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Regulation of Globin Gene Transcription by Heme in Erythroleukemia Cells: Analysis of Putative Heme Regulatory Motifs in the p45 NF-E2 Transcription Factor

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ABSTRACT

The function of the NF-E2 transcription factor, a p45/small Maf heterodimer, was analyzed in the erythroleukemia cell lines MEL and CB3. In contrast to MEL cells, CB3 cells are null for p45 and thus express only extremely low levels of adult globin transcripts upon induction by agents promoting erythroid differentiation. We investigated the response of erythroleukemia cells to hemin treatment. Hemin rapidly induces β-globin gene transcript levels in MEL cells, but not in CB3 cells. Stable expression of the large p45 NF-E2 subunit in CB3 cells restores hemin mediated β-globin gene transcription, suggesting that the presence of a functional NF-E2 is required for strong induction of β-globin mRNA levels by hemin in erythroleukemia cells. We performed mutagenesis of two potential heme-regulatory motifs (HRMs) in p45 NF-E2 and found that the mutated versions are expressed and can still recognize a NF-E2 DNA binding element. In addition, we showed that p45 NF-E2 HRM mutants are able to restore β-globin gene transcription in CB3 cells upon induction by hemin. Our results suggest that globin gene activation by heme appears to be independent of the putative HRMs in the p45 subunit of the NF-E2 transcription factor. *Antioxid. Redox Signal.* 8, 68–75.

INTRODUCTION

THE MECHANISMS GOVERNING the differentiation of erythroid cells have been studied in great detail and serve as a paradigm for the regulation of gene expression. The maturation of red blood cells requires the synthesis of large quantities of hemoglobin. The erythroid-specific activation of α- and β-globin genes is mediated by remote enhancer-like DNA sequences, termed locus control regions (LCRs) (37). LCR motifs recognized by the NF-E2 (nuclear factor-erythroid 2) basic-leucine zipper (bZIP) transcription factor are required for enhancer activity (37). NF-E2 is a heterodimer comprising a hematopoietic-specific subunit, the CNC protein p45, and a ubiquitously expressed small Maf subunit, either MafF, MafG, or MafK (3, 5, 10, 23). NF-E2 binds to NF-E2/MARE (Maf recognition element)-type DNA binding elements that contain a central AP-1 motif (2). Previous studies showed that NF-E2 promotes the opening of chromatin throughout the β-globin loci (6, 21, 43). Furthermore, it has been reported that binding of RNA polymerase II to the LCR is independent of the p45 NF-E2 subunit, but that recruitment of the polymerase to the hyperacetylated β-globin promoter requires p45 NF-E2 (24). Hence, NF-E2 appears to play an important role in the regulation of erythroid gene expression.

The murine erythroleukemia cell lines MEL and CB3 have been used to study the molecular events leading to the induction of globin chains, markers of erythroid differentiation. MEL cells can be induced to undergo erythroid differentiation by a variety of reagents including dimethyl sulfoxide (DMSO), hexamethylene bisacetamide (HMBA), hemin (oxidized heme),

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and butyrate (19, 27, 39, 41). Erythroid differentiation in MEL cells parallels the development from proerythroblasts to orthochromatic normoblasts *in vivo* and is manifested by induction of α- and β-globin gene transcription and heme biosynthesis enzymes (40, 42). A useful tool in the study of NF-E2 function has been the CB3 cell line. CB3 cells do not express the p45 subunit of NF-E2 due to a Friend leukemia virus insertion in one allele and the loss of the other (8, 28). Thus, CB3 cells express only minimal levels of α- and β-globin mRNA. Forced expression of p45 NF-E2, alone or covalently linked to MafK or MafG, restores globin gene induction by DMSO (9, 25, 28), implying a functional role of p45 NF-E2 in the differentiation of erythroleukemia cells.

