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Recognition of extracellular matrix proteins by *Paracoccidioides brasiliensis* yeast cells

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The adhesion of microorganism to host cells or extracellular matrix (ECM) proteins is the first step in the establishment of an infectious process. Interaction between *Paracoccidioides brasiliensis* yeast cells and ECM proteins has been previously noted. *In vivo*, in the chronic phase of experimental paracoccidioidomycosis (PCM), laminin and fibronectin have been detected on the surface of yeast cells located inside granulomatous lesions. The aim of the present study was to examine the ability of *P. brasiliensis* yeast cells to interact with extracellular matrix proteins (laminin, fibrinogen and fibronectin) and to establish which molecules were involved in this interaction. Immunofluorescence microscopy and flow cytometry demonstrated that all three ECM proteins tested were able to bind to the surface of *P. brasiliensis* yeast cells. Treatment with trypsin, chymotrypsin, chitinase, proteinase K or different sugars resulted in no change in laminin binding. In addition, ligand affinity assays were performed using different yeast extracts (total homogenates, β-mercaptoethanol, SDS extracts). These assays demonstrated the presence of 19 and 32-kDa proteins in the cell wall with the ability to bind to laminin, fibrinogen and fibronectin. This interaction could be important in mediating attachment of the fungus to host tissues and may consequently play a role in the pathogenesis of PCM.

Keywords adherence, fibrinogen, fibronectin, laminin, *Paracoccidioides brasiliensis*

Introduction

Paracoccidioidomycosis (PCM), a systemic mycosis restricted to Latin America and caused by the dimorphic fungus *Paracoccidioides brasiliensis*, has been shown to be acquired by inhalation of conidia produced by the mycelial form of the fungus in experimental animals; these propagules are sufficiently small to reach the alveoli [1]. The clinical manifestations of PCM are diverse, ranging from asymptomatic pulmonary lesions to systemic generalized infections. Most patients (about 50%) develop fibrotic sequelae that may severely hamper respiratory function [2–4].

Adhesion of pathogenic microorganisms to host tissues is regarded as a requirement for initial colonization and further dissemination. Very little information is available either on the mechanisms underlying the pathogenesis of PCM or the means by which the fungus persists in the lungs and disseminates to other organs [1]. It has been suggested that the ability of *P. brasiliensis* propagules to adhere to host cells and tissues may play an important role in the establishment of the infectious process [1].

Extracellular matrix (ECM) proteins have been implicated in the attachment of a variety of pathogens [5–10]. Of particular interest in this area is the

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identification of ECM-binding proteins on the surface of several fungi of clinical importance such as *Candida albicans* [6,11–13], *Aspergillus fumigatus* [7,14–17], *Histoplasma capsulatum* [18], *Cryptococcus neoformans* [19], *Pneumocystis carinii* [20,21], *Sporothrix schenckii* [22] and *Penicillium marneffei* [23,24].

Laminin is an extracellular matrix glycoprotein present in basement membranes and in the lungs; this glycoprotein can be exposed after tissue damage resulting from inflammatory processes. Laminin, the most abundant glycoprotein of the basement membrane, is composed of three polypeptide chains designated A, B1, B2, each containing six domains [25]. The multiple domains confer upon laminin many biological properties including promoting cellular adhesion, growth, differentiation, migration and matrix assembly. Laminin receptors have been demonstrated on metastatic tumor cells, granulocytes, lymphocytes and macrophages [15]. Fibrinogen, the major plasma glycoprotein, plays a key role in inflammatory reactions, and the recognition of fibrin (or fibrinogen) deposits in the surface of wounded epithelia constitutes a mechanism by which microbial attachment to mucosal surfaces does take place [15]. Fibronectin is present in the human host as a polymeric fibrilar network in the ECM and as soluble protomers in body fluids. Moreover, this glycoprotein mediates adhesion of several microorganisms to host tissues and also opsonizes *C. albicans* [26].

In the present study we sought to demonstrate the interaction of *P. brasiliensis* yeast cells with laminin, fibrinogen and fibronectin and to identify candidate fungal proteins that could be responsible for the interaction.

Materials and methods

Fungus

P. brasiliensis yeast cells (ATCC No. 60885) were grown in liquid brain heart infusion (BHI) plus glucose (1%) at 36°C with shaking for 4–5 days. Yeast cells were harvested by centrifugation and washed twice in phosphate-buffered saline (PBS) and the daughter cells disrupted by ultrasound to obtain as many single yeast cells as possible.

