7 Molecular Mechanisms of *Histoplasma* Pathogenesis

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I. Introduction

Histoplasma capsulatum is a primary fungal pathogen that causes respiratory and systemic disease. **The fungus is endemic to regions of North, Central, and South America as well as parts of Africa.** In the USA, *Histoplasma* causes more hospitalizations than all other endemic fungi (Chu et al. 2006; Baddley et al. 2011). Serology studies indicate that up to 80% of individuals in endemic areas of the USA have been infected (Edwards et al. 1969), yielding estimates of 200,000 infections annually (Ajello 1971). The severity of histoplasmosis is determined by the dose and by the immunological state of the host, with the majority of infections being self-limiting upon activation of cell-mediated immunity.

Histoplasmosis is initially a respiratory infection that results from inhalation of *Histoplasma* conidia. The infectious conidia are produced by and released from the environmental (mycelial) form of the fungus. Within the mammalian lung, elevated temperatures cause conidia to differentiate into the pathogenic yeast form. *Histoplasma* is thus a thermally dimorphic fungus with exclusively separate mycelial and yeast forms. Beyond fungal morphology, ***Histoplasma*'s dimorphism corresponds to alternate lifestyles, with the mycelia deriving nourishment from decaying organic matter but the yeast parasitizing host cells. *Histoplasma*'s dimorphism also reflects substantial transcriptional changes, which include expression of genes necessary for pathogenesis.**

Most fungi that cause human disease are opportunistic pathogens, their diseases being restricted to hosts with defects in innate and/or adaptive immunity. In contrast, ***Histoplasma* infections are not restricted to immunocompromised or immunosuppressed hosts.** Of clinical cases, less than half are associated with other comorbidities or known immunocompromising conditions (Chu et al. 2006; Baddley et al. 2011). The innate immune system is entirely unable to control *Histoplasma* infections until activation by cells of the adaptive immune system. **In the absence of cell-mediated immunity, *Histoplasma* infections nearly always progress to life-threatening, progressive disseminated histoplasmosis.** Even with T cell activation, *Histoplasma* infections may not be completely cleared and latent organisms can persist as a

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reservoir for reactivation histoplasmosis (Jain et al. 2006; Allen and Deepe 2006). This greater virulence of *Histoplasma* compared to opportunistic fungi indicates that *Histoplasma* must produce specific virulence factors that enable it to defeat immune defenses. Within the mammalian host, ***Histoplasma* is almost exclusively an intracellular pathogen with yeasts predominantly found in host macrophages**. As will be discussed in molecular detail, *Histoplasma*'s ability to reside within these immune cells that normally eliminate invading fungi is a function of (1) avoidance of detection by the host and (2) negation of host antimicrobial defenses.

Dissection of the molecular mechanisms that promote *Histoplasma* pathogenesis relies upon identification and characterization of the underlying virulence factors. Functional genetic studies have only recently been possible with molecular advances to eliminate or deplete specific gene functions. These include the development of a gene knockout strategy for a fungus with vanishingly low rates of homologous recombination (Sebghati et al. 2000), methodologies to deplete gene functions by RNA interference (RNAi) (Rappleye et al. 2004), and insertional mutagenesis techniques using Agrobacterium-mediated transformation (Sullivan et al. 2002; Youseff et al. 2009; Youseff and Rappleye 2012). In addition, modern platform technologies such as gene expression profiling (Hwang et al. 2003, 2012; Nittler et al. 2005) and proteomics (Albuquerque et al. 2008; Winters et al. 2008; Holbrook et al. 2011) have greatly enhanced identification and selection of candidate molecules for understanding *Histoplasma* virulence.

