Expression of the mRNA of Heme-Binding Protein 23 Is Coordinated with That of Heme Oxygenase-1 by Heme and Heavy Metals in Primary Rat Hepatocytes and Hepatoma Cells[†,‡]

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ABSTRACT: A 23-kDa protein with high affinity for heme (K_D = 55 nM), therefore termed heme-binding protein 23 kDa (HBP23), was purified from rat liver cytosol [Iwahara, S., et al. (1995) *Biochemistry 34,* 13398−13406]. Homology search of the cloned HBP23 cDNA revealed that this protein belongs to a recently recognized class of thiol peroxidases, the antioxidant peroxiredoxin family. Since HBP23 gene expression was highest in the liver, HBP23 mRNA regulation by heme and heavy metals was investigated in cultures of primary rat hepatocytes and mouse hepatoma Hepa 1-6 cells. In both cell cultures HBP23 mRNA levels were upregulated in a time- and dose-dependent manner by heme. Heme-dependent induction of HBP23 mRNA occurred coordinately with that of the heme-metabolizing enzyme heme oxygenase-1, which was recently identified as inducible by oxidative stress. Treatment of primary rat hepatocyte or hepatoma cell cultures with the heavy metals $CdCl_2$ (10 μM) and $CoCl_2$ (300 μM) induced in parallel HBP23 and HO-1 mRNA levels, in the case of $CdCl_2$ to even higher levels than heme. By contrast, mRNA expression of another heme binding protein, hemopexin, was not induced in hepatocyte cell cultures by heme or heavy metals. The data suggest that the expression of HBP23 and HO-1 mRNA is regulated by (a) similar mechanism(s) in liver and that both genes could play a common physiological role as antioxidants and/or in heme metabolism.

Heme-binding cytosol proteins such as heme binding protein/liver-fatty acid binding protein (Vincent & Muller-Eberhard, 1985; Vincent et al., 1987) and glutathione S-transferases (Pickett & Lu, 1989) may play a role in targeting heme to intracellular organelles and/or in oxidant defense mechanisms. The recently purified *Rhodnius prolixus* heme-binding protein was shown to inhibit heme-induced lipid peroxidation and may, therefore, protect against heme-dependent oxidative stress (Dansa-Petreski et al., 1995; Oliveira et al., 1995).

The accompanying article describes a heme-binding protein with a molecular mass of 23 kDa, termed heme-binding protein 23 (HBP23),[1] that was purified from rat liver cytosol (Iwahara et al., 1995). The HBP23 cDNA shows high sequence homology (96−97%) to three proteins recently cloned from human and mouse mammary cells or blood cells denoted MSP23 (Ishii et al., 1993), *pag* (Prosperi et al., 1993), and NKEF A (Shau et al., 1994). All four proteins belong to the peroxiredoxin family, which are peroxidases with protective functions against reactive oxygen species (Chae et al., 1994a,b). HBP23 mRNA is prominently expressed in liver and HBP23 mRNA expression changes during alterations of cellular heme metabolism (Iwahara et al., 1995). Heme degradation is governed by heme oxygenase (HO), which is the rate-limiting enzyme of heme degradation. Isozyme HO-1 is highly inducible by heme (Pimstone et al., 1971; Shibahara et al., 1985), whereas isozyme HO-2 is unresponsive to heme and is encoded by a separate gene (Maines et al., 1988; Cruse & Maines, 1988). HO-1 has been identified as a major stress protein that is implicated in antioxidant cellular defense mechanisms (Keyse & Tyrrell, 1989).

The main goal of the present study was to investigate the regulation of HBP23 mRNA expression during exposure to heme in cultures of primary rat hepatocytes and mouse hepatoma cells. We compared the induction pattern of HBP23 mRNA pattern with that of other genes involved in heme metabolism and transport, such as HO-1 and hemopexin, to gain initial understanding of the physiological function of HBP23 in heme metabolism and as an antioxidant.

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[‡] The nucleotide sequence reported in this paper has been submitted to the Genbank/EMBL Data Bank with the accession number D30035.

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[1] Abbreviations: ALA-S, 5-aminolevulinate synthase; bp, base pair; BSA, bovine serum albumin; GAP-DH, glyceraldehyde-3-phosphate dehydrogenase; HBP23, heme-binding protein 23 kDa; HO, heme oxygenase; MSP23, macrophage 23-kDa stress protein; NKEF A, natural killer cell enhancing factor A; pag, proliferation associated gene.