Data from a wealth of literature demonstrate that exogenous hemin increases the expression of globin genes and other erythroid-specific genes (14, 29, 40). In MEL as well as K562 human chronic myelogenous leukemia cells, hemin, but not other metalloporphyrins, can efficiently induce the expression of hemoglobin (13, 38). The response to hemin appears to require the presence of the NF-E2 binding site (34, 38, 45). A previous report also provided evidence that hemin increases the transactivation potential of a NF-E2 recognition element linked to a reporter gene in K562 cells (38), suggesting a direct effect of hemin on regulation mediated by transcriptional regulators binding to this DNA binding site. Recent studies showed that the CNC bZIP transcription factor Bach1, a homolog of the p45 subunit of NF-E2, is a heme-binding protein (11, 36, 48). Bach1 contains 6 heme-regulatory motifs (HRMs) and is negatively regulated by heme in that its DNA binding activity decreases in the presence of hemin (36). The upregulation of both α-globin and β-globin genes in MEL is mediated through disrupting the interaction of Bach1 with the MARE sites (50, 51). It was proposed that during erythroid differentiation in MEL cells a dynamic change from a repressing Bach1/small Maf complex to a transactivating p45/small Maf heterodimers (NF-E2) occurs (11, 36).

In this report, we analyzed the role of the NF-E2 heterodimer in the induction of erythroid differentiation by hemin. We noted the presence of two putative HRMs located in the amino terminus of the large p45 subunit of NF-E2. In our studies we found that hemin treatment leads to a strong increase of β-globin mRNA levels in MEL cells, but not in p45 NF-E2-deficient CB3 cells. We also show that stable expression of p45 NF-E2 in CB3 cells is able to confer a strong induction of β-globin transcripts by hemin. Further data suggest that this activation by hemin appears not to require the two putative HRMs in p45 NF-E2.

METHODS

Cell culture and treatments

MEL (murine erythroleukemia clone 745 GM86) and CB3 (p45-deficient murine erythroleukemia, a kind gift of Y. Ben-David, Toronto) (28) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum with addition of penicillin/streptomycin. For induction experiments, the cells were diluted to 2×10^5 cells/ml and treated for the indicated time with and without hemin (oxidized heme) at various concentrations. The hemin solution was prepared using ethanolamine as described (38).

Northern blot hybridization analyses

Total RNA was extracted by using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 10 μg of RNA was subjected to formalin-agarose gel electrophoresis (1% agarose), transferred onto a HYBOND-XL nylon membrane (Amersham Biosciences, Baie d'Urfé, QC), and hybridized (16) with ^{32}P-labeled probes specific for β_{major}-globin (7)and β-actin. After washing, the gel was exposed to x-ray film.

Plasmid constructs and site-directed mutagenesis

An EcoRI fragment comprising the p45 cDNA was cloned into the pEF1αneo expression vector. The EF1αneo construct is driven by the promoter of the human elongation factor 1α gene (31) and contains the neomycin resistance gene as a selectable marker. Site-directed mutagenesis of p45 was performed, according to the instructions of the manufacturer (QuickChange, Stratagene, La Jolla, CA). We mutated amino acids 4 or 66 from cytosine to alanine using the following primers:

5'-GGCACAGTAGGATGCCCCCGGCTCCTCCTCAG-CAG-3' (p45C4-F),

5'-CTGCTGAGGAGGAGCCGGGGGCATCCTACTGT-GCC-3' (p45C4-R),

5'-GCCACCTCCAACATATGCCCCCTGTTCAATTCA-TCC-3' (p45C66-F),

5'-GGATGAATTGAACAGGGGGCATATGTTGGAGG-TGGC-3' (p45C66-R)

For generation of the p45 double C4/C66 mutant we performed mutagenesis of the plasmid containing the C4 mutation using the p45C66-F and p45C66-R primers. The mutations were verified by sequence analysis.

Transfection experiments

Stable transfection of CB3 cells was performed by electroporation as follows: 1.5×10^7 cells/transfection were electroporated with 20 μg of pEF1αneo constructs using a Gene Pulser II (BioRad, Hercules, CA) at 280 mV and 960 μF. Stably transfected cells were selected in 1.5 mg/ml geneticin (GIBCO-BRL, Gaithersburg, MD).