The dimorphism of *Histoplasma*, and the built-in ability to correlate expression patterns specifically with the pathogenic phase, has helped propel virulence factor identification. Logically, factors uniquely produced by pathogenic-phase cells have been considered prime candidates that specifically increase the fitness of this phase. Because **differentiation of *Histoplasma* into the yeast phase is required for establishment of disease** (Medoff et al. 1986; Nemecek et al. 2006), yeast-phase factors are probably produced to facilitate *Histoplasma* pathogenesis. Although not virulence factors per se, a number of regulators of the transition to the yeast phase in response to mammalian body temperature have been identified. Transition to the yeast phase requires a histidine signaling kinase (Drk1) (Nemecek et al. 2006). In addition, the Ryp1 transcriptional regulator, which is related to the *Candida* Wor1 differentiation factor, binds to DNA upstream of yeast-phase genes and activates expression of approximately 740 genes of the yeast-phase regulon (Nguyen and Sil 2008). Two additional proteins, Ryp2 and Ryp3, are related to Velvet A-type regulators of filamentous fungi and are required for Ryp1 accumulation and for yeast-phase differentiation (Webster and Sil 2008). How the thermal stimulus is transduced into signals that involve these pathways is still unknown. Nevertheless, these yeast-phase-regulating factors contribute to pathogenesis indirectly by activating expression of many of the virulence mechanisms discussed below. Indeed, most virulence factors identified to date are characterized by yeast-phase-specific expression, and their functions tailored to ensuring *Histoplasma* survival as the yeasts interact with a fully functional host immune system.

II. Avoidance of Host Defenses

A. Adhesion to and Entry into Phagocytes

Infection of phagocytes involves intimate association of *Histoplasma* cells with the host cell surface to promote internalization, yet survival requires yeasts to minimize activation of phagocyte defense mechanisms (Fig. 7.1). Initial adherence of *Histoplasma* to macrophages is mediated by binding to macrophage heteromeric transmembrane β-integrins, chiefly the CD18-family complement receptors (CR): LFA-1 (CD11a+CD18), CR3/Mac-1 (CD11b+CD18), or CR4 (CD11c+CD18). Antibodies that block complement receptors substantially reduce attachment and phagocytosis of *Histoplasma* yeasts and conidia by macrophages, although blocking individual alpha subunits causes less impairment than blocking CD18 due to expression of multiple complement receptors on macrophages (Bullock and Wright 1987; Newman et al. 1990; Lin et al. 2010). Although *Histoplasma's* association with

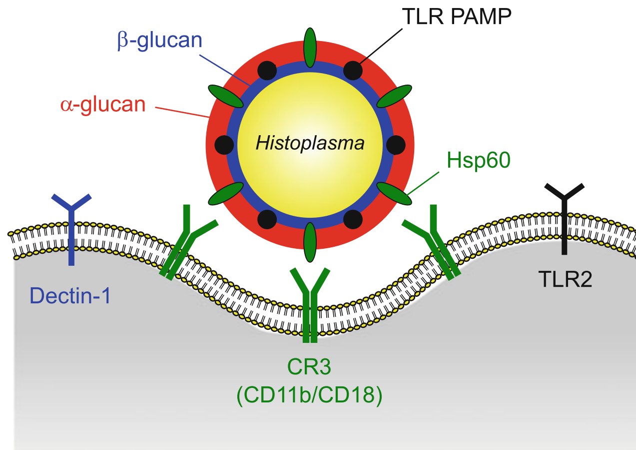

Fig. 7.1. Molecular mechanisms of detection avoidance during interaction of *Histoplasma* yeasts with macrophages. Adherence and phagocytosis of yeasts into macrophages is mediated by cell-wall-localized Hsp60 binding to CD18-family receptors (e.g., CR3) in the macrophage plasma membrane. At the same time, *Histoplasma* yeasts avoid detection by the macrophage β-glucan receptor Dectin-1 by concealing fungal β-glucans beneath an α-glucan polysaccharide layer in the yeast cell wall. In addition, macrophage TLR2 receptors do not detect undefined TLR2 PAMPs present in the yeast cell wall

