Heme Oxygenase in Candida albicans Is Regulated by Hemoglobin and Is Necessary for Metabolism of Exogenous Heme and Hemoglobin to α-Biliverdin* 

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Candida albicans is an opportunistic pathogen that has adapted uniquely to life in mammalian hosts. One of the host factors recognized by this yeast is hemoglobin, which binds to a specific cell surface receptor. In addition to its regulating the expression of adhesion receptors on the yeast, we have found that hemoglobin induces the expression of a C. albicans heme oxygenase (CaHmx1p). Hemoglobin transcriptionally induces the CaHMX1 gene independent of the presence of inorganic iron in the medium. A Renilla luciferase reporter driven by the CaHMX1 promoter demonstrated rapid activation of transcription by hemoglobin and (cobalt protoporphyrin IX) globin but not by apoglobin or other proteins. In contrast, iron deficiency or exogenous hemin did not activate the reporter until after 3 h, suggesting that induction of the promoter by hemoglobin is mediated by receptor signaling rather than heme or iron flux into the cell. As observed following disruption of the Saccharomyces cerevisiae ortholog, HMX1, a CaHMX1 null mutant was unable to grow under iron restriction. This suggests a role for CaHmx1p in inorganic iron acquisition. CaHMX1 encodes a functional heme oxygenase. Exogenous heme or hemoglobin is exclusively metabolized to α-biliverdin. CaHMX1 is required for utilization of these exogenous substrates, indicating that C. albicans heme oxygenase confers a nutritional advantage for growth in mammalian hosts. 

Candida albicans is an opportunistic pathogen that has adapted uniquely to its niche in the human host (1). It is a commensal organism in the normal gastrointestinal flora but becomes pathogenic following immunosuppressive chemotherapies for cancer, organ transplantation, and in AIDS patients (2). The switch from commensal colonization to invasive infection requires the exchange of specific signals between the pathogen and its host to allow survival and growth and to promote invasion of specific host tissues (1). 

We have identified hemoglobin (Hb) as one such host signal, based on the observation that Hb is a specific inducer of a high affinity fibronectin receptor (3, 4). This induction was specific for Hb in that other host proteins or ferriproteins were inactive. Intact Hb was required for this activity because globin or hemin did not induce the fibronectin receptor (3). However, substitution of CoPPIX* for the heme in globin restored activity, but coordination of CO, CN, and O2 as heme-axial ligands did not affect the activity of Hb (3). Hb bound saturably to the surface of Candida cells, which could be quantitatively inhibited by the Hb-binding protein haptoglobin (3). Signaling through the Hb receptor was independent of cellular iron status, because the fibronectin receptor was induced under conditions of iron sufficiency and preceded any detectable uptake of radioactive iron from Hb (3). Together these data indicate that, although heme iron can be utilized by the fungus after prolonged culture (5), Hb signaling through the cell surface Hb receptor is rapid and independent of iron acquisition from the protein. 

Because sensing of Hb may help the cells to recognize specific host tissue compartments, we examined gene regulation by Hb to gain insight into which fungal cellular functions depend upon this signaling pathway. We used a differential display to identify genes specifically regulated by Hb but not by inorganic iron. This analysis identified a C. albicans heme oxygenase gene (CaHMX1) that was shown recently to be regulated by iron and necessary for the organism to survive with heme as the sole iron source (5). 

Mammalian heme oxygenases are essential for normal heme protein turnover in the body and are directly responsible for recycling of Hb iron from normal turnover of senescent red cells or release because of trauma (6). Heme oxygenase catalyzes the oxidative cleavage of the α meso-edge of heme (Fig. 1). The reaction utilizes NADPH-reducing equivalents and a reductase to yield the open chain tetrapyrrrole α-biliverdin, CO, and iron (7–9). Both CO and α-biliverdin have cytoprotective activities (10, 11). 

