**THE YEAST HOMOLOG OF HEME OXYGENASE-1 AFFORDS CELLULAR ANTIOXIDANT PROTECTION VIA THE TRANSCRIPTIONAL REGULATION OF KNOWN ANTIOXIDANT GENES**

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Transcriptional regulation by heme oxygenase-1

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Heme oxygenase-1 (HO-1) degrades heme and protects cells from oxidative challenge. This antioxidant activity is thought to result from HO-1's enzymatic activity, manifested by a decrease in the concentration of the pro-oxidant substrate heme, and an increase in the antioxidant product bilirubin. Using a global transcriptional approach, and yeast as a model, we show that HO-1 affords cellular protection via upregulation of transcripts encoding enzymes involved in cellular antioxidant defense, rather than via its oxygenase activity. Like mammalian cells, yeast responds to oxidative stress by expressing its HO-1 homolog and, compared with the wild type, heme oxygenase null-mutant cells have increased sensitivity towards oxidants that is rescued by over-expression of human HO-1 or its yeast homolog. Increased oxidant sensitivity of heme oxygenase null-mutant cells is explained by a decrease in the expression of the genes encoding γ-glutamylcysteine synthetase, glutathione peroxidase, catalase and methionine sulfoxide reductase, since over-expression of any of these genes affords partial, and over-expression of all four genes provides complete, protection to the null mutant. Genes encoding antioxidant enzymes represent only a small portion of the 480 differentially expressed transcripts in heme oxygenase null mutants. Transcriptional regulation may be explained by the nuclear localization of heme oxygenase observed in oxidant-challenged cells. Our results challenge the notion that HO-1 functions simply as a catabolic and antioxidant enzyme. They indicate much broader functions for HO-1, the unraveling of which may help explain the multiple biological responses reported in animals as a result of altered HO-1 expression.

Heme oxygenase degrades heme to CO, Fe²⁺ and biliverdin (1). Mammalian cells contain heme oxygenase-1 (HO-1) and HO-2 that share 43% amino acid sequence homology (2). Compared with HO-2, HO-1 has a lower apparent $K_m$-value for heme (3), and the two enzymes are regulated differently and exhibit different physiological properties (4). The constitutively expressed HO-2 is implicated in oxygen sensing (5) and contains heme regulatory motifs that act as a thiol/disulfide redox switch, regulating the $K_d$ for ferric heme (6). In most tissues HO-1 expression is induced in response to different types of stress, including oxidative stress, heat shock and iron starvation (4). Increased expression of HO-1 is associated with a range of different cellular properties, including increased antioxidant protection and altered cell growth and signaling (4). In addition, there is mounting evidence suggesting that induction of HO-1 protects against various diseases (7, 8).

Prokaryotes and lower eukaryotes possess homologs of mammalian HO-1. In the case of *Saccharomyces cerevisiae*, the homolog Hmx1p was identified as a stress protein in response to iron deprivation (9), and in a genome-wide transcriptional investigation of the activator of ferrous transport (*AFT1*) regulon (10). Aft1p is an iron-dependent transcription factor (11) that induces the expression of several genes, including *HMX1*, in response to iron-limitation. Several lines of
evidence support a role for Hmx1p in the regulation of heme and iron homeostasis in yeast. Deletion of *HMX1* leads to the accumulation of heme and depletion of iron, as well as to the expression of *FET3*, a known Aft1p target gene that encodes a multi-copper oxidase (12) and that forms part of a high affinity iron transport complex (13). Loss of *HMX1* also leads to the induction of *CYC1* via the oxygen-sensing transcription factor Hap1p that itself is activated by heme (14). Heme acts as a positive and negative modulator of the transcription of aerobic and hypoxic genes, respectively (14).

It was recognized only recently that Hmx1p possesses classical heme oxygenase activity (15), raising the possibility that in addition to regulating cellular heme and iron levels, Hmx1p may also share some of the additional activities of mammalian HO-1. Here, we show that Hmx1p indeed is induced in response to different stresses in addition to iron starvation, and that it protects yeast cells against oxidant challenge in a glutathione-dependent manner and via transcriptional regulation of genes encoding known enzymes involved in cellular antioxidant defense.

**Experimental Procedures**

**Yeast strains and growth conditions**—Table S1 lists the *S. cerevisiae* strains used in this study. The HA-*HMX1* wild type strain, which expresses a triple copy of the hemagglutinin (HA) epitope at the N-terminus, was constructed by PCR epitope tagging as described (16) using the plasmid pMPY-3×HA (a kind gift from Dr. C.C. Philpott, National Institutes of Health, Bethesda, MD) and the following primers: 5′-CAGCACACATACTCACTCACACATAAAATAACCGCAAAAATAGGGACCAAACGCTGG-3′ and 5′-TAGCTCCTCCATGTCAHTGTGTGAGTGTATGATTGTATTGCTACTGTCCTTCAGGGCGAATTGGG-3′. Integration of the HA epitope was confirmed by PCR and by Western blotting. Strains were grown in rich YEPD medium (2% w/v glucose, 2% w/v bactopeptone, 1% yeast extract) or minimal synthetic-defined media (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% w/v glucose) supplemented with appropriate amino acids and bases: 2 mM L-leucine, 4 mM L-isoleucine, 1 mM L-valine, 0.3 mM L-histidine, 0.4 mM L-tryptophan, 1 mM L-lysine, 0.15 mM adenine, 0.2 mM uracil. Media were solidified by the addition of 2% (w/v) agar.

