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BRIEF COMMUNICATION

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Expression of Hsp60 and its cell location in *Paracoccidioides brasiliensis*

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ABSTRACT

Paracoccidioides species cause paracoccidioidomycosis (PCM), a systemic mycosis highly prevalent in Brazil. Therapy of PCM has some issues that make studies for new therapeutic and vaccine targets relevant, such as the *P. brasiliensis* 60-kDa-heat-shock protein (PbHsp60), an immunogenic antigen that induces protection in experimental mice infection. Here, we investigated the relative expression of mRNA for PbHsp60 in *P. brasiliensis* in the different morphotypes of *P. brasiliensis* and in morphological transition phases. In addition, antibodies to rPbHsp60 were produced and used to analyze the location of PbHsp60 in yeast and hyphae by electron microscopy. The analyses showed a substantial increase in the relative amounts of *HSP60* mRNA in yeast when compared to mycelium and an intermediate expression in transitional forms. Regarding the cell location, immunoelectron microscopy analysis revealed that PbHsp60 is within the cell wall. These observations suggest that this protein may be involved in the maintenance of the cell wall integrity and the interaction with the host for colonization, infection and pathogenesis.

KEYWORDS: *Paracoccidioides brasiliensis.* Heat-shock proteins. Hsp60. Subcellular localization.

INTRODUCTION

Besides *Paracoccidioides brasiliensis* and *P. lutzii*, which are undoubtedly recognized causative agents of paracoccidioidomycosis (PCM), Turissini *et al.*[1] have recently proposed to elevate three cryptic phylogenetic species to taxonomic species – PS2 for *P. americana*, PS3 for *P. restrepiensis*, and PS4 for *P. venezuelensis*. PCM is an endemic systemic mycosis in Latin America and highly prevalent in Brazil, where it is the leading cause of mortality among systemic mycoses and the eighth cause of deaths among chronic infectious diseases. In Brazil, about 49% of systemic mycoses were associated with PCM in the period between 1998 and 2006[2,3].

Cellular components from *Paracoccidioides* spp. have been widely studied as inducers of protective cellular immune response. Some of these components are immunogenic antigens that have been considered for the development of an alternative immunotherapeutic method, such as gp43 and its P10 peptide[4,5] and the heat shock protein 60 (Hsp60)[6,7]. Hsp60 has been successfully explored as a potential immunoprotective antigen against infections caused by *P. brasiliensis* and *P. lutzii*[6,8].

Regarding the fungal biology, Hsps have a prominent role, especially in dimorphic fungi, and are produced in response to shifts of temperature and pH, antifungal and oxidative stress, starvation and osmotic stress[9]. Additionally, Hsps

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have been implicated in cell protection, protein translocation across cellular membranes (chaperones), growth and differentiation of cells[10]. Different researchers have focused their analysis on Hsp60 from *P. brasiliensis* and *P.* lutzii[6-8,10]. Izacc *et al.*[10] showed higher Hsp60 expression in *P. brasiliensis* yeast cells than in mycelium by two-dimensional gel electrophoresis analysis. These authors suggest an essential role of this protein in the survival of fungi at host temperature. Furthermore, the authors showed increased Hsp60 expression during the transition from mycelium to yeast and decreased expression during the conversion to mycelium, i.e., Hsp60 appears to have a crucial role in *Paracoccidioides* morphogenesis[10].

Here, we quantified the heat-regulated *HSP60* mRNA relative expression in the different morphotypes (mycelium and yeast) and morphological transition phases (mycelium-to-yeast and yeast-to-mycelium) of *P. brasiliensis*. Moreover, we determined the cellular location of the Hsp60 protein in *P. brasiliensis* cell wall in mycelium and yeast morphotypes.

MATERIALS AND METHODS

Mice and ethics statement

Male BALB/c mice, 6-8 weeks old, weighing 20-25 g (n = 5/group) were obtained from the Animal Facility of University of Sao Paulo (USP) at Ribeirao Preto campus and maintained at the Animal House of Ribeirao Preto Medical School, USP. This study was performed following the ethical principles of animal research adopted by the Brazilian Society of Laboratory Animal Science and was approved by the Ethics Committee on Animal Use of the Ribeirao Preto Medical School, USP (protocol Nº 146/2007).