macrophages is mediated by complement receptors, binding is not dependent on complement opsonization of yeasts (Bullock and Wright 1987). **The cognate adhesin protein on yeasts that binds to CR3 is heat-shock protein 60 (Hsp60)** (Long et al. 2003). How Hsp60, a normally intracellular stress-response protein, becomes localized to the extracellular surface of yeasts is unknown. Consistent with its extracellular localization, antibodies to Hsp60 can opsonize yeast and promote Fc-dependent phagocytosis (Guimarães et al. 2009). Furthermore, vaccination with Hsp60 is protective to mice (Gomez et al. 1995; Deepe et al. 1996). In contrast to macrophages, attachment of *Histoplasma* yeasts to dendritic cells is facilitated by yeast cyclophilin A binding to dendritic cell-expressed VLA-5 receptors (Gildea et al. 2001; Gomez et al. 2008). No adhesin-receptor pairing has been determined yet for the interaction of yeasts with polymorphonuclear neutrophils (PMNs). Complement receptors seem unlikely candidates because surface expression of complement receptors on PMNs is minimal until PMNs are stimulated (Sengeløv et al. 1993; Videm and Strand 2004).

Binding to complement receptors for adherence also triggers internalization of yeasts into macrophages. Intracellular residence in macrophages enhances the virulence of *Histoplasma* by protecting yeast cells from pulmonary collectins, specifically surfactant proteins A (SP-A) and D (SP-D) (McCormack et al. 2003). Recent work has shown that SP-A and SP-D have direct fungicidal activity on *Histoplasma* by permeabilizing the yeast cells. SP-A knockout mice have increased susceptibility to *Histoplasma* infection in vivo (McCormack et al. 2003). This suggests that **phagocytic uptake is not only tolerated by *Histoplasma* but important for full virulence because it provides a shelter from soluble host defense molecules.** Unlike Fc-mediated phagocytosis of antibody-opsonized particles, complement receptor-mediated phagocytosis is generally noninflammatory unless accompanied by other stimulating signals (Aderem 2003).

B. Concealment of Pro-inflammatory Fungal Patterns

To reduce potential pro-inflammatory host responses, *Histoplasma* yeasts minimize cell wall β-glucan detection by concealing it beneath a layer of α-glucan. Macrophage-expressed

Dectin-1 is a critical pattern recognition receptor (PRR) for detection of fungal cell wall β-glucan and stimulates the antifungal responses of phagocytes (Goodridge et al. 2009; Drummond and Brown 2011). Dectin-1 detection of *Histoplasma* yeasts is necessary for pro-inflammatory cytokine production (Rappleye et al. 2007; Lin et al. 2010). However, most *Histoplasma* species synthesize α-linked glucan (α-glucan) in addition to β-glucans, and these two polysaccharides are organized in the yeast cell wall such that the β-glucans are concealed beneath the α-glucan layer (Rappleye et al. 2007). Functionally, **production of the yeast-specific α-glucan polysaccharide effectively masks the normally immunostimulatory cell wall β-glucans** (Fig. 7.1). Yeast cells unable to synthesize α-glucan are readily detected by Dectin-1 and these yeasts are severely attenuated in vivo (Rappleye et al. 2004, 2007).

Synthesis of α-glucan requires the function of an α-(1,3)-glucan synthase (Ags1) as well as an α-(1,4)-amylase (Amy1) (Rappleye et al. 2004; Marion et al. 2006). One species of *Histoplasma* (the North American type 2; NAm 2) lacks detectable α-glucan yet remains virulent. This species appears to express an alternate means of preventing β-glucan detection through an unknown mechanism (Edwards et al. 2011a).

Thus, the α-glucan polysaccharide produced by most species of *Histoplasma* facilitates stealthy entry of *Histoplasma* yeast into macrophages, thereby enhancing yeast survival.

