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Original article

Binding of extracellular matrix proteins to *Paracoccidioides brasiliensis*

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Abstract

Adhesion to extracellular matrix (ECM) proteins plays a crucial role in invasive fungal diseases. ECM proteins bind to the surface of *Paracoccidioides brasiliensis* yeast cells in distinct qualitative patterns. Extracts from Pb18 strain, before (18a) and after animal inoculation (18b), exhibited differential adhesion to ECM components. Pb18b extract had a higher capacity for binding to ECM components than Pb18a. Laminin was the most adherent component for both samples, followed by type I collagen, fibronectin, and type IV collagen for Pb18b. A remarkable difference was seen in the interaction of the two extracts with fibronectin and their fragments. Pb18b extract interacted significantly with the 120-kDa fragment. Ligand affinity binding assays showed that type I collagen recognized two components (47 and 80 kDa) and gp43 bound both fibronectin and laminin. The peptide 1 (NLGRDAKRHL) from gp43, with several positively charged amino acids, contributed most to the adhesion of *P. brasiliensis* to Vero cells. Synthetic peptides derived from peptide YIGRS of laminin or from RGD of both laminin and fibronectin showed the greatest inhibition of adhesion of gp43 to Vero cells. In conclusion, this work provided new molecular details on the interaction between *P. brasiliensis* and ECM components.

Keywords: *Paracoccidioides brasiliensis*; Extracellular matrix; Adhesins; gp43

1. Introduction

Paracoccidioides brasiliensis is a dimorphic fungus that causes systemic disease in humans. In paracoccidioidomycosis, clinical and experimental data have indicated the lung as the organ of entry of *P. brasiliensis*, through the inhalation of its conidia and its transformation into the pathogenic yeast form [1].

The spectrum of paracoccidioidomycosis ranges from benign and localized to severe and disseminated forms. The existence of different clinical forms of this disease and the occurrence of asymptomatic infection may be a result of host-related factors, immunological status and characteristics of the infecting agent, mainly its virulence [1]. The propagules that lodge in the alveoli adhere and invade the alveolar cells and/or the basal lamina. Alveolar basal lamina is composed of a specialized extracellular matrix (ECM), in which laminin, types IV and V collagen, entactin, proteoglycans of chondroitin sulfate and heparan sulfate, and fibronectin can be found [2]. In normal tissues, most ECMs are covered by epithelial

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or endothelial cells and hence are not available for binding. However, any type of trauma that damages host tissues may expose the ECM and enable microbial colonization and infection [3].

P. brasiliensis may actively penetrate the mucocutaneous surface and parasitize epithelial cells, thus evading the host defenses and reaching deeper tissues [4]. The ability of this fungus to adhere to and invade non-professional phagocyte cells has been reported by us [4]. It has also been demonstrated that a Pb isolate invaded HeLa and Vero cells, that the adherence phenomenon varies among strains and correlates with their virulence [5]. The virulence of *P. brasiliensis* can be attenuated or even lost after consecutive cycles of subculturing over long periods and can be reestablished after passage in animals and epithelial cell culture [6]. However, the putative factor involved in the virulence of these strains has not yet been identified. Some researchers have analyzed the 43-kDa glycoprotein that is also involved in *P. brasiliensis*, adhesion [5,7], but the other fungal components, such as the 19-, 30-, 32-kDa proteins, as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) participate in the adhesion process [4,8–10].

The ability of a microorganism to adhere and invade is recognized as an important factor of its pathogenicity [3]. Adherence implies that the fungus recognizes ligands on the surface of host cells or a constituent of ECM. Nevertheless, adhesion is only one factor among many that can promote the development of this infectious process. The mechanism of adherence has been studied extensively in pathogenic bacteria and fungi such as *Candida albicans*, *Aspergillus fumigatus*, *Sporothrix schenckii*, *Coccidioides immitis*, *Histoplasma capsulatum* and *Penicillium marneffei* [11–16], but little is known about the adherence mechanism in *P. brasiliensis*.

The aim of the present study was thus to further characterize the interaction of *P. brasiliensis* with ECM components (fibronectin, laminin and types I and IV collagen), using fluorescence microscopy, adherence tests and ligand affinity blotting, as well as to define the functional sites of gp43.

2. Materials and methods

2.1. Microorganism

Strain 18 of *P. brasiliensis* (Pb18) was isolated from a clinical case of paracoccidioidomycosis (PCM) and maintained in the Faculty of Medicine of the University of Sao Paulo (FM-USP), Brazil. During the current work, Pb18 was grown in PYG medium (peptone, yeast extract and glucose) at 35 °C and subcultured every 3–4 days, 72 times, to yield sample Pb18a.

2.2. Reisolation of P. brasiliensis

Pb18 was inoculated into male hamster testicles, using 0.2 ml of a standard suspension of 2.0×10^6 yeasts/ml per animal. After 30 days, the animals were sacrificed and the testicles macerated and cultured on Sabouraud agar with chloramphenicol at 25 °C until the development of characteristic *P. brasiliensis* mycelial colonies. These were identified and incubated at 35 °C in PYG medium, to obtain the yeast phase [17], and the resulting sample was labeled Pb18b.

