Estudos estruturais e funcionais sobre a glucuronoxilomanana dos patógenos fúngicos *Cryptococcus neoformans, Cryptococcus gattii* e *Trichosporon asahii*

Fernanda Lopes Fonseca

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências (Microbiologia), Instituto de Microbiologia Prof. Paulo de Góes da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Ciências Biológicas (Microbiologia)

Orientadores: Marcio Lourenço Rodrigues
Leonardo Nimrichter

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Aprovada por:

(Dra. Celuta Sales Alviano, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro)

(Dr. Marcio Lourenço Rodrigues, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro)

(Dr. Paulo Antônio de Souza Mourão, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro)

(Dra. Marilene Henning Vainstein, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul)

(Dr. André Luis Souza dos Santos, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro)

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2- Glucuronoxylomannan-mediated interaction of *Cryptococcus neoformans* with human alveolar cells results in fungal internalization and host cell damage. Microbes and Infection 8(2):493-502, 2006.

3- Binding of glucuronoxylomannan to the CD14 receptor in human A549 alveolar cells induces interleukin-8 production. Barbosa FM, Fonseca FL, Figueiredo RT, Bozza MT, Casadevall A, Nimrichter L, Rodrigues ML. Clinical and Vaccine Immunology, 14(1):94-8, 2007.

4- *Paracoccidioides brasiliensis* enolase is a surface protein that binds plasminogen and mediates of yeast forms with host cells. Nogueira SV, Fonseca FL, Rodrigues ML, Mundodi V, Abi-Chacra EA, Winters MS, Alderete JF, Soares CM. Infection and Immunity, July 2010.

RESUMO:

Estudos estruturais e funcionais sobre glucuronoxilomanana nos patógenos fúngicos *Cryptococcus neoformans, Cryptococcus gattii* e *Trichosporon asahii*

Fernanda Lopes Fonseca

Orientação: Marcio Lourenço Rodrigues e Leonardo Nimrichter

A glucuronoxilomanana (GXM) é um polissacarídeo encontrado na superfície celular de *Cryptococcus neoformans, Cryptococcus gattii* e *Trichosporon asahii*, fungos causadores de infecções com altas taxas de mortalidade. No complexo *Cryptococcus*, a GXM é o componente capsular majoritário, sendo considerado seu principal fator de virulência. São bem estudados os aspectos relacionados à função de GXM na imunopatogênese da infecção causada por *C. neoformans*. O papel desse polissacarídeo nas infecções causadas por *C. gattii* e *T. asahii*, entretanto, é desconhecido. Mecanismos relacionados à GXM, envolvidos na arquitetura da superfície celular dos patógenos acima mencionados, são também obscuros, assim como a relação entre estrutura e função do polissacarídeo. Nesse estudo, vários aspectos relacionados à estrutura e função da GXM de *C. neoformans*, *C. gattii* e *T. asahii* foram investigados. Nossos ensaios incluíram o estudo da arquitetura da GXM na superfície celular e também o impacto de várias amostras do polissacarídeo na interação desses fungos com células do hospedeiro.

Nos três patógenos estudados, a conexão da GXM com a parede celular envolveu moléculas derivadas de quitina, conforme determinado por microscopia de fluorescência usando marcadores específicos. Em *C. neoformans*, ensaios cromatográficos e físico-químicos demonstraram que quitina e oligômeros de quitina são capazes de se ligar ao polissacarídeo e formar complexos que interferem na organização capsular e na reatividade com um anticorpo monoclonal anti-GXM. A inibição da síntese de estruturas derivadas da quitina resultou em alterações expressivas na superfície celular, incluindo a geração de fibras de GXM com dimensões reduzidas.