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EXPERIMENTAL PROCEDURES

Materials. Media 199 and RPMI 1640 were obtained from GIBCO, and [α-^{32}P]dCTP was from Amersham-Buchler. Nitrocellulose filters were purchased from Schleicher and Schuell, multiprime labeling kit from New England Biolabs, and tissue culture dishes from Falcon, respectively. All other chemicals were purchased from Sigma.

Cell Culture. Hepatocytes were isolated from male Wistar rats by circulating perfusion with collagenase under sterile conditions as previously described (Muller-Eberhard et al., 1988). The cells were cultured under air/CO_2 (19/1) in medium 199 with Earle's salts containing bovine serum albumin (BSA; 2 g/L), $NaHCO_3$ (20 mM), HEPES (10 mM), streptomycin sulfate (117 mg/mL), penicillin (60 mg/L), insulin (1 nM), and dexamethasone (10 nM). Five percent fetal calf serum was present during the plating phase up to 4 h, and cell cultures were incubated in serum-free medium for another 24 h before treatment. Hepa 1-6 cells were from the American Type Culture Collection (Rockville, MD, catalog no. CRL 1830) and were cultured in RPMI 1640 medium containing 2% fetal calf serum until confluency of cell monolayers was reached. Confluent monolayers were incubated in serum-free medium 24 h before treatment.

RNA Isolation, Northern Blot Analysis, and Hybridization. Total RNA for Northern blotting was isolated by the one step method of Chomczynski and Sacchi (1987). Equal quantities of RNA were separated on 1.2% agarose/2.2 M formaldehyde gels. After electrophoresis, RNA was blotted onto BAS 85 nitrocellulose membranes and baked at 80 °C for 4 h. After prehybridization for 3−4 h at 42 °C, blots were hybridized overnight with [α-^{32}P]dCTP-radiolabeled cDNA probes at 42 °C. The hybridization solution contained 6× SSC, 5× Denhardt's solution [0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin (BSA)], 0.5% SDS, 50% formamide, and 100 μg/mL denatured salmon sperm DNA. Blots were washed subsequently with 2× SSC/0.1% SDS (once) and 0.1× SSC/0.1% SDS (twice) at 65 °C. Filters were exposed for up to 4 days to X-ray films (Kodak, X-OMAT RP). Autoradiographs were quantitated by densitometry using Gelimage software (Pharmacia). When nitrocellulose filters were sequentially hybridized with different cDNA probes, the ^{32}P-labeled cDNA was removed after autoradiography by two washing steps with boiling 0.05× SSC/0.1% SDS for 15 min and rehybridized.

cDNA Probes. The cDNA probes were a 883 bp *Eco*RI−*Hin*dIII fragment of pRH01, a plasmid with the full-length cDNA of rat HO-1 (Shibahara et al., 1985) and full-length cDNAs for rat HBP23 (Iwahara et al., 1995), rat hemopexin (Nikkilä et al., 1991), human ALA-S (a gift from Dr. D. Bishop, Mount Sinai Medical School, NY), and GAP-DH (a gift from Dr. K. Preissner, Bad Nauheim). Probes were labeled by the oligomer method (Feinberg & Vogelstein, 1983) with [α-^{32}P]dCTP using a multiprime DNA labeling kit according to the manufacturers' instructions.

RESULTS

HBP23 mRNA Levels Are Induced in a Time- and Dose-Dependent Manner in Primary Rat Hepatocytes and Mouse Hepa 1-6 Cell Cultures. To investigate whether the HBP23 gene is regulated by the ligand heme, HBP23 mRNA levels were determined in cultures of primary rat hepatocytes and mouse hepatoma Hepa 1-6 cells. Heme treatment of cell cultures was performed with heme bound to BSA considering that in hemolytic diseases by far most of the circulating heme is bound by albumin (Muller-Eberhard, 1988). The time course of HBP23 induction after addition of heme (Figure 1; see also Figures 3 and 4 for representative data) demonstrates that HBP23 mRNA levels were up-regulated within 1 h in both culture systems. The maximum HBP23 mRNA accumulation was observed in primary rat hepatocytes and Hepa 1-6 cells after 5 h of treatment with heme, and the maximum induction was slightly higher in Hepa 1-6 cells. Twenty-four hours after exposure to heme, HBP23 mRNA levels were still increased 2-fold over base line levels. The rate of HBP23 mRNA induction (Figure 1) was normalized to mRNA levels of the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAP-DH).

