



Heat Shock Proteins in *Histoplasma* and *Paracoccidioides*

Levi G. Cleare,  Daniel Zamith-Miranda, Joshua D. Nosanchuk

Department of Medicine (Division of Infectious Diseases) and Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA

ABSTRACT Heat shock proteins (Hsps) are highly conserved biomolecules that are constitutively expressed and generally upregulated in response to various stress conditions (biotic and abiotic). Hsps have diverse functions, categorizations, and classifications. Their adaptive expression in fungi indicates their significance in these diverse species, particularly in dimorphic pathogens. *Histoplasma capsulatum* and *Paracoccidioides* species are dimorphic fungi that are the causative agents of histoplasmosis and paracoccidioidomycosis, respectively. This minireview focuses on the pathobiology of Hsps, with particular emphasis on their roles in the morphogenesis and virulence of *Histoplasma* and *Paracoccidioides* and the potential roles of active and passive immunization against Hsps in protection against infection with these fungi.

KEYWORDS heat shock proteins, fungi, histoplasmosis, paracoccidioidomycosis, dimorphic fungal pathogen, immunotherapeutics, immunotherapy

Fungal diseases are a major global public health problem resulting in at least 1.5 million deaths annually (1), and morbidity and mortality are particularly increased in immunocompromised individuals (2) (<https://www.cdc.gov/fungal/global/index.html>). Certain fungi undergo morphogenesis from an environmental form to a distinctly different morphotype in hosts, which complicates the host responses to these pathogens. *Histoplasma capsulatum* and *Paracoccidioides* spp. are two major examples of dimorphic fungal pathogens that cause significant disease in both immunocompetent and compromised humans, with severe disease occurring more frequently in individuals with cellular immunodeficiencies. Heat shock proteins (Hsps) are ubiquitous proteins that can be activated during the morphogenic transitions in these pathogens, facilitating the adaptation of these fungi and promoting their capacity to evade host effector responses. This minireview focuses on the pathobiology of Hsps, specifically Hsp60, Hsp70, and Hsp90, with particular emphasis on their roles in morphogenesis and virulence of *Histoplasma* and *Paracoccidioides* and the potential roles of active and passive immunization against Hsps in protection against infection with these fungi.

HEAT SHOCK PROTEINS

Hsps are ubiquitously present in cells (in the cytosol, mitochondria, endoplasmic reticulum, nucleus, and cell membrane) (3). These highly conserved biomolecules are constitutively expressed and generally upregulated in response to various stress conditions (biotic and abiotic) (4). Hsps have diverse functions and are categorized based on both mass and function. The major roles of Hsps involve cell cycle progression and transcriptional and posttranslational processes, such as protein folding, stability, transportation, and degradation, and they are also involved in the activation of diverse key signal transducers in fungi (5, 6). Additionally, Hsps participate in regulatory pathways and behave as molecular chaperones for other cellular proteins (5).

The molecular mass ranges of fungal Hsps are from 15 to 110 kDa (5, 7). The larger Hsps are categorized as belonging to the 100-kDa, 90-kDa, 70-kDa, or 60-kDa family (8). The Hsps with molecular masses of less than 43 kDa are known as small Hsps. Based on

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Address correspondence to Joshua D. Nosanchuk, josh.nosanchuk@einstein.yu.edu.

functions, Hsps are categorized as chaperones (Hsp70 and -60), proteins with catalytic activity (proteases, Hsp100, ubiquitin, and tyrosine phosphatase), and proteins with unknown functions ("alpha"-crystalline and secreted glycoproteins) (9).

The Hsp60 protein family members, also called chaperonins, form a complex in the cytosol of prokaryotes and eukaryotes, as well as in several eukaryotic organelles (10). The family is also called the mitochondrial chaperones. Hsp60 protein is the yeast equivalent of *Escherichia coli* GroEL, a molecular chaperone that mediates the formation of the native conformation of proteins and in eukaryotes promotes folding of proteins in the mitochondrial matrix (11–13).

In fungal organisms, the Hsp70 protein family typically consists of at least 10 proteins with molecular masses of approximately 70 kDa. They are composed of two functionally separate domains, one of 44 kDa responsible for ATP binding (the N-terminal ATPase) and the other of 18 kDa (C-terminal protein binding domain) (10, 11). As chaperones, the Hsp70 proteins participate in several processes, which are important for both dimorphic and nondimorphic pathogenic fungi (10).