Heme oxygenases have also been identified in plants and several bacteria including Corynebacterium diphterithica (HnuO) (12) and Neisseria meningitidis (Hemo) (13). Null mutants of bacterial heme oxygenase (12, 13) as well as the CaHMX1 of C. albicans (5) have shown that a major role for microbial heme oxygenase is in the release of nutritional iron from heme and heme-protein complexes. Saccharomyces cerevisiae also expresses a protein containing the heme oxygenase protein signature (14, 15), but direct enzymatic activity could not be demonstrated (16). However, HMX1 is transcribed under conditions of iron deprivation, and its deletion leads to defects in iron accumulation and an increase in the intracellular heme pool (15). 

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1 The abbreviations used are: CoPPIX, cobalt protoporphyrin IX; EST, expressed sequence tag; FAS, ferrous ammonium sulfate; ORF, open reading frame; YNB, yeast nitrogen base; HPLC, high pressure liquid chromatography; contig, group of overlapping clones.
Hemoglobin Regulates Heme Oxygenase in C. albicans

FIG. 1. Chemical structures of heme and biliverdin. Greek letters indicate the four meso-edge positions susceptible to oxidative modifications. Heme oxygenases cleave at the α meso-edge of heme to form α-biliverdin, whereas chemical oxidations of heme yield all four isomers. V, vinyl; Pr, propionic acid.

In this paper we demonstrate the transcriptional regulation of CaHMX1 by mammalian Hb and show that this activation is iron-independent. CaHMX1 activation occurs rapidly following exposure to Hb and is additive with activation by iron deficiency. We additionally show that the CaHMX1 gene encodes a functional heme oxygenase enzyme and that the product of the reaction is α-biliverdin.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Conditions—Cell culture was conducted in a defined medium, yeast nitrogen base (YNB) with appropriate amino acid supplements (17). Iron-sufficient and -deficient media were supplied with or without 1.2 μM FeCl3, respectively (Q-Biogene, Carlsbad, CA). Cells were cultured at 30 °C by shaking at 250 rpm with appropriate additions as required. Bovine methemoglobin, FAS, ferrozine, fetuin, holotransferrin, apotransferrin, and casein were obtained from Sigma. Preparation and isolation of human Hb, CoPPIX-globin, and human apoglobin were described previously (3). Hemin and biliverdin standards were obtained from Frontier Scientific Porphyrin Products (Logan, UT). Biliverdin was quantified in acidic MeOH at 377 nm using ε = 66.2 μmol−1 cm−1 (18). Hemin and HB quantification have been described previously (3). C. albicans strain YJB6284 (19) is a prototrophic version of the parental strain YJB6284 (20) in which pyr4 was introduced as an episome (data not shown). The resulting strain, YJB6284-pyr4, was used for all reporter assays in this study. The correct promoter region, was confirmed by Northern hybridization using DNA from each EST clone as a radiolabeled probe.

Luciferase Assays—Reporter assays used C. albicans cells that were grown at 30 °C in minimal YNB medium with ammonium sulfate and 2% glucose. 5 × 10⁶ cells were harvested, cell extracts were obtained by glass bead lysis (22), and luminescence was determined using coelenterazine as a substrate (Promega). Luciferase activity is defined as the number of light units using 10⁷ cells in the absence of heme oxygenase and this buffer using a 5-μl volume for light measurement. To assess regulation of the promoter by iron, the ferrous iron chelator ferrozine was added to cell cultures in the presence of FAS to buffer iron concentrations as described (23) (also see Ref. 24). FAS added to 1 mM ferrozine at 10, 100, and 400 μM represents low, optimal, and high levels of iron, respectively. The effect of proteins other than Hb on the induction of the CaHMX1 promoter were tested using Ki-29 cells cultured for 15 min in iron-replete media containing each of the following at 1 mg/ml: Hb, CoPPIX-globin, holotransferrin, apotransferrin, fetuin, and casein.

