Analysis of the Paracoccidioides brasiliensis triosephosphate isomerase suggests the potential for adhesin function

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Abstract
Paracoccidioides brasiliensis is an important fungal pathogen. The disease it causes, paracoccidioidomycosis (PCM), ranges from localized pulmonary infection to systemic processes that endanger the life of the patient. Paracoccidioides brasiliensis adhesion to host tissues contributes to its virulence, but we know relatively little about molecules and the molecular mechanisms governing fungal adhesion to mammalian cells. Triosephosphate isomerase (TPI: EC 5.3.1.1) of P. brasiliensis (PbTPI) is a fungal antigen characterized by microsequencing of peptides. The protein, which is predominantly expressed in the yeast parasitic phase, localizes at the cell wall and in the cytoplasmic compartment. TPI and the respective polyclonal antibody produced against this protein inhibited the interaction of P. brasiliensis to vitro cultured epithelial cells. TPI binds preferentially to laminin, as determined by peptide inhibition assays. Collectively, these results suggest that TPI is required for interactions between P. brasiliensis and extracellular matrix molecules such as laminin and that this interaction may play an important role in the fungal adherence and invasion of host cells.

Introduction
The pathogenic fungus Paracoccidioides brasiliensis causes paracoccidioidomycosis (PCM), one of the most important systemic mycosis in Latin America. The severe nature of the disease and the occurrence of sequelae, frequently causing pulmonary dysfunction and other disabilities, render it a pathogen of considerable medical importance. The fungus is thermally dimorphic, grows as a yeast-like structure in the host tissue and as mycelium in the saprobic condition. The mycelial phase produces infective propagules that lodge in the host alveoli, and adhere and invade the alveolar cells and/or the basal lamina (Franco & Montenegro, 1994).

The attachment of microorganisms to a biological surface is a complex process involving specific interactions between adhesins and complex receptors on host tissues (Finlay & Cossart, 1997). During their evolution, fungal pathogens have acquired the ability to exploit cell surface matrix components as ligands for attachment (Rostand & Esko, 1997). The extracellular matrix of the alveolar basal lamina is a complex mixture of molecules, including fibronectin, laminin, and collagens. Although the matrix is not available for binding in normal cells, the components can be exposed after tissue damage resulting from either inflammatory process or lytic activity by toxins of the microorganism (Patti et al., 1994).

The adhesion of P. brasiliensis to cells is seen as an important determinant of pathogenesis. The fungal proteins functioning as adhesins in P. brasiliensis are just now coming to light. Some fungal proteins including a glycoprotein of 43 kDa (gp43), a 30 kDa protein, proteins of 19 and 32 kDa and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been identified and found to mediate adhesion to extracellular matrix components and human cells (Vicentini et al., 1994; Andreotti et al., 2005; Gonzáles et al., 2005; Barbosa et al., 2006; Mendes-Giannini et al., 2006).

Triosephosphate isomerase (TPI) is an enzyme catalyzing the conversion of dihydroxy acetone phosphate (DHAP) to glyceraldehyde-3-phosphate (G3P). The protein was well characterized in P. brasiliensis by immunoproteomic
analysis; it was originally described in our laboratory as a fungal antigen reactive with sera of PCM patients and sequences from N-terminal and internal peptides were obtained (Fonseca et al., 2001). Subsequently, the cognate cDNA was obtained and the recombinant protein was overproduced in a heterologous system (Pereira et al., 2004).

It has been reported that pathogen-derived TPI plays an important role in host–parasite interaction. The immunization of experimental animals with monoclonal antibodies to TPI, with the recombinant protein, as well as with plasmid DNA, led to a significant protection against schistosomiasis (Harn, 1987; Miao et al., 1998; Zhu et al., 2006). Recently, the enzyme was described as a novel binding protein of the integrin αIIb cytoplasmic domain (Liu et al., 2006).

Little is known about molecules related to fungal host-interaction, such as adhesion molecules and the molecular mechanisms governing *P. brasiliensis* adhesion to mammalian cells. With this in mind, we investigated the role of TPI as a putative novel molecule involved in the fungus interaction with the host. This work describes the production of an antirecombinant TPI (rPbTPI) polyclonal antibody that recognizes the native protein in yeast and mycelium, as well as recognizing the recombinant molecule. Immunoelectron microscopy was employed to define the TPI subcellular localization in *P. brasiliensis*. The PbTPI was detected in the cytoplasm and in the cell wall of the yeast phase of *P. brasiliensis*. TPI binds preferentially to laminin, as determined by peptide inhibition assays. Treatment of *P. brasiliensis* yeast cells with the anti-TPI polyclonal antibody promoted inhibition of interaction of *P. brasiliensis* to epithelial cells. Likewise, the treatment of *in vitro* cultured epithelial cells with the recombinant protein blocked binding of *P. brasiliensis*.

