Medical Mycology July 2012, **50,** 533–537

informa
healthcare

The hydrolase PbHAD32 participates in the adherence of Paracoccidioides brasiliensis conidia to epithelial lung cells

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Adherence of the dimorphic pathogenic fungus *Paracoccidioides brasiliensis* to lung epithelial cells is considered an essential event for the establishment of infection. We have previously shown that the *PbHAD32* hydrolase is important in this early stage of the host-*P. brasiliensis* yeast cells interaction. The aim of this study was to further elucidate the role of *PbHAD32* in conidial thermodimorphism and their interaction with lung epithelial cells. Analysis of the *PbHAD32* gene expression revealed higher mRNA levels during the conidia to mycelia (C–M) germination when compared to the conidia to yeast (C–Y) transition. Moreover, *PbHAD32* was consistently expressed at higher levels upon infection of lung epithelial cells, but to a greater extent when conidia germinated to produce mycelia. Interestingly, at this particular transitional stage, more conidia adhered to epithelial cells than when they were transiting to the yeast form. Altogether our data further corroborates the importance of *PbHAD32* during initial adherence to host cells and suggest that the 32-KDa hydrolase may also participate at different stages of the C–M and C–Y conversions.

Keywords *Paracoccidioides brasiliensis*, adherence, gene expression, down regulation

Introduction

Paracoccidioidomycosis (PCM) is an important systemic mycosis common in Latin America and is caused by the thermodimorphic fungus *Paracoccidioides brasiliensis* [1]. Fungal conidia, considered the infectious particles, have a low metabolic activity but are capable of germinating to mycelia at environmental temperatures (18–24°C) and to the pathogenic yeast forms at host temperatures (36–37°C) [2]. The infection is acquired when conidia, or small mycelial fragments, reach the lung alveoli where the initial interaction with host cells takes place, followed by phagocytosis and morphological transition to the yeast forms thanks to the increased body temperature [2]. During this process, the initial adherence of *P. brasiliensis* to lung epithelial cells is considered to be an essential event [3,4]. In fact, adherence has been described as a mechanism by which pathogenic fungi initially avoid entrapment by respiratory tract mucus and later on their elimination by mucigen-producing ciliary cells [4].

Several proteins have been shown to be involved in the adherence of *P. brasiliensis*, to host cells [5]. Among them is a 32-kDa protein present in cell wall protein extracts of *P. brasiliensis*. Gonzalez and co-workers showed that this 32-KDa protein is able to bind to extracellular matrix proteins (ECM), including laminin, fibronectin, and fibrinogen, and is involved in the initial conidial adherence to pulmonary epithelial cells, suggesting it acts as a bridge between host and fungal cell types [3,6]. Recently, our group characterized this 32-kDa protein as a member of the hydrolase family of proteins (gene PbHAD32) [7].

Received 31 May 2011; Received in final form 16 August 2011; Accepted 30 August 2011

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DOI: 10.3109/13693786.2011.619583

We proved that decreasing the expression of *PbHAD32* in *P. brasiliensis* yeast cells reduced their capability to adhere to human lung epithelial cells and significantly decreased virulence in a mouse model of infection [7]. Taking into account that conidia are considered as infectious particles, we aimed to further elucidate the role of *PbHAD32* during *P. brasiliensis* thermal dimorphism and interaction with human lung epithelial cells.

Materials and methods

Microorganisms and culture media

The *P. brasiliensis* strains used during this work were the wild-type strain ATCC 60855 (PbWt) and a strain previously generated in our laboratory by antisense RNA technology with an 80% reduced gene expression of *PbHAD32* (*PbHAD32*-aRNA) [7]. *P. brasiliensis* yeast and mycelial cells were maintained at 36°C and 18°C, respectively, by sub-culturing in brain heart infusion (BHI) solid media supplemented with 1% glucose (Becton Dickinson and Company, Sparks, MD, USA) and synthetic McVeigh Morton (SMVM) medium at 20°C, respectively [8]. *P. brasiliensis* conidia were produced as previously described using the glass-wool filtration protocol [9]. Cell quantification and viability were determined using Neubauer chamber counting and ethidium bromide-fluorescence staining procedures, respectively [10].

Morphological transition assays

The morphological transition was evaluated in both PbWt and *PbHAD32*-aRNA strains. The morphological changes were performed in BHI liquid medium stimulating C–M germination at 18°C and C–Y transition at 36°C during 24 and 48 h for both adherence and gene expression assays. All assays were carried out in 250 ml Erlenmeyer flasks with 100 ml of medium. Culture samples (20 ml) were collected for RNA extraction and quantification of *PbHAD32* gene expression during the transition process at defined time points [11].