Hsp90 protein is a specialized ATP-dependent molecular chaperone present both in bacteria, in which they are referred to as HtpG, and in eukaryotes. Hsp90 protein assists the folding of a select set of proteins, related primarily to signal transduction. The characteristic target proteins are steroid hormone receptors and protein kinases. Although the gene encoding HtpG is not an essential gene, all gene disruptions of eukaryotic Hsp90s have been found to be lethal, including in *Saccharomyces cerevisiae* and *Candida albicans*, providing clear evidence for the importance of this molecular chaperone in fungi (10, 14, 15). Hsp90 protein is a dimeric complex, and it is highly dependent on several cochaperones to perform its functions (10, 11).

DIMORPHIC FUNGI

Fungi encompasses a diverse kingdom involving filamentous and nonfilamentous organisms, which can be classified on the basis of morphology, growth and development, reproduction, evolution, and pathogenicity (16, 17). Some fungi are able to thrive only in unique environments, while others survive across extremely different environments. Dimorphism plays an important role in responding effectively to environmental changes. Fungi that qualify as dimorphic have the ability to convert between two discrete morphologies, each associated with specific environmental conditions. Certain dimorphic fungi are endemic to specific geographic regions and are referred to as endemic dimorphic fungi. The major medically important endemic dimorphic fungi include *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* (*Paracoccidioides* spp.), *Blastomyces dermatitidis*, *Coccidioides immitis* and *Coccidioides posadasii* (*Coccidioides* spp.), and *Talaromyces (Penicillium) marneffeii* (18). Acquisition, typically via inhalation, of dimorphic pathogens by humans may be asymptomatic or may cause disease ranging from self-limiting infections to systemic, life-threatening mycoses (18).

At ambient temperatures, typically between 20 and 25°C, dimorphic fungi normally exist as filamentous molds (mycelia) and grow as saprobes in the environment (18, 19). At the physiological temperature of the mammalian body (37°C), dimorphic fungi undergo a phase transition to nonfilamentous forms, with most adopting a yeast-like shape, except for *Coccidioides* spp., which form spherules. This unique correlation between fungal morphology and pathogenesis is the rationale behind many of the studies designed to identify fungal virulence factors. The virulence of dimorphic fungi is affected by factors that are responsible for the transition from the mycelial phase to the yeast form (18). As such, Hsps have a major role in pathogenesis because of their involvement in modulating cellular responses to changes in the temperature associated with the transition from soil to a mammalian host. Besides temperature shifts, fungal Hsps are also induced by changes in pH levels, oxidative stress, osmotic stress, starvation, or antifungal stress (18).

HISTOPLASMOSIS

Histoplasma capsulatum is a significant cause of mortality and morbidity on the global stage, and it is the most common cause of fungal pneumonia in the United States (20). Severe histoplasmosis occurs in both immunocompetent and immunocompromised individuals, although there is a greater incidence in immunosuppressed individuals (e.g., those affected by cancer, steroids, tumor necrosis factor alpha [TNF- α] inhibition, or HIV). Fortunately, the incidence of life-threatening histoplasmosis in individuals with HIV has fallen in the United States due to the broad availability of potent antiretroviral regimens (21). However, this mycosis remains a major killer in less-developed regions (22), particularly in Latin American countries, including Brazil (23, 24), Guatemala (25), and French Guiana (26). Similarly to *Paracoccidioides* spp., *H. capsulatum* grows as a multicellular saprobic mycelium at 25°C and as a unicellular pathogenic yeast at 37°C (27). Phase transition of *H. capsulatum* is necessary for its pathogenicity, and a heat shock-like phenomenon occurs during the mycelium-to-yeast transition of the pathogens, wherein Hsps are induced soon after the temperature shift (28).