All the data indicate that TPI present on the surface of *P. brasiliensis* is able to interact with host extracellular matrix proteins. This fungal cell-wall characterized protein could be crucial for the initial fungal adherence and invasion, thus promoting the fungal infection.

**Materials and methods**

*Paracoccidioides brasiliensis* isolates growth conditions and differentiation assays

*Paracoccidioides brasiliensis* Pb01 strain (ATCC-MYA-826) has been investigated by our laboratory and was cultivated for 7 days in semisolid Fava Netto’s medium at 36 °C in the yeast form and at 22 °C for its mycelia phase, as previously described (Barbosa et al., 2006). The differentiation was performed in the same medium above, without agar, by changing the temperature of the culture from 22 to 36 °C for the mycelium to yeast transition, as described (Moreira et al., 2004). The cells were previously grown in liquid medium for 18 h before changing the incubation temperature, which was maintained for 15 days. Aliquots were taken at different time intervals and processed for further analysis.

**Heterologous expression of *P. brasiliensis* TPI, recombinant protein purification and antibody production**

The production and purification of the recombinant TPI was performed as described (Pereira et al., 2004). Briefly, the cDNA was cloned into the EcoRI/XhoI sites of pGEX-4T-3 (GE Healthcare). The recombinant TPI was expressed in the soluble form by the bacteria and the protein was purified by affinity chromatography under nondenaturing conditions. The recombinant protein was used to generate specific rabbit polyclonal serum. The rPbTPI (300 μg) was injected into rabbit with Freud’s adjuvant, three times at 2-week intervals. The obtained serum, containing specific anti-TPI polyclonal antibodies, was sampled and stored at −20 °C.

**Preparation of fungal cell extracts**

Yeast and mycelial protein crude extracts were obtained as described (Barbosa et al., 2006). Two types of extracts were produced: (1) a total cell homogenate and (2) a cell-free homogenate. For the preparation of total cell homogenate, mycelium and yeast cells were frozen and exhaustively ground with mortar and pestle in the presence of protease inhibitors. The mixture was centrifuged (12 000 g) at 4 °C for 10 min, and the supernatant was used. The cell-free extracts of the yeast cells were obtained as described (Barbosa et al., 2006). In brief, yeast cells of *P. brasiliensis* (300 mg) were resuspended in 1.0 mL of 10 mM phosphate-buffered saline (PBS), pH 7.2, and vortexed for 30 s. The cells were centrifuged (5600 g) for 1 min, and the supernatant was collected and used for further analysis. The protein content of samples was determined.

**Determination of TPI specific activity**

The enzymatic activity was determined by coupling the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate, as described (Plaut & Knowles, 1972). The increase in the absorbance at 340 nm caused by the reduction of NAD was monitored. One activity unit (U) is defined as the conversion of 1 μmol substrate min⁻¹ at 25 °C. The reaction mixture was composed of 100 mM triethanolamine buffer, pH 7.6, 5 mM EDTA, 0.2 mM NAD, 20 μg mL⁻¹ glyceraldehyde-3-phosphate dehydrogenase, 1 mM dihydroxyacetone phosphate, and 20 μL (at a concentration of 1.5 mg mL⁻¹) of cell extracts. The substrate dihydroxyacetone phosphate was solubilized in triethanolamine buffer, as described above. The concentration of NADH and the specific enzymatic activity were estimated using the
extinction coefficient of the product (\(e_{340}=6220 \text{ M cm}^{-1}\)). Chemicals used in the enzymatic assay were from Sigma Chemical Co. (St Louis, MO).

**Western blot analysis**

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out and the proteins were electrophoretically transferred to a nylon membrane, according to standard protocols. TPI was detected with the polyclonal antibody (1 : 1000 diluted). The reaction was revealed with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT).

**Ultrastucture of the yeast cells and immunocytochemistry of TPI**

For the ultrastructural and immunocytochemistry studies, we employed the protocols previously described in Barbosa et al. (2006). After fixation of the yeast cells, ultrathin sections were stained with 3% (w/v) uranyl acetate and lead citrate.

