Analysis of *Paracoccidioides* secreted proteins reveals fructose 1,6-bisphosphate aldolase as a plasminogen-binding protein

Edilânia Gomes Araújo Chaves¹, Simone Schneider Weber¹, Sonia Nair Bao², Luiz Augusto Pereira¹, Alexandre Melo Bailão¹, Clayton Luiz Borges¹ and Célia Maria de Almeida Soares¹*

**Abstract**

**Background:** Despite being important thermal dimorphic fungi causing Paracoccidioidomycosis, the pathogenic mechanisms that underlie the genus *Paracoccidioides* remain largely unknown. Microbial pathogens express molecules that can interact with human plasminogen, a protein from blood plasma, which presents fibrinolytic activity when activated into plasmin. Additionally, plasmin exhibits the ability of degrading extracellular matrix components, favoring the pathogen spread to deeper tissues. Previous work from our group demonstrated that *Paracoccidioides* presents enolase, as a protein able to bind and activate plasminogen, increasing the fibrinolytic activity of the pathogen, and the potential for adhesion and invasion of the fungus to host cells. By using proteomic analysis, we aimed to identify other proteins of *Paracoccidioides* with the ability of binding to plasminogen.

**Results:** In the present study, we employed proteomic analysis of the secretome, in order to identify plasminogen-binding proteins of *Paracoccidioides*, Pb01. Fifteen proteins were present in the fungal secretome, presenting the ability to bind to plasminogen. Those proteins are probable targets of the fungus interaction with the host; thus, they could contribute to the invasiveness of the fungus. For validation tests, we selected the protein fructose 1,6-bisphosphate aldolase (FBA), described in other pathogens as a plasminogen-binding protein. The protein FBA at the fungus surface and the recombinant FBA (rFBA) bound human plasminogen and promoted its conversion to plasmin, potentially increasing the fibrinolytic capacity of the fungus, as demonstrated in fibrin degradation assays. The addition of rFBA or anti-rFBA antibodies was capable of reducing the interaction between macrophages and *Paracoccidioides*, possibly by blocking the binding sites for FBA. These data reveal the possible participation of the FBA in the processes of cell adhesion and tissue invasion/dissemination of *Paracoccidioides*.

**Conclusions:** These data indicate that *Paracoccidioides* is a pathogen that has several plasminogen-binding proteins that likely play important roles in pathogen-host interaction. In this context, FBA is a protein that might be involved somehow in the processes of invasion and spread of the fungus during infection.

**Keywords:** *Paracoccidioides*, Proteome, Secretome, Plasminogen-binding proteins, Fructose 1,6-bisphosphate aldolase

* Correspondence: cmasoares@gmail.com
¹Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, ICBl, Campus II, Universidade Federal de Goiás, 74001-970 Goiânia, Goiás, Brazil

Full list of author information is available at the end of the article.

© 2015 Chaves et al.; licensee BioMed Central. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background

The *Paracoccidioides* genus comprises a complex of pathogenic fungi, classified in at least four distinct phylogenetic lineages: S1, PS2, PS3 and Pb01-like [1-3]. These fungi are thermally dimorphic, growing at room temperatures as mycelium, which produces infectious conidia. The inhalation of conidia or mycelia propagules by the human host and their differentiation to yeast cells initiates paracoccidioidomycosis (PCM), a major health problem in South America. This human systemic mycosis is considered the tenth leading cause of chronic disease mortality among infectious and parasitic diseases, and the first among the systemic mycoses in Brazil (51.2% of cases of deaths) [4-6].

Pathogenic microorganisms are able to penetrate and colonize host tissues by establishing complex interactions with the host molecules. Some microorganisms degrade extracellular matrix components (ECM) by using proteins that subvert proteases of the host itself [7-9]. Reports have shown that pathogens can capture plasminogen (Plg) and its activation could substantially augment the organism's potential to tissue invasion and necrosis [10-20]. In eukaryotes, Plg is converted to its proteolytic form, plasmin, by physiological activators such as tissue type plasminogen activator (tPA) and urokinase type (uPA) [16]. Plasmin dissociates blood clots due to its role in the degradation of fibrin polymers and promotes the dissociation of the ECM components, which is relevant for dissemination of pathogens [17-22].

There is a variety of Plg-binding proteins and activation mechanisms used by pathogens. Besides the physiological activators, molecules produced by microorganisms, can also activate plasminogen. Studies describe various Plg-binding and activating proteins involved in the degradation of host tissues, components of ECM, which favors the spread and dissemination of different pathogens [14,23-25]. In bacteria, Plg-binding and activating proteins have been characterized [12-14,24,26-37]. Those proteins can increase the bacteria fibrinolytic activity, which favors tissue degradation and rapid progression of infection [35,38,39]. The importance of Plg in fungi is indicated by the Plg-binding properties of human pathogens, including *Candida albicans* [40,41], *Cryptococcus neoformans* [15], *Pneumocystis carinii* and *Aspergillus fumigatus* [42,43] that depict proteins at surface, which make them able to bind Plg, and improve their infectiveness.

The high dissemination of *Paracoccidioides* spp. from the site of infection to different tissues, underscores the importance of understanding the fungi virulence factors and their effects in human host. In a previous study developed by our group, we reported the recruitment of Plg and its activation into plasmin, by *Paracoccidioides*, *Pb01*, through tPA, in a process mediated by the protein enolase [10]. The enolase of *Paracoccidioides* is a surface associated protein that promotes an increase in the adhesion and invasion of the fungus to host cells in *in vitro* models of infection [10,44,45]. The recombinant *Paracoccidioides* enolase is able to adhere to some ECM components and to the surface of macrophages, reinforcing the role of this molecule in the host-pathogen interaction [46]. These data highlight that Plg-binding proteins increase the potential for invasion and pathogenicity of *Paracoccidioides* through the fibrinolytic activity of plasmin. Proteins with this ability may be transported to the surface of the fungus and secreted into the extracellular medium and promote plasmin formation, which also contributes to the pathogen dissemination [47]. In this sense, the enolase of *Paracoccidioides* is constitutively secreted by the yeast and mycelia phases [48], and is detected in the fungal cell wall [10].

In the present study, we employed proteomic analysis of the secretome, in order to identify Plg-binding proteins of *Paracoccidioides*, *Pb01*. Fifteen Plg-binding proteins were present in the fungal secretome. Proteins of the glycolytic pathway, such as phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and fructose 1,6-bisphosphate aldolase (FBA) were identified; the last was selected for further characterization. FBA has been described in various microorganisms as a Plg-binding protein, but its role has not been described in thermally dimorphic fungi. Here we show that *Paracoccidioides* binds Plg via FBA, that is found at the surface and secreted by the fungus. The protein binds human plasminogen (hPlg) and converts it into plasmin, in the presence of tPA. The interaction of the protein with hPlg, promoted increased fibrinolytic capacity of the fungus, as tested in fibrin degradation assays. The addition of recombinant FBA (rFBA) or anti-rFBA antibodies was capable of reducing the interaction between macrophages and *Paracoccidioides*, possibly by blocking the binding sites for FBA. These data reveal the possible participation of the FBA in the *Paracoccidioides* adhesion and invasion processes. The identification of novel surface/secreted proteins that can be involved in host-pathogen interaction is central to understand *Paracoccidioides* pathogenesis.

