Heme Positively Regulates the Expression of \(\beta\)-Globin at the Locus Control Region via the Transcriptional Factor Bach1 in Erythroid Cells*

The transcription factor Bach1 heterodimerizes with small Maf proteins to repress Maf recognition element (MARE)-dependent gene expression. The repressor activity of Bach1 is inhibited by the direct binding of heme. To investigate the involvement of Bach1 in the heme-dependent regulation of the expression of the \(\beta\)-globin gene, mouse erythroleukemia (MEL) cells were cultured with succinylacetone (SA), a specific inhibitor of heme biosynthesis, and the level of \(\beta\)-globin mRNA was examined. A marked decrease of \(\beta\)-globin mRNA in SA-treated cells was observed, and this decrease was reversed by the addition of hemin. An iron chelator, desferrioxamine, also lowered the level of \(\beta\)-globin mRNA. The heme-dependent expression of \(\beta\)-globin is a transcriptional event since the expression of the human \(\beta\)-globin gene promoter-reporter gene containing the micr ReSharper{L} control region (\(\mu LCR\)) was inhibited when human erythroleukemia K562 cells and MEL cells were cultured with SA. Hemin treatment restored the decrease in promoter activity caused by SA. The control of the \(\mu LCR-\beta\)-globin promoter reporter gene by heme was dependent on DNase I-hypersensitive site 2 (HS2), which contains MARE. The MARE binding activity of Bach1 in K562 and MEL cells increased upon SA treatment, and this increase was diminished by the treatment with hemin. Transient expression of Bach1 suppressed the \(\mu LCR\) activity, and this repressor activity was cancelled by treatment with hemin. The expression of a mutated Bach1 lacking heme-binding sites led to a loss in the heme responsiveness of the \(\mu LCR\). Furthermore, chromatin immunoprecipitation experiments revealed that Bach1 bound to the MARE of HS2 increased by the treatment of MEL cells with SA, and this was cancelled by hemin. We propose that heme positively regulates the \(\beta\)-globin gene expression by blocking the interaction of Bach1 with the MARE in the LCR.

The biochemical role of heme is related to either the transport or the utilization of oxygen, and therefore, heme exerts regulatory effects on various cell functions that sense oxygen. In addition, oxygen is an essential requirement of heme biosynthesis. In yeast, heme regulates the expression of genes involved in the respiratory chain, heme biosynthesis, and oxidative stress, at the transcriptional level, via heme-responsive transcription factors HAP1 and HAP2/3/4/5p (1). In mammals, heme has a profound effect on the proliferation and differentiation of hematopoietic progenitors. Heme not only is incorporated as a structural component of hemoglobin but also causes an increase in the expression of globin as well as enzymes of the heme biosynthetic pathway in erythroid cells (2–4). Hemin (the ferric chloride salt of heme) treatment also increases both the number of transferrin receptor and the ferritin content (5, 6). Thus, heme plays a key role in the coordinated expression of several genes during the differentiation of erythroid cells.

The human globin gene cluster spans a region of 70 kb containing five developmentally regulated genes including \(\gamma\)-, \(\delta\)-, \(\gamma\)-, \(\beta\)-, and \(\beta\)-globin genes (7). Among MARE-associated proteins, Bach1 is unique in that it has a BTB/POZ domain (17). Bach1 forms a multivalent DNA-binding complex, raising the possibility that it can act as an architectural component that is simultaneously able to crosslink multiple MAREs, resulting in the repression of transcription.
Expression of β-Globin Gene Regulated by Bach1

There are several key points from the document that highlight the regulation of β-globin expression by Bach1 in human erythroleukemia K562 cells. The gene encoding heme oxygenase (HO-1), a key enzyme in heme catabolism, is regulated by heme. The interaction of the LCR with Bach1 is positively regulated by heme, which leads to increased expression of the β-globin gene. This regulation is mediated by the HoxA9 transcription factor, which binds to the LCR in the presence of heme.

EXPERIMENTAL PROCEDURES

**MATERIALS**—[γ-32P]ATP, [α-32P]dCTP, and poly(dI-dC) were purchased from Amersham Biosciences. Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Co. and Toyobo Co. (Osaka, Japan) (Biolase Type B). The transfection reagent LipofectAMINE was from Invitrogen. Antibodies for c-Fos and p45 NF-E2 were purchased from Oncogene Science Co. and Santa Cruz Biotechnology Inc., respectively. Antibodies specific for Bach1 were purchased from Cell Signaling Technology.