**Western blot analysis**—Cell extracts were subjected to electrophoresis under reducing conditions on 4-12% NuPAGE mini-gels (Invitrogen) and proteins blotted onto nitrocellulose membranes (Amersham Biosciences). Cytosolic and nuclear extracts were prepared as described (17). Blots were incubated with either mouse monoclonal anti-HA (to localize Hmx1p) (Sigma, 1:5,000 dilution), rat anti-tubulin (loading control) (Abcam, 1:5,000), mouse anti-Pgk1 (cytosol marker) (Invitrogen, 1:5,000), mouse anti-Nop2 (nucleus marker) (Abcam, 1:5,000), mouse anti-Dpm1 (marker for ER and nuclear membrane-ER network) (Molecular Probes, 1:500) or rabbit anti-Kar2 antibody (ER marker) (Santa Cruz, 1:1,000), and bound antibody visualized by chemiluminescence (ECL, Amersham) following incubation with sheep anti-mouse immunoglobulin-horseradish peroxidase conjugate (Amersham, 1:5,000), sheep anti-rat immunoglobulin-horseradish peroxidase conjugate (Sigma, 1:5,000), or goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Abcam, 1:5,000).

**Sensitivity to oxidants**—Cells were grown to exponential phase (OD<sub>600nm</sub> = 1) in synthetic medium at 30 °C and treated with H<sub>2</sub>O<sub>2</sub>, diamide or menadione at the concentration and for the time indicated. Aliquots of cells were removed, diluted in fresh YEPD medium, plated in triplicate on YEPD plates, and the number of viable colonies counted after 3 d of culture.

**Plasmids**—A galactose-inducible multi-copy plasmid containing *HMX1* was constructed in JMB671, obtained as a generous gift from Dr. G. Perrone, (University of New South Wales, Sydney, Australia). The *HMX1-HA* coding sequence was amplified by PCR from the *HMX1-HAF* 5′-TTCTTGTCGACCATGTGATATACGAT-3′ and 3′-ATTGTCTGAGTCGACCATACTCACACATATAAAATAGGGACCAAACGCTGG-3′. Integration of the HA epitope was confirmed by PCR and by Western blotting. Strains were grown in rich YEPD medium (2% w/v glucose, 2% w/v bactopeptone, 1% yeast extract) or minimal synthetic-defined media (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% w/v glucose) supplemented with appropriate amino acids and bases: 2 mM L-leucine, 4 mM L-isoleucine, 1 mM L-valine, 0.3 mM L-histidine, 0.4 mM L-tryptophan, 1 mM L-lysine, 0.15 mM adenine, 0.2 mM uracil. Media were solidified by the addition of 2% (w/v) agar.

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Microarray hybridization and data analysis—Cells were grown in triplicate to exponential phase ($OD_{600nm} = 1$) in minimal SD media. Cells were broken in Trizol reagent (Gibco BRL, Life Technologies, MD) by three cycles of vigorous mixing in the presence of acid-washed glass beads (45 s) and placed on ice for 30 s. RNA was then extracted according to the manufacturer's instructions, and its quality determined by spectrophotometry (Nanodrop) and by Bioanalyser (Ramaciotti Centre for Gene Function Analysis, University of New South Wales, Australia). Preparation of cRNA, probes, and hybridization to whole yeast genome microarrays (YG-S98, Affymetrix) was performed at the Ramaciotti Centre. Affymetrix Yeast Genome 2.0 Arrays contain probe sets for $S.\ ceriseiae$ and Schizosaccharomyces pombe. The latter probes were excluded from the analysis and normalization was performed using the robust multi-array average (RMA) (18, 19) algorithm implemented in BioConductor (http://www.bioconductor.org/). For each individual $S.\ ceriseiae$ gene (probe set) on the array, fold-change, moderated t-statistics and corresponding p-value (20) were calculated. Candidate differentially expressed genes with a significant Bonferroni adjusted p-value < 0.05 and fold-change > 2.0 ($hmxl$ versus WT) were identified.

Relative mRNA levels of the differentially expressed antioxidant enzymes were determined by RT-PCR. Cells were prepared and RNA extracted as described above. cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer’s instructions. The resulting cDNA was then probed for GPX1, GSH1, CTT1, MXR1, GPX1, GPX2 and ACT1 by PCR, using the primers listed in Table S2. Resulting RT-PCR products were visualized by agarose gel electrophoresis and quantified using ImageJ (NIH).

Biochemical analyses—Total glutathione was determined based on the reduction of oxidized glutathione (GSSG) by GSSG-reductase and NADPH (21). Cells were harvested at exponential growth phase, washed three times with ice-cold PBS, and resuspended in ice-cold 8 mM HCl/1.3% (wt/vol) 5-sulfosalicylic acid. Cells were then broken as described above, the resulting mixture clarified by centrifugation (10 min, 13,000 x g, 4 °C), and total glutathione determined in the resulting supernate. Glutathione peroxidase activity was determined using tert-butyl hydroperoxide (Sigma) as substrate. Reactions were started by the addition of cell lysates and followed as the oxidation of NADPH coupled to GSSG reduction by glutathione reductase (22). Catalase activity was determined by the loss of added H$_2$O$_2$ (10 mM) in 50 mM K-phosphate buffer (pH 7.0) containing 0.5 mM EDTA after addition of cell extract (23). Blanks were run in the absence of H$_2$O$_2$ and activity calculated using $\varepsilon_{240nm} = 39.4 \text{ M}^{-1}\text{cm}^{-1}$.

Methionine sulfoxide reductase activity was determined as described previously (24). Briefly, the reaction mixture contained 0.4 mM NADPH, 5 mM free Met-R-SO (Sigma) and 5 µg thioredoxin and 0.5 µL thioredoxin reductase (both from E. coli, Sigma). The reaction was started by the addition of cell extract and allowed to take place for 15 min at 37 °C. Phosphate-buffered saline (200 µL) was then added and loss of NADPH determined immediately in a spectrophotometer using $\varepsilon_{340nm} = 6,220 \text{ M}^{-1}\text{cm}^{-1}$.