In addition to preventing Dectin-1 detection, ***Histoplasma* yeast cells do not trigger Toll-like receptors (TLRs) expressed on the macrophage surface.** The most relevant TLRs that recognize fungal pathogen-associated molecular patterns (PAMPs) are TLR2 and TLR4. One possibility is that *Histoplasma* yeasts lack TLR ligands altogether. Consistent with this possibility are in vitro studies in which TLR2 and TLR4 do not play a role in macrophage interactions with *Histoplasma* yeasts (Lin et al. 2010). Similarly, TLR2 does not contribute to the interferon (IFN)-β response of macrophages to *Histoplasma* conidia (Inglis et al. 2010). On the other hand, biochemical fractions of *Histoplasma* yeast cell walls can stimulate lipid body formation by macrophages (which is correlated with leukotriene production), and this is dependent on TLR2, indicating that cell wall fractions contain PAMP(s) recognized by TLR2 (Sorgi et al. 2009). Together, these data suggest that *Histoplasma* yeast have TLR ligands but that they are hidden from immune cells in intact, wild-type yeasts in much the same way that cell wall β-glucans are veiled (Fig. 7.1). All of these studies used cultured phagocytes so the role of TLRs in vivo remains undetermined. It is unknown whether α-glucan might also be the cell wall factor that masks the TLR ligands.

III. Negation of Host Defenses

A. Elimination of Antimicrobial Reactive Oxygen

Interaction of *Histoplasma* yeasts with phagocytes variably induces production of reactive oxygen species (ROS), which *Histoplasma* must detoxify in order to survive. Stimulatory signals, including PRR recognition of PAMPs, cytokines, and other phagocytic signals, trigger assembly of the NADPH-phagocyte oxidase (phox), a multimeric protein complex that produces the initial ROS, superoxide (O_2^-). In response to *Histoplasma* yeasts, PMNs readily produce ROS (Schaffner et al. 1986; Schnur and Newman 1990; Kurita et al. 1991a, b) but macrophages only produce significant ROS upon cytokine activation (Eissenberg and Goldman 1987; Wolf et al. 1987, 1989; Fleischmann et al. 1990; Wolf and Massof 1990). Regardless, *Histoplasma* survives phagocyte-derived superoxide in vitro and in vivo by expression of a yeast-phase-specific, **extracellular superoxide dismutase, Sod3** (Youseff et al. 2012). *Histoplasma* Sod3 is both secreted and anchored on the yeast cell wall, positioning it optimally to destroy phagocyte-derived superoxide (Fig. 7.2). Intracellular superoxide dismutase (i.e., *Histoplasma* Sod1) does not provide protection against extracellular superoxide because the charge on superoxide renders this ROS unable to pass through biological membranes. Without Sod3, *Histoplasma* virulence is significantly attenuated and *Histoplasma* yeast survival against PMNs and