P. brasiliensis culture

Yeast cells of *P. brasiliensis* strain 18 (Pb18) were cultured at 37 ºC in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St Louis, USA), under stirring at 100 rpm. Studies with the different morphological and transition phases were performed as described previously[11]. Briefly, yeast cells and mycelial forms were cultured at 37 °C and 25 ºC, respectively, for at least 7 days. The transition phase from mycelium-to-yeast was induced by culturing the mycelia at 37 °C for 24 h. The transition from yeast-to-mycelium was obtained by growing yeast at 25 °C for 24 h. One aliquot of each *P. brasiliensis* culture was analyzed by optical microscopy to verify the fungal morphology.

Differential expression of *HSP60* mRNA in *P. brasiliensis*

The *HSP60* gene expression profile in the different morphological and transition phases of *P. brasiliensis* was analyzed by real-time PCR (qPCR). The total RNA from the *P. brasiliensis* was extracted using TRIzol (Thermo Fisher Scientific, Waltham, USA) as described previously[11]. First-strand cDNA was synthesized using 1 µg of total RNA with oligo (dT)12−18 primers (Thermo Fisher Scientific, Waltham, USA) and SuperScript III reverse transcriptase (Thermo Fisher Scientific). Real-time PCR was performed using the Kit Platinum SYBR Green qPCR SuperMix-UDG with ROX (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instructions. Specific primers were used for the *HSP60* gene: 5'-GATACCAAGGCGCAGAAGGT-3' (sense) and 5'-GGTGAAAACAGT GGCGTTGG-3' (antisense). Fold changes in mRNA expression were calculated using the $2^{-\Delta Cq}$ formula, where ΔCq is the difference in the threshold cycle (Cq) between the Hsp60 (target) gene and the β-actin or α-tubulin reference genes. The primer sequence of the β-actin and α-tubulin genes were: 5'-GGATGAGGAGATGGATTATGG-3' (sense) and 5'-GA AACACTCGACGCACACGAC-3' (antisense); and 5'-GTGGACCAGGTGATCGATGT-3' (sense) and 5'-ACCCTGGAGGCAGTCACA-3' (antisense), respectively.

Production of anti-rPbHsp60 antibody

Recombinant PbHsp60 (rPbHsp60) was obtained from pET28a–*HSP60* vector-transformed *Escherichia coli*, as described previously.[7] Protein concentration was determined using the Pierce Coomassie Plus (Bradford) assay kit (Thermo Fisher Scientific) and the preparation homogeneity was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protean Tetra System (Bio-Rad, Hercules, CA, USA). Protein bands were excised and homogenized in phosphate-buffered saline, pH 7.2 (PBS), in a concentration of about 200 µg/mL, and emulsified in an equal volume of complete (CFA) or incomplete (IFA) Freund's adjuvant (Sigma-Aldrich). BALB/c mice were initially injected subcutaneously with 100 µL of antigen-CFA emulsion and afterward weekly with a booster of 100 µL of antigen-IFA emulsion. One week after the third boost, mice were bled and the sera containing polyclonal anti-rPbHsp60 antibodies were pooled and stored at -20 °C. Pre-immune sera were collected, pooled and used as the experiment control.

Preparation of total soluble antigens from *P. brasiliensis*

Yeast cells cultured as described above were washed with PBS by centrifugation at 7,000 × *g* at 4 °C for 10 min, resuspended in PBS and disrupted by ten sonication cycles on ice, each consisting of 1-min sonication at 200 W with 1-min resting interval. The supernatant from the sonicated sample, which was centrifuged at 7,000 × *g* at 4 °C for 10 min, was filtered (PbAgs) and analyzed by 12% SDS-PAGE.

Western Blotting

Following the electrophoresis, separated bands were transferred to polyvinylidene fluoride membranes (Hybond membranes Amersham Hybond P 0.45 PVDF, GE Healthcare, Little Chalfont, UK) at 150 V for 2 h. Membranes were blocked for 2 h at 25 °C in 3% bovine serum albumin (BSA) in TTBS (0.1% Tween-20, 20 mM Tris-HCl, 150 mM NaCl, pH 7.6), and incubated overnight at 4 ºC with anti-rPbHsp60 polyclonal antibody at 1:3,000 dilution in 1% BSA in TTBS. Then, membranes were washed five times with TTBS and incubated for 2 h at 25 °C with an anti-mouse secondary antibody IgG conjugated to peroxidase (Sigma-Aldrich) at 1:3,000 dilution in 1% BSA in TTBS. Blots were washed as described above and immersed in ECL reagent for 1 minute for the detection of protein using a ChemiDoc MP Imaging System (Bio-Rad).