2.3. Cell-free fungal extract and gp43

Cell-free fungal extracts were prepared from the 18a and b isolates in yeast form, as described elsewhere [18]. About 300 mg of *P. brasiliensis* cells was grown for 3–4 days on PYG solid medium, then scraped off and mixed with 1 ml of PBS, pH 7.2. This mixture was vortexed for 30 s and centrifuged at $560 \times g$ for 1 min. The supernatant (cell-free fungal extract) was removed and stored at −20 °C. The gp43 was purified from crude exoantigen of *P. brasiliensis* (strain 18) as previously described [19]. Briefly, the exoantigen was fractionated by affinity chromatography in columns of protein A-purified rabbit anti-gp43 IgG coupled to CNBr-Sepharose. The gp43 fraction was concentrated and further purified by gel filtration. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining showed a single band with a molecular weight of 43 kDa. The cell-free fungal extracts of *P. brasiliensis* (strains 18a and b) were also biotin-labeled by incubation with sulfo-NHS-biotin (ECL protein biotinylation module from Amersham Pharmacia Biotech®) following the manufacturer's instructions (the stock solution of labeled protein was 1 mg/ml).

2.4. P. brasiliensis antisera

Polyclonal antisera to *P. brasiliensis* cell-free fungal extract (Pb18b) and gp43 fraction were generated by immunizing rabbits with intradermal injections of 1.0 ml of protein (respectively, 1.2 and 1.0 mg/ml) mixed with 1.0 ml of Freund's complete adjuvant. Subsequent injections of protein 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 [19]. The immunoglobulin fractions of the antisera were separated by precipitation with ammonium sulfate and stored at −70 °C. These antisera reacted with *P. brasiliensis* cell-free fungal extracts and gp43, at titers of 1:200 and 1:100, respectively, by immunoblotting.

2.5. Cell culture

Monolayers of African green monkey Vero cells, obtained from the American Type Culture Collection, were cultured in 199 medium (Sigma, USA) supplemented with 10% (v/v) fetal calf serum (FCS; Cultilab, Brazil).

2.6. Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described and adapted [16], using suspensions of yeast forms of *P. brasiliensis* (10^7 cells/ml). Briefly, yeasts were resuspended in phosphate-buffered saline (PBS) 10 mM, pH 7.4, containing the ECM components at a concentration of 500 μg/ml (fibronectin, laminin, types I and IV collagen) or

bovine serum albumin (BSA) and incubated for 3 h at 37 °C. The suspensions were washed and resuspended with specific ECM antibodies (Sigma) diluted 1:10 in PBS, and finally incubated for 1 h at 37 °C. Suspensions were then washed and resuspended in goat anti-rabbit (1:20 dilution, Sigma) for laminin and fibronectin, and goat anti-mouse IgG FITC conjugate in PBS for types I and IV collagen, for 1 h at 37 °C. Finally, the suspensions were washed again and examined. Negative controls consisted of yeast suspensions incubated with BSA, anti-ECM protein antibodies and FITC-conjugated antibodies (all replaced with PBS).

2.7. P. brasiliensis *yeast-cell adherence assays*

Assays of ECM protein binding by indirect fluorescence [12]: suspensions of yeast containing about 10^7 cells/ml were washed once and resuspended with increasing amounts (10–500 μg/ml) of laminin, fibronectin, types I and IV collagen in 250 μl sterile PBS (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 and anti-fibronectin or mouse anti-types I and IV collagen (Sigma) diluted 1:100 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 (1:60 dilution made up in PBS), and incubated for 1 h at 37 °C. Negative controls consisted of yeast suspensions incubated with BSA, anti-ECM protein antibodies and FITC-conjugated antibodies (all replaced with PBS). The suspensions were washed once more, subsequently fixed in 1% paraformaldehyde solution in PBS and finally examined by flow cytometry. All flow cytometry analyses were performed on an EPICS XL (Coulter Eletronics, 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 Scatchard GraphPad Prism®. Analysis of the data provided the dissociation constants (K_d).

2.8. Cell-free fungal extract adherence assays

ECM proteins, represented by laminin, fibronectin, and types I and IV collagen, were immobilized on 96-well microtiter plates at 10 μg/ml. Some assays were performed with 120- and 40-kDa fibronectin fragments at 25 μg/ml. The plates were incubated for 1 h at 37 °C and overnight at 4 °C, washed in PBS-T and blocked with PBS, nonfat dried milk and BSA for 1 h at 37 °C. Finally, the plates were washed and biotin-labeled cell-free extracts of *P. brasiliensis* (strains 18a and b) were added to each well at 100 μg/ml, followed by incubation for 1 h at 37 °C. The wells were washed three times with PBS-T, followed by incubation with 50 μl of peroxidase-conjugated streptavidin (diluted 1:1000 in PBS) for 1 h at room temperature, and then washed again as described above. Semiquantitative analysis of bound cell-free fungal extracts was conducted by the addition of peroxidase substrate to the wells and determination of the optical density at 492 nm (OD492) [14]. In all experiments, a conjugate control was used, as well as a negative control without cells. Data shown are relative to the negative control (without cell-free fungal extract). These assays were performed in triplicate, and statistical analyses were conducted by ANOVA (*F* test followed by Duncan test).