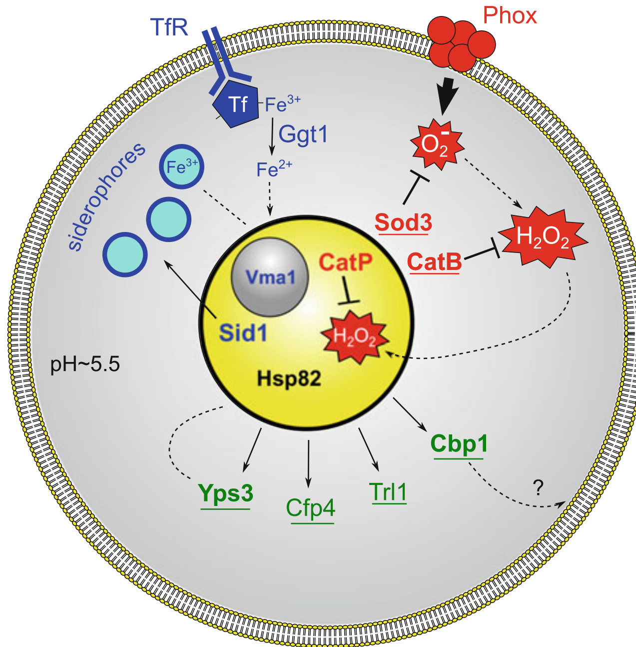

Fig. 7.2. Mechanisms facilitating *Histoplasma* yeast intracellular survival and proliferation within macrophage phagosomes. Demonstrated virulence factors are indicated in *bold* and those with pathogenic-phase-specific expression are *underlined*. *Histoplasma* yeasts extinguish phagocyte-produced ROS by production of extracellular oxidative stress defense factors (*red*). Assembly of the multimeric phagocyte NADPH-oxidase complex (Phox) in the phagosomal membrane leads to production of antimicrobial superoxide. This toxic ROS is eliminated by the extracellular *Histoplasma* superoxide dismutase (Sod3). Peroxide molecules are similarly destroyed by extracellular catalase (CatB). Residual peroxide that escapes CatB destruction and enters the yeast cell is eliminated by an intracellular catalase (CatP). *Histoplasma* yeasts have at least two strategies for essential iron acquisition (*blue*). Host iron is brought to the phagosome via transferrin after binding to the transferrin receptor (TfR). In one strategy, *Histoplasma* yeasts secrete a γ-glutamyl transferase (Ggt1), which utilizes GSH to reduce ferric to ferrous iron, promoting iron release from transferrin and allowing for import into yeast cells. In a second strategy, the *Histoplasma* ornithine monooxygenase (Sid1) initiates siderophore synthesis and siderophores are secreted into the phagosomal lumen to chelate iron. The action of the vacuolar proton-ATPase, of which Vma1 is a central subunit, maintains iron homeostasis in *Histoplasma* yeasts. *Histoplasma* yeasts also secrete several novel factors (*green*) with undefined roles in intracellular residence. Cbp1, a calcium-binding protein, is abundantly secreted by yeast cells and may interact with phagosomal lipids. Yps3 is a secreted factor that also binds to cell wall chitin. *Histoplasma* yeasts produce an extracellular thioredoxin reductase-like protein (Trl1) and the Cfp4 glycoprotein during infection of macrophages. Additionally, yeasts utilize the stress-response Hsp82 protein to ameliorate intracellular stresses

activated macrophages is reduced to similar levels as for wild-type *Candida*. Thus, **Sod3-dependent dismutation of host-derived superoxide contributes to the greater virulence of *Histoplasma* compared to opportunistic fungi.**

In addition to Sod3, *Histoplasma* yeasts express a yeast-phase-specific, **extracellular catalase, CatB**. CatB is equivalent to M-antigen, a major diagnostic exoantigen for histoplasmosis (Hamilton et al. 1990; Zancopé-Oliveira et al. 1999). Given the ability of CatB to destroy peroxide, many have assumed that CatB is essential to *Histoplasma* virulence. However, CatB-deficient *Histoplasma* yeasts are fully virulent (Holbrook et al. 2013). This is due to functional redundancy with the **intracellular *Histoplasma* catalase, CatP** (Holbrook et al. 2013). Production of either catalase is sufficient to protect *Histoplasma* from host-produced ROS. Nonetheless, expression of dual catalases may provide a more effective means of protecting ROS-sensitive intracellular targets (Fig. 7.2);

the phagocyte-derived ROS is efficiently destroyed by extracellular CatB before peroxide can enter the cell and affect intracellular targets. CatP, as an intracellular ROS defense, provides additional protection against any peroxide that escapes destruction by CatB. The combined activities of Sod3 and the dual catalase system effectively negate phagocyte-produced reactive oxygen defenses, thereby enabling yeast survival during infection.

B. Resistance to Reactive Nitrogen Compounds

Host defenses against fungal infections also include production of reactive nitrogen species (RNS), particularly by cytokine-activated phagocytes. Depletion or inhibition of the pro-inflammatory cytokines IFN-γ, TNF-α, and GM-CSF increases susceptibility to *Histoplasma* infection. This effect is correlated with impaired production of reactive nitrogen compounds, implicating RNS as one of the host defense mechanisms *Histoplasma* must combat (Lane et al. 1994; Allendoerfer and Deepe 1998; Deepe et al. 1999). Consistent with this, inhibition of RNS production by phagocytes in culture decreases their ability to control *Histoplasma* yeasts (Nakamura et al. 1994; Lane et al. 1994). However, the effect of phagocyte RNS on *Histoplasma* yeast is fungistatic, not fungicidal, suggesting that *Histoplasma* has defenses against the lethal effects of RNS. As candidate factors mediating this, 59 genes are upregulated in *Histoplasma* yeasts specifically in response to RNS treatment (Nittler et al. 2005). One of the induced genes is a mycelial-expressed nitric oxide reductase (Nor1) that, when ectopically expressed in yeasts, provides a slight benefit in vitro to yeast grown in the presence of nitric oxide. Whether Nor1 is necessary for the RNS resistance of *Histoplasma* yeasts remains to be determined.