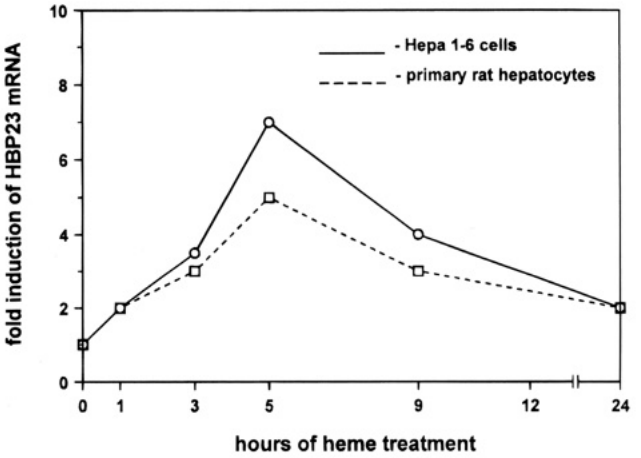

FIGURE 1: Time course of HBP23 mRNA expression in primary rat hepatocytes and Hepa 1-6 cells treated with heme-BSA. Primary rat hepatocytes and Hepa 1-6 cells, after cultivation for 24 h in serum free medium, were cultured for additional time intervals with 10 μM heme-BSA. Total cellular RNA (10 μg) was subjected to Northern blot analysis and probed with the ^{32}P-labeled cDNA of HBP23 and subsequently that of GAP-DH. Autoradiograms were quantitated by densitometry, and the signal of the GAP-DH band was used as an internal standard. Data are the means of duplicates from a representative experiment. Numbers show the fold induction rate relative to the basal HBP23 mRNA expression at 0 h.

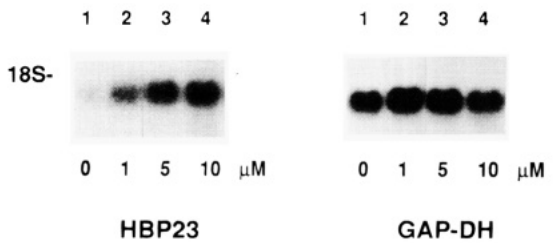

FIGURE 2: Dose dependency of HBP23 mRNA expression in Hepa 1-6 cells treated with heme-BSA. Hepa 1-6 cells were grown to confluency and incubated for 24 h in serum free medium and for another 5 h in serum free medium in the absence (lane 1) or presence of increasing concentrations of heme-BSA (lane 2−4; 1, 5, and 10 μM). Northern blot analysis of 10 μg of total cytosolic RNA was performed and probed with the ^{32}P-labeled cDNA of HBP23 and subsequently the cDNA of GAP-DH.

The dose-dependency pattern of the induction of HBP23 mRNA by heme-BSA in mouse Hepa 1-6 levels demonstrates that the maximum induction level was reached between heme-BSA concentrations of 5−10 μM (Figure 2). In all subsequent experiments 10 μM heme-BSA was added to cell cultures as the standard concentration.