Results and discussion

Identification of plasminogen-binding proteins of *Paracoccidioides*, *Pb01* yeast cells

In order to identify Plg-binding proteins in the secretome of *Paracoccidioides*, *Pb01*, we obtained 2-DE gels. The gels ran in parallel, were (i) stained with Coomassie brilliant blue or (ii) transferred to nitrocellulose membrane and reacted with Plg, in a Far-western blotting assay, as demonstrated in Figure 1, panel B. Image analysis were produced allowing the pairing of the proteins spots between the 2-DE gel and the membrane obtained by Far-western blotting.
The detected spots in the membrane (Figure 1B) were compared to the Coomassie blue partners in order to find their corresponding proteins spots in the 2-DE gel (Figure 1A). Subsequently, protein spots were manually excised of the gel, and identified by mass spectrometry. It was possible to identify in the secretome of yeast cells, 22 protein spots, which bound Plg, as depicted in Figure 1B. Figure 1, panel C, depicts the images of the negative control assay, in which the membrane was not previously incubated with Plg, indicating no cross-reactivity of the proteins with the antibody to Plg.

Spots identified as Plg-binding proteins were cut from the gel and subjected to tryptic digestion and mass spectrometry analysis. The data were used to search the Mascot, and provided the identification of 15 proteins/isoforms. Table 1 describes the secreted proteins of Paracoccidioides, identified as Plg-binding molecules. Several enzymes were detected in this category, some of them presenting several isoforms, such as homogentisate 1,2-dioxygenase (spots 4,5), NADP-specific glutamate dehydrogenase (spots 6,7), phosphoglycerate kinase (spots 8,16) 2-methylcitrate synthase (spots 9,10,11), FBA (spots 13,14,15) and malate dehydrogenase (spots 20,21). Thus, the 22 protein spots identified are summed up in 15 different proteins.

While much of the proteins described in this work are not annotated in the database Psort (http://www.genscript.com/psort/wolf_psort.html) as extracellular proteins, we found compatible data in other studies. The proteins: 2-methylcitrate synthase, FBA, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, formamidase, acetyl-CoA acetyltransferase and phosphoglycerate kinase were detected in the secretome of Paracoccidioides, Pb01 yeast and mycelia [48]. Other proteins were identified in the secretome of Paracoccidioides, Pb18: FBA, glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase [49]. These data corroborate the in silico analysis performed in the software Signal P and Secretome P, where we can observe that most of the proteins described here are secreted by nonclassical pathways (Table 1).

Some of the proteins identified in this study have also been described in other systems as Plg-binding proteins. In this way, acetyl-CoA acetyltransferase was identified in the bacteria Leptospira interrogans [50]; phosphoglycerate kinase was described in C. albicans [40], Streptococcus pneumoniae [51], as well as in C. neoformans [15]. In addition, FBA and glyceraldehyde 3-phosphate dehydrogenase were also described as Plg-binding proteins in C. albicans [40].

Formamidase is a highly abundant protein in Paracoccidioides, as previously described by our group [52,53]. The protein gp43 also detected in our binding assays, binds to laminin, putatively contributing to the fungus virulence and facilitating the process of infection [54,55].

The proteomic binding assays, also allowed the identification of enolase as a Plg-binding protein. The presence of glycolytic enzymes as Plg-binding proteins is reported in several pathogens, including bacteria and fungi. In Paracoccidioides, enolase is present at the yeast cells surface, where it binds and activates hPlg, presumably contributing to the fungus pathogenesis [10]. Other glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and FBA, were found here as Plg-binding proteins (Figure 1B, Table 1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), is a molecule that binds Plg and is present on the surface and secretome of bacteria [56-58] and fungi [40]. In C.
| Spot number | General information number (NCBI) | Protein description | pI theor/exp | MM (kDa) theor/exp | PMF score | Coverage sequence (%) | MS/MS Ions score | Matched peptides | Psort prediction | SignalP Value ≥ 0.5 | SecretomeP Value ≥ 0.5 | big-PI |
|-------------|---------------------------------|---------------------|-------------|-------------------|-----------|----------------------|-----------------|----------------|----------------|-----------------|----------------------|---------------|
| 1           | gi|226285916                     | aminomethyl transferase | 9.67/4.42  | 53.35/45.56       | 121       | 37                   | 84              | 4              | mito: 23.0       | NO                  | 0.516                 | NO            |
| 2           | gi|226278634                     | aldehyde dehydrogenase  | 5.92/6.94  | 54.69/45.97       | 94        | 59                   | 114             | 6              | cyto: 21.5       | NO                  | 0.562                 | NO            |
| 3           | gi|295668479                     | formamidase          | 6.10/7.13  | 46.14/45.71       | 144       | 44                   | 109             | 5              | cyto: 12.0       | NO                  | 0.565                 | NO            |
| 4           | gi|295658700                     | homogentisate 1,2-dioxygenase | 6.25/7.62  | 50.85/45.51       | 78        | 35                   | -               | -              | cyto: 13.0       | NO                  | 0.601                 | NO            |
| 5           | gi|295658700                     | homogentisate 1,2-dioxygenase | 6.16/7.76  | 50.86/45.35       | 76        | 26                   | 89              | 4              | cyto: 13.0       | NO                  | 0.621                 | NO            |
| 6           | gi|295659664                     | NADP-specific glutamate dehydrogenase | 7.66/8.48  | 50.38/45.35       | 102       | 53                   | 72              | 4              | cyto: 11.0       | NO                  | NO                    | NO            |
| 7           | gi|295659664                     | NADP-specific glutamate dehydrogenase | 7.17/8.75  | 50.46/45.45       | 101       | 56                   | 117             | 2              | cyto: 11.0       | NO                  | NO                    | NO            |
| 8           | gi|295669690                     | phosphoglycerate kinase | 6.48/9.49  | 45.31/44.54       | 83        | 61                   | 151             | 5              | cyto: 25.0       | NO                  | NO                    | NO            |
| 9           | gi|295666179                     | 2-methylcitrate synthase | 9.02/9.73  | 51.51/45.00       | -         | -                    | 226             | 6              | mito: 27.0       | NO                  | NO                    | NO            |
| 10          | gi|295666179                     | 2-methylcitrate synthase | 9.02/9.96  | 51.51/44.92       | 95        | 62                   | 95              | 4              | mito: 27.0       | NO                  | NO                    | NO            |
| 11          | gi|295666179                     | 2-methylcitrate synthase | 9.02/10.66 | 51.58/47.76       | 78        | 57                   | 88              | 4              | mito: 27.0       | NO                  | NO                    | NO            |
| 12          | gi|295658119                     | glyceraldehyde-3-phosphate dehydrogenase | 10.18/10.67 | 33.92/43.65       | -         | -                    | 93              | 2              | cyto: 27.0       | NO                  | 0.532                 | NO            |
| 13          | gi|29567120                      | fructose 1,6-bisphosphate aldolase | 6.09/6.41  | 39.72/41.29       | -         | -                    | 154             | 5              | cyto: 21.0       | NO                  | 0.505                 | NO            |
| 14          | gi|29567120                      | fructose 1,6-bisphosphate aldolase | 6.09/6.60  | 39.72/41.29       | -         | -                    | 670             | 5              | cyto: 21.0       | NO                  | 0.505                 | NO            |
| 15          | gi|29567120                      | fructose 1,6-bisphosphate aldolase | 6.09/6.88  | 39.72/40.94       | -         | -                    | 555             | 9              | cyto: 21.0       | NO                  | 0.505                 | NO            |
| 16          | gi|295669690                     | phosphoglycerate kinase | 6.48/7.75  | 45.31/42.67       | 86        | 59                   | 56              | 3              | cyto: 25.0       | NO                  | NO                    | NO            |
| 17          | gi|295668707                     | acetyl-CoA acetyltransferase | 8.98/7.88  | 46.65/42.67       | -         | -                    | 102             | 3              | mito: 24.5       | NO                  | 0.692                 | NO            |
| 18          | gi|11496183                       | immunodominant antigen Gp43 | 7.17/8.15  | 45.77/42.42       | 97        | 43                   | 102             | 4              | extr: 24.0       | NO                  | 0.746                 | NO            |
| 19          | gi|226285552                     | ketol-acid reductoisomerase | 9.12/8.46  | 44.86/42.17       | 172       | 62                   | 134             | 7              | mito: 27.0       | NO                  | 0.683                 | NO            |
| 20          | gi|295658218                     | malate dehydrogenase | 6.36/7.18  | 34.67/33.98       | 73        | 47                   | 69              | 5              | cyto: 17.0       | NO                  | 0.674                 | NO            |
| 21          | gi|295658218                     | malate dehydrogenase | 6.36/7.85  | 34.67/33.75       | 129       | 41                   | 344             | 9              | cyto: 17.0       | NO                  | 0.674                 | NO            |
| 22          | gi|226279168                     | 2,5-diketo-D-gluconic acid reductase A | 7.71/8.40  | 34.78/33.36       | 81        | 48                   | 50              | 3              | cyto: 20.5       | 0.5                | NO                    | NO            |

