UNIVERSIDADE FEDERAL DE GOIÁS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA TROPICAL E SAÚDE PÚBLICA

Edilânia Gomes Araújo Chaves

IDENTIFICAÇÃO E ANÁLISE DE PROTEÍNAS LIGANTES DE PLASMINOGÊNIO DE *Paracoccidioides*

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Medicina Tropical e Saúde Pública da Universidade Federal de Goiás para obtenção do Título de Mestre em Medicina Tropical e Saúde Pública, área de concentração Microbiologia.

Orientadora: Profª. Drª. Célia Maria de Almeida Soares

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ATA DA REUNIÃO DA BANCA EXAMINADORA DA DEFESA DE DISSERTAÇÃO DE EDILÂNIA GOMES ARAÚJO CHAVES - Aos vinte dias do mês de março do ano de 2013 (20/03/2013), às 14:00 horas, reuniram-se os componentes da Banca Examinadora: Profs. Drs. CÉLIA MARIA DE ALMEIDA SOARES, ANDRÉ CORREA AMARAL e ALEXANDRE MELO BAILÃO, para, sob a presidência da primeira, e em sessão pública realizada no INSTITUTO DE PATOLOGIA TROPICAL E SAÚDE PÚBLICA, procederem à avaliação da defesa de dissertação intitulada: **"IDENTIFICAÇÃO E CARACTERIZAÇÃO DE PROTEÍNAS LIGANTES DE PLASMINOGÊNIO EM *Paracoccidioides*"**, em nível de **MESTRADO**, área de concentração em **MICROBIOLOGIA**, de autoria de **EDILÂNIA GOMES ARAÚJO CHAVES,** discente do PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA TROPICAL E SAÚDE PÚBLICA, da Universidade Federal de Goiás. A sessão foi aberta pela Orientadora, Profa. Dra. CÉLIA MARIA DE ALMEIDA SOARES, que fez a apresentação formal dos membros da Banca e orientou a Candidata sobre como utilizar o tempo durante a apresentação de seu trabalho. A palavra a seguir, foi concedida ao autor da tese que, em 30 minutos procedeu à apresentação de seu trabalho. Terminada a apresentação, cada membro da Banca argüiu a Candidata, tendo-se adotado o sistema de diálogo seqüencial. Terminada a fase de argüição, procedeu-se à avaliação da defesa. Tendo-se em vista o que consta na Resolução nº. 1081/2012 do Conselho de Ensino, Pesquisa, Extensão e Cultura (CEPEC), que regulamenta o Programa de Pós-Graduação em Medicina Tropical e Saúde Pública a Banca, em sessão secreta, expressou seu Julgamento, considerando a candidata **Aprovada** ou **Reprovada**:

Banca Examinadora	**Aprovada / Reprovada**
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Data: 20/03/2013

“Ela acreditava em anjos. E, porque acreditava, eles existiam.”
A Hora da Estrela – Clarice Lispector

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SUMÁRIO

SÍMBOLOS, SIGLAS E ABREVIATURAS

°C: graus Celsius

%: porcentagem

BPBP: proteína ligante de plasminogênio de *Borrelia burgdorferi*

cDNA: ácido desoxiribonucleico complementar

CRASP-1: regulador complementar da proteína de superfície 1

ErpA: proteína extracelular A

ErpC: proteína extracelular C

ErpP: proteína extracelular P

FBA: frutose 1,6-bifosfato aldolase

GAPDH: gliceraldeído-3-fosfato desidrogenase

GP43: glicoproteína de 43 kilodaltons

Gpm1p: fosfoglicerato mutase 1

kDa: kilodalton

LenA: endostatina A de *Leptospira sp.*

Lsa20: adesina de superfície de 20 kDa de *Leptospira sp*.

Lsa30: adesina de superfície de 30 kDa de *Leptospira sp*.

MEC: matriz extracelular

Mhp182(P102): proteína de 182 kDa ligante de plasminogênio de *Mycoplasma hyopneumoniae*

OmpA: proteína A de superfície externa de *Leptospira sp*.

OmpL: proteína L de superfície externa de *Leptospira sp*.

OspA: proteína A de superfície externa

OspC: Proteína C de superfície externa

PavB: fator de aderência e virulência B de *Streptococcus pneumoniae*

***Pb*01:** isolado 01 de *Paracoccidioides lutzii*

***Pb18*:** isolado 18 de *Paracoccidioides brasiliensis*

***Pb*DFG5p:** proteína deficiente para o crescimento filamentoso de *Paracoccidioides*

Pbfba1: fructose 1,6-bifosfato aldolase 1 de *Paracoccidioides*

Pbfba2: fructose 1,6-bifosfato aldolase 2 de *Paracoccidioides*

***Pb*Had320:** hidrolase de aderência de 32 kDa de *Paracoccidioides*

PCM: paracoccidioidomicose

pH: potencial hidrogeniônico

Pla: proteína ativadora de plasminogênio

Plg: plasminogênio

Pra1: antígeno regulador de pH 1

PS2: espécie filogenética 2

PS3: espécie filogenética 3

rFBA: frutose 1,6-bifosfato aldolase recombinante

S1: espécie 1

TPI: triose fosfato isomerase

tPA: ativador de plasminogênio tecidual

uPA: ativador de plasminogênio urokinase

RESUMO

Paracoccicoidioides é o agente etiológico da paracoccidioidomicose (PCM), uma doença considerada como primeira causa de mortalidade entre as micoses sistêmicas no Brasil. O sucesso no estabelecimento da infeção está relacionado com a capacidade do fungo de aderir e degradar componentes da matriz extracelular (MEC). O plasminogênio (Plg) é uma proteína presente no plasma, a qual possui atividade fibrinolítica quando é ativado em plasmina. Muitos micro-organismos patogênicos são capazes de subverter o sistema plasminogênio/plasmina através de moléculas ligantes e promovem a degradação de barreiras teciduais. Neste trabalho foram identificadas, através de Far-Western e análise proteômica, um total de 15 proteínas secretadas por *Paracoccidioides* com habilidade de ligação ao plasminogênio. Essas proteínas são prováveis alvos da interação do patógeno com o hospedeiro, que contribui para o potencial invasivo do fungo. A frutose 1,6-bifosfato aldolase (FBA) foi descrita em outros organismos como ligante de plasminogênio e possui participação na adesão de *Paracoccidioides* às células do hospedeiro. Selecionamos esta proteína para ensaios de validação e foi observada sua presença na superfície e em vesículas secretoras do fungo. Confirmamos a capacidade da FBA de converter plasminogênio em plasmina na presença do ativador tecidual de plasminogênio (tPA) e essa interação promoveu a degradação de fibrina. Em ensaios de infecção, a adição de anticorpos que bloqueiam o sítio de ligação da FBA reduziu a interação do fungo com macrófagos e a interação de FBA com Plg aumentou o índice de invasão celular. Esses dados sugerem que a FBA pode contribuir no processo de adesão, invasão e disseminação do fungo.

ABSTRACT

*Paracoccicoidioide*s is the etiological agent of paracoccidioidomycosis (PCM), a disease considered one of the main causes of mortality among systemic mycoses in Brazil. The success in establishing of the infection is related with the ability of fungus to adhere and degrade components of the extracellular matrix (ECM). The plasminogen (Plg) is a protein of blood plasma of the host that presents fibrinolytic activity when activated into plasmin. Many pathogens are able to subvert the plasminogen/plasmin system using linker molecules and promote the degradation of tissue barriers. In this work, we identified through Far Western and proteomic analysis, a total of 15 proteins secreted of *Paracoccidioides* that are plasminogen binding proteins. Those proteins are probable targets of the interaction of the fungus with the host and could contribute to the invasiveness of the fungus. The fructose 1,6-biphosphate aldolase was described in other organisms such as plasminogen binder and presentes participation in the adherence of *Paracoccidioides* to host cells. This protein selected for validation tests and their presence was observed on the surface and secretory vesicles of the fungus. FBA confirm the ability to convert plasminogen to plasmin in the presence of tissue plasminogen activator (tPA) and this interaction promoted the degradation of fibrin. In infection assays, the addition of antibodies blocking the FBA binding site reduced the interaction of the fungus with macrophages and the interaction of FBA with Plg increased the rate of cell invasion. These data suggest that the FBA can contribute to adhesion, invasion and spread process of the fungus.