Immunoelectron microscopy

Mycelium and yeast cells of *P. brasiliensis* were cultured in DMEM (Sigma-Aldrich) at 25 °C and 37 °C, respectively, for 7 days. The cells were washed with PBS and fixed in 3.7% formaldehyde buffered with PBS at 25 °C for 1 h. Then, the cells were rinsed with PBS and resuspended in 0.05% glutaraldehyde buffered with PBS and incubated at 25 °C for 15 min. In the next step the cells were washed three times with PBS and resuspended in PBS containing 0.1% Triton X-100 and incubated at 25 °C for 45 min. The samples were recovered and washed with 1 mL of PBS. Cells were incubated with 1 mL PBS containing 1% BSA (PBS-BSA) at 25 °C. After 1 h, pre-immune serum or anti-Pb-Hsp60 polyclonal antibody (1:1000) was added for 1 h at 25 °C. After washing with PBS-BSA, colloidal gold-conjugated anti-mouse IgG (Nanoprobes Inc., NY, USA) was added to the cells for 1 h at 25 °C. The cells were washed five times with PBS, with 5 min of incubation for each wash and fixed with 2.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4) (EM Sciences, Hatfield, USA) at 25 °C for 1 h. Cells were washed with cacodylate buffer and incubated at 4 °C for 18 h, followed by washing five times with ultrapure water. Further processing of cells was performed as per manufacturer's instructions of Gold Enhancement (Nanoprobes). Subsequently, the cells samples were prepared to electron microscopy as described previously[11]. Finally, the sections were observed with a transmission electron microscope Jeol JEM-100 CXII (JEOL, Peabody, MA, USA).

Statistical analysis

The differences among means of the differential expression of the *HSP60* mRNA in the different morphologies and transition phases were defined using a one-way analysis of variance followed by Bonferroni's post-test. Differences were recognized as statistically significant at $P < 0.05$. All the experiments were repeated at least twice.

RESULTS AND DISCUSSION

Dimorphism is an adaptive mechanism of some fungi that grow saprophytically in ambient temperature and are able to adjust to the higher temperatures of their hosts. This ability has been driven many authors to search for the main virulence factors that correlate pathogenicity with the morphological transition[9]. The effect of temperature on the gene expression profile in several fungi is remarkable, among which we highlight the one of *HSP60*, which increases its expression in a temperature-dependent manner[12].

In the current study, we evaluated the *HSP60* mRNA expression in yeast and hyphal forms of *P. brasiliensis* as well as in their transition forms using qRT-PCR. *P. brasiliensis* was cultured at different temperatures to obtain yeasts, hyphae and transitional forms (mycelium-to-yeast and yeast-to-mycelium). These forms of *P. brasiliensis* were observed in light microscopy, and both mycelia and yeasts had the expected morphotypes. Moreover, the mycelium-to-yeast transitional phase had chlamydospore-like intercalary and terminal cells, and yeast-to-mycelium transition had cells with elongation (Figures 1A-1D), as already described[13]. The measurement of *HSP60* mRNA of mycelium and yeast of *P. brasiliensis* showed higher levels of expression of *HSP60* in yeast forms than those of mycelium (Figure 1E).

Interestingly, the mycelium-to-yeast transition form had increased the levels of relative expression of *HSP60* compared to the yeast-to-mycelium transition form (Figure 1E). A previous study suggested that fever can activate the *HSP60* gene expression in the host and subsequently modify the immunoregulatory process[14].