Immunocytochemistry and Confocal Microscopy—Cells were grown to exponential phase ($OD_{600nm} = 1$) in synthetic medium at 30 °C and treated for 6 h with H$_2$O$_2$ (4 mM). Cells were then washed and prepared for immunofluorescence microscopy as described (25), using anti-HA Alexa Fluor® 488 conjugated antibody (Molecular Probes) at 1:500 and the endoplasmic reticulum (ER) marker 3,3’-dihexyloxacarbocyanine iodide (DiOC$_6$(3), Invitrogen) at a final concentration of 10 µg/mL.

Images were captured using a confocal laser microscope (Zeiss LSM Meta 510) with a 100x oil objective, 1.4-numerical-aperture at 12-bit resolution in each channel. Yeast strains, without HA tag were used to control for background staining of the anti-HA Alexa conjugate. All captured images were converted to tagged image files, and Z-stacks collected at Z increments of 0.5 µm. Image processing 3D analysis and z projection was performed using ImageJ software (NIH).

Statistical analyses—Significant differences between treatments and controls purchased from Open Biosystems (Open Biosystems, Huntsville, AL).
were examined using the Wilcoxon-Mann-Whitney rank sum test. Where appropriate, data was analyzed by one- or two-way ANOVA with post-hoc Bonferroni test as indicated. Significance was accepted at $p < 0.05$.

**RESULTS**

**Expression of Hmx1p is induced in response to different oxidants**—As HO-1 is induced in response to oxidant stress (4), we tested whether Hmx1p expression is induced similarly in yeast cells, using an HA-tagged HMX1 strain and Western blotting with an anti-HA antibody (9). We noted modest expression of Hmx1p in control, unstressed cells (Fig. 1A, lane 1). Iron starvation increased Hmx1p expression (Fig. 1A, lane 2), confirming a finding reported previously by others (9). What is novel, however, is that Hmx1p was also induced when cells were exposed to H$_2$O$_2$, diamide (a membrane permeable, thiol-specific oxidant that reacts rapidly with reduced glutathione, GSH) and the redox-cycling drug menadione (that generates superoxide anion radicals) (Fig. 1A, lanes 3-5). Treatment of cells with H$_2$O$_2$ caused a time- (Fig. 1B) and oxidant concentration-dependent (Fig. 1C) increase in Hmx1p. Immunocytochemistry staining and confocal laser scanning microscopy confirmed low level of Hmx1p expression under standard, iron replete growth conditions (Fig. 2A, left panel arrows and boxed enlargements), and the increase in Hmx1p expression in cells exposed to iron starvation or H$_2$O$_2$ (Fig. 2A, B). Yeast strains without HA tag yielded low background staining (Fig. 2C). The extent of H$_2$O$_2$-mediated increase in Hmx1p expression was comparable for N- and C-terminus HA-tagged HMX1 strains (Fig. 2B). While both iron depletion and H$_2$O$_2$ increased Hmx1p expression visibly, the expression pattern was heterogeneous, with very high expression in a subset of cells, but low to non-detectable expression in most cells (Fig. 2A). Hmx1p expression was also increased in response to other known inducers of HO-1, such as rapamycin and heat shock (not shown). The observed similarity between induction of HO-1 and Hmx1p in response to oxidative stress supports the contention that Hmx1p is part of the antioxidant defense in yeast cells.

**HMX1 affects sensitivity to H$_2$O$_2$, diamide and menadione**—To test this possibility we compared the oxidant sensitivity of wild type and hmx1 mutant cells using concentration-response curves to H$_2$O$_2$, diamide and menadione. At all concentrations tested, the hmx1 mutant was more sensitive to the oxidants than the wild type strain (Fig. 3A and Fig. S1A). Conversely, over-expression of HMX1 increased the resistance of wild type and hmx1 mutant strains to H$_2$O$_2$ (Fig. 3B), diamide and menadione (Fig. S1B). For the hmx1 mutant strain, over-expression of HMX1 restored the oxidant resistance to that of wild type cells. Similarly, over-expression of HMX1-HA also fully rescued the oxidant sensitivity of the hmx1 null mutant (Fig. 3C and Fig. S1C), confirming that the HA-tagged Hmx1p used in our studies was functional. In addition, over-expression of recombinant human HO-1 similarly rescued the oxidant sensitivity of the hmx1 mutant (Fig. 3D).

Together, these data indicate that Hmx1p is induced by, and offers protection against, oxidative stress, similar to the situation with HO-1 in mammalian cells.

**The hmx1 mutant has altered expression of cellular antioxidant enzymes**—To determine if the transcriptional response of the hmx1 null mutant is altered compared with that of the wild type strain, Affymetrix microarray analyses were carried out. Loss of HMX1 significantly affected the transcriptome, with 265 open reading frames upregulated (Table S1) and 215 downregulated (Table S2) (Fig. 4A). Five gene ontologies were significantly over-represented in the upregulated transcripts: response to stress, sulphur metabolic process, transcription factor activity, antioxidant activity and transmembrane transporter activity. Two gene ontologies were significantly over-represented in the downregulated transcripts: RNA processing and ribosome biogenesis.

We further investigated five transcripts encoding enzymes contributing to cellular antioxidant defense (Fig. 4B), the altered transcription of which was confirmed by RT-PCR (Fig. 5). Of these, GSH1, GPX1, CTT1 and MXR1 were downregulated, while GPX2 was upregulated. GSH1 encodes $\gamma$-glutamylcysteine synthetase, which catalyzes the first step in the synthesis of GSH (26). It protects cells by scavenging oxidants and by acting as cofactor for several antioxidant
enzymes (27). GPX1 and CTT1 respectively encode a glutathione peroxidase that acts on phospholipid hydroperoxides and other organic peroxides (22, 28), and a cytosolic catalase that forms part of a H₂O₂-detoxification system and is redundant with the glutathione system (29). MXR1 encodes a peptide methionine sulfoxide reductase, which reduces methionine sulfoxide residues in proteins (30). GPX2 encodes an atypical 2-Cys peroxiredoxin, responsible for the reduction of hydroperoxides using thioredoxin rather than GSH as the preferred cofactor (31). This difference in cofactor preference may explain why GPX1 and GSH1 expression were downregulated, while GPX2 was upregulated in the hmx1 mutant. In addition to the five genes encoding known antioxidant enzymes, seven other transcripts with potential indirect participation in oxidative stress were also differentially expressed (TABLES S3 and S4). The role of these genes was not investigated further.