Assays of extracellular matrix proteins binding by indirect immunofluorescence: Suspensions of yeast containing about 10^7 cells/ml were washed once and resuspended in 250 µl sterile PBS containing laminin (derived from Engelbreth–Holm–Swarm mouse sarcoma), fibrinogen or fibronectin at 500 µg/ml (all ECM proteins obtained from Sigma Chemical, Poole, UK). After 3 h incubation at 37°C, the suspensions were washed three times in PBS and incubated for 1 h at 37°C with rabbit anti-laminin, rabbit anti-fibronectin or mouse anti-fibrinogen antibodies (Sigma) diluted 1:10 in PBS plus 1% BSA. The suspension was washed and resuspended in fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin antibody or goat anti-mouse immunoglobulin antibody (1:20 dilution made up in PBS) (Jackson Immunochemicals, West Grove, PA, USA), and incubated for 1 h at 37°C. The suspensions were washed once more and examined finally by immunofluorescence microscopy and flow cytometry. Negative controls consisted of yeast suspensions incubated in the absence of ECM proteins, anti-ECM protein antibodies and FITC-conjugated antibodies (all replaced with PBS) [23].

Flow cytometry assays: Binding of ECM proteins to *P. brasiliensis* yeasts was analyzed by flow cytometry. Aliquots (containing about 10^7 cells) of the yeasts suspension were incubated with increasing amounts (50 to 500 µg/ml) of the different ECM proteins in PBS, and immunofluorescence assays were performed by the procedure described above. The cells were subsequently fixed in 1% paraformaldehyde solution in PBS. All flow cytometry analyses were performed on an EPICS XL (Coulter Electronics, Hialeah, FL, USA) using an air-cooled argon-ion laser tuned at 488 nm and 115 mW. The flow rate was kept at approximately 10 000 events (cells) and green fluorescence was amplified logarithmically. Ten thousand events were collected as monoparametric histograms of log fluorescence, as well as list mode data files. The data were analyzed by the Windows Multiple Document Interface (WinMDI), version 2.8. As a negative control, equal number of cells were processed as described above.

In the enzymatic treatment experiments, the yeast suspensions were incubated with trypsin, chymotrypsin, proteinase K or chitinase (1000 µg/ml) for 1 h at 37°C. Proteolytic treatment was stopped by three washes with PBS containing 3% BSA or by adding phenyl methyl sulphonyl fluoride (Sigma) 100 mmol/l. After this treatment, the cells were washed again with PBS and assayed for laminin (as an exemplary case) binding by flow cytometry as described above, using in this case 500 µg/ml for the initial incubation.

Influence of carbohydrates on the binding of laminin: The possible role of carbohydrates in the recognition of laminin by *P. brasiliensis* yeast was studied by inhibition experiments. Different carbohydrates [i.e. glucose, galactose, mannose, *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc) and methyl-α-D-manopyranoside] were tested at 200 mmol/l.

Preparation of yeast extracts

Extracts were prepared according to the method previously described by Peñalver *et al*. [16] with minor modifications. Briefly, a very dense suspension (equivalent to 20 mg wet weight) of yeast form cells grown in BHI media for 5 days at 36°C were used. Yeast cells were mixed with lysis buffer [100 mmol/l Tris-hydrochloride, pH 7.4, containing a protease inhibitor cocktail (product no. P8215; Sigma); this cocktail was added at a ratio of 1 ml per 20 g wet weight]. Cell breakage was assessed by microscopic examination. The cells were frozen with liquid nitrogen and disrupted by mechanical maceration, and the cell walls were sediment (10 000 *g* for 30 min at 4°C) from the cell-free homogenate, washed three times with chilled distilled water; resuspended in 10 mmol/l phosphate buffer (pH 7.4) containing 1% (vol/vol) 2-mercaptoethanol (βME) plus a protease inhibitor cocktail as above (Sigma); and incubated for 30 min at 37°C in a rotary shaker. The cell walls were subsequently sediment and the supernatant fluid was recovered, dialyzed against distilled water at 4°C, and concentrated by assisted evaporation (βME extract). The evaporation procedure aims at concentrating the protein content of highly diluted antigen preparations by transferring the suspension into a dialysis membrane and placing it in front of an electric fan until a 10-fold reduction of the initial volume is achieved. βME-extracted walls were washed three times with chilled glass-distilled water and then boiled for 5 min in with a 2% sodium dodecyl sulfate (SDS) solution in glass-distilled water. After treatment, the suspensions were centrifuged at 10 000 *g* for 15 min. The proteins eventually present in the supernatant were precipitated with 75% (vol/vol) (final concentration) ethanol at 4°C for 16 h. The precipitates were recovered by centrifugation at 27 000 *g* for 30 min and resuspended in water (SDS extract). The protein content of the samples was determined by the Bradford method [27].

