— **Minireview** —

Heme-Regulated Transcription Factor Bach1

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Heme is believed to control expression of genes involved in the synthesis of globins and heme itself. However, heme-regulated transcription factor had not been identified in vertebrates. The mammalian transcription factor Bach1 functions as a repressor of the Maf recognition element (MARE) by forming antagonizing hetero-oligomers with the small Maf family proteins. Recently, we found that heme binds specifically to Bach1 and regulates its DNA binding activity. Deletion studies demonstrated that the heme binding region of Bach1 is confined within its C-terminal region that possesses four di-peptide cysteine-proline (CP) motifs. Mutations in all of the CP motifs of Bach1 abolished its interaction with heme. The DNA binding activity of Bach1 as a MafK hetero-oligomer was markedly inhibited by heme in gel mobility shift assays. The repressor activity of Bach1 was lost upon addition of hemin in transfected cells. These results suggest that increased level of intracellular heme inactivates the repressor Bach1, resulting in induction of a host of genes with MAREs.

Key words —— heme, Bach1, transcription factor

INTRODUCTION

Heme is an essential molecule that plays a central role in reactions with molecular oxygen including xenobiotic metabolism, as it being the prosthetic group of heme proteins. In the field of environmental sciences, the heme metabolism is known to be disturbed by many chemical hazards, for example, lead, dioxin, and trichloroethylene inhibit enzyme(s) in the heme biosynthetic pathway, while cadmium, dioxin and arsenate induce heme oxygenase (HO)-1, the key enzyme for heme catabolism. Thus, these chemicals tend to decrease the so-called "free (or regulatory) heme pool" as was observed in trichloroethylene intoxication[1] and halothane exposure.[2]

Preceding studies suggest that the free heme regulates genes encoding the rate-limiting enzyme of heme biosynthesis as well as catabolism, *i.e.*, δ-aminolevulinate synthase (ALAS)[3–5] and HO-1,[6,7] respectively. In addition, Goldberg *et al*. suggested that the deoxy form of heme activates erythropoietin gene.[8] It is therefore probable that a marked reduction in regulatory heme by chemical hazards cause development of symptoms via disorder(s) of heme-controlled genes.

Heme is known to participate in gene regulation as a ligand for transcription factors in prokaryotes[9] and yeast, such as Hap1.[10,11] In vertebrates, heme has also been proposed to fulfill similar regulatory roles as mentioned above,[12] however, no similar transcription factor has been identified. Elucidation of the heme-controlled transcription factor(s) in vertebrate is, therefore, important to understand development of signs and symptoms due to perturbation of regulatory heme by chemicals.

Based on the present study, we describe the molecular mechanism of Bach1 to regulate genes, a novel heme-regulated transcription mechanism in vertebrate. Bach1 is a basic-leucine zipper (bZip) protein that forms heterodimers with Maf-related oncoprotein family.[13] Like the erythroid transcription factor NF-E2 and its relating factor Nrf-1, -2, and -3,[13,14] their heterodimers with Maf proteins bind to the Maf recognition elements (MAREs).[15–19] MAREs are found in the regulatory region of various genes involved in heme metabolism such as

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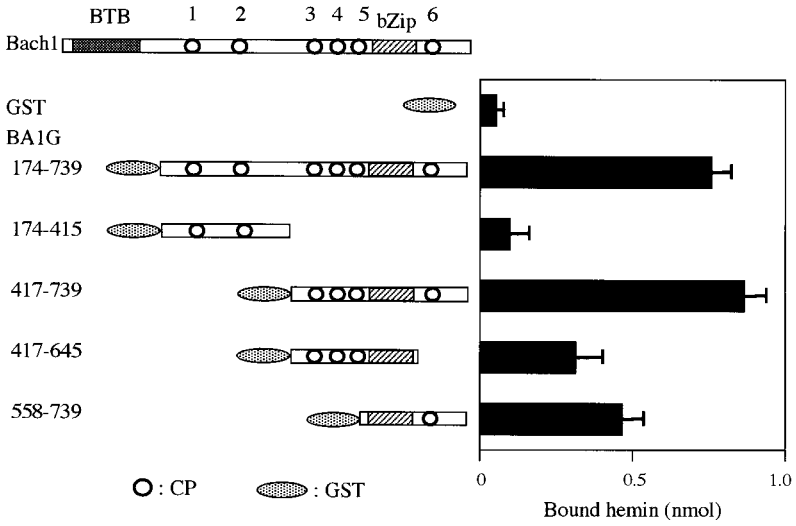