Over-expression of downregulated antioxidants rescues oxidant sensitivity of hmx1 mutant—To define the mechanism of antioxidant protection by Hmx1p, each of the downregulated antioxidant enzymes was over-expressed separately and the effect of this on rescue of hmx1 oxidant sensitivity determined. Over-expressing either GPX1 (Fig. 6A), GSH1 (Fig. 6B), CTT1 (Fig. 6C) or MXR1 (Fig. 6D) increased resistance of wild type cells to diamide, H₂O₂ and menadione. More importantly, over-expression of any of these genes also increased the resistance of the hmx1 mutant to each of the three oxidants tested (Fig. 6A-D). The extent of this increased resistance was less than that seen in the corresponding wild type cells, indicating that each of the four known antioxidant enzymes alone partially rescued the oxidant sensitivity of the hmx1 mutant strain. In contrast, simultaneous over-expression of all four transcripts encoding the antioxidant genes in the hmx1 mutant (Fig. S2) completely restored its oxidant resistance to that of wild type cells at all concentrations of H₂O₂ tested (Fig. 7).

HMX1 relates to cellular antioxidant activities—Consistent with the observed oxidant sensitivity, total glutathione concentration in the hmx1 mutant was only ~20% of the wild type strain value, and over-expression of HMX1 in the hmx1 mutant increased total glutathione to above wild type levels (TABLE 1). Similarly, in the hmx1 mutant, catalase and glutathione peroxidase activities were decreased compared with wild type cells, and over-expression of HMX1 increased the activity of both enzymes to above the corresponding wild type values (TABLE 1). The activity of methionine sulfoxide reductase was below the limit of detection in all strains, except wild type cells over-expressing MXR1. Together, these data show that the extent of HMX1 expression relates to the cellular activities of the differentially expressed antioxidant enzymes identified in the microarray experiments.

We next examined whether in hmx1 cells over-expression of each of the HMX1 related antioxidant enzymes affected the activities of the other downregulated antioxidants. As shown in Fig. 8, over-expression of GSH1 increased both the content of total glutathione and glutathione peroxidase activity. Also, over-expression of GPX1, but not CTT1 or MXR1, increased glutathione peroxidase activity (Fig. 8A). In the case of catalase, only over-expression of CTT1 increased the activity of this enzyme (Fig. 8B), while over-expression of GSH1 increased the levels of total glutathione (Fig. 8C). These results indicate that the differentially expressed antioxidant genes, at least in part, acted independently from each other.

The fact that over-expression of all four transcripts encoding antioxidant genes was required to completely restore the oxidant resistance of the hmx1 mutant (Fig. 7), suggested that the products of the reaction catalyzed by Hmx1p themselves did not provide substantial oxidant protection. Consistent with this interpretation, addition of biliverdin and the CO-releasing molecule CORM3, singly or together, failed to offer the hmx1 null mutant protection against 4 mM H₂O₂ (Fig. 9). Similar results were observed when cells were exposed to a lower H₂O₂ concentration (Fig. S3).

Dennery and co-workers recently reported HO-1 to localize to the nucleus of heme-treated mammalian cells and to activate transcription factors important in oxidative stress (32). In yeast, the nuclear and ER membranes are continuous (33), making it difficult to discriminate perinuclear from nuclear localization. We therefore use the term (peri)nuclear’ hereafter to refer to perinuclear or nuclear localization. We
observed (peri)nuclear localization of Hmx1p in cells exposed to 4 mM H$_2$O$_2$ for 6 h, as assessed by microscopy and biochemical analyses (Fig. 10). Similar results were observed with cells stressed with H$_2$O$_2$ for 1 h (Fig. S4), indicating that (peri)nuclear localization of Hmx1p occurred even after short periods of oxidant stress. In contrast, (peri)nuclear Hmx1p was not detected in cells in the absence of H$_2$O$_2$. Following oxidant treatment, (peri)nuclear expression of Hmx1p was observed in only a small subset of cells (Fig. 10A, B), with Hmx1p expressed more commonly in ER regions associated with membranes other than the nuclear membrane (Fig. S5). As in stressed mammalian cells nuclear localization has been reported to be preceded by calpain-mediated cleavage of the HO-1 C-terminus (32), we compared the extent of (peri)nuclear localization of Hmx1p in N- versus C-terminus HA-tagged \textit{H MX1} strains exposed to H$_2$O$_2$. We observed (peri)nuclear localization with both strains of yeast as assessed by confocal fluorescence microscopy (Fig. 10A) and biochemical analysis (Fig. 10C, Fig. S4). However, the extent was greater for N-terminus than C-terminus HA-tagged Hmx1p (Fig. 10B&D, Fig. S4). As expected from the close physical association of nuclear and ER membranes, the nuclear fraction contained ER markers (Dpm1 and Kar2 Fig. 10C), disallowing unambiguous localization of Hmx1p to the nucleus.

**DISCUSSION**

The ability of cells to respond to changes in environmental conditions, such as nutrient availability, determines their survival. Until now, Hmx1p, the yeast homolog of HO-1, was believed to be involved only in the cellular response to iron limitation (9). Here we provide evidence that an additional role of Hmx1p is in the cellular response to and protection against oxidative stress. Our studies, for the first time, show that this antioxidant activity of heme oxygenase is dependent on the upregulation of several genes encoding known antioxidant enzymes.