Affinity ligand assays

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described by Laemmli [28] using 12% resolving gels. Gels were stained with a silver stain (Bio-Rad, Hercules CA, USA) to detect the presence of proteins. Electrophoretic transfer of proteins from polyacrylamide gels to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore, Watford, UK) was carried out as described previously [16,29].

Blotted proteins were assayed for ECMP binding as follows. The PVDF membranes were blocked with 5% w/v bovine serum albumin (BSA) in Tris buffered saline (TBS) for 2 h at room temperature and then incubated overnight with gentle shaking in PBS containing 50 μg/ml of laminin (derived from Engelbreth–Holm–Swarm mouse sarcoma; Sigma), 100 μg/ml human fibronectin (Sigma) or 50 μg/ml bovine fibrinogen (Sigma). After being washed four times (10 min per wash) with TBS-Tween 0.05% (TBST), the PVDF membranes were incubated for 1 h with agitation with rabbit anti-mouse laminin (1:100 dilution); rabbit anti-human fibronectin antibodies (1:100) or goat anti-fibrinogen (1:500) (all of them from Sigma) in TBST plus BSA 1% (TBSTB). The blots were washed with TBST and incubated with alkaline phosphatase-labeled goat anti-rabbit or donkey anti-goat immunoglobulin (Jackson) at a 1:10 000 or 1:20 000 dilutions in TBSTB, respectively. Finally, the blots were washed again, and reactive bands were developed with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the chromogenic reagents.

Carbohydrate analysis

Cell wall SDS extracts were used to determine the presence of associated carbohydrates using a DIG Glycan Differentiation kit (Roche Molecular Biochemicals, Mannheim, Germany), in which specific lectins recognize different groups of sugars (or terminal carbohydrates) associated to proteins. The methodology was carried out following the manufacturer's instructions. In addition, 12% polyacrilamide gels were stained with Schiff reagent to determine the presence of sugars.

Results

Immunofluorescence microscopy

The surface of *P. brasiliensis* yeast cells demonstrated strong immunofluorescence labeling when incubated with laminin and fibrinogen (Fig. 1); there was no reactivity when fibronectin was used. No fluorescence signal was observed when incubation with anti-ECM proteins antibodies was omitted, demonstrating that fluorescence was dependent on the previous interaction of the cells with ECM proteins and their appropriate recognition. None of the remaining negative controls demonstrated fluorescent activity.

Flow cytometry analysis of extracellular matrix proteins binding to P. brasiliensis *yeast cells*

When yeast cells were incubated with the various concentrations of ECM proteins (50–500 μg/ml) it