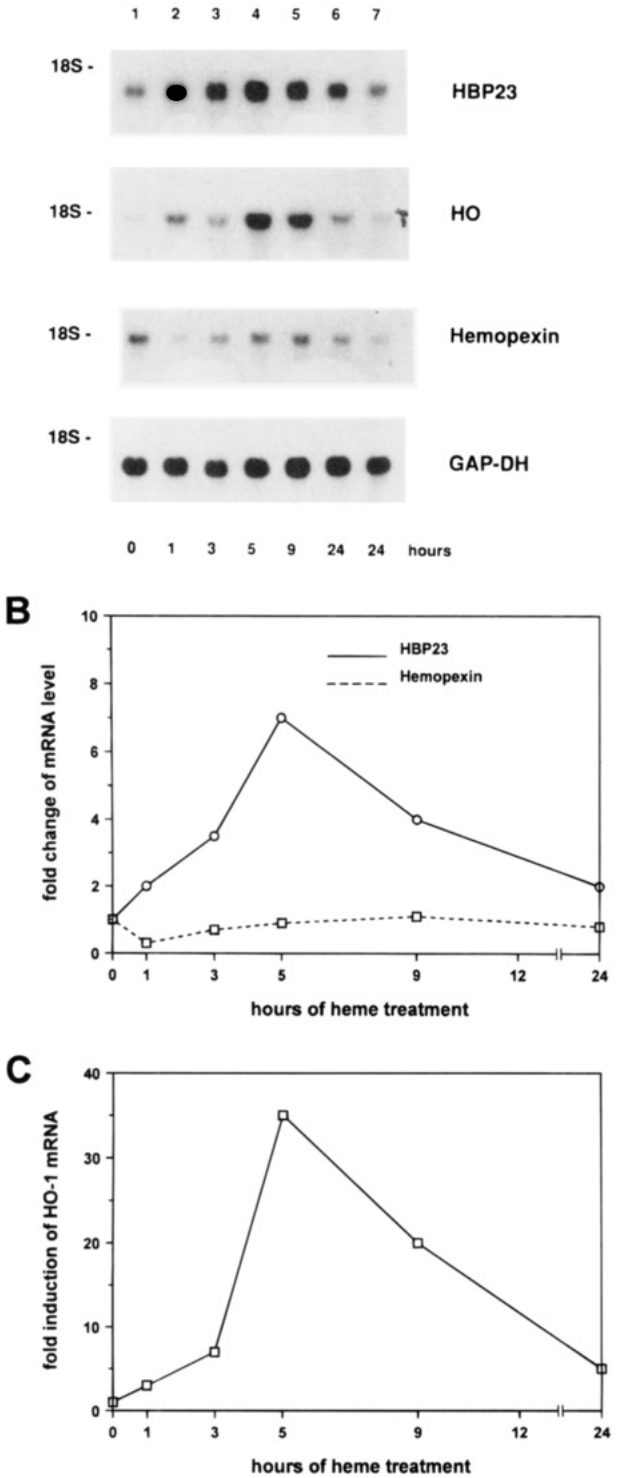

FIGURE 3: Coordinate time-dependent induction of HBP23 and HO-1 mRNA expression during treatment with heme-BSA in Hepa 1-6 cells. Hepa 1-6 cells were grown to confluency and incubated for another 24 h in serum free medium and for the times indicated in serum free medium in the absence (lane 1 and 7) or presence of 10 μM heme-BSA (lane 2−6). (A, top) Total RNA (10 μg) was analyzed by Northern blot and probed sequentially with the ^{32}P-labeled cDNAs of HBP23, HO-1, hemopexin, and GAP-DH. The size marker is the 18S ribosomal RNA band. (B) Graphic demonstration of mRNA levels of HBP23 and hemopexin and (C) HO-1 normalized to mRNA levels of GAP-DH. Please note the difference in the magnitude of fold induction on the ordinates in panels B and C.

HBP23 and HO-1 mRNA Expression Is Induced with a Similar Time-Dependent Regulatory Pattern during Heme Treatment. HO-1, the rate-limiting enzyme of heme degradation, is highly inducible by heme *in vivo* and *in vitro* (Pimstone et al., 1971; Shibahara et al., 1985). To compare the pattern of HO-1 mRNA regulation in cell cultures treated with heme to that of HBP23, mRNA expression of the two genes was determined during heme treatment of mouse Hepa 1-6 cell cultures. A representative experiment shows that the time course of HO-1 mRNA induction after heme exposure is similar to that observed for HBP23 mRNA induction (Figure 3A−C; see also Figure 1); the maximum mRNA expression of both genes was observed after 5 h of heme treatment. The mRNA induction rate relative to base line expression (lanes 1 and 7), however, was significantly higher for HO-1 mRNA (about 30−40-fold) than that for HBP23 mRNA (7-fold; compare Figure 3, panels B and C). The constitutive mRNA expression level of HO-1 in Hepa 1-6 cells was lower than that of HBP23 mRNA (Figure 3A). A slight decrease of mRNA levels after 1−3 h (Figure 3A,B) was observed for the heme-binding protein hemopexin, a β_2-glycoprotein that binds heme with very high affinity (Muller-Eberhard, 1988). In accordance with previous results (Hamilton et al., 1991), the mRNA levels of 5-aminolevulinate synthase (ALA-S), the rate-limiting enzyme of heme synthesis (May & Bawden, 1989), were significantly reduced during heme treatment (data not shown). Levels of GAP-DH mRNA showed no alteration during heme treatment.