PARACOCCIDIOIDOMYCOSIS

Paracoccidioidomycosis (PCM) is the most prevalent systemic mycosis in Latin America and is estimated to infect approximately 10 million people (29, 30). This disease is responsible for more than 50% of deaths associated with fungal infection in Brazil (23, 24). PCM is caused by fungi within the *Paracoccidioides* genus, which comprises four distinct phylogenetic lineages known as PS2, PS3, S1, and Pb01-like. Pb01 is morphologically and genetically distinct from the other lineages, which has led to its designation as *Paracoccidioides lutzii*, while the others are designated together as the species *Paracoccidioides brasiliensis*. However, the pathogenesis and disease manifestations of *P. brasiliensis* and *P. lutzii* are indistinguishable at present (31). *Paracoccidioides* spp. grow as a multicellular saprobic mycelium in the environment and as a unicellular pathogenic yeast *in vivo* (32, 33). During *in vitro* growth under experimental conditions, phase transitions are triggered when the incubation temperature is shifted from ambient to mammalian body temperature or in the reverse direction (34). The conversion of the inhaled airborne propagules to the yeast form occurs primarily within the lungs (35–37), and this transition is essential for the establishment of infection (38). After deposition in the lung, this alteration is driven largely by the shift in temperature. Hsps are expressed in large amounts by fungal cells in response to rapid increases in temperature and other types of environmental stress (39). These proteins are implicated in several biological processes, where the dimorphic transition is of particular interest. Another important issue concerning these proteins is their participation in the immunopathogenicity of disease (10). A database of mycelial and yeast tags, termed PbAESTs (*P. brasiliensis* assembled expressed sequence tags), from a partially sequenced transcriptome of *P. brasiliensis* has identified 438 ESTs (184 in mycelium and 253 in yeast) encoding *P. brasiliensis* molecular chaperones and their cochaperones, which were clustered in 48 genes. These genes have been classified in families, corresponding to three small chaperones, nine Hsp40s, 10 Hsp60s, seven Hsp70s, five Hsp90s, four Hsp100s, and 10 other chaperones (10).

FUNGAL MYCOSES AND HSPs

Hsp60. Members of the *Histoplasma* Hsp60 protein family have been implicated as immunoprotective antigens, and native and recombinant *Histoplasma* Hsp60 proteins can effectively immunize mice against recurrent challenges with the fungus (40, 41). In humans, Hsp60 protein has also been shown to induce a proliferative response in lymphocytes from histoplasmin-reactive individuals (42). Histoplasmin is a detergent extract from the cell wall and cell membrane of *H. capsulatum* that is antigen rich and includes significant quantities of an Hsp60 protein family member. These findings have important diagnostic and therapeutic implications. Hsp60 protein is the major cell recognition ligand between *H. capsulatum* yeast cells and CD11b/CD18 (CR3) on host

phagocytes (43), underscoring its importance in the pathogenesis of this fungus. Vaccination of three different mouse strains with *Histoplasma* Hsp60 protein conferred protection against subsequent lethal challenge with *H. capsulatum* yeast cells administered intravenously (44). Subsequently, immunization with *Histoplasma* rHsp60 was similarly shown to protect against lethal intranasal pulmonary histoplasmosis (41). The induction of the protective cellular responses by rHsp60 vaccination is linked to the activation of a subset of CD4⁺ T lymphocytes (44, 45), but during infection (effector phase), CD4⁺ and CD8⁺ subsets can have redundant roles in the control of disease (45).

In *Paracoccidioides* spp., a recombinant Hsp60 protein (rHsp60) has been investigated for its reactivity against sera from infected patients. That study looked at serum samples from patients with diagnosed mycoses (75 with PCM, 26 with histoplasmosis, 8 with sporotrichosis, 8 with aspergillosis, and 4 with cryptococcosis). Of the 75 serum samples from *P. brasiliensis*-infected patients tested against the rHsp60 from *P. brasiliensis*, 73 serum samples (97.3%) recognized rHsp60 protein (46). The rHsp60 did not react with sera from patients infected with aspergillosis, cryptococcosis, sporotrichosis, or tuberculosis but reacted with 3 of the 26 sera from patients with histoplasmosis (11.5%). This same recombinant *Paracoccidioides* Hsp60 protein (rHsp60) was investigated by a different group to evaluate the cross-reactivity between the mycoses mentioned above, and the results indicated that significant cross-reactivity (27%) occurred (47). This cross-reaction may have occurred due to the high conservation of Hsp genes and their resulting proteins in these fungi. Nevertheless, the high rate of false positivity suggests that the presented Western blot technique using *Paracoccidioides* Hsp60 protein was not a reasonable approach for PCM diagnosis.