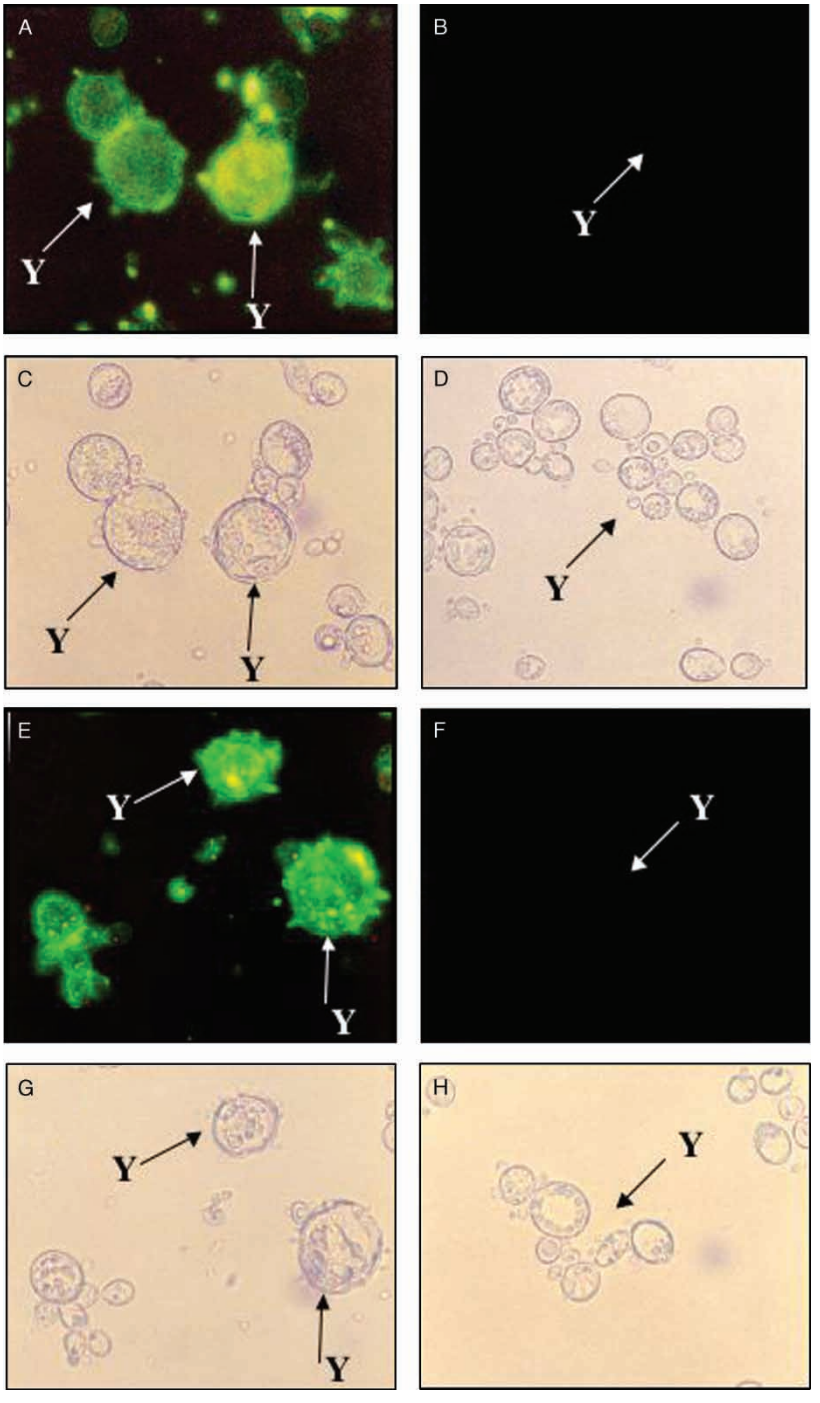

Fig. 1 Immunofluorescence identification of the binding of laminin (A–D) and fibrinogen (E–H) to *Paracoccidioides brasiliensis* yeast cells. Light (C, D, G, H) and immunofluorescence (A, B, E, F) microscopy of yeasts incubated with laminin or fibrinogen (500 μg/ml), anti-laminin or anti-fibrinogen antibodies, and FITC-labeled conjugated antibodies. Negative control, light (D, H) and immunofluorescence (B, F) microscopy in which the anti-laminin or anti-fibrinogen antibodies incubation were replaced by PBS. Magnification ×20.

was found that the ligand bound to the cells in a dose-dependent manner. The intensity of the fluorescence detected at the surface of yeast cells increased with the concentration of the ligand in solution, attesting to the saturability of binding. Representative histograms (Fig. 2A) showed that the incubation of yeast with laminin resulted in an intense labeling of a subpopulation of the cells. When the cells were exposed to fibrinogen uniform and intense labeling was observed (Fig. 2B). However, when the yeast cells were incubated with fibronectin in solution although an intense fluorescence was noted in a subpopulation, there was no difference between the ligand concentrations tested (Fig. 2C).

