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Enolase from *Paracoccidioides brasiliensis*: isolation and identification as a fibronectin-binding protein

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Paracoccidioides brasiliensis yeast cells can enter mammalian cells and may manipulate the host cell environment to favour their own growth and survival. Moreover, fibronectin and several other host extracellular matrix proteins are recognized by various components of the yeast cell extracts. The present study was designed to isolate and characterize a fibronectin-binding protein from *P. brasiliensis*. We also compared *P. brasiliensis* strain 18, tested before (Pb18a) and after (Pb18b) animal passage, in relation to its adhesion and invasion processes. Extracts from both samples, when cultured on blood agar solid medium, showed higher levels of protein expression than when the same samples were cultured on Fava-Netto solid medium, as demonstrated by two-dimensional electrophoresis and SDS-PAGE. Also, both Pb18a and Pb18b exhibited stronger adhesion to A549 epithelial cells when cultured on blood agar medium than when cultured on Fava-Netto medium. Ligand affinity binding assays revealed a protein of 54 kDa and pI 5.6 in *P. brasiliensis* cell-free extracts with the properties of a fibronectin-binding adhesin, which was characterized by tryptic digestion and mass spectroscopy as a homologue of enolase from *P. brasiliensis*. Antibody raised against this 54 kDa protein abolished 80 % of *P. brasiliensis* adhesion to A549 epithelial cells. Our results demonstrate that *P. brasiliensis* produces a fibronectin-binding adhesin, irrespective of the culture medium, and that this activity can be inhibited by a specific antibody and is involved in the adhesion of the fungus to pulmonary epithelial cells.

INTRODUCTION

Paracoccidioidomycosis is one of the most prevalent human systemic mycoses in Latin America and is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis* (Brummer *et al.*, 1993). The successful colonization of the host by this fungus is a complex event, generally involving interactions between ligands of the pathogen (adhesins) and cell receptors. It is known that during haematogenous dissemination, such interactions constitute the first stages in the development of innumerable infectious diseases (Mendes-Giannini *et al.*, 2005; Ofek *et al.*, 1996). In this way, the identification of the molecules involved could represent a step towards the discovery of efficient treatments for these systemic mycoses.

In recent years, several molecules with receptor-like characteristics have been described in pathogenic fungi (Mendes-Giannini *et al.*, 2000, 2005). Adhesins have been described in *P. brasiliensis* (Andreotti *et al.*, 2005; Barbosa *et al.*, 2006; Gonzalez *et al.*, 2005; Mendes-Giannini *et al.*, 2006; Vicentini *et al.*, 1994). Previous studies have shown the presence of a laminin receptor on the surface of the *P. brasiliensis* yeast form and a glycoprotein of 43 kDa (gp43) which has been shown to behave as an adhesin molecule (Hanna *et al.*, 2000; Vicentini *et al.*, 1994). Recently, a *P. brasiliensis* adhesin of 30 kDa, with a capacity to bind to laminin, was isolated. This protein was strongly

Abbreviation: ECM, extracellular matrix.

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expressed in the *P. brasiliensis* isolate Pb18, which exhibits higher adhesion capacity than most strains. Vero cells pre-treated with the 30 kDa molecule showed stronger inhibition of *P. brasiliensis* adhesion and invasion than those pre-treated with gp43. However, a combination of the 30 kDa and gp43 proteins decreased the epithelial cell adhesion and invasion indices significantly more than either protein alone (Andreotti *et al.*, 2005). *P. brasiliensis* also presents on its cell surface two proteins, of 32 and 19 kDa, that interact with various extracellular matrix (ECM) proteins, such as laminin, fibronectin and fibrinogen (Gonzalez *et al.*, 2005). The *P. brasiliensis* 39 kDa recombinant protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was capable of binding to laminin, collagen I and fibronectin. Either treatment of *P. brasiliensis* yeast forms with anti-GAPDH or treatment of pneumocytes with recombinant GAPDH led to inhibition of the fungal infection of epithelial cells (Barbosa *et al.*, 2006). Similar results were obtained with the triose phosphate isomerase (Pereira *et al.*, 2007). Paracoccin, a recently described adhesin, also interacted with laminin, in a dose-dependent manner. This interaction was inhibited by GlcNAc, followed by D-glucose and D-mannose, but not by D-galactose, *N*-acetyl-galactosamine or L-fucose (Coltri *et al.*, 2006).