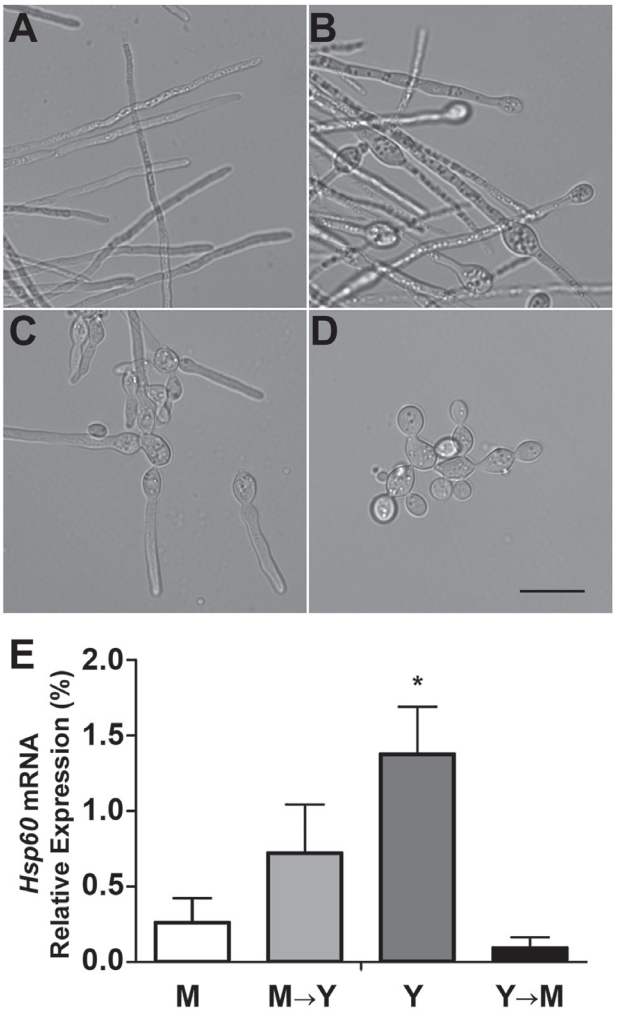

Figure 1 - Differential expression of the *HSP60* mRNA in the different morphologies and transition phases of *P. brasiliensis*. Optical microscopy of mycelia (A), mycelia-to-yeast (B), yeast-to-mycelia (C), yeast (D) of *P. brasiliensis*. The bars correspond to 10 µm. (E) Differential expression of *HSP60* mRNA in the different morphologies and transition phases performed by qPCR. The abbreviation M refers to mycelia, M→Y, mycelia-to-yeast, Y, yeast, and Y→M, yeast-to-mycelia. Data are expressed as the mean ± standard deviation of five samples per group. *P < 0.05 compared to the mycelia and yeast-to-mycelia groups.

These findings support that the temperature of 37 °C is critical for regulating the expression of *HSP60* in *P. brasiliensis*. Therefore, new roles that Hsp60 plays in the lifestyle of *P. brasiliensis* should be investigated.

The *H. capsulatum* Hsp60, which has high identity with *P. brasiliensis* Hsp60[15], is located on the yeast cell surface and is recognized by CD18/CD11b expressed in macrophages[16]. In general, the interaction between components of fungal cell walls and receptors of innate immune cells should be explored due to the effects on the regulation of the host immune response[17]. Besides, Holbrook and Rappleye[18] suggested that Hsp60 is an *H. capsulatum* adhesin that interacts with the CR3 molecule on phagocytes. These findings instigated us to investigate the cell location of PbHsp60 in yeast and mycelial forms. rPbHsp60 was expressed in *E. coli*, purified using HisTap column (Figure 2A), and used to immunize mice. An anti-PbHsp60 murine polyclonal antibody pAb-PbHsp60 was obtained, and it revealed only a protein band on Western blotting even when it was used in a preparation with multiples *P. brasiliensis* somatic antigens (Figure 2B). When the pAb-PbHsp60 was also used to identify the cell location of PbHsp60 in yeast and mycelial forms of *P. brasiliensis* by immunoelectron microscopy, we found that Hsp60 was located in the cytoplasm and within the fungal cell wall, where it had a prominent distribution on yeasts when compared with hyphae (Figure 3).