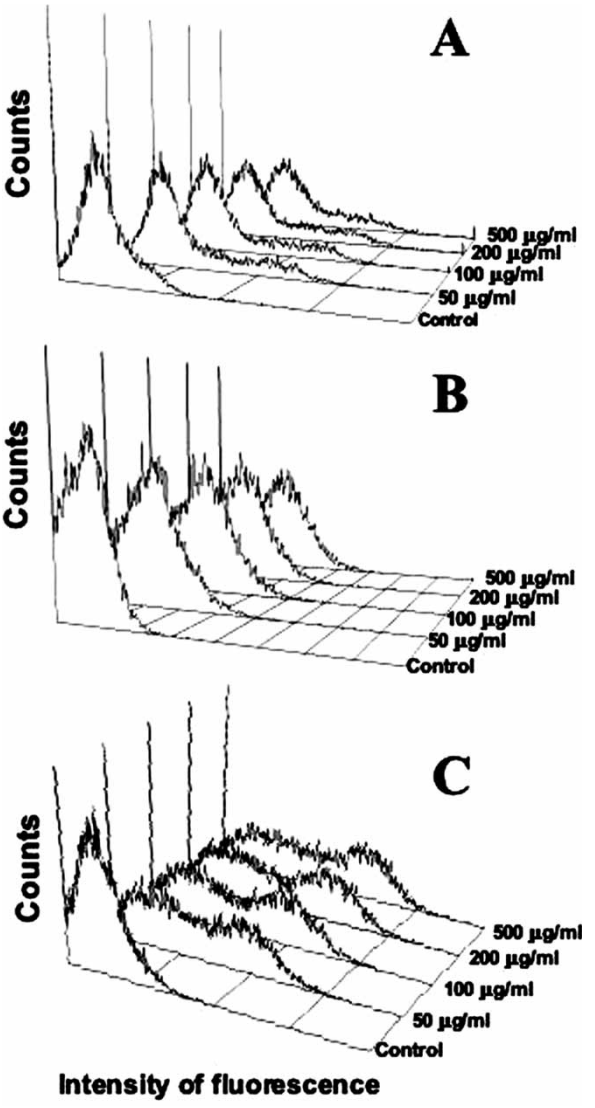

Fig. 2 Binding of *Paracoccidioides brasiliensis* yeast cells to soluble ECM proteins. Yeast cells were sequentially incubated in the presence of increasing concentrations of ECM proteins (50–500 µg/ml). (A) laminin; (B) fibrinogen; (C) fibronectin. The fluorescence intensity at the cell surface was then measured by flow cytometry. *x*-axis, fluorescence intensity; *y*-axis number of fluorescent cells. Control yeast cells (Ct), which had not been incubated with ECM proteins, are shown.

Influence of enzymatic treatment of P. brasiliensis *yeast cells on their ability to bind laminin*

Yeast cells pretreated with the different enzymes (trypsin, chymotrypsin, proteinase K or chitinase) were analyzed for their ability to bind laminin by flow cytometry (Table 1), in attempt to further characterize the biochemical nature of the binding sites. In this context, treatment with the various enzymes at their maximal concentration used (1000 µg/ml) resulted in no binding inhibition of laminin to *P. brasiliensis* yeast.

Table 1 Enzymatic effect on the binding of laminin to *Paracoccidioides brasiliensis* yeast

% Binding relative to the control	
Enzymes	1h
Sensitive	100
Trypsin 1000 µg/ml	103.4
Chymotrypsin 1000 µg/ml	101.7
Proteinase K 1000 µg/ml	97.7
Chitinase 1000 µg/ml	95.6

Effect of several carbohydrates on the binding of laminin to P. brasiliensis *yeast*

The effect of several sugars (glucose, galactose, mannose, GlcNac, GalNAc and methyl-α-D-manopyranoside) on the ability of yeast cells to bind to the soluble ligand was also assayed. The pretreatment of the cells with 200 mmol/l solutions of glucose, mannose and GlcNAc did not modify binding of *P. brasiliensis* yeast to laminin (Table 2). Pretreatment with galactose, GalNAc or methyl-α-D-manopyranoside at the same concentrations slightly reduced binding to soluble laminin by about 17.9, 13.5 and 14.3%, respectively (Table 2).

