Structural Requirements for Hemoglobin To Induce Fibronectin Receptor Expression in *Candida albicans*

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ABSTRACT: Hemoglobin (Hb) is a host factor that induces expression of a promiscuous receptor on *Candida albicans* for fibronectin (FN) and several other extracellular matrix proteins. FN receptor expression was induced by ferric (Hb^+Met and Hb^+CN), ferrous (HbCO and HbO_2), and cobalt−protoporphyrin derivatives of Hb, whereas globin was inactive. The Hb derivatives all exhibited saturable, dose-dependent kinetics of FN receptor induction, suggesting that Hb may be acting as a receptor ligand. Soluble Hb bound saturably to a low-affinity binding site [$K_d = (1.1 \pm 0.2) \times 10^{-6}$ M] on *C. albicans* blastospores. However, uptake of ^{55}FeHb revealed that heme or iron transport into the cell is not required for induction, since internalization of ^{55}Fe from Hb did not occur until after induction of FN binding. The serum Hb-binding protein, haptoglobin, specifically abrogated this response, indicating that protein structure rather than the heme ligand or iron is necessary for induction of this signaling pathway. *C. albicans* also adhered to immobilized Hb, which was sufficient to induce FN receptor expression, and to Hb polymers that formed in defined Hb liquid media in the presence of cells. Formation of Hb polymers in solution required metabolic energy, since the aggregation process was halted with azide addition. Collectively, these data demonstrate that *C. albicans* recognizes polymerized Hb through multivalent low-affinity interactions, and this may be a host environmental cue that triggers extracellular matrix receptor expression at a septic site.

Candida albicans is a normally commensal fungus that can, under certain conditions, rapidly overwhelm local host defenses to establish both mucosal and disseminated infections (*2*). An increase in the incidence of *C. albicans* infection over the past decade has been correlated with an increased number of patients immunocompromised through cancer chemotherapy, organ transplantation, and AIDS (*3*, *4*). These infections range from mucosal overgrowth, as seen in AIDS, to fatal systemic occurrences in severely neutropenic patients due to chemotherapy (*5*, *6*). Although the specific details for mucosal and systemic disease differ, in both cases fungal adherence to host surfaces such as the epithelium, endothelium, or extracellular matrix (ECM)[1] proteins is a prerequisite for colonization (*7*−*11*).

Possessing the flexibility to adhere and colonize in diverse host niches is central to the survival of pathogenic microorganisms (*12*). *C. albicans* is no exception and displays differential patterns of gene expression in response to environmental signals or cues encountered in the body (*2*, *7*, *10*). The most conspicuous of these patterns is filamentous differentiation from the yeast form that allows directed motility through endothelial cell layers and local invasion via expression of secreted aspartyl proteinases (*13*). The specific cues that govern expression of this phenotype are less obvious than adaptations of this organism to temperature (*2*) and pH (*14*). Nonetheless, included in all forms of adaptation are expression of adhesive structures which govern cell and ECM interactions (*15*, *16*).

Hemoglobin (Hb) is a specific host factor that regulates expression of a *C. albicans* fibronectin (FN) receptor (*17*). A single class of FN receptors with a K_d of 4.6×10^{-8} M is induced within several hours in defined media containing 1 mg/mL methemoglobin (Hb^+Met). Hb specifically enhanced binding to both immobilized and soluble FN as well as adhesion to endothelial cells. Induction of FN binding required cell growth in the presence of Hb and induced a specific set of cell surface proteins, one of which recognized FN (*18*). Heme, protoporphyrin IX, myoglobin, globin, bovine serum albumin (BSA), and the addition of iron salts did not increase the level of FN binding above background levels (*17*). These data indicate that Hb is not simply acting as an iron source, although *C. albicans* cells can acquire iron from both heme and Hb (*19*).

Interaction of Hb with the fungal cell surface initiates a signal transduction pathway that ultimately leads to FN receptor expression, but the molecular basis for this signaling is unknown. In this report, we examine the structural requirements for interaction of Hb with the fungal cell surface that are necessary for this process of gene induction.

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[1] Abbreviations: Hb^+Met, methemoglobin; HbN_3, azidomethemoglobin; HbO_2, oxyhemoglobin; HbCO, carbonmonoxyhemoglobin; Hb^+CN, cyanomethemoglobin; FN, fibronectin; FePPIX, iron protoporphyrin IX; CoPPIX, cobalt(III) protoporphyrin IX; cobaltHb, globin reconstituted with CoPPIX; globin, human apohemoglobin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; hemin, ferriprotoporphyrin IX; ECM, extracellular matrix; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; MS, mass spectrometry.

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EXPERIMENTAL PROCEDURES

Reagents. Organic solvents (J. T. Baker, Phillipsburg, NJ) and acids and bases (Mallinckrodt, Paris, KY) were all analytical reagent grade. All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified. Hemin, protoporphyrin IX, and Co(III) protoporphyrin IX chloride were purchased from Porphyrin Products (Logan, UT), and $^{55}FeCl_2$ (specific activity of 77.75 mCi/mg) was supplied by NEN Life Sciences (Boston, MA). Reagent concentrations were determined using the following extinction coefficients. The following values were used for to quantify porphyrins, their derivatives, and hemoglobin forms: hemin, $\epsilon_{350} = 90$ mM^{-1} cm^{-1} in 50 mM sodium borate ($Na_2B_4O_7$) (*20*); CoPPIX, $\epsilon_{424} = 180$ mM^{-1} cm^{-1} in 0.1 M a NaOH/pyridine/H_2O (3/10/17, v/v/v) (*21*); and $CoHbO_2$, $\epsilon_{422} = 120$ mM^{-1} cm^{-1} in 0.1 M sodium phosphate ($NaPO_4$) (*22*). Hb^+Met, HbO_2, and HbCO were all converted to Hb^+CN derivatives and quantified using an ϵ_{540} of 44.0 mM^{-1} cm^{-1} and a molecular weight for Hb of 64 458 (*23*).

Reagent Preparation. Human haptoglobin (Sigma) was purchased as a pooled preparation of phenotypes Hp1-1, -2-1, and -2-2 for cell culture experiments or as the Hp1-1 phenotype for chromatography of all three phenotypes and was suspended in PBS (pH 7.4). The protein content was quantified using the bicinchoninic acid method with BSA as the standard (*24*). Lyophilized bovine Hb (Sigma) was prepared in H_2O by stirring at 4 °C and then centrifuged at 26000*g* for 30 min to precipitate the insoluble material. The solution was then filter sterilized (0.22 μm) (Millex GV, Millipore) and stored at 4 °C. FN was purified from human plasma and iodinated using Iodogen (Pierce, Rockford, IL) as previously described (*25*). ^{55}FePPIX was synthesized following the method of Galbraith (*26*) to obtain material with a specific activity of 1 mCi/mmol.