For ultrastructural immunocytochemistry studies, the ultrathin sections were incubated for 1 h with the polyclonal antibody raised against the recombinant TPI (diluted 1 : 100) and for 1 h at room temperature with the labeled secondary antibody rabbit IgG, Au-conjugated (10 nm average particle size; 1 : 20 dilution). The grids were stained as described above and observed with a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan). Controls were obtained.

**Affinity ligand assays**

Far-Western assays were carried out as previously described (Guichet et al., 1997; Barbosa et al., 2006). Recombinant TPI was submitted to SDS-PAGE and blotted onto nitrocellulose membranes, which were assayed for laminin and fibronectin binding as follows. The membranes were incubated with 30 \(\mu\)g laminin mL\(^{-1}\) or 30 \(\mu\)g fibronectin mL\(^{-1}\), for 90 min. The blots were sequentially incubated with rabbit antibodies antilaminin or antifibronectin (1 : 100 diluted) and with peroxidase-labelled goat antirabbit immunoglobulin (1 : 1000 diluted). The reactive bands were developed with hydrogen peroxide and dianinobenzidine. Controls were obtained.

**Binding assays of recombinant PbTPI to in vitro cultured A549 pneumocytes and Vero cells**

Type II pneumocyte line A549 from the American Type Culture Collection (ATCC CCL185, Manassas, VA) and African green monkey kidney cells (Vero cells, ATCC CCL81) were used in the experiments. Briefly, the A540 pneumocytes and Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and Medium 199 (Sigma), respectively, both supplemented with 10% (v/v) fetal calf serum. Monolayers of cells were incubated with 50 \(\mu\)g mL\(^{-1}\) of *P. brasiliensis* recombinant TPI at 36 °C for 5 h. Distilled water was added for 4 h and the cell lysates were fractionated by electrophoresis (12% SDS-PAGE). Proteins in the gel were electrotransferred to membranes. Western blot analysis was performed as described before. Negative controls were obtained by analyzing the supernatant of lysed cells not preincubated with the recombinant TPI, as well as by coating the cell culture flask with 50 \(\mu\)g mL\(^{-1}\) of TPI.

**Interference of TPI and polyclonal antibody in interaction of *P. brasiliensis* to in vitro cultured cells**

A549 pneumocytes and Vero cells were incubated for 1 h at 36 °C with the recombinant TPI at 50 \(\mu\)g mL\(^{-1}\) in 10 mM PBS. After washing the cells in the respective culture media 1 x 10^8 yeast cells of *P. brasiliensis* were added to the cultures. Incubation was performed for 5 h at 36 °C, as previously described (Barbosa et al., 2006). In parallel, 1 x 10^8 yeast cells of *P. brasiliensis* were incubated for 1 h at 36 °C with the polyclonal antibody anti-TPI (1 : 1000 diluted). Cells were washed and allowed to interact with the A549 pneumocytes or Vero cells. Three types of control experiments were performed: (1) with yeast cells not preincubated with the recombinant TPI protein; (2) with yeast cells not preincubated with the anti-TPI antibody and (3) with yeast cells preincubated just with bovine serum albumin (BSA), at 50 \(\mu\)g mL\(^{-1}\). The number of *P. brasiliensis* yeast cells interacting with the in vitro cultured epithelial cells was determined, as described (Esquenazi et al., 2003; Mendes-Giannini et al., 2004). Results are presented as the means of counts performed three times with SDs included.

**Inhibitory effects of synthetic peptides on binding of TPI to pneumocytes**

A549 epithelial cells at 1 x 10^6 cells mL\(^{-1}\) were grown in microtitre plates, for 24 h in DMEM as described above. The cells were fixed in solution containing paraformaldehyde (v/v) 4%, washed and blocked in PBS containing 10% fetal calf serum for 1 h at room temperature. The cells were incubated with the recombinant TPI at 50 \(\mu\)g mL\(^{-1}\), in the presence and absence of individual synthetic peptides (200 \(\mu\)g mL\(^{-1}\)), (Manque et al., 2000). After 1 h at 36 °C, cells were washed with PBS and incubated with the polyclonal anti-TPI antibody (1 : 1000 diluted). After washing, the cells were incubated with the peroxidase-labelled goat antirabbit IgG (1 : 3000 diluted). Semi-quantitative analysis was conducted by the addition of the peroxidase substrate (hydrogen peroxide/diaminobenzidine) to the wells and by the determination of the OD_{492nm}. The peptide fragments Arg–Gly–Asp–Ser (RGDS) from fibronectin, Arg–Gly–Asp (RGD), from fibronectin and laminin and Tyr–Ile–
Gly–Ser–Arg (YIGSR) from laminin were used in the experiments. As a negative control, TPI was omitted and replaced by PBS.