Fig. 1. Binding of Hemin to Bach1 Subfragments

One nmol of GST-Bach1 fusions were immobilized onto sepharose beads and incubated with 1 μM hemin. Amounts of bound hemin were determined using spectrofluorometry.

oxidative stress responsive genes,[20] globin genes,[21,22] HO-1 gene,[23,24] and erythroid type ALAS (ALAS-E) gene.[25] Among MARE-binding factors, Bach1 as well as Bach2 function primarily as repressors.[13,21,22,26,27] The presence of a repressor class of molecules suggests that transcription regulation operating through MARE is based on the balance between activation and repression. Our findings clearly indicate that heme inhibits the DNA binding activity of Bach1/MafK heterodimer by direct interaction with Bach1 through cysteine-proline (CP) motifs,[13,28] which have been recognized as heme regulatory motifs (HRMs) in yeast transcriptional activator Hap1[10,11] and so forth.[29–34] Based on these studies, we propose for the first time that intracellular heme is one of the important factors of vertebrate transcription altering the DNA binding activity of Bach1.

Heme Binding Activity of Bach1

During the purification of Bach1 as a fusion protein with maltose binding protein (MBP-Bach1), we found that the purified protein samples showed a brownish color, while other MBP fusion proteins, *e.g.*, Nrf2, NF-E2 p45, and MafK, did not. To explore the possibility that Bach1 is a heme binding protein, we expressed various portions of it as fusion proteins with glutathione S-transferase (GST), and carried out heme binding assays.

Bach1 possesses at least two functional domains, BTB/POZ and bZip domains which are involved in protein interaction and DNA binding, respectively. To carry out a binding assay, we first expressed a portion of Bach1 (amino acid residues 174 to 739 lacking the BTB/POZ domain, see Fig. 1, Ref. 28) as a fusion protein with GST. Upon incubating BA1G174-739 with hemin, a typical hemoprotein spectrum with Soret band was observed by UV-visible spectroscopy,[28] indicating that Bach1 is in fact a heme binding protein. To estimate the affinity of Bach1 for heme, BA1G174-739 was immobilized onto sepharose beads and was incubated with ^{55}Fe-labeled hemin at various concentrations. Binding of hemin to BA1G174-739 was found to occur in a concentration-dependent and saturable manner with a *Kd* of 140 nM, while GST alone did not show any binding. Furthermore, non-labeled hemin competed with the binding of ^{55}Fe-labeled hemin to BA1G174-739, suggesting that the binding is specific.

Identification of Heme Binding Motif(s) in Bach1

To understand the molecular basis of heme binding of Bach1, we first attempted to identify a region of Bach1 responsible for heme binding. Toward this end, we generated several GST fusions of Bach1 derivatives (Fig. 1). First, two fragments (174–415 and 417–739) were tested for heme binding. The C-terminal BA1G417-739 showed a high affinity for heme that was comparable to BA1G174-739 whereas the N-terminal BA1G174-415 did not show any specific binding activity (Fig. 1). These results indicate that a putative heme binding domain is confined within the C-terminal region between amino acid residues 417 and 739. To define the binding