1. INTRODUÇÃO

1.1. *Paracoccidioides* e paracoccidioidomicose

O gênero *Paracoccidioides* compreende um complexo de, no mínimo, quatro espécies filogenéticas. Análises de diferentes isolados indicam que o gênero se divide em duas espécies: *Paracoccidioides brasiliensis* que compreende um complexo de, no mínimo, três grupos filogenéticos (S1, PS2, PS3) e *Paracoccidioides lutzii*, que compreende o isolado *Pb*01 (MATUTE et al, 2006; CARRERO et al, 2008; TEIXEIRA et al, 2009).

Membros do complexo *Paracoccidioides* são fungos termodimórficos que alternam entre as fases miceliana e leveduriforme de acordo com a temperatura (BRUMMER et al, 1993). O fungo é encontrado geralmente em solos, à temperatura ambiente, na forma de micélio caracterizado por hifas septadas e multinucleadas, a qual produz conídios infectantes. Caso os conídios sejam inalados, encontram nos pulmões do hospedeiro, condições que induzem a sua diferenciação em leveduras, como a temperatura de 36 °C (CAMARGO & TABORDA, 1993; SAN-BLAS, NINO-VEJA, ITURRIAGA, 2002). A fase leveduriforme apresenta múltiplos brotamentos formados por evaginações da célula mãe ganhando o aspecto de roda de leme de navio, uma de suas principais características microscópicas. A transição da forma infectante para a forma parasitária depende predominantemente da temperatura, mas necessita de várias outras mudanças moleculares, e somente isolados capazes de transitar para fase leveduriforme, conseguem estabelecer a infecção (SAN-BLAS et al, 2002).

Paracoccidioides é o agente etiológico da paracoccidioidomicose (PCM), uma micose sistêmica granulomatosa que foi descrita pela primeira vez em 1908 por Adolf Lutz. A inalação de propágulos do micélio geralmente ocorre durante a infância, no entanto o fungo pode passar por um longo período de latência. Assim, muitos pacientes infectados quando crianças, só apresentam sinais e sintomas da doença quando alcançam a idade adulta. Esta forma da PCM, classificada como fase crônica, é caracterizada por uma infecção sistêmica granulomatosa que atinge primeiramente os pulmões, podendo disseminar para outros órgãos e tecidos como fígado, baço e sistema nervoso central, via hematogênica e/ou linfática. Em casos de infecção aguda, a forma mais grave da doença, o paciente apresenta hipertrofia dos órgãos do sistema reticuloendotelial, disfunção da medula óssea, manifestações digestivas, hepatoesplenomegalia e lesões cutâneas, sendo potencialmente letal (LONDERO, 1986;

BRUMMER et al, 1993; BORGES-WALMSLEY et al, 2002; SHIKANAI-YASUDA et al, 2006).

A PCM é uma doença predominante em regiões tropicais e subtropicais, tendo alta prevalência nos países da América do Sul. Dados apontam uma distribuição que se estende desde o México até a Argentina destacando-se Brasil, Colômbia e Venezuela (FRANCO, 1987; SAN-BLAS et al, 2002; SHIKANAI-YASUDA et al, 2006). A PCM é a décima causa de mortalidade por doença crônica, entre as infecciosas e parasitárias, e a primeira entre as micoses sistêmicas no Brasil (PRADO et al, 2009). Acomete principalmente a população rural, provavelmente por ser um fungo saprobiótico. A forma aguda abrange cerca de 3 a 5% dos casos e acomete jovens de ambos os sexos (SAN-BLAS & NINO-VEGA, 2002). No entanto, a grande maioria dos indivíduos infectados são adultos do sexo masculino, numa proporção de 48 homens para cada mulher, provavelmente devido à ação do hormônio feminino β-estradiol. Foi observado que este hormônio inibiu a transição de micélio para fase leveduriforme de maneira dose dependente e favoreceu a resistência de fêmeas de rato no desenvolvimento inicial da PCM (RESTREPO et al, 1985; SANO et al, 1999; ARISTIZÁBAL et al, 2002; SHANKAR et al, 2011).

1.2. Participação de adesinas na relação parasito-hospedeiro

Para que os micro-organismos consigam estabelecer determinada infecção, inclusive a PCM, são necessários eventos cruciais para o processo de invasão, colonização e crescimento do patógeno no hospedeiro. A adesão do microorganismo é um dos eventos iniciais da infecção e ocorre através do reconhecimento de moléculas do hospedeiro (PATTI et al, 1994). A interação com componentes da matriz extracelular (MEC) tem sido correlacionada com a habilidade de adesão e invasão de diferentes organismos (WESTERLUND & KORHONEN, 1993; GIL et al, 1996; WASYLNKA & MOORE, 2000; LIMA et al, 2001; SINGH et al 2012). A MEC é formada por compostos como colágeno, laminina, fibronectina, vibronectina e proteoglicanos que participam da regulamentação de processos fisiológicos como migração e sinalização celular e transporte de solutos através das barreiras celulares (HYNES, 2009). A MEC circunda células epiteliais, endoteliais, tecido conjuntivo, estando presentes em diversos órgãos e tecidos do hospedeiro humano. O trato respiratório, foco primário da PCM, possui tecidos ricos em laminina e vários tipos de colágeno. Nos pulmões encontram-se fibroblastos alveolares que secretam diversos componentes da matriz extracelular, o que

favorece o processo de reconhecimento e interação do fungo aos tecidos do hospedeiro (AMENTA et al, 1988; DAVIDSON, 1990; HERZOG et al, 2008; SCHWARTZ, 2010; GEIGER & YAMADA, 2011).

Estudos sugerem que o processo de adesão e invasão da MEC é mediado por adesinas, que são proteínas de superfície dos patógenos que contribuem para o processo de ligação às moléculas do hospedeiro (PATTI et al, 1994; GOZALBO et al, 1998; VANLAERE & LIBERT, 2009; RAMANA & GUPTA, 2010). Algumas proteases secretadas por micro-organismos são citadas como adesinas que promovem a degradação da MEC exercendo também um papel importante na relação parasito-hospedeiro (MONOD et al, 1991; LEE & KOLATTUKUDY, 1995; GIL et al, 1996; DOS SANTOS & SOARES, 2005). Muitos estudos descrevem fungos patogênicos que apresentam proteínas de superfície ou secretadas que se ligam aos componentes da MEC. Podemos citar: *Aspergillus fumigatus* (MONOD et al, 1991; TRONCHIN et al, 1993; COULOT et al, 1994; LEE & KOLATTUKUDY, 1995; GIL et al, 1996; WASYLNKA & MOORE, 2000); espécies de *Candida* (LOPEZ-RIBOT et al, 1996; GAUR et al, 1999; DOS SANTOS & SOARES, 2005; PARNANEN et al, 2009; PORTELA et al, 2010), *Cryptococcus neoformans* (RODRIGUES et al, 2003), *Histoplasma capsulatum* (MCMAHON et al, 1995), *Pneumocystis carinii* (NARASIMHAN et al, 1994), *Penicillium marneffei* (HAMILTON et al, 1998, 1999), *Sporothrix schenckii* (LIMA et al, 2001) e *Trichophyton schoenleinii* (IBRAHIM-GRANET et al, 1996). Adesinas com esta habilidade também são descritas em bactérias como *Clostridium histolyticum* (SHI et al, 2010; PRUTEANU et al, 2011), *Porphyromonas gingivalis* (BABA et al, 2001; OLCZAK et al, 2001), espécies de *Pseudomonas* (DE BENTZMANN et al, 2000; SCHMIDTCHEN et al, 2003; MATSUMOTO 2004) e *Treponema denticola* (GRENIER et al, 1990; ISHIHARA, 2010).