HbO_2 was prepared from fresh human blood drawn into EDTA (*27*) and was used as the starting material for globin isolation. Human globin was prepared from HbO_2 by the methyl ethyl ketone procedure (*20*) and routinely resulted in preparations with <1% residual heme as indicated by an A_{405}/A_{280} ratio of <0.1. Incorporation of CoPPIX into globin was accomplished using the anerobic method outlined by Scholler et al. (*22*). The $CoHbO_2$ product was immediately quantified after synthesis and oxygenation using an ϵ_{422} of 120 mM^{-1} cm^{-1}. FePPIX-Hb was synthesized under aerobic conditions at 4 °C using a 0.5 μM globin solution (calculated as monomer). ^{55}FePPIX was incorporated into globin in an identical manner and resulted in a product with a specific activity of 0.01 mCi/mmol.

Hb^+CN was prepared from HbO_2 by oxidation with potassium ferricyanide in the presence of potassium cyanide (*23*). The ferrocyanide resulting from the oxidation was removed by gel chromatography using Sephadex G-25 (PD-10, Amersham Pharmacia Biotech). HbCO was prepared from HbO_2 by purging a 10% solution with CO gas in the presence of a trace amount of dithionite (*28*), and conversion to HbCO was monitored spectrophotometrically (*27*). Hb^+N_3 was prepared by the addition of NaN_3 to Hb^+Met at 24 °C in 0.01 M sodium phosphate (pH 7.4) (*29*).

Cultures and Growth Conditions. C. albicans ATCC 44807 (American Type Culture Collection, Rockville, MD) was routinely cultured in defined minimal medium composed of 4× yeast nitrogen base (YNB) (Becton Dickinson, Sparks, MD) containing 2% glucose. Cells were cultured at 28 °C in capped sterile tubes on a rocker platform at 30 cycles/min. These growth conditions did not lead to hyphal differentiation, and this was monitored on a regular basis by microscopic examination. Hb, Hb derivatives, and myoglobin (Mb) were added to cell cultures in sterile 10 mM sodium phosphate (pH 7.4) and incubated for 48 h at 28 °C to assess induction of FN binding activity.

Hb Extraction from Conditioned Media. Hb fibers formed during incubation of Hb-containing media with blastospores were extracted after a 30 s centrifugation at 5000*g* to pellet cells. The fibers were pulled from the top of the cell pellet with tweezers and washed twice in PBS by centrifugation at 13000*g* for 5 min. The pellet was solubilized in 4 M urea, 2% SDS, and 2% β-mercaptoethanol and processed for SDS−PAGE analysis.

Blastospore Binding to Immobilized Hb and Hb Complexes. Hb−haptoglobin complexes were prepared by incubating 50 μg of haptoglobin with increasing amounts of Hb^+Met for 10 min at 24 °C. These complexes and Hb (1−500 μg/mL) were coated onto eight-well glass chamber slides (Lab-Tek, Nalge Nunc International, Naperville, IL) in a final volume of 0.3 mL, and incubated overnight at 4 °C. The solutions were then aspirated, and the chambers were gently washed three times with Dulbecco's PBS. Log phase blastospores (2×10^6 cells/mL) were added in Dulbecco's PBS in a final volume of 0.3 mL, and incubated at 24 °C for 3 h. Cells were then aspirated followed by three washes each in Dulbecco's PBS and then H_2O. Adherent cells were then fixed in 2% glutaraldehyde for 15 min and stained with Diff-Quik (Dade Behring, Aguada, PR) according to the manufacturer's instructions. Adherent cells were counted at 450× using at least 30 fields per chamber. Samples were compared using one-way repeated measures ANOVA.

Cell Adhesion to Immobilized Hb and Assay of FN Receptor Induction. To assay FN receptor induction of cells adhering to immobilized Hb, Hb^+Met (1 mg/mL) was coated onto glass Petri dishes overnight and washed extensively with DPBS. Log phase blastospores were then added to either coated or uncoated plates and incubated at 28 °C for 24 h. After extensive washing, the adherent cells were assayed for induction of FN receptor induction using the assay described below.

Fibronectin and Hb Binding. Binding assays were carried out in triplicate as previously described (*25*). Briefly, 5×10^5 blastospores were washed three times in Dulbecco's PBS (pH 6.0) without calcium or magnesium and with 0.1% BSA and suspended in a buffer with the same composition. Cells were then added to tubes containing 500 ng [^{125}I]FN (2−10 μCi/μg), [^{125}I]Hb (2.2 mCi/mmol), or [^{55}Fe]Hb (0.01 mCi/mmol) and incubated for 3 h at room temperature on a gyrotory shaker at 160 cycles/min. The cells were then separated from the soluble material by centrifugation for 30 s through a 2/1 mixture of dibutyl phthalate and dioctyl phthalate. Oil mixtures were aspirated, and ^{125}I in the cell pellets was quantified directly. ^{55}Fe-labeled cell pellets were completely disrupted using the yeast protein extraction reagent (Y-PER, Pierce) and assayed for associated radioactivity by scintillation counting in Aquasol (New England Nuclear, Boston, MA). Binding of Hb was analyzed by nonlinear regression using LIGAND software (*30*, *31*).

^{55}Fe Uptake. Uptake of the ^{55}Fe label from Hb was assessed by incubation of 75 000 dpm of [^{55}Fe]Hb or $^{55}FeCl_2$ each with 1 mg/mL unlabeled compound and 2×10^6 log phase cells in 4× YNB medium (pH 5.4). At the appropriate times, proteinase K was added to samples at a concentration of 0.25 mg/mL and digested for 30 min at room temperature. Digested proteins and unincorporated label were separated from the cells by centrifugation through oil as described above. The cell pellets were then completely disrupted using Y-PER and assayed for cell-associated radioactivity by scintillation counting.