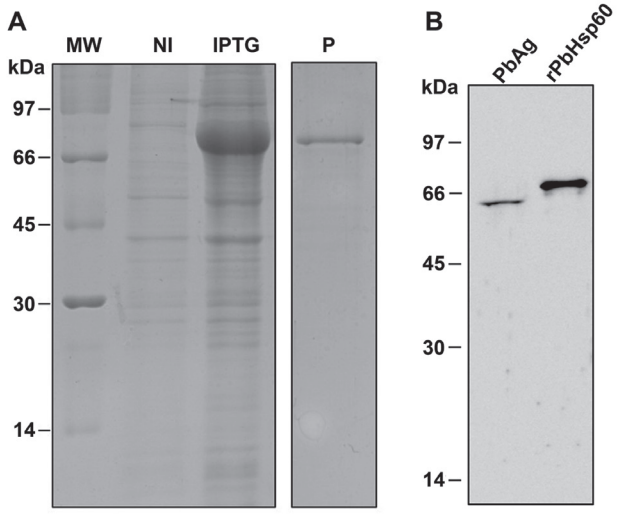

Figure 2 - Expression of rPbHsp60 in *P. brasiliensis* and analysis of the reactivity with anti-rPbHsp60 polyclonal serum: A) pET28a-*HSP60*-transformed *E. coli* were cultured in LB broth in the absence (NI) or presence (IPTG) of the inductor of the recombinant protein for 6 h at 37 °C under constant agitation. The recombinant protein was purified (P) on a *HisTrap* column. The preparations were analyzed in an SDS-PAGE and the proteins visualized by staining with Coomassie brilliant blue G250 (USB Corporation, Cleveland, OH, USA); B) Whole soluble antigens (PbAg) and recombinant protein (rPBHsp60) were separated by 12% SDS-PAGE, blotted blotted onto a PVDF membrane and probed with an anti-rPbHsp60 polyclonal antibody. The antigen-antibody reactions were revealed with the ECL reagent.

In summary, the current study showed the prevalence of Hsp60 expression in yeast and mycelium-to-yeast transition forms and identified it within the cell wall, which is critical in the host-pathogen interaction, and in this way, Hsp60 is a potential target to be explored from the therapeutic point of view. Our findings open perspectives to better understand the importance of Hsp60 for the lifestyle of *P. brasiliensis*, allowing for new approaches immunotherapy against PCM.

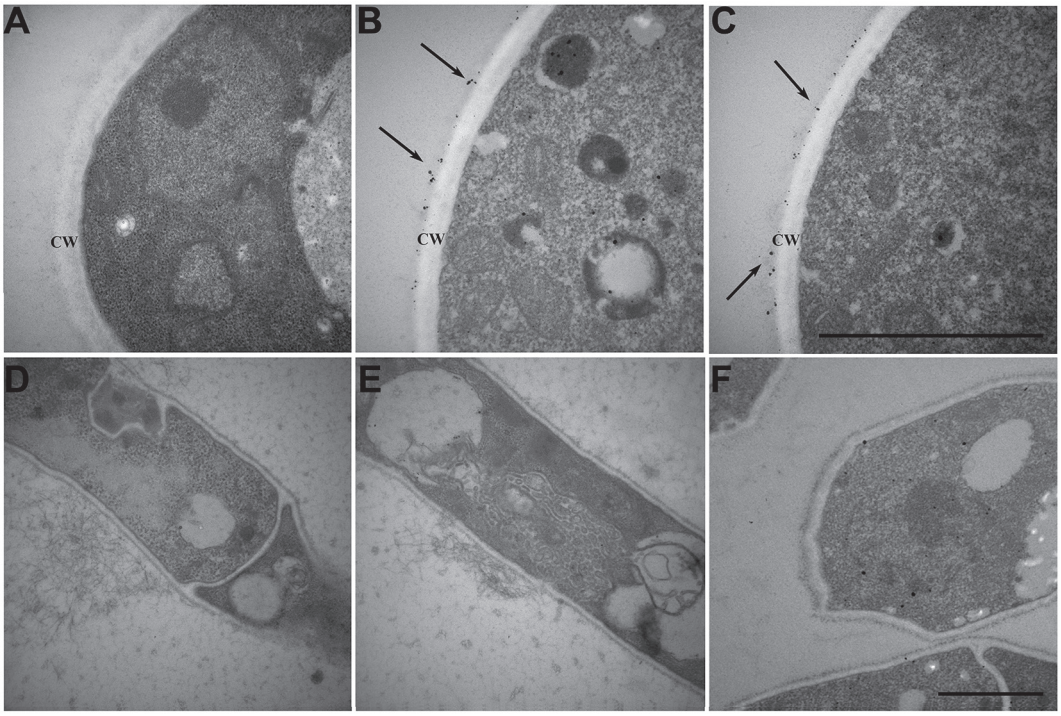

Figure 3 - Subcellular location of Hsp60 at *P. brasiliensis* by immunoelectron microscopy. (A-C) Yeast cells and (D-F) mycelium of *P. brasiliensis* were cultured in DMEM at 37 °C and 25 °C, respectively, for 7 days. Samples were fixed and incubated with (A and D) pre-immune serum or (B, C, E and F) anti-rPbHsp60 polyclonal antibody. The arrows show the PbHsp60 within the cell wall (CW) of *P. brasiliensis*. Scale bar = 2 µm.

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CONFLICT OF INTERESTS

On behalf of all of the authors, the corresponding author states that there is no conflict of interests.

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