1Spots numbers indicated in Figure 1A.
2NCBI database general information number (http://www.ncbi.nlm.nih.gov/).
3Isoelectric point (theoretical/experimental).
4Molecular Mass in kDa (theoretical/experimental).
5Mascot PMF score for fragmentation data (http://www.matrixscience.com/).
6Sequence coverage percentage.
7Mascot MS/MS score for fragmentation data (http://www.matrixscience.com/).
8Number of identified peptides (MS/MS).
9Subcellular localization prediction of proteins according Psort (http://www.genscript.com/psort/wolf_psort.html).
10Secretion prediction according to Signal P 3.0 server. The number corresponds to signal peptide probability (Score³ 0.5) (http://www.cbs.dtu.dk/services/SignalP/).
11Secretion prediction according to Secretome P 1.0 server; the number corresponds to neural network that exceeded a value of 0.5 (NN-score ≥ 0.50) (http://www.cbs.dtu.dk/services/SecretomeP/).
12GPI Modification Site Prediction of proteins according big-PI (http://mendel.imp.ac.at/gpi/gpi_server.html).

cyto: cytoplasm.
extr: extracellular.
mito: mitochondria.
albicans, this molecule is an adhesin that participates in the process of adherence to human cells, and binds to ECM components [40,59-61]. In studies conducted by our group, GAPDH is located at the surface of Paracoccidioides, where could mediate the adhesion and internalization of the fungus to host cells, binding to ECM components [62].

Phosphoglycerate kinase is an adhesin in both, bacteria [63] and fungi [15,40]. On the surface of group B streptococcus, phosphoglycerate kinase binds the host actin and Plg. Binding of ECM proteins to bacterial proteins, including phosphoglycerate kinase, promotes the activation of specific proteins on its surface, which induces bacterial adhesion [63,64]. Also, proteolytic degradation of ECM by phosphoglycerate kinase - recruited plasmin activity, promotes adherence to endothelial cells and bacterial dissemination in the host tissues [36]. In C. neoformans, phosphoglycerate kinase localizes to the fungal cell wall, where exhibits accessible carboxy-terminal lysine residues for Plg-binding [65].

FBA is cytoplasmic and also localized at the surface of several bacteria [66,67], as well as in pathogenic fungi [15,40] where it binds host molecules and depicts adhesion function, beyond its glycolytic activity. In this work, three isoforms of FBA were detected (Table 1, spots 13, 14 e 15). The FBA of Paracoccidioides, Pb01 was previously characterized in our laboratory [68,69]. The protein is as an antigenic molecule, reactive with sera of PCM patients, as demonstrated [68]. Studies revealed the role of FBA in cell adhesion and invasion [67]. The FBA-deficient mutant of Neisseria meningitidis was not affected in its ability to grow in vitro, but depicted a significant reduction in adhesion to human brain microvascular endothelial and HEp-2 cells, suggesting participation in adhesion of meningococci to human cells [67]. In C. neoformans, analysis of the Plg-binding proteins, allowed the identification of a FBA surface protein, that serves as a Plg receptor [15]. So, due to the relevance of FBA as an adhesin and a Plg-binding protein that promotes the virulence of microorganisms, the protein was selected for further investigation in Paracoccidioides.

Confirmatory assays of FBA as a plasminogen-binding protein
We selected FBA for further analysis, since the protein is a Plg-binding protein in several pathogens, as previously described [15,40,70]. To verify if the FBA of Paracoccidioides also has this ability, a recombinant protein was obtained by cloning the cDNA (GenBank Accession Number AY233454) into the expression vector pGEX-4 T-3 (GE Healthcare) as described in Material and Methods. The fusion protein was obtained in E. coli. As observed in Figure 2A, the recombinant protein was purified (lane 3) and cleaved from the fusion with GST by the addition of thrombin, rendering a 40-kDa protein (lane 4). A Far-western blotting with increasing concentrations of rFBA was obtained, and depicted in Figure 2B. Concentrations of 0.1 µg to 3 µg of the recombinant protein were subjected to Far-western, demonstrating a dose-dependent binding of the protein with Plg, showing that, in fact, the FBA of Paracoccidioides binds to the Plg.

Detection of FBA at the Paracoccidioides surface
In order to determine the localization of the FBA in Paracoccidioides, Pb01, we performed a western blotting with cellular fractions of Paracoccidioides and polyclonal antibodies raised in mice to the recombinant protein. As shown in Figure 3A, the FBA is present in the cytoplasm, secretome and cell wall (fractions 1 and 2). The fraction 1 contains proteins associated with the cell surface by non-covalent bonds or by disulfide bridges, as described [71,72]. The fraction 2 represents cell wall proteins sensitive to treatment with alkali (ASL-CWPs), including cell wall proteins with internal repeats (PIR-CWPs). Fraction 3 represents proteins with glycosyl-phosphatidylinositol (GPI) anchors linked to the wall.
(GPI-CWPs) [73,74], but rFBA was not detected in this fraction. Furthermore, the immunoelectron microscopy analysis revealed the presence of FBA in the cytoplasm, in vesicles in releasing process and at the cell surface, as depicted in Figure 3B, panel 2. The release of vesicles to the external environment is used by many pathogens to increase their invasive potential. Vesicles contain many virulence factors, including molecules that bind to and activate Plg [27,70,75]. The presence of FBA at the surface and vesicle of the fungus can allow the capture of hPlg and plasmin generation, forming a highly fibrinolytic layer around the fungal cell. These data suggest that FBA, can somehow influence fibrinolytic activity of yeast cells. Cell wall and secreted proteins, may participate in the process.

Paracoccidioides and rFBA bind and activate plasminogen, promoting fibrinolytic activity
We next investigated whether the capture of Plg by FBA, favors the generation of plasmin. Previous work from our group have demonstrated that yeast cells of Paracoccidioides bind to Plg [10]. As described in

![Figure 3 Detection of FBA in Paracoccidioides. (A) Western blotting analysis. Different protein samples (15 μg) of Paracoccidioides comprehending the soluble and secreted proteins, cell wall fractions 1, 2 and 3 were obtained by sequential treatments as described in Materials and Methods. For negative and positive controls, we employed 3 μg of samples of bovine serum albumin (BSA) and the rFBA, respectively. The immunoblot was probed with the polyclonal antibodies directed to the rFBA. (B) Immunoelectron microscopy. Panel 1 - Transmission electron microscopy of Paracoccidioides yeast cells showing the cell wall (w), intracytoplasmic vacuoles (v), nucleus (n) and mitochondria (m). Panel 2 - Gold particles are observed in the cytoplasm region (arrows) and vesicles in release process (arrowheads). * corresponds to the region which has been expanded from panel 2. Panels 3 and 4 - Negative controls with anti-rabbit-igG-Au-conjugated and rabbit non immune sera, respectively. The bars indicate: 1.0 μm (Panel 1), 1.0 μm (Panel 2), 0.5 μm (Panel 3), 1.0 μm (Panel 4) and 0.5 μm (Zoom panel).]
Materials and Methods the test was performed by fixation of yeast cells or the rFBA, followed by incubations with hPlg and tPA. In the presence of tPA, the yeast cells and the rFBA were able to generate plasmin. This ability was inhibited by the lysine analogue (εACA), which competes for the binding sites of Plg (Figure 4A). Competition experiments were developed by adding increasing concentrations of εACA, which inhibits plasmin generation in a dose dependent manner (Figure 4B). These data suggest that yeast cells, as well as the recombinant protein bind hPlg, converting into plasmin in the presence of tPA.