Immunoblot analysis

Nuclear extracts were prepared as described (4). The protein concentrations were determined using a protein assay kit (Bio Rad). 10 μg of the lysates were electrophoresed in 12% SDS-polyacrylamide gels (see Fig. 4A). Resolved proteins were transferred electrophoretically to a PVDF membrane (Immobilon, Millipore, Bedford, MA). After transfer, the membrane was blocked for 1 h at room temperature and was subsequently incubated overnight with p45 specific antiserum (3) in 1× TBS plus 5% milk, 0.05% Tween-20, and 350 m*M* NaCl. Following washing, the membrane was incubated for 1 h at

room temperature with secondary goat anti-rabbit antibodies conjugated to horseradish peroxidase (Pierce, Rockford, IL). The proteins were detected using a chemiluminescent reagent (Super-Signal West Pico, Pierce) following the manufacturer's instructions.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS

EMSA (electrophoretic mobility shift assay) of nuclear extracts was performed as described previously (3, 47). The NF-E2 binding site from the porphobilinogen deaminase (PBDG) promoter (3) was used as a probe. The preimmune (PI) or immune sera were added to the reactions, as indicated, at a concentration of 1.5 μl per 20 μl total reaction volume. An 800 molar excess of unlabeled oligonucleotide was used for competition. Anti-p45 antiserum has been described previously (3, 5). Protein-DNA-complexes were resolved on a 5% nondenaturing polyacrylamide gel in 0.5× TBE buffer. After electrophoresis the gel was dried and exposed to x-ray film.

RESULTS

Hemin induction in MEL erythroleukemia cells

Previously it had been shown that erythroleukemia cells can be induced by exogenous addition of oxidized heme (hemin). To study the role of the transcription factor NF-E2 in this induction, we first determined the dosage response and time course of hemin stimulation in our erythroleukemia cell model. We treated MEL cells with different concentrations of hemin and measured β-globin gene transcript levels by Northern analysis. Significant induction of β-globin mRNA expression levels was observed at 20 μ*M* and increased further at higher concentrations of hemin (Fig. 1A). High concentrations (100–200 μ*M*) appeared to be toxic to the cells causing lower viability (data not shown). We thus performed subsequent experiments at a concentration of 50 μ*M* hemin, resulting in strong induction of β-globin mRNA and lower toxicity. In time course experiments we found that activation of β-globin transcription by hemin is fast, showing an induction at 4 hours after treatment (Fig. 1B). We observed strong induction levels of β-globin mRNA by hemin at 24 and 48 hours (Fig. 1B). It is of interest to note that the response to the inducer DMSO is slower than observed for hemin. With DMSO, globin gene transcripts are notably induced only after a period of 24 hours, suggesting a different mode of action for both inducing agents (data not shown). In summary, our time course analysis suggests that hemin leads to the rapid induction of the erythroid differentiation program in MEL cells.

NF-E2 is required for hemin-mediated globin transcript induction in CB3 cells

We then asked if CB3 cells that do not express p45 NF-E2, respond to hemin as an inducer of erythroid differentiation. Northern analysis showed that unlike MEL cells, CB3 cells do not appear to express β-globin transcripts upon treatment with hemin, as has been reported previously for experiments

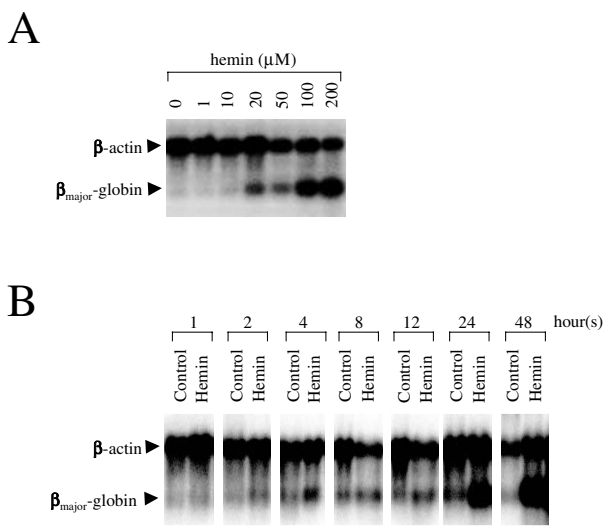

FIG. 1. Dosage response and time course analysis of β-globin transcript induction. (**A**) Effect of different doses of hemin on the induction of β-globin transcript levels in MEL cells. Northern blot analysis of total RNA prepared from untreated MEL cells or cells exposed to different concentrations of hemin for 48 h. 10 μg of total RNA was loaded per lane. β_{major}-globin and β-actin transcripts are indicated. (**B**) Time course analysis of induction of β-globin induction by hemin in MEL cells. Northern blot analysis of MEL cells cultured in the presence of 50 μ*M* hemin for various lengths of time. 10 μg of total RNA was loaded per lane. β_{major}-globin and β-actin transcripts are indicated.