**Statistical analysis**

Results are expressed as the mean ± SE of the mean of three independent experiments. Statistical analysis was performed using ANOVA (F-test followed by Duncan test). P-values of 0.05 or less were considered statistically significant.

**Results**

**Production of polyclonal antibody and analysis of TPI accumulation in *P. brasiliensis***

The cDNA encoding the *P. brasiliensis* TPI (GenBank accession number AY250089) was overexpressed into bacterial cells. After induction with IPTG, a 56-kDa fusion recombinant protein was detected in the bacterial lysates (Fig. 1a, lane 2). The fusion protein was cleaved by the addition of thrombin protease (Fig. 1a, lane 3). As observed, highly purified protein was obtained that migrated on SDS-PAGE as a single species of 29 kDa. The purified recombinant TPI was used to produce rabbit polyclonal antibody. The protein total extracts of *P. brasiliensis* yeast and mycelium (Fig. 1a, lanes 4 and 5, respectively) were visualized after Coomassie blue staining. Those samples, including the recombinant TPI before and after thrombin cleavage, were blotted onto membranes and reacted to the polyclonal antibody (Fig. 1a, lanes 6–9). As demonstrated, a single band of 29 kDa was detected in extracts of both yeast and mycelium (Fig. 1a, lanes 6 and 7). Recombinant TPI in the bacterial lysates and purified recombinant TPI were also recognized as a single band by the polyclonal antibody (Fig. 1a, lanes 8 and lane 9, respectively). No cross-reactivity to the rabbit preimmune serum was evidenced with the samples (data not shown).

Total cellular extracts from mycelium, mycelium in transition to yeast at days 1, 7 and 15 after the temperature shift, were taken and analyzed by one-dimensional gel electrophoresis (Fig. 1b, lanes 1–4, respectively). The samples were run in parallel and transferred to membrane to react with the polyclonal antibody (Fig. 1b, lanes 5–8). The native protein is weakly accumulated in the mycelia phase (Fig. 1b, lane 5) and its expression is progressively increased during the transition to yeast (Fig. 1b, lanes 6–8).

**Detection of TPI protein by immunoelectron microscopy of *P. brasiliensis* yeast cells**

Immunocytochemistry experiments were performed to define the cellular localization of the TPI protein in yeast cells. Electron microscopy of conventionally embedded cells revealed the ultrastructure of the *P. brasiliensis* yeast form (Fig. 2a). In yeast cells processed by the postembedding method,
gold particles were present in cytoplasm and the cell wall (Fig. 2b). Control samples not exposed to the polyclonal antibody, as well as sample incubated with the rabbit preimmune serum, prior to the incubation with the gold-conjugated antibody were free of label (data not shown).

### Enzymatic activity of native and recombinant TPI of *P. brasiliensis*

As the TPI of *P. brasiliensis* was found to be localized both at the cell wall and internally in the cytoplasm, we attempted to evaluate the enzymatic activity of the native protein in total cellular extracts of yeast and mycelium, as well as in the cell-free extract of yeast cells, which corresponds to the most superficial components of the cell wall. Also the recombinant protein was assayed for its enzymatic activity. Table 1 contains the results of these experiments. The TPI specific activity was substantially higher in the yeast cells when compared with mycelia, in agreement with the higher amount of the protein in the parasitic phase, as demonstrated by Western blot analysis. Additionally, the cell-free extract fraction exhibited a high specific activity of TPI, corroborating the high amount of gold particles found in the fungal cell wall by the immunoelectron microscopy experiments. The recombinant molecule presented the highest activity in the tested samples.

### Binding of recombinant TPI to extracellular matrix proteins and to *in vitro* cultured A549 pneumocytes and Vero cells

The ability of the recombinant TPI of *P. brasiliensis* to bind laminin and fibronectin was determined by far-

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**Table 1. Analysis of the enzymatic activity of the native and recombinant *P. brasiliensis* TPI**

<table>
<thead>
<tr>
<th>Protein source</th>
<th>PbTPI specific activity (U mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast ‘cell free’ extract</td>
<td>2.02 ± 0.0075</td>
</tr>
<tr>
<td>Yeast</td>
<td>1.9 ± 0.0059</td>
</tr>
<tr>
<td>Mycelium</td>
<td>0.6 ± 0.0038</td>
</tr>
<tr>
<td>Purified recombinant TPI (rPbTPI)</td>
<td>18.8 ± 0.0015</td>
</tr>
</tbody>
</table>