Adherence of P. brasiliensis *to A549 cells*

The human lung epithelial cell line A549 was obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). For the adherence assays, we used confluent monolayers obtained by adding 4×10^5 cells per well to 24-well tissue culture plates (Nunc, Kamstrup, Denmark). Cells were incubated for 24 h at 36°C with 5% CO_2 prior to evaluating interaction with PbWt or *PbHAD32*-aRNA cells. A549 cell monolayers were washed once with DMEM culture medium and co-cultured with *P. brasiliensis* conidia at a concentration of 4×10^5 conidia per well and incubated for 1 and 3 h at 36°C and 5% CO_2. Culture supernatant was then removed, the monolayers were lysed, conidia adherent to epithelial cells were collected, and dilutions of these suspensions were inoculated onto plates of BHI supplemented with 0.5% glucose, 4% horse serum and EDTA 300 mM [12]. These results were compared with the number of conidia that had been added to each well. Percentage of adherence was expressed as the number of CFUs obtained from each experimental well, (*P. brasiliensis* conidia cells and A549 cells) divided by the number of CFUs in the controls, (*P. brasiliensis* conidia cells alone). The viability of *P. brasiliensis* conidia was also evaluated after 24 h post-infection by determining CFUs and ethidium bromide-fluorescence staining as described above [10,12].

Gene expression analysis

Total RNA was obtained after interaction of PbWt and *PbHAD32*-aRNA conidia with A549 cells by treatment with TRIzol (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and tested using a conventional PCR with β-tubulin primers [13] to confirm absence of chromosomal DNA contamination. cDNA was synthesized using 1 μg of total RNA with SuperScript III reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad). Real-time PCR (qPCR) was performed using SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green, according to the manufacturer's instructions (Invitrogen). The CFX96 real-time PCR Detection System (Bio-Rad, Headquarters Hercules, CA, USA) was used to measure *PbHAD32* and β-tubulin (*TUB2*, house keeping gene) gene expression. Melting curve analysis was performed after the amplification phase to eliminate the possibility of nonspecific amplification or primer-dimer formation. Fold changes in mRNA expression were calculated using the $2^{\Delta\Delta CT}$ formula [14]. Each experiment was performed in triplicate and the expression level was measured three times.

Statistics

Data are reported as mean ± standard error of the mean. All statistical analysis was performed using SPSS statistics 17.0 program with analysis of variance between groups (ANOVA). A P value less than or equal to 0.05 was considered statistically significant.

Results

PbHAD32 *gene expression is higher during the conidial to mycelial (C–M) germination process*

We initially analyzed *PbHAD32*'s gene expression during the C–M germination and the C–Y transition processes.

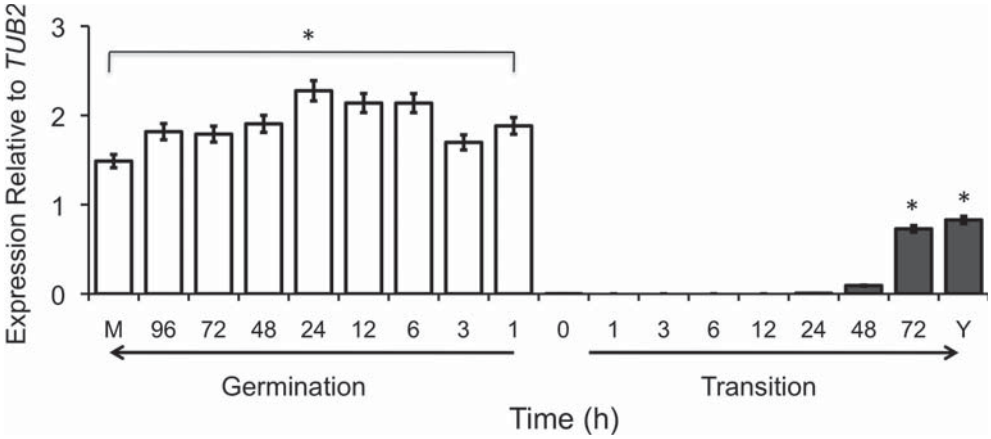

Fig. 1 Gene expression levels of *PbHAD32* during *P. brasiliensis* conidia-to-yeast (C–Y) transition and conidia-to-mycelia (C–M) germination (*$P < 0.05$ in comparison with conidia).

