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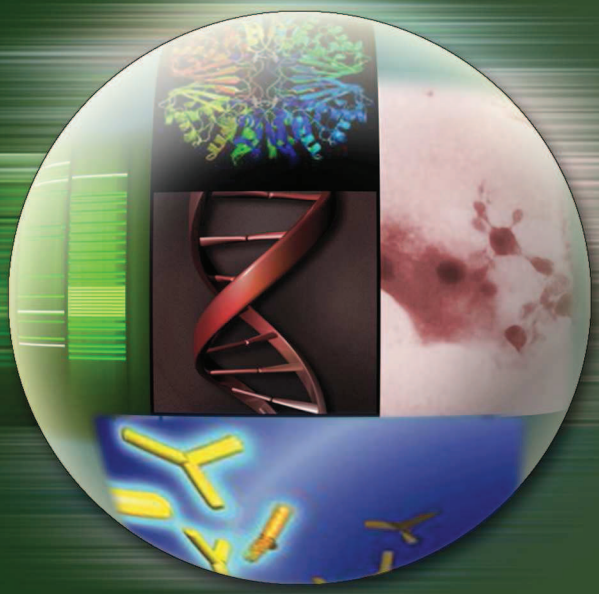
Instituto de Patologia Tropical e Saúde Pública
Programa de Pós-Graduação em Medicina Tropical

Tese de Doutorado

Análises transcricionais no processo de adesão por *Paracoccidioides brasiliensis* e caracterização funcional de adesinas



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SUMÁRIO

Lista de Abreviaturas

BSA – soro albumina bovina

cAMP – adenosina monofosfato cíclico

cDNA – DNA complementar

CoA – Coenzima A

DNA – ácido desoxirribonucléico

DTT – di-tiotreitol

EDTA – ácido etileno-diamino-tetra acético

EST – etiqueta de seqüência expressa

GAPDH – gliceraldeído 3-fosfato desidrogenase

GP – glicoproteína

GPI – glicosil-fosfatidil inositol

GST – glutationa S-transferase

HSP – proteína de choque térmico

IPTG – isopropil-β-D-tiogalactopiranosídeo

Kb – Kilobases

kDa – KiloDalton

MAPK – proteína quinase ativada por mitose

MEC – matriz extracelular

Mb – Mega base

NADH – nicotinamida adenina dinucleotídeo reduzido

NBT – nitro blue tetrazólico

NP40 – nonidete P-40

Nsdd – GATA fator de transcrição

PAGE – eletroforese em gel de poliacrilamida

*Pb*01, *Pb*03 e *Pb*18: isolados 01, 03 e 18 de *Paracoccidioides brasiliensis*

*Pb*ctr3– transportador de cobre de *Paracoccidioides brasiliensis*

*Pb*Dfg5p – proteína Dfg5 (deficiente para o crescimento filamentoso) de *P. brasiliensis*

*Pb*Eno – enolase de *P. brasiliensis*

PBS – solução de tampão fosfato

PCM – paracoccidioidomicose

PCR – reação em cadeia da polimerase

pH – potencial hidrogeniônico

pI – ponto isoelétrico

PKA – proteína quinase A

Plg – plasminogênio

PS – espécie filogenética

qRT-PCR: PCR quantitative acoplada à transcrição reversa

RDA – análise diferencial representacional

RNA – ácido ribonucléico

r*Pb*Eno – *Pb*Eno recombinante

rRNA – RNA ribossomal

S – espécie

SDS – dodecil sulfato de sódio

TPI – triose-fosfato isomerase

tPA – ativador do Plg tipo tecidual

uPA – ativador do Plg tipo uroquinase

RESUMO

Paraccidioides brasiliensis é o agente etiológico da paracoccidioidomicose (PCM), uma micose sistêmica, prevalente na América Latina. A matriz extracelular (MEC) é uma rede complexa formada por colágeno, laminina, fibronectina, entre outros componentes, que, quando exposta, é o local inicial de adesão do fungo. Nosso objetivo foi estudar genes envolvidos nesse processo de adesão utilizando Análise Diferencial Representacional (RDA). RDA é um método de subtração acoplado a PCR que permite o isolamento de genes diferencialmente expressos entre duas populações de cDNAs diferentes. Assim, cDNAs foram sintetizados a partir de RNAs extraídos de células leveduriformes de *P. brasiliensis* aderidos à colágeno e fibronectina para identificar genes super-expressos nestas condições. Genes envolvidos com vários processos celulares foram observados e *Pb*Ctr3 (transportador de cobre) e enolase (*Pb*Eno) foram escolhidos para análises adicionais. Um peptídeo sintético (*Pb*CTR3) e a proteína recombinante (r*Pb*Eno) foram utilizados, juntamente com o anticorpo policlonal anti-r*Pb*Eno em análises funcionais com componentes da MEC e plasminogênio. Os estudos sugerem que a enolase de *P. brasiliensis*, localizada na parede celular, é capaz de gerar plasmina a partir do plasminogênio mediada pelo ativador de plasminôgenio. Além disso, foi também demonstrado que esta proteína é secretada sendo capaz de promover a adesão e invasão do fungo a células. Esses estudos claramente estabelecem o papel da enolase na patogenicidade de *P. brasiliensis*.

ABSTRACT

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis (PCM), a human systemic mycosis, prevalent in Latin America. Extracellular matrix (ECM) is a complex net where collagens, laminin and fibronectin can be found and, when exposed, is the first site for the fungus adhesion. Our aim was to study genes involved in the adhesion process using Representational Difference Analysis (RDA). RDA is a PCR-coupled subtractive method that allows the isolation of genes differentially expressed in two different cDNA populations. Hence, cDNAs were synthesized from RNAs extracted from *P. brasiliensis* yeast cells adhered to collagen and fibronectin to identify overexpressed genes. Genes involved in a wide range of cellular process were found and *Pb*Ctr3 (cooper transporter) and enolase (*Pb*Eno) were chosen to further studies. A synthetic peptide (*Pb*CTR3) and the recombinant enolase (r*Pb*Eno) were utilized together with the anti-r*Pb*Eno polyclonal antibody in functional analysis with ECM components and plasminogen. The studies suggest that *P. brasiliensis* enolase, in the surface, is able to generate plasmin from plasminogen by plasminogen activator. Therefore, it was also demonstrated that this protein is secreted and able to promote fungus adhesion and invasion to cells. These findings clearly establish the role of enolase in the patogenicity of *P. brasiliensis*.

INTRODUÇÃO

I – INTRODUÇÃO

I. 1 – A Paracoccidioidomicose e o fungo *Paracoccidioides brasiliensis*

O fungo *Paracoccidioides brasiliensis*, isolado pela primeira vez em 1908 por Adolpho Lutz, é o agente etiológico da paracoccidioidomicose (PCM) (Franco, 1987), uma doença sistêmica que primariamente envolve os pulmões e, em seguida, se dissemina a outros órgãos (Brummer *et al.*,1993). A PCM é uma micose prevalente na America Latina (Theodoro *et al.*, 2007) sendo que 85% dos casos ocorrem no Brasil, representando, assim, um sério problema de saúde em nível nacional. A PCM representa a principal causa de morte entre as micoses sistêmicas (Prado *et al.*, 2009) e a oitava entre as doenças infecciosas e parasitárias (Coutinho *et al.*, 2002; Bagagli *et al.*, 2006).

A infecção é causada pela inalação dos propágulos da fase miceliana do fungo, mas o longo período de latência da doença e a ausência de picos epidêmicos geram dificuldades para determinar sob que circunstâncias a infecção primária ocorre (Restrepo *et al.*, 2008). As lesões secundárias freqüentemente aparecem nas membranas mucosas, pele, linfonodos e glândulas adrenais. Tanto a apresentação clínica quanto o curso da doença variam de paciente para paciente, dificultando o pronto diagnóstico clínico (Brummer *et al.*,1993).

A interação entre fatores do hospedeiro, virulência do fungo e condições do ambiente podem alterar o equilíbrio levando ao desenvolvimento da PCM (Franco, 1987; Rappleye & Goldman, 2006). Quando a micose está estabelecida, os pacientes apresentam uma gama de sinais e sintomas que têm sido a base para a classificação das formas clínicas. Há duas formas clínicas principais da PCM: uma aguda ou subaguda (tipo juvenil) e uma crônica (tipo adulto), embora em ambas, a apresentação clínica e o curso da doença, variem de paciente para paciente. A forma juvenil atinge crianças de ambos os sexos, tem evolução mais rápida e é mais severa levando a taxas de mortalidade significantes, afetando principalmente o sistema retículo endotelial. A forma adulta, por sua vez, é altamente prevalente entre adultos do sexo masculino, tem progressão lenta e compromete primeiramente os pulmões podendo disseminar para outros órgãos e tecidos formando lesões secundárias (Franco, 1987).

A alta incidência da PCM em adultos masculinos sugere que fatores hormonais possam desempenhar uma função na patogênese da doença (Sano *et al.* 1999). Estudos

mostraram que o hormônio 17-β-estradiol é capaz de inibir a transição de micélio para levedura de maneira dose-dependente (Restrepo *et al*. 1985). E ainda, Aristzabal e colaboradores (2002) observaram, *in vivo*, a participação do hormônio feminino na resistência de fêmeas de rato ao desenvolvimento inicial da PCM.

O fungo *P. brasiliensis* apresenta dimorfismo térmico, ou seja, cresce na forma de levedura nos tecidos infectados ou quando cultivado *in vitro* a 36 °C e como micélio em condições saprobióticas no ambiente, ou quando cultivado em temperaturas inferiores a 28 °C (Kanetsuna *et al.*, 1972; Bagagli *et al.*, 2006). As células leveduriformes são multinucleadas, multiplicam-se por brotamento polar ou multipolar e dão à estrutura uma aparência de roda de leme, sendo esta a mais importante característica taxonômica e diagnóstica de *P. brasiliensis.* A forma miceliana cresce à temperatura ambiente mostrando hifas septadas com aparência de fios entrelaçados (San-Blas & Niño-Vega, 2004).

Em fungos dimóficos, a regulação da expressão gênica é relevante uma vez que mudanças morfogenéticas estão interligadas com estratégias adaptativas e de sobrevivência. Eventos moleculares relacionados a genes que controlam transdução de sinal, síntese da parede celular e fatores de virulência parecem estar envolvidos na transição dimófica (Felipe *et al.*, 2005b; Bastos *et al.*, 2007). A conversão morfológica de micélio para a forma de levedura é requerida para a virulência. Essa alteração fenotípica resulta não somente na mudança na forma da célula, mas também na mudança na composição da parede celular, na expressão de moléculas antigênicas e na expressão de fatores de virulência. Em *P. brasiliensis*, assim como outros fungos, lipídeos, quitina, glicanas e proteínas são os principais constituintes da parede celular. Durante a transição de micélio para levedura, ocorre uma substituição gradual do polímero de β-1,3-glicana para α-1,3-glicana (Kanetsuna *et al.*, 1969). O conteúdo de glicana está correlacionado com o nível de virulência (Klein & Tebbets, 2007), pois, em comum com *Blastomyces dermatitidis*, linhagens mutantes de *P. brasiliensis* que possuem menor conteúdo de α-1,3-glicana apresentam virulência diminuída (Hogan & Klein, 1994).

Embora os eventos bioquímicos que regulam a transição dimórfica não sejam totalmente conhecidos, algumas informações relevantes já foram estabelecidas através de estudos transcricionais em *P. brasiliensis*. De acordo com Felipe e colaboradores (2005b), o perfil transcricional da fase miceliana sugere que o piruvato seja utilizado no

metabolismo aeróbio, uma vez que a expressão dos transcritos que codificam enzimas do ciclo do ácido tricarboxílico é induzida na fase miceliana. Em contraste, o perfil transcricional da fase leveduriforme sugere o desvio do piruvato da via glicolítica para o metabolismo anaeróbico. Esta observação está de acordo com a ocorrência de baixos níveis de oxigênio nos tecidos infectados. A habilidade de *P. brasiliensis* de produzir etanol sugere uma possível via anaeróbia para *P. brasiliensis*, que é dependente do estado metabólico da célula. A temperatura do ambiente parece ser o principal regulador do envio do produto final da glicólise para o metabolismo aeróbio ou anaeróbio.

A identificação de componentes da via de sinalização cAMP/PKA no transcriptoma de *P. brasiliensis* sugere um possível mecanismo de envolvimento de cAMP na transição dimórfica, dependente da temperatura (Felipe *et al.*, 2005a; Fernandes *et al.*, 2005). Este tópico também foi objeto de estudo de Chen e colaboradores (2007), segundo os quais a transição morfológica em *P. brasiliensis* foi controlada por mudanças nos níveis de cAMP. Assim, há evidências científicas que reforçam que a ativação da via de sinalização do cAMP seja importante durante o processo de transição dimórfica. No fungo dimórfico *Histoplama capsulatum*, foi demonstrado que, durante a transição de levedura para micélio, a qual é dependente da mudança da temperatura de 37° C para 25 °C, há um aumento dos níveis intracelulares de cAMP associado à alteração morfológica (Sacco *et al.*, 1981). Em *P. brasiliensis* também foi demonstrado que cAMP exógeno inibe a diferenciação de micélio para levedura (Paris & Duran, 1985).

Com base em estudos de filogenia molecular, *P. brasiliensis* é descrito como sendo pertencente ao reino Fungi, filo Ascomycota, classe Plectomyceto, subclasse Euascomycetidae, ordem Onygenales, família Onygenaceae, subfamília Onygenaceae Anamórficos, gênero *Paracoccidioides*, espécie *Paracoccidioides brasiliensis* (San-Blas *et al*, 2002). Evidências experimentais sugerem a ocorrência de um complexo com várias espécies filogenéticas em *P. brasiliensis.* Em estudos usando abordagem filogenética, Matute e colaboradores (2006) propuseram três espécies filogenéticas distintas: PS2 (espécie filogenética 2), que compreende cinco isolados distribuídos nos estados de Minas Gerais e São Paulo e um isolado da Venezuela; PS3 (espécie filogenética 3), com 21 isolados, geograficamente restrita à Colombia; e S1 (espécie 1), com 38 isolados, distribuída pelo Brasil, Argentina, Paraguai, Peru e Venezuela. Contudo, Carrero e colaboradores (2008) sugerem a possibilidade de mais de três

espécies filogenéticas de *P. brasiliensis*, pois o isolado *Pb*01 não foi enquadrado em nenhuma destas espécies. Recentemente, com o estudo de mais isolados de *P. brasiliensis*, foi identificado um grupo com 17 isolados genotipicamente similares, entre eles *Pb*01, que se distancia do grupo S1/PS2/PS3, reforçando a existência de uma nova espécie, semelhante a *Pb*01 (Teixeira *et al.*, 2009).

Embora nas últimas décadas abordagens moleculares tenham ampliado a visão da organização genômica de *P. brasiliensis*, definições conclusivas estavam longe de serem alcançadas. A carência de informação sobre a composição genética deste fungo se deve à ausência de um estágio teleomórfico reconhecido, o que dificulta as análises da espécie usando estratégias de investigação da genética clássica (Cano *et al.*, 1998). Um projeto genoma comparativo foi realizado visando examinar a diversidade entre três isolados de *P. brasiliensis* (*Pb*01, *Pb*03 e *Pb*18) e determinar os aspectos comuns e únicos de cada isolado. O projeto, denominado "Genômica Comparativa de *Coccidioides* e Outros Fungos Dimórficos", foi responsável pelo seqüenciamento dos genomas. O comprimento da seqüência do genoma completo de *Pb*01 foi de 32,94 Mb com um total de 9.132 genes identificados. O isolado *Pb*03 apresentou um genoma de 29,06 Mb com 7.875 genes identificados e *Pb*18 possuia um genoma de 29,95 Mb, contendo 8.741 genes identificados (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html).

I. 2 – Matriz Extracelular e Adesinas

Um passo necessário na colonização e, em última instância, no desenvolvimento de doenças por patógenos, está associado à sua habilidade de se aderir às superfícies do hospedeiro. A capacidade de aderência é um fenômeno biológico vastamente distribuído, compartilhado por organismos diversos para capacitá-los a colonizar seus respectivos habitats. Muitos fungos, especialmente os patogênicos, são capazes de aderir ao tecido do hospedeiro, sendo este o primeiro passo no processo de invasão. Uma colonização bem-sucedida geralmente é um evento complexo e deve envolver proteínas da superfície do fungo e receptores celulares (Sohn *et al.*, 2006). Dessa forma, o desenvolvimento da PCM depende de interações entre o fungo e componentes celulares do hospedeiro.

A matriz extracelular (MEC) é uma rede complexa de macromoléculas entrelaçadas, situada abaixo das células epiteliais e endoteliais, circundando as células do tecido conectivo (Black *et al.*, 2003). A MEC é responsável pela integridade dos tecidos e oferece uma plataforma para as células se aderirem. Os vertebrados possuem tecidos conectivos especializados, como ossos e cartilagem, que são especialmente ricos em MEC. Essa matriz também forma estruturas organizadas, como as membranas basais que geralmente separam as camadas de células epiteliais dos tecidos mesenquimais subjacentes (Heino *et al.*, 2008). A MEC desempenha, dessa forma, um papel essencial na sobrevivência, migração e proliferação das células (Meredith *et al.*, 1993; Vakonakis & Campbell, 2007). A MEC é constituída de proteínas fibrosas (colágeno e elastina) e proteínas estruturais ou adesivas (fibronectina e laminina) embebidas numa espécie de gel de polissacarídio contendo várias glicosaminoglicanas (Pelosi *et al.*, 2007).

Moléculas de adesão celular são encontradas na supefície de todas as células e desempenham papel nas interações célula-célula e célula-MEC. Além de fornecerem uma ligação mecânica entre a MEC e o citoesqueleto, as moléculas de adesão celular estão envolvidas na sinalização entre o interior e o exterior da célula. Assim, elas atuam em processos como crescimento, proliferação, organização espacial e migração. Esses processos também ocorrem em condições patológicas como cicatrização celular, inflamação, neoplasia, invasão tumoral e metástase. Sob estas condições, vários constituintes da MEC sofrem mudanças que estão envolvidas no remodelamento da matriz (Lyons & Jones, 2007).

A MEC serve como substrato não somente para a adesão das células ao organismo, mas também para a adesão de micro-organismos. Muitos organismos expressam proteínas na superfície celular que podem mediar a sua adesão à MEC dos tecidos do hospedeiro (Patti *et al.*, 1994a). As interações com o hospedeiro são mediadas por moléculas complementares em ambas as superfícies dos micro-organismos e das células hospedeiras. As moléculas que atuam nessas interações foram designadas adesinas, e o grande repertório de adesinas exibidas pelos fungos são um reflexo da variedade de sítios que eles podem invadir no hospedeiro (Hostetter, 1994; López-Ribot *et al.*, 1996). A expressão diferencial dos vários genes que codificam para as adesinas capacita os fungos a rapidamente adaptarem suas propriedades adesivas a um ambiente em particular. Enquanto as condições exatas que induzem a adesão não são totalmente entendidas, muitas cascatas de sinalização, incluindo Ras/cAMP/PKA e MAP Kinase, são empregadas para assegurar a regulação apropriada deste fenótipo importante (Verstrepen & Klis, 2006). Juntas, essas vias podem desencadear a expressão de fatores envolvidos com adesão em resposta a estresse, quantidade limitada de nutrientes, entre outros (Gagiano *et al.*, 2002). Dessa forma, estes mecanismos diferentes fazem da adesão fúngica um dos fenótipos mais versáteis, ressaltando a necessidade do fungo de adaptar seu comportamento de adesão ao ambiente e a constantemente explorar novas oportunidades de infecção (Verstrepen & Klis, 2006).

Embora as matrizes extracelulares sejam cobertas com células epiteliais e endoteliais, injúria celular pode ocorrer durante infecções levando à exposição de componentes da MEC (Klotz & Maca, 1988; Lima *et al.*, 2001). Várias moléculas de micro-organismos diversos, *Toxoplasma gondii* (Furtado *et al.*, 1992), *Mycobacterium avium* (Sato *et al.*, 2003), *Candida albicans* (Gozalbo *et al.*, 1998), *Penicillium marneffei* (Hamilton *et al.*, 1999), *P. brasiliensis* (Barbosa *et al.*, 2006), entre outros, foram identificados por se ligarem à componentes da MEC.