Fibrinogen is a major substrate of plasmin in vivo and for that, we examined plasmin activity in jellified matrices containing fibrinogen (Figure 4C). Fibrin degradation tests were performed in triplicate (data not shown), where yeast cells were incubated in the presence of hPlg and tPA. It was observed the formation of hydrolysis haloes within the jellified-fibrin-containing matrix (Figure 4C, panel 3). In an attempt to block the receptor of plasminogen on the surface of the fungus, yeast cells were incubated with anti-rFBA polyclonal antibodies (Figure 4C, panel 4). A decrease in the hydrolysis halo comparing the panels 3 to 4, can be observed. The addition of protease inhibitors resulted in no halo formation, due to inactivation of plasmin activity (Figure 4C, panels 5 and 6). Negative controls are presented in panels 1 and 2, whereas positive control is presented in panel 7. Thus, we can conclude that FBA of Paracoccidioides may have an important role in the host tissues invasion by the fungus, besides participating in metabolic processes. Corroborating other studies on this subject, the secondary role of this protein makes it an important virulence factor. By capturing and activating Plg, FBA can promote the spread of the fungus, certainly by matrix degradation, paving the way for infection toward internal organs.

Figure 4 Plasminogen-binding and activation and fibrin degradation assays. (A) Paracoccidioides yeast cells and the rFBA were incubated with hPlg in the presence or absence of tPA and εACA. We used a plasmin substrate (D-valyl-L-lysyl-p-nitroaniline hydrochloride) (Sigma-Aldrich) to dose the amidolytic activity of the reaction of converting plasminogen into plasmin. (B) In competition experiments we added to the wells increasing concentrations of εACA (50 mM to 1 M), followed by the addition of hPlg. Experiments were performed in triplicate as described in Materials and Methods. The error bars indicate the standard deviations between the results. *: results significantly different from control, at a p value < 0.05. (C) The fibrinolytic activity of Paracoccidioides was analysed by the observation of clear hydrolysis haloes within the opaque jellified-fibrin-containing matrix. Panel 1: Paracoccidioides yeast cells in the absence of hPlg; 2: the fungus after binding to hPlg; 3: Similar to 1 and 2, but reflecting the presence of tPA. The fungus was incubated in the presence of hPlg and tPA, with the addition of anti-rFBA (panel 4) and proteases inhibitors, aprotinin and PMSF (panels 5 and 6). Controls consisting of plasminogen and tPA (panel 7).
rFBA influences the interaction of *Paracoccidioides* with macrophages

The rFBA of *Paracoccidioides* behaved as an adhesin in a binding assay between J774 and rFBA. Macrophages were able to bind/internalize the rFBA after 5 h incubation (Figure 5A, line 2). Control is depicted in Figure 5A, line 1, in which no reaction was obtained in macrophages not incubated with rFBA. Positive (rFBA, Figure 5A, line 3) and negative (BSA, Figure 5A, line 4) controls, are depicted. Next, we investigated the putative role of FBA in the interaction between *Paracoccidioides* and macrophages. Data represent the percentage of CFUs recovered from infected macrophages, in relation to the control (Figure 5B). The results show that infection of J774 by *Paracoccidioides* was reduced by 79% when the macrophages were pre incubated with rFBA, and 86% when the yeast cells were pre incubated with anti-rFBA antibodies. The data strongly suggest a role for the FBA in the infective process in macrophages.

Similar experiments with other proteins such as glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase, that promoted reduced interaction of *Paracoccidioides* with pneumocytes and Vero cells, were reported [62,76]. Regarding to Plg, the pre-incubation with *Paracoccidioides*, in the presence of tPA, promoted increased macrophage infection (Figure 5B). The addition of the antibodies to rFBA and Plg, prompted inhibited the macrophage infection. This data is consistent with the role of FBA in activating Plg to plasmin, as previously demonstrated in Figure 4C. Our data suggest that binding of the FBA to Plg, may increase the virulence of this pathogen.

**Conclusions**

Many microorganisms express proteins that are able to subvert the host proteases and use them in their favor. Once activated to plasmin, Plg acquires fibrinolytic activity. Pathogens able to capture Plg can increase their potential for dissemination in the host tissues. This work identified several secreted proteins of *Paracoccidioides* with ability to bind to hPlg. These proteins are probable targets of the interaction of the fungus with the host, probable acting as mediators of plasmin formation, which may contribute to the invasion of the fungus in the host tissues. The FBA, was detected at the *Paracoccidioides* surface and secretory vesicles, in addition to the conventional cytoplasmatic localization. The protein can bind to hPlg, converting it to plasmin in the presence of tPA. This interaction promoted increased fibrinolytic capacity of the fungus, as demonstrated in fibrin degradation assay. Moreover, we demonstrated that FBA adhered to macrophages and contribute in some way to the interaction of the fungus with these defense cells. These data suggest that FBA is a Plg-binding protein, and may be important virulence factor involved in the process of adhesion, invasion and spread of the fungus.

**Methods**

**Strains and media**

*Paracoccidioides, Pb*01 (ATCC MYA-826) was used in all experiments. The yeast phase was maintained in vitro by sub culturing at 36°C during 7 days in Fava Netto's solid medium [1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.3% (w/v) proteose peptone, 0.5% (w/v) beef extract, 0.5% (w/v) NaCl, 4% (w/v) glucose, 1.2% (w/v) agar, pH 7.2].

**Preparation of Paracoccidioides protein fractions**

To obtain the secreted proteins, the yeast cells of *Paracoccidioides, Pb*01 were inoculated in Fava Netto's liquid medium and cultured at 36°C for 24 h with shaking at 200 rpm, as previously described [48]. The proportion of cells used to obtain the inoculum was 2.5 g wet weight of yeast cells per 50 mL of liquid medium, or 50 mg/mL. After the incubation for 24 h, microscopic analysis was...
performed to check fungal viability, followed by cells centrifugation at 10,000 \( \times \) g, for 30 min at 4°C. The supernatant was used for obtain the secreted proteins [48]. The culture supernatant was sequentially filtered through 0.45 mm-pore and 0.22 mm-pore membrane filters. Culture filtrates were concentrated and subsequently washed three times with ultrapure water via centrifugation 10,000 \( \times \) g through a 10-kDa molecular weight cut off membrane (Amicon ultra centrifugal filter, Millipore, Bedford, MA, USA). The obtained pellet, which contains the yeast cells, was used to the extraction of *Paracoccidioides* soluble [77] and cell wall proteins. Briefly, yeast cells were washed five times with 10 mM Tris–HCl, pH 8.5, 2 mM CaCl\(_2\) added of the 1:1000 protease inhibitor phenyl methyl sulfonyl fluoride (PMSF) and centrifuged at 10,000 \( \times \) g for 30 min at 4°C. The cells were frozen in liquid nitrogen and disrupted by maceration. Subsequently, the precipitate was resuspended in lysis buffer (20 mM Tris–HCl pH 8.8; 2 mM CaCl\(_2\) added of the protease inhibitor PMSF (1:1000) and glass beads; the mixture was agitated for 1 h. After centrifugation 10,000 \( \times \) g for 30 min at 4°C, the supernatant and pellet were used to obtain the *Paracoccidioides* soluble and cell wall proteins, respectively. The cell wall proteins were extracted by sequential treatments according to the type of connection that these proteins establish with other cell wall components, as previously described, with some modifications [10,71,74,78]. Briefly, the pellet was washed 5 times as following: with cold sterile distilled water; with 5% (w/v) NaCl, with 2% (w/v) NaCl and with 1% (w/v) NaCl. After the washes, the pellet was treated with extraction buffer [50 mM TrisHCl, pH 7.8, 2% (w/v) SDS, 100 mM EDTA and 40 mM β-mercaptoethanol] for 10 min at 100°C. The supernatant from centrifugation constitutes the first fraction (Fraction 1). The pellet resistant to extraction with SDS was washed 5 times with 0.1 M sodium acetate pH 5.5. The obtained solution was centrifuged at 10,000 \( \times \) g for 30 min at 4°C and the pellet was treated with 30 mM NaOH for 24 h at 4°C, to obtain the second fraction, that after centrifugation at 10,000 \( \times \) g for 30 min at 4°C, constituted the fraction 2. The pellet was treated with pyridine-hydrogenated fluoride (HF-pyridine) on ice for 24 h to give the third fraction (Fraction 3).