2.9. gp43 and synthetic peptide inhibition assays

Vero cells (1×10^6 cells/ml) were grown in microtiter plates, fixed with paraformaldehyde, washed and blocked with PBS and 10% FCS for 1 h at room temperature and then treated with gp43 (10 μg/ml) in the presence and absence of individual synthetic peptides (200 μg/ml), as described by Manque et al. [20]. After 1 h at 37 °C, cells were washed with PBS and incubated with anti-gp43 (1:100) and peroxidase-labeled goat anti-rabbit IgG (1:3000) in PBS–FCS. The ELISA protocol was completed as described above. As a negative control, gp43 was omitted and replaced by PBS. The peptide fragments Arg-Gly-Asp-Ser (RGDS), from fibronectin and laminin, and Tyr-Ile-Gly-Ser-Arg (YIGSR) and Cys-Asp-Pro-Gly-Try-Ile-Gly-Ser-Arg-NH_2 (CDPGYIGSR-NH_2) from laminin (Sigma) were used, as well as synthetic fragments that represent specific sequences of gp43: Asn-Leu-Gly-Arg-Asp-Ala-Lys-Arg-His-Leu (PEP 1 = NLGRDAKRHL), Ser-Ala-Gln-Gln-Lys-Lys-Asp-Thr-Leu-Arg-Tys-Ile (PEP 2 = SAQQKKDTLRYI), Ile-Thr-Glu-Asp-Asp-Phe-Lys-Asn-Ile-Ala (PEP 3 = ITEDDF-KNIA) and Lys-Gln-Thr-Leu-Ile-Ala-Ile-His-Thr-Leu-Ala-Ile-Arg-Tyr-Ala-Asn (PEP 4 = KQTLRTAHTLAIRYAN) (Genosys Biotechnologies).

2.10. Molecular modeling

The objective of molecular modeling is to evaluate the presence of these peptides on molecular surface and solvent accessibility to predict protein–protein interaction permission. The sequence of the mature immunodominant antigen gp43 (β-glucanase-like protein from *P. brasiliensis*), comprising 381 residues, was extracted from the sequence deposited in TrEMBL Protein Databank under accession number Q01575. Exo-B-(1,3)-glucanase from *C. albicans* (PDB ID 1EQP, 1.90 Å resolution; 1cz1a, 1.85 Å resolution and 1eqc, 1.85 Å resolution) and Exo-B-(1,3)-glucanase from *Saccharomyces cerevisiae* (PDB ID 1H4P, 1.75 Å resolution) were used in the modeling procedure in Swiss model, being submitted for a first-approach model, and visualized by SwissPDB Viewer v3.7. The PROCHECK suite of programs was used for model validation [21].

2.11. Immunoblotting

Adhesins involved in *P. brasiliensis*–ECM interactions were characterized by immunoblotting. Proteins of the fungal extract from *P. brasiliensis* 18b isolate were separated by

SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting analysis was carried out as previously described [12]. Briefly, membrane strips were prepared, blocked with 1% BSA in PBS for 4 h, and then probed with laminin (10, 20 and 30 μg/ml), fibronectin (10 and 20 μg/ml) and type I collagen (10 μg/ml) in PBS-T–BSA for 90 min at room temperature. The membrane strips were washed and then incubated overnight with rabbit anti-laminin, anti-fibronectin and anti-type I collagen antibodies (1:100) in PBS-T–BSA. After further washing, they were incubated with a goat anti-rabbit peroxidase conjugate (Sigma, St. Louis. MO, USA) diluted 1:1000 in PBS-T–BSA and washed again. Peroxidase was visualized with 0.05% diaminobenzidine in Tris–HCl buffer (pH 7.4) and H_2O_2 (0.1%) as substrate. The specificity of the reaction was assessed by omitting the ECM proteins or by using a nonspecific immune serum instead of the ECM antisera. The positive control was developed from paracoccidioidomycosis patient serum (1:40).

2.12. Statistical analysis

Analysis of variance (ANOVA), Fisher's exact probability test (SAS System) and an unpaired *t* test were used to compare the binding capabilities of the cell-free fungal extracts and ECM proteins as well as to determine differences among the treatments, samples and proteins, $p < 0.05$ being considered significant.

3. Results

3.1. Binding of ECM components to P. brasiliensis yeast cells

The surface of *P. brasiliensis* yeast cells (Pb18b) demonstrated strong immunofluorescent labeling when incubated with laminin, fibronectin and types I and IV collagen, indicating clear interactions between the yeast cells and these ECM components (Fig. 1). Laminin and fibronectin fluorescence was scattered, granular and diffuse all over the whole fungus cell wall (Fig. 1A and B). Type I collagen expressed a dominant linear pattern on the surface, with some granular deposits (Fig. 1C). The type IV collagen fluorescence tended to be more linearly arranged, with no clear spatial localization (Fig. 1D). No reactivity was evident when the cells were incubated in the absence of ECM components (data not shown), demonstrating that the immunofluorescence depended on prior interaction of the cells with ECM proteins and the appropriate recognition. None of the other negative controls demonstrated any fluorescent activity.