Hb Aggregation Assay. Log phase blastospores were inoculated into 4× YNB medium containing 1 mg/mL Hb^+-Met and incubated in capped tubes at 28 °C. The amount of residual Hb in the supernatant fraction was determined by centrifugation of a culture sample at 13000*g* for 3 min to pellet cells and Hb aggregates. The supernatant was made 2 mM in NaN_3, and the Hb concentration was determined spectroscopically at 417 nm. The effects of NaN_3 on aggregation during cell growth were determined by the addition of NaN_3 to a final concentration of 2 mM at 0, 3, and 5 h during incubation. Cells were also cultured in the presence of NaN_3 in 4× YNB medium lacking Hb to show metabolic inhibition by azide alone.

Size Exclusion Chromatography and Formation of the Hb−Haptoglobin Complex. Formation of the Hb−haptoglobin complex was assessed using an HPLC system consisting of a Superdex 200 column (1.5 cm × 20 cm) (Amersham Pharmacia Biotech) controlled by a Peak Net Chromatography Workstation (Dionex, Sunnyvale, CA). The PBS mobile phase (pH 7.4) at 0.6 mL/min was monitored at 405 nm to detect Hb and Hb−haptoglobin complexes and 280 nm for non-heme proteins. Column calibration used the following proteins as standards: apohaptoglobin, phenotype Hp1-1 (M_r = 98 200), holotransferrin (M_r = 72 000), and horse myoglobin (M_r = 16 000). Hb titration with haptoglobin used a mixture of all three haptoglobin phenotypes (Hp1-1, Hp2-1, and Hp2-2) and was accomplished using 36 μg aliquots of either human HbO_2 or Hb^+Met combined with 33 μg of haptoglobin aliquots (pooled preparation) immediately before column loading.

LC−MS Identification of Protein Bands. Protein bands identified in SDS−polyacrylamide gels were excised as gel slices, washed twice with methanol and ammonium bicarbonate buffer, dried in vacuo, and then treated overnight with trypsin. The resulting peptides were extracted, separated, and analyzed on a Finnigan LCQ LC−MS system. The resulting run files were analyzed using Sequest database searching software.

RESULTS

Hb Induction of FN Binding Is Dose-Dependent and Relies on Hb Protein Structure. Our laboratory previously reported the specific induction of a *C. albicans* cell surface FN receptor by culturing blastospores in the presence of Hb. This effect was Hb-specific in that neither heme, free porphyrin, myoglobin, nor globin increased the level of expression of the FN receptor (*17*, *18*, *32*). Maximal levels of soluble FN binding occurred near 24 h with 1 mg/mL bovine Hb^+Met, and the level of adhesion to immobilized FN and corneal endothelial cells was increased as well (*17*). To further characterize the interaction of Hb with the cell surface, we examined the dose dependence of Hb induction using a soluble FN binding assay. *C. albicans* blastospores were incubated in defined minimal medium in the presence of varying doses of human Hb at 28 °C for 48 h. Expression of soluble FN binding was saturable and reached a maximal response between 0.5 and 1 mg/mL Hb (Figure 1), and half-maximal induction was obtained at 2.5×10^{-6} M. This indicates that the effect of Hb on the expression of the FN receptor depends on the specific recognition of Hb present in the medium and suggests that Hb interacts with a specific receptor.

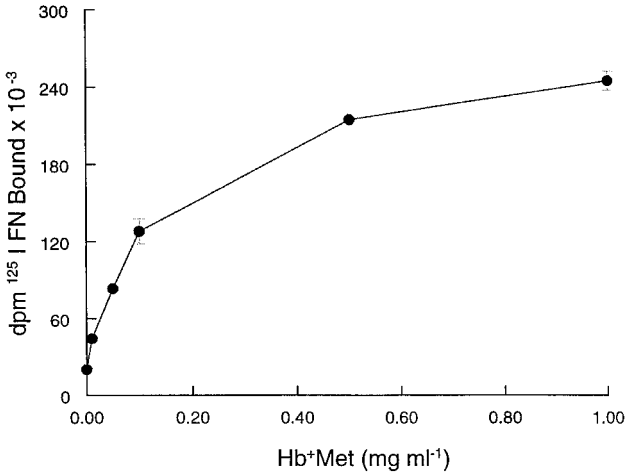

FIGURE 1: Hb induction of FN binding to *C. albicans* 44807 is dose-dependent. Blastospores were cultivated in defined media at 28 °C with varying doses of Hb, and binding of [^{125}I]FN was assessed after 48 h. Nonspecific binding in this assay is within 10% of binding without Hb. Results throughout are reported as mean ± the standard deviation (n = 3) except where noted.

Many human pathogenic microorganisms, especially bacterial pathogens, can utilize Hb as an iron source in the otherwise iron-deficient host environment (*33*). We therefore considered whether Hb may induce the FN receptor by delivering iron to the cells, since. *C. albicans* can utilize iron from both heme and Hb (*19*). Yan et al. had previously shown that iron salts added to a *C. albicans* culture did not induce FN binding to the cell surface, implying that expression of this FN receptor is not directly regulated by iron (*17*). However, other possibilities exist such as the delivery of iron by transport of heme, Hb, or a proteolytic fragment into the cell to alter gene expression and were not excluded. To distinguish among these possibilities, we followed the fate of the radiolabel from ^{55}FePPIX-Hb when cultured with *C. albicans* in defined minimal medium and compared this to the uptake of inorganic iron detected using an $^{55}FeCl_3$ tracer. Globin was isolated from fresh human Hb and used for the incorporation of ^{55}FePPIX as described in Experimental Procedures. Mid-log phase yeast cells were inoculated into medium containing equimolar amounts of either labeled Hb or $FeCl_3$. After defined incubation periods, samples were treated with proteinase K, free peptides and the radiolabel were separated from cells, and the cell pellets were completely disrupted and assayed for cell-associated radioactivity (*25*).

Uptake of the ^{55}Fe label from the ferric salt occurred with rapid kinetics and reached maximal levels within 12 h. In contrast, significant accumulation of label from Hb did not

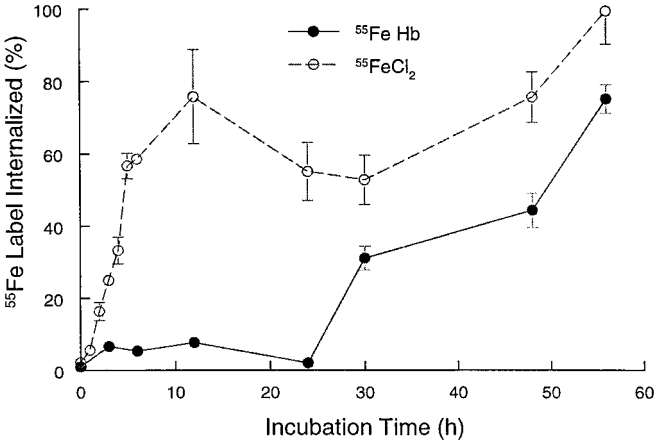

FIGURE 2: Uptake of ^{55}Fe from Hb occurs after FN receptor induction. Blastospore cultures were sampled at the indicated times after addition of $^{55}FeCl_2$ (○) or ^{55}FePPIX-Hb (●) and digested with proteinase K for 30 min. Extracellular label and peptides were separated from the cells by centrifugation through a dibutyl phthalate/dioctyl phthalate mixture (2:1), and the cell pellet was assayed for internalized label by scintillation counting.