Os estudos iniciais sobre a habilidade de *P. brasiliensis* de se aderir aos componentes da MEC foram descritos por Vicentini e colaboradores (1994). Naquele estudo, foi mostrado um aumento de adesão fúngica às células MDCK (Madin-Darby canine kidney) quando células levedurifomes foram pré-incubadas com laminina, sugerindo a existência de proteínas de ligação à laminina na superfície fúngica. No mesmo estudo, foi demonstrado que a gp43, o principal componente antigênico de *P. brasiliensis*, presente na superfície celular, era capaz de se ligar à laminina. Além disso,

Hanna e colaboradores (2000) mostraram o envolvimento de gp43 na adesão de *P. brasiliensis* à células Vero. Posteriormente, González e colaboradores (2005), demonstraram que *P. brasiliensis* possui na sua superfície duas proteínas com massas moleculares de 19 kDa e 32 kDa, que interagem com diferentes proteínas da matriz extracelular como laminina, fibronectina e fibrinogênio. E ainda, Andreotti e colaboradores (2005) isolaram e caracterizaram uma proteína de 30 kDa que se ligou à laminina.

Na tentativa de caracterizar novas moléculas relevantes para a interação fungo-hospedeiro, nosso grupo tem estudado a interação de proteínas de *P. brasiliensis* com proteínas da MEC. A proteína gliceraldeído 3-fosfato desidrogenase (GAPDH), que está associada à parede celular, parece mediar o processo de adesão e internalização de *P. brasiliensis* em células cultivadas *in vitro*, sendo, desse modo, importante no estabelecimento da doença (Barbosa *et al.*, 2006). Pereira e colaboradores (2007) identificaram e purificaram uma proteína de 29 kDa, a triose fosfato isomerase (TPI), que é capaz de interagir com laminina e fibronectina, o que faz dela um possível candidato envolvido na adesão inicial do fungo e posterior invasão tecidual. Castro e colaboradores (2008), buscando conhecer o papel desempenhado pela proteína *Pb*Dfg5p na interação deste fungo com as células do hospedeiro, mostraram que ela está presente na superfície celular de *P. brasiliensis* e tem a capacidade de se ligar à laminina, fibronectina e colágenos tipo I e tipo II. E, ainda, a proteína malato sintase foi purificada e se mostrou capaz de se ligar a fibronectina, e colágenos tipo I e IV (Neto *et al.*, 2009). Isso mostra que adesinas de *P. brasiliensis* estão envolvidas nas interações do fungo com o hospedeiro.

I. 2.1 – Colágeno

Os colágenos são os principais constituintes da MEC. Tradicionalmente, o papel atribuído a essas proteínas era apenas estrutural. Os vertebrados possuem pelo menos 15 tipos de colágenos, que são encontrados em padrões de distribuição tecido-específicos e exibem propriedades funcionais diferentes. Além disso, os colágenos estão envolvidos na adesão e diferenciação celular, como agentes quimiotáticos, como antígenos em processos imunopatológicos e como componentes de defesa em certas condições patológicas. O tipo mais abundante de colágeno isolado de tecidos conectivos como

pele, ossos, tendões e córnea é o colágeno tipo I. As membranas basais são constituídas por uma variedade de classes de moléculas, incluindo os colágenos, sendo o colágeno tipo IV o principal deles (Hay, 1991). Como constituintes das matrizes extracelulares, os colágenos já foram descritos como alvos para adesão de células tumorais (Kazarian *et al.*, 2003) e de proteínas de micro-organismos como *Leptospira interrogans* (Atzingen *et al.*, 2008), *Bartonella henselae* (Dabo *et al.*, 2006) e *P. brasiliensis* (Barbosa *et al.*, 2006). Portanto, os colágenos têm um papel importante no aumento da capacidade de invasão de células tumorais e de micro-organismos.

I. 2.2 – Fibronectina

A fibronectina é uma glicoproteína presente nas superfícies celulares, nos tecidos conectivos, no sangue e em outros fluidos corpóreos. Há evidências de que a fibronectina da superfície celular seja capaz de mediar adesão entre as células. Ela foi a primeira proteína da MEC descrita por atuar como substrato para adesão de células eucarióticas (Oh *et al.*, 1981; Patti *et al.*, 1994b). A molécula de fibronectina é composta por dois polipeptídeos que se associam através de duas pontes dissulfeto perto da extremidade carboxi-terminal para formar um dímero com massa molecular de aproximadamente 550 kDa. A função biológica primária da fibronectina é relacionada com sua habilidade de servir como substrato para a adesão de células eucarióticas, um processo que envolve a ligação de receptores específicos de superfícies celulares à domínios na molécula de fibronectina (Ruoslahti *et al.*, 1988). Como a fibronectina desempenha um papel vital numa variedade de processos biológicos normais, alguns micro-organismos fazem dela um alvo estratégico no estabelecimento, manutenção e disseminação da infecção no hospedeiro (Schwarz-Linek *et al.*, 2004).

I. 2.3 – Laminina

A laminina é uma glicoproteína de 900 kDa da MEC e tem importância no desenvolvimento e manutenção da organização celular (Beck *et al.*, 1990). Ela é uma estrutura flexível consistindo de três braços curtos e um braço longo, formando uma estrutura complexa de três cadeias polipeptídicas geneticamente diferentes, uma cadeia A e duas cadeias menores B (Yurchenco & Schittny, 1990). A laminina é um dos

componentes mais importantes da membrana basal com funções estruturais e regulatórias diversas, que surgem das interações de seus vários domínios a receptores celulares e outros ligantes (Tzu & Marinkovich, 2008). A laminina exibe uma variedade de atividades biológicas, incluindo promoção da adesão celular, crescimento e diferenciação de células e múltiplas interações com outros componentes da membrana basal (Beck *et al.*, 1990). Ela também está implicada na ligação de uma variedade de patógenos intra e extracelulares às células do hospedeiro, podendo aumentar a adesão dos taquizoítos de *T. gondii* às células J774 (Furtado *et al.*, 1992) e de *H. capsulatum* aos componentes da membrana basal (McMahon *et al.*, 1995). Já foi demonstrado que células de *P. brasiliensis* se ligam à laminina através de gp43, aumentando a patogenicidade das células fúngicas em murinos (Vicentini *et al.*, 1994).

I. 3 – Enolase

A enzima enolase (2-fosfo-D-glicerato hidroliase, EC 4.2.1.11) cataliza a desidratação de 2-fosfo-D-glicerato (2-PG) à fosfoenolpiruvato (PEP) na segunda metade da via glicolítica. A enolase é uma das enzimas citoplasmáticas mais abundantemente expressas em muitos organismos (Pancholi, 2001). Assim, por muitos anos, a enolase foi vista como uma enzima glicolítica solúvel, presente exclusivamente no citoplasma. Contudo, vários estudos têm mostrado que esta enzima metabólica possui atividades funcionais adicionais (Pancholi & Fischetti, 1998; Sriram *et al.*, 2005; López-Villar *et al.*, 2006).

Em células de mamíferos, já foram identificadas três isoformas de enolase, α (ENO1), β (ENO3) e γ (ENO2). ENO1 é vastamente distribuída em uma variedade de tecidos, enquanto ENO2 e ENO3 são encontradas exclusivamente nos tecido neuroendócrino e músculo, respectivamente (Chang *et al.*, 2006). Além de sua função glicolítica, ENO1 já foi encontrada na superfície de monócitos e neutrófilos atuando como um receptor de plasminogênio, sugerindo um possível papel na invasão tecidual (Redlitz *et al.*, 1995). Em situações de hipóxia, a enolase também atua como uma proteína de estresse que poderia proteger as células aumentando o metabolismo anaeróbico (Jiang *et al.*, 1997). Num estudo em pacientes com câncer de pulmão, 65% dos indivíduos mostraram superexpressão do gene ENO1 nos tumores, comparado com células epiteliais normais de pulmão (Chang *et al.*, 2006). A ativação de enzimas glicolíticas é comum numa variedade de cânceres. Em resposta à hipóxia, células normais aumentam a expressão gênica de enzimas glicolíticas para se adaptar ao estresse do ambiente através da ativação de fator de transcrição induzido por hipóxia. Mudanças no metabolismo de energia são propriedades fundamentais de células cancerosas que fazem com que elas sobrevivam num estado de estresse gerado por hipóxia seguida de indução de angiogênese e aumento da invasão local ou metástases. Ainda, níveis aumentados da expressão do gene *ENO*1 parece ser uma conseqüência inevitável durante tumorigênese (Chang *et al.*, 2006).

Apesar de sua função ser primariamente metabólica, a enolase também já foi implicada em várias doenças uma vez que anticorpos anti-enolase têm sido encontrados em várias condições autoimunes incluindo doença inflamatória de Bowel e lúpus eritematoso discóide (Yousefi *et al.*, 2000). Diferentemente de outros genes codificantes

de enzimas glicolíticas que são expressas continuamente, a expressão da enolase pode ser induzida após estimulação com mitógenos (Giallongo *et al.*, 1986), hipóxia (Semenza *et al.*, 1996), citocinas (Sousa *et al.*, 2005), entre outros. Nos estudos de Yousefi e colaboradores (2000), a ativação da enolase refletiu a atividade metabólica aumentada em neutrófilos estimulados, proporcionando uma visão sobre os mecanismos de como as citocinas mudam a atividade funcional e transcricional de granulócitos diferenciados.

Em fungos, bem como em outros organismos procariotos e eucariotos, proteínas secretadas possuem peptídeos sinais típicos na extremidade N-terminal que as dirigem para fora da célula. Esse processo envolve o aparato de translocação do retículo endoplasmático e se processa por vesículas secretórias derivadas do complexo de Golgi que se fusionam com a membrana plasmática para liberar o seu conteúdo de proteínas no espaço extracelular. Esse é considerado o mecanismo canônico para a secreção de proteínas e o reconhecimento de uma seqüência peptídeo sinal é tido como uma clara indicação de que o produto correspondente é exportado pela célula. Entretanto, estudos já mostraram que um número significativo de proteínas, como enzimas glicolíticas e proteínas sem um peptídeo sinal N-terminal, alcançam a superfície da célula fúngica (Lópes-Villar *et al.*, 2006; Martínez *et al.*, 1998).

Moléculas sem peptídeo sinal característico foram identificadas em vesículas secretórias em fungos patogênicos humanos, como TPI em *H. capsulatum* (Albuquerque *et al.*, 2008). E ainda, Rodrigues e colaboradores (2008) identificaram várias moléculas relacionadas à funções diversas, como GAPDH e enolase, nas frações vesiculares de *Cryptococcus neoformans.* Algumas das proteínas dessas vesículas foram reconhecidas pelo soro de pacientes com criptococose, sugerindo que essas proteínas são produzidas durante a infecção em humanos. Albuquerque e colaboradores (2008) mostraram que *H. capsulatum* produz vesículas heterogêneas que são secretadas extracelularmente. Uma variedade de moléculas, incluindo fosfolipídeos e proteínas associadas à resposta a estresse, patogênese, arquitetura da parede celular e virulência estão presentes nas vesículas de *H. capsulatum.*

Apesar da ausência de uma seqüência sinal requerida para secreção e motivos de ancoramento à membrana, experimentos de microscopia imunoeletrônica indicaram a presença da enolase na superfície de pneumococos encapsulados e não encapsulados (Bergmann *et al.*, 2004) e na superfície de outros micro-organismos como *Neisseria*

meningitidis (Knaust *et al.*, 2007), *Staphylococcus aureus* (Carneiro *et al.*, 2004), *Streptococcus pyogenesis* (Severin *et al.*, 2007) e *Echinostoma caproni* (Marcilla *et al.*, 2007), lugar em que a enolase poderia interagir com o hospedeiro através de sua habilidade de se ligar a componentes da MEC e ao plasminogênio. Conforme estudos de Lópes-Villar e colaboradores (2006), 169 aminoácidos da extremidade N-terminal foram suficientes para direcionar a enolase de *Saccharomyces cerevisae* para a superfície celular. E, segundo Nakada e colaboradores (2005), uma região conservada na extremidade N-terminal da enolase de *Trichinella spiralis* também poderia estar atuando como um peptídeo sinal.

I. 4 – O Sistema Plasminogênio

O sistema plasminogênio (Plg) desempenha duas funções gerais na defesa do hospedeiro. Em primeiro lugar, ele é a via central para a dissolução de coágulos de fibrina, que é essencial para a manutenção da hemostasia. Em segundo lugar, o sistema Plg facilita a migração celular por ajudar na penetração através de barreiras protéicas. Sob condições fisiológicas, essa ativação é finamente regulada. Os ativadores do Plg são serina-proteases que catalizam a conversão do Plg à plasmina e foram classificados como sendo de dois tipos: ativador do Plg tipo tecidual (tPA) e ativador do Plg tipo uroquinase (uPA) (Plow *et at.*, 1995; Coleman & Benach, 1999).

A penetração através de membranas basais é um passo importante na patogênese de muitos micro-organismos. Alguns patógenos podem adquirir atividade proteolítica pela ligação do Plg a sua superfície celular onde ele é ativado pelo tPA do hospedeiro. Eberhard e colaboradores (1999) demonstraram que plasmina ligada à superfície celular é capaz de promover a disseminação de *Streptococcus pneumoniae* através da membrana basal. Vieira e colaboradores (2009) mostraram que *Leptospira interrogans* se liga ao Plg e que a plasmina gerada na superfície foi capaz de degradar fibronectina, revelando-se como um fator essencial na patogênese desse micro-organismo.

Vários patógenos possuem em suas superfícies adesinas e receptores de Plg para promover a invasão através dos tecidos. Pancholi & Fischetti (1998) identificaram a proteína enolase como a principal molécula de ligação ao plasminogênio na superfície de *S. pyogenes*. Yavlovich e colaboradores (2007) descreveram também a presença da enolase na superfície de *Mycoplasma fermentas* onde ela é capaz de se ligar ao plasminogênio.

A ligação do Plg às superfícies celulares é mediada por cinco domínios, denominados *kringle*, que possuem afinidade por resíduos de lisina. Mundodi e colaboradores (2008) demonstraram que a proteína enolase, presente na superfície de *Trichomonas vaginalis*, se liga ao Plg através de resíduos de lisina, uma vez que houve diminuição dessa ligação na presença do ácido aminocapróico, análogo da lisina.

I. 5 – RDA

A Análise Representacional Diferencial (RDA) é um processo de subtração acoplado à amplificação, originalmente desenvolvido para uso com DNA genômico como um método capaz de isolar as diferenças entre dois genomas complexos. Esta técnica elimina aqueles fragmentos presentes em ambas as populações, deixando apenas as diferenças. O RDA genômico se baseia na geração, por digestão com enzima de restrição e amplificação por PCR (reação em cadeia da polimerase), de versões simplificadas dos genomas sob investigação conhecidas como "representações". Se um fragmento de restrição amplificável (o alvo) existe numa representação (*tester*) e está ausente em outra (*driver* – controle), um enriquecimento cinético do alvo pode ser alcançado por hibridização subtrativa do *tester* na presença de um excesso de *driver*. Seqüências com homólogos no *driver* não são amplificadas, enquanto o alvo hibridiza apenas com ele mesmo e retém a habilidade de ser amplificável por PCR. Interações sucessivas da subtração e o processo de PCR produzem fragmentos de DNA visíveis num gel de agarose correspondendo ao alvo enriquecido (Fig. 1) (Hubank & Schatz, 1994). A técnica de RDA é flexível porque as populações de cDNA podem ser fracionadas por um número de enzimas de restrição com seqüências curtas de reconhecimento para produzir conjuntos de cDNAs. Este aspecto do RDA melhora grandemente as chances de se clonar com sucesso espécies diferencialmente expressas. Além disso, pelo fato de que cada cDNA é restringido no seu comprimento para produzir fragmentos menores, o procedimento de RDA oferece múltiplas chances de se recuperar um gene de interesse (Pastorian *et al.*, 2000).

No intuito de se conhecer genes que poderiam contribuir para a adaptação e sobrevivência de *P. brasiliensis* durante a infecção, Bailão e colaboradores (2006) utilizaram a técnica de RDA para identificar genes induzidos durante o processo infectivo num modelo murino de infecção e em condições que imitam a rota hematológica da disseminação fúngica. Os transcritos diferencialmente expressos nesse estudo eram predominantemente relacionados com remodelamento de parede celular e síntese da parede celular. Através de RDA foi também observada a influência do plasma humano na expressão gênica de *P. brasiliensis,* sugerindo genes que poderiam ser essenciais na adaptação do fungo no hospedeiro (Bailão *et al.*, 2007). Foi demonstrado neste estudo que o plasma ativa significativamente a expressão de transcritos associados

com biossíntese de proteínas, facilitadores de transporte, degradação de ácidos graxos, remodelamento de parede e defesa celular.

A identificação de genes expressos durante o processo de adesão de *P. brasiliensis* pode contribuir para um melhor entendimento das interações entre o fungo e o hospedeiro. No presente estudo, foi usada a técnica de RDA para a identificação de genes diferencialmente expressos em *P. brasiliensis* durante o processo de adesão ao colágeno tipo I e fibronectina.

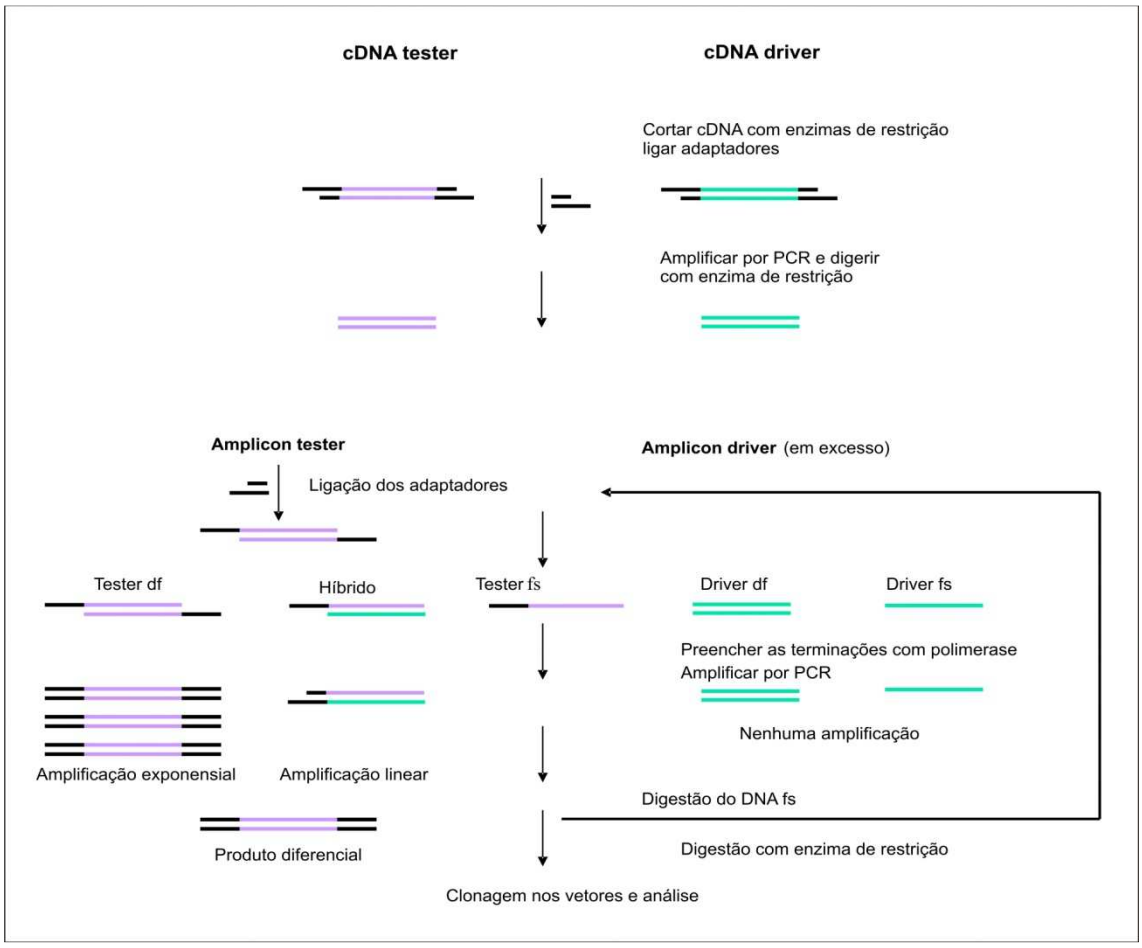

Fig.1 – Diagrama esquemático da metodologia do RDA. Os cDNAs são digeridos com enzima de restrição *Sau*3AI para gerar fragmentos eficientemente amplificáveis por PCR e com sítios de restrição para ligar os adaptadores. Os produtos da digestão são purificados em sistema comercial GFX (GE Healthcare, Chalfont St. Giles, UK), ligados aos adaptadores (16 h a 16 °C) e amplificados por PCR (25 ciclos de 45 s a 95 °C e 4 min a 72°C, cada). Os produtos finais da reação de PCR são purificados com o sistema comercial GFX. Ambos, *tester* e *driver*, são digeridos com *Sau*3AI para remoção dos adaptadores e purificados antes da ligação de um novo par de adaptadores somente no *tester*. Para a geração do primeiro produto diferencial, *driver* e *tester* são hibridizados, numa relação de 10:1, por 16 h a 67 °C e amplificados por PCR (7 ciclos de 45 s a 95 °C e 3 min a 72 °C, cada). Os produtos são submetidos a uma nova etapa de amplificação (20 ciclos) em que os *teste*rs dupla fita (df) são exponensialmente amplificados, e os cDNAs fita simples (fs) são removidos. Para geração de um segundo produto diferencial, novos adaptadores são ligados ao primeiro produto diferencial, que é hibridizado ao *driver* numa relação de 100:1.