Em *Paracoccidioides* são descritas diversas proteínas que interagem com componentes do hospedeiro favorecendo o processo infeccioso. Uma proteína de 30 kDa foi descrita como adesina devido a sua capacidade de aderir a componentes da MEC e invadir células Vero (MENDES-GIANNINI et al, 1994; HANNA et al, 2000; MONTEIRO et al, 2001; ANDREOTTI et al, 2005). Uma glicoproteína de 43 kDa (gp43) presente na superfície de *Paracoccidioides* atua como molécula potencialmente mediadora da ligação do fungo à laminina e participa da invasão e inibição de macrófagos (VICENTINI et al, 1994; ALMEIDA et al, 1998; POPI et al, 2002;

KONNO et al, 2009; 2012). A proteína gliceraldeido-3-fosfato desidrogenase (GAPDH) e a triose fosfato isomerase (TPI) do fungo são capazes de ligar à laminina, colágeno I e fibronectina (BARBOSA et al, 2006; PEREIRA et al, 2007). *Paracoccidioides* apresenta ainda duas proteínas de superfície, uma de 32 kDa e outra 19 kDa, que interagem com a laminina, fibronectina e fibrinogênio (GONZÁLEZ et al, 2005, 2008). A proteína de 32 kDa também foi descrita em outros trabalhos como uma hidrolase de aderência (*Pb*Had320) que promove um aumento no potencial do fungo em invadir células do hospedeiro (HERNANDEZ et al, 2010). Castro e colaboradores (2008) descrevem uma proteína defeituosa para o crescimento filamentoso (*Pb*DFG5p) que está presente na parede de *Paracoccidioides* e que também pode se ligar a componentes da MEC, podendo ser relevante para as etapas iniciais que conduzem a fixação e colonização do fungo nos tecidos do hospedeiro. A malato sintase também foi caracterizada como adesina por mediar adesão do fungo aos tecidos hospedeiros e a disseminação da infecção (NETO et al, 2009).

Como descrito, muitos agentes patogênicos tem a capacidade de se ligar aos componentes da MEC, o que potencializa o processo de adesão e o rompimento das barreiras celulares (GONZÁLEZ et al, 2008; OSHEROV, 2012; SINGH et al, 2012). Assim diversos patógenos, incluindo o *Paracoccidioides*, conseguem penetrar na superfície muco cutânea e parasitar células evadindo-se da resposta imune celular do hospedeiro e alcançando tecidos mais profundos (WESTERLUND & KORHONEN 1993; MENDES-GIANNINI et al, 2000). Embora *Paracoccidioides* não seja considerado um patógeno tipicamente intracelular, estudos têm demonstrado que células leveduriformes do fungo são capazes de aderir, invadir e se multiplicar no interior de células do hospedeiro (MOSCARDI-BACCHI et al, 1994; VICENTINI et al, 1994; HANNA et al, 2000; MONTEIRO et al, 2001; MENDES- GIANNINI et al, 2004, 2008; ANDREOTTI et al, 2005; BARBOSA et al, 2006; PEREIRA et al, 2007; DONOFRIO et al, 2009). Além disso, a PCM apresenta diferentes manifestações clínicas, variando de benigna e localizada para forma grave e disseminada, a qual atinge outros órgãos além do pulmão (RESTREPO & TOBON, 2005; MARTINEZ et al, 2006; SHIKANAI-YASUDA et al, 2006). Essas evidências destacam o potencial de adesão, invasão e disseminação do fungo, o que pode estar relacionado com a produção de enzimas, adesinas e proteases que facilitam o estabelecimento da infecção.

1.3. Sistema plasminogênio-plasmina

Alguns micro-organismos são capazes de degradar a MEC através da produção ou "sequestro" de proteases do hospedeiro (SINGH et al, 2012). O plasminogênio (Plg) é exemplo de protease do hospedeiro que possui atividade fibrinolítica e diversos patógenos tem seu potencial de invasão e disseminação aumentado através da ligação essa molécula (LAHTEENMAKI et al, 1995; COLEMAN & BENACH, 1999; BERGMANN & HAMMERSCHMIDT, 2007; BHATTACHARYA et al, 2012; NOMURA, 2012; SINGH et al, 2012).

O plasminogênio é uma pró-enzima de cadeia única com uma massa molecular de 92 kDa, abundante no plasma e em fluidos extracelulares. A ligação do Plg às superfícies de patógenos é mediada por cinco domínios, denominados kringle, que possuem afinidade por resíduos de lisina (VASSALLI et al, 1991; PLOW et al, 1995; MUNDODI et al, 2008). Em eucariotos o plasminogênio é convertido em sua forma proteolítica, a plasmina, através de ativadores fisiológicos como o ativador de plasminogênio tipo tecidual (tPA) e tipo urokinase (uPA) (CASTELLINO & PLOPLIS, 2005). Ambos os ativadores hidrolisam uma única ligação peptídica (Arg560-Val561) resultando na formação de plasmina. A plasmina dissocia coágulos sanguíneos em função de seu papel na degradação de polímeros de fibrina, sendo capaz de dissociar componentes da MEC (CHAPMAN, 1997; FRANCO, 2001; LAHTEENMAKI et al, 2005).

Estudos demonstram que alguns patógenos têm seu potencial de invasão aumentado na presença de plasmina (LAHTEENMAKI et al, 1995; ITZEK et al, 2010; BARTHEL et al, 2012). Muitos micro-organismos patogênicos são capazes de subverter plasminogênio do hospedeiro através de receptores encontrados em sua superfície e por moléculas secretadas para o meio externo. O plasminogênio ligado à superfície do patógeno pode ser ativado pelos ativadores de Plg do hospedeiro ou por proteínas produzidas pelo próprio micro-organismo, formando sobre ele um envoltório protéico com atividade fibrinolítica. As proteínas secretadas pelos agentes patogênicos, que são capazes de converter plasminogênio em plasmina, também abrem caminho para a disseminação desses micro-organismos (KORHONEN et al, 1997; STIE et al, 2009; BHATTACHARYA et al, 2012; FIGUERA et al, 2012; TOLEDO et al, 2012). Assim, o potencial fibrinolítico do sistema plasminogênio-plasmina, que o torna uma via central para a manutenção da hemostasia, pode, em contrapartida, ser utilizado para a migração de micro-organismos facilitando sua penetração através das barreiras protéicas, o que

constitui um aspecto importante durante vários processos infecciosos (VASSALLI et al, 1991; PLOW et al, 1995; WALKER et al, 2005).