Molecular Biology Techniques—Total yeast RNA was prepared using the acid-phenol method (25). Yeast transformations were carried out by the lithium acetate technique (26). Northern and Southern analyses, DNA manipulations, and sequence analysis used standard methods (27). Differentially expressed genes were identified by RNA primed PCR following the recommendations of the manufacturer (Stratagene, La Jolla, CA) using RNA from C. albicans strain ATCC 44897 cells grown with or without 1 mg/ml Hb. Briefly, C. albicans cells were incubated into YNB broth with or without 62 μM (expressed as iron equivalents) hemoglobin or ferrous sulfate and grown at 26 °C for 24 h. Under these growth conditions, no germination was found upon microscopic examination. For induction of the hyphal form of C. albicans, cells grown in YNB were resuspended into RPMI 1640 supplemented with 2 mM glutamine in the absence of heme oxygenase and incubated 24 h at 37 °C with shaking at 250 rpm. A 1-h incubation converted nearly 100% of candidal cells to hyphae or pseudohyphae by microscopic examination. First strand cDNA was synthesized using the arbitrary primers 5′-AACCTGAGCTCTCCTCTC-3′, 5′-AATCTAGAGCTCTCCTC-3′, 5′-ATCTAGAATCTGGCAAG-3′, and 5′-CACACGCCCAGGGA-3′. Differentially expressed products were analyzed by standard procedures (27). A total of 35 ESTs that expressed increased expression in Hb cultures, but not when supplemented with an equivalent molar concentration of iron, were cloned and sequenced. Seven were ESTs from carboxypeptidase Y; 13 were multiple hits of four genes, and the remainder were from discrete ORFs. Differential expression of the genes induced by Hb was confirmed by Northern hybridization using DNA from each EST clone as a radiolabeled probe.

Heme Oxygenase Assay—Coupled oxidation of human Hb to generate biliverdin α and β isomers followed the methods of O’Carra and Colleran (28). Briefly, 50 mg of human Hb was made to 5 mg/ml in 0.1 mM sodium phosphate buffer, pH 7.0, and incubated with 20 mg of sodium ascorbate for 2 h at 37 °C with vigorous agitation. The sample was extracted twice with equal volumes of anhydrous ethyl ether to remove unreacted heme. Biliverdin in the aqueous phase was extracted with CHCl3 and concentrated by evaporation under a stream of nitrogen at room temperature. The blue-green residue was dissolved in MeOH and made to 60% with aqueous 0.1 mM ammonium acetate (pH 7.5) in preparation for HPLC analysis. Coupled oxidation was confirmed by NMR analysis of the biliverdin isomers followed the methods of O’Carra and Colleran (28). Hemin (29) (Hem) (5 mg/ml) was made to 50% with 5 mM sodium phosphate buffer, pH 7.0, with 10 mg of ascorbate and incubated for 16 h at 37 °C. The mixture was then acidified with HCl and the biliverdin isomers were extracted twice into CHCl3 and concentrated by evaporation under nitrogen. The compounds were further purified on a C-18 Sep-Pak column (Millipore, Bedford, MA) as described below.

Biliverdin was extracted from cell pellets by suspension in an equal volume of MeOH, vortexing for 30 s, and centrifuging for 10 min at 5000 × g at room temperature. The supernatant of this extraction was mixed with 50% with aqueous 0.1 mM ammonium acetate, pH 7.5, and 5 ml of MeOH, 5 ml of H2O, and 15 ml of Buffer A (60% 0.1 mM ammonium acetate, pH 5.2, 40% MeOH v/v). The column was then washed with 5 ml of 0.1 mM ammonium acetate, pH 5.2, 5 ml of Buffer A,
Identification of CaHMX1 and Its Regulation by Hemoglobin—Genes specifically induced by Hb but not by equivalent molar concentrations of inorganic iron were identified using random arbitrarily primed PCR (Fig. 2A). RNA isolated from cells cultured for 24 h at 24 °C in the presence or absence of Hb identified a total of 33 ESTs that exhibited increased expression in Hb cultures but not when supplemented with iron. One such EST (Fig. 2A, arrow), which was verified by Northern hybridization to be induced by Hb and not by iron or hyphal differentiation (Fig. 2B), overlapped the 3′-end of the CaHMX1 gene in the C. albicans genomic data base (Fig. 2D).