All the obtained protein extracts described above were concentrated and washed three times with ultrapure water via centrifugation through a 10 kDa molecular weight cut off in ultracel regenerated membrane (Amicon ultra centrifugal filter, Millipore, Bedford, MA, USA). The protein concentrations were determined by the Bradford assay using bovine serum albumin as standard [79].

### Two-dimensional gel electrophoresis

Two-dimensional fractionation (2-DE) of secreted proteins was performed, as described [77,80]. The 2-DE gels were obtained in duplicates, using 500 µg of proteins, for each one. The samples were treated with the commercial system of purification 2D Clean-up Kit (GE Healthcare, Uppsala, Sweden) for removing interferences according to the manufacturer’s instructions, before protein isoelectric focusing. Proteins samples were treated with 250 µL of buffer containing 7 M urea, 2 M thiourea, 130 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.002% (w/v) dithiothreitol (DTT), ampholyte-containing buffer (IPG gel, GE Healthcare), and trace amounts of bromophenol blue. Then the samples were loaded onto a 13 cm Immobiline™ DryStrip gel (GE Healthcare) with a pH range of 3–11 for separation of proteins according to their isoelectric points (pI) with an electric current of 50 µA / strip at 20°C. In order to perform the first separation of secreted proteins, isoelectric focusing was conducted as following: 30 V for 1 h, 250 V for 1 h (step), 1 kV for 1 h (step), 2 kV for 1 h (step), 5 kV for 3 h (gradient), 8 kV for 8 h (gradient) and 8 kV for 1 h (step). Strips were reduced with 18 mM DTT (dithiothreitol) and alkylated with 135 mM iodoacetamide in equilibration buffer [50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS) and 0.002% (w/v) bromophenol blue] during 40 min. The second dimension was performed in 12% polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) in running buffer [25 mM Tris–HCl, 192 mM glycine, 0.1% (w/v) SDS], using a vertical system (GE Healthcare) at 12°C during 1 h at 150 V, and after at 250 V. Two gels were stained by Coomassie brilliant blue (Plus One Coomassie Tablets Phast Gel Blue R-350, GE Healthcare) according to manufacturer’s instructions to visualize the proteins.

### Far-western

For the Far-western experiments, the 2-DE gels were produced in duplicates. The secreted proteins, after one or two-dimensional fractionation, were transferred to nitrocellulose membranes for ligand binding with Plg, to identify Plg-binding receptors. The results were compared to the protein pattern of the Coomassie blue stained counterpart. The membranes were incubated in blocking buffer [0.1% (v/v) Tween 20, 5% (w/v) skimmed powder milk, in 10 mM PBS (0.14 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3)] for 1 h at room temperature. Subsequently, the membranes were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T) and, except for the negative control, the membranes were incubated with 35 µg/mL of hPlg (Sigma-Aldrich) diluted in blocking buffer, for 1 h under shaking, as described [10]. Subsequently, the membranes were washed three times with PBS-T and incubated with 1 µg/mL anti-human plasminogen (Anti-hPlg) produced in mice (Sigma-Aldrich) diluted 1:100 in blocking buffer.
After three washes in PBS-T, the membranes were incubated with the secondary antibody (anti-mouse IgG coupled to alkaline phosphatase) (Sigma-Aldrich) diluted 1:5000 in blocking buffer, for 1 h. After that, the membranes were washed and the reaction was developed using 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetr bazolium (NBT).

Expression of the rFBA by Escherichia coli, purification of the recombinant protein and polyclonal antibodies production

The cDNA that encodes FBA of Paracoccidioides, Pb01 (GenBank Accession Number AY233454) was previously obtained [69]. Oligonucleotide primers were designed: sense S (5′-GAATTCCATGGGCGTGAAAGACA-3′) and antisense AT (5′-GGGGCCGGCTTACAACTGGTTAGA-3′) in order to obtain the cDNA. The cDNA product obtained by RT-PCR was cloned into the expression vector pGEX-4 T-3 (GE Healthcare) and transformed into Escherichia coli XL1 blue competent cells. Bacterial cells were grown in Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin under agitation at 37°C until the OD reaches an absorbance of 0.6 at a wavelength of 600 nm. The reagent Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the growing culture to a final concentration of 0.1 mM. After 16 h incubation at 15°C, the bacterial cells were harvested by centrifugation at 10,000 x g for 10 min and resuspended in PBS. Soluble proteins were obtained by sonication, followed by centrifugation at 10,000 x g during 10 min. FBA linked to GST (glutathione-S-transferase) was affinity purified using glutathione Sepharose 4B resin (GE Healthcare). The resin was washed 10 times in PBS and the GST was cleaved by addition of thrombin (50 U/ml) (Sigma-Aldrich). The purity and size of the recombinant protein were assessed by 12% SDS-PAGE followed by staining with Coomassie Blue.

The rFBA was used for production of polyclonal antibodies in mice. The purified protein (300 µg) was injected subcutaneously three times at intervals of 15 days. Serum containing polyclonal antibodies was collected and stored at −20°C.

Western blotting analysis

For western blotting analysis, the Paracoccidioides protein samples were probed using polyclonal antibodies produced to the rFBA. Protein samples were loaded onto a 12% SDS-PAGE gel and separated by electrophoresis. The gels were run at 150 V for approximately 2 h and the proteins were transferred to nitrocellulose membranes at 30 V for 16 h in a buffer containing 25 mM Tris–HCl (pH 8.8), 190 mM glycine and 20% (v/v) methanol. The gels were stained with Ponceau red to verify complete protein transfer. Next, each membrane was incubated in blocking buffer [1X PBS, 1.4 mM KH₂PO₄, 8 mM Na₂HPO₄, 140 mM NaCl, 2.7 mM KCl (pH 7.3), 3% (w/v) nonfat dried milk and 0.1% (v/v) Tween 20] for 2 h. The membranes were washed with PBS-T, and incubated with anti-rFBA polyclonal antibody (1:1000), followed by washing in blocking buffer three times, during 15 min each wash. The membranes were incubated with the secondary antibody anti-mouse immunoglobulin G (IgG) coupled to alkaline phosphatase (Sigma Aldrich) diluted 1:5000 in blocking buffer, for 1 h. After that, the membranes were washed and the reaction was developed using 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetr bazolium (NBT).

