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Comparative transcriptome analysis of *Paracoccidioides brasiliensis* during in vitro adhesion to type I collagen and fibronectin: identification of potential adhesins

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Abstract

Paracoccidioidomycosis is caused by the dimorphic fungus *Paracoccidioides brasiliensis*. The extracellular matrix (ECM) plays an important role in regulation of cell adhesion, differentiation, migration and proliferation of cells. An in vitro binding assay of *P. brasiliensis* yeast cells adhering to type I collagen and fibronectin was performed in order to identify novel adhesins. Representational difference analysis (RDA) was employed to identify genes upregulated under adhesion-inducing conditions. Expressed sequence tags (ESTs) from cDNA libraries generated by the RDA technique were analyzed. Genes related to functional categories, such as metabolism, transcription, energy, protein synthesis and fate, cellular transport and biogenesis of cellular components were upregulated. Transcripts encoding the *P. brasiliensis* protein enolase (*Pb*Eno) and the high-affinity cooper transporter (*Pb*Ctr3) were identified and further characterized. The recombinant enolase (r*Pb*Eno) and a synthetic peptide designed for *Pb*Ctr3 were obtained and demonstrated to be able to bind ECM components. Immunofluorescence assays demonstrated that r*Pb*Eno specifically binds to the macrophage surface, reinforcing the role of this molecule in the *P. brasiliensis* interaction with host cells. In addition, upregulation of selected genes was demonstrated by qRT-PCR. In synthesis, the strategy can be useful in characterization of potential *P. brasiliensis* adhesins.

Keywords: *Paracoccidioides brasiliensis*; Adhesin; RDA; Enolase; Cooper transporter

1. Introduction

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis (PCM), a human systemic mycosis prevalent in South America (Restrepo et al., 2001). In the soil, the fungus grows as a saprobic mycelium, resulting in formation of propagules. After reaching the host, the fungus must convert to the yeast form, a fundamental step in successful establishment of the infection (San-Blas et al., 2002). The mycelial propagules adhere to and invade alveolar cells and the basal lamina, the latter of which is composed of a specialized extracellular matrix (ECM) in which laminin, collagen and fibronectin can be found (Dunsmore and Rannels, 1996; Gonzaléz et al., 2008; Hanna et al., 2000).

Adherence of the pathogens to host cells is considered an essential step in the establishment of infection (Carneiro et al., 2004; Marchais et al., 2005). *P. brasiliensis* has been shown to adhere to ECM proteins. Several studies have established the role of certain *P. brasiliensis* proteins in the adherence process. An antigenic component of *P. brasiliensis*, glycoprotein gp43,

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binds laminin, thereby increasing the pathogenicity of the yeast cells (Vicentini et al., 1994). Proteins with molecular masses of 19 and 32 kDa are present on the fungal surface and interact with laminin, fibronectin and fibrinogen (Gonzaléz et al., 2005). The 32 kDa protein (*Pb*Had32p) was characterized as a hydrolase that influences *P. brasiliensis* pathogenicity (Hernández et al., 2010). In addition, Andreotti et al. (2005) demonstrated that a *P. brasiliensis* 30 kDa protein is able to bind laminin. We characterized several *P. brasiliensis* adhesins such as *Pb*Dfg5p (defective for filamentous growth protein Dfg5p), which was detected by electron microscopy in the cell wall of the fungus and binds laminin, fibronectin and types I and IV collagen (Castro et al., 2008). In addition, triosephosphate isomerase (*Pb*TPI) which binds laminin and fibronectin (Pereira et al., 2007), and glyceraldehyde-3-phosphate dehydrogenase (*Pb*GAPDH), which binds fibronectin, type I collagen and laminin (Barbosa et al., 2006), were found in the *P. brasiliensis* cell wall mediating fungal adherence to in vitro cultured cells. Malate synthase (*Pb*MLS) binds fibronectin and types I and IV collagen and is present in the *P. brasiliensis* cell wall (Neto et al., 2009). In addition, *P. brasiliensis* enolase is a fibronectin and plasminogen binding protein (Donofrio et al., 2009; Nogueira et al., 2010). Therefore, *P. brasiliensis* seems to possess several proteins involved in adhesion, and knowledge of these proteins could advance our understanding of the first steps in establishment of the infection.