Gene expression was significantly increased in PbWt cells throughout the 96 h of C–M germination (Fig. 1). In contrast, *PbHAD32* mRNA was undetected during the first 48 h of the C–Y transition process, only reaching significant levels at 72 h, but always at lower levels than in the mycelial form during the C–M germination. In addition, *PbHAD32* expression was significantly higher in the mycelia than in the yeast cells. Regarding *PbHAD32*-aRNA conidia, PbHAD32 mRNA was undetectable throughout the C–Y transition or C–M germination processes (data not shown).

PbHAD32 *plays a role in adherence of* P. brasiliensis *conidia to human epithelial lung cells*

To help clarify the role of *PbHAD32* in the adherence of *P. brasiliensis* conidia to host cells, we infected A549 epithelial human lung cells with both PbWt and *PbHAD32*-aRNA conidia. The adherence of *PbHAD32*-aRNA conidia to epithelial cells was significantly decreased 1 and 3 h post-infection when compared to those of PbWt (Fig. 2A). Moreover, we evaluated *PbHAD32* gene expression in the presence and absence of epithelial cells. Interaction with these cells significantly increased *PbHAD32* gene expression in PbWt conidia after 3 h (Fig. 2B). On the other hand, *PbHAD32*-aRNA conidia maintained low gene expression levels either in the presence or absence of epithelial cells.

The induction of C-M germination in PbWt increases cellular adherence and PbHAD32 *gene expression*

To evaluate the relevance of the initial steps of thermodimorphism of *P. brasiliensis* conidia in their adherence to human epithelial lung cells, we independently induced the C–Y transition and the C–M germination for 24 and 48 h prior to infection of A549 cells. As shown previously in this work (Fig. 2A), we consistently observed a significant reduction in adherence of *PbHAD32*-aRNA conidia when

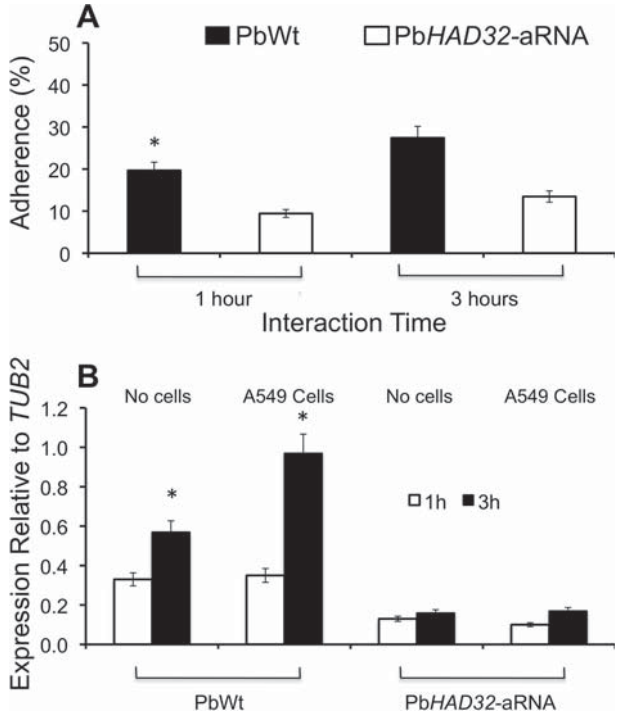

Fig. 2 *PbHAD32* plays an important role during interaction of non-stimulated *P. brasiliensis* conidia and human epithelial lung cells. (A) Adherence percentage of PbWt and *PbHAD32*-aRNA conidia to epithelial cells after 1 and 3 hours of interaction. (B) Gene expression level of *PbHAD32* in PbWt and *PbHAD32*-aRNA conidia after 1 and 3 hours in the absence and the presence of epithelial cells (*$P < 0.05$ in comparison with the *PbHAD32*-aRNA strain).

