Vol. 207, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 6, 1995 Pages 160-167

DIFFERENTIAL CONTROL OF THE METAL-MEDIATED ACTIVATION OF THE HUMAN HEME OXYGENASE-1 AND METALLOTHIONEIN IIA GENES

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Received December 18, 1994

Heme oxygenase-1, an essential enzyme in heme catabolism,and metallothionein IIA, a small metal-binding protein with clusters of cysteins,are remarkably induced in HeLa cells following the treatment with cadmium or zinc. Both proteins are considered to be involved in the defense system against metal toxicity. Here we showed by transient expression assays that the cadmium-responsive element (CdRE) of the human heme oxygenase-1 gene is not responsive to zinc, whereas a metal-regulatory element (MRE) of the human metallothionein IIA gene is able to respond to either cadmium or zinc. The CdRE is recognized by a certain nuclear protein(s) which is however unable to bind to an MRE of the metallothionein IIA gene. These results suggest that the metal-selective activation of each gene promoter is mediated by a separate mechanism.

Heme oxygenase (E.C.1.14.99.3) is an essential enzyme in heme catabolism, cleaving heme to release carbon monoxide, iron, and biliverdin (1), the latter of which is subsequently converted to bilirubin by biliverdin reductase (2). Recently it was suggested that an inducible type of heme oxygenase, heme oxygenase-1, also functions as a defense system against oxidative stress, since biliverdin or bilirubin produced locally may work as a physiological antioxidant (3). Indeed, heme oxygenase activity is highly inducible by its substrate heme/hemin (2, 4, 5) and by various environmental derangements (reviewed in Ref. 6), including heavy metals (7, 8) and heat shock (9). It was already shown that hemin or cadmium increased the transcription of the heme oxygenase-1 gene (10, 11). However, in contrast to heme/hemin, a biological role of cadmium has not been defined yet, although cadmium is present in minute traces in living organisms. Because cadmium is able to bind various cellular components such as free sulfhydryl groups and nucleic acids (reviewed in Ref. 12), cadmium may simply mimic or modify a physiological mediator that is involved in a signal transduction system leading to activation of the heme oxygenase-1 gene transcription.

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Abbreviations: CdRE, cadmium-responsive element; MRE, metal-regulatory element; MEM, minimum essential medium; SV40, simian virus 40.

0006-291X/95 $5.00

Metallothioneins constitute a family of the cystein-rich proteins with a low molecular weight and are involved in multiple cellular processes such as metal homeostasis, adaptation to stress, and heavy metal detoxification (reviewed in Refs. 13, 14). The expression of the metallothionein genes is controlled mainly at the transcriptional level and can be induced by a wide range of different stimuli, including exposure to heavy metals such as zinc and cadmium (15-17). In contrast to human or rat heme oxygenase-1 lacking a cysteine residue (18, 19), metallothioneins are directly involved in the detoxification of cadmium.

Heme oxygenase-1 and metallothionein genes provide attractive models for studying the regulation of gene expression in animal cells. Recently, we have identified the cadmium-responsive element (CdRE) of the human heme oxygenase-1 gene that is responsible for induction by cadmium (11). The metal-regulatory elements (MREs) of the metallothionein genes, responsible for induction by heavy metals such as cadmium and zinc, have been well established (16, 17, 20). However, the CdRE of the human heme oxygenase-1 gene, TGCTAGATTT (11), is different from the consensus sequence of the MRE, TGCRCNC (R, purine; N, any nucleotides) (16), except for the TGC trinucleotides at its 5' end. In this study, we compared the function of CdRE with that of an MRE of the metallothionein IIA gene promoter. The data indicate that the mechanism for the metal-mediated induction of the heme oxygenase-1 gene is different from that for the metallothionein IIA gene, suggesting that heme oxygenase-1 is a member of a hitherto unknown defense system against metal toxicity.

Experimental Procedures

Materials. Restriction endonucleases were purchased from Takara Shuzo, Boehringer Mannheim and New England BioLabs; Klenow enzyme, T4 DNA polymerase, T4 polynucleotide kinase and T4 DNA ligase from Takara Shuzo; [α-^{32}P]dCTP from Amersham; [γ-^{32}P]ATP from ICN.