The composition of the ECM varies in different tissues and during phases of injury, inflammation and repair (Kottom *et al.*, 2003). Fibronectin is a disulfide-linked dimeric glycoprotein present in a soluble form in blood plasma and other body fluids and in a fibrillar form in the ECM. The main function of fibronectin is probably related to its ability to mediate adhesion to mammalian cells, a process that involves the binding of specific cell surface receptors to discrete domains in the fibronectin molecule (Pearlstein *et al.*, 1980; Ruoslahti, 1988). A previous study showed that cell-free components prepared from *P. brasiliensis* strain Pb18, subcultured before (Pb18a) and after (Pb18b) animal inoculation, adhered differently to 40 kDa and 120 kDa fibronectin fragments. While Pb18a adhered more strongly to whole fibronectin and the 120 kDa fragment than to the 40 kDa fragment, the Pb18b extract adhered more strongly to the 40 kDa fragment (Mendes-Giannini *et al.*, 2006).

The aim of the work described here was thus to isolate and characterize a fibronectin-binding protein related to adhesion by comparing two samples of *P. brasiliensis*, tested before (Pb18a) and after (Pb18b) animal inoculation, both cultured on two different media.

METHODS

Micro-organism. *P. brasiliensis* strain 18 (Pb18) was isolated from a clinical case of paracoccidioidomycosis and maintained at the Faculty of Medicine of the University of Sao Paulo (FM-USP), Brazil. Pb18a was grown on Fava-Netto solid medium (Fava-Netto, 1961) at 35 °C and subcultured every 3–4 days, 72 times. Pb18 was inoculated into a male hamster intratesticularly, and after 30 days the animal was sacrificed and the testicles were macerated and cultured on Sabouraud agar with chloramphenicol at 25 °C until the development of characteristic *P. brasiliensis* mycelial colonies (Andreotti *et al.*, 2005). These were identified and incubated at 35 °C on Fava-Netto medium, to obtain the yeast phase, and the resulting sample was labelled Pb18b. This sample was subcultured three to four times for the assays. Subsequently, the samples Pb18a and Pb18b in the yeast phase were grown for 3–4 days at 35 °C on blood agar medium containing 5 % sheep blood.

Cell-free extract. Cell-free extracts were prepared from *P. brasiliensis* strain 18 in yeast form, subcultured before (Pb18a) and after (Pb18b) animal passage, as described elsewhere (Blotta & Camargo, 1993). About 300 mg (6×10^4 cells ml^{-1}) *P. brasiliensis* was grown for 3–4 days at 35 °C on Fava-Netto solid medium or blood agar medium with 5 % sheep blood, then scraped off and mixed with 1 ml PBS (pH 7.2). This mixture was vortexed for 30 s and centrifuged at 560 ***g*** for 1 min. The four supernatant cell-free extracts (Pb18a and Pb18b grown on each of the two media) were removed and stored at –20 °C.

Anti-cell-free and anti-54 kDa polyclonal sera. Polyclonal antisera to *P. brasiliensis* cell-free extract (Pb18) and 54 kDa protein were generated by immunizing rabbits with intradermal injections of 1.0 ml extracts (respectively, 1.2 and 1.0 mg ml^{-1}) mixed with 1.0 ml Freund's complete adjuvant. Subsequent injections of extract or 54 kDa protein mixed with incomplete adjuvant were given weekly for a period of 4 weeks and then monthly for a period of 3 months. The rabbits were bled 7 days after the last dose. The immunoglobulin fractions of both sera were separated by precipitation with ammonium sulfate (1.56 M; pH 6.5) and stored at –70 °C.

Fractionation and purification of fibronectin-binding adhesins from *P. brasiliensis*. Firstly, Pb18a was cultured in yeast form on blood base agar (BBA) with 5 % sheep blood. Cell-free extract was prepared from the sample and semi-purified in a gelatin-agarose affinity column (Bio-Rad). The semi-purified fractions were pooled and dialysed against PBS (0.01 M; pH 7.4) overnight, as described by Klotz *et al.* (1993), then analysed by SDS-PAGE (using 10 % gels) (Laemmli, 1970) and immunoblotting (Towbin *et al.*, 1979). After electrophoretic analysis, the gel was stained with Coomassie brilliant blue R-250 and the 54 kDa protein species was cut from the gel, purified by electroelution (Neophytou *et al.*, 1996; Sa-Pereira *et al.*, 2000) and then analysed by two-dimensional electrophoresis.

Epithelial cells. The type II pneumocyte cell line A549 was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in HAM F-12 Nutrient (Cultilab) supplemented with 10 % heat-inactivated fetal calf serum (Cultilab) at 36.5 °C.

Assay of *P. brasiliensis*–mammalian cell interaction. Cells were cultured at 36.5 °C in 24-well plates for 24 h, with the well bottoms covered with coverslips, and adjusted to 1.0×10^6 cells per well. Next, the *P. brasiliensis* samples, Pb18a and Pb18b, were cultured on Fava-Netto solid medium and BBA with 5 % sheep blood for 3–4 days at 35 °C. A suspension of each sample was resuspended in sterilized PBS (0.01 M; pH 7.4) at 1.0×10^6 yeast cells ml^{-1} and added to the epithelial cells and incubated for various times at 36.5 °C to observe adhesion, total infection and invasion processes. After each period of incubation, the medium was discarded, the cells were washed with PBS and the coverslips were fixed with 2 % paraformaldehyde and stained in May-Grünwald Giemsa. The coverslips were examined by conventional microscopy, the adherent fungal cells were counted and a graph was produced to analyse the kinetics of interaction of *P. brasiliensis* with cell line A549. All experiments were performed in triplicate. The mean and standard deviation of at least three separate experiments were determined. Data were analysed statistically by

Student's *t*-test. *P*-values of 0.05 or less were considered to indicate significant differences.