CaHMX1 had been identified by Santos et al. (5) as an iron- and hemin-regulated gene that is essential for survival with hemin as the sole iron source. We therefore characterized CaHMX1 by measuring its steady state mRNA in the presence and absence of Hb under iron sufficiency to verify the results obtained through RNA arbitrarily primed PCR screening. C. albicans SC5314 cells (30) in early stationary phase growth were transferred to Hb-containing medium, and samples were harvested for RNA isolation at the indicated times (Fig. 2C). An increased mRNA level was evident as early as 30 min after Hb addition, and the level increased 10–15-fold at 3 h (Fig. 2C). These data indicate that accumulation of CaHMX1 mRNA is increased by hemoglobin under iron sufficiency.

CaHMX1 Transcription Is Regulated by Hemoglobin as well as Iron Deficiency—Accumulation of CaHMX1 mRNA within 30 min following Hb addition suggested transcriptional regulation (Fig. 2C). To measure active transcription during the early stages of Hb exposure, we constructed a luciferase reporter driven by the CaHMX1 promoter. A 1.4-kb region upstream of the CaHMX1-predicted translational start site (Fig. 2D) was cloned in the Renilla luciferase reporter plasmid pCRW3 (22). This region contained four HAP1 consensus sites (31) as well as a single predicted iron-responsive element (32) (Fig. 2D, Fe RE). The plasmid was initially introduced into the C. albicans strain Red 3/6 (22) by recombination into the neutral Ade2 locus. However, expression from this construct could not be detected (data not shown). We therefore recombined the reporter plasmid into the genome CaHMX1 to generate the knock-in strain CAMP Ki-29 (see “Experimental Procedures”). In this strain, the entire genomic region upstream of the CaHMX1 ATG could serve to supply promoter elements for the introduced Renilla luciferase (Fig. 2D and data not shown). Similar knock-ins have been used successfully in C. albicans (33).

Strain CAMP Ki-29 was first tested for responsiveness to Hb in iron-sufficient medium. Within 2.5 min following the addition of Hb, luciferase activity increased more than 10-fold over the non-induced control, and this level was sustained almost to the end of the test period (Fig. 3A). These data indicate that Hb binding to its cell surface receptor (3) induces a signal that rapidly increases transcription to the CaHMX1 promoter under iron-replete conditions.

To ensure that this induction is specific for Hb, we tested several control proteins for their ability to stimulate luciferase activity in strain Ki-29 (Table I). The cobalt analog of Hb, CoPPIX-globin, had equivalent activity to Hb, but apoglobin was inactive. Therefore, the iron in Hb is not essential for activity, but the native conformation of globin induced by porphyrin binding is required. These results are consistent with our previous report that CoPPIX-globin stimulated expression of a fibronectin receptor in C. albicans to the same extent as Hb, whereas globin was inactive (3). None of the other proteins tested significantly induced luciferase activity above the control, but the ferroprotein transferrin somewhat decreased the basal activity of the CaHMX1 promoter (Table I). These proteins also failed to induce the expression of the fibronectin receptor in C. albicans (4).

Although the iron in Hb is not required to induce CaHMX1 transcription, steady state CaHMX1 mRNA was shown previously to be increased by iron deficiency and by hemin at 5 h post-transfer to new medium (5). We therefore tested promoter
activity over an extended time course using Hb under iron-sufficient and -deficient conditions. Hb rapidly increased promoter activity independent of the iron status of the medium (Fig. 3B, circles). Neither iron deficiency nor hemin addition increased promoter activity at the early time points, but both showed induction after a lag time of 3 h (Fig. 3B). Taken together, Table I and Fig. 3 indicate that the effects of Hb, iron deficiency, and hemin on CaHMX1 transcription are separable events.

CaHMX1 was initially identified as a Hb-regulated gene by RNA arbitrarily primed PCR analysis using RNA isolated 24 h after the addition of Hb (see "Experimental Procedures"). To examine the iron dependence of the Hb response for CaHMX1
the closely related S. cerevisiae protein, His-25 is altered to Ala — a meso-edge hydrophobic contact; arrows, interactions with heme propionate residues at γ meso-edge; inverted open triangles, polar residues involved in ligand discrimination.

at later times, we duplicated these conditions with the CAMP Ki-29 reporter strain (Fig. 4). After cell culture for 24 h, iron-replete conditions maintained the promoter in an inactive state, but the addition of Hb stimulated activity — 40-fold at this time (Fig. 4A). When ferrozine was added to generate iron deficiency, a similar induction of promoter activity was seen (Fig. 4A). However, ferrozine and Hb together produced an additive effect and resulted in transcriptional activity greater than either compound added alone (Fig. 4A). This additivity further indicated that Hb and iron depletion are distinct signals that regulate the CaHMX1 promoter.