1.4. Proteínas ligantes de plasminogênio

As adesinas produzidas pelos micro-organismos podem reconhecer diversas moléculas do hospedeiro, incluindo o plasminogênio humano (PATTI et al, 1994; GOZALBO et al, 1998; RAMANA & GUPTA, 2010). Proteínas ligantes de plasminogênio são intensamente investigadas quanto a sua participação na disseminação de microorganismos durante as infecções. Em bactérias elas contribuem na atividade fibrinolítica, favorecendo a degradação do tecido e a rápida progressão da doença (MALKE et al,1994; GRENIER, 1996; PANCHOLI & FISCHETTI, 1998; BERGMANN et al, 2001; LAHTEENMAKI, 2001b; CARNEIRO et al, 2004; DERBISE et al, 2004; KOLBERG et al, 2006; FLODEN et al, 2011). Estudos desenvolvidos com *Borrelia burgdorferi* descrevem a presença de proteínas ligantes de plasminogênio na superfície e em membranas de vesículas secretadas pela bactéria, a exemplo as proteínas de superfície - "outer surfasse protein" A (OspA) e C (OspC), proteínas extracelulares P, A e C (ErpP, ErpA e ErpC), uma proteína de ligação ao plasminogênio de 70 kDa (BPBP), um regulador complementar da proteína de superfície 1 - "complement regulator acquiring surfasse protein 1" (CRASP-1) e enolase (FUCHS et al 1996; HU et al, 1997; BRISSETTE et al, 2009; HALLSTROM et al, 2010; ONDER et al, 2012; TOLEDO et al, 2012). Tais proteínas permitem o recrutamento de plasminogênio na superfície da bactéria e a degradação de tecidos, componentes da matriz extracelular e membrana basal. Experimentos *in vivo* mostram que a ligação da bactéria ao Plg favorece sua disseminação e sobrevivência em carrapatos (vetor) e em camundongos (COLEMAN et al, 1995, 1997, 1999; KLEMPNER et al, 1996; GEBBIA et al, 1999; NORDSTRAND et al, 2001; GRAB et al, 2005; NOGUEIRA et al 2012).

Bactérias do gênero Leptospira possuem algumas proteínas de superfície, como um receptor presente na superfície da bactéria "leptospiral endostatin-like protein A" (LenA), proteínas de superfície - leptospiral surface adhesin 20 (Lsa20) e 30 (Lsa30), proteínas da membrana externa - "outer membrane protein" L (OmpL) e A (OmpA) que atuam como receptores de Plg, favorecendo a formação de plasmina e a degradação de fibronectina (VERMA et al, 2010; VIEIRA et al, 2010; MENDES et al, 2011; OLIVEIRA et al, 2011; SOUSA et al, 2012; FERNANDES et al, 2012). Em *Yersinia*

pestis foi detectada uma proteína ativadora de plasminogenio (Pla), que é capaz de ativar plasminogênio em plasmina (RUBACK et al, 2012). Muitas proteínas bacterianas são descritas como ligantes de plasminogênio, entre elas: estreptoquinase A de *Streptococcus* spp, estafiloquinase de *Staphylococcus aureus*, proteínas ativadoras de plasminogênio *Streptococcus uberis* (PauA e SUPA) (PARRY et al, 2000; LAHTEENMAKI et al, 2001a, 2001b; ZHANG et al, 2012), proteínas da família M que são ligantes de plasminogênio de *Streptococci* dos grupos A, C e G, aspartase de *Haemophilus influenzae* e fímbrias e flagelos de *Escherichia coli* e *Salmonella* (LI et al, 1999; LAHTEENMAKI et al, 2001a, 2001b), um fator de aderência e virulência B de *Streptococcus pneumoniae* (PavB) (JENSCH et al, 2010), proteínas putativas de *Francisella tularensis* (CLINTON et al, 2010), proteína skizzle de *Streptococcus agalactiae* (WILES et al, 2010), endostatin-like proteína A e proteínas putativas, hipotéticas e conservadas de *Leptospira interrogans* (VIEIRA et al, 2010; OLIVEIRA et al, 2011), uma proteína mediadora de internalização de *Streptococcus pyogenes* em queratinócitos (SIEMENS et al, 2011), proteínas de *Streptococcus dysgalactiae* ssp. (BERGMANN et al, 2011), proteína E de *H. influenzae* (BARTHEl et al, 2012) e uma proteína ligante de plasminogênio de *Mycoplasma hyopneumoniae* (Mhp182 (P102)) (SEYMOUR et al, 2012).

Fungos também apresentam proteínas com habilidade de associar-se ao sistema plasminogênio-plasmina. Em *C. albicans* foram detectadas oito proteínas ligantes de plasminogênio: fosfoglicerato mutase, proteína antioxidante tiol-específica, catalase peroxisomal, GAPDH, fator de elongação, fosfoglicerato kinase, álcool desidrogenase e frutose bifosfato aldolase (CROWE et al, 2003). Foram relatadas também as proteínas enolase, antígeno regulador de pH 1 (Pra1) e fosfoglicerato mutase 1 (Gpm1p) que aumentaram o potencial de invasão celular do fungo pela ligação ao Plg (JONG et al, 2003; POLTERMANN et al, 2007; LUO et al, 2009). *Pneumocystis carinii*, *Aspergillus fumigatus* e *Cryptococcus neoformans* são capazes de recrutar plasminogênio e ativá-lo em plasmina através de proteínas de superfície que promovem o processo de invasão à MEC (FOX & SMULIAN 2001; BEHNSEN et al, 2008; ZAAS et al, 2008).

O potencial de *Paracoccidioides* em recrutar plasminogênio humano para invasão e virulência tem sido investigado. Estudos demonstram que este fungo é capaz de captar plasminogênio e ativá-lo em um processo mediado pela proteína enolase. Esta proteína promove um aumento na adesão e invasão do fungo em modelos *in vitro* de infecção mediando sua atividade fibrinolítica (DONOFRIO et al, 2009; NOGUEIRA et

al, 2010; MARCOS et al, 2012). A enolase recombinante de *Paracoccidioides* é capaz de aderir aos componentes da matriz extracelular e à superfície de macrófagos, reforçando o papel dessa molécula na interação do fungo com células do hospedeiro (BAILÃO et al, 2012).

Esses dados demonstram que proteínas ligantes de plasminogênio aumentam o potencial de invasão e patogenicidade de *Paracoccidioides* através da atividade fibrinolítica da plasmina. Proteínas com esta habilidade podem ser transportadas para a superfície do fungo e secretadas para o meio exterior e promover a formação de plasmina, o que também contribui para sua disseminação. Em função do exposto, verifica-se a importância da identificação e análise dessas proteínas para estender o estudo de fatores de virulência do fungo.

2. JUSTIFICATIVA

Proteínas ligantes de plasminogênio tem sido alvo de estudos em diversos micro-organismos. Essas adesinas aumentam o potencial dos patógenos em aderir e disseminar nos tecidos do hospedeiro durante a infecção. A habilidade do fungo *Paracoccidioides* em ligar ao plasminogênio humano está potencialmente relacionada ao processo de adesão e invasão tecidual, como recentemente mostrado em estudos realizados em nosso laboratório. Foi identificada a proteína enolase na superfície do fungo, a qual contribui significativamente para ligação ao plasminogênio e sua conversão em plasmina, além de participar do processo de degradação de componentes da MEC e adesão a macrófagos. Dessa forma, é importante identificar e analisar outras proteínas ligantes de plasminogênio de *Paracoccidioides*, sendo uma relevante contribuição para o estudo da virulência do fungo.

3. OBJETIVOS

3.1. Geral

- Identificar e analisar proteínas ligantes de plasminogênio em *Paracoccidioides.*

3.2. Específicos

- Detectar proteínas secretadas de *Paracoccidioides* que são ligantes de plasminogênio, através de ensaios de Far Western e análises proteômicas;
- Identificar as proteínas ligantes de plasminogênio por espectometria de massas;
- Promover ensaios de validação de ligação e ativação de plasminogênio e ensaios de degradação de fibrina;
- Realizar ensaios de infecção de macrófagos na presença de anticorpos que bloqueiam os sítios de ligação de proteínas ligante de plasminogênio;
- Avaliar a influência do plasminogênio durante a infecção de macrófagos.