Two-dimensional electrophoresis protein separation. The semi-purified Pb18a extract was subjected to isoelectric focusing, as described by O'Farrell (1975). The second dimension was performed on a 5–15 % gradient polyacrylamide gel, as described by Laemmli (1970). Gels were washed and the proteins were stained with Coomassie blue.

Affinity ligand assays. Cell-free extracts of Pb18a grown on BBA media with and without 5 % sheep blood, as well as one of the fractions of semi-purified extract of the Pb18a grown on the same medium with 5 % sheep blood, were characterized by immunoblotting. SDS-PAGE was performed basically as described by Laemmli (1970) and electrophoretic transfer blotting to a nitrocellulose membrane was carried out as described previously by Towbin *et al.* (1979). Blotted proteins were assayed for fibronectin affinity as follows. The membranes were incubated with 1 % BSA in 10 mM PBS (pH 7.4) containing 0.9 % NaCl for 4 h at room temperature and then for 90 min in PBS-T-BSA (PBS, 1 % BSA, 0.05 % Tween 20) containing human fibronectin (30 μg ml^{-1}; Gibco). After washing four times in PBS buffer containing 0.05 % Tween 20 (PBS-T buffer), the sheets were incubated for 1 h with rabbit anti-fibronectin antibody (diluted 1 : 1000) (Dako). As a control, the blots were incubated with anti-fibronectin antibody, in the absence of ECM protein (fibronectin). Positive controls were provided by serum from a paracoccidioidomycosis patient (1 : 20) and anti-*P. brasiliensis* cell-free (1 : 20) serum.

Biotinylation of the protein. Pb18a cell-free extracts from cultures grown on BBA with 5 % sheep blood were biotinylated with the ECL protein biotinylation kit (GE Healthcare, Amersham Pharmacia Biotech) as recommended by the manufacturer. Monolayers of A549 cells were incubated with the biotinylated proteins at 37 °C overnight and washed with PBS to remove unbound protein. Next, double-distilled water was added and the cells were incubated for 4 h at room temperature to obtain total lysis. The lysates were centrifuged at 1400 ***g*** for 5 min, and the supernatant was subjected to electrophoresis by SDS-PAGE. Proteins in the gel were transferred to a nitrocellulose membrane and strips of the membrane were incubated with blocking buffer (PBS-T with 2 % BSA) for 4 h at room temperature. Patterns were revealed with the Catalysed Signal Amplification System kit (Dako). The supernatant of A549 cells that had not been incubated with the biotinylated protein was processed similarly as a negative control.

***In situ* trypsin digestion and MS.** The protein separated by SDS-PAGE was subjected to *in situ* gel band trypsin digestion with 0.5 μg modified trypsin (Promega). The tryptic peptides were desalted on a Vydac C18 micro-tip reversed-phase column and eluted in 70 % methanol, 0.2 % formic acid for MS analysis. The MS analysis of tryptic peptides was carried out in a Quattro II electrospray triple-quadrupole mass spectrometer (Micromass) by direct infusion (300 nl min^{-1}) under the following conditions: capillary voltage maintained at 2.8 kV, cone voltage 40 V, cone temperature set to 100 °C. The parameters for MS1 scanning and daughter ion scanning were optimized with synthetic peptide for the highest signal-to-noise ratio and calibrated with PEG (50–2000 Da). In the daughter ion scanning mode, the collision energy was set to 25–35 eV, and argon was used as collision gas with a partial pressure of 3.0×10^{-3} mTorr. The spectrum was collected as a mean of 20–50 scans (2–5 s per scan) and processed by using the MassLynx software v.3.3 (Micromass). The sequence of tryptic peptides was deduced by collision-induced dissociation MS, employing Biolynx software (Micromass). The data were compared with the NCBI databases (http://www.ncbi.nlm.nih.gov/BLAST/), Swiss-Prot (http://prospector.ucsf.edu/) and Mascot (http://www.matrixscience.com/search_form_select.html) to identify the protein.