3.2. Adherence of Pb yeast cells to ECM components

The ability of *P. brasiliensis* (Pb18b) yeast cells to adhere to the ECM components (laminin, fibronectin, types I and IV collagen) is illustrated in Fig. 2. Several yeast cell concentrations were tested, and 10^7 cells/ml gave a linear and more inoculum-dependent correlation in the flow cytometry assay

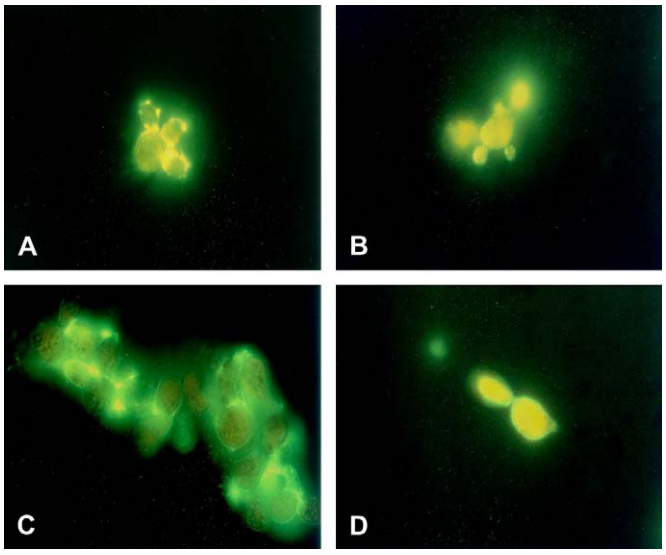

Fig. 1. Binding of laminin (A), fibronectin (B), type I collagen (C) and type IV collagen (D) on the yeast cell surface of *P. brasiliensis* sample 18b detected by indirect immunofluorescence assay. Yeast cells were incubated with ECM proteins, then with specific ECM antibodies, and finally with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin to laminin and fibronectin and anti-mouse immunoglobulin to types I and IV collagen.

(data not shown). When yeast cells were incubated with the various concentrations of ECM proteins (10–500 μg/ml), it 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. The cells adhered to types I and IV collagen and laminin immobilized at concentrations ranging from 10 to 100 μg/ml. In the fibronectin assay, a higher concentration (10–500 μg/ml) was needed to obtain similar behavior. Analysis of these data provided the dissociation constants (K_d). The fibronectin showed the highest value ($K_d = 19.48$), suggesting a weak attachment of the fungal cells to this protein (Fig. 2A).

3.3. Adherence of cell-free components of Pb to ECM

This fungal extract preparation corresponds to the most superficial part of the fungal cell and is probably the one that enters most directly in contact with the cells of the host. The ability of the two fungal extracts to bind ECM proteins is demonstrated in Fig. 3. By applying ANOVA (*F* test followed by Duncan test), different interactions were observed between the two fungal extracts and ECM components. As shown in Fig. 3, Pb18b extract bound more intensely to the ECM than did the Pb18a extract ($p < 0.005$). Laminin was most reactive, followed by type I collagen, fibronectin and type IV collagen, in relation to Pb18b extract. In contrast, Pb18a extract exhibited a different pattern of binding strength (in descending order: laminin, type I collagen, type IV collagen and fibronectin). The interaction of both extracts with laminin was