**RESULTS**

**Effects of SA and Hemin on β-Globin mRNA in MEL Cells**—SA is known as a competitive inhibitor of δ-aminolevulinic acid dehydratase, the second enzyme of the heme biosynthetic pathway. MEL cells were cultured in the presence of 1 μM SA or an iron chelator, DFO (100 μM), for 16 h, after which the content of heme in the cells was measured. The heme content of SA- and DFO-treated cells decreased to about 70 and 85%, respectively, of that of control cells (Fig. 1A). The increase in β-globin mRNA expression by the inhibition of hem biosynthesis was reversed by culture with 50 μM of SA- and DFO-treated cells. When MEL cells were exposed to x-ray film at −80°C, RNA Blot—Total RNA was isolated from the cells by the guanidium isothiocyanate method (23). The RNA (20 μg) was applied to a 1.0% agarose gel, electrophoresed, and then blotted with a nylon membrane (Biolase Type B) for hybridization with biotin-labeled antisense β-globin and actin RNAs. The filters were hybridized and washed, according to the method of Suzuki et al. (24). Hybridized DNA was immersed in alkaline phosphatase-conjugated avidin, washed, and then exposed to x-ray film at −80°C.

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Expression of β-Globin Gene Regulated by Bach1

Involvement of HS2 of the LCR in the Regulation of Hemin-dependent Expression in K562 and MEL Cells—Among the DNase I-hypersensitive sites (HSs), HS3 and HS4 harbor single MAREs, and HS2 contains tandem duplicated MAREs. It was shown previously that heme induces the enhancer activity of the tandem MAREs within the HS-2 (previously referred to as the NF-E2 site) in K562 cells (15, 16). To verify that the tandem MAREs are involved in heme-dependent regulation of the reporter gene, activities of a reporter plasmid pGLCRhβHS2mut carrying the mutated MAREs of HS2 in the LCR were compared using cells treated with SA or heme. As shown in Fig. 3A, the basal activity of pGLCRhβHS2mut in K562 cells was significantly less than that of the wild-type control pGLCRhβ. Moreover, the reporter activity of pGLCRhβHS2mut was not affected by SA or heme. When MEL cells were induced to differentiate with 2% Me2SO for 24 h, the reporter activity of pGLCRhβ was markedly increased as compared with that in uninduced cells (Fig. 3B). The reporter activity of pGLCRhβ was inhibited by SA, and the effect of SA was cancelled by simultaneously adding heme. In contrast, the reporter activity of pGLCRhβHS2mut did not change significantly in the presence of SA or SA plus heme (Fig. 3B). These results indicate that a deficiency of heme in the cells leads to the suppression of the enhancer activity of the LCR and that this regulation by heme involves the tandem MAREs within the HS2.

Regulation of Hemin-dependent Expression of β-Globin Gene in Erythroid Cells by Bach1—The above results confirm previous observations that the tandem MAREs were essential for the Me2SO-induced expression of the LCR reporter. Bach1 interacts with the tandem MAREs in vitro and competes for the sites with p45 NF-E2 (18). Since Bach1 acts as a repressor of MARE-dependent transcription and its DNA binding activity is inhibited by heme (18, 20, 21), it may be involved in the regulation of the enhancer activity of HS2 by heme. We then conducted a conventional gel-shift assay using a synthetic oligonucleotide probe containing a MARE from the chicken β-globin 3’ enhancer to examine whether the DNA binding activity of endogenous Bach1 is indeed regulated by heme. To characterize the binding of Bach1 and p45 NF-E2, the MARE probe was incubated with nuclear extracts of untreated and Me2SO-treated MEL cells. As reported previously (18), we observed two specific retarding bands (Fig. 4A, lane 2). The upper band disappeared upon preincubation of the nuclear extracts with anti-MafK and Bach1, indicating that the binding complex was composed of Bach1 and small Maf protein. The DNA binding activity of the Bach1/small Maf complex was slightly and markedly decreased in 48- and 72-h-treated cells, respectively (Fig. 4A). The lower band, NF-E2 (p45 and small Maf heterodimer), which disappeared with anti-p45 NF-E2 antibodies, intensified upon Me2SO-induced differentiation. The specific band corresponding to Bach1 intensified when MEL cells were treated with 1 mM SA (Fig. 4B). The DNA binding activity of Bach1 was decreased by treating the cells with SA plus 50 μM heme. In this experiment, a weak band corresponding to NF-E2 was found, and the activity remained unchanged by a 16-h incubation with the addition of 50 μM heme. To confirm these observations in other cells, MEL cells were transfected with pGLβp and pGLCRhβ, and the effects of SA and hemin on the reporter activity were also examined. As shown in Fig. 2B, the decrease in pGLCRhβ activity caused by SA, and its cancellation by heme, was similar to that in K562 cells. In contrast, when BALB/3T3 cells were examined, neither the μLCR-dependent activation nor the SA-dependent inhibition of the β-globin gene reporter was observed (Fig. 2C). Thus, heme induces the expression of the β-globin gene by stimulating the enhancer activity of the β-globin LCR in erythroid cells.
In Me₂SO-treated MEL cells, SA caused an increase in the Bach1 activity but inhibited the increase in the NF-E2 activity (Fig. 4A). When nuclear extracts from K562 cells were used, the probe containing MAREs exhibited a binding pattern similar to that seen with MEL cells (Fig. 4C). No change in the bindings of Bach1 and NF-E2 to the MARE occurred by the addition of SA in vitro (data not shown).