To obtain and characterize new molecules involved in the adhesion process in *P. brasiliensis*, we used cDNA representational difference analysis (cDNA-RDA) to identify genes induced during incubation of *P. brasiliensis* yeast cells with ECM components. Fibronectin, a multifunctional extracellular matrix and plasma protein that plays a central role in cell adhesion (Ruoslahti, 1988), and collagens, as the most common matrix molecules (Lyons and Jones, 2007), represent targets for microorganism adherence. Therefore, in this study, we investigated involvement of type I collagen and fibronectin in the adherence process of *P. brasiliensis* and described several putative novel adhesins.

2. Materials and methods

2.1. P. brasiliensis *growth conditions*

P. brasiliensis Pb 01 (ATCC MYA-826) is being studied at our laboratory (Bailão et al., 2006; Barbosa et al., 2006). This isolate was cultivated at 36 °C in Fava-Netto's medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 4% (w/v) glucose; 1% (w/v) agar; pH 7.2] for 4 days.

2.2. *Adherence assay on polystyrene flasks*

The adherence assays were performed as described by Penalver et al. (1996) with several modifications. Briefly, polystyrene flasks (Corning Ultra-Low Attachment 75 cm^2 rectangular canted-neck cell-culture flask) were coated with type I collagen or fibronectin at 50 μg/ml in coating buffer ($NaHCO_3$, Na_2CO_3, [pH 9.6]) and incubated for 1 h at 37 °C and overnight at 4 °C. The plates were blocked by adding PBS (1 mM $Na_2HPO_4.2H_2O$, 1 mM $NaH_2PO_4.H_2O$, 50 mM NaCl, pH 7.4)−1% (w/v) BSA and washed three times with PBS−0.1% (v/v) Tween 20 before a yeast cell suspension (10^8/ml) in PBS was added. The control yeast cells were incubated in PBS−1% (w/v) BSA. The plates were incubated for 1 h at 37 °C and washed three times with PBS−0.1% (v/v) Tween 20 following RNA isolation.

2.3. *RNA isolation*

Total RNAs from *P. brasiliensis* were obtained by the Trizol method according to the manufacturer's instructions (GIBCO, Invitrogen, Carlsbard, CA, USA). DNA contamination was extinguished by treating total RNA with RNAse free DNAse (Promega Corporation®). The RNAs were used to construct double-stranded cDNAs.

2.4. *Subtractive hybridization and generation of subtracted libraries*

Subtractive hybridization was performed as previously described by Bailão et al. (2006). Briefly, 1.0 μg of total RNA was used to produce cDNA. The synthesis of the first strand was performed with SuperScript II reverse transcriptase (Invitrogen Life Technologies); this product was then used as a template to synthesize double-stranded cDNA. The resulting cDNAs were digested with restriction enzyme *Sau3*AI. The subtracted cDNA libraries were constructed using driver cDNAs (from RNAs extracted from the control) and tester cDNAs (synthesized from RNAs extracted from *P. brasiliensis* adhered to type I collagen or fibronectin). The resulting products were purified using a GFX kit (GE Healthcare, Chalfont St. Giles, UK). The tester-digested cDNA was ligated to adapters (a 24-mer annealed to a 12-mer) and amplified by PCR. The amplicons were digested with *Sau3*AI to remove the adapters that had been incorporated into the cDNAs and, after spin-column purification, a new 24-mer adapter was ligated onto the cDNA tester and a different DNA molecule was ligated onto the cDNA driver. The cDNA driver was PCR-amplified and, after cleavage to remove the adapters, it was purified and quantified.

For generation of the differential products, tester and driver cDNAs were mixed, hybridized at 67 °C for 18 h and amplified by PCR with the 24-mer adapter. Two successive rounds of subtraction and PCR amplification using hybridization tester-driver ratios 1:10 and 1:100 were performed. The adapters used for subtractive hybridizations are listed in Table 1 in supplementary material.

After the second subtractive reaction, the final amplified cDNAs were cloned into a pGEM-T Easy vector (Promega, Madison, USA). *Escherichia coli* XL1 Blue competent cells were transformed with the ligation products. Selected colonies were picked and grown in microliter plates and plasmid DNA was prepared. To generate expressed sequence tags (ESTs),