JUSTIFICATIVAS

II – JUSTIFICATIVA

A PCM é uma doença de distribuição geográfica restrita à America Latina, sendo que mais de 80% dos casos notificados ocorrem no Brasil. A incidência de doenças fúngicas sistêmicas tanto em indivíduos saudáveis como nos imunocomprometidos mostra um padrão crescente em todo mundo nos últimos anos, convertendo as doenças fúngicas em um importante campo de pesquisa médica. Na última década, estudos genômicos têm se mostrado um marco na caracterização de fatores de virulência fúngica, tornando-se um ponto de partida para o conhecimento da patogênese desses micro-organismos.

A aderência a células do hospedeiro é, para muitos patógenos, o passo inicial no estabelecimento da infecção. Uma vez que os mecanismos de adesão e infecção de *P. brasiliensis* ainda são pouco conhecidas, faz-se relevante a utilização de uma abordagem subtrativa para identificação de genes potencialmente envolvidos na adesão do fungo a componentes da MEC do hospedeiro. A caracterização desses genes pode trazer maior conhecimento sobre a interação do fungo com o hospedeiro, proporcionando bases moleculares e bioquímicas para que novos métodos diagnósticos e tratamentos mais eficazes sejam desenvolvidos.

OBJETIVOS

III – OBJETIVOS

II. 1 – Objetivo geral do projeto

O presente trabalho teve como objetivo a identificação de genes envolvidos no processo de adesão de *P.brasiliensis* em modelo experimental *in vitro*.

III. 2 – Objetivos específicos do protejo

- Identificar os cDNAs superexpressos em modelos de adesão *in vitro*.

- Analisar a expressão dos transcritos através de RT-PCR em Tempo Real.

- Promover a expressão heteróloga de proteínas recombinantes ou síntese de peptídeos sintéticos.

- Caracterizar funcionalmente potenciais adesinas.

IV – ESTRATÉGIAS EXPERIMENTAIS

As etapas experimentais realizadas neste estudo estão resumidas na Fig. 2.

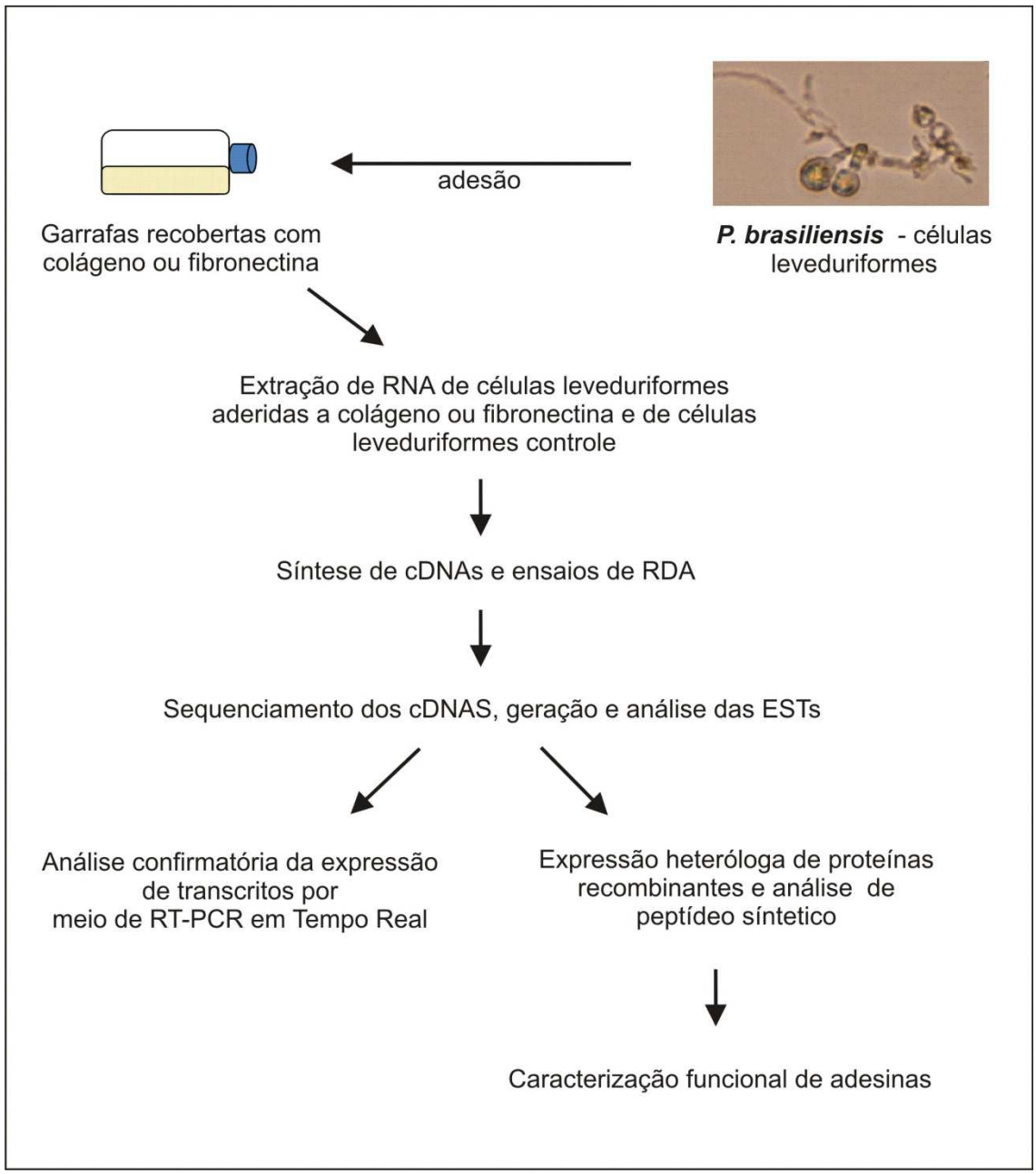

Fig.2 – Síntese das etapas experimentais. Garrafas para cultura de células foram cobertas com colágeno tipo I [50 µg/ml] ou fibronectina [50 µg/ml] diluídos em tampão carbonato ($NaHCO_3$ 0,2 M, Na_2CO_3 0.2 M, [pH 9,6]) e incubadas por 1 h a 37°C e por 16 h a 4°C. Em seguida, foram realizadas três lavagens com PBS 1X com 0,1% de Tween 20 e uma suspensão de 10^8 células leveduriformes de *P.brasiliensis* foi adicionada às garrafas. Após uma hora de incubação, a suspensão foi retirada e as garrafas foram lavadas com PBS 1X-Tween 20. Procedeu-se a extração de RNA das células aderidas e das células controle. A primeira e a segunda fitas de

cDNA foram sintetizadas e usados na técnica de RDA. O seqüenciamento dos cDNAs diferenciamente expressos foi realizado e as ESTs foram obtidas. Após a análise das ESTs, cDNAs foram selecionados para a expressão de proteínas recombinantes em sistema heterólogo e caracterização funcional de potenciais adesinas. Foi também obtido um peptídeo cognato sintético para *Pb*Ctr3 (como descrito por Dantas *et al.*, 2009) que foi utilizado em ensaio de ligação a componentes de matriz extracelular.


MANUSCRITOS

A comparative transcriptome analysis of *Paracoccidioides brasiliensis* during *in vitro* adhesion to type I collagen and fibronectin: identification of potential adhesins

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Keywords: ***Paracoccidioides brasiliensis*****, adhesin, RDA, enolase, cooper transporter**

Abstract

Paracoccidioidomycosis is caused by the dimorphic fungus *Paracoccidioides brasiliensis*. Extracellular matrix (ECM) plays an important role in the regulation of cell adhesion, differentiation, migration and proliferation of cells. An *in vitro* binding assay of *P. brasiliensis* yeast cells adhered to type I collagen and fibronectin was performed in order to identify novel adhesins of *P. brasiliensis*. Representational Difference Analysis (RDA) was employed to identify genes up regulated in the *in vitro* adhesion condition. Expressed sequence tags (ESTs) from the cDNA libraries generated by RDA technique were analysed. Genes related to functional categories such as metabolism, transcription, energy, protein synthesis and fate, cellular transport, biogenesis of cellular components were up regulated. Transcripts encoding *P. brasiliensis* enolase and cooper transporter were identified and further characterized. Recombinant enolase and a synthetic peptide designed to the cooper transporter in *P. brasiliensis* were able to bind ECM components. Additionally, the up regulation of selected genes was demonstrated by qRT-PCR. In synthesis, the strategy has resulted adequate to characterize potential *P. brasiliensis* adhesins.

Keywords: *Paracoccidioides brasiliensis*, adhesin, RDA, enolase, cooper transporter

1. Introduction

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis (PCM), a human systemic mycosis, prevalent in South America (Restrepo et al., 2001). In the soil the fungus grows as saprobic mycelium, resulting in the formation of propagules. After reaching the host, the fungus must convert to the yeast form, a fundamental step for the successful establishment of the infection (San-Blas & Nino-Vega, 2002). The propagules adhere to and invade the alveolar cells and the basal lamina (Hanna et al., 2000; Gonzalez et al., 2008). Alveolar basal lamina is composed of a specialized extracellular matrix (ECM), in which laminin, collagen and fibronectin can be found (Dunsmore & Rannels, 1996).

Adherence of the pathogens to the host cells is considered an essential step in the establishment of infection (Marchais et al., 2005; Sillanpää et al., 2009). *P. brasiliensis* has been shown to adhere to extracellular matrix proteins. Several studies have established the role of some *P. brasiliensis* proteins in the adherence process. An antigenic component of *P. brasiliensis*, gp43, is a glycoprotein that binds laminin, leading to increased pathogenicity of yeast cells (Vicentini et al., 1994). Gonzalez et al. (2005) demonstrated that two proteins with molecular masses of 19 and 32 kDa are present on fungal surface and interact with laminin, fibronectin and fibrinogen. Also, Andreotti et al. (2005) demonstrated that a *P. brasiliensis* 30 kDa protein is able to bind laminin. We have characterized some *P. brasiliensis* adhesins such as *Pb*Dfg5p (defective for filamentous growth protein Dfg5p) that was detected by electron microscopy in the cell wall of the fungus and binds laminin, fibronectin and types I and IV collagen (Castro et al., 2008). Also, triosephosphate isomerase (*Pb*TPI) which binds laminin and fibronectin (Pereira et al., 2007) and glyceraldehyde-3-phosphate dehydrogenase (*Pb*GAPDH) which binds fibronectin, type I collagen, and laminin

(Barbosa et al., 2006) were found in *P. brasiliensis* cell wall mediating fungal adherence to *in vitro* cultured cells. Malate sinthase (*Pb*MLS) binds fibronectin and types I and IV collagen and also is present in the *P. brasiliensis* cell wall (Neto et al., 2009). Therefore, *P. brasiliensis* seems to have several proteins involved in adhesion and the knowledge of them could help in the understanding of the first steps in the pathogenicity of this fungus.

In order to obtain a more comprehensive view on the adhesion process of *P. brasiliensis*, we used cDNA representational difference analysis (cDNA-RDA) to identify genes induced during incubation of *P. brasiliensis* yeast cells with ECM components. Fibronectin is a multifunctional extracellular matrix and plasma protein that plays a central role in cell adhesion (Ruoslahti, 1988). Collagens are the commonest matrix molecule. Over 20 genetically distinct collagens have been identified (Lyons and Jones, 2007). For members of the Streptococcus group, adherence to collagen type I was found to be the most common phenotype exhibited by 76% of isolates, followed by collagen type IV (53%), fibrinogen (47%), collagen type V (35%) and fibronectin (35%) (Sillanpää et al., 2008). Hence, we demonstrated in this study the involvement of these ECM proteins in the adherence of *P. brasiliensis* to the host and described some putative adhesins.

2. Materials and Methods

2.1. Fungal isolate and growth conditions

P. brasiliensis isolate Pb 01 (ATCC MYA-826) has been studied at our laboratory (Barbosa et al., 2006; Bailão et al., 2006). It was cultivated at 36 °C, in Fava-Netto´s medium [1% (w/v) peptone; (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5%

(w/v) beef extract; 0.5% (w/v); 0.5% (w/v) NaCl; 4% (wt/vol) glucose; 1% (w/v) agar; pH 7.2] for 4 days.

2.2. Adherence assay on polystyrene flasks

Adherence assays were performed essentially as described by Penalver et al. (1996) with some modifications. Briefly, polystyrene flasks (Corning® Ultra-Low Attachment 75cm² Rectangular Canted Neck Cell Culture Flask) were coated with type I collagen or fibronectin at 50 µg/ml in coating buffer ($NaHCO_3$, Na_2CO_3, [pH 9,6]) and incubated for 1 h at 37 °C and then overnight at 4 °C. The plates were blocked by adding PBS (1 mM $Na_2HPO_4.2H_2O$, 1 mM $NaH_2PO_4.H_2O$, 50 mM NaCl, pH 7.4) -1% BSA (w/v), washed three times with PBS-0.1% Tween 20 (v/v) and yeast cell suspension (10^8/ml) in PBS was added. Control yeast cells were incubated in PBS-1% BSA. The plates were incubated for 1 h at 37 °C and washed three times with PBS-0.1% Tween 20 (v/v) following RNA isolation.

2.3. RNA isolation

Total RNA from *P. brasiliensis* was obtained by the Trizol method, according to manufacturer's instructions (GIBCO, Invitrogen, Carlsbard, CA, USA). The RNAs were used to construct double-stranded cDNAs.

2.4. Subtractive hybridization and generation of subtracted libraries

Subtractive hybridization was performed as previous described by Bailão et al. (2006). Briefly, 1.0 µg of total RNAs was used to produce cDNA. The synthesis of the first-strand was performed with SuperScript II reverse transcriptase (Invitrogen Life Technologies) and used as template to synthesize double stranded cDNA. The resulting cDNAs were digested with the restriction enzyme *Sau3*AI. Subtracted cDNA libraries

were constructed using driver cDNA from RNAs extracted from control and tester cDNAs synthesized from RNAs extracted from *P. brasiliensis* adhered to type I collagen or fibronectin. The resulting products were purified using a GFX kit (GE Healthcare, Chalfont St. Giles, UK). The tester-digested cDNA was ligated to adapters (a 24-mer annealed to a 12-mer) and amplified by PCR. The amplicons were digested with *Sau3*AI to remove the adapters that had been incorporated into cDNAs and, after spin-column purification, a new 24-mer adapter was ligated onto the cDNA tester and a different one was ligated onto the cDNA driver. The cDNA driver was PCR amplified and, after cleavage to remove the adapters, it was purified and quantified.

For the generation of the differential products, tester and driver cDNAs were mixed, hybridized at 67 °C for 18 h and amplified by PCR with the 24-mer adapter. Two successive rounds of subtraction and PCR amplification using hybridization tester-driver ratios 1:10 and 1:100 were performed. The adapters used for subtractive hybridizations are listed in Table 1, supplementary material.

After the second subtractive reaction, the final amplified cDNAs were cloned into pGEM-T Easy (Promega, Madison, USA). *Escherichia coli* XL1 Blue competent cells were transformed with the ligation products. Selected colonies were picked and grown in microliter plates, and plasmid DNA was prepared. In order to generate expressed sequence tags (ESTs), single-pass, 5'-end sequencing of cDNAs by standard fluorescence labeling dye-terminator protocols with T7 flanking vector primer was performed. Samples were loaded onto a MegaBACE 1000 DNA sequencer (GE Healthcare) for automated sequencing analysis.

2.5. EST processing pipeline, annotation and sequence analysis

The EST sequences were preprocessed using Phred (Ewing & Green, 1998) and Crossmatch programs (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm) and were then assembled into contigs by using CAP3 (Huang & Madan, 1999), all these tools integrated in a specific pipeline (http://www.lbm.icb.ufg.br/pipelineUFG/). Only sequences with at least 75 nucleotides and PHRED quality greater or equal to 20 were considered. ESTs were screened for vector sequences against the UniVec data. The clustered sequences were compared using Blast X against the GenBank non-redundant (nr) database from National Center for Biotechnology Information (NCBI) and the nucleotide database generated from *P. brasiliensis* structural genome (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/ MultiHome.html). The database sequence matches were considered significant at E-values $\leq 10^{-10}$.

The search for functional categories was performed by using the bioinformatic tool Blast2GO that joints in one application GO annotation based on similarity searches with statistical analysis and highlighted visualization on directed acyclic graphs (Conesa et al., 2005). The Blast2GO annotation algorithm already took multiple parameters into account such as sequence similarity, BLAST HSP (highest scoring pair) length and e-values, the GO hierarchical structure and GO term evidence codes (Conesa et al., 2005; Götz et al., 2008). Sequences were grouped in functional categories according to the classification of the MIPS functional catalog (Munich Center for Protein Sequences; http://mips.gst.de/).

2.6. Quantitative analysis of RNA transcripts by reverse transcription real-time (qRT-PCR)

This assay was performed to confirm the RDA results and the reliability of our approaches. Total RNA from *P. brasiliensis* control yeast cells and from yeast cells adhered to type I collagen or fibronectin were obtained as previously described, in independent experiments from that used in RDA analysis. Total RNAs treated with DNAse were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo $(dT)_{15}$ primer. qRT-PCR was performed in triplicates, with samples from three independent experiments in the StepOnePlus™ real time PCR system (Applied Biosystems, Foster City, CA). The PCR thermal cycling was 40 cycles of 95 °C for 15 s; 60 °C for 1 min. The SYBR green PCR master mix (Applied Biosystems) was used as reaction mixture, added of 10 pmol of each specific primer and 40 ng of template cDNA, in a final volume of 20 µl. A melting curve analysis was performed to confirm a single PCR product. The data were normalized with the transcript for α-tubulin amplified in each set of qRT-PCR experiments. A non-template control was included. A cDNA for a relative standard curve was generated by pooling an aliquot from each cDNA sample. The standard curve was serially diluted 1:5, and a standard curve was generated using five samples from the pooled cDNA. Relative expression levels of genes of interest were calculated using the standard curve method for relative quantification (Bookout et al., 2006). The specific primers, sense and antisense were described in Table 1, supplementary material.

2.7. Cloning the cDNA encoding enolase into expression vector and purification of the recombinant protein

The complete enolase cDNA (GenBank accession number EF558735.1), obtained from a library from yeast cells of *P. brasiliensis* (Costa et al., 2007), was amplified by PCR employing primers, as described in Table 1, supplementary material. The PCR product was cloned in-frame with the glutathione S-transferase (GST) coding region of the pGEX-4T3 vector to yield the GST-*Pb*Eno construct. The *Escherichia coli* strain BL21 pLys competent cells were transformed with the expression construct, as previously described (Nogueira et al., 2010, in press).

Bacteria transformed with the GST-*Pb*Eno construct were grown in Luria Bertani (LB) medium supplemented with ampicillin (100 μg/ml) and glucose (20 mM/ml) at 37°C, 200 rpm. At an A_{600} of 0.6, protein production was induced by addition of isopropyl-β-d-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. Growth was proceeded for 16 h, at 15 °C and the cells were harvested by centrifugation. The *E. coli* bacterial pellets were resuspended in PBS, incubated on ice for 30 min and sonicated 15 times during 60 s each, on ice. The GST- *Pb*Eno protein was affinity purified using glutathione Sepharose 4B (GE Healthcare) according to manufacturer's protocol and *Pb*Eno was released from GST-*Pb*Eno by the addition of thrombin (Sigma Aldrich). The cleavage reaction was stopped by freezing the sample at – 20 °C. The purity and integrity of the protein were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining.

2.8. Affinity ligand assays and dot blot analysis

Far-Western assays were carried out as previously described (Barbosa et al., 2006; Castro et al., 2008) [10, 8]. Recombinant enolase was submitted to SDS-PAGE and

blotted onto nitrocellulose membranes. Blotted protein was assayed for laminin, fibronectin, type I and type IV collagen binding as following. The blotted membranes were blocked for 4 h with PBS-1% BSA (w/v) and 5% (w/v) milk, incubated with laminin (30 µg/ml), fibronectin (30 µg/ml), type I collagen (20 µg/ml), or type IV collagen (20 µg/ml) diluted in PBS-1% BSA (w/v) for 90 min and then washed three times with PBS-0.1% Tween 20 (v/v). The membranes were incubated overnight with rabbit antibodies antilaminin, antifibronectin, anti-type I collagen or anti type IV collagen (diluted 1:100). The blots were washed with PBS-0.1% Tween 20 (v/v) and incubated with peroxidase-labeled goat anti-rabbit immunoglobulin (diluted 1:1000) for 2 h. The blots were washed with PBS-0.1% Tween 20 (v/v), and the reactive bands were developed with hydrogen peroxide and diaminobenzidine (Sigma) as the chromogenic reagent. As a negative control, r*Pb*Eno was incubated only with peroxidase-labeled goat anti-rabbit immunoglobulin, in the absence of the ECM proteins (laminin, fibronectin and type I and IV collagen). Additional control was obtained by incubating r*Pb*Eno with BSA.