We next examined whether Bach1 affects the hemin-dependent activity of the 3′LCR. The reporter activity was measured in K562 cells in the presence of the wild-type and heme-insensitive Bach1-expression plasmids. The Bach1 mutant (Bach1mCP1–6) carrying multiple changes in the heme-binding cysteine-proline (CP) motifs does not bind heme, and its DNA binding activity is not inhibited by heme (20, 21). As shown in Fig. 5A, the reporter activity of pGLCRhβ was suppressed by the wild-type Bach1. Consistent with the previous results using a synthetic reporter and HO-1 reporter (20, 21), the repressor activity of Bach1 was lost by treating cells with hemin (Fig. 5A). In contrast, the repressor activity of Bach1mCP1–6 was not affected by hemin treatment (Fig. 5A). As shown in Fig. 5B, the repressor activity of Bach1 further dropped in SA-treated cells. Unlike the case for the wild-type Bach1, the reporter activity was not further reduced by SA in Bach1mCP1–6 expressing cells (Fig. 5B). When Bach1 was expressed in MEL cells, a heme-dependent regulation of the LCR activity, similar to that in the case of K562 cells, was observed (data not shown). These results suggest that Bach1 regulates the heme-dependent activity of the HS2 enhancer and that the repressive ability of Bach1 on the LCR activity is independent of the heme responsiveness.
We finally examined the recruitment of Bach1 and NF-E2 to the MARE of HS2 in MEL cells treated with SA and hemin. The status of the endogenous transcription complexes present on the HS2 was determined using chromatin immunoprecipitations. The presence of the HS2 in the chromatin immunoprecipitates was analyzed by semiquantitative PCR using specific pairs of primers spanning the HS2 of the LCR. As shown in Fig. 6A, the PCR product increased dependent on 22–28 cycles, and then we analyzed the products with 25 cycles of PCR to evaluate the binding of Bach1 and NF-E2 to HS2. Bach1 and NF-E2 were equally bound to the HS2, and an increase in the binding of Bach1 was observed when cells were treated with 1 mM SA (Fig. 6B). The increase of Bach1 was cancelled by treatment with 50 μM hemin, whereas the binding of NF-E2 to the HS2 increased in the same cells.

**DISCUSSION**

The present study showed that Bach1 plays an important role in the differentiation of erythroid cells and directly regulates the expression of β-globin, mediated by hemin. The expression of β-globin mRNA was reduced when the content of heme in uninduced and Me2SO-treated MEL cells was decreased by treatment with SA or DFO. Exogenously added hemin restored the level of β-globin mRNA. These findings were similar to previous observations that the expression of globin chains in MEL and human erythroid progenitor cells was reduced when these cells were cultured with SA (31, 32) and support the concept of the positive effect of heme on β-globin synthesis (33, 34). Using a promoter assay of β-globin, the present study confirmed that the expression of β-globin mRNA is regulated by hemin at the transcriptional level. There are also many reports that heme induces erythropoiesis by the coordinate expression of α-, and β-globin, transferrin receptors, ferrochelatase, and erythroid-specific δ-aminolevulinic acid-synthase (4, 32, 35). In erythroid cells, it is reported that heme acts as not only a transcription factor NF-Y for ferritin synthesis (36) but also as a transcription factor for kinases including the heme-regulated inhibitor kinase, heme-regulated eIF2α kinase (37). It is also known that heme-regulated eIF2α kinase mRNA increases in induced MEL cells but decreases when the heme biosynthesis is blocked (38). Furthermore, dominant negative heme-regulated eIF2α kinase mutant expressed in MEL cells resulted in the increase in hemoglobin synthesis (38). Thus, heme is well known to regulate the synthesis of globin chains at the translational step. We have shown for the first time that the hemin-dependent activation of β-globin expression at the transcriptional level occurred at the HS2 of the LCR, where it interacted with Bach1.