Northern blot analysis. HeLa cells were cultivated in minimum essential medium (MEM) containing 10% fetal calf serum. For induction experiments, HeLa cells were treated for 3 h with $CdCl_2$ (5 and 50 μM) or $ZnCl_2$ (50 and 100 μM) in MEM supplemented with 10% fetal calf serum, and harvested for RNA preparation. Total RNA was subjected to Northern blot analysis as described previously (18). The probes used were the XhoI/XbaI fragment (-64/923) derived from a human heme oxygenase-1 cDNA pHHO-1 (19), the PvuII/HinfI fragment (750/906) of the human metallothionein IIA gene (21) obtained from American Type Culture Collection, and the SmaI/ScaI fragment (124/1050) of a β-actin cDNA (22).

Plasmids construction. A fusion gene pHHOL5, containing the 282-bp promoter region of the human heme oxygenase-1 gene upstream from the firefly luciferase gene (11), was linearized with HindIII and ClaI. The resulting larger fragment was then ligated to the following two fragments: the HindIII/BamHI fragment (-764/+76) containing the 5'-flanking region of the human metallothionein IIA gene derived from p84H (21) and the XhoI/ClaI fragment containing the luciferase gene derived from pHTL10 (23). Both BamHI and XhoI sites had been converted to blunt ends prior to ligation. A construct phMTIIAL thus obtained contains the firefly luciferase gene under the human metallothionein IIA promoter. The double-stranded synthetic oligonucleotides, containing the CdRE of the human heme oxygenase-1 gene (11) and MREb, an MRE of the human metallothionein IIA gene (16), were inserted in the upstream of the enhancerless simian virus 40 (SV40) promoter of pSVLE(-) (11), yielding pSVLCd2 and pSVLMREb, respectively (see Fig.3).

Transient expression analysis of fusion genes. HeLa cells, about 70% confluent in 6-cm dishes, were transfected by the calcium phosphate method (11, 24) with modifications; namely, cells were incubated with plasmid DNA precipitated with Ca phosphate for 16 h

refed with fresh medium, and incubated for 24 h. The DNA used for cotransfection was 7 μg of each fusion gene and 1 μg of β-galactosidase expression vector pCH110 (Pharmacia LKB Biotechnology Inc.), containing the SV40 early promoter, as an internal control. Following the 24 h incubation, cells were incubated with $CdCl_2$ (5 and 50 μM) or $ZnCl_2$ (50 and 100 μM) in MEM supplemented with 10% fetal calf serum. After incubation for 5 h, cells were lysed by repeated freezing and thawing in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol, and centrifuged at 10,000 g for 10 min. The supernatants were assayed for luciferase activity (25) and for β-galactosidase activity (26). To normalize the variability in transfection efficiency, each luciferase activity was divided by β-galactosidase activity (relative luciferase activity). In all cases, the data shown are means ± standard deviations for at least three independent experiments (Fig.2 and Fig.3).

Gel mobility shift assay. Nuclear extracts were prepared by the modified method of Lassar et al. (27). HeLa cells, seeded in 15 cm-diameter dishes, were incubated in serum-free MEM containing 5 μM $CdCl_2$ or no addition for 90 min. Other conditions were the same as detailed previously (11). Nuclear extracts were incubated with 0.5-1 ng of ^{32}P-labeled probe (5,000-10,000 cpm) in a reaction buffer containing 20 mM HEPES (pH 7.9), 5% glycerol, 50 mM KCl, 1.5 mM $MgCl_2$, 1.2 μg poly (dI-dC) and either with or without competitors. The probe used was the double-stranded oligonucleotide Cd2 containing CdRE, and the competitors were the synthetic oligonucleotides (see Fig.3A). After 30 min incubation at room temperature, the reaction mixture was loaded onto 4 % polyacrylamide gels containing 6.7 mM Tris-HCl (pH 7.9), 3.3 mM acetic acid and 1 mM EDTA, and was electrophoresed at 150 V for 3 h at 4 °C.

Results and Discussion

To analyze the metal-mediated induction of heme oxygenase-1 and metallothionein IIA mRNAs, HeLa cells were treated with $CdCl_2$ or $ZnCl_2$ in MEM supplemented with 10% fetal calf serum. Both mRNAs were hardly detectable in untreated cells, but remarkably induced by either cadmium (5 and 50 μM) or zinc (50 and 100 μM) in a dose-dependent manner (Fig.1). In contrast to the effects of cadmium, a lower concentration of zinc (5 μM) did not increase the levels of heme oxygenase-1 mRNA (data not shown) as described previously (11). It is noteworthy that a higher concentration of zinc is required to obtain the maximal induction of both mRNAs. The larger species of heme oxygenase-1 RNA seen may represent the unspliced or partially spliced transcripts. These aberrant transcripts were detected when transcription of the heme oxygenase-1 gene was remarkably activated (11, 22).