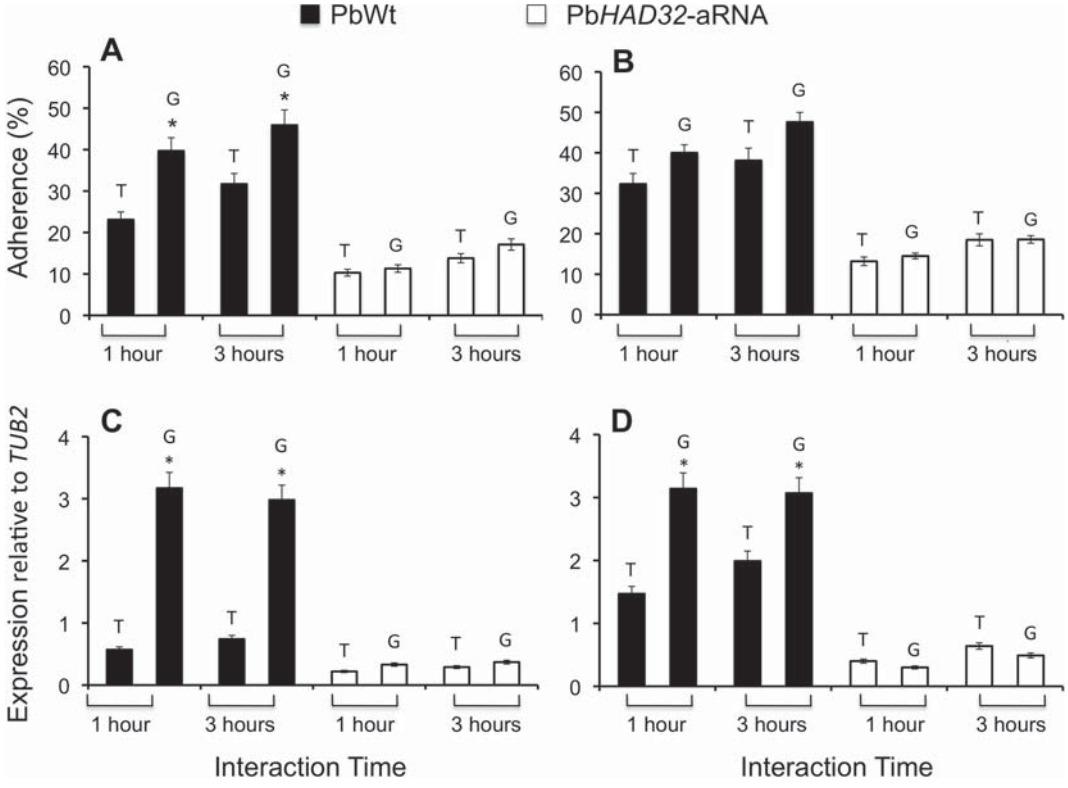

Fig. 3 Stimulation of *P. brasiliensis* conidia to transit to yeast cells or germinate mycelia alters adherence and *PbHAD32* gene expression upon interaction with human epithelial lung cells. (A) Adherence percentage of conidia stimulated to transit to yeast or germinate to mycelia during 24 hours before interaction with epithelial cells. (B) Adherence percentage of conidia stimulated to transit to yeast or germinate to mycelia during 48 hours before interaction with epithelial cells. (C) *PbHAD32* gene expression of conidia stimulated to transit to yeast or and germinate to mycelia during 24 hours before interaction with epithelial cells. (D) *PbHAD32* gene expression of conidia stimulated to transit to yeast or and germinate to mycelia during 48 hours before interaction with epithelial cells. (*$P < 0.05$ in comparison with the *PbHAD32*-aRNA strain).

compared to PbWt. PbWt and *PbHAD32*-aRNA conidia that were previously stimulated during 24 and 48 h to either germinate to mycelia or convert to yeasts, exhibited an increased adherence to epithelial cells after 1 and 3 h (Fig. 3A, 3B) when compared to non-stimulated PbWt conidia (Fig. 2A). Interestingly, PbWt conidia germinating to mycelia exhibited a significantly increased adherence when compared to conidia converting to the yeast form (Fig. 3A, 3B).

We also measured *PbHAD32* gene expression under the same conditions (Fig. 3C, 3D). As shown previously (Fig. 2B), the absence of epithelial lung cells did not induce *PbHAD32* gene expression in either PbWt or *PbHAD32*-aRNA conidia (data not shown). Moreover, *PbHAD32* gene expression levels were at low values for *PbHAD32*-aRNA conidia under all the experimental conditions tested. On the other hand, *PbHAD32* mRNA levels were significantly increased in PbWt conidia subjected to either germinating (C–M) or transiting (C–Y) conditions prior to infection of epithelial cells. More importantly, PbWt conidia germinating to mycelia expressed significantly higher *PbHAD32* levels in comparison with conidia converting to the yeast form (Fig. 3C, 3D).

Discussion

The interaction of clinically relevant dimorphic fungi, such as *P. brasiliensis*, with host cells and their subsequent adherence constitute an important step in their avoiding elimination by the host and thereby allowing their internalization into the target cells, the establishment of the initial focus of infection and dissemination to other organs [4]. We have previously suggested that the 32-KDa hydrolase, encoded by *PbHAD32*, plays an important role in adherence of *P. brasiliensis* yeast cells to epithelial lung cells [7]. The aim of the present study was to evaluate the relevance of *PbHAD32* in adherence of conidia to epithelial lung cells. Although conidia are generally accepted as primary infectious particles in paracoccidioidomycosis, experimental designs using conidia in *P. brasiliensis* research have been restricted to few laboratories, thus hampering the elucidation of the infectious process. This is the first study

using *P. brasiliensis* conidia with gene expression modulated by aRNA technology.