A peptide was synthesized based on the deduced sequence of *Pb*CTR3 (GenBank accession number DQ534496) towards amino acids 90 to 130 (Dantas et al. 2009) and dot blot analysis was also performed to assay the reactivity of this peptide to ECM proteins. Reactions were performed as described above for the affinity ligand assay.

2.9. Statistical analysis

Experiments were performed in triplicates with samples in triplicates. Results were presented as means (±) standard deviation. Statistical comparisons were performed using Student´s *t* test. Statistical significance was accepted for $P < 0.05$.

3. Results

3.1. Expression profile of *P.brasiliensis* yeast cells adhered to type I collagen and fibronectin

The RDA approach was performed with RNAs obtained from three conditions: (a) *P. brasiliensis* yeast cells adhered to type I collagen; (b) *P. brasiliensis* yeast cells adhered to fibronectin; and (c) control *P. brasiliensis* yeast cells. The first and the second conditions were used independently as tester cDNA populations and the third was used as driver cDNA population. Subtraction hybridization was performed by incubating the driver and each tester. Selection of the cDNAs was achieved by construction of subtracted libraries.

For comparative analysis, the 535 ESTs from cells adhered to type I collagen were grouped in 65 clusters, represented by 30 contigs and 35 singlets. Most of the annotated ESTs (34%) corresponded to energy. A high proportion of the ESTs found in the type I collagen condition (55%) exhibited sequence similarity to genes of unknown function or encoding hypothetical proteins (Supplementary Fig. 1A). A broad view of the nature of the adaptations made by *P.brasiliensis* during adherence to type I collagen was obtained by classifying the ESTs into seven groups of functionally related genes (Table 1).

The ESTs from cells adhered to fibronectin were grouped in 62 clusters, represented by 25 contigs and 37 singlets. The analysis of 583 ESTs revealed that most of the annotated ESTs (42%) corresponded to transcripts related to cell rescue, defense and virulence (Supplementary Fig 1B) and 31% of the ESTs found in the fibronectin-binding condition did not show similarity to known *P. brasiliensis* genes. The annotated ESTs fell in nine different MIPS category, indicating a wide range of processes probably involved in *P. brasiliensis* adhesion mechanism to fibronectin (Table 2).

3.2. qRT-PCR assays in analysis of gene expression

For further confirmatory data about the expression level from EST redundancy analysis, assessment of *P. brasiliensis* alcohol dehydrogenase (*Pb*Adh), hexokinase (*Pb*Hxk), sexual development transcription factor (*Pb*NsdD), enoyl-CoA hydratase (*Pb*Enoyl-CoA), arginine N-methyltransferase (*Pb*Skb1), heat shock protein 70 *(Pb*Hsp70), cooper transporter (*Pb*Ctr3) and enolase (*Pb*Eno) were provided by qRT-PCR analysis. *Pb*Adh, *Pb*Eno and *Pb*Skb1 were confirmed to be upregulated in yeast cells adhered to type I collagen and fibronectin. *Pb*Enoyl-CoA was upregulated in cells adhered to collagen and *Pb*Ctr3, *Pb*Hsp70, *Pb*Hxk and *Pb*NsdD were upregulated in the cells adhered to fibronectin (Fig. 1).

3.3. Binding assays of r*Pb*Eno and *Pb*Ctr3

The full-length cDNA encoding enolase consisted of 1684 bp with an open reading frame encoding 438 amino acids with a calculated molecular mass of 47 kDa. The cDNA encoding the *P. brasiliensis* enolase was cloned into the expression vector pGEX-4T-3 to obtain the recombinant fusion protein in *E. coli*. After induction with IPTG, a recombinant protein was detected in bacterial lysates (Fig.2A, lane 2). The fusion protein was affinity purified and r*Pb*Eno was obtained by digestion with thrombin (Fig. 2A, lane 3).

The ability of the r*Pb*Eno to bind laminin, fibronectin and type I collagen was determined by far-Western blotting assays, as shown in Fig. 2B. The r*Pb*Eno presents the ability to bind to laminin (lane 3), fibronectin (lane 4) and type I collagen (lane 5). There was no detectable reaction with type IV collagen (lane 6). Negative controls were

obtained by incubating r*Pb*Eno in the absence of the ECM proteins (lane 1), as well by using BSA (lane 2).

Also, the synthetic peptide (*Pb*Ctr3) (Fig. 2C), reacted with type I collagen (lane 2), type IV collagen (lane 3) and fibronectin (lane 4). There was no reactivity with BSA (negative control) (lane 1) and laminin (lane 5).

4. Discussion

Our objective in the present work was uncovering potential adhesins that could be expressed during the adhesion process of *P. brasiliensis* that occurs during infection. For that, in vitro adherence assays were performed. Among the identified transcripts, it was detected some encoding for described adhesins. Alcohol dehydrogenases (ADH) are oxidoreductases that catalyse the reversible oxidation of aldehydes or ketones, with the concomitant reduction of NAD^+ or $NADP^+$ (de Smidt et al., 2008). Screening a cDNA expression library of *C. albicans* yeast cells with polyclonal antiserum to human fibronectin, Klotz et al. (2001) isolated cDNA clones which encoded ADH, suggesting that this protein is found on the cell surface of this fungus and could be a receptor for fibronectin. Also, Crowe et al. (2003) in an attempt to identified *C. albicans* proteins involved in plasminogen binding, identified ADH in the cell wall protein extracts of this fungus. In addition, Albuquerque et al. (2008) found ADH as one of the protein components of *Histoplasma capsulatum* vesicles showing that this fungus can utilize a trans-cell wall vesicular transport secretory mechanism to promote virulence.

The transcript encoding enolase, was induced in both conditions. The molecule is a cell surface protein in *Staphylococcus aureus* and mediates the binding of this microorganism to laminin, potentially playing a critical role in its pathogenesis

(Carneiro et al, 2004). In addition, Esgleas et al. (2008) showed the surface localization of *Streptococcus suis* enolase and its ability to bind fibronectin. Donofrio et al. (2009) showed that *P. brasiliensis* enolase is a fibronectin binding protein. Moreover, Castaldo et al. (2009) using immune electron microscopy also showed the cell surface localization of *Lactobacillus plantarum* enolase where it can bind fibronectin and mediates the adhesion of this commensal bacteria to the human intestinal cells. We have demonstrated the presence of *P. brasiliensis* enolase at the fungus surface and cytoplasm and it binds to and activate plasminogen. Also, exposure of epithelial cells and phagocytes to *P. brasiliensis* enolase was associated with an increase expression of surface sites of adhesion (Nogueira et al., 2010, in press).

Molecular chaperones were up regulated during *P. brasiliensis* in vitro adhesion to fibronectin. DnaJ is a member of the Hsp40 family of molecular chaperones, which is also called the J-protein family, the members of which regulate the activity of Hsp70s (Walsh et al., 2004). Batista et al. (2006), reported the presence of a member of the J-domain protein family, Mdj1, in the cell surface of *P. brasiliensis*. Other chaperones have already been found in cell surface, such as Hsp60, which has been detected in small clusters at discrete points on the *H. capsulatum* cell wall, and it has been shown to mediate attachment of the fungus to macrophages via CD11/CD18 receptors (Long et al., 2006). Also, Hsp70 was found to be present on the cell wall of *C. albicans* (Eroles et al., 1997). Likewise, Hsp30, Hsp60, Hsp70 were found in secretory vesicles in *H. capsulatum* (Albuquerque et al., 2008).

Although Enoyl-CoA hidratase and C-5 sterol desaturase were not described to be involved with adhesion, they were found in secretory vesicles. The former was identified in *H. capsulatum* vesicles (Albuquerque et al., 2008), and sterols are

components of extracellular vesicles in *Criptococcus neoformans* (Rodrigues et al., 2007).

In this study, genes encoding for some enzymes involved in metabolic processes were up regulated. According to Pancholi and Chhatwal (2003) housekeeping enzymes are constitutively expressed in all organisms to perform essential metabolic functions for the purpose of survival and can be important as virulence factors for a vast variety of pathogens, interacting with host components, such as fibronectin, collagen and plasminogen. But to do so, they must be located on the surface. And, although they do not have the typical signal sequence or membrane anchoring mechanisms, they do get secreted and are displayed on the surface, probably by reassociation.

Some of the annotated hypothetical proteins have transmembrane domains with signal peptide (PAAG_01303.1, PABG_01874.1, PAAG_02061.1, PAAG_07480.1, PABG_00089.1) and one of them (PAAG_07033.1 - dynamitin) (Lien et al., 2008) was described to be involved in adhesion (tables 1 and 2).

The transcript enconding the cooper transporter *Pb*Ctr3 was up regulated in adhesion of yeast cells to fibronectin. The *Pb*Ctr3 transcript had already been shown to be over expressed in *P. brasiliensis* yeast cells derived from infected tissues (Bailão et al., 2006) and was also recognized by sera of PCM patients (Dantas et al., 2009) clearly indicating its role in the infection process. Although *Pb*Ctr3 has not been described before as an ECM-binding component, its probable localization at the cell surface should enable its binding capacity.

In conclusion, this study provides a better knowledge of proteins which can be involved in the adhesion process of *P. brasiliensis*. Indeed, some of them have already been described in the pathogenesis of this and others microorganisms and the

elucidation of the role of some hypothetical proteins could reveal more information of the molecules involved in adherence and pathogensis.

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Figure Legends

Supplementary Figure 1 – Functional classification of ESTs from *P. brasiliensis* yeast cell adhered to type I collagen (A) and fibronectin (B). The classification was performed according to the functional categories of the MIPS (http://mips.gsf.de/) functional annotation scheme.

Figure 1 – Average of gene expression of *Pb*Adh, *Pb*Eno, *Pb*Skb1, *Pb*Ctr3, *Pb*Enoyl-CoA, *Pb*Hxk, *Pb*NsdD and *Pb*Hsp70 uniformizar nomes de genes e proteínas as determined by quantitative real time RT-PCR. (**A**) qRT-PCR plot of *Pb*Adh, *Pb*Eno and *Pb*Skb1expression levels in yeast cells adhered to type I collagen and fibronectin. (**B**) qRT-PCR plot of *Pb*Enoyl-CoA expression levels in yeast cells adhered to type I collagen. (**C**) qRT-PCR plot of *Pb*Ctr3, *Pb*Hxk, *Pb*Hsp70 and *Pb*NsdD expression levels in yeast cells adhered to fibronectin. The values of expression were standardized using the values of expression of the constitutive gene

encoding to α-tubulin. The expression level was calculated by relative standard curve method. The standard deviations are presented from three independent experiments.

Figure 2 – Binding of *Pb*Eno and *Pb*Ctr3 to extracellular matrix components. (A) SDS-PAGE analysis of *P. brasiliensis* recombinant enolase (r*Pb*Eno). *E.coli* cells harboring the pGEX-4T-3-enolase plasmid were grown at 37 °C to an A600 of 0.6 and harvested before (lane 1) and after (lane 2) 16 h incubation at 15 °C with 0.1 mM IPTG. The cells were lysed by extensive sonication. Lane 3, purified r*Pb*Eno (after cleavage with thrombin). The protein extracts were fractionated by one-dimensional gel electrophoresis and stained by Coomassie blue. **(B)** Recombinant enolase (0.5 µg) was subjected to SDS-PAGE and electroblotted. Membranes were reacted with laminin (lane 3), fibronectin (lane 4), type I collagen (lane 5) and type IV collagen (lane 6), and subsequently incubated with rabbit IgG antilaminin, antifibronectin, anti-type I collagen and anti-type IV collagen antibodies respectively. Use of peroxidase-conjugated anti-rabbit IgG revealed the reactions. The negative control was obtained by incubating the r*Pb*Eno with no ECM component (lane 1) and using BSA (lane 2). **(C)** Reactivity of the synthetic peptide from *Pb*CTR3 with type I collagen (lane 2), type IV collagen (lane 3), fibronectin (lane 4), laminin (lane 5). The negative control was obtained by using BSA (lane 1).

Supplementary Figure 1

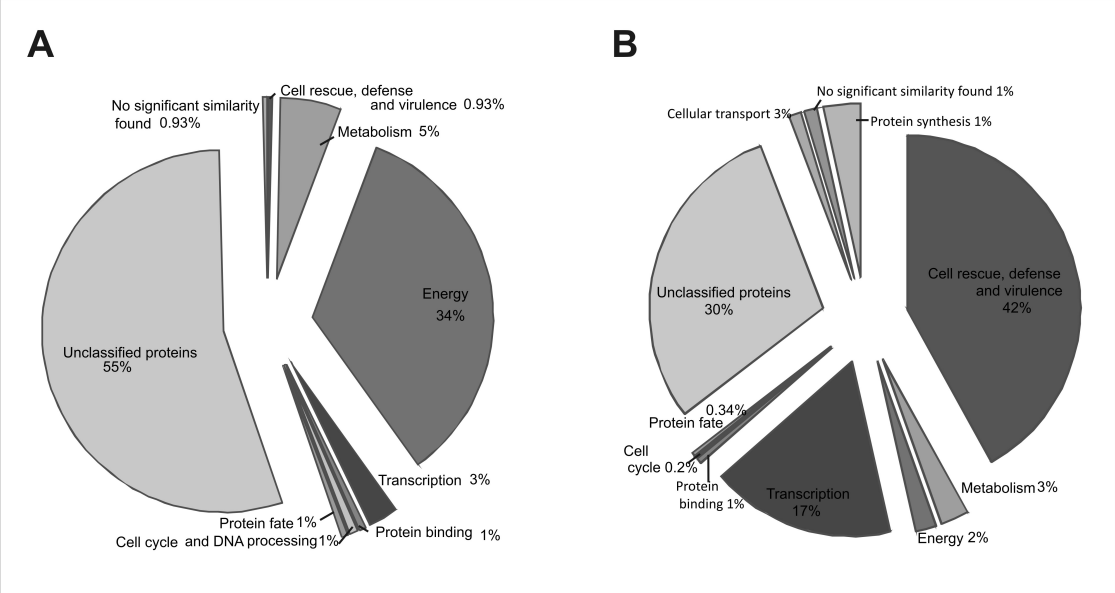

Figure 1

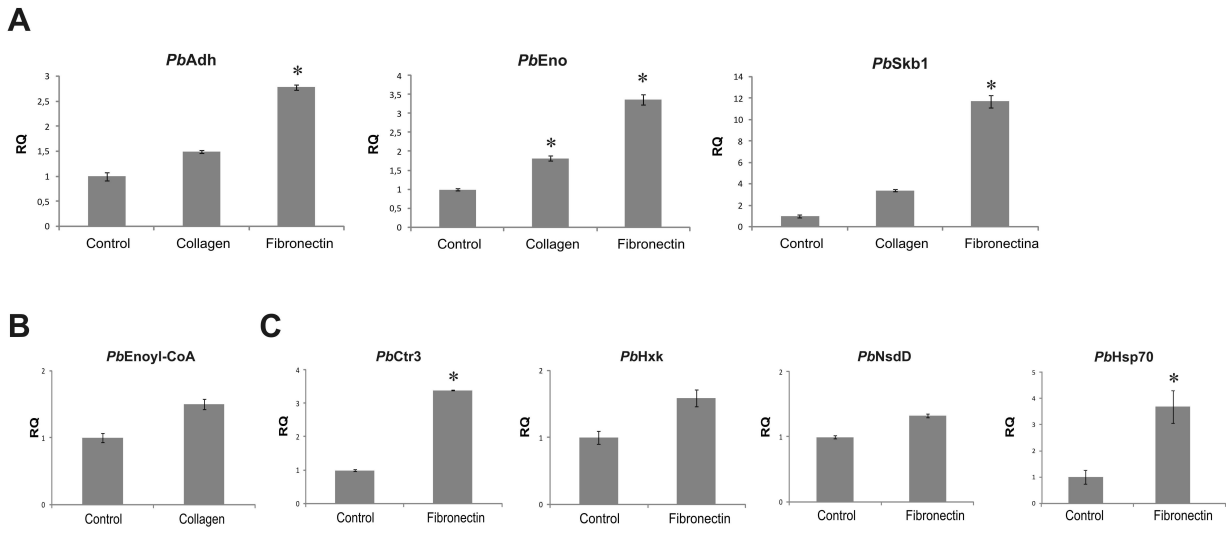

Figure 2

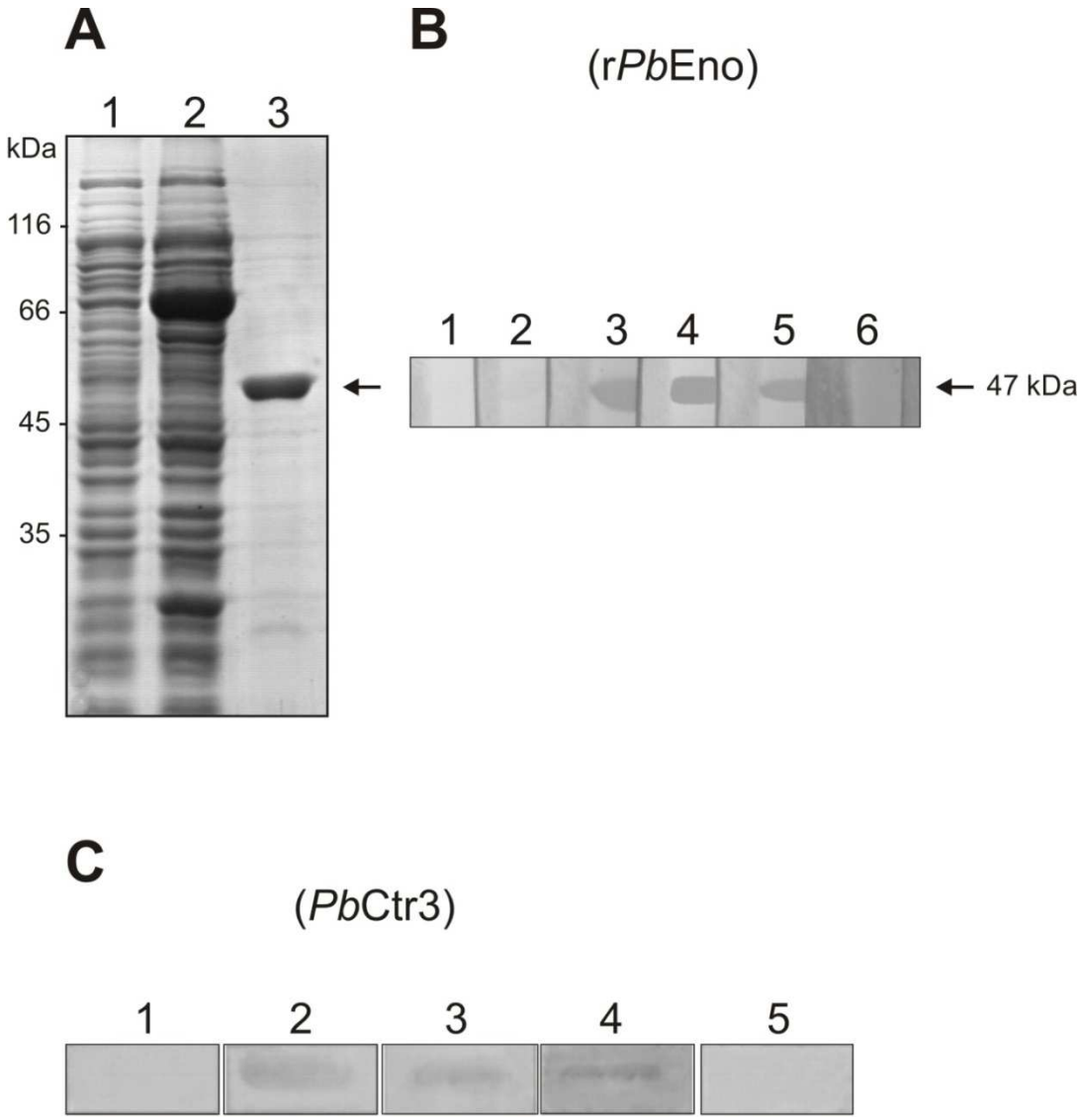

Table 1 - Annotated ESTs with high abundance in yeast cells during adhesion to collagen versus control yeast cells.