4. ARTIGO PUBLICADO

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

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

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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*, *Pb*01. 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

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Background

The *Paracoccidioides* genus comprises a complex of pathogenic fungi, classified in at least four distinct phylogenetic lineages: S1, PS2, PS3 and *Pb*01-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 ther 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*, *Pb*01, 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 external 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*, *Pb*01. 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*, *Pb*01 yeast cells

In order to identify Plg-binding proteins in the secretome of *Paracoccidioides, Pb*01, 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.

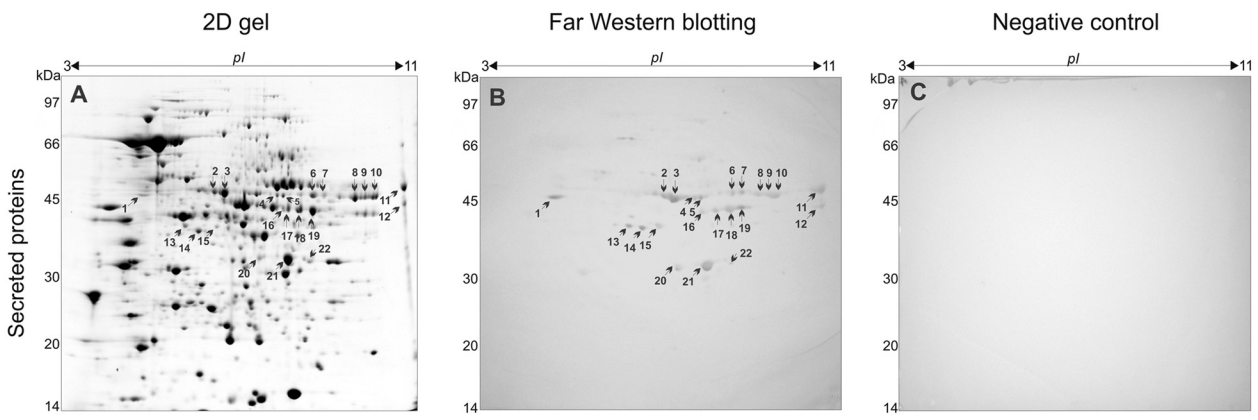

Figure 1 Detection of plasminogen-binding proteins of *Paracoccidioides*, *Pb*01. Secreted proteins (500 μg) of *Paracoccidioides, Pb*01, were subjected to 2-DE [first dimension: IEF pH range 3–11 non-linear, second dimension: 12% (w/v) SDS-PAGE)]. 2-DE gels were stained using Coomassie brilliant blue **(Panel A)** or transferred to nitrocellulose membranes that were processed for Far-western blotting **(Panel B)**. Negative controls include a membrane not incubated with Plg **(Panel C)**. Membrane in panel B was incubated with 35 μg/ml of hPlg, followed by the incubation with the anti-hPlg antibody (1 μg/mL) and the reaction was developed with BCIP/NBT. To identify in the 2-DE gel, the proteins spots that were visualized in the membrane, a pairing of proteins spots was performed, using Image master 2D Platinum software (GE Healthcare). The pH gradient is shown above the gel, and the molecular mass protein standards (kDa) are indicated to the left of the gels.

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, formamidase, acetyl-CoA acetyltransferase and phosphoglycerate kinase were detected in the secretome of *Paracoccidioides, Pb*01 yeast and mycelia [48]. Other proteins were identified in the secretome of *Paracoccidioides, Pb*18: 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.*

Table 1 Secreted proteins of *Paracoccidioides* that bind plasminogen

Spot number[1]	General information number (NCBI)[2]	Protein description	*pI*theor/exp[3]	MM (kDa) theor/exp[4]	PMF score[5]	Coverage sequence (%)[6]	MS/MS Ions score[7]	Matched peptides[8]	Psort prediction[9]	SignalP Value ≥ 0.5[10]	SecretomeP Value ≥ 0.5[11]	big-PI[12]
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|295671120	fructose 1,6-bisphosphate aldolase	6.09/6.41	39.72/41.29	-	-	154	5	cyto: 21.0	NO	0.505	NO
14	gi|295671120	fructose 1,6-bisphosphate aldolase	6.09/6.60	39.72/41.29	-	-	670	5	cyto: 21.0	NO	0.505	NO
15	gi|295671120	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

[1]Spots numbers indicated in Figure 1A.
[2]NCBI database general information number (http://www.ncbi.nlm.nih.gov/).
[3]Isoelectric point (theoretical/experimental).
[4]Molecular Mass in kDa (theoretical/experimental);
[5]Mascot PMF score for fragmentation data (http://www.matrixscience.com).
[6]Sequence coverage percentage.
[7]Mascot MS/MS score for fragmentation data (http://www.matrixscience.com).
[8]Number of identified peptides (MS/MS).
[9]Subcellular localization prediction of proteins according Psort (http://www.genscript.com/psort/wolf_psort.html).
[10]Secretion prediction according to Signal P 3.0 server. The number corresponds to signal peptide probability (Score^3 0.5) (http://www.cbs.dtu.dk/services/SignalP/).
[11]Secretion prediction according to Secretome P 1.0 server; the number corresponds to neural network that exceeded a value of 0.5 (NN-score ^{3} 0.50) (http://www.cbs.dtu.dk/services/SecretomeP/).
[12]GPI 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 components 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 carboxyl-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 adhesin 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*, *Pb*01 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 meningitides* 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.

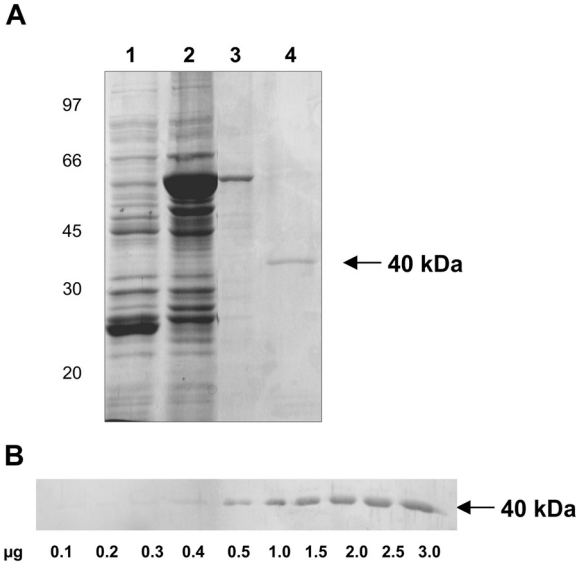

Figure 2 *Paracoccidioides* FBA is a plasminogen-binding protein. **(A)** SDS-PAGE analysis of the rFBA of *Paracoccidioides*. The recombinant protein was obtained by heterologous expression in *E. coli*. The bacteria total protein extract, before (lane 1), and after (lane 2) the induction with IPTG; the recombinant protein fused to gluthatione S-transferase (GST) (lane 3) and the purified rFBA (lane 4). **(B)** Far-western analysis. Increasing concentrations of the rFBA (0.1 to 3.0 μg) were fractionated by SDS-PAGE (12%) and transfered to a nitrocellulose membrane that was subsequently incubated with hPLg, anti-hPlg antibodies produced in mouse and anti-mouse immunoglobulin G (IgG) coupled to alkaline phosphatase. The reaction was developed using 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT).

