RESEARCH ARTICLE

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* (*Pb*TPI) 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 *in 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 mycoses 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 (r*Pb*TPI) 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 *Pb*TPI 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 Pb*01 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 r*Pb*TPI (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 ($\varepsilon_{340} = 6220\ M\ cm^{-1}$). 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).

Ultrastructure 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 diaminobenzidine. 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) heat-inactivated 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 mediums 1×10^8 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, 1×10^8 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 1×10^6 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). Semiquantitative analysis was conducted by the addition of the peroxidase substrate (hydrogen peroxide/diaminobenzidine) to the wells and by the determination of the $OD_{492\,nm}$. 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.

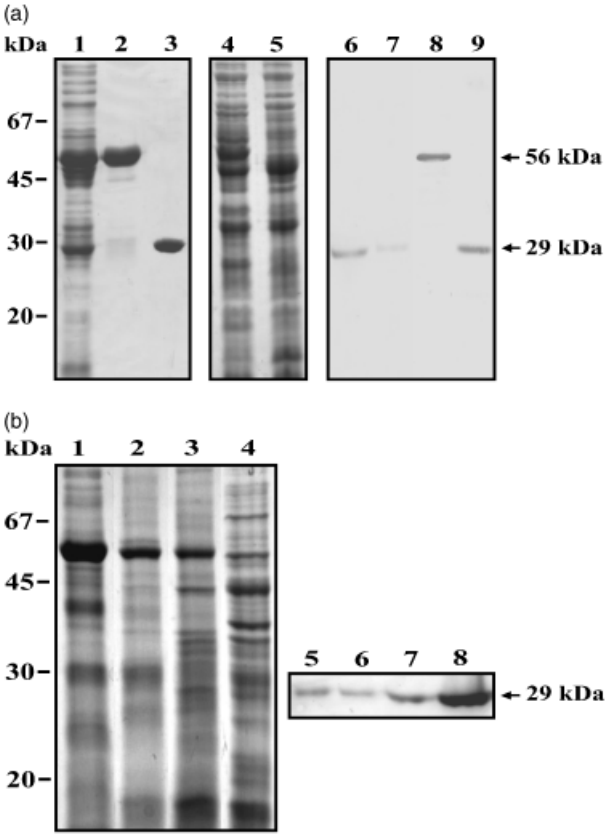

Fig. 1. Recognition of *P. brasiliensis* TPI by the rabbit polyclonal antibody and protein expression during fungal dimorphic transition. (a) Recombinant and native TPI are recognized by the polyclonal antibody. *Escherichia coli* XL1-Blue cells harbouring the pGEX-4T-3-TPI plasmid were grown at 30 °C to an $A_{260\,nm}$ of 0.6 and harvested after (lane 1) a 2 h incubation with 0.1 mM IPTG. Lane 2 – the affinity-isolated recombinant TPI. Lane 3 – the recombinant fusion protein cleaved by thrombin. Lane 4 – protein extracts from yeast (30 μg), Lane 5 – protein extracts from mycelium (30 μg). The proteins were fractionated by one-dimensional gel electrophoresis and stained using Coomassie blue. Lanes 6–9 – Western blot analysis of the native and recombinant TPI. The same samples and quantities of proteins were fractionated (12% SDS-PAGE) and transferred to membrane. Lane 6 – protein extracts from yeast. Lane 7 – protein extracts from mycelium. Lane 8 – *E. coli* transformed with pGEX-4T-3-TPI total extract; Lane 9 – the recombinant fusion protein cleaved by thrombin. The blots were reacted to the polyclonal anti-TPI antibody. Molecular size markers are indicated. Arrows indicate the recombinant fusion protein and the native TPI. (b) Analysis of the expression of TPI during the transition from mycelium to yeast in *P. brasiliensis*. The proteins (30 μg) were fractionated by one-dimensional gel electrophoresis and stained using Coomassie blue. Lane 1 – mycelium. Lanes 2–4 – mycelium in transition to yeast after 1, 7 and 15 days of the temperature shift, respectively. Lanes 5–8 – The same protein extracts (lanes 1–4) were probed to the anti-*Pb*TPI polyclonal antibody. Reactions were performed as described.