Inhibition assays. The anti-54 kDa serum fraction was employed in the adherence inhibition assay. A standard suspension of *P. brasiliensis* (Pb18a and Pb18b samples cultivated on Fava-Netto solid medium and solid BBA with 5 % sheep blood) was incubated for 1 h at 37 °C with rabbit preimmune serum or rabbit anti-54 kDa serum diluted 1 : 100 in PBS (pH 7.2). After this incubation, 300 μl of each sample was transferred to the plate coated with epithelial cells and incubated for 2 and 5 h at 37 °C, as described above. As positive control, epithelial cells were assayed with *P. brasiliensis* without previous treatment and a negative control was included consisting of epithelial cells assayed with *P. brasiliensis* with treatment with rabbit preimmune serum diluted 1 : 100 in PBS. The percentage of infected cells was determined by randomly counting a minimum of 300 cells on each duplicate coverslip, and experiments were repeated at least three times. The adhesion index was calculated by multiplying the mean number of attached fungi per pneumocyte cell by percentage of infected cells, observed by microscopic examination with an oil-immersion objective. The endocytic index was determined in a similar way, except that the mean number of ingested yeasts was used (Esquenazi *et al.*, 2003). All experiments were performed in triplicate. The mean and standard deviation of at least three separate or independent experiments were determined. Data were analysed statistically by Student's *t*-test. *P*-values of 0.05 or less were considered to indicate significant differences.

Statistical analysis. The results of the interaction of *P. brasiliensis* (Pb18a and Pb18b) samples with cell line A549 and the results of the adherence inhibition assays are expressed as mean ± standard deviation. Statistical comparisons were performed using Student's *t* test data. Statistical significance was accepted for $P < 0.05$.

RESULTS

Electrophoretic analysis of *P. brasiliensis* cell-free extracts

Extract from Pb18b, grown on Fava-Netto solid medium, showed a higher total protein expression than Pb18a (Fig. 1a). However, both extracts (Pb18a and Pb18b) from the samples cultured on medium with blood showed higher protein expression than the same samples cultured on common medium (Fava-Netto) or medium without blood (solid base agar). The highly expressed proteins in the Pb18a and Pb18b isolates had molecular masses of 100, 76, 67, 54 and 30 kDa, as demonstrated in Fig. 1(b). The 54 kDa protein was expressed more strongly in both samples when cultured on blood medium. Apparently culturing on blood medium has the same effect on 54 kDa protein expression in cultures obtained before and after animal passage. For this reason, all subsequent experiments were performed with the Pb18a isolate cultured on BBA with blood.

Characterization of adhesin-like proteins

Several proteins (72, 60, 58, 54, 50, 43 and 26 kDa) from the Pb18a extract were able to bind to fibronectin when this yeast was cultured on BBA with 5 % blood, and when this isolate was cultured without blood a small number of

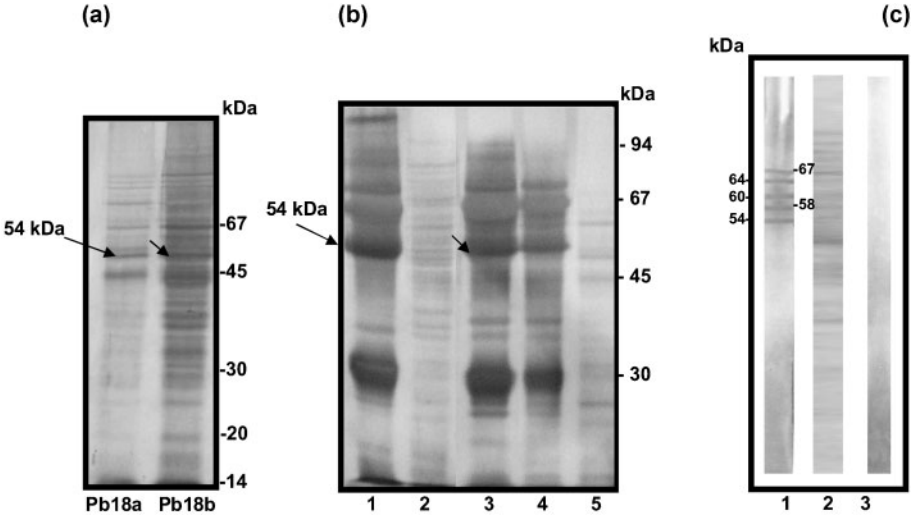

Fig. 1. (a) SDS-PAGE of *P. brasiliensis* cell-free extracts of cultures of strain Pb18 isolated before (Pb18a) and after (Pb18b) passage in hamster, both subcultured on Fava-Netto solid medium, stained with silver stain. (b) SDS-PAGE of cell-free extracts of Pb18 isolates cultured on solid medium with and without sheep blood. Lanes: 1, Pb18a (pre-passage) cultured on BBA with 5 % blood; 2, Pb18a cultured without blood; 3, Pb18b (passaged in hamster) cultured on BBA with 10 % blood; (4) Pb18b (passaged in hamster) cultured on BBA with 5 % blood; 5, Pb18b cultured without blood. (c) Characterization of some proteins from cell-free extracts as adhesins. Lanes: 1, Lysed pulmonary cells treated with biotinylated cell-free extracts from Pb18a cultured on BBA solid medium; 2, positive control: cell-free extract biotinylated; 3, negative control: lysed pulmonary cells not treated with biotinylated extracts.

proteins (58, 54 and 50 kDa) retained the capacity to bind to this ECM component (data not shown). The proteins of 67, 64, 60, 58 and 54 kDa behaved as adhesins, binding to the pulmonary cells in the biotinylation experiment (Fig. 1c).