Detection of FBA at the *Paracoccidioides* surface

In order to determine the localization of the FBA in *Paracoccidioides*, *Pb*01, 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 glycosylphosphatidylinositol (GPI) anchors linked to the wall

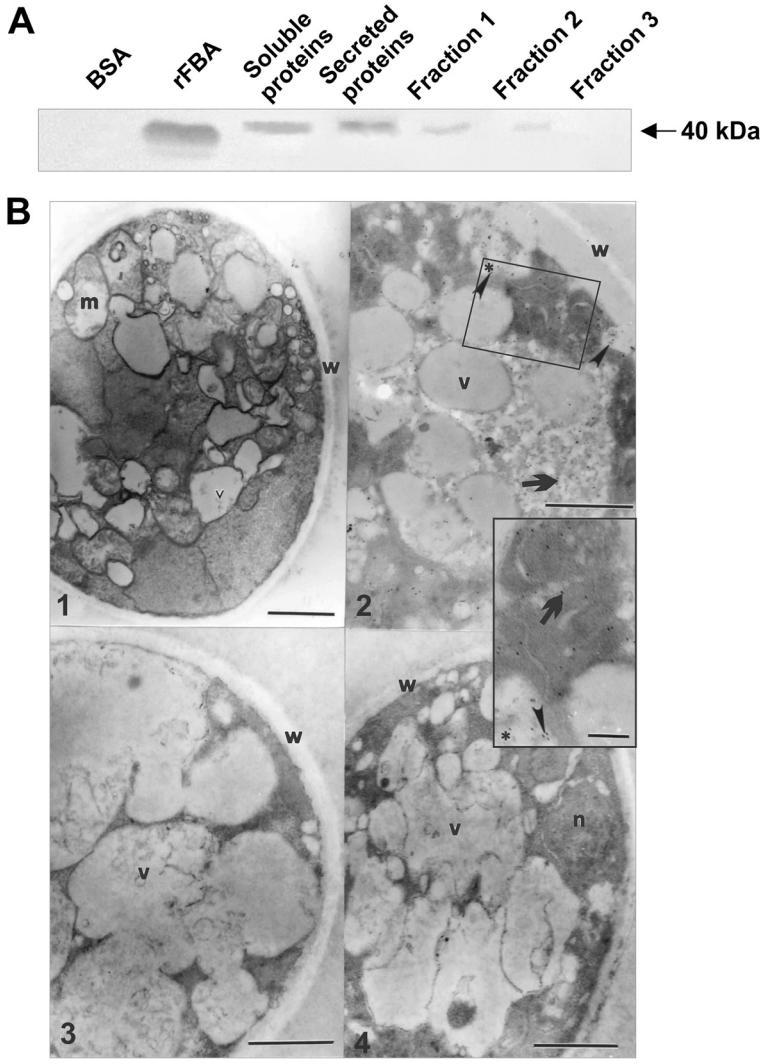

Figure 3 Detection of FBA in *Paracoccidioides*. (A) Western blotting analysis. Different protein samples (15 μg) of *Paracoccidioides* comprehending the soluble and secreted proteins, cell wall fractions 1, 2 and 3 were obtained by sequential treatments as described in Materials and Methods. For negative 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).

(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

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.

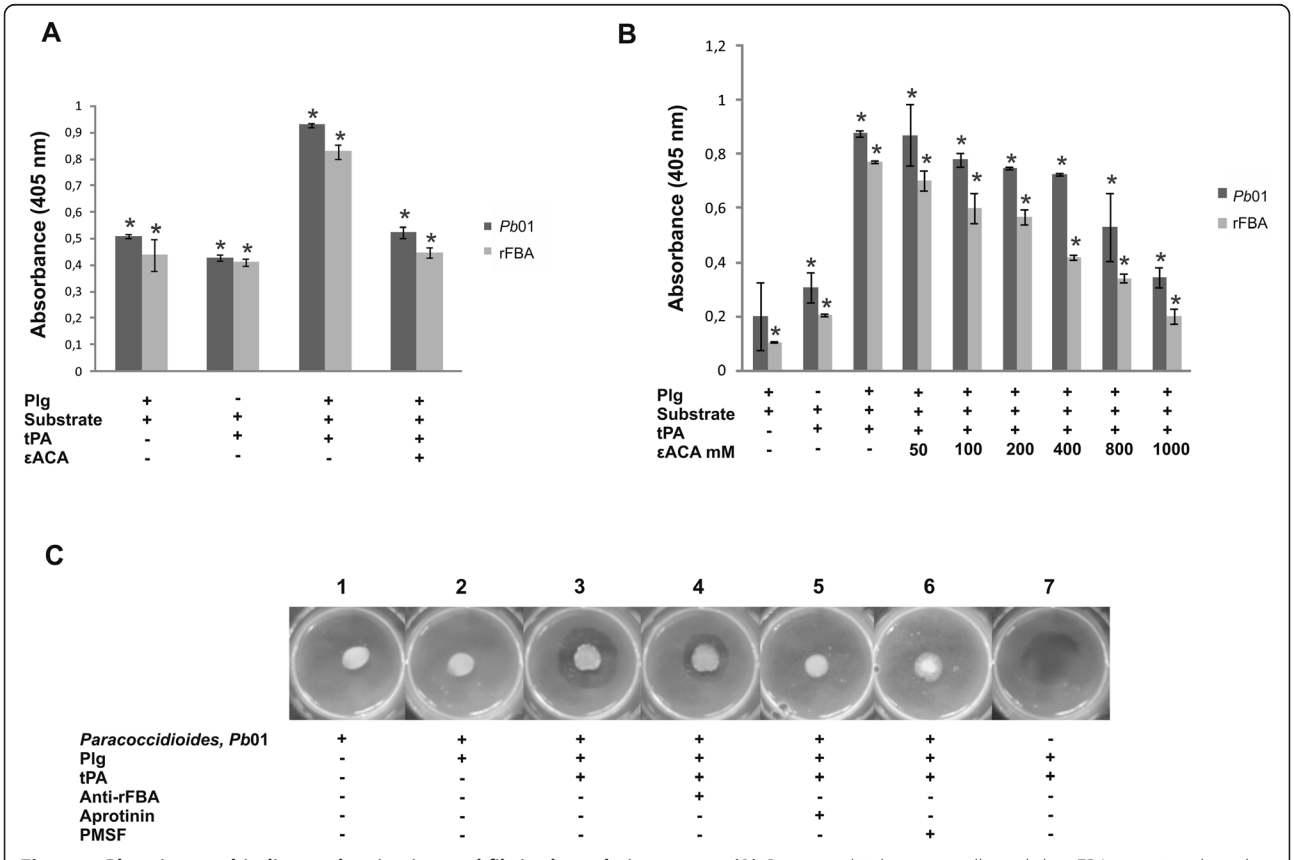

Figure 4 Plasminogen-binding and activation and fibrin degradation assays. (A) *Paracoccidioides* yeast cells and the rFBA were incubated with hPlg in the presence or absence of tPA and εACA. We used a plasmin substrate (D-valyl-L-lysyl-p-nitroaniline hydrochloride) (Sigma-Aldrich) to dose the amidolytic 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.