The presence of four HAP1 consensus sites (31) 5’ of CaHMX1 (Fig. 2D) suggested that hemin could play a direct role in the regulation of this promoter. However, hemin addition to CAMP Ki-29 cells increased activity only about 2-fold after 24 h, in contrast to the high activity of the combination of Hb and ferrozine (Fig. 4B). Surprisingly, however, the inducing activity of Hb combined with ferrozine was suppressed by the addition of hemin and resulted in only a 2.5-fold increase in activity over the iron-replete control (Fig. 4B). Therefore, hemin is unlikely to be the mediator of CaHMX1 induction by Hb and may be a negative regulator of CaHMX1 under some conditions.

CaHMX1 Is Necessary for Growth under Iron Deficiency — Acquisition of iron is clearly essential for cell survival (34). The interplay of iron and Hb regulation at the CaHMX1 promoter suggested a role for this gene in cellular iron metabolism. The S. cerevisiae ortholog HMX1 plays a role in the mobilization of iron from internal heme stores, indicating a direct connection of HMX1 to iron metabolism (15). To test whether cell growth depended upon CaHMX1 activity, we compared survival of parental and homozygous deletion strains under various levels of iron sufficiency. The deletion mutant grew at an equivalent rate and produced a stationary cell density equivalent to those of the parental strain in iron-replete medium (Fig. 5 and data not shown). Growth of both strains was suppressed in the presence of ferrozine to generate iron deficiency. However, titration of iron into the medium by the addition of FAS (15) permitted growth of the parental strain but not the CaHMX1 deletion mutant under iron restriction (Fig. 5). Addition of 100 μM FAS approximates optimal physiological iron conditions (35). Therefore, a step in iron assimilation that becomes rate-limiting at low iron concentrations requires CaHmx1p.

CaHmx1 Displays a Heme Oxygenase Signature in Its Primary Sequence — The CaHmx1p coding region has 36% identity and 49% similarity to the hypothetical heme oxygenase in S. cerevisiae (Hmx1, data not shown) (15). A comparison to the human isoform-1, for which the crystal structure had been determined, showed 25% identity and 38% similarity. The landmarks comprising a heme oxygenase signature in the primary sequence were highly conserved (Fig. 6). In the heme-binding pocket in heme oxygenases, heme is positioned between the proximal and distal helices (36). The proximal region of the heme-binding pocket, which contains residues that make direct heme contact, was present in the CaHMX1 crystal structure. However, the ability to demonstrate biliverdin synthesis catalyzed by the reconstituted enzyme was not possible (Fig. 6, filled ovals). This particular mutation in the human HO-1 protein renders the enzyme non-functional (37) and may account for an inability to demonstrate biliverdin synthesis catalyzed by the
The mobile phase consisted of 40% MeOH in 0.1M ammonium and chromatographed by HPLC using a C-18 Alltech absorbosphere at 665 nm. Cell extracts were further purified using a C-18 solid support length of absorbance maxima except for the indicated plateau starting filled circles and CAMP 50 (CaHMX1rental) or CAMP 50 (CaHMX1/H9262solid trace) with aeration in the presence of hemin (25/H11002dotted trace) in iron-deficient conditions. Cell pellets were extracted after centrifugation of the culture, the majority of the blue compound remained associated with the cell pellet. Extraction of the cell pellets with MeOH and determination of their visible spectra in acidic MeOH demonstrated an increase in absorbance beyond 550 nm that was maximal at 650–700 nm, which is typical of biliverdin in this solvent (39) (Fig. 7A).