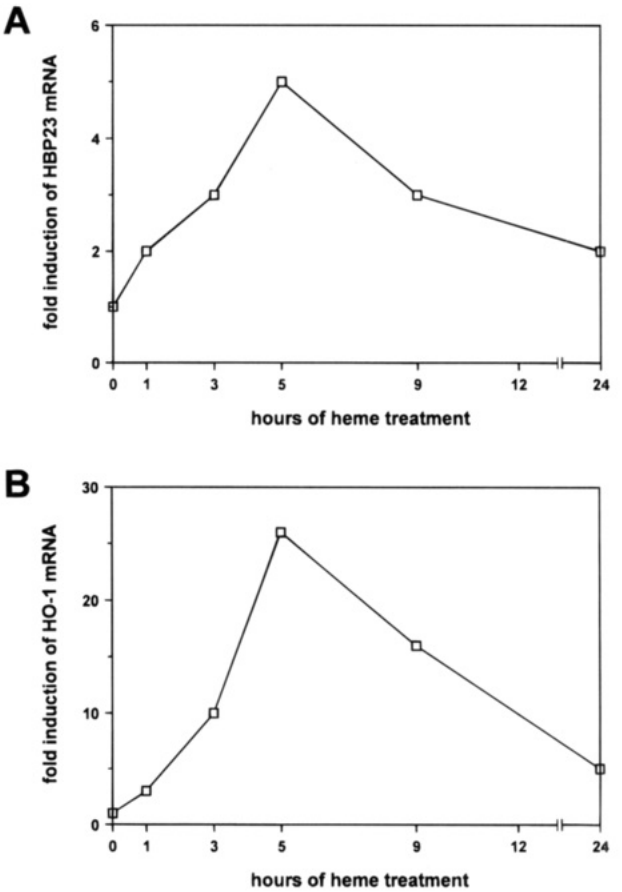

FIGURE 4: Time-dependent induction patterns of HBP23 and HO-1 mRNA expression after heme-BSA treatment of cultured primary rat hepatocytes. Primary rat hepatocytes were cultured as described in Experimental Procedures. Total cellular RNA (10 μg) was analyzed by Northern blotting and sequentially hybridized with ^{32}P-labeled cDNAs of HBP23, HO-1, and GAP-DH. (A) Graphic demonstration of the induction of HBP23 mRNA and (B) of HO-1 mRNA, both normalized to GAP-DH mRNA.

The pattern of induction of the levels of HBP23 and HO-1 mRNA in mouse Hepa 1-6 cells (Figure 3) was qualitatively similar in primary rat hepatocytes (Figure 4).

HBP23 and HO-1 mRNA Levels Are Induced in Parallel by $CdCl_2$ and $CoCl_2$ in Hepatic Cell Cultures. HO-1 enzyme activity and mRNA expression are not only induced by heme but also by heavy metals such as $CdCl_2$ and $CoCl_2$ (Sardana et al., 1982; Alam et al., 1989). Therefore, HBP23 and HO-1 mRNA expression in the presence of $CdCl_2$ and $CoCl_2$ in Hepa 1-6 cells and primary rat hepatocytes was investigated. In dose-dependency experiments it was determined that the minimum concentration of $CoCl_2$ for induction of HO-1 and HBP23 mRNA in Hepa 1-6 cells was 50−100 μM and that the maximum induction rate was reached at 300 μM. Similar observations for inducibility of Hepa 1-6 cells by cobalt were made previously (Alam et al., 1989). $CdCl_2$ had a maximum inducing effect at 10 μM, and concentrations higher than 20 μM were toxic (as determined by microscopic observation and lactate dehydrogenase leakage). Treatment of Hepa 1-6 cell cultures with $CoCl_2$ (300 μM) resulted in a rate of HBP23 mRNA induction to levels about equal to treatment with heme (Figure 5A,B). The rate of induction of HBP23 mRNA elicited by $CdCl_2$ was even higher than that of heme (Figure 5A,B). The patterns of HBP23 mRNA induction during treatment with $CdCl_2$ and $CoCl_2$ resembled that found for HO-1 mRNA (Figure 5). Under identical conditions hemopexin was not regulated; neither was regulation of HBP23 mRNA levels observed after exposing cells to $SnCl_2$ and $ZnCl_2$ (data not shown). Similar induction rates for HBP23 and HO-1 mRNA as found for Hepa 1-6 cells were observed for primary rat hepatocyte cultures (data not shown).