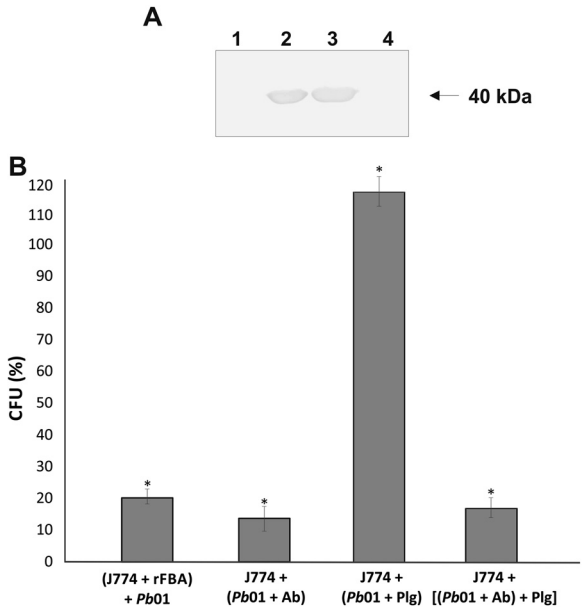

Figure 5 *Paracoccidioides* adhesion/internalization by macrophage: Effects of plasminogen and FBA. (A) Western blotting analysis of binding of J774 macrophages and rFBA. Macrophages were incubated with 50 μg rFBA (line 2). Additionally controls were performed with 5 μg of rFBA for the positive control (line 3), and 5 μg of BSA for negative control (line 4). Line 1 depicts macrophages, without incubation with the rFBA. The immunoblot was probed with the polyclonal antibodies directed to the rFBA. **(B)** Macrophages were or not pre-incubated with the rFBA (50 μg) for 1 h, before infection. Yeast cells (*Pb*01) were or not treated with the antibodies to the rFBA (Ab, 1:1000 diluted), with hPlg (50 μg) and tPA (50 ng), for 1 h, or with the antibodies to the rFBA (Ab, 1:1000 diluted) and subsequently with Plg (50 μg), and tPA (50 ng), for 1 h. Macrophages were incubated with the yeast cells above for 12 h at 36°C. * : results significantly different from control, at a p value < 0.05.

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 × 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 × 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 × 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 × 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 × 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 × 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 buffer, GE Healthcare), and trace amounts of bromophenol blue. Then the samples were loaded onto a 13 cm Immobiline^{TM} 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 14 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 Commassie 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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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 Tetrazolium (NBT).

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

The cDNA that encodes FBA of *Paracoccidioides, Pb*01 (GenBank Accession Number AY233454) was previously obtained [69]. Oligonucleotide primers were designed: sense S (5'-GAATTCCATGGGCGTGAAAGACA-3') and antisense AT (5'-GCGGCCGCCTACAACTGGTTAGA A-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 × g for 10 min and resuspended in PBS. Soluble proteins were obtained by sonication, followed by centrifugation at 10,000 × 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 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 into mice along with Freund's adjuvant three times at intervals of 15 days. Serum containing polyclonal antibodies was collected and stored at −20°C.

Western blotting

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 KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl (pH 7.3), 5% (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 antibodies (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 Tetrazolium (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 DDT in 25 mM ammonium bicarbonate (NH_4HCO_3), and alkylated with 55 mM iodoacetamide in 25 mM NH_4HCO_3, 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 NH_4HCO_3. 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 chromatographic 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 reflectron 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: carbamidomethylation 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 Secretome P (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 multitier plates were coated with 1 μg of rFBA or fixed with 1×10^8 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 1×10^7 cells of *Paracoccidioides*, *Pb*01 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

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) (Vitrocell Embriolife,) containing IFN-γ (1U per mL) and MEM non-essential amino acid solution (Sigma Aldrich, Missouri, USA) at 36°C and 5% CO_2, 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 anti-mouse 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 5×10^6 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% CO_2, in RPMI medium containing IFN-γ (1U per mL). The J774 cells were pre incubated for 1 h at 36°C with the rFBA (50 μg/ml), or the yeast cells were pre- incubated with anti-rFBA antibody (1:1000) and then the infection was performed. In parallel, yeast cells were incubated with the polyclonal antibody anti-rFBA (1:1000 diluted) for 1 h at 36°C, and then

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. SSW obtained protein samples, assisted in the production of 2-DE gels, cooperated in the identification and *in silico* analysis of proteins. SNB performed the immunoelectron microscopy assay. LAP was responsible for the production of bacteria clones expressing the rFBA 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.

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5. DISCUSSÃO

No presente estudo, analisamos o secretoma do fungo *Paracoccidioides*, *Pb*01 com o objetivo de identificar e analisar as proteínas ligantes de plasminogênio. Foi possível detectar, através de ensaios de Far Western e espectrometria de massa, um total de 22 spots com habilidade de ligar ao plasminogênio dentre as proteínas secretadas pelo fungo. Várias enzimas detectadas nesta categoria apresentaram isoformas, tais como homogentisato 1,2-dioxigenase, glutamato desidrogenase, fosfoglicerato quinase, 2-metilcitrato sintase, FBA e malato desidrogenase. Dessa forma, os 22 spots identificados foram resumidos em 15 diferentes proteínas. Muitas dessas proteínas foram previamente descritas no secretoma de *Paracoccidioides*. Dentre as 15 proteínas identificadas neste trabalho, 11 foram descritas no secretoma de *Paracoccidioides, Pb*01 nas formas levedura e micélio, sendo elas: 2-metillcitrato sintase, FBA, gliceraldeido 3-fosfato desidrogenase, formamidase, acetil-CoA acetiltransferase e fosfoglicerato quinase (WEBER et al, 2012). Também foram encontradas entre as proteínas extracelulares de *Pb*18: FBA, gliceraldeído 3-fosfato desidrogenase e fosfoglicerato quinase (VALLEJO et al, 2012). Esses dados corroboram com a análise *in silico* em que foi utilizado o Software Signal P e Secretome P, onde podemos observar que a maioria das proteínas aqui descritas são secretadas por vias não clássica.

Algumas das proteínas identificadas neste estudo também foram descritos em outros sistemas como proteínas de ligação ao Plg. Desta forma, a FBA e gliceraldeído 3-fosfato desidrogenase foram descritas como proteínas ligantes de Plg em *C. albicans* (CROWE et al, 2003). A acetil-CoA acetiltransferase foi identificada na bactéria *L. interrogans* (VIEIRA et al, 2010) e a fosfoglicerato quinase foi descrita em *C. albicans* (CROWE et. al., 2003) e em *C. neoformans* (STIE et al, 2009). A fosfoglicerato quinase é descrita como adesina tanto em bactérias (BOONE & TYRRELL, 2012) como em fungos (CROWE et al, 2003; STIE et al, 2009). Na superfície de espécies de *Estreptococo* do grupo B, a fosfoglicerato quinase é capaz de ligar-se à moléculas do hospedeiro como actina e plasminogênio. A interação de componentes da MEC a com proteínas de bactérias, incluindo fosfoglicerato quinase, promove a ativação de proteínas específicas em sua superfície as quais induzem a adesão bacteriana (HOFFMANN et al, 2010; BOONE & TYRRELL, 2012). Além disso, a degradação proteolítica da MEC por fosfoglicerato quinase, aderida à plasmina, promove a adesão às células endoteliais e promove a disseminação bacteriana nos tecidos do hospedeiro

(FULDE, STEINERT, BERGMANN, 2013). Em *C. neoformans*, a fosfoglicerato quinase localiza-se na parede celular fúngica, onde exibe resíduos de lisina na região carboxi-terminal, formando um acesso para a ligação de Plg (MILES et al, 1991).