The metal-mediated induction of both genes is controlled mainly at the transcriptional level (11, 16, 17), and we intended to clarify whether a common regulatory mechanism is responsible for each induction. The CdRE of the human heme oxygenase-1 gene has been identified about 4 kb upstream from the transcriptional initiation site (11). The human metallothionein IIA gene contains multiple copies of MREs in its 5'-flanking region (16). To examine the effects of cadmium and zinc on the human heme oxygenase-1 and metallothionein IIA promoter activities, we performed transient expression assays of the fusion genes containing a luciferase reporter linked to each gene promoter (Fig.2A). Expression of the luciferase activities in the HeLa cells transfected with pHHOL15 was induced by cadmium in a dose-dependent manner but was not induced at all by zinc (Fig.2B), suggesting that separate regulatory elements are involved in the cadmium- and zinc-mediated induction of the human heme oxygenase-1 gene. It is therefore conceivable that a zinc-responsive element of the human heme oxygenase-1 gene may not be present in the 4.5-kb 5'-flanking region carried by pHHOL15 and may be located in the 5'- or 3'- flanking region or in

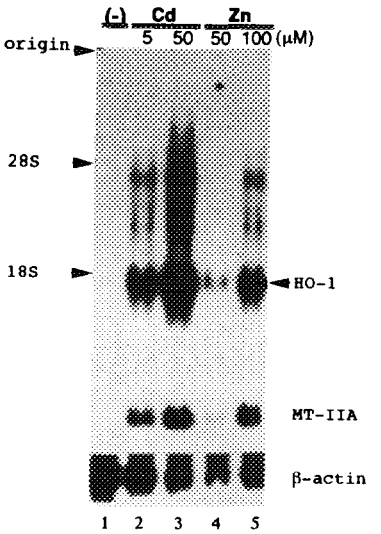

Fig.1. Induction of heme oxygenase-1 and metallothionein IIA mRNAs by cadmium or zinc. HeLa cells were treated for 3h with either $CdCl_2$ or $ZnCl_2$ and were harvested for RNA preparation. Each lane contained 10 μg of total RNA prepared from untreated cells (lane 1) or from the cells treated with 5 and 50 μM of $CdCl_2$ (lanes 2 and 3) or with 50 and 100 μM of $ZnCl_2$ (lanes 4 and 5). Heme oxygenase-1 (HO-1) and metallothionein IIA (MT-IIA) mRNAs are shown at the *top* and the *bottom*, respectively. β-actin mRNA is also shown as an internal control, representing the amounts of RNA loaded. The size markers were human rRNAs.

the introns not examined in this study. In contrast, either cadmium or zinc induced the luciferase activities in the cells transfected with a construct phMTIIAL containing the metallothionein IIA promoter. The magnitude of activation by each heavy metal was in a dose-dependent manner, consistent with the results of Northern blot analysis (see Fig.1). These results suggest the functional difference between the CdRE of the heme oxygenase-1 gene and MREs of the metallothionein IIA gene.

We thus designed the experiments focused on the CdRE and on the one of MREs of the human metallothionein IIA gene, MREb (positions -57/-43) (16), to analyze whether metal-selective activation is dependent on the presence of each element. MREb has been well characterized (16, 28, 29). Double-stranded oligonucleotides containing either CdRE or MREb were inserted in the upstream of the SV40 promoter connected to the luciferase gene (Fig. 3A). The luciferase activity was induced by cadmium in the cells transfected with pSVLCd2 or pSVLMREb by two- to three-fold (Fig.3B), whereas zinc increased the luciferase activity in HeLa cells transfected with pSVLMREb but not with pSVLCd2. In the case of pSVLMREb, luciferase activity was increased noticeably in a dose-dependent manner by zinc. This confirms that the CdRE of the heme oxygenase-1 gene is not responsive to zinc, although MREb is responsive to either cadmium or zinc.