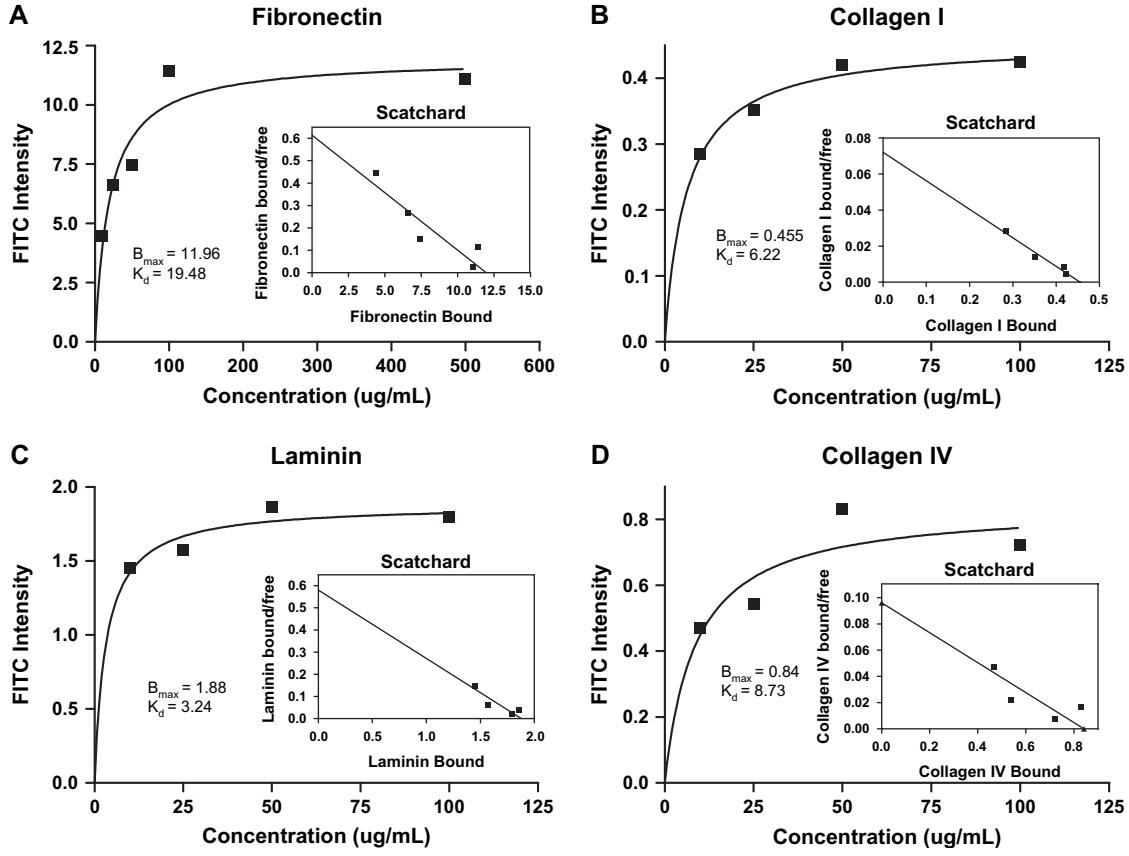

Fig. 2. *P. brasiliensis* (Pb18b) adhesion (10^7 cells/ml) to ECM proteins (10–500 μg/ml). A. Fibronectin; B. Collagen I; C. Laminin and D. Collagen IV. The interaction was assayed by indirect immunofluorescence and analyzed by flow cytometry. Ten thousand events were collected as monoparametric histograms of log fluorescence, as well as list mode data files. The data were analyzed by Scatchard Graphpad Prism. Analysis of the data provided the dissociation constants (K_d).

significantly different from those with all other ECM components ($p < 0.05$), and remarkably different interactions were observed between fibronectin and the two different fungal extracts ($p \leq 0.0005$).

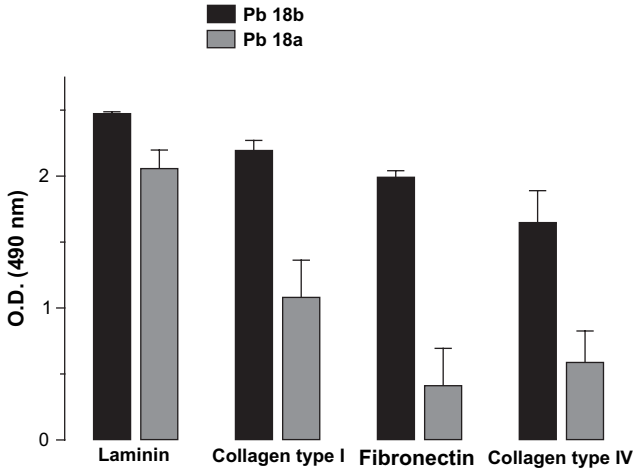

Fig. 3. *P. brasiliensis* 18a and 18b biotin-labeled cell-free fungal extract (100 μg/ml) interaction with immobilized types I and IV collagen, fibronectin and laminin (10 μg/ml). The interaction was revealed by ELISA with peroxidase-conjugated streptavidin. The results were expressed in absorbance units and correspond to mean values ± SD of triplicate experiments.

3.4. Fibronectin adherence to P. brasiliensis *cell-free fungal extracts*

Since many fibronectin-binding proteins are known to interact with specific regions of fibronectin, additional experiments were undertaken with two purified fibronectin proteolytic fragments, one comprising the RGD cell-binding domain (120 kDa), and the other the heparin-binding domain (40 kDa). Cell-free fungal extracts of the two *P. brasiliensis* isolates (18b and a) adhered to fibronectin in different ways. Conversely to Pb18a extract, Pb18b extract exhibited stronger interaction with the 120-kDa fragment than with the 40-kDa fragment ($p < 0.05$), while Pb18a extract reacted more with the 40- than the 120-kDa fragment. Binding of the extracts to the fragments was only observed when coating was performed with solutions of at least 25 μg/ml of the fragments bound to the plate, whereas 10 μg/ml intact fibronectin promoted adhesion (Fig. 4).

3.5. Immunoblotting assay

The cell-free extract of Pb18b contained a complex array of polypeptide chains ranging from 14 to 106 kDa. Laminin binding was observed with four major components whose molecular masses were approximately 30, 34, 38, 43 and 68 kDa: fibronectin

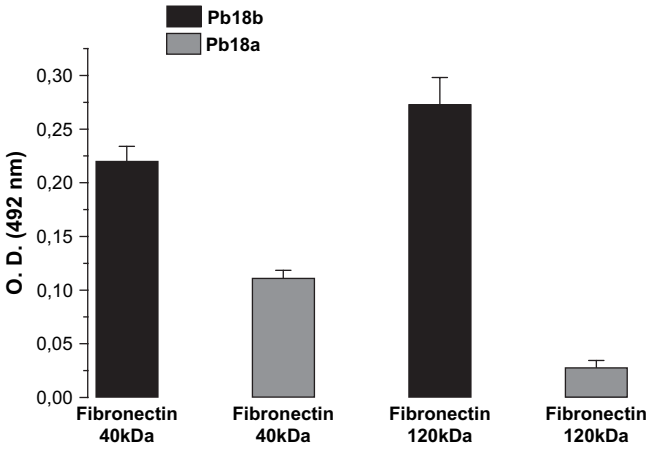

Fig. 4. *P. brasiliensis* biotin-labeled cell-free fungal extract (100 μg/ml) interaction with fibronectin and the immobilized fragments at 25 μg/ml. The interaction was revealed by ELISA with peroxidase-conjugated streptavidin. The results were expressed in absorbance units and correspond to mean values ± SD of triplicate experiments.

with five components, of 15, 28, 34 and 43 kDa and type I collagen with two components, of 47 and 80 kDa (data not show). gp43 was recognized by both laminin and fibronectin. Controls performed by omission of the ECM proteins were negative, attesting the specificity of these reactions.