A proteína enolase tem sido descrita em alguns trabalhos realizados com *Paracoccidioides*. Foi assim demonstrado que o fungo é capaz de recrutar plasminogênio e ativar o sistema plasminogênio-plasmina em um processo mediado por essa proteína, a qual pode ser encontrada na superfície do fungo. A enolase produzida em sistema heterólogo promove um aumento da adesão e invasão do fungo em modelos *in vitro* de infecção, um processo que parece estar associado à capacidade da proteína em modificar a superfície de células do hospedeiro (NOGUEIRA et al, 2010; MARCOS et al, 2012). Adicionalmente, a enolase de *Paracoccidioides* participa do processo de adesão, ligação aos componentes da MEC e internalização do fungo a células do hospedeiro (DONOFRIO et al, 2009; BAILÃO et al, 2012; MARCOS et al, 2012). Esta proteína também foi descrita como ligante de plasminogênio em organismos como *S. pneumoniae* (BERGMANN et al, 2001), *C. albicans* (JONG et al, 2003), *Streptococci sp.* (PANCHOLI & FISCHETTI 1198; DERBISE et al, 2004), *P. carinii* (FOX & SMULIAN 2001; KOLBERG et al, 2006), *Trichomonas vaginalis* (MUNDODI et al, 2008), *C. neoformans* (STIE et al, 2009), *S. pyogenes* (KORNBLATT, KORNBLATT, HANCOCK, 2011), *Leishmania mexicana* (FIGUERA et al, 2012), *Lactobacillus plantarum* (VASTANO et al, 2012), *Salmonella* sp. (CHAUHAN et al, 2012) e *B. burgdorferi* (FLODEN et al, 2011; TOLEDO et al, 2012; NOGUEIRA et al, 2012).

A gliceraldeído 3-fosfato desidrogenase (GAPDH) também foi descrita como adesina em outros organismos. Em *Neisseria meningitidis* ela participa do processo adesão às células humanas e em *T. vaginalis* é capaz de se ligar aos componentes da MEC (LAMA et al, 2009; TUNIO et al, 2010). Esta proteína também foi caracterizada em *C. albicans* e em *E. coli* como antígeno de superfície e como proteína ligante de fibronectina, laminina e plasminogênio (GIL-NAVARRO et al, 1997; GOZALBO et al, 1998; CROWE et al, 2003; EGEA et al, 2007; LUO et al, 2013). Em trabalhos realizados por nosso grupo esta molécula foi detectada na superfície e no citoplasma de *Paracoccidioides* participando da adesão e invasão através da ligação aos componentes da MEC (BARBOSA et al, 2006). Trabalhos desenvolvidos com a proteína GP43 de *Paracoccidioides* descrevem seu efeito inibitório sobre macrófagos e sobre a resposta inflamatória. A GP43 é capaz de se ligar à laminina, o que aumenta a patogenicidade do

fungo, facilitando o processo de infecção (VICENTINI et al, 1994; POPI et al, 2002; KONNO et al, 2009; 2012).

A frutose 1,6-bifosfato aldolase de *Paracoccidioides* foi previamente caracterizada em nosso laboratório (FONSECA et al, 2001; CARNEIRO et al, 2005). A proteína foi primeiramente identificada como uma proteína imunogênica reativa a soros de pacientes com PCM por Western blotting. A proteína foi caracterizada como FBA por meio de digestão com endoproteinase Lys-C e degradação de Edman (FONSECA et al, 2001). Subsequentemente, Carneiro et al, (2005) clonaram e caracterizaram dois cDNAs que codificam dois homólogos da frutose 1,6-bifosfato aldolase de Classe II de *Paracoccidioides* (*Pbfba1* e *Pbfba2*), que demonstram 70% de identificação entre si.

No presente estudo encontramos evidências de que FBA pode exercer papel importante na patobiologia do fungo. Há indícios de sua função como adesina e de sua importância na relação parasito-hospedeiro. Alguns estudos sugerem a participação da FBA na conexão entre adesinas da família TRAP com o citoesqueleto de patógenos e na motilidade de alguns parasitas (BURUCOA et al, 1995; MCCARTHY et al, 2002; JEWETT & SIBLEY, 2003). Em estudos com *C. albicans* foram descritas oito proteínas ligantes de plasminogênio, entre elas a frutose 1,6-bifosfato aldolase (CROWE et al, 2003). Neste trabalho, FBA apresentou habilidade de ligar ao plasminogênio e promover a geração de plasmina na presença de tPA. Esta ligação favoreceu a degradação de fibrinogênio, que é substrato da plasmina, o que revela sua participação na atividade fibrinolítica do fungo. FBA foi primeiramente detectada no secretoma, como foi demonstrado nos ensaios de Far Western e espectometria de massa, assim como na porção citoplasmática e na parede do fungo, inclusive em uma vesícula em processo de secreção, como foi observado nos ensaios de Western blotting e imunocitoquímica. O lançamento de vesículas para o ambiente externo é usado por muitos patógenos para aumentar seu potencial invasor. Vesículas secretoras são ricas em fatores de virulência, incluindo moléculas que ligam e ativam Plg (TOLEDO et al, 2012; FIGUERA et al, 2013). Atuando na superfície do patógeno, a FBA pode captar plasminogênio e em ação conjunta com o tPA do hospedeiro, pode convertê-lo em plasmina, o que favorece o processo de adesão e disseminação do fungo.

Esta hipótese foi reforçada após ensaios de infecção de macrófagos J774 por *Paracoccidioides*. Os resultados mostram que o índice de infecção foi reduzido em 79% quando os macrófagos foram pré-incubados com rFBA e 86% quando as células de

levedura foram pré-incubadas com anticorpos anti-rFBA, ou seja, após o bloqueio dos sítios de interação entre o fungo e células de defesa do hospedeiro. Experimentos semelhantes com outras proteínas, tais como proteínas recombinantes de gliceraldeído 3-fosfato desidrogenase e triose fosfato isomerase promoveram redução da interação de *Paracoccidioides* com pneumócitos e células Vero (BARBOSA et al, 2006; PEREIRA et al, 2007). Em relação ao plasminogênio, a pré-incubação com leveduras do fungo, na presença de tPA, promoveu um aumento da infecção por macrófagos. Enquanto que a adição de anticorpos para rFBA e Plg inibiu a internalização do fungo pelos macrófagos. Esses dados sugerem fortemente um papel de FBA no processo infeccioso de macrófagos e são consistentes com o papel de FBA na activação de Plg em plasmina, sugerindo a interação da FBA com plasminogênio humano, pode aumentar a virulência deste patógeno.

6. CONCLUSÃO

Muitos micro-organismos expressam proteínas capazes de subverter as proteases do hospedeiro e usá-las em seu favor. Uma vez ativado em plasmina, o plasminogênio adquire atividade fibrinolítica. Patógenos capazes de capturar Plg podem potencializam sua disseminação nos tecidos hospedeiros. No presente estudo objetivamos identificar e analisar as proteínas secretadas pelo fungo *Paracoccidioides*, *Pb*01 com habilidade de ligar ao plasminogênio humano. Através de ensaios de Far Western e espectrometria de massa, foi possível detectar um total de 22 spots representados por 15 proteínas. Essas proteínas são prováveis alvos da interação patógeno-hospedeiro e atuam como mediadoras da formação de plasmina, o que pode contribuir para no processo invasivo do fungo. Selecionamos a proteína frutose 1,6-bifosfato aldolase para ensaios de validação e encontramos evidências de que FBA pode exercer papel importante na patobiologia do fungo. Além do secretoma, essa proteína também foi detectada em vesículas secretoras e nas frações proteicas da parede e citoplasmática do fungo. FBA apresentou habilidade de ligar ao plasminogênio e promover a geração de plasmina na presença de tPA. Esta ligação favoreceu a degradação de fibrinogênio, que é substrato da plasmina, o que revela sua participação na atividade fibrinolítica do fungo. Além disso, demonstramos que a interação de FBA ao plasminogênio humano contribui para a interação do fungo com células de defesa, como observado durante ensaios de infecção de macrófagos. Em suma, esses dados sugerem que a frutose 1,6-bifosfato aldolase é uma proteína de ligação ao plasminogênio e pode estar envolvida no processo de aderência, invasão e disseminação de *Paracoccidioides*.

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