Functional category	Gene Product	Best Hit/GenBank accession number* or *P. brasiliensis* genome lócus**	e-value	Number of occurences
Metabolism	Acetamidase	*P.brasiliensis*/ PAAG_03626.1**	1 e-55	12
	Transketolase	*P. brasiliensis*/ PAAG_04444.1**	1 e-55	5
	enoyl-CoA hydratase	*P. brasiliensis*/ PABG_02862.1**	1 e-38	2
	Mitochondrial protein potentially involved in regulation of respiratory metabolism	*Saccharomyces cerevisiae*/ NP_690845.1*	3 e-11	8
	Alcohol dehydrogenase[a]	*P.brasiliensis*/ PAAG_04541.1**	1 e-51	1
Energy	NADH dehydrogenase (integral membrane protein)	*P. brasiliensis*/ PAAG_04760.1**	1 e-26	176
	Enolase[a]	*P. brasiliensis*/ PAAG_00771.1**/ EF558735.1*	1 e-56	3
Transcription	transcription factor MetR	*P. brasiliensis*/ PAAG_04371.1**	1 e-14	5
	endoribonuclease ysh1 (Bzip)	*P. brasiliensis*/ PAAG_08788.1**	1 e-76	6
	SWI/SNF transcription activation complex subunit	*P. brasiliensis*/ PAAG_06542.1**	1 e-52	1
	protein krueppel	*P. brasiliensis*/ PAAG_06709.1**	1 e-27	1
	Pre mRNA splicing factor prp1	*P. brasiliensis*/ PAAG_00995.1**	1 e-26	1
Protein binding	FAD-linked sulfhydryl oxidase	*P. brasiliensis*/ PAAG_06132.1**	1 e-35	2
	cytosolic Fe-S cluster assembling factor NBP35	*P. brasiliensis*/ PAAG_03944.1**	1 e-112	1
Cell cycle and DNA processing	DNA polymerase epsilon subunit c	*P. brasiliensis*/ PAAG_00002.1**	1 e-10	4
Cell rescue, defense and virulence	Hsp98/hsp104	*P. brasiliensis*/ PAAG_02130.1**	1 e-49	2
Protein fate (folding, modification, destination)	arginine N-methyltransferase skb1[a]	*P. brasiliensis*/ PAAG_02402.1**	1 e-85	3
Unclassified proteins	senescence associated protein	*Pisum sativum* / BAB33421.1*	1 e-30	32
	conserved hypothetical protein	*P. brasiliensis*/ PAAG_08039.1**	1 e-19	2
	conserved hypothetical protein	*P. brasiliensis*/ PABG_01516.1**	1 e-13	1

	conserved hypothetical protein	*P. brasiliensis*/ PAAG_04760.1**	1 e-26	1
	conserved hypothetical protein	*P. brasiliensis*/ PAAG_01303.1**	1 e-34	1
	conserved hypothetical protein	*P. brasiliensis*/ PAAG_07033.1**	1 e-13	16
	conserved hypothetical protein	*P. brasiliensis*/ PABG_03557.1**	1 e-34	2
	conserved hypothetical protein	*P. brasiliensis*/ PABG_07127.1**	1 e-18	6
	hypothetical protein	*P.brasiliensis*/ PABG_06807.1**	1 e-27	167
	hypothetical protein	*P. brasiliensis*/ PAAG_07288.1**	1 e-36	49
	hypothetical protein	*P. brasiliensis*/ PABG_01874.1**	1 e-64	4
	hypothetical protein	*P. brasiliensis*/ PAAG_03580.1**	1 e-49	3
	hypothetical protein	*P. brasiliensis*/ PAAG_02061.1**	1 e-19	2
No significant similarity found				2

[a] – transcripts overexpressed in the presence of type I collagen and fibronectin

Table 2 - Annotated ESTs with high abundance in yeast cells during adhesion to fibronectin versus control yeast cells.

Functional category	Gene Product	Best Hit/GenBank accession number* or *P. brasiliensis* genome lócus**	e-value	Number of occurences
Metabolism	alanine-glyoxylate aminotransferase	*P. brasiliensis*/ PAAG_03138.1**	1e-105	5
	Betaine aldehyde dehydrogenase	*P. brasiliensis*/ PAAG_05392.1**	1e-63	7
	Mitochondrial NADP specific isocitrate dehydrogenase	*P. brasiliensis*/ PAAG_08351.1**	1e-57	1
	Alcohol dehydrogenase[a]	*P. brasiliensis*/ PAAG_00403.1**	1e-59	1
	C-5 sterol desaturase	*P. brasiliensis*/ PAAG_03651.1**	1e-68	1
Energy	Enolase[a]	*P. brasiliensis*/ PAAG_00771.1**/EF558735.1*	1e-43	10
	hexokinase-1	*P. brasiliensis*/ PAAG_01377.1**	1e-15	1
Transcription	C2H2 transcription factor (Seb1)	*P. brasiliensis*/ EEH47059.1*	1e-21	4
	Sexual development transcription factor NsdD	*P. brasiliensis*/ PAAG_05818.1**	1e-47	74
	C2H2 transcription factor (Con7)	*Ajellomyces dermatitidis*/ EEQ91999.1*	1e-52	1
	C6 transcription factor (Ctf1B)	*P. brasiliensis*/ PAAG_01359.1**	1e-12	2
	NF-X1 finger transcription factor	*Ajellomyces dermatitidis*/ EEQ87210.1*	7e-89	15
	APSES transcription factor	*Aspergillus fumigatus*/ EDP51876.1*	1e-41	1
	forkhead box protein D1	*P. brasiliensis*/ PAAG_07388.1**	1e-14	1
	transcription factor atf1	*P. brasiliensis*/ PAAG_01945.1**	1e-22	2
Protein binding	SCP-like extracellular	*P. brasiliensis*/XP_752604.1*	1e-50	1
	ribosomal protein mrp4	*P. brasiliensis*/ PAAG_07873.1**	1e-70	1
	Hsp90 binding co-chaperone (Sba1)	*P. brasiliensis*/ PAAG_05226.1**	1e-16	1
Cell cycle and DNA processing	cell cycle inhibitor Nif1	*Ajellomyces capsulatus*/ EER43226.1*	1e-15	1
Cell rescue, defense and virulence	HSP70	*P. brasiliensis*/ PAAG_08003.1**	1e-37	231
	HSP60	*P. brasiliensis*/ PAAG_08059.1**	1e-56	7
	HSP30	*P. brasiliensis*/ PAAG_00871.1**	1e-62	5
	DnaJ domain protein Psi	*P. brasiliensis* /PAAG_00478.1**	1e-24	1
Cellular transport, transport facilities and	*Pb*Ctr 3- high affinity copper transporter	*P. brasiliensis*/ PAAG_05251.1**/EU530695*	1e-92	15

transport routes				
	Mechanosensitive ion channel family	*P. brasiliensis*/ PAAG_01645.1**	1e-84	2
	Golgi membrane protein (Coy1)	*P. brasiliensis*/ PAAG_05425.1**	1e-53	1
	Benomyl/ methotrexate resistance protein	*P. brasiliensis*/ PAAG_07478.1**	1e-84	2
Protein fate (folding, modification, destination)	galactosyltransferase	*P. brasiliensis*/ PADG_00117.1**	1e-66	1
	arginine N-methyltransferase skb1[a]	*P. brasiliensis*/ PAAG_02402.1**	1e-61	1
Protein synthesis	CAP20	*P. brasiliensis*/ PAAG_06538.1**	1e-79	7
Unclassified proteins	Urg3	*P. brasiliensis*/ PABG_03978.1**	1e-89	3
	conserved hypothetical protein	*P. brasiliensis*/ PAAG_08906.1**	1e-23	103
	conserved hypothetical protein	*P. brasiliensis*/ PADG_08537.1**	8e-45	40
	conserved hypothetical protein	*P. brasiliensis*/ PAAG_05634.1**	0.0	1
	conserved hypothetical protein	*P. brasiliensis*/ PAAG_03559.1**	0.0	2
	conserved hypothetical protein	*P. brasiliensis*/ PAAG_07480.1**	0.0	1
	conserved hypothetical protein	*P. brasiliensis*/ PAAG_00128.1**	0.0	1
	hypothetical protein	*P. brasiliensis*/ PAAG_01169.1**	6e-26	8
	hypothetical protein	*P. brasiliensis*/ PAAG_00089.1**	0.0	3
	hypothetical protein	*P. brasiliensis*/ PAAG_08515.1**	1e-33	3
	hypothetical protein	*P. brasiliensis*/ XP_002484510.1*	1e-44	1
	hypothetical protein	*Shewanella oneidensis*/ NP_717361.1*	3e-11	3
	hypothetical protein	*P. brasiliensis*/ PAAG_03092.1**	0.0	1
	hypothetical protein	Gibberella zeae/ XP_382291.1*	1e-11	1
No significant similarity found				7

[a] – transcripts overexpressed in the presence of type I collagen and fibronectin

Supplementary Table 1 – Oligonucleotide primers used in RDA analysis, DNA cloning and sequencing and qRT-PCR analysis.

Oligonucleotides	**Sequence**	**Purpose**
cDNA	5’ AGCAGTGGTATCAACGACAGAGTACGCGGG 3’	cDNA first strand synthesis
CDS	5’ AAGCAGTGGTATCAACGCAGAGTACT(30)N1N 3’	cDNA first strand synthesis
PCRII	5’ AAGCAGTGGTATCAACGCAGAGT 3’	cDNA first strand synthesis
JBam12	5’ GATCCGTTCATG 3’	Adapter1 (RDA)
JBam24	5’ ACCGACGTCGACTATCCATGAACG 3’	Adapter 1 (RDA)
NBam12	5’ GATCCTCCCTCG 3’	Adapter 2 (RDA)
NBam24	5’ AGGCAACTGTGCTATCCGAGGGAG 3’	Adapter2 (RDA)
RBam12	5’ GATCCTCGGTGA 3’	Adapter 3 (RDA)
RBam24	5’ AGCACTCTCCAGCCTCTCTCACCGAG 3’	Adapter 3 (RDA)
T7	5’ GTAATACGACTCACTATAGGGC 3’	DNA sequencing
ENO Sense	5’-<u>GTC GAC</u> ATG GCT ATC ACC AAA ATC CAC G-3’ *	enolase cDNA amplification
ENO Antisense	5’-<u>GCG GCC GC</u>T TAC ATA TTA ATA GCTVGCC C-3’ *	enolase cDNA amplification
Tubulina Sense	5’-ACAGTGCTTGGGAACTATACC-3’	qRT-PCR
Tubulina Antisense	5’-GGGACATATTTGCCACTGCC-3’	qRT-PCR
*Pb*Adh Sense	5’-ATCATACGACGGGGCTTCTG-3’	qRT-PCR
*Pb*Adh Antisense	5’-AGTGGTAAAAGTTGGATGATTG-3’	qRT-PCR
*Pb*Enoyl-CoA Sense	5’- TACCCCTGTCATCGCTGCC-3’	qRT-PCR
*Pb*Enoyl-CoA Antisense	5’-TCTTCCCAATTGCTCTCGTGA -3’	qRT-PCR
*Pb*NsdD Sense	5’-CAAAAAACGACGAGGGAAAGC-3’	qRT-PCR
*Pb*NsdD Antisense	5’-ACTTCCGGGTTAACTTGGCG-3’	qRT-PCR
*Pb*skb1 Sense	5’-CGCAGGAGGGGGATTATGA-3’	qRT-PCR
*Pb*skb1 Antisense	5’-GGTGTCAAAAAGGTATCATCAG-3’	qRT-PCR
*Pb*Eno Sense	5’-GATTTGCAGGTTGTCGCCGA-3’	qRT-PCR
*Pb*Eno Antisense	5’-TGGCTGCCTGGATGGATTCA-3’	qRT-PCR
*Pb*Ctr3 Sense	5’-CATGTCATCAATGCCTGCTTC-3’	qRT-PCR
*Pb*Ctr3 Antisense	5’-TGCAGGCGGGTCGGGAGA-3’	qRT-PCR
*Pb*Hxk Sense	5’-GTCAATACCGAACTTAGCATGT-3’	qRT-PCR
*Pb*Hxk Antisense	5’-ATGACCAACCGCACGATCTC-3’	qRT-PCR

* *Sal*I and *Not*I restriction sites (underlined letters), respectively.

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Paracoccidioides brasiliensis enolase is a surface protein that binds plasminogen and mediates interaction of yeast forms with host cells

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Abstract

Paracoccidioidomycosis (PCM), caused by the dimorphic fungus *Paracoccidioides brasiliensis*, is a disseminated, systemic disorder that involves lungs and other organs. The ability of the pathogen to interact with host components, including extracellular matrix (ECM) proteins, is essential to further colonization, invasion and growth. Previously enolase (EC 4.2.1.11) was characterized as a fibronectin-binding protein in *P. brasiliensis*. Interaction of surface-bound enolase with plasminogen has been incriminated in tissue invasion for pathogenesis in several pathogens. In this paper enolase was expressed in *Escherichia coli* as a recombinant GST fusion protein (r*Pb*Eno). The *P. brasiliensis* native enolase (*Pb*Eno) was detected at the fungus surface and cytoplasm by immunofluorescence using an anti- r*Pb*Eno antibody. Immobilized purified r*Pb*Eno bound plasminogen in a specific, concentration-dependent fashion. Both native and r*Pb*Eno activated conversion of plasminogen to plasmin through tissue plasminogen activator. The association between *Pb* Eno and plasminogen was lysine dependent. In competition experiments, purified r*Pb*Eno, in its soluble form, inhibited plasminogen binding to fixed *P. brasiliensis*, suggesting that this interaction required surface-localized *Pb*Eno. Plasminogen coated *P. brasiliensis* yeast cells were capable of degrading purified fibronectin providing *in vitro* evidence for the generation of active plasmin on the fungus surface. Exposure of epithelial cells and phagocytes to enolase was associated with an increased expression of surface sites of adhesion. In fact, the association of *P. brasiliensis* with epithelial cells and phagocytes was increased in the presence of r*Pb*Eno. The expression of *Pb*Eno was up regulated in yeast cells derived from mouse-infected tissues. These data indicate that surface associated *Pb*Eno may contribute to the pathogenesis of *P. brasiliensis*.

47 **Keywords: *Paracoccidioides brasiliensis*, enolase, plasminogen activation,**

48 **fibrinogen degradation, adhesion.**

49

Introduction

Microbial adhesion to host tissues is the initial event of most infectious process (39). Interaction with extracellular matrix (ECM) proteins has been correlated with the invasive ability of different organisms (40; 28). ECM underlines epithelial and endothelial cells and surrounds connective tissues and its major components are the collagens, laminin, fibronectin and proteoglycans (52). After adherence the next step must be to overcome the barriers imposed by epithelial tissues and ECM. The proteolytic activity achieved by subversion of host proteases by pathogens, such as plasmin, has been shown to be important during many infections process (51, 47).

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis (PCM), a human systemic mycosis that constitutes a major health problem in South America (44). Clinical manifestations of PCM are related to chronic granulomatous reactions with involvement of the lung, reticulo-endothelial system, as well as mucocutaneous areas and other organs (22). In the soil the fungus grows as saprobic mycelium, resulting in the formation of infectious propagules. After penetrating the host, the fungus differentiates into its yeast form, a fundamental step for the successful establishment of the disease (46).

Although not traditionally considered as a typical intracellular pathogen, independent studies have demonstrated that *P. brasiliensis* yeast cells have the capacity to adhere and invade host cells (4, 24, 31). *P. brasiliensis* may actively penetrate the mucocutaneous surface and parasitize epithelial cells, thus evading the host defenses and reaching deeper tissues.

Fungal ECM-binding adhesins have been characterized in different models, including *P. brasiliensis*. Vicentini et al. (49) showed specific binding of the protein gp43 to laminin, which is correlated to the fungus´ adhesiveness *in vitro* as well as to an enhancement of

pathogenic potential. We have been systematically searching for new adhesion proteins in *P. brasiliensis* with potential to play roles in the fungal virulence and proteins such as *Pb*MLS (malate synthase) (34), *Pb*Dfg5p (defective for filamentous growth protein) (9), triosephosphate isomerase (*Pb*TPI) (41) and glyceraldehyde-3-phosphate dehydrogenase (*Pb*GAPDH) (4) were found to associate with ECM components. In particular, enolase from *P. brasiliensis* is a fibronectin-binding protein, as characterized by affinity ligand assays (17).

The importance of plasminogen in infectious diseases is supported by the fact that many pathogens manifest the ability to bind plaminogen (47, 13). Plasminogen is a single-chain glycoprotein with a molecular mass of 92 kDa. Protein structure comprises an N-terminal preactivation peptide, five consecutive disulfide-bonded triple-loop *kringle* domains, and a serine-protease domain containing the catalytic triad (48). The kringle domains of plasminogen mediate its attachment to cells surfaces by binding proteins with accessible carboxyl-terminal or internal lysine residues. The plasminogen system displays a unique role in the host defense by dissolving fibrin clots and serving as an essential component to maintain homeostasis (43). Activation of the fibrinolytic system is dependent on the conversion of plasminogen to the serine protease plasmin by the physiological activators urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA) (10). Plasmin is involved in fibrinolysis homeostasis and degradation of the extracellular matrix and basement membrane. The mammalian plasminogen-plasmin proteolytic system plays a crucial role in extracellular matrix degradation which is exploited by invasive pathogens, including fungi (25, 47). Microbial derived plasminogen conversion to plasmin may promote dissemination of the pathogen within the host (1).

Among several proteins enolase has been found to play a major role in microbial recruitment of plasminogen (32). By serving as a key surface receptor for plasminogen recruitment enolase has been shown to function as mediator of microbial virulence (6, 15). The potential of *P. brasiliensis* to recruit human plasminogen for invasion and virulence has not been studied until date. In this report we demonstrated for the first time that *P. brasiliensis* is capable of recruiting plasminogen and activating the plasminogen fribrinolytic system in a process, at least in part, mediated by the cell wall-localized enolase. Furthermore, r*Pb*Eno promoted an increase in the adhesion/invasion of *P. brasiliensis* in *in vitro* models of infection, a process that seems to be associated with the enolase ability of modifying the surface of host cells. These data suggest that *Pb*Eno may play a role in mediating the *P. brasiliensis* recruitment of plasminogen as well as in attachment and internalization of the fungus to host tissues, potentially playing a role in the establishment of PCM.

Materials and Methods

Fungal isolate and growth conditions.

Yeast cells were obtained by growing the *P. brasiliensis* isolate 01 (ATCC MYA-826) in Fava-Netto´s medium for 4 days at 36 °C, as described previously (4).

Cloning cDNA containing the complete coding region of enolase into expression vector.

The enolase cDNA (GenBank accession number EF558735.1), obtained from a library from yeast cells of *P. brasiliensis* (14), was amplified by PCR using oligonucleotide sense (5'-GTC GAC ATG GCT ATC ACC AAA ATC CAC G-3'; *Sal*I restriction site underlined) and antisense (5'-GCG GCC GCT TAC ATA TTA ATA GCT GCC C-3';

*Not*I restriction site, underlined) primers. The PCR product was cloned in-frame with the glutathione S-transferase (GST) coding region of the pGEX-4T-3 vector (GE Healthcare) to yield the pGEX-4T-3-*Pb*Eno construct. The *Escherichia coli* strain BL21 pLys competent cells were transformed with the expression construct.

Expression and characterization of the recombinant enolase.

Bacteria transformed with the pGEX-4T-3-*Pb*Eno construct were grown in LB medium supplemented with ampicillin (100 µg/ml) and glucose (20 mM) at 37 °C, 200 rpm. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The GST-*Pb*Eno protein was affinity purified using glutathione Sepharose 4B (GE Healthcare) and the GST was cleaved by the addition of thrombin (Sigma Aldrich).

Antibody production.

The purified r*Pb*Eno was used to generate specific rabbit polyclonal serum. Rabbit preimmune serum was obtained and stored at -20°C. The purified protein was injected into rabbit with Freund's adjuvant three times at 2-weeks intervals. The serum, containing monospecific anti- r*Pb*Eno polyclonal antibodies (5.3 µg/µl), was stored at -20 °C.

Preparation of *P. brasiliensis* protein fractions.

The *P. brasiliensis* crude protein extract was obtained by disruption of frozen yeast cells in the presence of protease inhibitors: 50 µg/ml *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 1 mM 4-chloromercuribenzoic acid (PCMB), 20 mM leupeptin, 20 mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM iodoacetamide in homogenization

buffer (20 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$). The mixture was centrifuged at 12000 × *g* at 4 °C for 10 min, and the supernatant was used for further analysis of proteins. Cell wall fractionation was performed basically as described previously (42). The culture filtrate was processed as described previously (33) with modifications. Yeast cells were harvested from the solid medium and transferred to Fava Netto's liquid medium. After 1 day of growth at 37 °C with gentle agitation, the proteins from the supernatant were precipitated with 10% (wt/vol) trichloroacetic acid (TCA) during overnight (o/n) incubation at 4 °C. The precipitate was centrifuged for 10 min at 10000 × *g*. The pellet was washed twice with acetone and air dried prior to resuspending in electrophoresis dissolving buffer. Protein samples were then subjected to SDS-PAGE. The protein content was quantified using the Bradford assay (8).