using the inducer DMSO (28) (Fig. 2). Next, we analyzed CB3 cells stably transfected with the expression construct pEF1αneo-p45 coding for p45 NF-E2. In contrast to parental CB3 cells and cell clones with vector alone, the CB3 cell clones stably transfected with a construct coding for p45 NF-

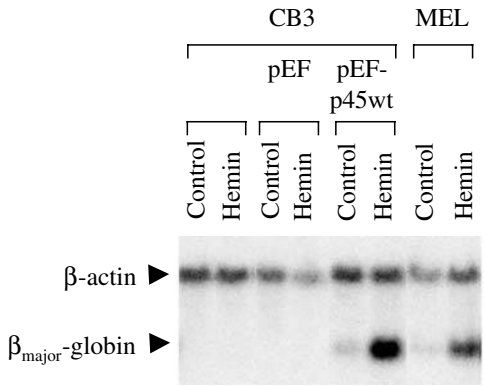

FIG. 2. Hemin induction of β-globin gene transcription in CB3 depends on the presence of p45 NF-E2. Northern blot analysis of total RNA of erythroleukemia cells treated with 50 μ*M* hemin for 48 h. We analyzed MEL cells, untransfected CB3 cells as well as CB3 cell clones that were stably transfected with the pEF1αneo (pEF) expression vector alone or with the pEF1αneo construct coding for p45 wild-type (wt). 10 μg of total RNA was loaded per lane. β_{major}-globin and β-actin transcripts are indicated.

E2 are clearly responding to hemin as β-globin gene transcription is strongly induced (Fig. 2).

Hemin signaling is independent of the two potential heme regulatory motifs in p45 NF-E2

We showed that induction of globin gene transcription in CB3 cells by hemin is dependent on the presence of p45 NF-E2 (Fig. 2). We speculated that hemin could regulate p45 activity in various ways. Previous studies have shown that the CNC protein Bach1, a homolog of p45 NF-E2 and a repressor of globin gene transcription, is a heme binding protein and is negatively regulated through the presence of HRMs (36). Comparison among different HRM motif containing proteins previously suggested that only the cysteine-proline (CP) dipeptide is absolutely conserved (52). This is further supported by the alignment of HRM sequences found in various proteins that have been shown to confer regulation by heme (Fig. 3A) (22, 26, 30, 33, 36, 46, 52). We noted two putative HRMs, both comprising a CP dipeptide, in the amino terminus of p45 NF-E2 that are conserved among different species (Fig. 3B). We thus performed site-directed mutagenesis of the two CP dipeptides in p45 NF-E2 by mutating the cysteines to alanine at position 4 and/or 66. Using EMSA we confirmed that these mutated p45 NF-E2 versions A4 and A66 are still able to bind to a typical NF-E2 DNA binding site by analyzing extracts of 293HEK cells co-transfected with expression vectors coding for either of the mutant versions and the small Maf protein MafG (data not shown). We concluded that the mutated p45 NF-E2 proteins are not impaired in their DNA binding and dimerization activities. We thus stably transfected the pEF1αneo expression vector coding for the various p45 NF-E2 HRM mutants, including an A4/A66 double mutant, into CB3 cells to determine whether they can rescue β-globin gene transcription. Immunoblot analysis confirmed that the stably transfected CB3 clones express the wild-type and mutant p45 NF-E2