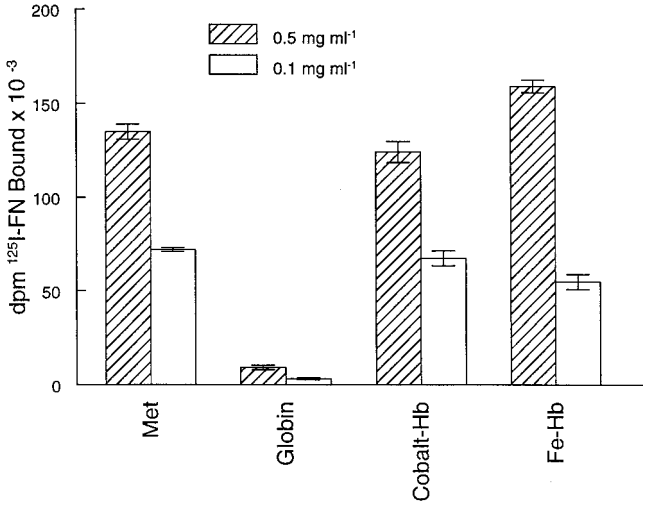

FIGURE 3: Replacement of iron with cobalt in Hb does not affect induction of FN binding. Hb$^+$Met, globin, and globin reconstituted with cobaltPPIX and FePPIX were compared for FN receptor induction at 0.5 (hatched bars) and 0.1 mg/mL (white bars).

appear inside the cells until after 24 h (Figure 2). This indicated that if Hb or heme had been transported intracellularly, it occurred only after the time when FN receptor expression was near maximal levels (*17*, *34*).

These experiments suggested that neither iron nor heme released from Hb was involved in the induction of FN binding, unless they exerted their effects at the cell surface. This indicated that Hb protein structural features may play a direct role in induction, perhaps through a receptor−ligand interaction. Yan et al. found that globin did not induce expression of FN binding above background levels (*17*). However, since globin is unstable and denatures at room temperature (*20*), we incorporated cobalt−PPIX into globin to conserve the structural features of the protein and examined the effects on induction of soluble FN binding activity. Incorporation of either cobalt−PPIX or Fe−PPIX into this globin preparation resulted in a full restoration of the ability of globin to induce FN binding activity (Figure 3). Globin alone at 0.5 mg/mL showed only a slight increase above background levels and was approximately 25-fold less active than the induction seen with Hb$^+$Met at the same protein concentration. Additionally, at a 5-fold lower dose (0.1 mg/mL), the globin control exhibited background levels of induction (Figure 3).

These data indicate that heme or heme iron is not essential for induction of FN receptor expression. Instead, protein structural features or O_2 bound to the ferrous or cobaltous Hb complexes could mediate the induction of FN expression. Since the ferric Hb derivatives do not bind O_2, we investigated this matter further by preparing the Hb$^+$CN, HbCO, HbO_2, and Hb$^+$Met forms of human Hb and compared their dose−response profiles using a single [^{125}I]FN preparation. The dose−response profiles were similar for all four derivatives, indicating that neither the oxidation state nor the heme ligand plays an obvious role in FN receptor induction (Figure 4A).

The HbO_2 response displayed a decreased level of FN binding at each dose level relative to the other three Hb forms in several independent preparations (Figure 4A and data not shown). To test whether this deviation is an inherent difference due to O_2 binding, we prepared HbCO from a fresh HbO_2 preparation. As such, both forms were identical in composition and concentration, and any differences noted in their dose−response patterns would be related to structural changes caused by substitution of CO for O_2. We found no significant difference between the two Hb forms at all dose levels that were tested (Figure 4B). This supports the idea that slightly decreased activity was due to the relative instability of HbO_2 in storage, which results in an underestimation of the actual effector molecule concentration in Figure 4A when calculated on a per heme basis.

Haptoglobin Inhibits Hb Induction of FN Binding. Haptoglobin is a serum α-2-glycoprotein that binds Hb dimers with a 1:1 stoichometry in an essentially irreversible reaction ($K_a > 10^{15}$ M^{-1}) (*35*, *58*) and is a physiological substrate for Hb released by red blood cell hemolysis (*36*). To test whether Hb−haptoglobin complexes were still active in FN receptor induction, we first empirically determined the extent of haptoglobin saturation. This was necessary since haptoglobin is composed of three phenotypic forms with M_r values of 98000−283000 (*1*),[2] and this made precise stoichometric calculations impossible. Increasing amounts of apohaptoglobin were added to a fixed amount of Hb, and Hb−haptoglobin complexes were identified by gel permeation chromatography. Hb−haptoglobin complexes eluted as two major peaks: the Hp1-1 isoform complex at 17 min and the heterogeneous Hp2-1 and -2-2 isoform complexes near 15 min (Figure 5A). A decrease in the levels of HbO_2 at 23−25 min with haptoglobin addition illustrates that the relative extent of haptoglobin saturation can be calculated using this method.

Haptoglobin−Hb complexes were added to *C. albicans* cultures to test for the induction of soluble FN binding. Haptoglobin added to Hb at a 1:1 mass ratio effectively and completely blocked FN receptor induction (Figure 5B, inset).

[2] Each haptoglobin phenotype designated Hp1-1, Hp2-1, and Hp2-2 uses a common β subunit (40 000) and combinations of α^1 and α^2 subunits (9100 and 15 000, respectively) with the following general formulas: Hp1-1, $2\alpha^1 2\beta$; Hp2-1, $(\alpha^1\beta)_2 \times (\alpha^2\beta)_n$ ($n = 0, 1, 2, ...$); Hp2-2, $(\alpha^2\beta)_m$ ($m = 3, 4, 5, ...$).