Two-dimensional (2D) gel electrophoresis and MALDI-TOF mass spectrometry analysis.

Samples containing 200 µg of *P. brasiliensis* yeast protein crude extract were separated by isoelectric focusing, as described by O'Farrell (1975) (37). The second dimension was performed as described by Laemmli (1970) (27). Protein spots were excised from the gel, submitted to reduction, alquilation and in-gel digestion with trypsin (Promega, Madison, WI). The resulting tryptic peptides were extracted and submitted to MS analysis. The protein tryptic fragments were analyzed using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer (Reflex IV, Bruker Daltonics, Karlsruhe, Germany). The peptide mass list obtained for each spectrum was searched against the SwissProt database (http://expasy.org/sprot) using MASCOT (http://www.matrixscience.com).

Western blot and ligand binding analysis.

Proteins fractionated by gel electrophoresis were transferred to nylon membranes. Blots were sequentially incubated with the rabbit polyclonal anti-r*Pb*Eno antibodies, anti-rabbit immunoglobulin G (IgG) coupled to alkaline phosphatase (Sigma), and developed with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP-NBT). Rabbit preimmune serum was used as a negative control.

For ligand blot analysis, the membrane was washed three times with 0.1 % (vol/vol) Tween 20 in PBS and incubated o/n with human plasminogen (hPlg, Sigma) (35 µg/ml) in PBS 1% BSA (wt/vol). The blot was washed and incubated with mouse anti-human plasminogen MAb (1 µg/ml) (R&D Systems) in PBS 1% BSA (wt/vol) for 1 h at room temperature. The membrane was next incubated with anti-mouse IgG coupled to alkaline phosphatase (Sigma). The blots were developed with BCIP/NBT.

Immunofluorescence detection of *Pb*Eno and plasminogen on the surface of *P.brasiliensis* yeast cells.

Immunofluorescence assay was performed using a modification of a described procedure (33). Briefly, 2×10^7 yeast cells were fixed with 4% (vol/vol) paraformaldehyde in PBS for 10 min. The cells were washed twice in PBS and blocked with BSA for 1 h at room temperature prior to incubation with the following: (i) rabbit anti- r*Pb*Eno antibodies or (ii) plasminogen followed by incubation with anti-plasminogen monoclonal antibodies. The cells were then washed three times with PBS and treated for 1 h at 37 °C with affinity-purified fluorescein isothiocyanate-conjugated goat anti-rabbit IgG or anti-mouse IgG (Sigma) diluted 1:1000. Finally, yeast cells were washed twice with PBS and visualized using the Olympus BX41 microscope at ×100 magnification.

Plasminogen binding assay.

Cellular assays were performed after coating the wells of multititer plates with fungal cells followed by fixation. Briefly, 1×10^8 yeast cells in PBS were added to the wells, incubated for 1 h and glutaraldehyde was added to the wells to a final concentration of 1% (vol/vol) for 10 min. After three washes with PBS, the wells were blocked with 1% (wt/vol) BSA in PBS for 1 h. Different amounts of hPlg (0.05 a 1.0 µg) were added to the wells which were incubated for 1 h. Competition experiments were performed by the addition of increasing concentrations (0.5 µg to 3 µg) of r*Pb*Eno for 1 h prior to the addition of 1 µg of hPlg. Binding was determined by incubation with anti-plasminogen monoclonal antibody. Plates were washed three times with 0.1% Tween 20 (vol/vol) in PBS. Horseradish peroxidase was added to the wells and incubated for 1 h. The absorbance was measured at A_{405} using a microplate reader (Bio Tek Instruments Inc., Winooski, VT).

In another set of experiments, wells of multititer plates were coated with 1 µg of r*Pb*Eno diluted in carbonate buffer. After blocking and washing, as described above, different amounts of hPlg (1 µg to 4 µg) were added to the plates. Alternatively, the plates were coated with 1 µg of hPlg diluted in carbonate buffer and incubated o/n at 4 °C. A range of concentrations (1 µg to 4 µg) of r*Pb*Eno diluted in 1% BSA (wt/vol) in PBS were added to the hPlg coated wells, incubated for 1 h and washed with 0.1% (vol/vol) Tween 20 in PBS. Protein-protein interactions were determined by incubation with anti-r*Pb*Eno polyclonal antibodies. Competition experiments were performed by the addition of increasing concentrations (5 mM to 20 mM) of the lysine analogue ε-aminocaproic acid (ε-ACA) (Sigma) to the r*Pb*Eno coated wells. The wells were incubated for 1 h followed by the addition of hPlg. Another set of competition experiments included addition of specific r*Pb*Eno rabbit polyclonal antibodies prior to the addition of hPlg.

All reactions were carried out at 37 °C. Binding was determined by incubation with anti-plasminogen monoclonal antibody. Following three washes, the wells were developed as described above. All final volumes for the ELISA reactions were 100 µl.

Plasminogen activation assay.

Plasminogen activation was performed by measuring the amidolytic activity of the generated plasmin. Wells of multititer plates were coated with 1 µg of r*Pb*Eno or fixed *P. brasiliensis*, and incubated with 1 µg hPlg (Sigma), 3 µg of plasmin substrate (D-valyl-L-lysy-l*p*-nitroaniline hydrochloride) (Sigma), and 15 ng of tissue plasminogen activator (tPA) (Sigma). Control experiments were performed by measuring the generation of plasmin in either the absence of tPA or in the presence of ε-ACA. Plates were incubated at room temperature for 2 h and read at A_{405}.

Degradation of fibrin in jellified matrices.

Fibrinolysis was assayed using previously described methods with minor modifications (25). Briefly, 10^7 *P. brasiliensis* cells were pre-incubated with hPlg (50 µg) for 3 h in the presence or absence of tPA (50 ng) and the serine proteinase inhibitors aprotinin (1 µg) and PMSF (50 mM) in a final volume of 1 ml. Thereafter, the mixtures were washed three times with PBS to remove free plasminogen. The resulting cell pellets were placed in wells of a fibrin substrate matrix gel that contained 1.25 % (wt/vol) low-melting-temperature agarose, hPlg (100 µg) and fibrinogen (Sigma, 4 mg) in a final volume of 2 ml. Controls consisted of untreated cells (no plasminogen incubation) or incubations systems where no cells were added. The jellified matrix was incubated in a humidified chamber at 37 ºC for 12 h. Plasmin activity was detected by the observation of clear hydrolysis haloes within the opaque jellified-fibrin containing matrix.

Influence of enolase on the interaction of *P. brasiliensis* with host cells.

Human type II alveolar cells (A549 lineage) and murine macrophage-like cells (RAW 264.7 lineage) were obtained from the American Type Culture Collection (ATCC). Cultures were maintained and grown to confluence in 25 cm^2 culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) at 37 °C and 5% CO_2. To evaluate the effects of r*Pb*Eno on the interaction of *P. brasiliensis* with host cells, RAW and A549 lineages were first exposed to the enzyme and then probed with succinylated wheat germ agglutinin (S-WGA). S-WGA has affinity for β1,4 *N*-acetylglucosamine (GlcNAc) oligomers, which are recognized by the *P. brasiliensis* adhesin paracoccin (23). Mammalian cells were placed in a 24-well plate (10^5 cells/well) and treated with varying concentrations of r*Pb*Eno (1 and 50 µg/ml) for 1 h at 37 °C. Controls were exposed to medium alone for the same amount of time. The cells were detached from plastic surfaces, fixed and blocked as described previously (3) and then incubated for 30 min at 37°C in 100 µl of a 5 µg/ml solution of TRITC-labeled S-WGA (EY Laboratories). The cells were washed in PBS and analyzed by flow cytometry as previously described (3). To analyze the effects of r*Pb*Eno on the interaction of *P. brasiliensis* with host cells, the culture medium of RAW or A549 cells was replaced with fresh media for further incubation with *P. brasiliensis* yeast cells. For flow cytometry experiments, the cell wall of *P. brasiliensis* was stained with 0.5 mg/ml fluorescein isothiocyanate (FITC, Sigma) (3, 11) in PBS (25 °C) for 10 min (11). Fungal suspensions were prepared in DMEM to generate a ratio of 10 yeasts per host cell. Interactions between fungal and host cells occurred at 37 °C and 5 % CO_2 for 18 h. Cells were washed three times with PBS to remove non-adherent yeasts. Fungi-host cell complexes were treated for 10 min at 25 °C with trypan blue (200 µg/ml) to discriminate between surface-associated and

intracellular yeast cells (3, 11). After removal from the plastic surface with a cell scrapper, the cells were analyzed by flow cytometry as described previously (3). Control preparations were developed as described above using uninfected cells and non-stained yeast (data not shown). For analysis of the morphological aspects of infected cells, the complexes were fixed with paraformaldehyde and stained with 25 µM calcofluor white (Invitrogen™, Life Technologies). Control or infected cells were finally observed with an Axioplan 2 (Zeiss, Germany) fluorescence microscope, following conditions previously described (3).

Infection of mice with *P. brasiliensis* and RNA extraction.

Mice were infected as described previously (16). Female BALB/c mice were infected intraperitoneally with 1×10^8 yeast cells and intranasally with 5×10^7 yeast cells and killed on the 7 th day after infection; livers, spleens were removed from mice infected intraperitoneally and lungs were removed from mice infected intranasally. One hundred milliliters of this suspension were plated onto BHI Agar (Becton-Dickinson, MD, USA), supplemented with 1% (wt/vol) glucose. After 7 days total RNA was extracted from the yeast cells (1×10^{10}). Control cDNA was prepared by removing *P. brasiliensis* yeast cells from Fava-Netto cultures and plating to BHI Agar as above.

Quantitative analysis of RNA transcripts by reverse transcription real-time (qRT-PCR).

Total RNAs were treated with DNAse and cDNA was prepared using Superscript II reverse transcriptase (Invitrogen) and oligo $(dT)_{15}$ primer. qRT-PCR analysis was performed on a StepOnePlus™ real time PCR system (Applied Biosystems, Foster City, CA) in triplicate. Values were averaged from three biological replicates. PCR thermal

cycling was performed at 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. Ten pmol of each primer and 40 ng of template cDNA in a total volume of 25 µL SYBR green PCR master mix (Applied Biosystems) were used for each experiment. A melting curve analysis was performed to confirm a single PCR product. The data were normalized with transcript encoding tubulin amplified in each set of qRT-PCR experiments. A non-template control was also included. Relative expression levels of the genes of interest were calculated using the standard curve method for relative quantification (7).

Statistical analysis.

Experiments were performed in triplicates with samples in triplicates. Results were presented as means (±) standard deviation. Statistical comparisons were performed using Student´s *t* test. Statistical significance was accepted for $P < 0.05$.

Results

Expression and purification of the *P. brasiliensis* enolase and production of polyclonal antibodies.

Previous studies identified *Pb*Eno (EC 4.2.1.11) as a fibronectin-binding protein. In the present work to further investigate the role of *Pb*Eno in fungus-host interaction we first expressed the protein in order to create antibodies specific for *Pb*Eno. The cDNA encoding the *PbENO* (GenBank accession number EF558735.1) was cloned into the expression vector, pGEX-4T-3, to obtain the recombinant fusion protein GST-*Pb*Eno. The fusion protein was affinity purified, and the 47-kDa r*Pb*Eno was obtained by digestion with thrombin (data not shown). The purified r*Pb*Eno was used to generate rabbit polyclonal antibodies.

Antibody specificity was evaluated in serological tests using protein extracts from cell lysates resolved by 2D gel electrophoresis. A protein with pI of 5.67 was recognized by polyclonal antibodies raised to r*Pb*Eno (Fig. 1A and 1B). The protein was analyzed by mass spectrometry (Fig. 1C). Experimental masses were searched against public gene databases using MASCOT. The peptides obtained (Table 1) matched for *Pb*Eno.

Detection of *Pb*Eno on fungal surface.

In order to determine the cellular distribution of *Pb*Eno we probed different fractions of fungal cells by western blot analysis. *Pb*Eno was detected in total protein extract (Fig. 2A, lane 3), cell wall enriched fraction (Fig. 2A, lane 4), cytoplasmic fraction (Fig. 2A, lane 5) and in the culture filtrate (Fig. 2A, lane 6). Bovine serum albumin (Fig. 2A, lane 1) and the r*Pb*Eno (Fig. 2A, lane 2) were employed as negative and positive controls, respectively. Altogether, these results suggest that *Pb*Eno is associated to the cell wall and is secreted to the extracellular space, besides its expected intracellular distribution in *P. brasiliensis*.

To further validate *Pb*Eno's association with the fungal surface, immunofluorecence was performed. As shown in figure 2B (panel 4), rabbit polyclonal antibodies reacted with the surface of the organism. Plasminogen antibodies (panel 6) also reacted with the surface of plasminogen-treated organisms, suggesting an ability of *P. brasiliensis* to recognize this molecule. No fluorescence and immunoreactivity were detected when yeast cells were incubated with the secondary antibody alone (panel 2).

***P. brasiliensis* and *Pb*Eno binds plasminogen.**

By the immunofluorecence assay, we discovered that *P. brasiliensis* binds to hPlg. To further characterize this phenomenon, *P. brasiliensis* yeast cells were fixed to the wells

of multititer plates and increasing concentrations of hPlg were added. Figure 3A shows a dose dependent pattern of binding of hPlg to fixed fungal cells. The addition of increasing concentrations of r*Pb*Eno decreased hPlg binding to *P. brasiliensis* in a dose-dependent manner (Fig. 3B). These data further confirmed that enolase was involved in *P. brasiliensis* binding to hPlg. To support this supposition, we performed a ligand blot assay using crude protein extracts, cell wall enriched fraction and r*Pb*Eno (Fig. 3C). Different *P. brasiliensis* proteins, including enolase, interacted with hPlg. The presence of several proteins in the ligand blot assay implicated the existence of other plasminogen-binding proteins in *P. brasiliensis,* as described in other organisms (15). Therefore the ability of r*Pb*Eno to bind hPlg was tested in ELISA. Increasing concentrations of hPlg bound to immobilized r*Pb*Eno in a dose-dependent fashion (Fig. 3D). The same increasing pattern was observed when the wells were coated with hPlg and increasing concentrations of r*Pb*Eno were added (Fig. 3E).

Previous work has shown that enolase binds to hPlg through lysine residues (33, 47). We thus examined if binding of r*Pb*Eno was lysine dependent using competitive antagonism with the lysine analog ε-ACA. The results shown in Figure 3F indicate that lysine residues present on r*Pb*Eno may have a role in plasminogen recruitment by *P. brasiliensis*.

To further validate r*Pb*Eno binding to hPlg, competition experiments were also performed by the addition of specific r*Pb*Eno rabbit polyclonal antibodies to the experimental system. The presence of enolase specific antibodies, dose-dependently decreased hPlg binding to r*Pb*Eno (Fig. 3G). In the presence of preimmune sera no effects were observed (data not shown). These data confirmed that enolase specifically binds hPlg.

Plasminogen activation and fibrinolysis.

Once yeast cells and r*Pb*Eno were formerly seen to bind hPlg we would expect that this interaction could also activate hPlg. For this reason an ELISA was performed to determine the ability of *P. brasiliensis* and r*Pb*Eno to produce plasmin from hPlg. In the presence of tPA, r*Pb*Eno was able to generate plasmin (Fig. 4A). The addition of ε-ACA inhibited plasmin generation. hPlg activation was evaluated in assays using fixed fungal cells in the presence of tPA, confirming that interaction with fixed *P. brasiliensis* also result in hPlg activation (Fig. 4B). The addition of increasing concentrations of ε-ACA to the experimental system inhibited plasmin generation in a dose-dependent manner. These results suggest that *P. brasiliensis* and r*Pb*Eno mediate activation of plasminogen to plasmin and that lysine residues are involved in binding and activation of hPlg (Fig. 4B).

Fibrinogen is one of the major substrates of plasminogen/plasmin *in vivo*, and jellified matrices containing fibrinogen have been used to examine plasmin activity (25, 1). As demonstrated in Figure 4C, the association of *P. brasiliensis* with plasminogen and tPA promoted increased fibrinolysis (Fig. 4C, lane 3). Aprotinin (lane 4) and PMSF (lane 5) inhibited proteolysis, indicating the specificity of the reaction. No proteolysis was observed when either *P. brasiliensis* yeast cells were used alone or in the presence of plasminogen (lanes 1 and 2, respectively). Lane 6, control consisting of plasminogen and tPA.

Enolase influences the interaction of *P. brasiliensis* with host cells.

Previous studies had demonstrated that antibodies raised to a 54-kDa enolase from *P. brasiliensis,* isolate Pb18, abolished 80% adhesion to A549 epithelial cells. In this work, the participation of enolase in the infection of host cells by *P. brasiliensis* was evaluated

398 by flow cytometry and fluorescence microscopy. Exposure of human epithelial cells
399 (Figure 5A, panel a) and murine phagocytes (Figure 5A, panel b) to r*Pb*Eno resulted in
400 an expressive increase in their reactivity with WGA , suggesting that the enzyme
401 modifies the surface of host cells to promote an enhanced exposure of GlcNAc residues,
402 which are recognized by a *P. brasiliensis* adhesin (23). We therefore asked whether
403 exposure to the enzyme would turn mammalian cells more susceptible to infection by *P.*
404 *brasiliensis*.

405 Incubation of FITC-stained *P. brasiliensis* yeast cells with epithelial or
406 macrophage-like cells under control conditions resulted in high levels of infection.
407 Approximately 85% of the epithelial cells became fluorescent after interaction with
408 fungi. This index corresponded to almost 90% of infected cells when phagocytes were
409 used. Pre-treatment of the cells with r*Pb*Eno caused an increase in the percentage of
410 infected cells to approximately 97% in both epithelial and macrophage-like systems.
411 More importantly, the intensity of fluorescence in infected cells clearly increased when
412 they were first exposed to r*Pb*Eno (Fig. 5B, panels a and d). Although this characteristic
413 was common to both systems of infections, a dose-response profile of fluorescence
414 increase after exposure to the enzyme was clearer in the macrophage system (Fig. 5B,
415 panels b and e).

416 To evaluate if *P. brasiliensis* yeast cells were internalized by epithelial cells, the
417 fungus were treated with trypan blue. Exposure to this dye caused an expressive
418 decrease in the levels of fluorescence of infected A549 cells, suggesting that fungal cells
419 adhered but were not internalized by alveolar epithelial cells. In contrast, the
420 fluorescence levels of infected macrophages were barely affected by exposure to trypan
421 blue. This indicates that internalization of *P. brasiliensis* by the phagocytes, and
422 consequent protection against fluorescence quenching, occurred efficiently. Results

shown in Fig. 5B are representative of two independent experiments producing the same fluorescence profile in flow cytometry measurements. Statistics were not included because, although the profiles described in Fig. 5B were similar in all experiments, the absolute fluorescence values may differ considerably in different assays, impairing calculation of reliable average values. Data interpretation was confirmed by fluorescence microscopy (Fig. 5B, panels c and f). In either system, the viability of host cells was not affected by the fungal infection (data not shown).

Assessment of *PbEno* by real-time PCR in models of infection.

If *Pb*Eno was required for efficient fungal attachment and invasion of host cells we speculated that the upregulation of the gene during infection would be necessary. Relative quantification of gene transcripts were examined by real-time PCR in yeast cells of *P. brasiliensis* derived from infected mice lung, spleen and liver (Fig. 6). Enolase expression was upregulated in yeast cells derived from tissues at 7 days post inoculation.