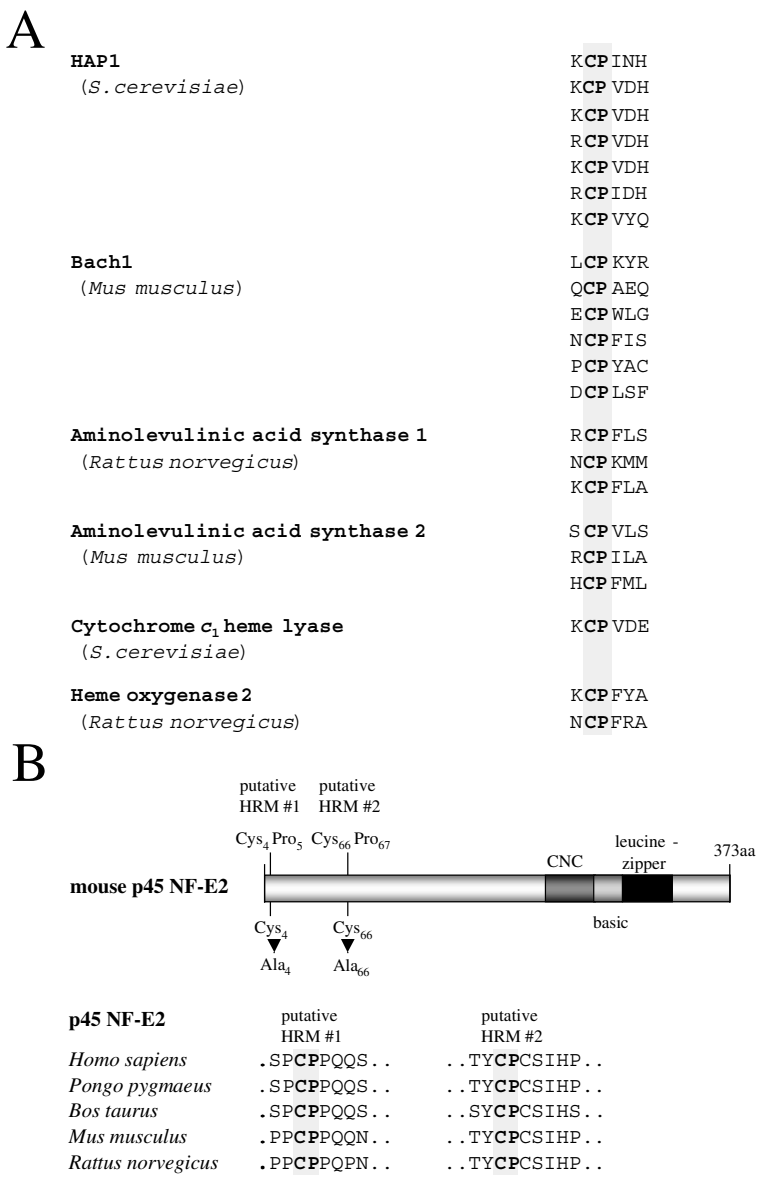

FIG. 3. Putative HRM motifs in p45 NF-E2. (**A**) Alignment of HRM motifs in proteins that have been reported to confer regulation by heme. This includes the HAP1 (22) and Bach1 (36) transcription factors as well as the enzymes aminolevulinic acid synthase 1 (33), aminolevulinic acid synthase 2 (26), cytochrome c_1 heme lyase (46), and heme oxygenase 2 (30). (**B**) Diagram illustrating the putative HRMs comprising the CP dipeptide in the p45 subunit of the NF-E2 transcription factor. The cysteine to alanine mutations introduced by site-directed mutagenesis are shown. CNC, basic and leucine zipper (ZIP) domains are indicated. Conservation of the CP dipeptide in the p45 NF-E2 protein of different species is shown.

versions, whereas in a CB3 cell clone transfected with vector alone no p45 NF-E2 was detectable (Fig. 4A). It is of interest to note that the levels of the recombinant wild-type and mutant versions appeared to exceed the levels of endogenous p45 NF-E2 protein in MEL cells (Fig. 4A). To confirm the DNA binding of the mutant p45 NF-E2 proteins in nuclear extracts of CB3 cell clones, we carried out EMSA analysis. We observed strong binding to a labeled oligonucleotide corresponding to a NF-E2 binding site in extracts from cells stably transfected with an expression vector coding for wild-type p45 and the mutated versions (Fig. 4B). The presence of the p45 subunit was confirmed by addition of a p45 antiserum to the binding reaction, resulting in the disappearance of the complex (Fig. 4B). We performed Northern analysis of stably transfected CB3 cells using RNA prepared from uninduced cells or cells induced with hemin for 48 h (Fig. 4C). We examined multiple clones and the ones that showed DNA binding activity in the EMSA were also able to restore β-globin mRNA induction in CB3 cells (Fig. 4C), suggesting that hemin signaling is independent of the two putative HRMs in p45.