C. Stress Response

In addition to ROS- and RNS-based host defenses, *Histoplasma* yeasts must contend with other stresses related to intracellular growth and host infection. A **heat-shock protein (Hsp82)**, which is homologous to proteins of the 90 kDa stress-response family, **facilitates intracellular growth as well as full virulence in vivo** (Edwards et al. 2011b). Although it is assumed that heat-shock proteins are essential for thermally dimorphic fungal adaptations, yeasts with reduced Hsp82 function grow normally at mammalian body temperature (37 °C). At febrile temperatures, *hsp82*-mutant yeasts are more inhibited than wild-type yeast cells. Thus, Hsp82 is not required for yeast growth at 37 °C but instead ameliorates stresses associated with infection that are not limited to high temperature growth.

IV. Essential Nutrients for Intracellular Growth

A. Iron Acquisition

Like all cells, *Histoplasma* yeasts require iron for essential life processes and, during infection, must acquire this element from the host. Host iron restriction, enhanced by IFN-γ that is produced during cell-mediated immunity or administration of iron chelators, restricts *Histoplasma* replication (Lane et al. 1991; Newman et al. 1994, 1995). To obtain limited iron, *Histoplasma* relies upon a number of iron acquisition strategies. Maintenance of the phagosomal pH at around 5.5 is thought to allow release of one of the two iron atoms from transferrin, yet this pH is not acidic enough for activation of lysosomal hydrolases. In addition, *Histoplasma* yeasts produce and secrete iron-scavenging hydroxamate siderophores, which can acquire iron from holotransferrin (Howard et al. 2000) (Fig. 7.2). Recent genetic studies have confirmed the importance of siderophore production in *Histoplasma* virulence; the **loss of siderophore production reduces *Histoplasma* yeast growth under iron-limiting conditions in vitro, and impairs yeast replication in cultured macrophages** by 50–75% (Hwang et al. 2008; Hilty et al. 2011).

Conflicting evidence exists for the requirement of siderophores in vivo. In one study, loss of siderophore production in the genetically tractable Panama strain

does not impair infection and replication of *Histoplasma* in murine lungs until 15 days post infection (Hwang et al. 2008). On the other hand, depletion of siderophores in the NAm 2 strain caused a nearly 100-fold reduction in lung fungal burden at 7 days post infection (Hilty et al. 2011). This discrepancy may result from the operation of alternative iron acquisition mechanisms in some *Histoplasma* strains. In support of this, the genome of the Panama strain, but not the NAm 2 strain, contains *FET3* and *FTR1* genes that encode a high-affinity iron transport system (Hilty et al. 2011).

Although iron is an essential cofactor, excess iron has detrimental effects on cells, and consequently iron acquisition must be tightly regulated. Iron limitation induces both transcriptional (Hwang et al. 2008) and protein level changes (Winters et al. 2008) in *Histoplasma* yeast. A cluster of genes repressed by the iron-responsive GATA transcription factor, Sre1, encode enzymes responsible for siderophore synthesis in response to iron limitation (Hwang et al. 2008, 2012). The enzyme ornithine monooxygenase (encoded by the *SID1* gene) that catalyzes the first committed step in siderophore biosynthesis is included in this iron-regulated gene cluster. Iron homeostasis in *Histoplasma* also depends on vacuolar pH because loss of the vacuolar ATPase subunit Vma1 or treatment with chemicals that block vacuolar acidification (e.g., bafilomycin) impair growth under iron-limiting conditions (Hilty et al. 2008).