Discussion

The present study describes characterization of enolase as a plasminogen-binding molecule on the surface of *P. brasiliensis*. The presence of enolase on the surface of cells is not without precedent. Pitarch et al. (42) analyzed cell wall fractions of *C. albicans* and concluded that enolase can be loosely associated with the cell surface, as it was released when the cells were treated with SDS. The enzyme was also found to be tightly entrapped within the glucan-chitin network, which is consistent with the identification of enolase as a glucan-associated integral component of the cell wall of *C. albicans* (2). The question of how proteins lacking any signal peptide are exported on

448 the cell surface is unresolved. It is clear, however, that fungal cells express many
449 molecules with apparently conflicting functions (35). The nuclear histone-like protein
450 H2B, for instance, is also found at the cell wall of *Histoplasma capsulatum*, where it
451 functions as a target for protective antibodies (36). In *P. brasiliensis*, the mitochondrial
452 protein Mdj1p and the cytosolic enzymes GAPDH and TPI were also characterized as
453 cell wall components (5, 4, 41). This multiplicity in cellular distribution and functions is
454 also common to enolase because this protein functions in sugar metabolism, but is also
455 present at the cell surface (30) and in secretory vesicles that reach the extracellular
456 space (45). Enolase has been also described as a cell wall component in bacteria (26),
457 where it mediates the interaction of *Streptococcus pneumoniae* with human
458 plasminogen (13). The dual location in the cytosol and on cell surface indicated the
459 pivotal role of enolase in glycoslysis and pathogenesis, respectively. However, an
460 important and challenging important issue that needs to be addressed further is to
461 discern the mechanism of its export to the cell surface.
462 *Pb*Eno has previously been characterized as a 54-kDa fibronectin binding protein (17).
463 Differences in molecular mass related in the previous work could be related to the
464 potential sites for glycosylation and myristolation, present in the protein deduced
465 sequence (data not shown). We demonstrated that *Pb*Eno was not the only adherence
466 protein, but it is involved in *P. brasiliensis* binding to plasminogen. Similarly, enolase is
467 a predominant plasminogen binding and cell wall protein in *C. albicans*, *Aspergillus*
468 *fumigatus*, and *Pneumocystis carinii* (25, 30, 19, 21). Plasminogen is abundant in the
469 circulation and its activation by invasive pathogens could increase the organism's
470 potential of tissue invasion. The binding of plasminogen to mammalian and bacterial
471 cells is mediated by its five kringle domains, which have affinity for lysine (43). Lysine
472 dependent binding is characteristic of the plasminogen pathogen interaction (50). We

observed that the lysine analogue, εACA, inhibited plasminogen binding to both *P. brasiliensis* and r*Pb*Eno while also inhibited activation to plasmin. These data suggest that plasminogen binding to the surface of *P. brasiliensis* might involve lysine residues since the majority of all of the plasminogen-receptor proteins identified have carboxy-terminal lysine residues (38, 25, 33). Taken together we hypothesized that *P. brasiliensis* may take advantage of the plasminogen-clotting system during invasion of host tissues.

In addition to the localization and functional characterization of *Pb*Eno, we also described the fibrinolytic potential of *P. brasiliensis* mediated by the surface-associated enolase. Our studies with jellified matrices provided more evidence that plasminogen can perform proteolytic activity while bound to *P. brasiliensis*. Functional studies to address the significance of plasminogen binding in the invasiveness of *Cryptococcus neoformans* demonstrated that plasmin-coated organisms possess an increased potential to penetrate the ECM, *in vitro* (47). Remarkable are the studies showing that host susceptibility to invasive aspergillosis is strongly influenced by the plasminogen system and that plasminogen activation on the surface of both *A. fumigatus* and *C. albicans* promotes ECM invasion (25, 53). In agreement with this finding, Esgleas et al. (20) showed that enolase was important for the adhesion and invasion of brain microvascular endothelial cells by *Streptococcus suis*. Although multiple factors contribute to fungal virulence, including the expression of extracellular proteases, morphological switching and adherence, the ability of fungal pathogens to subvert the host plasminogen system suggests that plasminogen binding may be an additional mechanism used by fungi to promote dissemination and tissue invasion during infection (25, 30, 19, 21, 53). The capture of plasminogen by adhesins such as enolase and its conversion to plasmin has been in fact described for different pathogens (20).

498 In the current study, exposure of epithelial cells and phagocytes to r*Pb*Eno enhanced the
499 efficacy of *P. brasiliensis* association with host components. Treatment of host cells
500 with enolase caused an increase in exposure of surface N-acetylglucosamine. Although
501 the mechanisms connecting exposure to enolase with changes in surface carbohydrates
502 are unclear, this observation echoes previous findings showing that animal infection
503 with *S. pneumoniae*, an enolase-producing pathogen (6), results in an increased surface
504 exposure of N-acetylglucosamine residues by host tissues (29). Since *P. brasiliensis*
505 uses N-acetylglucosamine as surface sites of adhesion in host cells (12, 18, 23), we
506 hypothesized that treatment of host cells with enolase could result in increased
507 infectivity.
508 Yeast cells preferentially adhered to epithelial cells and were internalized by
509 phagocytes. The mechanisms explaining how an enzyme could alter surface interactions
510 of a fungal pathogen with host cells are still obscure. Although the mechanisms by
511 which enolase interferes with steps of the interaction of pathogens with host cells are
512 unknown, it is evident from the current literature that this enzyme may be involved in
513 adhesion and infection of microbes to host elements. Our analysis demonstrated that
514 enolase is a surface secreted protein in *P. brasiliensis* as it is in *C. neoformans* (45). In
515 this way, release of the enzyme to the extracellular space, as demonstrated here, could
516 somehow increase the availability of adhesion sites in host cells. This putative
517 phenomenon would result in higher efficacy of association of fungi with host cells, as
518 currently described in our manuscript.
519 The results described in this paper expand current knowledge on the adhesion and
520 invasion processes by *P. brasiliensis*. The transcript encoding *Pb*Eno was up regulated
521 yeast cells derived from infected-mice tissues. Overall the present work is the first
522 study, to our knowledge, to demonstrate the plasminogen binding and activation activity

of *P. brasiliensis* enolase, and shows that, similar to other microbes, enolase may contribute to the virulence of *P. brasiliensis*. In summary we have shown that *P. brasiliensis* can borrow the plasminogen system from the host in a process mediated by the surface protein enolase.

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758
759 **Figure Legends**
760
761 FIG. 1. Identification of enolase in the *P. brasiliensis* proteome by two-dimensional gel
762 electrophoresis. (A) Proteins staining with Coomassie blue. (B) Reactivity of the *P.*
763 *brasiliensis* total extract with rabbit polyclonal antibodies to enolase raised to the
764 recombinant protein. Numbers in the left side of A and B refer to the molecular mass of
765 the enolase . At the top is indicated the isoelectric point of the protein. Arrows point to
766 enolase. (C) Peptide Mass spectrum generated from tryptic digestion of the *Pb*Eno. The
767 protein reacting with polyclonal antibodies was removed from the gel and submitted to
768 mass spectrometry analysis after trypsin digestion. The black stars indicate the peaklist

769 for enolase and each peak correspond to a peptide. Experiments represent three gels
770 from independent protein preparations.
771
772 FIG. 2. Detection of *Pb*Eno and plasminogen binding at the cell surface of *P.*
773 *brasiliensis*. (A) Western blot analysis of Bovine Serum Albumin (BSA lane 1),
774 r*Pb*Eno (lane 2), *P. brasiliensis* crude protein extract (lane 3), cell wall enriched fraction
775 proteins (lane 4), soluble cytoplasmic fraction (lane 5) and secreted proteins (lane 6)
776 blotted onto a nylon membrane and detected with rabbit polyclonal anti-recombinant
777 enolase antibodies. Arrow indicates enolase. (B) Paraformaldehyde-fixed,
778 nonpermeabilized cells were incubated with r*Pb*Eno antibodies, (panels 3-4) or treated
779 with human plasminogen followed by incubation with an antibody raised to this protein
780 (panels 5-6). Control systems were obtained with anti-rabbit immunoglobulin G (IgG)
781 coupled to alkaline phosphatase antibody only (panels 1-2). Bright-field microscopy is
782 shown in left panels. The same cells are shown under the fluorescence mode in the right
783 panel. Experiments in A and B were performed in triplicates.
784
785 FIG. 3. Plasminogen binding assays. Microtiter plates were coated with fixed *P.*
786 *brasiliensis* yeast cells as detailed in Materials and Methods. (A) Plasminogen (0.05 to
787 1.0 µg) binds to fixed *P. brasiliensis* in a concentration-dependent manner. (B) In a
788 competition assay, binding of plasminogen is inhibited by increasing amounts of
789 r*Pb*Eno (0.5 to 3.0 µg). (C) Binding of *P. brasiliensis* proteins to plasminogen. *P.*
790 *brasiliensis* crude protein extract (lane 1), cell wall enriched fraction proteins (lane 2),
791 r*Pb*Eno (lane 3) and hPlg (lane 4) were sequentially incubated with plasminogen and a
792 mouse monoclonal anti-human plasminogen antibody. The numbers on the left side are
793 molecular size markers. (D) Plasminogen (1 to 4 µg) binds to r*Pb*Eno (1 µg)

794 immobilized on microtiter well plates in a concentration-dependent manner. ELISA
795 assays were developed at A_{405} using the antibody anti-plasminogen. (E) r*Pb*Eno (1 to 4
796 µg) binds to immobilized plasminogen (1 µg) in a similar fashion. The assay was
797 developed at A_{405} using antibodies to r*Pb*Eno. (F) Effects of different ε-ACA
798 concentrations (5 to 20 mM) on plasminogen binding. (G) Plasminogen binding to
799 immobilized r*Pb*Eno is specifically inhibited by anti-r*Pb*Eno. Microtiter plates were
800 coated by overnight incubation with 1µg of r*Pb*Eno. After blocking, the wells were
801 incubated with decreasing concentrations of rabbit polyclonal r*Pb*Eno antibodies.
802 Reactions were developed after incubation with hPlg, the anti-plasminogen antibody
803 followed by secondary antibodies. A, B, D, E, F and G are the averages of three
804 independent experiments performed in triplicates. The error bars indicate the standard
805 deviations from three independent experiments performed in triplicates. *, significantly
806 different from control, $P < 0.05$.
807
808 FIG. 4. Plasminogen activation assays. (A) The r*Pb*Eno (1 µg) generates plasmin from
809 plasminogen in the presence of tPA and in the absence of ε-ACA. (B) *P. brasiliensis*
810 converts plasminogen into plasmin in the presence of tPA. Various concentrations of ε-
811 ACA (50 mM to 1000 mM) were added to wells containing fixed *P. brasiliensis*,
812 followed by the addition of plasminogen, and ELISA was performed as described in
813 Materials and Methods. The error bars indicate the standard deviations from three
814 independent experiments performed in triplicates. *, significantly different from control,
815 $P < 0.05$. (C) Fibrinolytic activity of plasminogen-bound *P. brasiliensis*. Lane 1, *P.*
816 *brasiliensis* cells in the absence of plasminogen; lane 2, *P. brasiliensis* cells after
817 binding to plasminogen. Lanes 3, 4 and 5 are similar to lane 2, except for the presence

818 of tPA, tPA plus aprotinin, and tPA plus PMSF, respectively. Lane 6, controls
819 consisting of plasminogen and tPA.
820
821 FIG. 5. Exposure of host cells to r*Pb*Eno enhances the efficacy of association of *P.*
822 *brasiliensis* to host cells. A. Treatment of RAW phagocytes (a) or A549 epithelial cells
823 (b) resulted in increased reactivity with WGA, indicating enhanced exposure of GlcNAc
824 residues. B. Effects of r*Pb*Eno on the infection of host cells by *P. brasiliensis*. Panels a
825 and d demonstrate that *P. brasiliensis* (Pb) efficiently infects epithelial (A549) and
826 macrophage-like (RAW) cells. Histograms of control cells (non-infected) are shown in
827 red. Exposure of host cells to r*Pb*Eno (10 µg/ml, green histogram) results in their
828 increased association with fungi, as determined by the comparison with infection
829 systems prepared in the absence of enolase (black histograms). Exposure of cells
830 infected with FITC-*P. brasiliensis* to trypan blue (b and e) resulted in an accentuated
831 reduction of fluorescence levels in A549 cells, but not macrophages. The suggestive
832 internalization of *P. brasiliensis* by macrophages, but not by epithelial cells, was
833 supported by fluorescence microscopy (c and f). In this analysis, yeast fluorescence
834 appears in blue.
835
836 FIG. 6. Analysis of enolase transcripts by quantitative real time RT-PCR. qRT-PCR plot
837 of *PbEno* expression levels of transcripts from yeast cells of *P. brasiliensis* derived
838 from lung , liver and spleen of mice, after seven days of infection. Control systems,
839 consisted of yeast cells from cultures inoculated in BHI agar. The primers were as
840 following: sense 5'- GATTTGCAGGTTGTCGCCGA -3', antisense 5'-
841 TGGCTGCCTGGATGGATTCA-3'. The values of expression were standardized using
842 the values of expression of the constitutive gene encoding to the protein tubulin. The

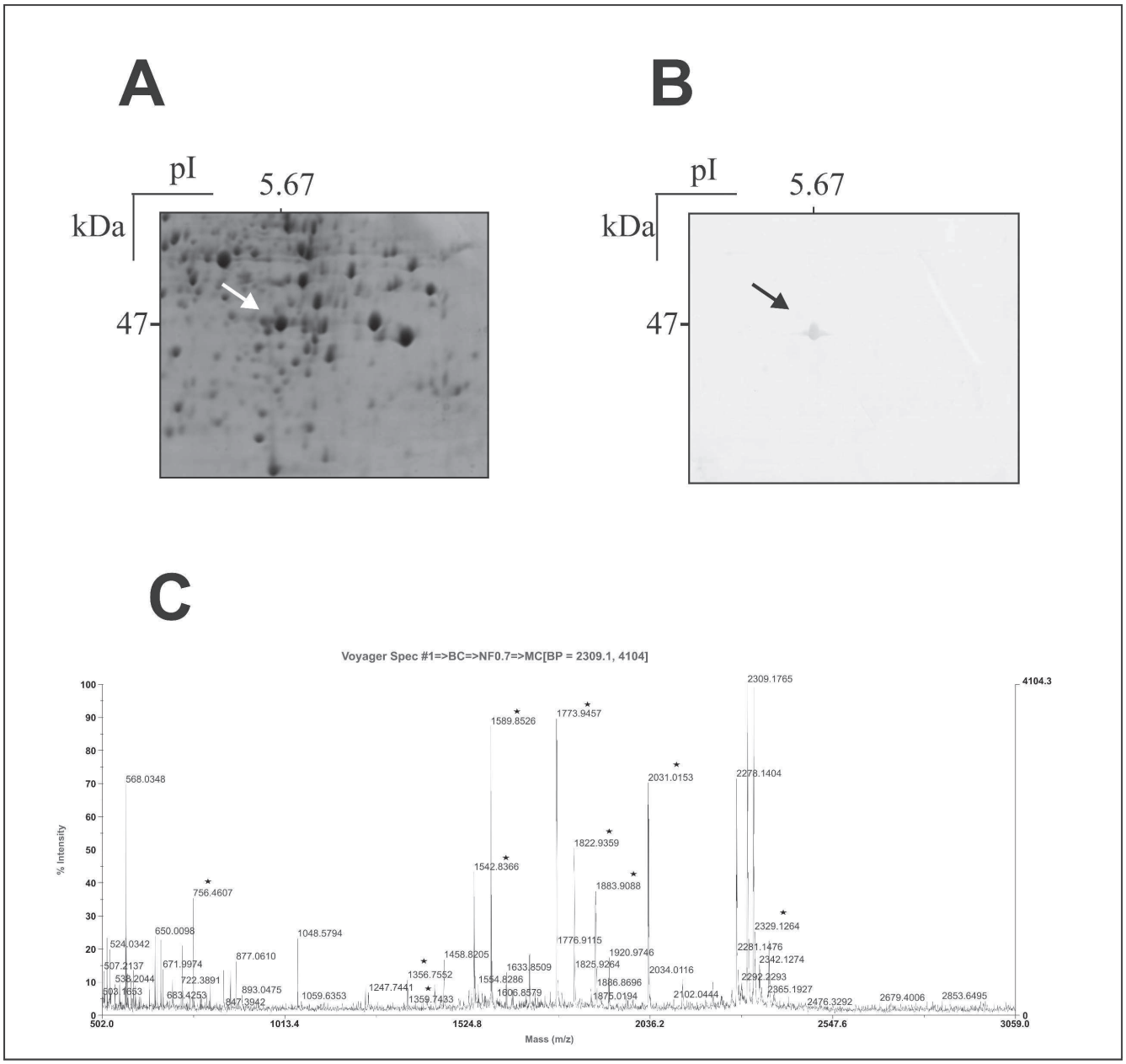
RQ (relative quantification) of the experiment was performed in triplicates. The error bars indicate the standard deviations from three independent experiments performed in triplicates *, significantly different from control, P <0.05.

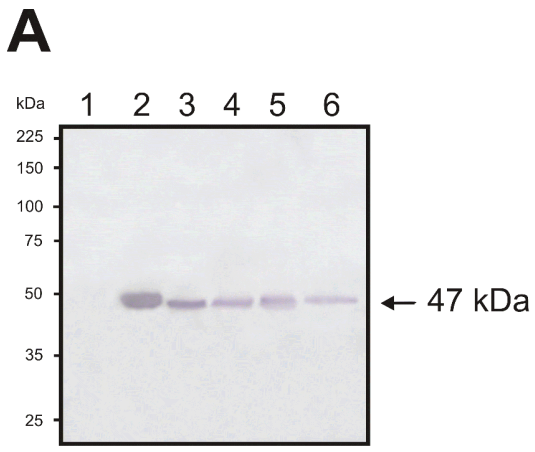
TABLE 1. Identification of *P. brasiliensis* enolase by peptide mass fingerprint [a,b]

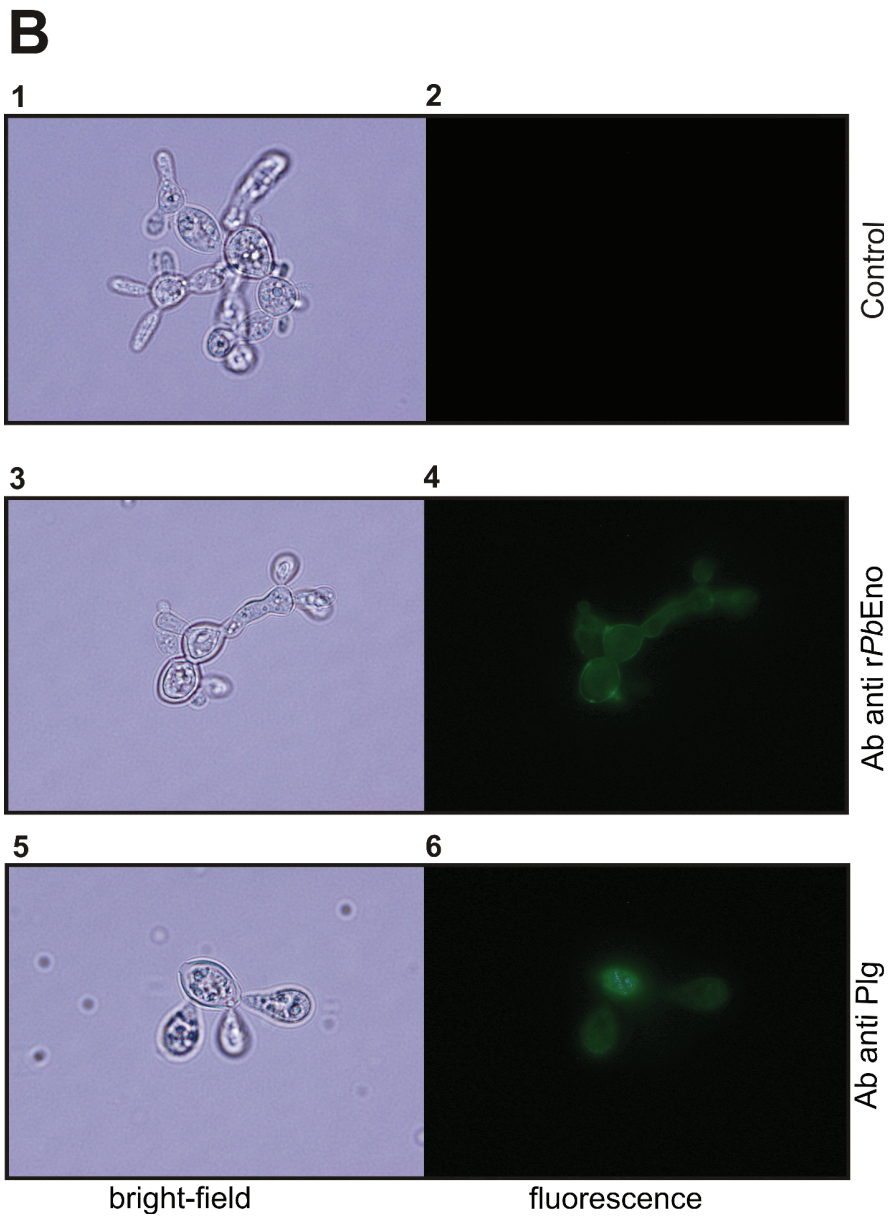
Position	Identified amino acid sequence	Experimental mass (*in-gel* digestion)	Theoretical mass (*in silico* digestion)
16 - 32	R.GNPTVEVDVVTETGLHR.A	1822.9489	1822.9293
33-50	R.AIVPSGASTGQHEACELR.D	1882.9548	1825.8861
90 -103	K.VDEFLNKLDGTPNK.S	1589.8751	1588.8678
106-120	K.LGANAILGVSLAIAK.A	1410.9979	1410.8678
164-184	R.LAFQEFMIVPTAAPSFSEALR.Q	2325.2178	2325.1947
243-254	K.IALDIASSEFYK.A	1356.7701	1356.7045
274-285	K.WLTYEQLADLYK.K	1542.8513	1542.7838
314-331	K.TCDLQVVADDLTVTNPIR.I	2030.0239	1973.0008
377 - 393	R.SGETEDVTIADIVVGLR.A	1773.9489	1773.9228
411 - 416	K.LNQILR.I	756.4464	756.4726