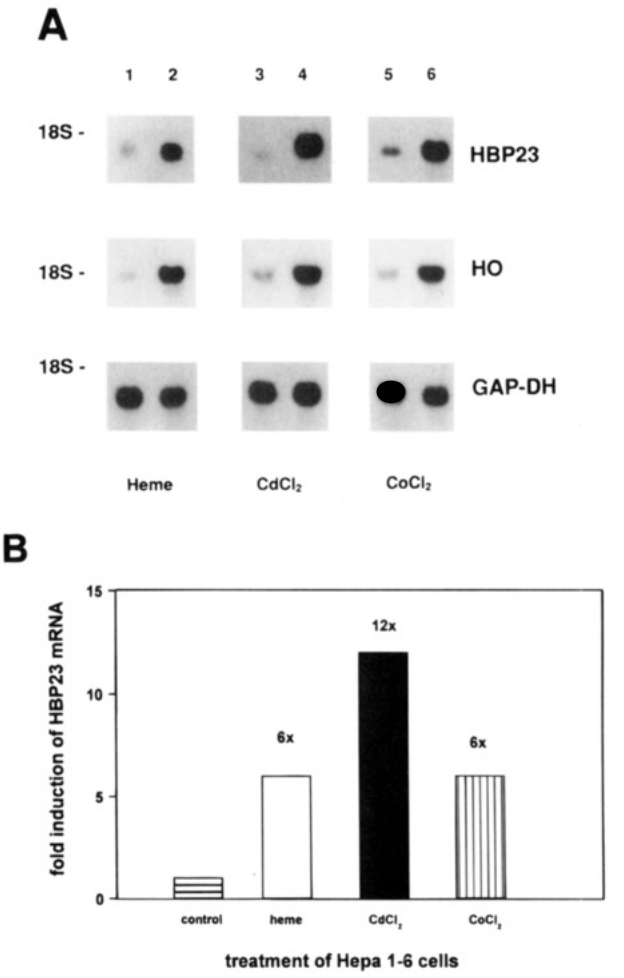

FIGURE 5: Parallel induction of HBP23 and HO-1 mRNA levels by heme, $CdCl_2$ and $CoCl_2$, in Hepa 1-6 cells. (A) Confluent Hepa 1-6 cells were cultured for 5 h in serum free medium with 10 μM heme-BSA (lane 2), 10 μM $CdCl_2$ (lane 4), 300 μM $CoCl_2$ (lane 6), or control medium (lanes 1, 3, and 5). Total RNA (10 μg) was isolated, subjected to Northern blot analysis, and probed sequentially with ^{32}P-labeled cDNAs of HBP23, HO-1, and GAP-DH. (B) The results in panel A were quantitated by densitometry and normalized with GAP-DH. The bar graphs indicate the induction rate of the HBP23 mRNA levels obtained for the respective treatments. Numbers of columns indicate the fold induction rate relative to the control.

DISCUSSION

The liver cytosolic protein HBP23 that is characterized in the accompanying paper (Iwahara et al., 1995) is a heme-binding protein induced by the ligand heme; the manner of induction is similar to that found for the heme metabolizing enzyme HO-1.

The coordinated regulatory pattern of the mRNA levels of HBP23 and HO-1 induced by heme (Figures 3 and 4) or by the heavy metals $CdCl_2$ and $CoCl_2$ (Figure 5) suggests that both genes are induced by (a) similar mechanism(s). HO-1 is known to be transcriptionally upregulated by heme and heavy metals (Alam et al., 1989). However, whether this induction is mediated by a common or different mechanism(s) is still under investigation. A regulatory element in the HO-1 5′-flanking promoter region which is prominently induced by $CdCl_2$, but not by heme, has been demonstrated, therefore suggesting that disparate regulatory mechanisms for heme and cadmium exist (Takeda et al., 1994). From recent data obtained *in vivo*, it has been derived that the heavy metal-dependent induction of HO-1 may be caused by increased H_2O_2 levels in the liver, which in turn induce expression of stress proteins such as HO-1 (Llesuy et al., 1994). This finding is supported by preliminary observations from our laboratory in hepatocyte cultures, where H_2O_2 induced both HBP23 and HO-1 mRNA expression.