Histoplasma yeasts preferentially utilize ferrous iron (Zarnowski et al. 2008b) and have multiple ferric ion reducing systems (Fig. 7.2). Each of these ferric reducing systems is extracellular, consistent with reduction of ferric ions to promote release from extracellular siderophores and host iron-carrying molecules such as transferrin. Early biochemical studies indicated that yeasts produce three extracellular ferric reductases, two soluble and one cell-associated (Timmerman and Woods 1999, 2001). The secreted glutathione-dependent ferric reductase has now been identified as a γ-glutamyl transferase (Ggt1), which is produced by *Histoplasma* yeast cells and catalyzes a two-step, pH-independent reduction of ferric iron (Zarnowski et al. 2008a) Depletion of Ggt1 by RNAi impairs the ability of *Histoplasma* yeasts to replicate and kill cultured phagocytes but the requirement for Ggt1 in vivo remains to be determined.

B. De Novo Vitamin Biosynthesis

Growth of *Histoplasma* yeasts in the nutrient-limiting phagosome of phagocytes requires de novo vitamin synthesis. Growth in chemically defined, minimal media confirms that ***Histoplasma* yeasts can synthesize the essential cofactors riboflavin, niacin, pantothenate, pyridoxine, biotin, and folate** (McVeigh and Morton 1965). On the other hand, mammals are unable to synthesize these vitamins and must acquire them from dietary sources. Yeast cells unable to synthesize their own riboflavin are severely compromised for intracellular growth (Garfoot et al. 2013). These yeasts are similarly unable to replicate following lung infection, indicating that dietary vitamins of the host are unavailable to intracellular yeasts in vivo, therefore mandating that *Histoplasma* yeasts synthesize them de novo. This suggests that targeting vitamin biosynthetic enzymes is a viable strategy for development of new therapeutics.

V. Secretion of Novel Factors

To influence the outcome of the host–pathogen interaction, *Histoplasma* yeasts secrete several novel factors with as yet unidentified functions (Fig. 7.2). Each of these factors is preferentially expressed by pathogenic-phase cells compared to nonpathogenic mycelia, arguing that their function is fundamentally linked to pathogenesis. The secreted **Cbp1 protein was the first virulence factor identified** and, without it, yeasts are significantly attenuated in their ability to establish respiratory infection (Sebghati et al. 2000). Originally described as a calcium-binding protein (Batanghari and Goldman 1997), Cbp1 has nanomolar affinity for calcium (Beck et al. 2008). The Cbp1 protein is a homodimer that is held in a compact structure by

intramolecular disulfide bonds, making Cbp1 highly resistant to protease treatment, a characteristic that suits it well for function in the macrophage phagosome (Beck et al. 2008). Indeed, Cbp1 is secreted into the lumen of the phagosome by yeast cells (Kügler et al. 2000). The α-helices of Cbp1 have structural similarity to lipid-binding saposin-family proteins, which suggests that Cbp1 interacts with host membranes or glycolipids of the phagosome (Beck et al. 2009).

Histoplasma yeasts secrete the **Yps3 protein,** although production is restricted to the North American (NAm 2) phylogenetic group (Keath et al. 1989; Keath and Abidi 1994; Bohse and Woods 2007a). Restricted production results from transcriptional regulation because replacement of the promoter of the *YPS3* gene with a constitutive promoter restores production of Yps3 in *Histoplasma* cells that normally do not synthesize it (Bohse and Woods 2007a). Yps3 has homology to the Bad1 *Blastomyces* adhesin protein and, like Bad1, Yps3 is both secreted and associated with the cell wall through interactions with chitin (Weaver et al. 1996; Bohse and Woods 2005). However, Yps3 does not share the adhesin functions of Bad1 and, although Yps3 has internal tandem repeats, Yps3 lacks the tryptophan-enriched tandem repeat domains of Bad1 that have immunomodulatory function. Functionally, Yps3 is dispensable for pulmonary colonization, but depletion of Yps3 by RNAi impairs the ability of yeasts to establish disseminated infections (Bohse and Woods 2007b). At the cellular level, no function has yet been identified for either soluble or cell-associated Yps3.