Several lines of evidence support the conclusion that Hmx1p affords cellular antioxidant protection, and that this is via the transcriptional regulation of known antioxidant genes rather than its oxygenase activity. First, the expression of Hmx1p, like that of HO-1, is induced in cells exposed to different oxidants. Secondly, deletion of \textit{H MX1} renders cells more sensitive to oxidant challenge while over-expression of \textit{H MX1}, like human HO-1, restores oxidant resistance of the hmx1 null mutant to that of wild type cells. Thirdly, microarray analyses revealed GS\textit{H}1, GP\textit{X}1, CT\textit{T}1 and MX\textit{R}1 that encode well-established antioxidant defense enzymes to be downregulated in the \textit{hmx1} null mutant compared with wild type cells. Fourthly, over-expression of each of the downregulated antioxidant genes partially rescues oxidant sensitivity of the \textit{hmx1} null mutant, while over-expression of all four downregulated antioxidant genes provides complete protection to the null mutant. Furthermore, the levels of \textit{H MX1} transcript mirrored (at least in the case of total glutathione, catalase and glutathione peroxidase) the antioxidant activities of the genes downregulated in the \textit{hmx1} null mutant. Therefore, changes to cellular glutathione and GSH-related antioxidant activities are likely key mechanisms by which Hmx1p protects cells against oxidants. This interpretation is consistent with the fact that in human cells one general mechanism of HO-1 induction is via modulation of cellular glutathione status (34).

It is now well established that HO-1 protects mammals against oxidative stress. For example, mice deficient in HO-1 have increased susceptibility to oxidative stress (35), and their cells are less capable to withstand an oxidative challenge than the corresponding wild type cells (36), while HO-1 over-expression increases cellular resistance to oxidants (37). However, the mechanism underlying the antioxidant protection provided by HO-1 is not well understood. Early studies ascribed the antioxidant action to HO-1’s ability to simultaneously decrease the concentrations of the pro-oxidant heme and increase the levels of the antioxidant bilirubin (38). Bilirubin is an efficient oxidant scavenger \textit{in vitro} (39), and when added at micromolar concentrations both bilirubin and the HO-1 substrate hemin protect cells against oxidants (40). However, there is little direct evidence that bilirubin produced from endogenous heme as a consequence of increased HO-1 activity acts as a cellular antioxidant (41), and the amounts and sources of cellular heme available for degradation by HO-1 remain unknown.
Indeed, addition of the products of heme oxygenase, biliverdin and CO, had no measurable effect on the sensitivity of the \textit{hmx1} null mutant to H$_2$O$_2$ challenge (Fig. 9). Together, our results indicate that the antioxidant protection afforded by the yeast homolog of HO-1 is via an adaptive response that involves the transcriptional control of antioxidant genes, rather than directly via its oxygenase activity (Fig. 11). Transcriptional regulation by Hmx1p was observed with millimolar concentrations of H$_2$O$_2$, raising the question of physiological relevance. However, ‘extrapolating’ oxidant concentrations from yeast to mammalian cells is complicated because of several species differences, including the oxidant resistance of the cell wall compared with plasma membrane (42), and redundancies in H$_2$O$_2$ metabolism (29).

In addition to catalyzing heme degradation and providing antioxidant protection, it is increasingly appreciated that HO-1 participates in the regulation of many biological processes, including inflammation, cell growth, vascular tone and angiogenesis (4) that can translate into protection against various diseases (7, 43, 44). This raises the intriguing question of how HO-1 achieves these various activities. Using a global microarray analysis approach and yeast as a model, our studies revealed an unexpectedly large number of heme oxygenase-dependent, differentially expressed genes, the products of which are involved in several previously unrecognized processes, such as RNA processing, ribosome biogenesis, transcriptional regulation and membrane transport (Fig. 4). In fact, of the differentially expressed genes, only a small number, corresponding to \textasciitilde1\%, relate to antioxidant defense. This indicates that at least in yeast, antioxidant protection may represent a relatively minor function of heme oxygenase, and that the enzyme likely participates in many presently unappreciated processes. For example, consistent with a regulatory role of HO-1 in the growth of mammalian cells, loss of \textit{HMX1} increased the growth of yeast cells, and this was abrogated by over-expression of \textit{HMX1} (not shown). This may relate to the differentially expressed genes involved in RNA processing and ribosome biogenesis (TABLES S3 and S4). The downregulation of transcripts encoding proteins involved in sulphur metabolism has been linked to increased oxidative stress (45) and hence may help explain the oxidant sensitivity of the \textit{hmx1} mutant. Likewise, mammalian HO-1 has been reported to affect the activity of transcription factors (32), so that the downregulation of transcription factors may help explain the inability of the \textit{hmx1} mutant to mount an adequate response to oxidative stress.

Our microarray analyses also raise the question of how an ER protein can participate in transcriptional regulation. One possibility is that ‘diffusible’ product(s) of heme oxygenase activity are involved. Indeed, a recent report suggested that in cardiomyocytes HO-1-derived CO regulates mitochondrial biogenesis via transcriptional regulation of nuclear respiratory factor-1 (46). Inconsistent with this notion, however, transfection of cells with a mutant HO-1 that lacks enzymatic activity still increased cellular resistance to H$_2$O$_2$ (37), and we observed no protective effect of CO on the sensitivity of the \textit{hmx1} null mutant to H$_2$O$_2$ challenge (Fig. 9). We note however that our experimental design may not have adequately reflected local production of biliverdin/CO, perhaps close to or within the nucleus. An alternative explanation for the ability of HO-1 to regulate gene transcription has been provided by Dennery and colleagues (32) who reported that a C-terminus truncated form of HO-1 migrates to the nucleus in response to hypoxia, hemin and heme-hemopexin. We observed that in oxidant stressed yeast cells, Hmx1p localized to the (peri)nuclear region where it could conceivably participate in the transcriptional regulation. We observed greater (peri)nuclear localization with N-terminus than C-terminus tagged Hmx1p, suggesting that translocation to the perinuclear region may precede cleavage. However, our results do not unambiguously establish nuclear localization or cleavage of Hmx1p. Indeed, integral ER proteins can enter the nucleus without a need for proteolytic cleavage (33). Clearly, additional studies are required to elucidate the mechanism by which Hmx1p affects transcriptional regulation.