Analysis by atomic force microscopy revealed large looped DNA structures between MAREs located in different regulatory elements within the human β-globin LCR formed by Bach1/MafK heterodimer (19). Based on the observations that the formation of these loops required the Bach1 BTB/POZ protein interaction domain (19), Bach1 would function as an architectural factor and cause the repression of the enhancer activity of the LCR. We confirmed that Bach1 suppresses the activity of the LCR and demonstrated that the heme binding ability of Bach1 contributes to the hemin-dependent induction of the LCR enhancer (Fig. 5). Consistent with previous findings that the formation of a multimetric and multivalent DNA complex with multiple MAREs occurs simultaneously (18) and that β-globin LCR holocomplex can be formed by structurally con-
necting the MAREs present in HS2, HS3, and HS4 of the LCR (9, 19), Bach1 contributes to the generation of a multiprotein complex as a repressosome among the HS of the LCR (39). The present data by gel-shift assay and chromatin immunoprecipitations clearly showed the physiological role of the interaction of Bach1 with HS2 in the regulation of the expression of β-globin.

Recently, chromatin immunoprecipitation experiments with anti-MafK and anti-p45 NF-E2 showed that the LCR was occupied by small Maf proteins in uninduced MEL cells where the synthesis of globin is suppressed (29) and that p45 NF-E2 was recruited to the LCR as well as the active globin promoters on erythroid differentiation. Using chromatin immunoprecipitations, we found that the recruitments of Bach1 and NF-E2 to the MARE of HS2 were regulated by the intracellular level of heme, indicating that Bach1 and NF-E2 share MAREs and effectively compete with them. Furthermore, the present study and a previous study (18) showed that the DNA complex with Bach1 was down-regulated at the late stage of Me2SO-induced MEL cell differentiation, whereas the NF-E2 complex was induced to form by Me2SO treatment. Bach1 may be involved in the assembly of the LCR complex at the early stages of hematopoietic cell differentiation, whereas p45 NF-E2 contributes to the activation of globin genes per se at the late stage. Based on the fact that the level of intracellular heme was markedly elevated in Me2SO-treated cells and that a marked decrease in Bach1 activity was observed in hemin-treated cells, heme contributes to the loss of function of Bach1 during erythroid differentiation. This is supported by the finding that the expression of Bach1 and Bach1mCP1−6 led to a decrease in the activity for the LCR enhancement, but the hemin-dependent restoration of the LCR activity was only observed in wild-type Bach1-expressing cells (Fig. 5). In fact, the treatment of MEL cells with Me2SO increased NF-E2 activity significantly (40), which is also supported by the present observation that the reporter activity of pGLCRhβ in Me2SO-induced MEL cells was markedly increased as compared with that in uninduced cells (Fig. 3B). Thus, the induction of the p45 NF-E2 binding activity during erythroid differentiation implies that the replacement of the Bach1 associated with small Maf molecules with the

![Fig. 4. Gel-shift assay of the Bach1-MARE binding activity in induced and uninduced MEL cells and in K562 cells.](image-url)
NF-E2 complex occurs dependent on the stage of the differentiation. Similarly, a recent study (21) clarified the mechanisms involved in the induction of HO-1 by hemin. Namely, Bach1 represses the expression of HO-1 under physiological conditions, and an increased level of heme displaces Bach1 from the enhancers by inhibiting DNA binding, allowing activators to bind the enhancers. Thus, heterodimers of small Maf- and p45 NF-E2-related activators including Nrf2 are most likely the form binding to the HO-1 enhancers upon transcriptional activation.

We now provide evidence of a new function of heme directly regulating the transcription of genes. For a long time, it has been considered that intracellular heme has a significant role in the transcriptional up-regulation of several erythroid-specific proteins, including globin chains, transferrin receptors, ferritin, and enzymes of the heme biosynthetic pathway (3). We identified the LCR-globin gene as one of the targets of Bach1 where heme acts as a positive regulator of the transcription of the β-globin gene by regulating the interaction of Bach1 with the MARE region of the LCR (HS2). We propose that transcriptional regulation of β-globin is a direct sensing of heme levels during the terminal differentiation of erythroid cells. Since Bach1 is ubiquitously expressed in a variety of tissues, there may be activation systems for various genes involving the replacement of the repressor Bach1 with some enhancer protein, the event being triggered by heme.

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