Image analyses

The comparative analysis between the images of the proteins stained with Coomassie Blue and the membranes of the Far Western assay were performed using the Image Master 2D Platinum software v7.0 (GE Healthcare) in order to identify in the 2-DE gels the protein spots that were visualized in the membranes through the pairing. The gels and membranes were aligned and the spots were compared according to their isoelectric points and molecular masses.

Mass spectrometry analysis

The spots of interest were manually excised from the 2-DE gels and treated with trypsin as previously described [48,77,80]. The spots were removed, washed three times with ultrapure water, resuspended in 100 µl of 100% acetonitrile (ACN) and dried in a vacuum centrifuge. Subsequently, the samples were reduced with 10 mM DTT in 25 mM ammonium bicarbonate (NH₄HCO₃), and alkylated with 55 mM iodoacetamide in 25 mM NH₄HCO₃ protected from light. The supernatant was removed and the gel pieces were washed with 100 µl of a solution containing 25 mM ammonium bicarbonate/50% ACN (v/v), vortexed for 5 min, and centrifuged. Enzymatic digestion was performed by incubation at 37°C for 16 h in buffer containing trypsin (12.5 ng/µl) and 25 µl of 25 mM NH₄HCO₃. The supernatant was transferred to a new tube and the gel pieces were shaken for 30 min in 50% ACN (v/v), and 1% trifluoroacetic acid (TFA) (v/v), followed by sonication for 5 min, after which the supernatant was combined with the one obtained in the previous step. The dried samples were resuspended in 10 µl of ultrapure water and subsequently purified using a pipette tip with a bed of chromato graphic media (ZipTips® C18 Pipette Tips, Millipore, Bedford, MA, USA). Two microliters of each peptide sample were deposited onto a matrix-assisted laser desorption ionization quadrupole time-of-flight mass spectrometry (MALDI-Q-TOF MS) target plate. Next, 2 µL of matrix solution (10 µg/µL a-cyano-4-hydroxyciannamic acid matrix in 50% (v/v) ACN and 5% (v/v)
TFA) was added. The mass spectra were performed in the positive reflection mode on a MALDI-Q-TOF mass spectrometer (SYNAPT, Waters Corporation, Manchester, UK). The MS/MS and PMF analysis was performed using Mascot software v. 2.4 (http://www.matrixscience.com) (Matrix Science, Boston, USA). The ion search parameters were: tryptic peptides with one missed cleavage allowed; fungi taxonomic restrictions; fixed modifications: carboxymethylation of Cys residues; variable modifications: oxidation of methionine and a tolerance of 0.6 Da. In silico analyzes were performed to validate the results obtained in vitro. In order to predict the location of proteins we used the program Psort (http://www.genscript.com/psort/wolf_psort.html). The software big-PI Fungal Predictor (http://mendel.imp.ac.at/gpi/fungi_server.html) was used to predict glycosylphosphatidylinositol (GPI) protein anchors. In order to predict proteins to be secreted we employed the Signal P (http://www.cbs.dtu.dk/services/SignalP/) that predicts the classical pathway secretion and SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP/) that predicts nonclassical pathway secretion.

Ultrastructure of the yeast cells and immunocytochemistry of FBA

For the ultrastructural and immunocytochemistry studies, previously described protocols were employed [76,81,82]. The yeast cells were fixed in solution containing 2% (v/v) glutaraldehyde, 2% (w/v) paraformaldehyde, and 3% (w/v) sucrose in 0.1 M sodium cacodylate buffer pH 7.2. Ultrathin sections were stained with 3% (w/v) uranyl acetate and lead citrate. For ultra-structural immunocytochemistry studies, the ultrathin sections were incubated for 1 h with the polyclonal antibodies raised against the rFBA (diluted 1:100) and for 1 h at room temperature with the labeled secondary antibody anti mouse IgG, Au-conjugated (10 nm average particle size; 1:20 dilution). The grids were stained as described above, and observed with a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan). Controls were incubated with mouse preimmune serum (1:100 dilution).

Plasminogen activation assay

The wells of multterior plates were coated with 1 µg of rFBA or fixed with 1 × 10⁶ yeast cells during 1 h. After that, the wells were incubated with 1 µg of hPlg (Sigma-Aldrich), followed by incubation with 3 µg of plasmin substrate (D-valyl-L-lysyl-p-nitroaniline hydrochloride) (Sigma-Aldrich) and 15 ng of tPA (Sigma-Aldrich). Competition and control experiments were performed by blocking the generation of plasmin in the absence of tPA (Sigma-Aldrich) or in the presence of the lysine analogue ε-aminocaproic acid (εACA). The amidolytic activity of the generated plasmin was measured at 405 nm.

Fibrin matrix-gel degradation analysis

The matrix gel contained 1.25% (w/v) low-melting-temperature agarose, 100 µg of hPlg (Sigma-Aldrich) and 4 mg of fibrinogen (Sigma-Aldrich) in a final volume of 2 mL. To detect fibrinolysis activity, a total 1 × 10⁷ cells of Paracoccidioides, Pb01 were incubated with 50 µg of hPlg for 3 h in the presence or absence of tPA (50 ng). The yeast cells were also incubated with the serine proteinase inhibitors aprotinin (1 µg), PMSF (50 mM) and with anti-rFBA antibodies in a final volume of 1 mL. Thereafter, the mixtures were washed three times with PBS and the pellets were placed in wells of a fibrin substrate matrix gel. Plasmin activity was detected by the observation of clear hydrolysis haloes within the opaque jellified fibrin-containing matrix, after incubation in a humidified chamber at 37°C for 12 h.

Binding assays of the rFBA to in vitro cultured macrophages

The J774 1.6 macrophages (Rio de Janeiro Cell Bank – BCRJ/ UFRJ, accession number 0273) were used for phagocytosis assays. The J774 1.6 cells were cultured in RPMI medium containing bovine fetal serum 10% (v/v) (Vitro, Missouri, USA) at 36°C and 5% CO₂ until complete confluence. The macrophages were incubated with 50 µg/mL of rFBA, at 36°C for 5 h, and washed. Next, the cells were lysed by incubating with distilled water for 1 h. The lysate was centrifuged at 1,400 × g for 5 min. The proteins contained in the supernatant were submitted to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated blocking buffer [PBS 1X with 5% (w/v) nonfat dried milk and 0.1% (v/v) Tween 20] for 2 h, and then successively with anti-rFBA polyclonal antibodies (1:1000) and with the antimouse immunoglobulin G (IgG) coupled to alkaline phosphatase (Sigma Aldrich). The reactions were developed with BCIP-NBT.

rFBA and anti rFBA-antibodies decrease Paracoccidioides macrophages interaction

We tested the interference of the rFBA and antibodies to adhesion/infection of Paracoccidioides in macrophages. In addition, we tested the ability of Plg-treated yeast cells to adhere/infect macrophages. A total of 5 × 10⁶ yeast cells, per well, were added to the macrophages, reaching a yeast:macrophages cells ratio of 5:1, followed by incubation for 12 h at 36°C, in 5% CO₂ in RPMI medium containing IFN-γ (1U per mL) and MEM non-essential amino acid solution (Sigma Aldrich, Missouri, USA) at 36°C and 5% CO₂, until complete confluence. The macrophages were incubated with 50 µg/mL of rFBA, at 36°C for 5 h, and washed. Next, the wells were incubated with distilled water for 1 h. The lysate was centrifuged at 1,400 × g for 5 min. The proteins contained in the supernatant were submitted to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated blocking buffer [PBS 1X with 5% (w/v) nonfat dried milk and 0.1% (v/v) Tween 20] for 2 h, and then successively with anti-rFBA polyclonal antibodies (1:1000) and with the anti-mouse immunoglobulin G (IgG) coupled to alkaline phosphatase (Sigma Aldrich). The reactions were developed with BCIP-NBT.
incubated with plasminogen (50 μg) and tPA (50 ng) for 1 h at 36°C. After that, the yeast cells were washed three times in PBS 1X and incubated with the macrophages.