In summary, our data show that in yeast HO-1 homolog provides antioxidant protection to cells indirectly via the differential expression and activities of several known antioxidant enzymes. Our findings challenge the paradigm that the cellular/biological effects of HO-1 are
explained solely by its enzymatic activity. Instead, they suggest that at least some of HO-1’s cellular/biological effects are the result of an adaptive response by the cells (Fig. 11). Clearly, \textit{HMX1} regulates the expression of a large number of genes involved in numerous functions unrelated to heme oxygenase or antioxidant activities. Elucidating the relationship of heme oxygenase with these differentially expressed genes will likely unravel a multitude of novel functions of HO-1.

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FOOTNOTES

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The abbreviations used are: CTT1, gene encoding cytosolic catalase; GPX1, gene encoding a glutathione peroxidase that acts on phospholipid hydroperoxides/non-phospholipid and other organic peroxides; GPX2, gene encoding an atypical 2-Cys peroxiredoxin; GSH1, gene encoding γ-glutamylcysteine synthetase; GSSG, oxidized glutathione; HA-HMX1p, N-terminus HA-tagged Hmx1p; Hmx1p, yeast heme oxygenase protein; HMX1, gene encoding yeast heme oxygenase; HMX1-HAp, C-terminus tagged Hmx1p; HO-1, heme oxygenase-1; HO-2, heme oxygenase-2; MXR1, gene encoding peptide methionine sulfoxide reductase; GSH, reduced glutathione; H2O2, hydrogen peroxide
FIGURE LEGENDS

Fig. 1. Hmx1p is induced in response to oxidative stress.  A) Cells grown to exponential phase in iron-replete (1) or iron-deprived (2, positive control) conditions, or in iron-replete condition for 6 h in the presence of 3 mM H$_2$O$_2$ (3), 3 mM diamide (4) or 10 mM menadione (5) were lysed, and the expression of Hmx1-HAp assessed by Western blotting relative to that of tubulin.  B, C) Cells grown to exponential phase in iron-replete conditions were treated with (B) 4 mM H$_2$O$_2$ for the indicated time, or (C) the indicated H$_2$O$_2$ concentration for 6 h.  Following oxidant exposure, cells were lysed and the expression of Hmx1-HAp and tubulin assessed by Western blotting.  A-C) The upper panels show results typical of three separate experiments.  The lower panels show quantitative data expressed as mean ± SEM from the three separate experiments for each of the three conditions, with the respective density ratios of Hmx1p to tubulin determined using Quantity One, and with the respective ratio for (A) iron-replete non-stressed cells, (B) time 0, and (C) 0 H$_2$O$_2$ set at 1.

Fig. 2. Heterogenous expression of Hmx1p in response to stress.  A) Confocal fluorescence images of yeast cells expressing Hmx1-HAp (top) or HA-Hmx1p (bottom) and stained with anti-HA Alexa 488 (green) and DAPI (red).  Cells were grown at 30 °C in synthetic medium under iron replete (control, left panel) or iron deplete (middle panel) conditions, or treated for 6 h with 4 mM H$_2$O$_2$ (right panel) before being prepared for immunofluorescence microscopy.  For each experimental setting several Z-stacks were collected at Z increments of 0.5 µm.  Arrows denote control cells that display HA expression at low levels.  Boxes show enlargements of selected cells.  Scale bars 10 µm.  B) Hmx1 expression is increased upon iron deprivation or H$_2$O$_2$ treatment.  Quantification of HA expression of cultures represented in (A).  Following 3D image stack acquisition, the collected composite files of the z-stacks were analyzed and orthogonal planes were projected with the Image J software.  Cells were counted that displayed notable staining with the anti HA Alexa 488 antibody.  Quantification of fluorescence images represents data (mean ± SEM) from a single experiment with 3-8 independent z-stacks per treatment taken with identical settings.  C) Images of HA-tagged control cells.  Parallel control cultures not expressing any HA-tagged constructs were prepared and microscopy performed with identical imaging settings as in (A).  Transmitted light (TL), anti-HA Alexa 488 (green) and DAPI (red) images are shown.

Fig. 3. Loss of HMX1 renders the cell sensitive to H$_2$O$_2$, whereas over-expression of HMX1, HMX1-HA or human HO-1 rescues the hmx1 sensitivity to oxidant challenge.  A) Wild type (●) and hmx1 mutant strains (○) were grown to exponential phase in SD medium and treated for 1 h with H$_2$O$_2$ at the concentration indicated.  Following treatment, cells were diluted and plated in triplicate onto YEPD medium to assess cell viability.  B-D) Wild type and hmx1 mutant strains were transformed with a galactose-inducible multi-copy plasmid containing HMX1 (B, triangles), HMX1-HA (C, diamonds), or the human HO-1 gene (D, squares).  Cells were grown to exponential phase in raffinose- (non-induced, open symbols) or galactose-containing medium (induced, closed symbols) before treatment with H$_2$O$_2$.  For human HO-1 over-expression (D), the hmx1 mutant strain was used only.  Survival is expressed as percentage of that seen with untreated control cells.  Results show mean ± SEM of a single experiment performed in triplicate, with standard error bars smaller than the symbols.  *P<0.05 compared with wild type (A) or non-induced conditions (B-D) (two-ANOVA with Bonferroni correction).