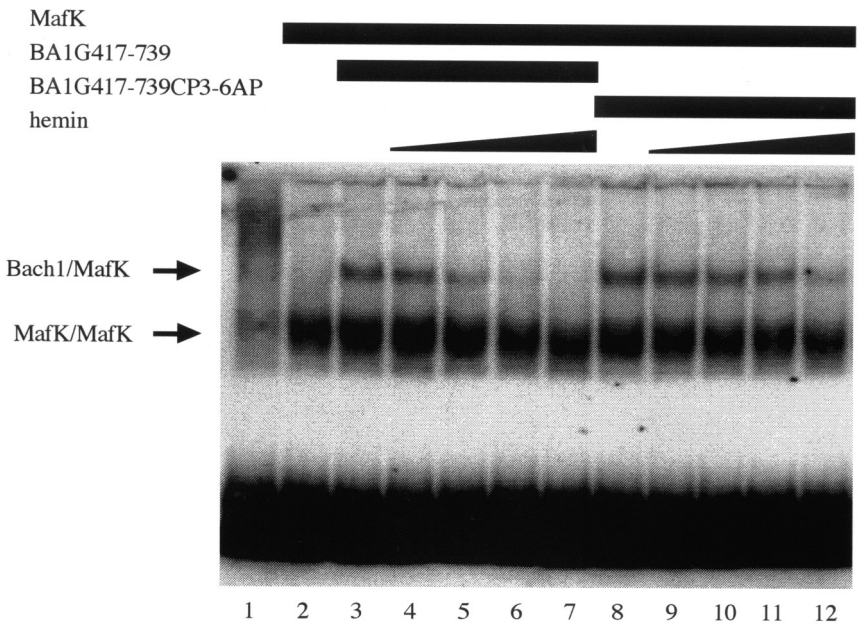

Fig. 2. Involvement of CP Motifs in Regulation of DNA Binding Activity of Bach1 by Heme

BA1G417-739 or BA1G417-739CP3-6AP and MafK were incubated in the presence or absence of various concentrations of hemin. Binding to MARE-containing probe was examined by EMSA. Hemin concentrations were 0.1 μM (lanes 4 and 9), 0.3 μM (5 and 10), 0.5 μM (6 and 11), and 1.0 μM (7 and 12).

region(s) more precisely, we prepared two sub-fragments from the heme binding fragment (417–739). To our surprise, both sub-fragments (417–645 and 558–739) showed comparable heme binding activities.

CP motif(s) has been found in HRMs[10,11,29–34] and plays an important role in heme binding in various proteins. Interestingly, Bach1 contains six CP motifs (Fig. 1). Namely, there are two CP motifs downstream of the BTB domain (CP1, CP2), three CP motifs upstream of the bZip domain (CP3, CP4, CP5), and one CP motif downstream of the bZip domain (CP6). Among these CP motifs, CP3–CP6 are present within the putative heme binding region. To test the possibility that these CP motifs are involved in heme binding, we prepared GST fusion proteins with single or multiple mutations within the motifs in the context of BA1G417-739 by changing cysteine residue to alanine. Single amino acid substitutions did not influence the heme binding. In contrast, alteration of the three clustered CPs (BA1G417-739CP3-5AP) significantly decreased the heme binding activity. Most importantly, replacement of all four CPs (CP3–CP6, BA1G417-739CP3-6AP) abolished the high affinity binding to heme.[28] The *Kd* values were 170 nM and 1220 nM for BA1G417-739 and BA1G417-739CP3-6AP, respectively. These results indicate that there is cooperativity among the four CP motifs of Bach1 for heme binding.

Inhibition of DNA Binding Activity of Bach1 by Heme

The specific binding of heme to Bach1 strongly suggests that heme regulates certain functions of Bach1. Since the four CP motifs (CP3–CP6) that are involved in the heme binding surround the bZip domain (Fig. 1), we examined the effects of heme on DNA binding using electrophoretic mobility shift assays (EMSAs). Addition of hemin to the binding reactions resulted in a concentration-dependent inhibition of DNA binding by the Bach1/MafK heterooligomer. Even 0.03 μM hemin caused a slight but reproducible inhibition, and one micromolar hemin almost completely inhibited the DNA binding activity of Bach1.[28]

We next investigated whether the CP motifs that are essential for heme binding may also be involved in the inhibition of DNA binding. We used a Bach1 derivative that harbored cysteine to alanine substitutions in the four CP motifs (BA1G417-739CP3-6AP, Fig. 2, Ref. 28). Comparison of the amounts of DNA binding complex generated in the presence of various concentrations of hemin clearly indicated that the CP3-6AP substitutions rendered Bach1 less sensitive to hemin. On the other hand, in contrast to

its essential lack of its heme binding, the CP3-6AP form still retained some sensitivity toward heme. Taken together, these results indicate that the four CP motifs are important for both Bach1-heme interaction and heme-mediated inhibition of DNA binding.

Heme Abrogates Transcription Repression by Bach1 in Transfected Cells

To probe the functional consequences of the interaction between heme and Bach1 within a cell, we compared regulatory activities of Bach1 and a Bach1 derivative with multiple CP mutations. The Bach1 mCP1-6 harbors substitutions of cystein to alanine in all of the six CP motifs, *e.g.*, CP1 through CP6. Each of the expression plasmids was transfected into 293 cells along with a synthetic MARE-dependent reporter plasmid, and their effects on the reporter gene expression in the presence or absence of 10 μM hemin in the medium were compared.[28] Without addition of hemin, both Bach1 and Bach1 mCP1-6 repressed the reporter gene expression. When hemin was added to the medium, repression by Bach1 was markedly abrogated.[28] It should be noted that the amount of hemin was similar to those utilized in experiments to examine heme-mediated gene induction in cultured cells.[24] In contrast, Bach1 mCP1-6 remained effective in transcription repression even in the presence of hemin.[28] Taken together with the fact that the CP motifs play an important role in Bach1-heme interaction, these results indicate that repressor activity of Bach1 within a cell is negatively regulated by heme.