Identification of cell components with the ability to bind to extracellular matrix proteins

In order to identify which components from the fungal cells bound to ECM proteins, different extracts from yeast cells were examined using ligand affinity binding assays. On silver-stained 12% SDS–PAGE gels, a complex array of proteins was observed in all fungal extracts (total homogenates, cell wall β-mercaptoethanol extracts and cell wall SDS extracts) (Fig. 3A). However, binding of the three ECM proteins (laminin, fibrinogen and fibronectin) was detected only in the

Table 2 Effect of carbohydrates on the binding of laminin to *Paracoccidioides brasiliensis* yeast

Carbohydrate (200 mM)	% Binding relative to the control
None	100
Glucose	96.7
Galactose	82.1
Mannose	90.3
GlcNAc	93.4
GalNAc	86.5
Methyl-a-D-Manopyranoside	85.7

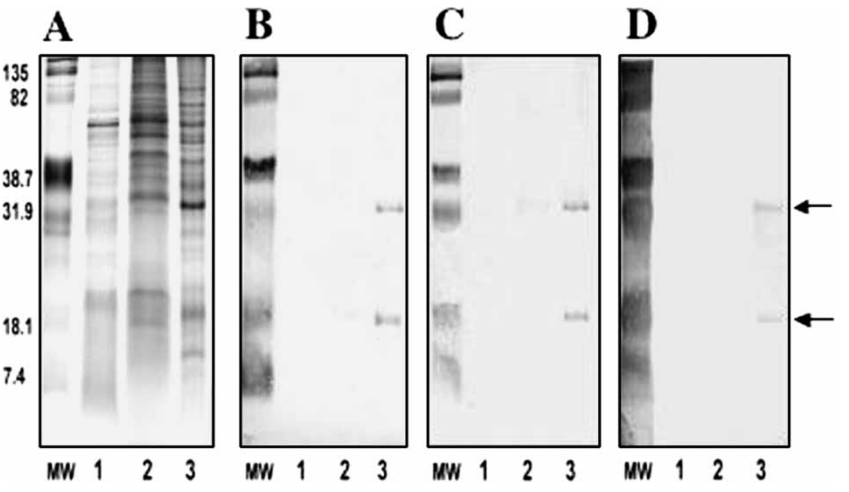

Fig. 3 Identification of *Paracoccidioides brasiliensis* proteins able to bind to ECM proteins. (A) SDS–PAGE gel stained with silver; (B–D) ligand affinity assays using laminin (B), fibronectin (C), and fibrinogen (D). MW, molecular weight markers (shown in kDa). Different extracts from yeast (tracks 1–3) are shown; total homogenates (tracks 1), cell wall β-mercaptoethanol extracts (tracks 2) and cell wall SDS extracts (tracks 3). The arrows indicate two proteins with molecular masses of 19 and 32 kDa that recognize the three ECM proteins, which were present solely on cell wall SDS extracts.

SDS cell wall extracts by two components having relative molecular masses of 19 and 32 kDa (Fig. 3B–D). The reaction was specific as indicated by the absence of bands following exposure to the specific anti-ECM protein antibodies, when previous incubation of the blots in the ECM protein solution had been omitted (data not shown).

Carbohydrates analysis

The presence of sugars groups (or terminal carbohydrates) associated to *P. brasiliensis* in either the 32- and 19-kDa protein was not observed when specific lectins or Schiff reagent were used. However, when the presence of sugars on the cell wall SDS extracts was analyzed, species with molecular masses of about 38 and 82 kDa recognizing terminal mannose [α(1-3), α(1-6) or α(1-2) linked to mannose] were observed, different for the 19- and 32-kDa proteins (data not shown).

Discussion

This work has clearly demonstrated the presence of ECM protein binding sites on the surface of *P. brasiliensis* yeast cells. These binding sites were represented by two polypeptides with molecular masses of 19 and 32 kDa which recognize laminin, fibrinogen and fibronectin. Similar interactions between ECM proteins and several other pathogenic fungi have previously been reported; thus in several cases these ECM proteins appear to share common receptors. In this manner, in *P. marneffei*, ligand affinity assays have revealed a 20-kDa protein that binds to laminin and fibronectin [23,24]. In *A. fumigatus* the existence of receptors with the capacity to recognize more than one ligand have also been demonstrated [15]. These molecules exhibit a diverse array of molecular weights with 23, 30, 37 and 72 kDa species recognizing ECM proteins such as laminin and fibrinogen [7,15,16,30,31]. In *C. albicans* molecules of 37, 58 and 67 kDa which bind to laminin and fibrinogen have also been described [13,32]. In addition, integrin-like and lectin-like receptors have been shown in yeast cells and germinal tubes of *C. albicans* and are involved in adherence to vitronectin, collagen type IV and also to human endothelial cell lines [33–35]. In *H. capsulatum* a molecule of 50-kDa that confers capacity to bind to laminin has also been identified [18]. Recently, it has been demonstrated that *C. neoformans* interacts with fibronectin through two polypeptides of 25 and 35 kDa present on total homogenates and cell wall preparations, respectively [19].