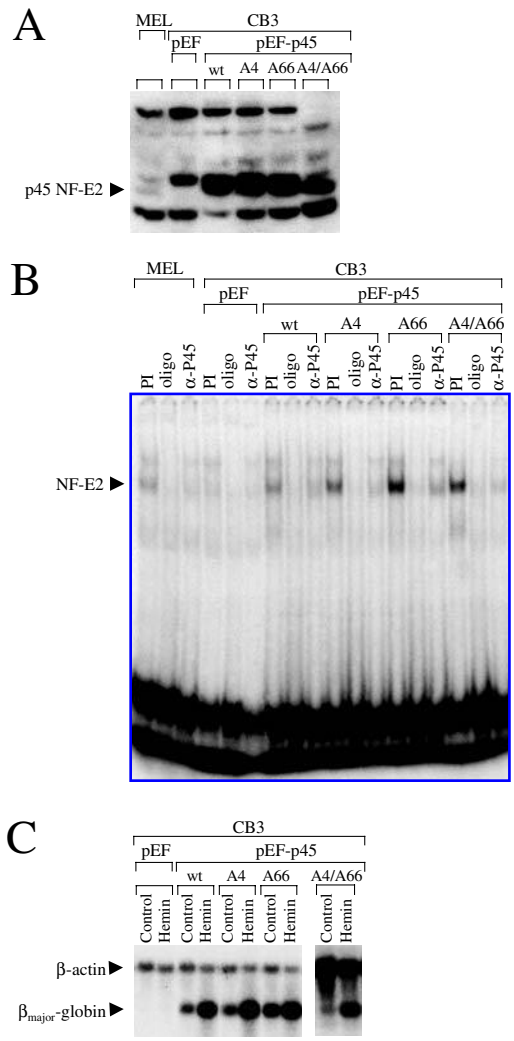

FIG. 4. Analysis of putative HRMs in p45 NF-E2. (**A**) Immunoblot analysis of nuclear extracts prepared from MEL cells and CB3 cell clones stably transfected with the pEF1αneo expression vector without insert (pEF) or coding either for p45 wild-type (wt), p45-A4, p45-A66 or p45-A4/A66. For immunoblot analysis a p45 NF-E2 specific antiserum was used (3). Equal protein amounts (10 μg) have been loaded per lane. The p45 NF-E2 protein is indicated by an *arrow*. (**B**) EMSA analysis of nuclear extracts from MEL cells and CB3 cell clones. An oligonucleotide corresponding to the NF-E2 in the porphobilinogen deaminase promoter has been used as a probe. Nonlabeled NF-E2 binding site competitor oligonucleotide (oligo), pre-immune (PI) or anti-p45 NF-E2 serum (α-p45) was added to the reaction as indicated. (**C**) Northern blot analysis of total RNA prepared from MEL cells and CB3 cell clones. 10 μg of total RNA was loaded per lane. β_{major}-globin and β-actin transcripts are indicated.

DISCUSSION

Recent studies have shown that heme can directly bind to transcription factors and thus regulate their activity (17, 36). Here, we analyzed the link between heme signaling and the NF-E2 transcription factor in MEL cells. We found that β-globin mRNA levels in MEL cells start to increase within hours of hemin treatment, in agreement with earlier studies (35). In contrast, we and others found that with DMSO as inducing agent β-globin mRNA levels are not detected until 24 h after treatment (35, and data not shown). We speculate that induction by hemin, a lipophilic compound that readily enters the cell (44), is more rapid than activation by DMSO, because treating the cells with the endproduct of the heme biosynthesis pathway circumvents the need to stimulate pathways ultimately leading to increased cellular heme levels, which then exert their effect on transcriptional regulators. An important question is whether the presence of higher intracellular heme levels is the crucial factor or only part of the requirement to initiate the late stages of the erythroid differentiation program?

We then asked whether the transcription factor NF-E2, a heterodimer comprised of a large p45 subunit and small Maf protein, is essential for hemin-mediated induction of erythroid differentiation. To this end, we used CB3 erythroleukemia cells that do not express p45 NF-E2, resulting in a drastic reduction of adult globin gene expression (28). Reintroduction of p45 NF-E2 as well as covalently linked p45/MafK or p45/MafG heterodimers can restore globin gene transcription in DMSO-induced CB3 cell clones (10, 25, 28). We found that parental CB3 cells treated with hemin do not appear to express β-globin gene transcripts. We thus generated CB3 cells stably expressing wild-type p45 NF-E2 and showed that β-globin mRNA levels can be induced in these clones following hemin treatment. In contrast to MEL cells, some of these stably transfected CB3 cell clones exhibit high basal levels of β-globin transcripts. This observation hints at a possible "priming" towards the erythroid differentiation program through the presence of p45 NF-E2. This effect may be explained by the higher expression levels of the recombinant p45 NF-E2 in stably transfected CB3 cell clones when compared to endogenous p45 NF-E2 levels in MEL cells. It is of interest that the CB3 cell clones expressing higher basal levels are still inducible by exogenous hemin. Our results strongly suggest that NF-E2 is essential for the induction of globin gene transcription by