Hsp70. Early during the mycelium-to-yeast transition upon temperature shift, levels of *Histoplasma* Hsp70 are rapidly elevated during the first hour, followed by a return to constitutive levels by about 6 h, implying that Hsp70 protein may be required only for the initial adaptation to higher-temperature growth in this fungus (19, 48). Indeed, Hsp60 and Hsp70 protein family members are among the fungal antigens that are most highly recognized by antibodies in sera from histoplasmosis and PCM patients, confirming fungal expression and immunogenicity of Hsps during infection (18, 39, 41, 46, 49, 50). Because of their immunogenicity, Hsp60 and Hsp70 proteins are suggested to be important proteins that are expressed in the pathogenesis of these fungi and have also been discussed as potential candidates for immunotherapy, as they are major targets of the host immune response during infections (51). Splenocytes from mice immunized with the rHsp70 gene from *H. capsulatum* present a proliferative response to subsequent challenge with the recombinant protein. Although rHsp70 is able to induce a lymphoproliferative response in previously immunized mice, it fails to confer protection to a second challenge with *H. capsulatum* (52).

In *Paracoccidioides* spp., Hsp70 protein is constitutively expressed during sporulation as well as induced during heat shock (53). The Hsp70 gene of *P. brasiliensis* has high sequence homology to other Hsp70 genes from dimorphic fungi such as *H. capsulatum*. *P. brasiliensis* has low levels of Hsp70 mRNA in the mycelium form at 26°C but has the same amount of protein in both cell types, mycelium and yeast (53). Hsp70 protein appears to be expressed optimally at normal human temperature, ~37°C. Interestingly, increasing the yeast incubation temperature to 42°C results in downregulation of the expression of most cellular proteins, but there is a predominant expression of only four proteins that could be Hsps of different families, with the 70-kDa protein by far being the major species (53). The temperature-induced mycelium-to-yeast transition is concomitant with an increase in the level of Hsp70 mRNA transcripts and protein. This behavior suggests that Hsp70 protein expression is probably related to both cellular differentiation and a heat shock response as a result of temperature shift (53). The alterations in the expression of Hsp70 protein having a direct relation to cellular differentiation suggests that this process can be targeted for antifungal drug research.

Hsp90. In *H. capsulatum*, a heat-inducible Hsp82 (an Hsp90 protein) that is most actively transcribed at 37°C during morphogenesis initiated from the mycelium phase

has been identified (54). Screening of a mutant *H. capsulatum* library revealed that yeast cells with disruptions in the Hsp82 gene had relatively normal growth phenotypes at 37°C and under salinity and acid stressors, but the mutant yeast cells were significantly attenuated in cocultures with macrophages (55), which suggests the importance of the Hsp82 protein for survival during host-pathogen-associated stress and, thus, in *H. capsulatum* virulence.

Hsp90 protein has been detected on the cell surfaces of hyphae, including those of *P. brasiliensis* (11, 56). In *Paracoccidioides* spp., Hsp90 protein regulates the proliferation and adaptation of the fungus to different environmental conditions (57, 58), and it may assist yeast cells in coping with a variety of physiological stresses, including temperature, host interaction, and oxidative injury (15). For example, exposure of *Paracoccidioides* spp. to oxidative agents induces the expression of Hsp90 protein (15, 57, 59). This fungus encounters similar conditions after phagocytosis and residence within a macrophage's phagolysosome (15), which suggests that Hsp90 protein plays an important role for *Paracoccidioides* spp. through the regulation of molecular events that enhance the fungus's capacity to combat harsh intracellular conditions, such as low pH and oxidative stress. This notion is strengthened by the findings that mRNA expression of the Hsp90 gene is higher in *Paracoccidioides* yeast than in mycelia and that gene expression is upregulated during the early phase of the mycelium-to-yeast transition (60). Although Hsp90 protein regulates reactive oxygen species (ROS) levels from the fungal cell, additional factors have been identified. One such mechanism is through calcineurin-dependent functions. Pharmacological blocking of Hsp90 proteins and/or calcineurin revealed that Hsp90 proteins and calcineurin act synergistically to regulate morphogenesis from the mold to the yeast form of *P. brasiliensis*, whereas neither is required for the yeast-to-mold transition (60). Blocking of Hsp90 proteins also inhibits yeast cell proliferation but not mycelial development. Hsp90 protein is an immunodominant antigen in *Paracoccidioides* spp. and is associated with responses that facilitate cellular survival in the setting of various environmental stresses (15, 57, 58, 60, 61). The fact that Hsp90 protein is linked to the transition from mycelium to yeast further supports the targeting of this protein in therapeutics.