Two-dimensional electrophoretic analysis of *P. brasiliensis* cell-free extracts

The cell-free extract from the Pb18a sample, cultured on BBA with 5 % blood, was fractionated on a gelatin-agarose affinity column. The resulting semi-purified fraction of the cell-free extract was analysed by two-dimensional gel electrophoresis and the protein species of 60 kDa/pI 6.3, 58 kDa/pI 5.9 and 54 kDa/pI 5.6 were expressed strongly, as shown in Fig. 2(a). These proteins display the capacity to bind to fibronectin, as shown in Fig. 2(b), and this reaction is specific, as demonstrated by a negative control (Fig. 2c).

Purification of the 54 kDa protein by electroelution and reactivity of antiserum

After electrophoretic analysis, the 54 kDa protein was excised from the gel and extracted from the polyacrylamide gel by electroelution. The electroelution buffer was analysed by two-dimensional electrophoresis (isoelectric focusing for separation by pI, followed by gradient PAGE for size focusing), revealing a protein species of 54 kDa and pI 5.6 (Fig. 2d). This protein was subjected to SDS-PAGE and *in situ* trypsin digestion. Peptides were analysed by MS.

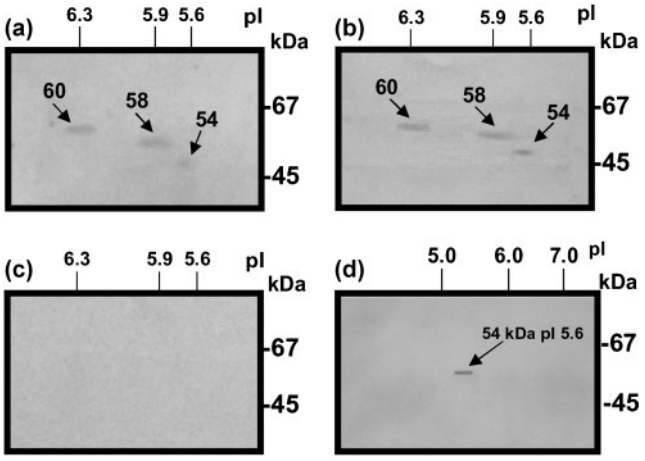

Fig. 2. (a) Two-dimensional gel electrophoresis of the semi-purified fraction from Pb18a cultured on BBA solid medium with 5 % blood after fractionation on a gelatin-agarose affinity column. Gel was stained with Coomassie brilliant blue G250. Arrows point to the 60 kDa (pI 6.3), 58 kDa (pI 5.9) and 54 kDa (pI 5.6) proteins. (b) Immunoblotting membrane of the same fraction treated with fibronectin and anti-fibronectin serum and peroxidase-conjugated anti-rabbit IgG. (c) Negative control: blot was incubated with anti-fibronectin antibody in the absence of ECM protein (fibronectin). (d) Analysis of the 54 kDa protein by two-dimensional gel electrophoresis after electroelution purification.

The sequence of tryptic peptides was deduced by collision-induced dissociation MS using the software Biolynx (Micromass).

A BLAST search revealed 100 % identity between this sequence and that of enolase from *P. brasiliensis* (GenBank accession no. ABQ45367.1). The protein was also similar to enolases from *Coccidioides posadasii* (GenBank accession no. ABH10638.1), with an identity of 92 %, *Aspergillus nidulans* (GenBank accession no. XP_663350.1) and *Aspergillus terreus* (GenBank accession no. XP_001212080.1), with an identity of 89 %, and *Aspergillus oryzae* (GenBank accession no. Q12560), *Penicillium chrysogenum* (GenBank accession no. Q76KF9) and *Aspergillus fumigatus* (GenBank accession no. AAK49451.1), with an identity of 88 % (Table 1).

Kinetics of interaction of *P. brasiliensis* with epithelial cell culture

The adhesion of Pb18a and Pb18b cells to epithelial cells was observed after 30 min of contact. The two samples exhibited numerical differences in relation to adhesion, as demonstrated in Fig. 3(a, b), irrespective of the culture medium. Pb18b adhered to pulmonary cells more efficiently than Pb18a.

Pb18a and Pb18b cultured on blood medium exhibited significantly greater numbers of adhering cells ($P < 0.05$) than Pb18a and Pb18b cultured on common medium (Fava-Netto), as demonstrated in Fig. 3(a, b).