Stuart *et al.* have shown that MREs of the mouse metallothionein I promoter are composed of TGCRCNC (16), the sequence of which is also contained in MREb. The CdRE of the human heme oxygenase-1 gene, TGCTAGATTT (11), is different from this consensus except for the TGC trinucleotides at its 5' end. In particular, the G residue at position 390 of

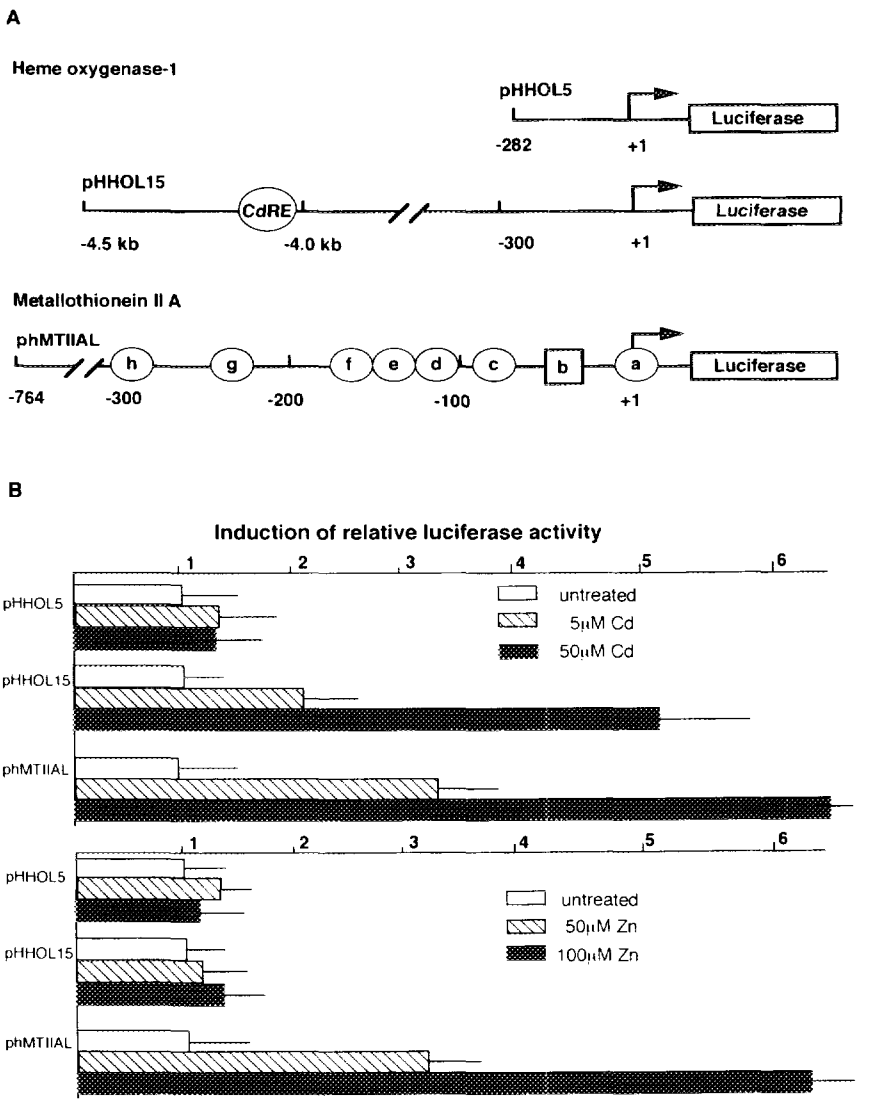

Fig.2. Activation of heme oxygenase-1 and metallothionein IIA promoters by cadmium or zinc. A. The 5'-flanking regions of the human heme oxygenase-1 and metallothionein IIA genes. The 5'-flanking regions are shown as *bold bars*, and the *arrows* indicate the transcription initiation sites. The CdRE of the heme oxygenase-1 gene (11) and the eight MREs of the metallothionein IIA gene (16) are schematically indicated. The MREb is indicated by *closed box*. The *numbers* shown are the nucleotide residues from the transcription initiation sites. B. Activation of the promoter function by cadmium or zinc. The plasmids used for transfection are shown to the *left*. The magnitude of induction, shown to the *right*, represents the ratio to the relative luciferase activity obtained with pHHOL5.

the CdRE is crucial for its function (11), whereas the equivalent position in the consensus MRE of the metallothionein genes can accept any nucleotides (16). On the other hand, both CdRE and MREb share the 6-bp sequence, TTTTGC (see Fig.3A). To explore whether a common nuclear protein(s) binds to CdRE and to MREb, we performed the gel mobility shift assays using HeLa nuclear extracts. Nuclear protein(s) prepared from untreated cells did bind