The blue-green pigment was subjected to HPLC analysis to confirm its identity as biliverdin. The HPLC elution profile revealed two peaks for the parental strain extract (Fig. 7B) but only a single peak for the CaHMX1 deletion strain (Fig. 7C). Therefore, CaHMX1 is necessary for the generation of the product in peak I. Under the same growth conditions, no reaction was observed using S. cerevisiae strains S150-2B (40) and YPH 499 (15) (data not shown), indicating the species specificity of the reaction.

Comparison of the elution times of these peaks with commercial biliverdin and hemin standards confirmed that peaks I and V represented biliverdin and hemin, respectively (Fig. 8C). To identify the isomer of biliverdin in peak I, we performed coupled oxidation reactions of heme in pyridine to produce all four isomers (Fig. 8A) and of Hb to generate a mixture of α and β isoamides (28) (Fig. 8B, peaks I and II, respectively). The product isolated from the cells coincided with the biliverdin α meso-isomer peak (compare Fig. 7B with Fig. 8, A and B). The addition of Hb alone to YJB6284 cell cultures in iron-replete medium also generated the α-biliverdin isomer (Fig. 8D). Thus, strain YJB6285 cells can utilize either exogenous heme or Hb to exclusively produce the α isomer of meso-biliverdin. This confirms that C. albicans possesses a true heme oxygenase activity and that the CaHMX1 gene encodes this enzyme.

### DISCUSSION

The α meso-edge of heme is positioned near a hydrophobic wall comprising Met-34, Phe-37, and Phe-214 (36). All are represented in the Candida sequence as substitutions to smaller hydrophobic residues (Fig. 6, filled circles), although Met-34 in human isoform-2 possesses a Val substitution (36). Similar substitutions were found to occur in the functional HmuO protein from C. diptheriae except for Phe-214, which was retained (12). The γ meso-edge of the heme and the heme propionate residues interact with residues of the distal helix. Most all of the participating basic amino acids are represented in the Candida sequence as conservative changes of K22R, K179R, and R183K (Fig. 6, arrows), although a non-conservative substitution occurs at K18N (Gln in S. cerevisiae). Y137 is fully conserved (Fig. 6, arrow).

A highly polar region of the human heme oxygenase pocket involved in ligand discrimination (36) had the following substitutions: N210D (identical in HmuO and Hmx1p), R136M (Leu in Hmx1p), D140L (Leu in Hmx1p), and the conservative substitutions Y58F and Y114P (Fig. 6, inverted open triangles). In all of the regions that define the heme oxygenase signature at the level of the primary sequence, 58% of the C. albicans residues are identical to the human isoform-1, and this increased to 79% when conservative substitutions were included. The 21% of the residues not conserved were primarily located in the polar region of the pocket (Fig. 6).

CaHmx1p Possesses Heme Oxygenase Activity and Generates α-Biliverdin Exclusively—To determine whether CaHmx1p possesses heme oxygenase activity, we generated genomic deletions of both CaHMX1 alleles in C. albicans strain BWP17 (20) to produce a negative control strain. The culture of parental (YJB6284) and null CAMP 50 (CaHMX1/-/-) cells overnight in the presence of Hb and hemin resulted in media with distinct colors. Although the null strain retained the brown-green color of the added heme and Hb, YJB6284 cells generated a distinctly blue-green medium (data not shown). After centrifugation of the culture, the majority of the blue compound remained associated with the cell pellet. Extraction of the cell pellets with MeOH and determination of their visible spectra in acidic MeOH demonstrated an increase in absorbance beyond 550 nm that was maximal at 650–700 nm, which is typical of biliverdin in this solvent (39) (Fig. 7A).

The blue-green pigment was subjected to HPLC analysis to confirm its identity as biliverdin. The HPLC elution profile revealed two peaks for the parental strain extract (Fig. 7B) but only a single peak for the CaHMX1 deletion strain (Fig. 7C). Therefore, CaHMX1 is necessary for the generation of the product in peak I. Under the same growth conditions, no reaction was observed using S. cerevisiae strains S150-2B (40) and YPH 499 (15) (data not shown), indicating the species specificity of the reaction.

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Heme oxygenases have been identified in several microorganisms and are clearly important for scavenging of iron from heme proteins, but this is the first evidence that this gene can be regulated in a pathogen by a specific host protein.