Inhibition of *P. brasiliensis* interaction by a specific polyclonal antibody

The 54 kDa protein was used to generate a polyclonal antibody. A pool of cell-free extracts from Pb18a and Pb18b (cultured on 5 % blood medium) and the purified protein were subjected to SDS-PAGE separation, blotted on to nitrocellulose membrane and allowed to react with the polyclonal antibody (Fig. 4). As demonstrated, a single band of 54 kDa reacted with the purified protein and *P. brasiliensis* cell-free extract (Fig. 4, lanes 1 and 2). No cross-reactivity to the rabbit preimmune serum was detected.

The adhesion and endocytic indices obtained by the interaction of *P. brasiliensis* yeast cells, pretreated with the rabbit anti-54 kDa polyclonal antibody, with A549 cells were compared with those of the control to calculate the percentage adhesion and invasion inhibition by this polyclonal antiserum (Fig. 5). Both Pb18a and Pb18b cells pretreated with anti-54 kDa antibody, irrespective of the culture medium employed, were markedly inhibited (up to 82 %) in their adhesion to pulmonary cells after 2 h and 5 h of interaction, as well as in the endocytic process (~45 %). No inhibition was observed with rabbit preimmune serum.

DISCUSSION

The ability of *P. brasiliensis* to interact with host surface structures is essential to further colonization, invasion and growth (Mendes-Giannini *et al.*, 2000).

This study was designed to isolate and characterize a fibronectin-binding adhesin and to relate it to the capacity of this fungus to adhere to the host, by comparing *P. brasiliensis* samples taken before (Pb18a) and after (Pb18b) passage in animal hosts and cultured on Fava-Netto solid medium and on BBA medium with 5 % blood. Studies have been published indicating that supplementing a chemically defined medium with haemoglobin induces the expression of fibronectin-binding adhesin (Pendrak *et al.*, 2000; Yan *et al.*, 1998). When Pb18a and Pb18b samples were cultured on medium with 5 % blood, higher protein expression was observed than with the same samples cultured on Fava-Netto medium or the base agar medium without blood. Apparently, the use of medium with blood has a similar effect to animal passage.

The cell-free extracts of Pb18a cultured on BBA medium with 5 % blood were fractionated and analysed by two-dimensional electrophoresis and ligand affinity binding

Table 1. Analysis of an *in situ* trypsin digest of the 54 kDa gel band by MS and comparison of the most-similar proteins from the BLAST search

Enolase	Sequence*	Identity (%)†
Paracoccidioides brasiliensis (this study)	1 MAITKIHARSVYDSRGNPTVEVDVVTETGLHRAIVPSGASTGQHEACELRDGDQSKWLGK 60	
Paracoccidioides brasiliensis	1 MAITKIHARSVYDSRGNPTVEVDVVTETGLHRAIVPSGASTGQHEACELRDGDQSKWLGK 60	100
Coccidioides posadasii	1 MAITKIHARSVYDSRGNPTVEVDVVTETGLHRAIVPSGASTGQHEACELRDGD++ WLGK 60	92
Aspergillus nidulans	1 M I+KIHARSVYDSRGNPTVEVDVVTETGLHRAIVPSGASTGQHEA ELRDGD+ KWLGK 60	89
Aspergillus terreus	1 M I+KIHARSVYDSRGNPTVEVDVVTETGLHRAIVPSGASTGQHEACELRDGD++ W GK 60	89
Aspergillus oryzae	1 M ITKIHARSVYDSRGNPTVEVDVVTETGLHRAIVPSGASTGQHEA ELRDGD++ W GK 60	88
Penicillium chrysogenum	1 M I+KIHARSVYDSRGNPTVEVDVVTETGLHRAIVPSGASTG HE ELRDGD++KW GK 60	88
Aspergillus fumigatus	1 M I+KIHARSVYDSRGNPTVEVDV TETGLHRAIVPSGASTGQHEA ELRDGD+++W GK 60	88

*Identical residues are underlined.

†Percentage sequence identities in amino acid overlap.