At the end of the infection, the adhered macrophages were washed, lysed by addition of distilled water and centrifuged. The pellet was diluted 1:10 and plated in solid BHI medium supplemented with inactivated fetal calf serum (4% v/v). After 7 days at 37°C the number of CFU’s was counted.

Statistical analysis

The experiments were performed in triplicate, with samples in triplicates. Results are presented as means ± standard deviations. Statistical comparisons were performed using Student’s t test and the statistical significance was accepted for P value of < 0.05.

Authors’ contributions

EGAC participated in the design of the study, participated actively in all experiments and drafted the manuscript. SSP obtained protein samples, assisted in the production of 2-DE gels, cooperated in the identification and in silico analysis of proteins. SBN performed the immunoelectron microscopy assay. LAP was responsible for the production of bacteria clones expressing the pFBA and the production of polyclonal antibodies. CLB and AMB helped in the preparation of the figures of the article, in analysis and suggestions during the course of the experiments and critically revised the manuscript. CMAS designed the experiments, provided guidance during all parts of the work, and wrote the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

The authors wish to thank the funding agencies: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG) by financial support.

Author details

1. Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, ICBII, Campus II, Universidade Federal de Goiás, 74001-970 Goiânia, Goiás, Brazil.
2. Laboratório de Microscopia, Departamento de Biologia Celular, Instituto de Ciências Biológicas, Universidade de Brasília, Brasília, Distrito Federal, Brazil.

Received: 19 August 2014 Accepted: 18 February 2015

Published online: 27 February 2015

References

1. Matute DR, Sepulveda VE, Quesada LM, Goldman GH, Taylor JW, Restrepo A, et al. Microsatellite analysis of three phylogenetic species of Paracoccidioides brasiliensis. J Clin Microbiol. 2006;44(6):2153–7.
2. Carrero LL, Nino-Vega G, Teixeira MM, Carvalho MJ, Soares CMA, Pereira M, et al. New Paracoccidioides brasiliensis isolates reveals unexpected genomic variability in this human pathogen. Fungal Genet Biol. 2008;45(5):605–12.
3. Teixeira MM, Theodoros RC, de Carvalho MJ, Fernandes L, Paes HC, Hahn RC, et al. Phylogenetic analysis reveals a high level of speciation in the Paracoccidioides genus. Mol Phylogenet Evol. 2005;2(2):273–83.
4. San-Bas G, Nino-Vega G, Itumia T. Paracoccidioides brasiliensis and para-coccidioidomycosis: molecular approaches to morphogenesis, diagnosis, epidemiology, taxonomy and genetics. Med Mycol. 2002;40(3):225–42.
5. Shikhamai- Yasuda MA, Telles Filho Fde Q, Mendes RP, Colombo AL, Moretto ML. Guidelines in paracoccidioidomycosis. Rev Soc Bras Med Trop. 2006;39(3):297–310.
6. Prado M, Silva MB, Laurenti R, Travassos LR, Taborda CP. Mortality due to systemic mycoses as a primary cause of death or in association with AIDS in Brazil: a review from 1996 to 2006. Mem Inst Oswaldo Cruz. 2009;104(3):513–21.
7. Westerlund B, Korhonen TK. Bacterial proteins binding to the mammalian extracellular matrix. Mol Microbiol. 1993;9(4):687–94.
8. Mendes-Giannini MJ, Taylor ML, Bouchara JB, Burger E, Calich VL, Escalante ED, et al. Pathogenesis II: fungal responses to host responses: interaction of host organs with fungi. Med Mycol. 2000;38 Suppl 1:113–23.
9. Fumikazu U, Siebert-Gulle K, Rossgrenert R, Soostak MF. Factors influencing the cell adhesion and invasion capacity of Mycoplasma gallisepticum. Acta Vet Scand. 2013;55:63.
10. Nogueira SV, Fonseca FL, Rodrigues ML, Mundodí V, Abi-Chacra EA, Winters MS, et al. Paracoccidioides brasiliensis enolase is a surface protein that binds plasminogen and mediates interaction of yeast forms with host cells. Infect Immun. 2010;78(9):4040–50.
11. Nogueira SV, Smith AA, Qin JH, Pal U. A surface enolase participates in Borella burgdorferi-plasminogen interaction and contributes to pathogen survival within feeding ticks. Infect Immun. 2012;80(1):82–90.
12. Nogueira SV, Backstedt BT, Smith AA, Qin JH, Wunder Jr EA, Ko A, et al. Leptospirosis interrogans enolase is secreted extracellularly and interacts with plasminogen. PLoS One. 2013;8(10):e78150.
13. Agarwal V, Kuchipudi A, Fulke M, Riesbeck K, Bergmann S, Blom AM. Streptococcus pneumoniae endopeptidase O (PePO) is a multifunctional plasminogen- and fibronectin-binding protein, facilitating evasion of innate immunity and invasion of host cells. J Biol Chem. 2013;288(10):6949–63.
14. Magalhaes VA, Andrade EB, Alves J, Ribeiro A, Kim KS, Lima M, et al. Group B Streptococcus hijacks the host plasminogen system to promote brain endothelial cell invasion. PLoS One. 2013;8(5):e63244.
15. Ste J, Bruni G, Fox D. Surface-associated plasminogen binding of Cryptococcus neoformans promotes extracellular matrix invasion. PLoS One. 2009;4(6):e5780.
16. Castellino FJ, Ploplis VA. Structure and function of the plasminogen/plasmin system. Thromb Haemost. 2005;93(6):567–54.
17. Bhattacharya S, Ploplis VA, Castellino FJ. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. J Biomed Biotechnol. 2012;2012:482006.
18. Chapman HA. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr Opin Cell Biol. 1997;9(5):714–24.
19. Flow EF, Herren T, Redzic A, Miles LA, Hoover-Plow JL. The cell biology of the plasminogen system. PASEB J. 1995;9(10):339–45.
20. Gonzalez A, Gomez BL, Munoz C, Arizabalo BH, Restrepo A, Hamilton AI, et al. Involvement of extracellular matrix proteins in the course of experimental paracoccidioidomycosis. FEMS Immunol Med Microbiol. 2008;53(1):114–25.
21. Lähteenmäki K, Edelman S, Korhonen TK. Bacterial metastasis: the host plasminogen system in bacterial invasion. Trends Microbiol. 2005;13(2):79–85.
22. Geiger B, Yamada KM. Molecular architecture and function of matrix adhesions. Cold Spring Harb Perspect Biol 2011, 3(5).
23. Önder O, Humphrey PT, McOmber B, Korobova F, Francella N, Greenbaum DC, et al. OsipC is potent plasminogen receptor on surface of Borella burgdorferi. J Biol Chem. 2012;287(20):16860–8.
24. Vieira ML, Atzingen MV, Oliveira R, Mendes RS, Domingos RF, Vasconcellos SA, et al. Plasminogen binding proteins and plasmin generation on the surface of Leptospira spp. the contribution to the bacteria-host interactions. J Biomed Biotechnol. 2012;2012:758513.
25. Barthel D, Singh B, Riesbeck K, Zipfel PF. Haemophilus influenzae uses the surface protein E to acquire human plasminogen and to evade innate immunity. J Immunol. 2012;188(1):379–85.
26. Ferreira Ede O, Teixeira FL, Cordeiro F, Araujo Lobo L, Rocha ER, Smith JC, et al. The Bp60 surface adhesion is an extracellular matrix and plasminogen protein interacting in Bacteroides fragilis. Int J Med Microbiol. 2013;303(8):492–7.
27. Toledo A, Coleman JL, Kuhlow CJ, Crowley JT, Benach JL. The envelope of Borella burgdorferi is a plasminogen receptor released in outer membrane vesicles. Infect Immun. 2013;81(1):359–68.
28. Koenigs A, Hammerschmidt C, Jutras BL, Pogoryelov D, Barthel D, Smith JC, et al. The BBA70 of Borella burgdorferi is a novel plasminogen-binding protein. J Biol Chem. 2013;288(35):25229–43.
29. Furuya H, Ikeda R. Interaction of triosephosphate isomerase from Staphylococcus aureus with plasminogen. Microbiol Immunol. 2011;55(12):855–62.
30. Domingos RF, Vieira ML, Romero EC, Gonçalves AP, de Moraes ZM, Vasconcellos SA, et al. Features of two proteins of Leptospira interrogans with potential role in host-pathogen interactions. BMC Microbiol. 2012;12:250.