Além de ser um componente estrutural, a GXM também é secretada para o meio extracelular, onde é responsável por vários efeitos imunomoduladores no hospedeiro. Em nosso estudo, demonstramos que a GXM de *C. neoformans* e *C. gattii* são capazes de ativar a resposta celular mediada por heterodímeros de receptores do tipo Toll (TLR), como TLR2/1 e TLR2/6. O polissacarídeo obtido de uma cepa de *C. gattii* de sorotipo B mostrou a maior eficiência na ativação de receptores TLR, além de uma maior capacidade de estimular a

produção de óxido nítrico (NO) por macrófagos murinos. Várias propriedades estruturais foram analisadas nas amostras de GXM citadas acima. Para amostras de *C. gattii* (sorotipo B), foi observada uma clara correlação entre a estimulação de respostas celulares e dimensões reduzidas das fibras de GXM.

As propriedades da GXM em *C. neoformans* e seu efeito na modulação das defesas do hospedeiro são bem conhecidas. Entretanto, pouco se sabe sobre aspectos estruturais e funcionais da GXM de *T. asahii*. Nossos ensaios demonstraram que a GXM desse fungo compartilha determinantes antigênicos com o polissacarídeo criptococócico. Entretanto, a GXM de *T. asahii* apresentou dimensões reduzidas e teor de carboidratos distinto. Pela primeira vez na literatura, demonstramos que a GXM de *T. asahii* apresenta capacidade de proteger células fúngicas contra fagocitose como ocorre com *C. neoformans*. Essa observação sugere que o polissacarídeo possui funções compartilhadas em diferentes gêneros fúngicos.

Nessa tese, descrevemos novos aspectos relacionados a estrutura e função de GXM em três espécies fúngicas. O estudo das propriedades estruturais e funcionais desse importante polissacarídeo é promissor para o entendimento de mecanismos de patogenicidade e, possivelmente, na elaboração de novas estratégicas terapêuticas e de prevenção das infecções causadas por *C. neoformans*, *C. gattii* e *T. asahii*.

Palavras-chave: Glucuronoxilomanana, *Cryptococcus*, Cápsula, Arquitetura Capsular, Quitina, *Trichosporon*, Resposta imune

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ABSTRACT:

Structural and functional studies of glucuronoxylomannan in the fungal pahogens *Cryptococcus neoformans, Cryptococcus gattii* and *Trichosporon asahii*

Fernanda Lopes Fonseca

Orientação: Marcio Lourenço Rodrigues e Leonardo Nimrichter

Glucuronoxylomannan (GXM) is a surface polysaccharide of *Cryptococcus neoformans, Cryptococcus gattii* and *Trichosporon asahii*, fungal pathogens associated with high mortality rates. In the complex *Cryptococcus*, GXM is a major capsular component that also represents its main virulence factor. The role of GXM in the immunopathogenesis of cryptococcal infections has been widely studied. However, functions of the polysaccharide in infections caused by *C. gattii* and *T. asahii* are virtually unknown. Mechanisms involved in the GXM-related cell surface architecture of the pathogens mentioned above are still obscure, which is also true for the relation between structure and functions of the polysaccharide. In this work, several functional and structural aspects of *C. neoformans*, *C. gattii* and *T. asahii* GXMs were studied. Our experiments included the study of the architecture of GXM at the cell surface and also the impact of several polysaccharide samples in the interaction of these fungi with host cells.

In all three pathogens, GXM connections with the cell wall involved chitin-like molecules, as determined by fluorescence microscopy using specific staining reagents. In *C. neoformans*, chromatographic and physical-chemistry assays showed that chitin and chitooligomers can bind to the cryptococcal polysaccharide and form complexes that interfere with capsular assembly and reactivity with a monoclonal antibody to GXM. Inhibition of the synthesis of chitin-like structures resulted in marked alterations of the cell surface, including the generation of GXM fibers with reduced dimensions.

Besides being a structural component of the cellular surface, GXM is also secreted into the extracellular environment space, where it is responsible for several immunomodulatory effects during infection of the host. In our study, we demonstrated that *C. neoformans* and *C. gattii* GXMs activate cellular responses mediated by heterodimers of Toll-like receptors (TLR), as TLR2/1 and TLR2/6. A polysaccharide sample from *C. gattii* (serotype B) showed the most effectiveness in activating the TLR-mediated response. This sample was also the most highly efficient in eliciting the production of NO by murine macrophages. Several structural properties were analyzed in the GXM samples mentioned above. For *C. gattii* samples (serotype B), a

correlation between stimulation of cellular responses and reduced dimension of GXM fibers was observed.