Exclusive production of the α isomer of biliverdin and loss of this activity following disruption of the gene confirms that CaHMX1 encodes a functional heme oxygenase. Regiospecific cleavage of the α bridge is a hallmark of mammalian heme oxygenases (7–9). The mechanism for heme binding to human heme oxygenase has been defined from the crystal structure (36). The heme is orientated with the α meso-edge coordinated with specific internal heme contact residues and the distal γ edge, containing the propionic acids, oriented away from the α cleavage site. The result of this positioning is the exclusive production of the α meso-isomer of biliverdin. The residues that comprise the heme oxygenase signature are all appropriately positioned in the C. albicans enzyme, consistent with the observed isomeric specificity of heme cleavage in C. albicans (Fig. 7).

The existence of heme oxygenase enzymes in microorganisms and are clearly important for scavenging of iron from heme proteins, but this is the first evidence that this gene can be regulated in a pathogen by a specific host protein.

Figure 8. CaHmx1p catalyzes the formation of α-biliverdin from Hb. Biliverdin isomers for standards were generated by coupled oxidation reactions with ascorbate (see “Experimental Procedures”). A, hemin-coupled oxidation products. B, Hb-coupled oxidation products. C, standards, biliverdin (25 nmol) and hemin (12.5 nmol). Peak identification: I, α-biliverdin; II, β-biliverdin; III and IV, a mixture of δ- and γ-biliverdin isomers; V, hemin; γ and δ isomers could not be resolved in this solvent system. D, MeOH extract from strain YJB6284 grown in the presence of Hb (25 μM) in iron-sufficient media for 48 h.
from the local environment, reduce the local redox potential, and reduce levels of reactive oxygen species (11). CO also has potent anti-inflammatory effects on monocytes and macrophages (11), which could be advantageous to fungal survival in a disseminated infection.

The only fungal heme oxygenase ortholog that has been described is the S. cerevisiae Hmx1p (14, 15). A HMX1 deletion alters iron availability from internal heme pools during iron deficiency (15). Because heme is transported very inefficiently in S. cerevisiae (15), Hmx1p activity in this fungus may be limited to the mobilization of internal iron stores. In support of this, Hb cannot be used as an iron source by S. cerevisiae. Hb signaling does not occur in S. cerevisiae (47), and a true heme oxygenase activity could not be demonstrated for Hmx1p (16).

Thus, the regulation of heme oxygenase we identified for Hmx1p in C. albicans is not conserved in a fungus that lives independent of a mammalian host. Interestingly, heme uptake by C. albicans is much more robust (5), suggesting evolution not only in the pathway for heme catabolism but also for acquisition of heme from exogenous heme proteins.

We have identified four potential Hap1p consensus sites in the CaHMX1 promoter (see Fig. 2D). An increase in heme catabolism would be a logical step to increase iron availability under iron deprivation. This would result in an increase in CaHMX1 transcription mediated presumably through a Hap1p-heme complex. However, exogenous hemin was not a robust inducer of transcription either during log phase or early stationary phase growth. Surprisingly, exogenous hemin inhibited CaHMX1 transcription when stimulated by either exogenous Hb or iron deficiency (Fig. 3B). Whether this results from intracellular transport of hemin or from cell surface binding remains to be determined. These results imply that extracellular release of heme does not mediate the observed regulation of CaHMX1 by Hb but also suggest C. albicans has evolved to limit its acquisition of iron from Hb when it is exposed to exogenous hemin. This may prevent accumulation of toxic levels of iron.

CaHMX1 is one of several Hb-regulated genes in C. albicans. Exposure to Hb may be enhanced during invasive infection and may be exacerbated by C. albicans hemolysins (48, 49). Hb signaling may provide information about spatial positioning within the host and proximity to locations where host defenses may be encountered. The response of CaHMX1 transcription is very rapid, suggesting that a rapid signaling pathway is controlled by the as yet undefined Hb receptor. Therefore, CaHmx1p may be a useful target for novel antifungals to regulate growth in the iron-restricted environment of a mammalian host and to limit the ability of C. albicans to survive in specific host microenvironments.

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