3.6. Peptide inhibition

To further characterize the cell-binding site of gp43, we performed binding–inhibition experiments by incubating Vero cells with gp43 protein in the presence of individual gp43 peptides. Peptide 1 (NLGRDAKRHL) had the strongest inhibitory effect on the adhesion of gp43 to Vero cells (57%) ($p < 0.05$), followed by the peptides PEP 2 (SAQ QKKDTLRYI), PEP 3 (ITEDDFKNIA) and PEP 4 (KQTLRTAHTLAIRYAN) (Fig. 5A), with 29.2%, 27.0% and 24.5% inhibition, respectively. Besides being present in ECM, fibronectin and laminin are associated with epithelial cells. Accordingly, we asked whether these ECM components might serve as receptors in the interactions between gp43 and cultured epithelial cells. In order to probe the molecular mechanisms involved in gp43 adherence, competitive binding was performed with synthetic peptides corresponding to the adhesive recognition sequences of laminin and fibronectin. Synthetic peptides YIGSR and CDPGYIGSR-NH2, from laminin, respectively, inhibited by 42.5% ($p < 0.0001$) and 51.5% ($p < 0.0001$) the binding of gp43 to Vero cells. On the other hand, RGDS peptide showed an inhibitory effect of 43.5% ($p < 0.0001$) (Fig. 5B).

3.7. Molecular modeling

The gp43 was modeled by computer in order to locate the position of peptide 1 in the protein structure. The structure of gp43 resembled fungal beta glucanases (Fig. 6E). The molecular model of gp43 was obtained with 381 aas, started in valine 32 and finished in arginine 416. Valine 32 until Q 35 is located in the leader peptide region [22]. The model shows the same number of α-helices and β-strands as the homologous protein. Approximately 90% of the residues were plotted in the most favored regions (Fig. 6A–C) of the Ramachandran plot. The region corresponding to PEP 1 was found on the surface of the protein and showed high solvent accessibility (Fig. 6D). Fig. 6A is the gp43 model demonstrating secondary structures. Fig. 6B–D shows the PEP 1 on the surface of the protein and accessible to protein interaction with ECM. This model was representative of the structure of the protein, and it was constructed using protein homologues (Fig. 6E), which were crystallized. In fibronectin, the RGD motif was present on the surface of the molecule, and the secondary structure was a loop [23,24]. PEP 1 was a loop, and it was present on surface, as observed in the RGD motif. Consequently, the PEP 1 showed characteristics important for protein–protein interaction, as observed in the RGD motif (Fig. 6F).

4. Discussion

Adhesion of microorganisms to host cells and tissues represents a critical step in the process of infection [13,15,16]. We previously demonstrated that *P. brasiliensis* was capable of adhering to and invading epithelial cells. By comparing several independent isolates of *P. brasiliensis*, it was also observed

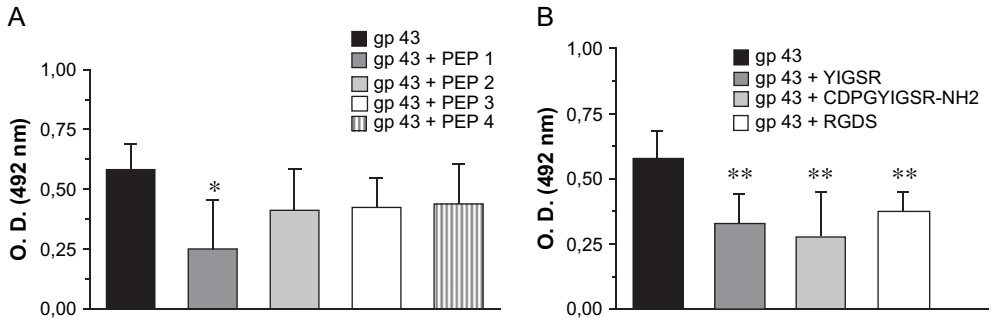

Fig. 5. Competitive assay with gp43 and peptides during the interaction with Vero cells. (A) Vero cells were incubated with gp43 (10 μg/ml), gp43 peptides (200 μg/ml) and anti-gp43 antiserum (1:100). (B) cells incubated with gp43 (10 μg/ml); gp43 (10 μg/ml) and the synthetic peptides of laminin and fibronectin, respectively, CDPGYIGSR-NH2, YIGSR and RGDS, and anti-gp43 antiserum (1:100). The interaction was visualized by ELISA. The results are expressed in absorbance units and correspond to mean values ± SD of triplicate experiments. (*) = $p < 0.05$ and (**) = $p < 0.0001$.