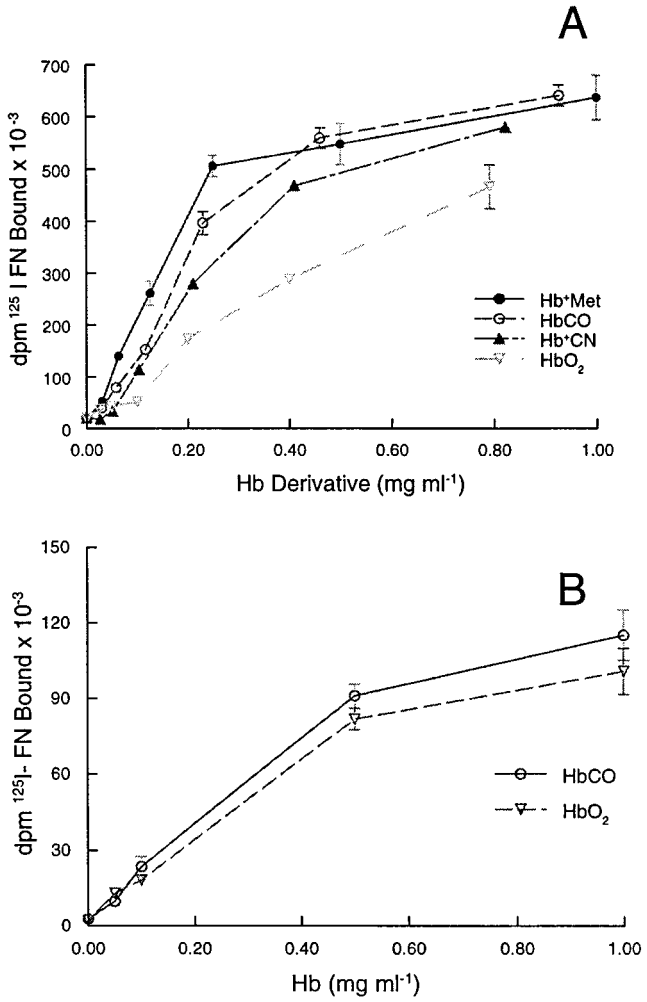

FIGURE 4: Induction of FN receptor expression by Hb is independent of iron oxidation state and heme ligand. (A) Ferric and ferrous Hb derivatives were tested for their ability to induce FN receptor expression in the standard 48 h assay: Hb^+Met (●), Hb^+CN (▲), HbCO (○), and HbO_2 (▽). (B) HbO_2 was isolated from freshly drawn blood and converted to the carbon monoxide form using CO gas and then tested for induction of FN receptor expression: HbO_2 (▽) and HbCO (○).

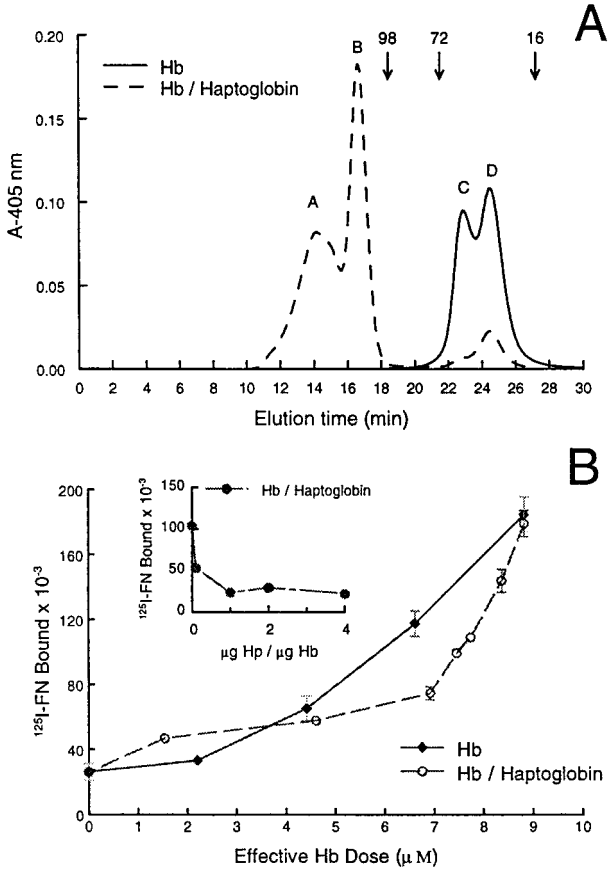

FIGURE 5: Haptoglobin antagonizes Hb induction of FN receptor expression. (A) Size exclusion chromatography of haptoglobin complexes. The extent of haptoglobin saturation with Hb was monitored at 405 nm using a Superdex 200 column (M_r = 200 000 cutoff). Elution positions of calibration standards are noted by labeled arrows: apohaptoglobin isoform Hp1-1 (98 200), holotransferrin (72 000), and horse myoglobin (16 000). Increasing amounts of apohaptoglobin were added to a fixed amount of Hb to determine the mass needed for 100% haptoglobin saturation. A representative titration end point is depicted where HbO_2 (36 μg) was chromatographed either alone (—) or after incubation with 33 μg of apohaptoglobin (– – –, >95% saturation). The apo forms of Hp2-1 and Hp2-2 elute as broad peaks near 17 min (data not shown). The labeled peaks are as follows: A, Hb−haptoglobin isoforms 2-1 and 2-2; B, Hb−haptoglobin isoform 1-1; C, Hb tetramer; and D, Hb dimer. (B) Haptoglobin inhibits Hb-induced FN receptor expression. Hb or haptoglobin−Hb complexes were tested for induction of FN receptor expression in the standard 48 h FN binding assay. The effective Hb doses were calculated on the basis of the relative haptoglobin saturation as judged by size exclusion chromatography: Hb (●) and Hb−haptoglobin complex (○). The inset shows the Hb titration with Hb based upon mass. Hb and haptoglobin were combined at the indicated ratios, added to *C. albicans* cultures, and assayed for FN receptor binding. Apohaptoglobin added at 0.5 mg/mL in the absence of Hb gave background levels of induction (data not shown). This figure depicts two separate experiments.

Apohaptoglobin added alone at 2 mg/mL did not alter FN binding (data not shown). We next calculated the amount of free Hb theoretically present based on the amount of Hb bound.[3] The calculated effective dose of free Hb remaining after haptoglobin binding exhibited a clear dose dependency similar to that observed in the Hb dose curve (Figure 5B). Together, these results illustrate that haptoglobin can completely titrate the Hb response and confirm that the effector molecule for the induction of the FN receptor signaling pathway is Hb.