A more systematic effort to identify the proteins comprising the secreted proteome of *Histoplasma* yeasts has revealed additional factors with potential roles in *Histoplasma* virulence (Holbrook et al. 2011). The secreted proteome includes the Sod3, CatB, and Cbp1 factors described above. Additional prominent factors in the extracellular fraction include glycanases (exo- and endoglucanases, Exg1 and Eng1, respectively) as well as a chitinase (Chs1), which probably contribute to yeast cell wall remodeling. The secreted proteome has at least five novel culture filtrate proteins (Cfp). Chief among these is Cfp4, a copiously produced, heavily glycosylated factor uniquely expressed by yeast-phase cells. *Histoplasma* yeasts also secrete a thioredoxin reductase-like protein (Trl1), which is distinct from intracellular thioredoxin reductases. Although no functional role has been determined for the glycanases, Cfp4, or Trl1, expression of each factor is linked to the pathogenic phase and expression is maintained during infection (Holbrook et al. 2011). Given that these factors share characteristics common to the vast majority of demonstrated virulence factors (i.e., extracellular localization and pathogenic-phase regulated expression), the identified secreted proteome is a rich source of candidate factors that promote *Histoplasma* virulence. In addition to the canonical eukaryotic secretion pathway, *Histoplasma* also secretes vesicles (Albuquerque et al. 2008), which may be a secondary means of delivering factors to the extracellular environment. Nevertheless, not all secreted factors are necessarily important for virulence, as shown by the lack of any attenuation when the secreted dipeptidyl peptidase (DppIV) is depleted (Cooper and Woods 2009).

VI. Conclusions

Histoplasma capsulatum's success as a pathogen of mammals is due first and foremost to its ability to survive host immune defenses. The intracellular nature of *Histoplasma* pathogenesis provides a shelter from soluble immune molecules but also presents challenges because phagocytes are the primary effector cells for elimination of fungi. The virulence factors recently identified though molecular genetic advancements in *Histoplasma* reveal **a twofold strategy used by yeast cells to overcome this challenge: avoidance of host defenses and neutralization of antifungal reactive oxygen.** Stealthy entry into macrophages relies upon binding of yeasts to complement receptors while at the same time using an α-glucan polysaccharide to conceal fungal molecules from the phagocyte pattern recognition receptors. Protection from phagocyte-derived reactive oxygen

critically depends on expression of ROS-destroying enzymes by *Histoplasma* yeasts. These major mechanisms all employ factors that are characterized by pathogenic-phase-specific production as these tasks are unique requirements for the pathogenic phase. Furthermore, the extracellular localization of these factors positions them to directly affect the host–pathogen interaction. Identification of additional factors that share these two features will probably reveal additional mechanisms of *Histoplasma* pathogenesis.

Once the task of survival has been met, *Histoplasma* pathogenesis depends on acquisition of essential nutrients for growth and replication. Identification of siderophore production and a number of iron reductases that are necessary for full virulence highlight the central role iron restriction plays in host defense and the mechanisms employed by *Histoplasma* yeasts to attain this critical element. Beyond iron, we know little about the metabolism of yeasts within the nutrient-poor phagosome, but continued identification of genes required for intracellular replication should help decipher the metabolic process and the intracellular resources exploited by *Histoplasma* to enable intracellular growth. Various novel factors such as Cbp1 and the Cfp proteins will require additional characterization to uncover the roles they play in pathogenesis. Nonetheless, their yeast-phase-specific expression and their secretion from yeasts suggests that they will somehow affect the host cell or host cell defenses. Mechanistic studies on these factors and application of functional tests on additional candidates discovered will continue to reveal the mechanisms underlying *Histoplasma*'s success as an intracellular primary fungal pathogen.

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