Fig. 4. Functional categories of differentially expressed genes in hmx1 deletion mutant compared to wild type cells.  Three separate cultures of each wild type and hmx1 mutant strains were grown to exponential phase, their RNA extracted and whole changes in the transcriptome analyzed using Affymetrix whole yeast genome microarrays (YG-S98), as described in Materials and Methods.  A) Genes up or downregulated in hmx1 cells were sorted into groups according to the Gene Ontology Term Finder, provided by the Saccharomyces genome database (www.yeastgenome.org), with all data shown in Supplemental Tables S3 & S4.  B) Antioxidant genes differentially expressed in hmx1 cells.
Deletion of \textit{HMX1} or over-expression of \textit{HMX1} affects the expression of antioxidant genes. Wild type (empty bars) and \textit{hmx1} mutant strains (grey bars) without (no stripes) and with over-expression of the multi-copy plasmid containing \textit{HMX1} (striped) were grown to exponential phase, the RNA extracted, and cDNA generated. The resulting cDNA was then probed for \textit{GSH1}, \textit{GPX1}, \textit{CTT1}, \textit{MXR1}, \textit{GPX2} and \textit{ACT1} as described in Materials and Methods, and antioxidant gene expression shown relative to that of \textit{ACT1}. Results represent mean ± SEM of three separate experiments. *P<0.05 compared with the corresponding \textit{HMX1} over-expressing strain (one-way ANOVA with Bonferroni correction).

**Over-expression of \textit{GPX1}, \textit{CTT1}, \textit{GSH1} or \textit{MXR1} rescues oxidant sensitivity of \textit{hmx1} deletion mutant.** Wild type and \textit{hmx1} mutant strains were transformed with a galactose-inducible multi-copy plasmid containing \textit{GPX1} (A), \textit{GSH1} (B), \textit{CTT1} (C), or \textit{MXR1} (D). Cells were grown to exponential phase in raffinose- (non-induced, open bars) or galactose-containing medium (induced, closed bars) and treated for 1 h with 4 mM diamide, 4 mM \textit{H2O2}, or 15 mM menadione. Survival is expressed as percentage of that seen with control cells. Results shown are mean ± SEM of a single experiment performed in triplicate. *P<0.05 compared with the corresponding non-induced strain (one-way ANOVA with Bonferroni correction).

**Simultaneous over-expression of \textit{GPX1}, \textit{CTT1}, \textit{GSH1} and \textit{MXR1} completely rescues oxidant sensitivity of \textit{hmx1} deletion mutant.** Wild type and \textit{hmx1} mutant strains were transformed with 4 galactose-inducible multi-copy plasmids containing \textit{GSH1}, \textit{GPX1}, \textit{CTT1} and \textit{MXR1}. Cells were grown to exponential phase in raffinose- (O) or galactose-containing medium (●) and treated for 1 h with \textit{H2O2} at the indicated concentration. Survival is expressed as percentage of that seen with control cells. Results shown are mean ± SEM of a single experiment performed in triplicate, with standard error bars smaller than the symbols. *P<0.05 compared with the corresponding non-induced strain (two-way ANOVA with Bonferroni correction). There is no significant difference between wild type and \textit{hmx1} mutant strains with all four antioxidant genes induced.

**Altering the levels of antioxidants alters the activity of some but not all antioxidants.** \textit{hmx1} mutant strains were transformed with a galactose-inducible multi-copy plasmid containing \textit{GSH1} (1), \textit{GPX1} (2), \textit{CTT1} (3), or \textit{MXR1} (4). Cells were grown to exponential phase in raffinose- (non-induced, open bars) or galactose-containing medium (induced, closed bars) before the activity of (A) glutathione peroxidase or (B) catalase, and (C) total glutathione was determined as described in the Materials and Methods sections. Results shown represent mean ± SEM of three separate experiments. *P<0.05 compared with the corresponding wild type strain (one-way ANOVA with Bonferroni correction). There were no differences between non-induced \textit{hmx1} mutant and (A) the \textit{hmx1} mutant with \textit{CTT1} or \textit{MXR1} induced, (B) \textit{GSH1}, \textit{GPX1} or \textit{MXR1} induced, and (C) \textit{GPX1}, \textit{CTT1} or \textit{MXR1} induced.

**Addition of biliverdin or CORM3 fails to rescue \textit{hmx1} oxidant sensitivity.** Wild type (●) and \textit{hmx1} (O) mutant strains were grown to exponential phase in SD medium and treated with 4 mM \textit{H2O2} for 1 h in the absence and presence of the indicated concentration of biliverdin (A), CORM3 (B) or both (C). Following treatment, cells were diluted and plated in triplicate onto YEPD medium to assess cell viability. Results shown represent mean ± SEM of three separate experiments. Where SEM bars cannot be seen, they are smaller than the symbol size.

**Heme oxygenase-1 translocates to the (peri)nuclear region in response to \textit{H2O2}.** A) Confocal microscopy images of HA-tagged Hmx1p cells treated with 4 mM \textit{H2O2} for 6 h and then stained as described in Experimental Procedures. Arrows denote areas (white) that show staining with all three markers, DAPI (blue), anti-HA (green) and ER marker DiOC6(3) (red), indicating (peri)nuclear localization of Hmx1p. Scale bars 10 or 5 µm, as indicated. B) Quantification of the experiments shown in A). The extent of (peri)nuclear localization of Hmx1-HAp (open bars) and HA-Hmx1p (closed bars) in \textit{H2O2}-treated cells was compared by counting cells that displayed distinctive (peri)nuclear staining, as assessed by acquisition and analysis of z-stacks taken of >1,000 cells for each of two separate experiments. C) Cells were treated as in (A), followed by cell
fractionation into cytosolic (C) and nuclear fraction (N), and Western blotting using antibodies
directed against HA (Hmx1p), Pgk1 (cytosol), Nop2 (nucleus), Dpm1p (ER) and Kar2 (ER). The
results shown are representative of three separate experiments. D) Quantification of the data obtained
in (C). The extent of (peri)nuclear/ER-localized Hmx1-HAp (open bars) and HA-Hmx1p (closed
bars) in cells treated with H2O2 (4 mM, 6 h) was assessed by determining the respective density ratios
of Hmx1p to either Nop2, Dpm1 or Kar2 from three separate experiments using ImageJ, with the
value for the respective ratio for Hmx1-HAp set at 1. *P<0.05 compared with corresponding Hmx1-
HAp (Wilcoxon Rank Sum Test).