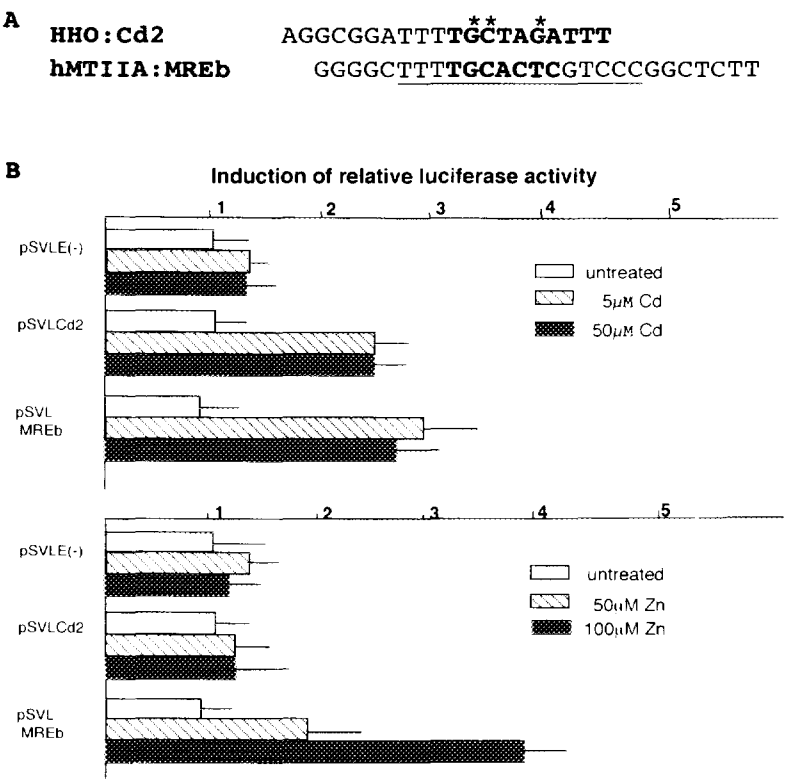

Fig.3. Functional analysis of the two types of metal-regulatory elements. A. The nucleotide sequences of CdRE (*top*) and MREb (*bottom*). The Cd2 oligonucleotide represents the region at positions 375 to 394, tentatively numbered (11) and the MREb oligonucleotide represents the promoter region (-62/-36) of the metallothionein IIA gene (21). Bold letters indicate the CdRE and a core sequence of MRE, and asterisks indicate the crucial residues for the function of CdRE. MREb (16) is underlined. B. Functional analysis of CdRE and MREb using a heterologous promoter. The magnitude of induction represents the ratio to the relative luciferase activity obtained with pSVLE(-).

to CdRE but was unable to bind to MREb (Fig.4). The CdRE-binding activities were not apparently affected even if nuclear proteins were prepared from the cadmium-treated cells (11). In contrast, no protein/MREb complexes were detected under the conditions used (data not shown). These results also support the functional difference between CdRE and MREb.

Both heme oxygenase-1 and metallothioneins differ in their structures, but share the common properties, such as ubiquitous expression and metal-inducibility. Here we show that the CdRE of the human heme oxygenase-1 gene is a novel type of the *cis*-acting element that is responsive only to cadmium and suggest that CdRE and MREb are recognized by separate *trans*-acting factors. Thus, a signal transduction system leading to the induction of heme oxygenase-1 by cadmium may be different from that for metallothionein induction. It appears reasonable that living organisms have acquired the two independent systems involved in detoxification of heavy metals during evolution. Elucidation of the regulatory mechanism of the heme oxygenase-1 gene expression will facilitate our understanding of a newly recognized defense system against metal toxicity.

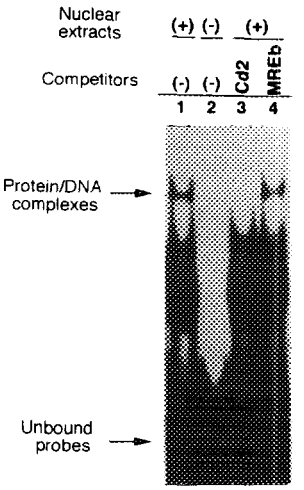

Fig.4. Binding of nuclear proteins to the CdRE of the heme oxygenase-1 gene. Gel mobility shift assay was performed using nuclear extracts prepared from untreated HeLa cells. A DNA probe was the Cd2 oligonucleotide containing the CdRE of the human heme oxygenase-1 gene. The competitors used were the Cd2 oligonucleotide (lane 3) and the oligonucleotide containing MREb (lane 4) as presented in Fig.3A.

Acknowledgments

We thank S. Subramani for providing the luciferase gene and T. Yamamoto for a β-actin cDNA. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture of Japan.

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