expression of erythropoietin receptor (30). These changes of NF-E2 activity were absent in DR cells, which also fail to undergo erythroid differentiation (Figs. 1 and 2). These findings suggest that the up-regulation of NF-E2 activity is an important regulatory event in erythroid differentiation.

NF-E2 can be phosphorylated by a cAMP-dependent protein kinase in vitro, and prolonged activation of this kinase increases the amount of NF-E2-DNA complex in MEL cells (15). Our findings in this study show that inhibition of serine/threonine phosphorylation results in marked reduction of Me₂SO-mediated induction of a NF-E2-DNA complex as well as the enhancer activity of the NF-E2 site (Fig. 4, A and D), suggesting that NF-E2 activity may be regulated by serine/threonine phosphorylation. Inhibition of serine/threonine phosphorylation by 2-AP was also accompanied by marked reduction in the levels of β-globin mRNA, ALAS-E mRNA (Fig. 5) and heme content. These findings suggest that serine/threonine phosphorylation plays a critical role in the expression of erythroid genes in MEL cell differentiation.

In contrast, inhibition of tyrosine phosphorylation augmented the enhancer activity of the NF-E2 site, without influencing DNA binding activity of NF-E2 (Fig. 4, A and D). It is unclear at present why genistein increased the enhancer activity of the NF-E2 site, but it is possible that transactivation activity of the NF-E2 molecule might be inhibited indirectly or by tyrosine phosphorylation. It is also possible that other factors that can interact with the NF-E2 site might be activated by genistein.

Consistent with such a hypothesis, our results demonstrated that the activity of NF-E2 reconstituted in fibroblasts is under the control of the Ras signaling cascade. Namely, a constitutively active form of Ras and MAPKK, a downstream protein kinase, potentiated DNA binding as well as transactivation of NF-E2 (Figs. 6A and 7). Transactivation assays using GAL4DBD-p45 fusion constructs also demonstrated that the active form of Ras stimulates the p45 transactivation domain (Figs. 7B and 8A). The amino-terminal transactivation domain of p45 also possesses two consensus sequences for phosphorylation by MAPK (31, 32). The insertion of a 2-amino acid substitution into the consensus sequences, however, did not affect the reporter gene activity in transactivation assays (data not shown). These findings suggest that either coactivators essential for the activation domain of p45 may be modified by phosphorylation, or other phosphorylation sites of the p45 activation domain may be involved in such regulation.

We showed previously that the small Maf family proteins can function as transcriptional repressors of NF-E2 site-dependent transcription in the absence of p45 (3, 5). Because MafK possesses an active transcription repression domain in its b-zip region (Fig. 10B), it can exert effects not only by competition for binding sites but also by an active repression mechanism that might involve protein interaction with other factors such as corepressors. Furthermore, the fact that an excess amount of MafK repressed the MAPKK-, or Ras-induced cis-regulatory activity of the NF-E2 site even in the presence of p45 (Fig. 10A) suggests that the amount of MafK homodimer may be crucial in determining the level of NF-E2 site-dependent gene expression. In other words, certain combinations of MafK and heterodimerizing partners may predispose cells to a particular response upon stimulation of the Ras cascade.

To provide a hypothesis for further testing, a model for the role of the Ras signaling cascade in NF-E2 activation is pre-
sentent in Fig. 11. This predicts that the Ras signaling cascade is activated during erythroid differentiation, resulting in the increase of the erythroid-specific gene expression, which is caused in part by an increase in NF-E2-DNA binding and in part by an increase in p45 transactivation activity. One feature that is MaFK functions as both a signal transducer and a signal blocker, depending on its amount as well as on the presence of p45. The fact that DR cells show constitutive expression of p45. The fact that DR cells show constitutive expression of mafK mRNA and no increase in p45 mRNA by Me2SO treatment is consistent with the view that signal transduction resulting in the activation of NF-E2 function by the Ras signaling cascade may be inhibited by an excess amount of MaFK homodimer in these cells.

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