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,

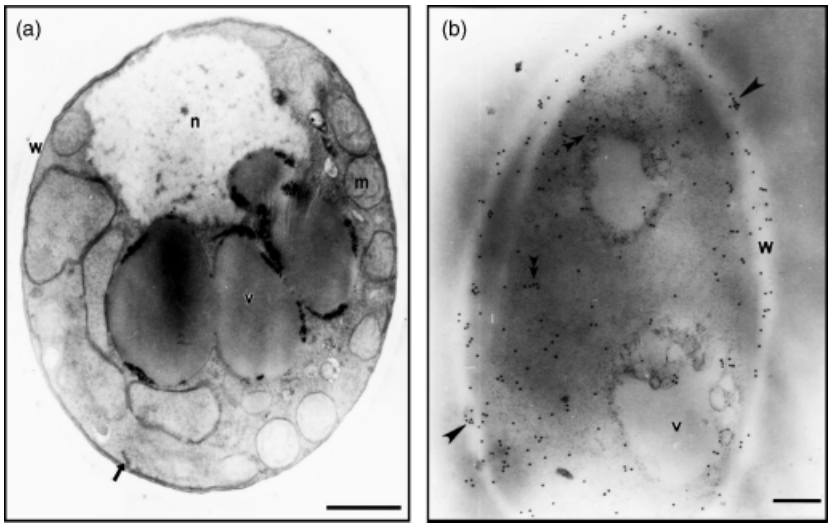

Fig. 2. Immunoelectron microscopy detection of TPI in *P. brasiliensis* yeast cells by postembedding methods. (a) Transmission electron microscopy of *P. brasiliensis* yeast cells; nucleus (n), intracytoplasmic vacuoles (v) mitochondria (m). The plasma membrane (arrow) and cell wall (w) are also shown. (b) Gold particles (arrowheads) are observed at the fungus cell wall (w) and in the cytoplasm (double arrowheads). Bars, 1 μm (a), 0.2 μm (b).

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.

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

Protein source	PbTPI specific activity (U mg^{-1} protein)*
Yeast 'cell free' extract	2.02 ± 0.0075
Yeast	1.9 ± 0.0059
Mycelium	0.6 ± 0.0038
Purified recombinant TPI (r*Pb*TPI)	18.8 ± 0.0015

*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.

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-Western blotting assays (Fig. 3a). The recombinant protein manifests the ability to bind to laminin (Fig. 3a, lane 2) and fibronectin (Fig. 3a, lane 3). The positive control was developed with the anti-TPI polyclonal antibody (Fig. 3a, lane 1). Negative controls were obtained by incubating the recombinant molecule with just the secondary antibody or in the absence of the extracellular matrix proteins (data not shown). An additional control demonstrated the specificity of the binding of TPI to the extracellular matrix proteins, as no reactivity between BSA and the extracellular matrix proteins was demonstrated and the polyclonal antibody to TPI did not present cross-reactivity to BSA (data not shown). The adhesin characteristic of TPI was also evaluated by interaction of the recombinant protein with pneumocytes and Vero cells (Fig. 3b and c, respectively). The purified protein behaved as an adhesin, binding to the *in vitro* cultured cells (Fig. 3b and c, lane 2). Negative and positive controls were developed, respectively, with pneumocytes A549 and Vero cells not incubated with the recombinant TPI (Fig. 3b and c, respectively, lane 3), as well

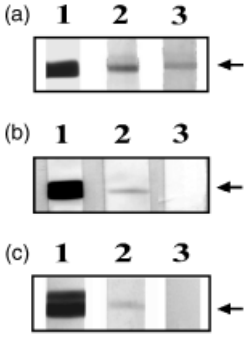

Fig. 3. Interaction of the r*Pb*TPI to extracellular matrix components and to the *in vitro* cultured A549 pneumocytes and Vero cells. (a) The r*Pb*TPI (0.5 μg) was subjected to SDS-PAGE and electroblotted. The membranes were reacted with laminin (lane 2) and fibronectin (lane 3) and subsequently incubated with the rabbit IgG antilaminin and antifibronectin antibodies, respectively. Use of peroxidase-conjugated antirabbit IgG revealed the reactions. The positive control was obtained by incubating the recombinant protein with the anti-TPI polyclonal antibody (lane 1). (b and c) Cultured pneumocytes (b) and Vero cells (c) were incubated with 50 μg of recombinant TPI for 5 h at 36 °C. The cells were lysed, the supernatant was fractionated by SDS-PAGE and proteins were transferred to membranes. Detection was performed by incubation with rabbit anti-TPI polyclonal antibody and subsequent reaction with alkaline phosphatase-coupled anti-rabbit IgG. The reaction was developed with BCIP/NBT. Positive control, recombinant protein transferred to the membranes (lane 1); supernatant of pneumocytes or Vero cells after incubation of the cells with the recombinant TPI (lane 2); supernatant of pneumocytes and Vero cells not incubated with the recombinant TPI (lane 3).

as with the recombinant protein added to the cell culture flask, in the absence of the cells monolayer (Fig. 3b and c, lanes 1).