Interactions between *P. brasiliensis* yeasts and ECM proteins have previously been noted. In experimental paracoccidioidomycosis during the chronic phase, laminin and fibronectin have been detected on the surface of yeast cells inside the granulomatous lesions, probably as part of the granuloma architecture [36]. In addition, Vicentini *et al*. [37] observed that the coating of *P. brasiliensis* yeast cells with laminin before injection into hamster testicles enhanced the fungus virulence, resulting in a faster and more severe granulomatous disease; *in vitro* it has been shown that laminin binds specifically to *P. brasiliensis* yeast cells and enhances adhesion of the fungus to the surface of epithelial Madin–Darby canine kidneys cells [37]. The latter authors suggested that this interaction is

mediated by the 43-kDa extracellular glycoprotein (gp43), which has been previously identified as a main diagnostic antigen of *P. brasiliensis* [38]. However, the present study has demonstrated that the interaction between *P. brasiliensis* yeast cells and ECM proteins was mediated by two polypeptides with molecular masses of 19 and 32 kDa with the capacity to bind laminin and also fibrinogen and fibronectin. These components could only be detected in the SDS yeast extract, suggesting that they are present in the cell wall. In addition, characterization of the *P. brasiliensis* cell wall SDS extracts revealed that the 19- and 32-kDa proteins had no associated carbohydrates indicating that these molecules are not glycoproteins. In spite of the fact that gp43 has been shown to be an important molecule responsible for interaction with laminin, the evidence presented here indicates that there are another two molecules that could play an important role in the yeast's interactions with the ECM proteins. It is possible that this difference could be attributed to the isolates utilized. The first line of evidences suggesting that gp43 is responsible for laminin binding came from isolate Pb339 while we used isolate ATCC 60855. For instance, Moura-Campos *et al*. [39] demonstrated the different expression and isoforms of gp43 in various strains of *P. brasiliensis*. They studied 13 *P. brasiliensis* isolates and found that when their exoantigens were analyzed by SDS–PAGE, eight presented high amounts of gp43, two presented small amounts and in three gp43 was undetectable. In addition, the eight isolates that presented high amounts of this glycoprotein were submitted to isolectric focusing and revealed different isoform profiles [39].

Recently, André *et al*. [40] showed that previous treatment with laminin does not enhance *P. brasiliensis* pathogenicity in a pulmonary model of infection, even when low infecting doses of virulent yeasts (Pb18) or a low-virulence isolate (Pb265) were used. Treatment with laminin led to a less severe pathologic process as reveled by histopathology of the Pb18-infected group, as well as by diminished colony forming units (c.f.u.) in the lungs of mice infected with the low-virulence isolate. These investigators speculated that previous *in vitro* laminin treatment covered laminin-binding epitopes on gp43 thus preventing their *in vivo* interaction with the corresponding molecules present in damaged alveolar spaces, resulting in lower adherence of fungal cells. It was also suggested that isolates with different degree of virulence could express a diverse pattern of adhesion or even produce varying concentrations of the same fungal adhesin [39].

Enzymatic treatment did not alter the yeasts' ability to bind to laminin, but this may not indicate that such receptors are not proteinaceous in nature. It may be possible that by adhering to the yeast cell wall glucans, the three dimensional conformation of these proteins changes becoming encrypted and non-available to the action of lytic enzymes. In addition as the composition of the yeast cell wall contains α-glucan ranging between 40 and 3% [41], the presence of this polysaccharide could act as a mantle that interferes or protects these protein moieties from enzymatic attack. Likewise, the various carbohydrates tested did not inhibit (glucose, mannose and GlcNAc) or slightly reduced (Galactose, GalNAc and methyl-α-D-manopyranoside) binding of laminin to yeast cells. Although these observations support the contention of a yeast receptor or binding site for laminin, the possibility of the presence of other molecules or of sugars different from those tested cannot be completely ruled out.

Given that the parasitic form of *P. brasiliensis* (yeast cells) expresses two proteins with the capacity to bind to extracellular matrix proteins, it is possible that this interaction could play an important role in the dissemination process and in the pathogenesis of PCM.

Ongoing work seeks to address the precise nature of the interaction among these extracellular matrix proteins based on recognition systems; attempts to purify these receptors will be the first step in their full characterization.

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