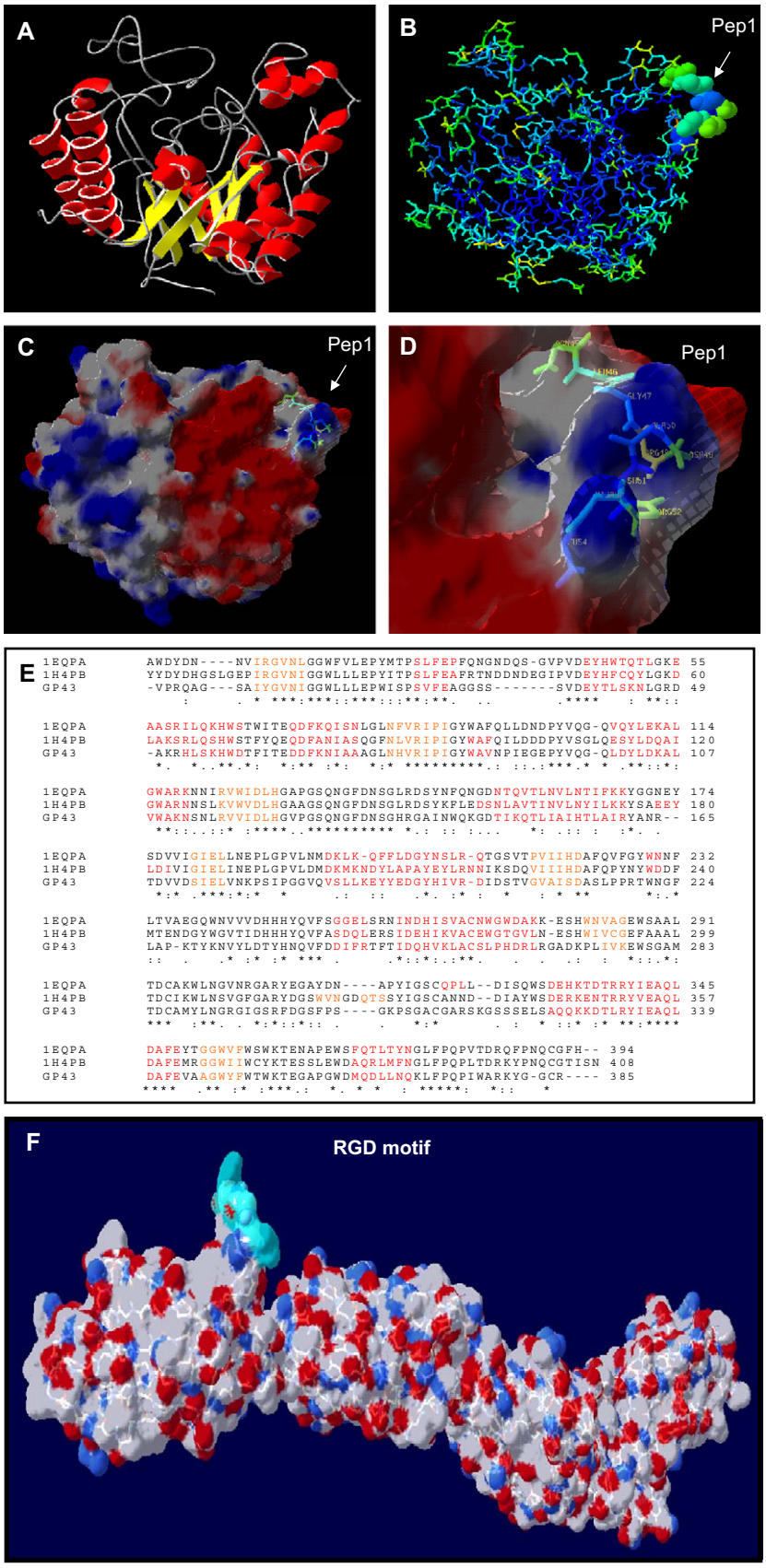

Fig. 6. Panel A: gp43 molecular model ribbon representation colored for secondary structure (red, α helices; yellow, β strands; grey, loops. Panel B: gp43 structure colored for solvent accessibility. Panel C: The surface of gp43 was mapped. The surface was made transparent to allow the perception of the internal protein architecture and colored according to the Coulombic electrostatic potential: red, negative; grey, neutral; and blue, positive. Panel D: Top view of PEP 1 on gp43 structure. The secondary structure, solvent accessibility and molecular surfaces were calculated in SwissPDB Viewer v3.7. Panel E: Multiple sequence alignment of glucanases from *C. albicans*, *S. cerevisiae* and *P. brasiliensis* used in the construction of the model. Secondary elements are represented in orange (β strands) and in red (α helices). Panel F: Molecular model of fibronectin indicating the surface position of the RGD motif.

that strains that were most virulent in animals exhibited enhanced adhesion *in vitro*. In particular, we found that strain 18, described as the most virulent in animals, had the strongest adhesion [5]. *P. brasiliensis* strain 18 lost the capacity to adhere after many consecutive cycles of subculturing over long periods [8], but reestablished it after infecting animals or animal cell cultures. In the last three decades, several studies have demonstrated that some infectious agents produce molecules that bind to components of the ECM. In the present work, the interactions of two isolates, Pb18a and b were investigated with Vero cells and ECM proteins. Fungal cells were bound to immobilize ECM proteins, and the immunofluorescence labeling clearly demonstrated the presence of laminin, fibronectin and types I and IV collagen-binding sites on the surface of *P. brasiliensis* yeast cells.

ECM proteins were recognized by both strains in ELISA assay, although they exhibited different adhesion patterns when the tests were performed with Pb18a and b extracts. Probably, distinct recognition components enhance the differences in adhesion and virulence between the two isolates. As already mentioned, *P. brasiliensis* strains are not homogeneous in their behavior, a fact noted by several authors [4,5,25]. The reisolated sample (18b) demonstrated a higher capacity to adhere to ECM proteins than the subcultured one (18a). Singer-Vermes et al. [25] showed that the higher virulence of strain 18 compared to other strains was probably related to antigenic differences between strains. Probably, adhesion is one important factor among several that promotes the development of this infectious process.