Low-Affinity Binding Characterizes Hb Interactions with C. albicans Blastospores. The association of Hb with *C. albicans* cells indicated that receptor−ligand interactions may be part of the process leading to FN receptor expression. To quantify this interaction, we measured the constants for binding of soluble Hb using [^{125}I]Hb and competitive displacement by the unlabeled ligand. Three separate experiments revealed saturable, low-affinity binding of soluble Hb [$K_d = (1.1 \pm 0.2) \times 10^{-6}$ M] with approximately 1.4×10^7 sites per cell (Figure 6).

Blastospore Culture with Hb Leads to Macroscopic Fiber Formation. During the course of these experiments, we observed the formation of macroscopic filamentous particles within the first hours of blastospore incubation with Hb-containing media (data not shown). Under the light microscope, two general particle types could be distinguished: (i)

[3] An estimate of 109 000 was used for the haptoglobin mass capable of binding two Hb dimers. This calculation was made assuming (i) an average value for the mass of the three α-chain types based on their relative abundance and (ii) cross-linking of the Hp2-1 and Hp2-2 phenotypes does not affect the quantity of Hb dimer loading (*1*).

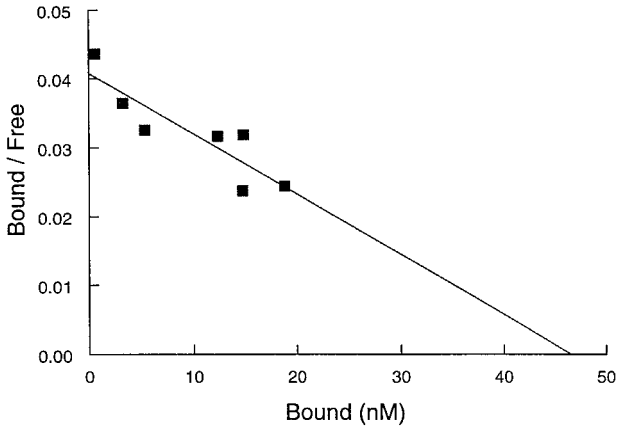

FIGURE 6: Scatchard plot for competitive displacement of Hb^+-Met binding to *C. albicans* blastospores. Unlabeled Hb was used as an inhibitor of [^{125}I]Hb binding to *C. albicans* blastospores (5×10^5) prepared from log phase cultures grown in 4× YNB medium. Cells were incubated with labeled and unlabeled ligand ($7.8-780 \times 10^{-8}$ M) for 3 h and then separated from unbound ligand by a 30 s centrifugation through oil. Binding constants were determined by nonlinear regression using the LIGAND program (*30*, *31*) to analyze displacement at each dose in triplicate. Similar results were obtained from three separate experiments, and mean values from a representative experiment are shown. Constants were calculated on the basis of a single-site model [$K_d = (1.1 \pm 0.2) \times 10^{-6}$ M with $(1.4 \pm 0.2) \times 10^7$ sites per cell].

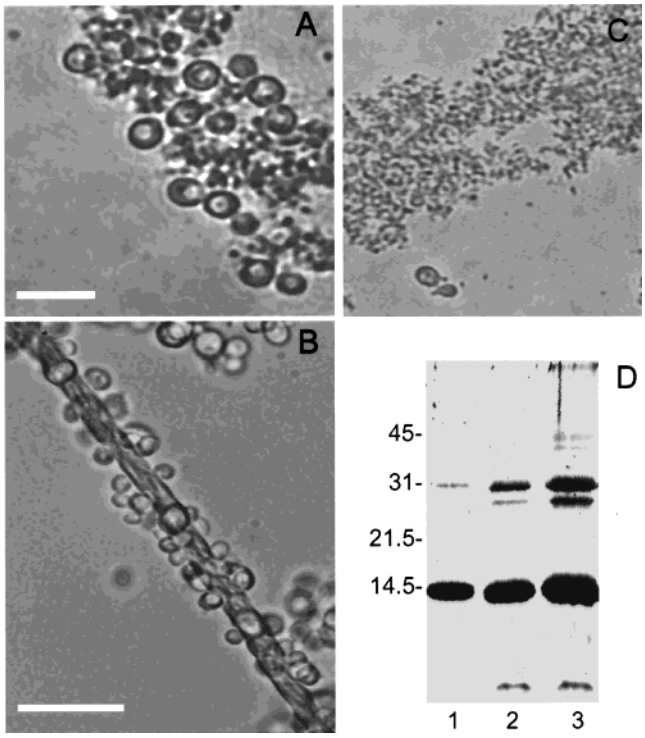

FIGURE 7: Light micrographs showing attachment of *C. albicans* blastospores to Hb polymers. (A) Amorphous Hb−blastospore aggregates which predominated in the cultures. The bar is 10 mm long. (B) Hb fibers isolated as floating particles in the medium. This photo illustrates the regular arrangement of blastospores attached along the length of the fibers. The bar is 25 mm long. (C) Blastospores do not bind to myoglobin polymers. Mb (1 mg/mL) was incubated with cells under standard culture conditions for 48 h. A typical Mb aggregate is shown and demonstrates that these polymers did not display blastospore binding sites and remained evenly dispersed and showed no signs of aggregation. The scale is the same as that in panel B. (D) Fibers from *C. albicans* Hb cell cultures contain intact Hb subunits. Fibers extracted from *C. albicans* cultured in the presence of Hb^+Met were analyzed on SDS−acrylamide gels (15%) under reducing conditions: lane 1, Hb^+Met stock (starting material for cell culture); lane 2, Hb fiber taken from cells grown in Hb media and not exposed to direct cellular contact; and lane 3, Hb fiber extracted from cell culture and solubilized with 4 M urea and 2% SDS. Molecular weight standards are indicated ($\times 10^{-3}$). The three major Hb bands are at 32 000, 28 000, and 16 000 (M_r), representing the dimer, partial dimer, and monomer, respectively. Hemin migrates at the leading edge of the gel in lanes 2 and 3.

amorphous brown aggregates tightly packed with cells and (ii) 50−500 mm × 5−10 mm regular fibers with cells attached along their length (panels A and B of Figure 7, respectively). The majority of the cells cultivated with Hb were found to be associated with the amorphous type structures (Figure 7A). The light brown color of the particles indicated the presence of Hb, and this was confirmed by fiber solubilization with urea and SDS and visualization by SDS−PAGE (Figure 7D). Primary sequence determination by LC−MS of the three major protein bands (M_r = 32 000, 28 000, and 16 000) confirmed that the only significant components of these fibers are intact Hb subunits (data not shown). Myoglobin incubated with cells under the same conditions also resulted in protein aggregation. However, these myoglobin polymers did not demonstrate blastospore binding, and the cells remained dispersed as single cells (Figure 7C). This indicates that cell binding to Hb fibers is specific for a Hb protein structural determinant.