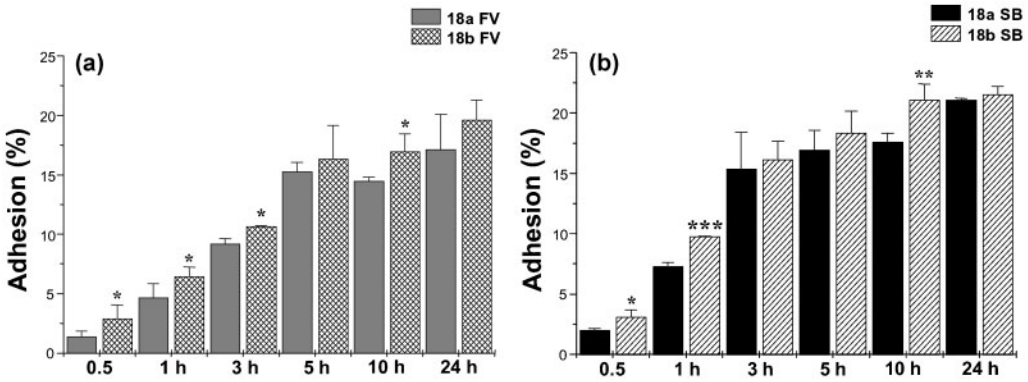

Fig. 3. Percentage of A549 cells showing yeast adhesion plotted against time (h) of incubation with Pb18a and Pb18b isolates grown on common medium (Fava-Netto) or solid blood medium. Data represent means and standard deviations of three independent experiments. One asterisk denotes values statistically different from samples ($P < 0.05$), two asterisks denote $P < 0.01$ and three asterisks denote $P < 0.001$. 18a FV, Pb18a subcultured on Fava-Netto solid medium; 18b FV, Pb18b after passage in hamster and cultured on Fava-Netto solid medium; 18a SB, Pb18a subcultured on BBA with 5 % blood; 18b SB, Pb18b after passage in hamster and cultured on BBA with 5 % blood.

assays. One of the semi-purified fractions was rich in three proteins, with these electrophoretic properties: 60 kDa and pI 6.3; 58 kDa and pI 5.9; and 54 kDa and pI 5.6. All three behaved as fibronectin-binding adhesins. Further purification was performed in order to isolate and characterize the 54 kDa (pI 5.6) protein, highly expressed in both Pb18a and Pb18b, as well as in other isolates, especially strains Pb113 and Pb339 (data not shown), when cultivated on medium with 5 % blood. It was confirmed that this protein behaves as a fibronectin-binding adhesin.

The sequence of the tryptic peptides of the 54 kDa protein revealed similarity with enolases from various fungi, especially with those of *P. brasiliensis*, *C. posadasii*, *A. nidulans*, *A. terreus*, *A. oryzae*, *P. chrysogenum* and *A. fumigatus*. This enolase appears to be conserved in *C. posadasii*, *A. nidulans*, *A. terreus* and *A. oryzae*. These results suggest a new function for this protein (adhesin), which may participate in the adhesion of *P. brasiliensis*. Enolase is a ubiquitous glycolytic enzyme (2-phospho-D-glycerate hydrolase) that catalyses the dehydration of 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP) in glycolysis; the same enzyme catalyses hydration of PEP to PGA during gluconeogenesis. For many years, enolase was regarded solely as a soluble cytosolic glycolytic enzyme. However, recent studies have shown that enolase is a protein with diverse distribution and biological functions (Pancholi, 2001). There is a growing list of proteins which are expressed in more than one location in both eukaryotes and prokaryotes and which exhibit quite distinct biological functions. For example, enolase is involved in laminin binding in *Staphylococcus aureus* (Carneiro *et al.*, 2004) and alpha-enolase is an anchorless, surface-associated glycolytic enzyme in *Trichomonas vaginalis* (Mundodi *et al.*, 2008), *Streptococcus pneumoniae* (Bergmann *et al.*, 2001) and *Fasciola hepatica* (Bernal *et al.*, 2004).

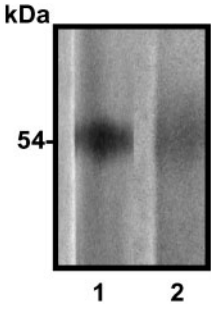

Fig. 4. Immunoblotting analysis of purified protein and *P. brasiliensis* cell-free extract. Lanes: 1, purified protein; 2, pooled cell-free extracts of Pb18a and Pb18b cultured on BBA with 5 % blood, fractioned by one-dimensional gel electrophoresis, blotted onto a nitrocellulose membrane and detected with rabbit anti-54 kDa polyclonal antibody.

The ability of some fungi to adhere to host surfaces and components of the ECM is an important factor in pathogenesis. Therefore, protein ligands of fibronectin can contribute to the pathogenesis of the infection and maintenance of the fungus in tissues by mediating adhesion to host ligands. From these results, we suggest that *P. brasiliensis* may use this protein to adhere to and maybe invade host cells; it probably acts as a virulence factor, as described for other pathogens, and may exhibit an adhesive function involving plasminogen binding (Bergmann *et al.*, 2001; Bernal *et al.*, 2004; Pancholi, 2001). The ability of pathogenic micro-organisms to bind plasminogen has been associated with invasive properties (Coleman *et al.*, 1997; Lähteenmäki *et al.*, 1995; Mundodi