DISCUSSION

Heme plays various critical roles within cells because of its ability to bind molecular oxygen and to mediate electron transfer. Besides these functions, heme is known to induce expression of ALAS-E,[3] globin,[4,35] and HO-1 genes[6,7] while it decreases the mRNA of non-specific ALAS (ALAS-N).[3,5] These proteins are involved in metabolism of heme or hemoglobin synthesis, and the proper control of their expression is important to survive under various environmental stress. These observations led to the conjecture that heme plays a role in gene regulation.[12] While this hypothesis predicts the presence of a heme-regulated transcription factor, the yeast transcription factor Hap1 has been the only known example in eukaryotes[10,11] with no other factors with such function known in higher eukaryotes. We found the biochemical characterization of heme-Bach1 interaction. Most importantly, DNA binding and repressor activities of Bach1 are negatively regulated by heme. These results suggest that heme may regulate gene expression by shifting the balance between activators and repressors that bind to MARE (s).

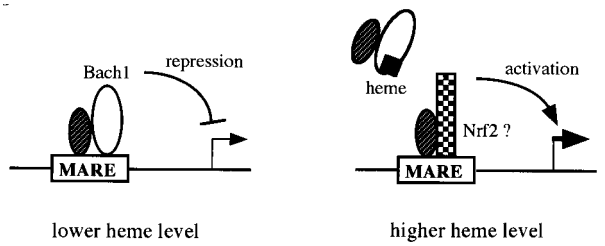

Fig. 3. Transcription Regulation by Heme through Bach1
A model based on our observations. For detail, see Discussion.

Despite the fact that Bach1 is not related to Hap1, there is a resemblance between them in that they interact with hemin through the CP motifs; namely, both proteins bind to heme with high affinity via their CP motifs, no single CP motif of Bach1 is essential for heme binding, indicating their functional redundancy. Consistent with this notion, each CP motif on Hap1 is known to bind to heme when tested using a synthetic peptide.[34] Thus, it is reasonable to assume that each such motif on Bach1 possesses the potential to interact with heme. This may explain the heme binding activity of the C-terminal fragment containing one CP motif (BA1G417-739CP3-5AP and BA1G558-739).

There is still a remaining question whether the CP3-6 and their clustering are the sole determinant for heme binding. This question stems from the observation that hemin exerts a regulatory effect on Bach1 *in vitro* at relatively lower concentrations as compared with other mammalian heme-regulated proteins. For example, heme at 5 to 25 μM is known to inhibit mitochondrial transport of ALAS-E via its CP cluster in the C-terminal region.[32]

Based on our observation and previous reports, we propose a hypothetical mechanism in which heme functions as an inducer of gene expression by inhibiting the repressor molecule Bach1 (Fig. 3). In this model, Bach1 represses genes with MAREs in their regulatory regions in the presence of lower levels of heme by competing with activators like Nrf2[20,24] and NF-E2 p45[21] for binding to MAREs. Higher levels of heme cause inhibition of DNA binding activity of Bach1, resulting in dissociation of Bach1 from the enhancers and derepression of the target genes.

The next question will be identification of physiological target genes for Bach1. Bach1 targets will be found among genes that possess MARE(s) in their regulatory regions and are induced by heme. It may worth noting that the locus control region of the β-globin genes[21,22] and the heme-responsive enhancers of HO-1[23,24] contain multiple MAREs. Since Bach1 forms multivalent DNA binding complexes through BTB domain that can cooperatively bind to reiterated MARE motifs,[22] β-globin and HO-1 enhancers may allow effective binding by Bach1. Together with the facts that these genes are directly or indirectly regulated by heme,[4,6,7,35] β-globin and HO-1 genes are the prime candidates. Further studies are obviously necessary to test this hypothesis. Nonetheless, our observations provide for the first time a molecular mechanism for the long-standing contention that intracellular free heme regulates gene expression in mammalian cells.

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