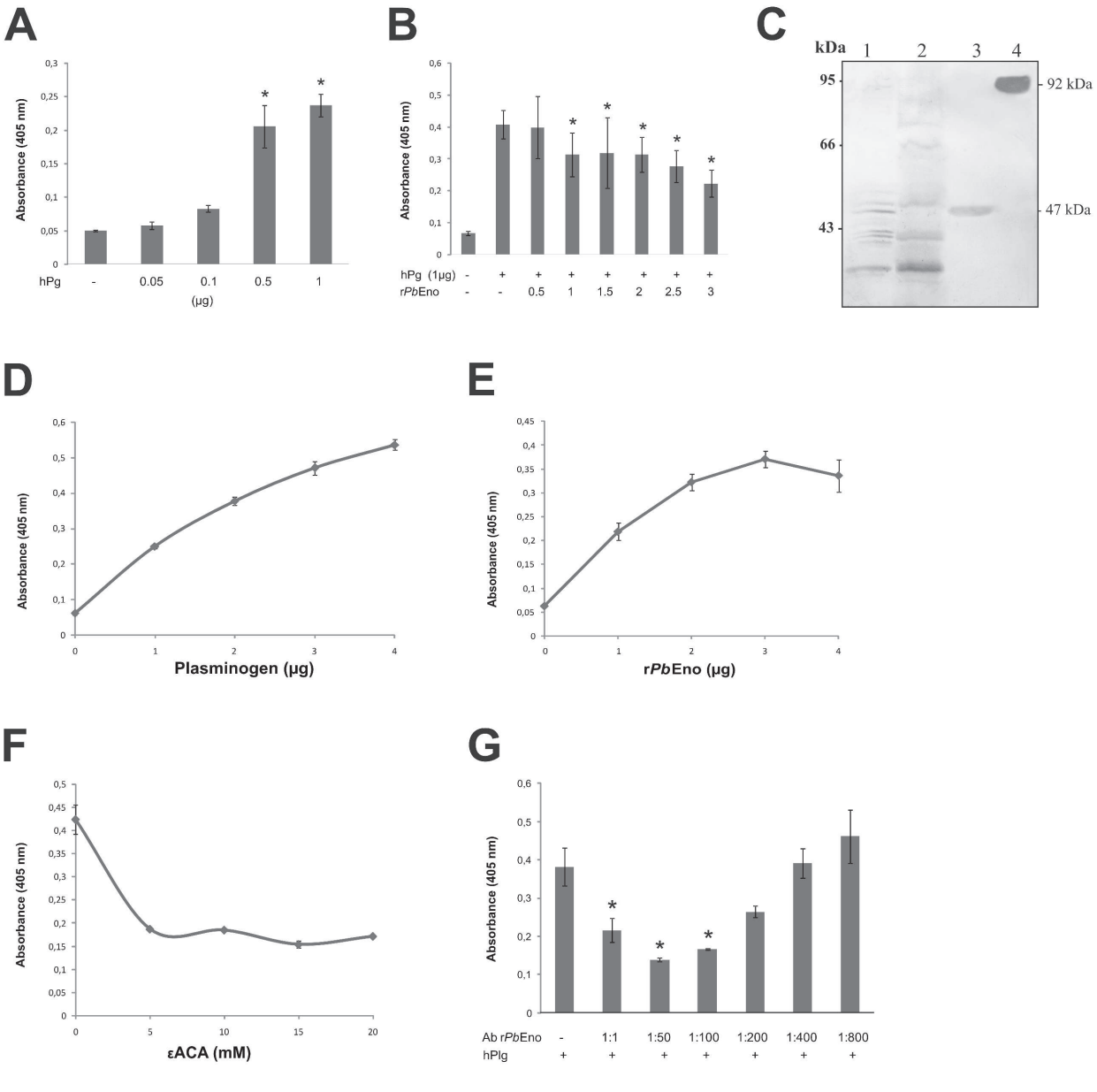
a – Protein scores higher than 76 are significant (p<0.05).

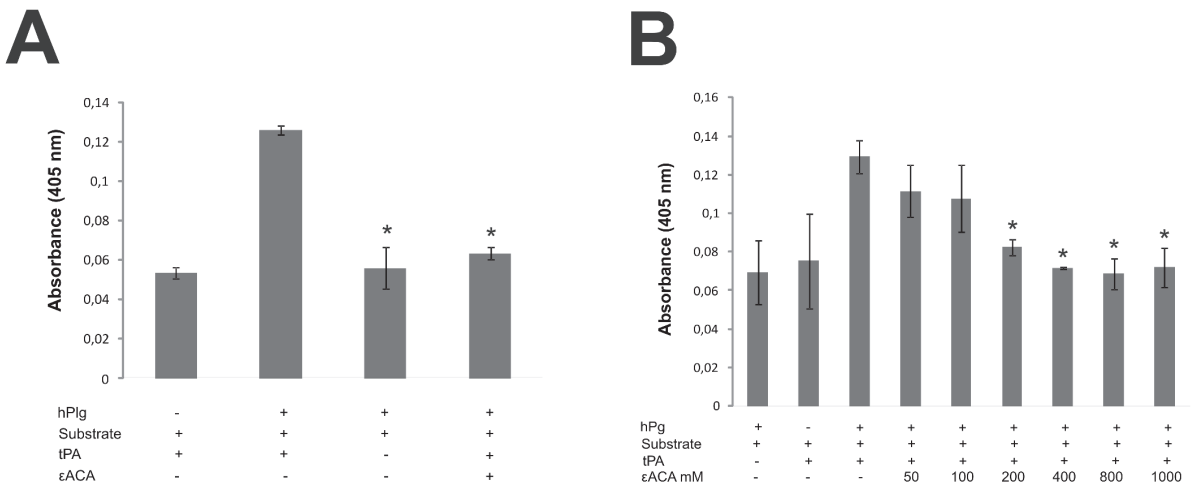
b – Peptide masses matched with *Pb*Eno (GenBank accession number EF558735.1) , presenting a score of 113 and coverage of 34.25% of the whole deduced sequence.

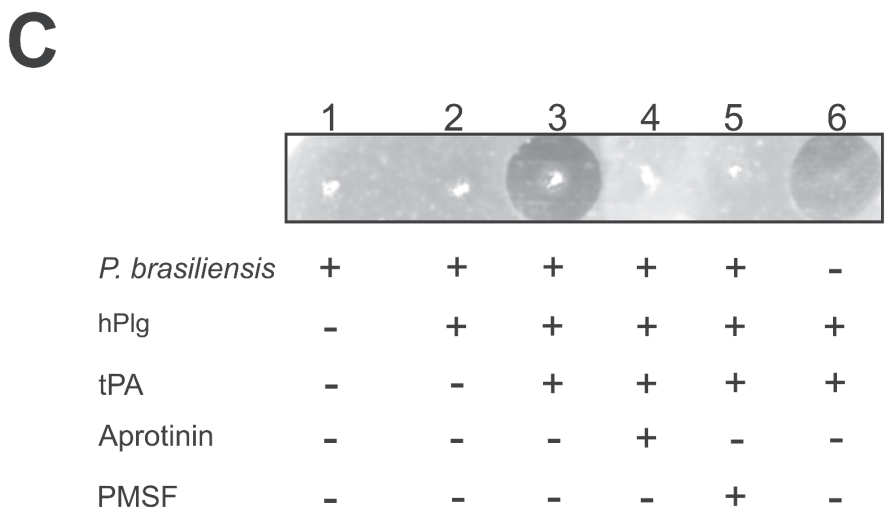


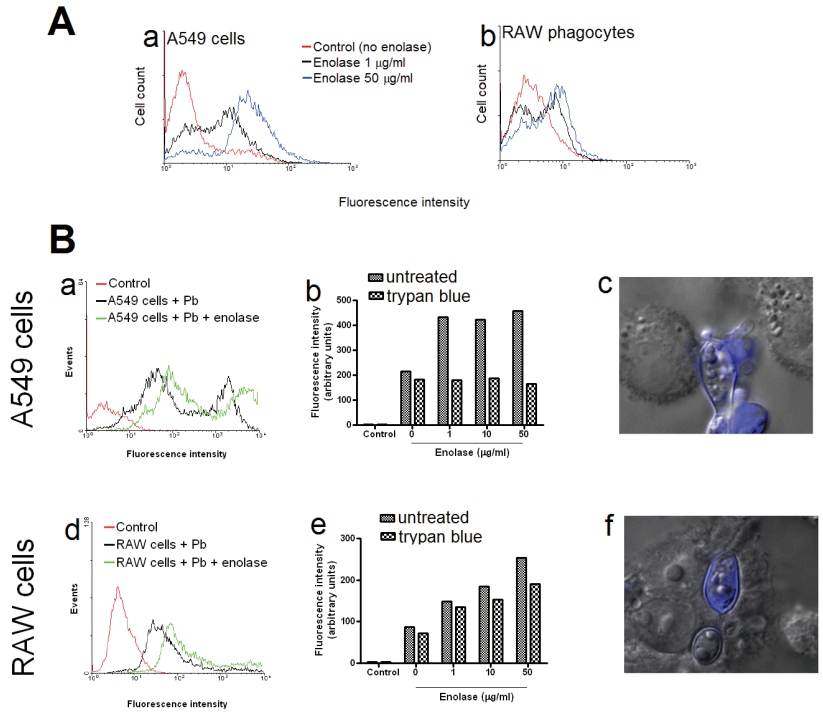


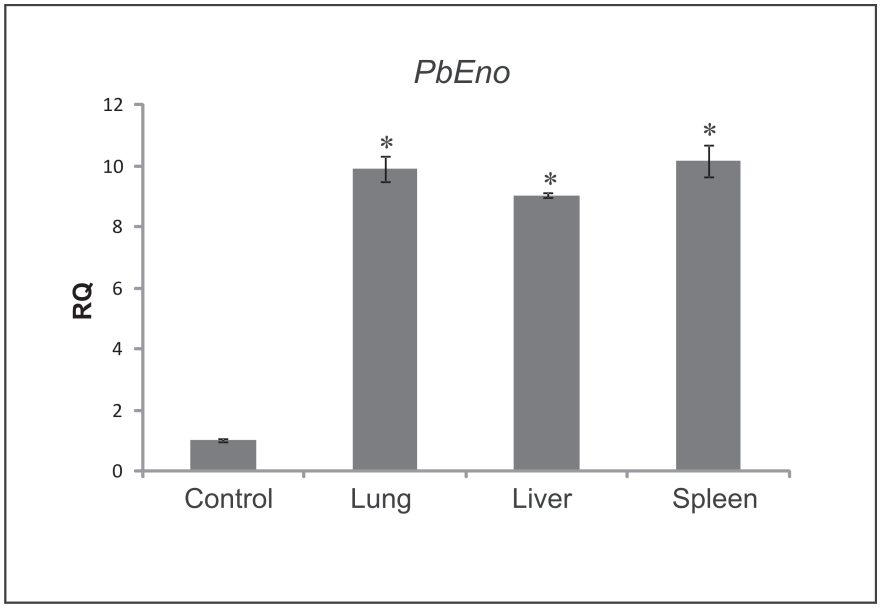













DISCUSSÃO

VI – DISCUSSÃO

No intuito de se ampliar o conhecimento do processo de adesão de *P. brasiliensis* aos componentes da matriz extracelular, utilizou-se a técnica de RDA. A anotação dos genes diferencialmente expressos permitiu a classificação dos transcritos em diferentes categorias funcionais, evidenciando alterações globais na expressão gênica associada com a aderência do fungo.

Com relação às ESTs encontradas na condição de adesão ao colágeno, as principais alterações foram associadas com metabolismo, transcrição, energia, ciclo celular e processamento de DNA. Em *C. albicans*, análises com microarranjo foram realizadas para elucidar os mecanismos envolvidos no processo de adesão (Marchais *et al*., 2005) e genes envolvidos em vários processos celulares, como organização e transporte celular, metabolismo e transcrição, também foram encontrados. Já foi demonstrado que genes codificando enzimas envolvidas no metabolismo de proteínas e lipídeos têm um papel importante na patogênese em *C. albicans* (Hube *et al.*, 1997; Hube *et al.*, 2000).

Dentre as ESTs encontradas na condição de adesão à fibronectina, várias correspondem a cDNAs relacionados com metabolismo, transcrição e transporte celular. Ainda, algumas proteínas envolvidas com choque térmico foram superexpressas. Segundo Albuquerque e colaboradores (2008), várias proteínas de choque térmico estavam presentes em vesículas secretórias de *H. capsulatum.* O papel dessas proteínas não se restringe a uma resposta ao choque térmico, mas pode ser parte do processo de adaptação para a sobrevivência do parasita no hospedeiro (Burnie *et al.*, 2005). Soltys & Gupta (1999) revisaram estudos mostrando a presença de proteínas classicamente mitocondriais em localizações celulares inesperadas. Nesse estudo, Hsp60, Hsp70 e ainda DnaJ foram encontradas em locais diferentes daqueles em que elas originalmente estariam, inclusive em vesículas secretórias, sugerindo a existência de uma via de secreção e também múltiplas funções para estas proteínas.

Os genes que codificam para a enolase, álcool desidrogenase e arginina metiltrasferase foram superexpressos nas duas condições. A expressão diferencial desses genes e ainda de *Pb*Ctr3, *Pb*NsdD, *Pb*Hxk, *Pb*Enoyl-CoA e *Pb*HSP70 observada pelas análises de RDA foram confirmadas por RT-PCR Tempo Real, ressaltando e a relevância desta técnica nas condições experimentais utilizadas.

O transcrito condificante para o transportador de cobre de alta afinidade (*Pb*Ctr3) foi superexpresso na condição de adesão a fibronectina. Bailão e colaboradores (2006) já haviam observado a superexpressão desse transportador em células leveduriformes de *P. brasiliensis* derivadas de tecidos infectados, ressaltando a necessidade da regulação da captação de cobre durante a infecção. E ainda, Dantas e colaboradores (2009) demonstraram que *Pb*Ctr3 é reconhecido por soros de pacientes com PCM. Em nosso estudo, *Pb*Ctr3 se ligou à componentes de ECM. A provável localização desse transportador na superfície celular ressalta a sua importância na patogênese de *P. brasiliensis.*

Muitas das enzimas metabólicas, em especial as da via glicolítica, são chamadas *housekeeping* pelo fato de desempenharem funções essenciais e pelo fato da expressão dessas enzimas não estar sob o controle de nenhuma maquinaria regulatória tecido-específica. Contudo, vários estudos já demonstraram que essas enzimas citoplasmáticas clássicas, que não possuem sequências sinalizadoras para a superfície celular ou mecanismos de ancoramento às membranas, foram encontradas na superfície de patógenos, desempenhando uma variedade de funções aparentemente não relacionadas àquelas originalmente descritas (Pancholi & Chhatwal, 2003), sendo, por isso, denominadas proteínas *moonlighting*. Sriram e colaboradores (2005) estudaram enzimas *moonlighting* envolvidas com doenças humanas e descreveram a participação dessas proteínas em diversos eventos celulares distintos, como transdução de sinal, regulação transcricional, apoptose, crescimento, motilidade e até funções estruturais. Segundo os autores, a casualidade tem sido o fator determinante em várias dessas descobertas, pois algumas dessas funções foram identificadas quando as enzimas em questão não eram sequer o alvo inicial do estudo.

Dentre os transcritos diferencialmente expressos na condição de adesão ao colágeno e à fibronectina, foi detectado aquele codificante para enolase a qual já foi descrita para outros micro-organismos patogênicos como uma molécula de interação com componentes da ECM. Neste estudo, o cDNA codificante para a proteína enolase, de massa molecular de 47 kDa e pI 5,67, foi obtido e, assim, foi possível expressar proteína recombinante que foi usada para a produção de anticorpo policlonal. A presença da enolase em extratos protéicos de células leveduriformes de *P. brasiliensis* foi confirmada por ensaios de *Western blotting*, eletroforese em gel de poliacrilamida bidimensional e espectrometria de massas.

Barbosa e colaboradores (2004) demonstraram a presença de GAPDH em maiores quantidades na fase leveduriforme de *P. brasiliensis* do que na fase miceliana, sugerindo um possível papel de GAPDH na fase parasitária deste fungo. Além disso, esta proteína está localizada na parede celular de *P. brasiliensis* onde ela foi capaz de interagir com os componentes da ECM, como laminina, fibronectina e colágeno tipo I. Ainda, GAPDH parece ser importante nos estágios precoces da infecção fúngica. O tratamento de pneumócitos com GAPDH e a incubação de células leveduriformes de *P. brasiliensis* com o anticorpo policlonal anti-GAPDH resultou na inibição da adesão e da infecção das células epiteliais (Barbosa *et al.*, 2006). A proteína TPI recombinante e o anticorpo policlonal anti-TPI também foram capazes de interferir na interação *in vitro* de *P. brasiliensis* com cultura de células epiteliais (Pereira *et al.*, 2006).

Neste estudo, foi demonstrada a propriedade adesiva da enzima glicolítica enolase pela observação da sua interação com laminina, fibronectina e colágeno tipo I através de experimentos de adesão dessas moléculas com a enolase recombinante imobilizada. De maneira semelhante, Esgleas e colaboradores (2008) expressaram a enolase de *Streptococcus suis* e demonstraram que a proteína estava presente na superfície celular, sendo capaz de se ligar à fibronectina e ao plasminogênio.

Carneiro e colaboradores (2004) sugeriram que a enolase poderia mediar a ligação de *Staphylococcus aureus* à laminina funcionando, assim, como um mecanismo de orientação, inicialmente permitindo a aderência de *S. aureus* à matriz extracelular, colonização tecidual e, em seguida, ativação do plasminogênio e degradação da laminina em áreas restritas. Também foi sugerido que a habilidade de *S. aureus* de se ligar a ambas, laminina e fibronectina, representaria um mecanismo importante pelo qual o micro-organismo poderia aderir e colonizar diferentes tecidos no hospedeiro. Do mesmo modo, a laminina parece ser importante para a adesão de *P. brasiliensis* (Mendes-Giannini *et al.*, 2006), podendo participar na disseminação e invasão tecidual por parte desse fungo (Andreotti *et al.*, 2005; Vicentini *et al.*, 1994).

A enolase é uma das mais abundantes enzimas expressas no citoplasma de muitos organismos (Pancholi, 2001) e, embora nenhuma seqüência peptídeo-sinal para secreção ou endereçamento para a membrana externa tenha sido identificada, enolases localizadas na membrana externa de bactérias e eucariotos já foram descritas (Pancholi & Fischetti, 1998; López-Villar *et al.*, 2006). Há várias proteínas endereçadas a mais de uma localização em ambos procariotos e eucariotos com funções biológicas variadas.

Pal-Bhowmick e colaboradores (2007) demonstraram que, em *Plasmodium yoelii*, a enolase está associada às membranas celulares e à frações nucleares e do citoesqueleto onde poderia desempenhar funções diversas. Em *P. brasiliensis* foi demonstrado no presente trabalho a localização da enolase na superfície de células leveduriformes.

A presença da enolase na superfície de células eucarióticas pode mediar ligação celular ao plasminogênio, levando ao aumento da sua ativação e localização na superfície celular da atividade proteolítica da plasmina (Agarwal *et al.*, 2006). *P. brasiliensis* e a enolase recombinante de *P. brasiliensis* se ligam ao plasminogênio, que é ativado pelo tPA. Dessa forma, o fungo seria capaz de adquirir atividade proteolítica, pois a plasmina gerada é uma enzima chave do sistema plasminogênio e contribui para a degradação de uma variedade de constituintes da matriz. A ligação ao plasminogênio e a sua conversão à plasmina (uma serina protease) pode contribuir para a patogenicidade de *P. brasiliensis* por facilitar invasão tecidual no hospedeiro.

Segundo Bergmann e colaboradores (2001), a enolase de *Streptococcus pneumoniae* foi secretada e capaz de se reassociar com a superfície da bactéria aumentando a ligação desta ao plasminogênio. A incubação de células epiteliais e fagócitos com enolase recombinante de *P. brasiliensis* tmabém levou a um aumento da interação do fungo com estes sistemas celulares. Neste estudo, foi mostrado que a proteína enolase é secretada em *P. brasiliensis*, sugerindo um importante papel na patogenicidade do fungo.

Esses resultados sugerem que a enolase está potencialmente envolvida nos mecanismos de adesão e disseminação, requeridos durante o processo infectivo de *P. brasiliensis*, e podem levar a uma melhor compreensão da interação de *P. brasiliensis* com os tecidos do hospedeiro e da patogênese da PCM.

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