The results of this study taken together with data of previous studies on HO-1 (Keyse & Tyrrell, 1989; Llesuy et al., 1994) and the extensive sequence homology of HBP23 with the recently cloned protein family of thioredoxin reductases (Chae et al., 1994a,b) strongly support the hypothesis that HBP23 and HO-1 have common physiological functions as antioxidants and/or in heme metabolism. The parallel pattern of the regulation of HBP23 and HO-1 mRNA expression in hepatic cells (Figures 3 and 4) suggests that HBP23 could constitute an important cellular defense mechanism against intracellular oxidative damage, as previously shown for HO-1 (Keyse & Tyrrell, 1989). HO-1 is induced in various tissues by stimuli causing oxidative stress such as H_2O_2, UV-light, sulfhydryl reactive agents (Keyse & Tyrrell, 1989) or reperfusion injury (Paschen et al., 1994). In addition, HO produces the catabolites biliverdin and bilirubin which have been shown to be potent antioxidants (Stocker et al., 1987).

A sequence comparison demonstrates that HBP23 has 74% homology with thioredoxin peroxidases. Thioredoxin peroxidases belong to the peroxiredoxin family (Chae et al., 1994a,b), that has been conserved from bacteria to human and exhibits a protective function against oxidative damage

by thiol groups (Chae et al., 1994a,b). HBP23 and three other recently cloned proteins with 96−97% amino acid sequence homology to HBP23 [the mouse protein MSP23 (Ishii et al., 1993) and the human proteins NKEF A (Shau et al., 1994) and pag (Prosperi et al., 1993)] are members of this protein entity. HBP23 was cloned from liver whereas MSP23 and NKEF A were cloned from blood cells; MSP23 is also inducible by various stress stimuli in macrophages (Ishii et al., 1993).

HBP23 may also have a functional role in heme metabolism. Heme is the prosthetic group of many hemoproteins, such as hemoglobin, myoglobin, and cytochromes, but can also be detrimental if not contained in proteins (Muller-Eberhard & Fraig, 1993). Heme has been implicated in the generation of hazardous oxidant species in several experimental systems including ischemia/reperfusion injury (Sadrzadeh et al., 1987; Balla et al., 1991). In addition, heme iron and cellular iron are believed to be essential for the transformation of superoxide to highly toxic hydroxyl radical compounds via the iron-catalyzed Fenton reaction (Halliwell & Gutteridge, 1984). Both HO-1 and HBP23 may be functional in containing heme either by enzymatic degradation of heme (HO-1) or by binding heme (HBP23). Thereby, the hazardous properties of "free" heme are inhibited as shown for the *R. prolixus* heme-binding protein that is able to decrease *in vitro* the heme-induced lipid peroxidation of linolenic acid liposomes and lipophorin (Dansa-Petreski et al., 1995; Oliveira et al., 1995). This is consistent with previous observations on heme-induced lipid peroxidation that is inhibited by heme-binding proteins (Vincent et al., 1988). In addition, it was previously demonstrated that heme mediated lysis of endothelial cells or heme-stimulated chemiluminescence in perfused rat lungs is greatly prevented by the presence of the heme-binding protein hemopexin (Balla et al., 1991; Barnard et al., 1993).

HBP23 could be an intracellular heme transporter, e.g., a vehicle from the cell membrane to cell organelles (Muller-Eberhard & Vincent, 1985). A recent study on heme binding to membranes of various cell types and cell organelles indicates that a specific process may be necessary to release heme from the membrane to which it is bound (Liem et al., 1994). Such a transporter function has been suggested previously for two other intracellular heme-binding proteins, heme-binding protein/liver-fatty acid binding protein and glutathione-S-transferases (Vincent et al., 1985, 1987; Pickett & Lu, 1989). Among the heme-binding proteins that are candidates for participating in intracellular heme transport HBP23 is unique in that it is induced by the ligand heme.

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