Inhibitory effects of TPI and polyclonal antibody on *P. brasiliensis* 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

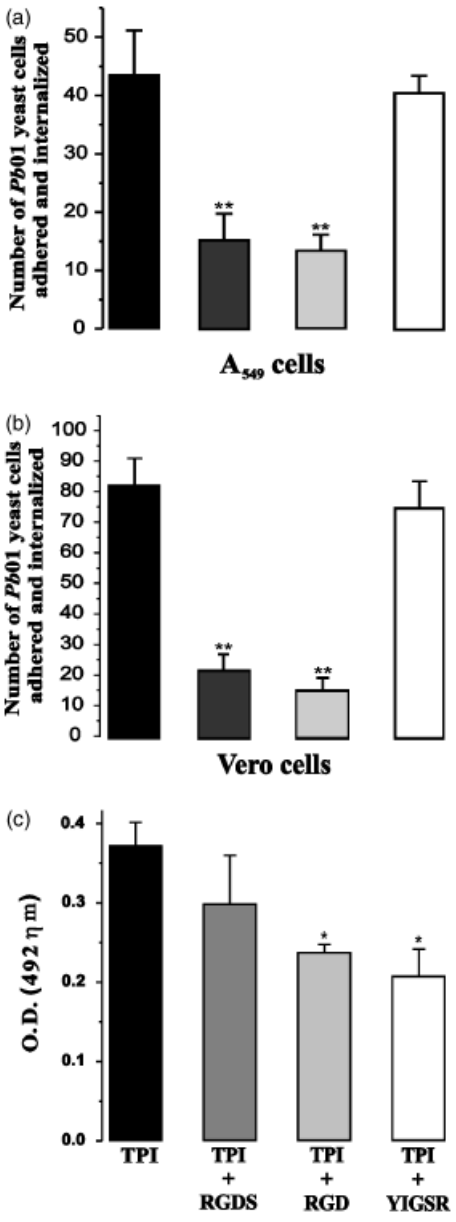

Fig. 4. Interaction assay data and competitive assay with *Pb*TPI and peptides during the interaction with pneumocytes. (a and b) Histograms showing the interaction (adhesion plus internalization) of *P. brasiliensis* yeast cells to pneumocytes A549 and Vero cells, respectively. Prior to each assay, the yeast cells were treated for 1 h with the anti-TPI polyclonal antibody (1 : 1000 diluted). In another set of experiments the A549 and Vero cells were pretreated for 1 h with 50 μg mL^{-1} of the r*Pb*TPI. The interaction of *P. brasiliensis* to pneumocytes and Vero cells was analyzed after 5 h (a and b, respectively). Black bars, control; dark grey bars, epithelial cells treated with the recombinant TPI; light grey bars, *Paracoccidioides brasiliensis* yeast cells treated with the anti-TPI polyclonal antibody; white bars, cells treated with BSA. The values represent the mean ± SD of three independent experiments performed in triplicate. Asterisks denote values statistically different from control at $P < 0.0001$. Vertical bars indicate SD. (c) Pneumocyte A549 cells were incubated with TPI (10 μg mL^{-1}); TPI (10 μg mL^{-1}) and the synthetic peptides of fibronectin (RGDS and RGD), and laminin (RGD and YIGSR) and anti-*Pb*TPI antiserum (1 : 1000). The interaction was visualized using ELISA. The results were expressed in absorbance units and correspond to mean values ± SD of triplicate experiments. *$P < 0.05$.

P. brasiliensis to those cells. In addition, the treatment of *P. brasiliensis* yeast cells with the polyclonal antibody resulted in 69% inhibition of the adherence and internalization to pneumocytes (Fig. 4a). Similar results were obtained when Vero cells were infected with *P. brasiliensis*. The treatment of the epithelial cells with the recombinant TPI resulted in 73% inhibition of fungal adherence and internalization. We observed an 85% inhibition of adhesion/internalization with the treatment of the yeast cells with the polyclonal antibody (Fig. 4b).

As laminin and fibronectin bind to immobilized TPI we attempted to evaluate whether peptides of those molecules had the ability to inhibit TPI binding to *in vitro* cultured pneumocytes. Competitive binding was performed with synthetic peptides corresponding to the adhesive recognition sequences of fibronectin and laminin, as shown in Fig. 4c. The peptide RGDS from fibronectin exhibited a small inhibitory effect ($P > 0.05$) on the binding of TPI to *in vitro* cultured pneumocytes. Synthetic peptide RGD, from fibronectin and laminin, inhibited the interaction of TPI with the cultured pneumocytes by 36.0% ($P < 0.05$), as shown. The peptide YIGSR, from laminin, showed an inhibitory effect of 44.1% ($P < 0.05$).

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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