Fig. 11. Proposed new paradigm for cellular and biological functions of HO-1. A) Conventional
view: cellular/biological effects of HO-1 result from its catalytic activity, i.e., the degradation of heme
to CO, biliverdin/bilirubin (BV/BR) and Fe2+. B) Proposed new paradigm: cellular/biological effects
of HO-1 result from an adaptive response of cells to HO-1, independent (broken line) and/or
dependent on HO-1 enzymatic activity (solid line).
**TABLE 1. Glutathione content and activities of antioxidant enzymes the genes of which are differently expressed in wild type and hmx1 mutant strains**

| Strain          | Total glutathione (µmol/min/mgp) | Catalase (µmol/min/mgp) | GSH peroxidase (µmol/min/mgp) | Methionine sulfoxide reductase (nmol/min/mgp) |
|-----------------|----------------------------------|-------------------------|-------------------------------|-----------------------------------------------|
| wt              | 1.0 ± 0.1                         | 87.0 ± 6.3              | 13.8 ± 1.1                    | ND                                            |
| wt HMX1 non-induced | 1.2 ± 0.1                         | 95.5 ± 1.8              | 14.7 ± 0.4                    | ND                                            |
| wt HMX1 induced | 5.7 ± 0.4*                        | 368.6 ± 15.9*           | 37.2 ± 3.3*                   | ND                                            |
| hmx1            | 0.2 ± 0.1*                        | 27.6 ± 2.4*             | 12.1 ± 0.3                    | ND                                            |
| hmx1 HMX1 non-induced | 0.2 ± 0.1*                      | 29.1 ± 1.3*             | 10.1 ± 0.7*                   | ND                                            |
| hmx1 HMX1 induced | 1.7 ± 0.1*                        | 214.4 ± 4.8*            | 18.4 ± 1.0                    | ND                                            |
| wt MXR1 induced | NT                               | NT                      | NT                            | 1.3 ± 0.2                                     |

Cells transformed with a galactose-inducible multi-copy plasmid containing HMX1 or MXR1 were grown to exponential phase in raffinose- (non-induced) or galactose-containing medium (induced), lysed and total glutathione and antioxidant enzyme activities determined as described in Experimental Procedures. Results show mean ± SEM of three separate experiments. *P<0.05 compared with corresponding data from wild type strain (one-way ANOVA with Bonferroni correction). ND, not detectable (detection limit, 0.1 nmol/min/mgp); NT, not tested.
Figure 1
Figure 2

A

Hmx1-HAp

Control

Iron deplete

H$_2$O$_2$

Anti-HA

DAPI

merge

B

Cells expressing Hmx1p (%)

20

15

10

5

0

Hmx1-HAp

HA-Hmx1p

Ctrl

Iron

H$_2$O$_2$
deplete

Ctrl

Iron

H$_2$O$_2$
deplete

C

No HA tag control

Anti-HA

DAPI

merge

TL
Figure 3
Figure 4

A

| P value | GO:0006950 | Response to stress |
|---------|-------------|--------------------|
| 0.003   | NS          | Sulphur metabolic process |
| 10^{-13} | NS          | RNA Processing |
| NS      | 10^{-3}     | Ribosome biogenesis |
| NS      | NS          | Protein localization in mitochondrion |
| NS      | NS          | Protein targeting to mitochondrion |
| 0.03    | NS          | Mitochondrion component |
| 0.02    | NS          | Transcription factor complex |
| 0.02    | NS          | Transcription factor activity |
| 0.02    | NS          | Antioxidant activity |
| 0.02    | NS          | Transmembrane transporter activity |

B

| Fold | MXR1 | CTT1 | GPX2 | GPX1 | GSH1 |
|------|------|------|------|------|------|
| 6    | 20   | 10   | 0    | 0    | 0    |
| 4    | 0    | 0    | 0    | 0    | 0    |
| 2    | 0    | 0    | 0    | 0    | 0    |
| 0    | 0    | 0    | 0    | 0    | 0    |

Figure 4

A

| Fold | Down-regulation | Up-regulation |
|------|----------------|---------------|
| 6    | 20             | 0             |
| 4    | 10             | 0             |
| 2    | 0              | 0             |
| 0    | 0              | 0             |

B

| Fold | MXR1 | CTT1 | GPX2 | GPX1 | GSH1 |
|------|------|------|------|------|------|
| 6    | 20   | 10   | 0    | 0    | 0    |
| 4    | 0    | 0    | 0    | 0    | 0    |
| 2    | 0    | 0    | 0    | 0    | 0    |
| 0    | 0    | 0    | 0    | 0    | 0    |
Figure 5
Figure 6
Figure 7

Survival (%) vs. H$_2$O$_2$ (mM) for WT and hmx1 strains.
Figure 8

**A**
- GPx Activity (µmol/min/mgp)
  - WT
  - hmx1

**B**
- Catalase Activity (µmol/min/mgp)
  - WT
  - hmx1

**C**
- Total glutathione (µmol/min/mgp)
  - WT
  - hmx1

Note: The bars with asterisks (*) indicate statistical significance.
Figure 9
Figure 10

A

### Anti-HA

- Hmx1-HAp
- HA-Hmx1p

DiOC6

- 10 µm
- 5 µm

DAPI

- 10 µm
- 5 µm

Merge

### (Peri)nuclear HA staining (% cells)

- Exp 1
- Exp 2

B

### Ctrl

- Hmx1-HAp
- HA-Hmx1p

C

### Ctrl

- Hmx1-HAp
- HA-Hmx1p

### H2O2

- Hmx1-HAp
- HA-Hmx1p

### Fold change

- Nop2
- Dpm1
- Kar2

### Fold change

- 3
- 2
- 1
- 0

D

### *
Figure 11
The yeast homolog of heme oxygenase-1 affords cellular antioxidant protection via the transcriptional regulation of known antioxidant genes

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