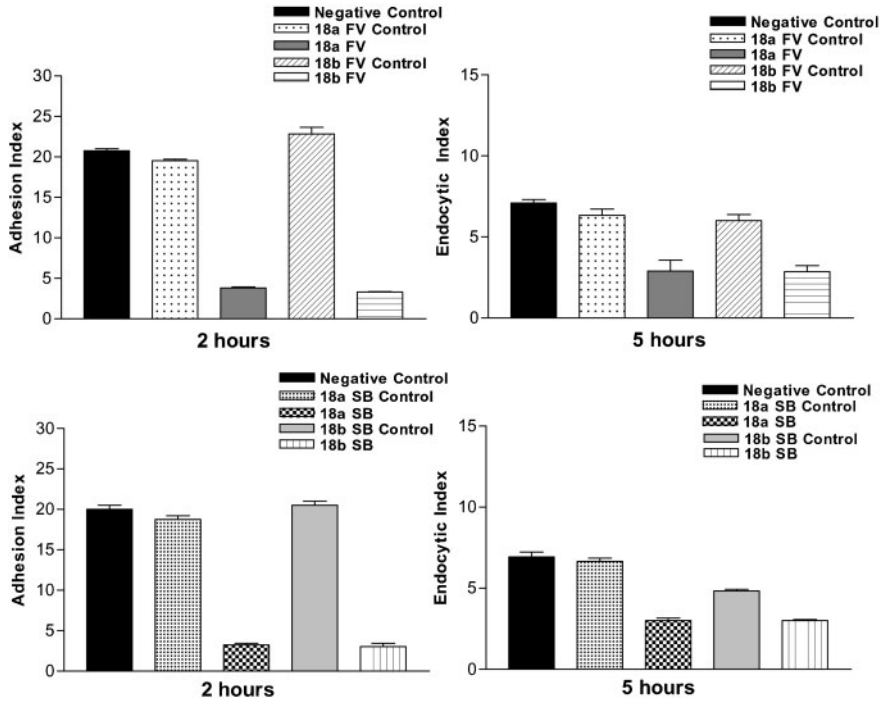

Fig. 5. Assay of inhibition of the interaction between *P. brasiliensis* yeast cells (pretreated with anti-54 kDa polyclonal antibody) and A549 cells. Pb18a and 18b were pretreated or not (control) for 1 h with antiserum, before the interaction with pulmonary cells for 2 and 5 h. Data represent mean and standard deviations of three independent experiments. 18a FV, Pb18a subcultured on Fava-Netto solid medium; 18b FV, Pb18b after passage in hamster and cultured on Fava-Netto solid medium; 18a SB, Pb18a subcultured on BBA with 5 % blood; 18b SB, Pb18b after passage in hamster and cultured on BBA with 5 % blood. 18a FV control, epithelial cells with Pb18a FV without previous treatment. 18b FV control, epithelial cells with Pb18b FV without previous treatment. 18a SB control, epithelial cells with Pb18a SB without previous treatment. 18b SB control, epithelial cells with Pb18b SB without previous treatment. Negative control, epithelial cells pretreated with rabbit preimmune serum.

et al., 2008) and could facilitate their penetration to the basement membrane to permit associations with fibronectin and laminin. Although *P. brasiliensis* is considered an extracellular pathogen, fibronectin-binding adhesins may also mediate invasion of the cells. Further experiments may provide evidence of the role of the 54 kDa fibronectin-ligand in the invasion process.

We also studied another factor involved in adhesion by comparing Pb18a and Pb18b cultured on Fava-Netto medium and BBA with 5 % blood. Our experiments with A549 cells revealed that Pb18b, cultured either on Fava-Netto medium or on medium with 5 % blood, was able to adhere and to invade more efficiently than Pb18a grown on the same medium. These data confirm previous findings of Andreotti *et al.* (2005) and Mendes-Giannini *et al.* (2006). Pb18a and Pb18b cultured on medium with blood exhibited significant differences ($P < 0.05$) in adhesion for 1, 3 and 10 h, relative to Pb18a and Pb18b cultured on Fava-Netto medium. On the other hand, Pb18a cultured on the blood medium has an adhesion profile similar to that of Pb18b, showing that the haemoglobin component is important for higher expression of proteins involved in adhesion as described for *Candida albicans* (Yan *et al.*, 1998; Pendrak *et al.*, 2000).

In this work, inhibition of the adhesion of *P. brasiliensis* to the epithelial cell cultures was achieved by treatment with rabbit anti-54 kDa polyclonal antibody. This antiserum abolished 82 % of the binding activity of *P. brasiliensis*, irrespective of the medium and sample used. Previously, gp43 antiserum also inhibited the adhesion of this fungus to Vero cells (Hanna *et al.*, 2000). Further studies are envisaged in which both antisera will be used in an attempt to abolish the infective process.

We report here that *P. brasiliensis* enolase is a newly identified surface-associated protein that can be inhibited by a specific antibody, influencing the adhesion of the fungus to pulmonary cells.

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