Laminin, the main component of the basement membranes [26] seems to be important in Pb18 adhesion. Attachment to the ECM component laminin might be responsible for triggering the initiation of *P. brasiliensis* infection, since tissue injury that damages the epithelial layer would expose the laminin-rich basement membrane. In addition, *P. brasiliensis* attachment to laminin may play a role in fungus dissemination and tissue invasion [7]. However, Andre et al. [27] showed that treatment with laminin did not enhance *P. brasiliensis* pathogenicity in a pulmonary model of infection, even when low infecting doses of the virulent yeast (Pb18) or a low-virulence isolate (Pb265) was used. Here it was confirmed that *P. brasiliensis* uses this protein in the process of adhesion to the host cells, as previously described in several studies [7–10].

The present results also indicate that *P. brasiliensis* interacts with human fibronectin. In fact, both *P. brasiliensis* 18a and 18b fungal extracts were adhered to the fibronectin fragments, although they presented distinct recognition patterns. Fungal extract 18b interacted more strongly with whole fibronectin and more with the 120-kDa fragment than with the 40 kDa one, in contrast to Pb18a fungal extract, which interacted more with the 40-kDa fragment than with the 120-kDa fragment. These results suggest that *P. brasiliensis* strain 18 links differently to the two fibronectin fragments, and more intensely to the entire molecule. Likewise, Penn and Klotz [28] demonstrated that *C. albicans* interacted more strongly with the 120-kDa fragment, which contains the cell-linking sequence (RGD). The existence of multiple binding to fibronectin fragments may be a characteristic of pathogens interacting with proteins of the extracellular matrix. On the other hand, *A. fumigatus* conidia preferentially bound to the non-glycosylated 40-kDa fragment, which contains the glycosaminoglycan (GAG)-binding domain [29]. This difference could be explained by the distinct molecules, which participate in the pathogen interaction among ECM components. Binding to the RGD sequence appears to guarantee a stronger molecular interaction than carbohydrate linkages, which are commonly weaker.

Collagen is the main constituent of the ECM and represents the main target for the binding of many species of microorganisms [3]. The recognition of type I collagen was notable, and these data are consistent with those of Kerr et al. [30], where the synthesis of extracellular matrix after injury started with the deposition of the fibrin-like material. Such comparative histopathological analysis emphasizes the involvement of collagen in the pathogenesis of *P. brasiliensis* [4]. In addition, Klotz [31] also showed that *C. albicans* bound to immobilized types I and IV collagen.

Analysis by SDS-PAGE and ligand blotting with ECM components identified, besides gp43, other fungal extract components that bind laminin and fibronectin. On the other hand, gp43 had significant homologies to the fibronectin molecule (data not shown), which may reinforce this interaction. Type I collagen was recognized by 47- and 80-kDa proteins. Thus, we can speculate that laminin and fibronectin may mediate the adhesion of gp43 to Vero cells. Multifunctional adhesins have been described at the surface of numerous pathogens [4,32], as observed for us.

In recent years, several molecules with receptor-like characteristics have been described in pathogenic fungi [32]. Most of these microbial molecules are glycoproteins present in the cell wall and are known as adhesins, displaying properties analogous to integrins. They generally recognize RGD-containing peptides in various tissues [3]. Competitive assays with RGDS, which is part of the fibronectin and laminin molecules, reduced by approximately 43.5% the binding of gp43 to the Vero cells. On the other hand, CDPGYIGSR-NH2 and YIGSR derived from the laminin reduced the binding by 51.5% and 42.5%, respectively. RGD peptide was shown to be a competitive ligand to other fungi [33]. On the basis of our data one can speculate that the proteins laminin and fibronectin mediate the adhesion of gp43 to the Vero cells; certainly, we know that *P. brasiliensis* cell-free fungal extracts can bind several ECM proteins, including collagen, fibronectin and laminin.

Four peptides have been delineated within the sequence of gp43, three of which have hydrophobic, and one, hydrophilic structure. Peptide 1 (NLGRDAKRHL), corresponding to residues 76–85, competed with gp43 and significantly inhibited the adhesion of *P. brasiliensis* to the Vero cells by approximately 60%. This gp43 segment contains several positively charged amino acids. Apparently, the greater contribution that PEP 1 makes to the inhibition of gp43 binding to the cells may be correlated with these positively charged residues, in accordance with a previous study in *Trypanosoma cruzi*,

whose binding of gp82 to HeLa cells involved strong adhesion by several positively charged amino acids [20]. Furthermore, the external localization of this peptide on the native molecule suggests its importance in the ECM interaction, indicating that this site could participate in protein–protein interaction in the native protein and can be responsible for adhesion to ECM proteins. The most interesting evidence in this model is that the region corresponding to PEP 1 forms a loop and in solution adopts the same conformation. In fibronectin, for example, the RGD motif is present on the molecule's surface and the secondary structure is a loop [23,24]. Consequently, the PEP 1 shows characteristics important for protein–protein interaction, as observed with the RGD motif. Besides, this peptide has a GRD sequence, that is a truncated version of the RGD motif. This structure could have an influence on the adhesion of fungi in vitro. In conclusion, this study has provided new molecular details of the interaction between *P. brasiliensis* and ECM components, using *in vitro* cellular models and adherence assays.

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