Our culture conditions repress secreted aspartyl protease expression (*13*, *37*), and the addition of pepstatin A to cultures did not affect the induction of FN receptor expression by Hb (data not shown). This indicates that the major secreted aspartyl proteases are probably not involved in fiber formation. Fibronectin is also a target of a surface metalloproteinase (*38*), but FN can be recovered intact from cells grown under similar conditions (*25*). Therefore, proteolysis is probably not necessary for Hb fiber formation, although limited proteolysis by terminal amino acid peptidases would not have been detected by our SDS−PAGE analysis.

Immobilized Hb Is an Adhesion Substrate. The extensive binding of blastospores to Hb fibers and aggregates in the liquid cultures and the low affinity of soluble Hb binding suggested that Hb binding to cells may be facilitated by multivalent interactions. We therefore tested blastospore adhesion to Hb immobilized on glass slides. Cells adhered to immobilized Hb in a dose-dependent manner (Figure 8A, dark bars), but at 10-fold higher concentrations than needed for comparable adherence to immobilized FN (*17*). This difference in dose dependence is consistent with the approximately 20-fold lower K_d value for the FN receptor (*17*, *25*). Haptoglobin complexes also inhibited Hb-mediated adhesion in a dose-dependent manner (Figure 8A, hatched bars). This indicated that immobilized Hb presents adhesive surfaces that maintain the haptoglobin-sensitive structural features. These common features of Hb are necessary for both induction of FN receptor expression and cell adhesion to Hb polymers.

Hb Adhesion Stimulates FN Receptor Expression. Cell adhesion to higher-order Hb suggested that multivalent Hb binding mediates the induction of FN receptor expression. We tested this by coating glass plates with Hb and incubating cells on Hb-coated or uncoated plates for 24 h. Nonadherent cells were removed by washing; the adherent cells were

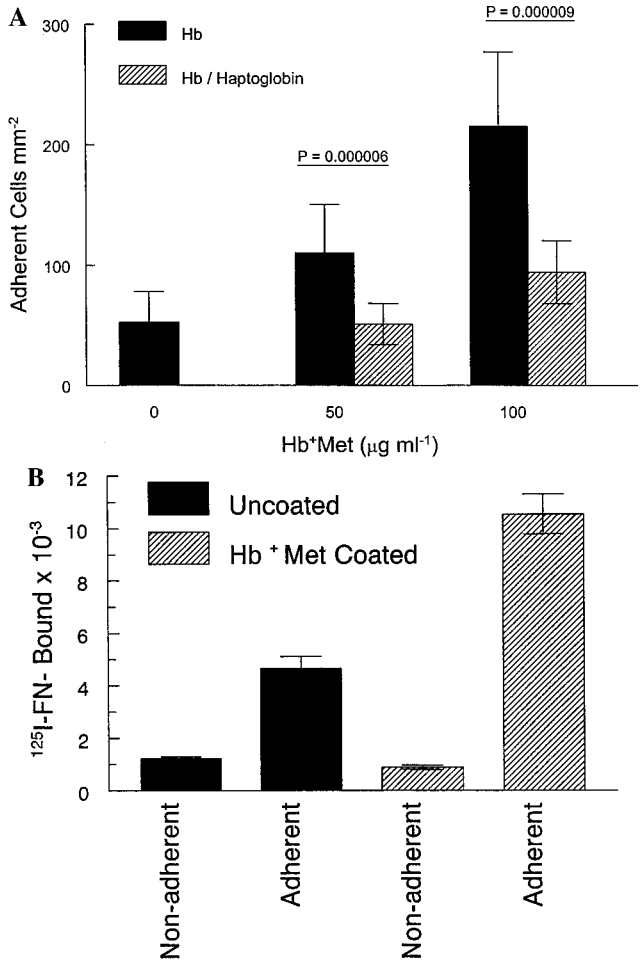

FIGURE 8: Immobilized Hb is a substrate for cell adhesion and induction of FN receptor expression. (A) Adhesion of *C. albicans* to immobilized Hb is dose-dependent and can be inhibited by haptoglobin. Hb^+Met or Hb−haptoglobin complexes were coated on glass slides overnight at 4 °C and tested for adhesion of *C. albicans* blastospores. The concentrations indicate the amount of Hb added either alone (black bars) or with haptoglobin at 50 μg/mL (hatched bars). The *P* values were calculated using one-way repeated measures ANOVA using cell counts from at least 30 high-power (450×) fields for each concentration and are representative of three independent experiments. (B) Adhesion of yeast phase cells to immobilized Hb induces FN receptor expression. Glass plates were coated overnight at 4 °C with Hb^+Met and then incubated with log phase *C. albicans* cells for 24 h at 28 °C. Nonadherent cells were removed by extensive washing, and adherent cells were then scraped from the plates. Both adherent and nonadherent cells were then tested for induction of FN binding. This figure is representative of two independent experiments.

isolated by plate scraping, and both groups were used for the standard [^{125}I]FN binding assay. Nonadherent cells displayed only background levels of soluble FN binding (Figure 8B). Although the level of glass-adherent cells was somewhat increased in FN binding, there was a significant further enhancement when Hb was the adhesion substrate (Figure 8B). These data indicate that adhesion to immobilized Hb can induce FN receptor expression and imply that formation of higher-order Hb structures and induction of FN binding are related.