31. Fernandes LG, Vieira ML, Krichgau K, Alves U, de Moraes ZM, Vasconcellos SA, et al. Ompl1 is an extracellular matrix- and plasminogen-interacting protein of Leptospira spp. Infect Immun. 2012;80(10):3679–92.

32. Souza NM, Vieira ML, Alves U, de Moraes ZM, Vasconcellos SA, Nascimento AL. Lsa30, a novel adhesin of Leptospira interrogans binds human plasminogen and the complement regulator C4bp. Microb Pathog. 2012;53(3):4125–34.

33. Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, Demoll E, et al. Leptospira interrogans endostatin-like outer membrane proteins bind host fibrinectin, laminin and regulators of complement. PLoS One. 2007;2(11):e1188.

34. Bergmann S, Schoenen H, Hammerschmidt S. The interaction between bacterial enolase and plasminogen promotes adherence of Streptococcus pneumoniae to epithelial and endothelial cells. Int J Med Microbiol. 2013;303(8):452–62.

35. Lahteenmaki K, Kuusela P, Korhonen TK. Bacterial plasminogen activators and receptors. FEMS Microbiol Rev. 2001;25(5):531–52.

36. Fulde M, Steiner M, Bergmann S. Interaction of streptococcal plasminogen binding proteins with the host fibrinolytic system. Front Cell Infect Microbiol. 2013;3:85.

37. Salazar N, Cantiblanco-Valencia MM, da Silva LB, de Castro I, Monaris D, Masuda HP, et al. Staphylococcus aureus manganese transport protein C (MntC) is an extracellular matrix- and plasminogen-binding protein. PLoS One. 2014;9(11):e112730.

38. Colberg J, Aase A, Bergmann S, Herstad TK, Rodal G, Frank R, et al. Streptococcus pneumoniae enolase is important for plasminogen binding despite low abundance of enolase protein on the bacterial cell surface. Microbiology. 2006;152(Pt 5):1307–17.

39. Floden AM, Watt JA, Brissette CA. Borelia burgdorferi enolase is a surface-exposed plasminogen binding protein. PLoS One. 2011;6(11):e27502.

40. Crowe JD, Slewrick IK, Auld GC, Moore NR, Gov N, Booth NA. Candida albicans binds human plasminogen: identification of eight plasminogen-binding proteins. Mol Microbiol. 2003;47(6):1637–51.

41. Poltermann S, Kunert A, von der Heide M, Eck R, Hartmann A, Zipfel PF. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase of Candida albicans is a surface antigen. J Bacteriol. 1997;179(16):4992–9.

42. Gozalbo D, Gil Navarro J, Gil ML, Casanova M, O’Connor JE, Martinez JP, Gozalbo D. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase of Candida albicans is a surface plasminogen receptor. FEMS Yeast Res. 2012;12(5):557–70.

43. Boche TJ, Tynrell GJ. Identification of the plasminogen and plasmin binding regions of group B streptococcal phosphoglycerate kinase. J Biol Chem. 2002;277(52):37557–64.

44. B好不好Im, Hartmann A, Schmaler J, Gehrke A, Brakhage AA, Zipfel PF. The moonlighting protein fructose-1,6-bisphosphate aldolase, an essential enzyme produced by Candida albicans: isolation and identification as a fibrinectin-binding protein. Microb Pathog. 2004;37(5):321–31.

45. Marcos CM, de Fatima da Silva J, Alves BH, da Silva RA M, Oncini I, Conde JPB, et al. Identification of the actin and plasminogen binding domains of the fungal glyceraldehyde-3-phosphate dehydrogenase. Mol Microbiol. 2013;89(4):1060–71.

46. Bailao AM, Nogueira SV, Rondon Caixeta Bonfim SM, de Castro KP, de Oliveira HC, da Silva RA M, et al. Detection of a homotetrameric structure and protein-protein interactions of the pathogen Leptospira interrogans. PLoS One. 2010;5(6):e11259.

47. Fulde M, Bernardo-Garcia N, Rohde M, Nachtrigel N, Frank R, Preissner KT, et al. Pneumococcal phosphoglycerate kinase interacts with plasminogen and its tissue activator. Thromb Haemost. 2014;111(3):401–16.
71. Pitarch A, Sanchez M, Nombela C, Gil C. Sequential fractionation and two-dimensional gel analysis unravels the complexity of the dimorphic fungus *Candida albicans* cell wall proteome. Mol Cell Proteomics. 2002;1(12):967–82.
72. Pitarch A, Nombela C, Gil C. Cell wall fractionation for yeast and fungal proteomics. Methods Mol Biol. 2008;425:217–39.
73. Kapteyn JC, Van Den Ende H, Klis FM. The contribution of cell wall proteins to the organization of the yeast cell wall. Biochim Biophys Acta. 1999;1426(2):373–83.
74. de Groot PW, de Boer AD, Cunningham J, Dekker HL, de Jong I, Hellingwerf KJ, et al. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. Eukaryot Cell. 2004;3(4):655–65.
75. Eddy JL, Gieda LM, Caulfield AJ, Rangel SM, Lathem WW. Production of outer membrane vesicles by the plague pathogen *Yersinia pestis*. PLoS One. 2014;9(9):e107002.
76. Pereira LA, Bao SN, Barbosa MS, da Silva JL, Felipe MS, de Santana JM, et al. Analysis of the *Paracoccidioides brasiliensis* triosephosphate isomerase suggests the potential for adhesion function. FEMS Yeast Res. 2007;7(3):1381–8.
77. Parente AF, Bailao AM, Borges CL, Parente JA, Magalhaes AD, Ricart CA, et al. Proteomic analysis reveals that iron availability alters the metabolic status of the pathogenic fungus *Paracoccidioides brasiliensis*. PLoS One. 2011;6(7):e22810.
78. da Silva CN, Barbosa MS, Maia ZA, Bao SN, Felipe MS, Santana JM, et al. Characterization of *Paracoccidioides brasiliensis* PbDfg5p, a cell-wall protein implicated in filamentous growth. Yeast. 2008;25(2):141–54.
79. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
80. Rezende TC, Borges CL, Magalhaes AD, de Sousa MV, Ricart CA, Bailao AM, et al. A quantitative view of the morphological phases of *Paracoccidioides brasiliensis* using proteomics. J Proteomics. 2011;75(2):572–87.
81. Castro N da S, de Castro KP, Orlandi I, Feitosa Ldos S, Silva LK R e, Vainstein MH, et al. Characterization and functional analysis of the beta-1,3-glucanosyltransferase 3 of the human pathogenic fungus *Paracoccidioides brasiliensis*. FEMS Yeast Res. 2009;9(1):103–14.
82. de Sousa LP, Bailao EF, Silva MG, Castro Nida S, Bao SN, Orlandi I, et al. Characterization of the *Paracoccidioides* beta-1,3-glucanosyltransferase family. FEMS Yeast Res. 2012;12(6):685–702.