Previous works showed that the 32-KDa protein is a constitutive component of the cell wall of conidia, mycelia, and yeast cells [6]. Our data demonstrate that *PbHAD32* transcription level is higher in mycelia and during the C–M germination than in yeast cells or during the C–Y transition. For all studied scenarios, *PbHAD32* gene expression and adherence to epithelial lung cells was significantly higher for conidia germinating to mycelia than for conidia converting to yeast. These results seem to concur with our data showing an increased expression of *PbHAD32* during the C–M process. These data suggest that the germinating conidia may respond more efficiently to the host cell stimuli that trigger the molecular mechanisms behind the adherence process of *P. brasiliensis* cells.

Altogether, our results not only further support the relevance of *PbHAD32* during the initial interaction with host cells, but also raise important issues regarding the role of this protein during *P. brasiliensis* life cycle and adaptation to different environments. Future studies may be conducted to elucidate to what extent *PbHAD32* is vital during the initial steps of infection with *P. brasiliensis* conidia with aRNA-modulated gene expression.

Acknowledgements

This work was supported by COLCIENCIAS Colombia, Project No. 2213-343-19183, the Corporación para Investigaciones Biológicas, and the Instituto de Biología of the Universidad de Antioquia. The National Doctoral Program of COLCIENCIAS 2008 supported Orville Hernández Ruiz. We thank Fernando Rodrigues for his initial support with the project from the School of Health Sciences, University of Minho, Braga, Portugal.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

This paper was first published online on Early Online on 10 October 2011.

References

1 Restrepo A, McEwen JG, Castaneda E. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? *Med Mycol* 2001; **39**: 233–241.
2 Brummer E, Castaneda E, Restrepo A. Paracoccidioidomycosis: an update. *Clin Microbiol Rev* 1993; **6**: 89–117.
3 Gonzalez A, Caro E, Munoz C, *et al*. *Paracoccidioides brasiliensis* conidia recognize fibronectin and fibrinogen which subsequently participate in adherence to human type II alveolar cells: involvement of a specific adhesin. *Microb Pathog* 2008; **44**: 389–401.
4 Mendes-Giannini MJ, Monteiro da Silva JL, de Fatima da Silva J, *et al*. Interactions of *Paracoccidioides brasiliensis* with host cells: recent advances. *Mycopathologia* 2008; **165**: 237–248.
5 Vicentini AP, Gesztesi JL, Franco MF, *et al*. Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. *Infect Immun* 1994; **62**: 1465–1469.
6 Gonzalez A, Gomez BL, Diez S, *et al*. Purification and partial characterization of a *Paracoccidioides brasiliensis* protein with capacity to bind to extracellular matrix proteins. *Infect Immun* 2005; **73**: 2486–2495.
7 Hernández O, Almeida AJ, Gonzalez A, *et al*. A 32-kilodalton hydrolase plays an important role in *Paracoccidioides brasiliensis* adherence to host cells and influences pathogenicity. *Infect Immun* 2010; **78**: 5280–5286.
8 Restrepo A, Jimenez B. Growth of *Paracoccidioides brasiliensis* yeast phase in a chemically defined culture medium. *J Clin Microbiol* 1980; **12**: 279–281.
9 Restrepo A, Salazar M, Cano L, Patino M. A technique to collect and dislodge conidia produced by *Paracoccidioides brasiliensis* mycelial form. *J Med Vet Mycol* 1986; **24**: 247–250.
10 Calich VL, Purchio A, Paula CR. A new fluorescent viability test for fungi cells. *Mycopathologia* 1979; **66**: 175–177.
11 Garcia AM, Hernández O, Aristizabal BH, *et al*. Gene expression analysis of *Paracoccidioides brasiliensis* transition from conidium to yeast cell. *Med Mycol* 2010; **48**: 147–154.
12 Kurita N, Sano A, Coelho KI, *et al*. An improved culture medium for detecting live yeast phase cells of *Paracoccidioides brasiliensis*. *J Med Vet Mycol* 1993; **31**: 201–205.
13 Goldman GH, dos Reis Marques E, Duarte Ribeiro DC, *et al*. Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. *Eukaryot Cell* 2003; **2**: 34–48.
14 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402–408.