hemin in these erythroleukemia cells. These data are supported by recent chromatin immunoprecipitation (ChIP) studies showing that occupancy of α- and β-globin regulatory elements by p45 NF-E2 is increased after hemin induction (50, 51), suggesting regulation of NF-E2 DNA binding activity by intracellular heme levels.

Based on our results we hypothesized that hemin might act directly on p45 NF-E2, as it has been shown for the p45 homolog, Bach1 (36). The DNA binding activity of Bach1, a heme binding protein that comprises 6 HRMs, is negatively regulated by heme (36). In fact, Bach1 is functioning as a repressor of α- and β-globin gene transcription and this repressing activity can be cancelled by treatment with hemin (50, 51). The alignment of different HRM motif containing proteins had indicated that only a CP dipeptide in the motif is completely conserved (52). We noted the presence of two CP dipeptides in the amino terminus of p45 NF-E2, at amino acids 4/5 and 66/67. We thus performed site-directed mutagenesis to verify whether these potential HRM motifs are required for heme signaling in our erythroleukemia cell model. We found that the presence of single or double mutations of the HRMs does not interfere with the ability of p45 NF-E2 to restore β-globin gene transcription in CB3 cells, strongly suggesting that heme signaling via NF-E2 occurs independently of the CP dipeptide. It should be noted that these data do not exclude the possibility of heme binding to these sites. Similar mutational analysis was performed for the enzyme heme oxygenase-2 (HO-2) which contains two HRMs, showing that these motifs are not required for the catalytic activity of the enzyme. Intriguingly, using spectrophotometric analysis, HO-2 was still found to be a hemoprotein, with heme binding to these HRMs (30). It was proposed that these motifs may act as "heme sensors". The authors speculated that the heme binding to these sites is protected from degradation, and that when heme exceeds the binding capacity of the HRMs in HO-2 the excess heme is available for catalysis. Thus by controlling heme levels, HO-2 could regulate the expression of genes that are responsive to heme, although the enzyme itself is not controlled by heme (30). It is also possible that p45 NF-E2 function does not rely on binding of heme to the transcription factor, but heme may regulate p45 NF-E2 activity indirectly through other proteins. Several non-bZIP proteins including CBP and WW domain-containing proteins have been shown to interact with distinct domains of p45 NF-E2 (1, 15, 18, 20, 32). Previous studies have also shown that p45 NF-E2 is phosphorylated, although the specific phosphorylation sites are not known (12). Hence, heme may act on NF-E2 through signaling molecules or cofactors. Importantly, several recent reports provided evidence that induction of erythroid differentiation in MEL cells is mediated by a switch from a Bach1/small Maf heterodimer that represses globin gene transcription to a p45/small Maf (NF-E2) complex that leads to the induction of globin gene expression (11, 36, 50, 51). It has been shown that hemin is able to repress the DNA binding activity of Bach1 via its HRMs (36). Further studies suggested that hemin treatment results in the nuclear export of Bach1 via the exporter CRM1 (49). Thus, the activity of Bach1, but possibly not that of p45 NF-E2, is regulated by direct interaction with heme during erythroid differentiation.

Our data here suggest that the presence of p45 NF-E2 is essential for induction of globin transcript levels in erythroleukemia cells by heme, but this process does not appear to require the putative HRMs present in the amino terminus of p45 NF-E2. In contrast, it has been shown that the p45 NF-E2 antagonist Bach1 is directly controlled by heme binding to its HRMs. Nevertheless, the control of both, the Bach1 and NF-E2 transcription factor activities, appears to be critical for globin gene transcription and erythroid differentiation in erythroleukemia cells. Elucidating the exact mechanisms of this dynamic interplay between these bZIP transcription factors will be important for a better understanding of the regulation of erythroid differentiation by intracellular heme levels.

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ABBREVIATIONS

C-P, cysteine-proline; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; HO-2, heme oxygenase-2; HRM, heme-regulatory motif; LCR, locus control region; NF-E2, nuclear factor-erythroid 2.

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