ANTIFUNGALS AND HSPs

In *Candida albicans*, Hsp90 protein plays a fundamental role in the control of cellular morphology. The inhibition of Hsp90 protein sensitizes the fungi to azoles. Fluconazole, which is fungistatic to *C. albicans*, becomes fungicidal when Hsp90 protein is absent or impaired. The proper functioning of Hsp90 protein in *C. albicans* enables the development of a drug-resistant phenotype, while its inhibition abrogates the emergence of resistance (60, 62). In fact, Hsp90 proteins have been targeted with a recombinant antibody (Mycograb), which shows intrinsic antifungal activity and synergy with amphotericin B *in vitro* against both *Candida* spp. (63, 64) and *Cryptococcus neoformans* (65) and provided a significant survival benefit in a clinical trial for systemic candidiasis (66).

MAbs AGAINST HSPs

As represented by the work with Mycograb, since Hsps are conserved immunodominant antigens that can evoke cellular and humoral responses during infection, they have emerged as a potential therapeutic target of monoclonal antibodies (MAbs) for dimorphic fungi, as some are expressed on the fungal cell surface (31, 46, 51, 67). Notably, protective MAbs against surface components of several important fungi, such as *Cryptococcus neoformans*, *Aspergillus fumigatus*, *C. albicans*, and *H. capsulatum*, have been successfully identified (31, 68, 69).

A panel of MAbs against *H. capsulatum* Hsp60 proteins that effectively modify the pathogenesis of murine histoplasmosis to the benefit of the host has been generated (70). Interestingly, a single nonprotective isotype IgG2b, named MAb 7B6, enhances *Histoplasma* replication after being phagocytosed by macrophages. In contrast, the protective IgG1 and IgG2a MAbs, named 6B7, increase the rate of phagolysosomal

fusion, resulting in enhanced fungal killing, antigen processing, and immune activation (70), which are activities similarly achieved by MAbs to certain other cell surface antigens (71, 72).

Another study examined how these same antibodies could affect the contents of *H. capsulatum* extracellular vesicles when bound to Hsp60 protein. Diverse fungal species release extracellular vesicles, indicating that this process is linked to a common pathway for the delivery of molecules to the extracellular space (73). Fungal extracellular vesicles have been recognized as important structures for the trans-cell-wall transport of virulence factors that modulate the host's immune responses (74). The results reveal that treatment of *H. capsulatum* with MAb 6B7 or 7B6 results in differences in the quantities of specific enzymes in vesicles, as well as protein and lipid contents, suggesting that these antibodies modulate the production, trafficking, and release into the extracellular space of important fungal virulence factors. The alteration of several proteins concomitantly is important, as modification of single proteins may not significantly impact pathogenicity (73).

Given the high level of conservation among the dimorphic fungal Hsp family, and particularly between *H. capsulatum* and the *Paracoccidioides* sp. complexes (31, 75), testing the effects of MAbs generated to *H. capsulatum* Hsp60 protein in an experimental *P. lutzii* infection model was also evaluated. Interestingly, MAbs that were either protective (IgG2a) or nonprotective (IgG2b) in *H. capsulatum* were both protective in a validated experimental PCM experimental model (31). Hence, MAbs to Hsp on fungal cell surfaces have been demonstrated to be effective in different pathogen and host models, which supports further investigation of these proteins as therapeutic targets.

CONCLUSION

Hsps are highly conserved proteins that have diverse regulatory functions, several of which are clearly linked to the pathobiology of important dimorphic fungal pathogens. Given their roles, particularly in response to heat shock stressors, they are intimately involved in the phase transition of dimorphic pathogenic fungi. Hsp functions have been definitively associated with fungal virulence, and targeting of Hsps through vaccination approaches that activate host effector responses or through passive immunization with MAbs has been shown to protect hosts against dimorphic fungi in several animal models. Continued investigations targeting Hsps and their functions are necessary for the development of better therapeutic approaches to these diseases, which continue to carry significant rates of morbidity and mortality.

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