Hb Aggregation by C. albicans Requires Metabolic Energy. As noted above, visible fiber or aggregate formation was apparent in cell cultures within 3−6 h. To quantify the extent of aggregation, log phase cells were inoculated in 4× YNB medium with Hb^+Met (1 mg/mL) in the presence or

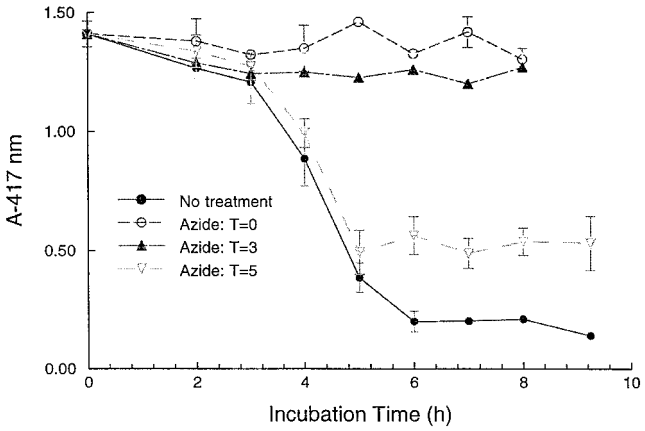

FIGURE 9: Hb aggregation by *C. albicans* blastospores requires metabolically active cells. Blastospores and Hb aggregates were precipitated by centrifugation, and residual Hb in the supernatant was assessed by conversion to the HbN_3 form and quantified at 417 nm. Samples were taken at designated intervals from cultures in the absence of metabolic inhibitors (●) or with NaN_3 added to a final concentration of 2 mM at 0 (○), 3 (▲), or 5 h (▽).

absence of 2 mM NaN_3 to determine whether aggregation is the result of an active metabolic process. The amount of soluble Hb remaining after defined time periods was determined by centrifugation of cells and associated Hb polymers. Hb in the supernatant was quantified by conversion to the HbN_3 form and measured at 417 nm. Figure 9 shows that Hb aggregation by the cells proceeded rapidly after an initial lag period of approximately one cell doubling, and that greater than 90% of the soluble Hb had been precipitated after 6 h. However, treatment with NaN_3 for 10 min before inoculation resulted in a complete inhibition of aggregation (Figure 9, azide $T = 0$). NaN_3 addition after 3 or 5 h intervals rapidly stopped any further aggregation after addition, indicating that the factor(s) responsible for this effect required metabolic energy (Figure 9).

DISCUSSION

Hb is utilized by many pathogenic microorganisms as a source of heme, iron, or amino acids (*43*−*46*). In these instances, a nutritional purpose is served for the survival of the organism. However, in the case of *C. albicans*, Hb can also function as a signaling molecule to initiate a pathway leading to fibronectin receptor expression. The interaction of Hb with *C. albicans* is a receptor-mediated process as indicated by the dose dependence of FN receptor expression, the strict requirement for porphyrin, the saturable binding of soluble Hb, and inactivation by binding of Hb to haptoglobin. However, binding of soluble Hb to the cell surface was characterized by low-affinity interactions [$K_d = (1.1 \pm 0.2) \times 10^{-6}$ M], indicating that a higher-order Hb structure may be the most efficient form for inducing FN receptor expression. *C. albicans* blastospores avidly bound to immobilized Hb and to macroscopic Hb fibers or aggregates that formed rapidly (within 3 h) under standard culture conditions with Hb. Adhesion to immobilized Hb was also specific, in that haptoglobin inhibited this process and that myoglobin polymeric structures did not contain cell binding sites. Since both immobilized Hb and the Hb fibers are multivalent, they should facilitate adhesion of *C. albicans* mediated by its low-affinity, high-copy number receptor. Indeed, the estimated number of Hb binding sites per cell is

30-fold more than the number of FN binding sites (*32*) and indicates that the Hb receptor is a relatively abundant surface structure. Adhesion to immobilized Hb also stimulated FN receptor expression, suggesting a functional link between multivalent Hb binding and initiation of a signaling pathway culminating in FN receptor expression.

Although *C. albicans* can utilize Hb as an iron source (*19*, *47*), we have shown that it also recognizes this host protein as a signaling molecule. The outcome of this recognition process is the elaboration of a FN receptor ($K_d = 4.6 \times 10^{-8}$ M) (*18*, *32*) and activation of other Hb-response genes whose functions have yet to be determined.[4] The studies of Hb-induced gene activation in *C. albicans* to date have been carried out under iron-replete conditions (*17*, *18*, *25*, *32*, *34*). This is particularly interesting since Hb is recognized and utilized as an iron source by virtually all major bacterial pathogens (*33*) and as a protein source for a number of mammalian parasites (*46*). The lowest Hb concentration used in our experiments was sufficient to restore growth under iron-deficient conditions (*47*), which implies that Hb induction is not related to iron uptake. Indeed, we have shown that uptake of iron from Hb is minimal under conditions promoting maximal binding of soluble FN. Although we have shown a strictly signaling function for Hb, this does not exclude an additional role as an iron source during infection.

The ability of haptoglobin to inhibit FN receptor expression suggests a physiological role for this molecule in the host response to *Candida* infections. In mammals, haptoglobin is an acute phase reactant generated by the liver in response to tissue injury, infection, inflammation, or tumor growth (*57*). Circulating levels of haptoglobin in humans are approximately 0.5−2 mg/mL, so hemolysis from as little as 2 mL of red cell lysis would cause a total depletion (*56*). However, the Hb levels needed to induce FN receptor expression (0.01 mg/mL) represent a 12000−18000-fold dilution of the typical Hb content in blood of a normal human subject. This implies that the potential for competition for Hb between the fungus and circulating haptoglobin may exist in a healthy individual and thereby prevent induction of the FN receptor.

Hb fiber formation in liquid media could be rapidly and completely blocked with NaN_3. This uncoupling agent restricts cells to nonoxidative pathways for ATP generation (*49*) and indicates that fiber or aggregate formation is an active process but probably not the result of proteolysis (*39*−*42*). One potential explanation is the secretion of a soluble heme or Hb binding (transport) protein capable of extracting heme from Hb. Heme chelators have been identified in *Serratia marscens* (*50*, *51*), *Hemophilus influenzae* (*52*, *53*), and *Pseudomonas aeruginosa* (*54*) and rely on energy-dependent ABC transporters for their secretion. In our system, both the energy dependence of aggregation and the soluble nature of the process are suggestive of a similar type system. Whether the secreted hemolysin produced by this organism (*47*, *55*) plays a role in this process is not known. However, iron uptake through such a system is clearly not required to induce the FN receptor.

In summary, we have defined several structural features of Hb that are necessary for the induction of FN receptor expression in *C. albicans* and demonstrated a protective activity of haptoglobin. The interaction of Hb with the cell to induce gene expression was dose-dependent and independent of the iron oxidation state and heme ligand. Hb aggregation generates a structure compatible with adhesion of blastospores mediated by low-affinity, multivalent interactions, and resulting in FN receptor induction. Also, Hb aggregation induced by *C. albicans* requires an intact cellular metabolism. The identification of the molecules responsible for Hb structural alterations and how this relates to *Candida* infection may lead to therapeutic approaches for treatment or prevention of disseminated candidiasis.

[4] S. Yan et al., manuscript in preparation.

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