*One activity unit (U) is defined as the conversion of 1 μmol substrate min⁻¹ at 25 °C. Activities are means of three independent determinations.*
Inhibitory effects of TPI and polyclonal antibody on *P. brasilensis* interaction to cells and competitive assay with TPI and peptides during interaction with pneumocytes

Yeast cells were assayed for the interaction with *in vitro* cultured pneumocytes and Vero cells, as shown in Fig. 4a and b, respectively. *Paracoccidioides brasiliensis* yeast cells were treated with the antibody anti-TPI prior to interaction with the *in vitro* cultured cells; pneumocytes and Vero cells were pretreated with recombinant TPI prior to the interaction with *P. brasiliensis* yeast cells. As demonstrated, the treatment of pneumocytes with recombinant TPI resulted in 65% inhibition of the adherence and internalization of as with the recombinant protein added to the cell culture flask, in the absence of the cells monolayer (Fig. 3b and c, lanes 1).
**Discussion**

In the present study we have investigated the role of TPI as an adhesin putatively involved in host cell binding. The fungal cell wall is the initial site of interaction between fungal cells and their host and thus is a key structure for entry and infection. Although not formally considered an intracellular pathogen, *P. brasiliensis* can enter epithelial cells (Hanna et al., 2000; Mendes-Giannini et al., 2000). Endocytosis of this fungus requires intact epithelial cell microfilaments and microtubules and triggers host-cell apoptosis (Mendes-Giannini et al., 2004). Although the invasion of nonphagocytic cells is likely central to the pathogenesis of the disease, there is a paucity of knowledge about the fungal surface proteins that induce invasion, as well as the host-cell receptors to the fungal adhesins.

In addition to a cytoplasmic location, the TPI of *P. brasiliensis* is present at the fungal cell wall. Despite its external location, the protein lacks an N-terminal signal peptide, as previously demonstrated (Fonseca et al., 2001; Pereira et al., 2004). Evidence has been accumulating using as current models *Saccharomyces cerevisiae* and *Candida albicans*, clearly showing that many proteins that lack an N-terminal peptide also reach the cell surface. Of special note, many of those surface proteins lacking the N-terminal signal peptide are also found in the cytoplasm, where they perform well-known functions (Nombela et al., 2006). Some of those nonconventionally secreted proteins are involved in binding to host components (Gozalbo et al., 1998). In regard to *P. brasiliensis*, we have demonstrated that two adhesins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Barbosa et al., 2006) and TPI, described here, do not present canonical sequences to surface trafficking. In addition to its probable binding to host-cell components mediating fungal adhesion and invasion, TPI exhibits enzymatic activity at the cell wall, as demonstrated in this work.

Adhesion encoding genes are not constitutively expressed, but in general are activated by diverse environmental triggers (Cheng et al., 2005). We demonstrated that the expression of TPI is developmentally regulated in *P. brasiliensis*, with expression increasing as the fungus adopts the pathogenic yeast-like morphology. Data on TPI enzymatic activity corroborate the Western blot result.

The ability of *P. brasiliensis* to colonize host tissues may be facilitated by fungal surface proteins with high affinity to extracellular matrix molecules, and the outcome of such colonization depends largely on the receptor/ligand interactions between the host cells and the fungus. As it has been proposed that initial infection of *P. brasiliensis* originates in the lung following inhalation of airborne conidia (Franco et al., 1994), the fungal ability to initiate infection may be due to the adhesion of its spores to both extracellular matrix molecules and lung epithelial cells. The TPI can be involved in the interaction of *P. brasiliensis* with the extracellular matrix components laminin and fibronectin, as inferred by the adhesion experiments of those molecules with immobilized TPI. The recombinant TPI and the polyclonal antibody raised against the molecule were able to interfere with the interaction of *P. brasiliensis* to *in vitro* cultured epithelial cells. Competitive assays with the RGD peptide, part of the laminin and fibronectin molecules, reduced by 36.0% the adhesion of TPI to pneumocytes. On the other hand, YIGSR derived from the laminin reduced the binding by 44.1% and RGDS did not reduce significantly the adhesion of TPI to pneumocytes. On the basis of those findings we can speculate that laminin mainly mediates the adhesion of TPI to the epithelial cells.

Recognition and binding to the host cells is a key step in the pathogenesis of many fungi, and consequently the characterization of novel adherence molecules and identification of the molecular basis of *P. brasiliensis* attachment to host cells remain important objectives.

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