The properties of cryptococcal GXMs and their effects on host defenses are well known. Structural and functional aspects of the *T. asahii* GXM, however, remain elusive. Our assays demonstrated that the trichosporal GXM shares antigenic determinants with the criptococcal polysaccharide. However, polysaccharide fractions from *T. asahii* showed reduced dimensions and distinct monosaccharide composition. For the first time in the literature we demonstrated that the trichosporal GXM protects fungal cells against phagocytosis, as observed for *C. neoformans*. This observation suggests that the polysaccharide has shared functions in different fungal genera.

In this thesis, we describe new functional and structural of GXMs in three fungal species. We believe that studies on this important polysaccharide may contribute to the understanding of pathogenic mechanisms shared by different pathogens and, possibly, to the development of new strategies aiming at control and prevention of infections caused by *C. neoformans*, *C. gattii* and *T. asahii*.

Key-words: Glucuronoxylomannan, *Cryptococcus*, Capsule, Capsular Architecture, Chitin, *Trichosporon*, Immune response

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LISTA DE SIGLAS E ABREVIATURAS:

- AIDS (*Acquired Immunodeficiency Syndrome*): Síndrome da Imunodeficiência Adquirida
- ConA: Concanavalina A
- CTAB (*Cetyl trimethylammonium bromide*): brometo de hexadecil trimetilamônio
- DMSO: Dimetilsulfóxido
- EDTA (*Ethylenediamine tetraacetic acid*): Ácido etilenodiamino tetracético
- FACE (*Fluorescent-assisted carbohydrate electrophoresis*): Eletroforese de carboidratos assistida por fluoróforo
- GalXM: Galactoxilomanana
- GC-MS (*Gas Chromatography-Mass Spectrometry*): Cromatografia gasosa acoplada à espectrometria de massas
- GlcA (*Glucuronic acid*): Ácido glucurônico
- GXM: Glucuronoxilomanana
- GXMGal: Glucuronoxilomananogalactana
- HAART (*Highly Active Antiretroviral Therapy*): Terapia anti-retroviral de alta atividade
- HIV (*Human Immunodeficiency Vírus*): Vírus da imunodeficiência humana
- IL: Interleucina
- INFγ: Interferon-gama
- LPS: Lipopolissacarídeo
- MP: Manoproteína
- NF-κB (*Nuclear factor Kappa B*): fator nuclear Kappa B
- NO (*Nitric oxid*): óxido nítrico
- Nva-FMDP: L-norvalil- N^3-(4-metoxifumaroil)-L-2,3-ácido diaminopropanóico
- PMN (*Polymorphonuclear leukocytes*): Leucócitos polimorfonucleares
- RMN: ressonância magnética nuclear
- SPH (S*ummer-Type Hypersensivity Pneumonitis*): Pneumonia de hipersensibilidade do verão
- TLR (*Toll-like receptors*): Receptores do tipo *Toll*
- TNF-α (*Tumor necrosis factor*- α): Fator de necrose tumoral-alfa
- UTI: Unidade de terapia intensiva
- WGA (*Wheat germ agglutin*): Lectina do germe de trigo
- β-1,4-GlcNAc: β-1,4-*N*-acetilglucosamina

I – Introdução:

1- Infecções fúngicas: um problema mundial.

Nas últimas três décadas, as infecções fúngicas tornaram-se uma das maiores causas de doenças em indivíduos imunocomprometidos ou hospitalizados com sérias doenças de base (PERLROTH, CHOI & SPELLBERG, 2007; KOLLEF *et al.*, 2008; RICHARDSON & LASS-FLORL, 2008; HORN *et al.*, 2009; NEOFYTOS *et al.*, 2009). Esse tipo de infecção, associada a altas taxas de mortalidade (SANCHEZ & LARSEN, 2007), vem aumentando progressivamente (PFALLER & DIEKEMA, ; REINGOLD *et al.*, 1986; REES *et al.*, 1998; WILSON *et al.*, 2002; PFALLER & DIEKEMA, 2007). Um estudo epidemiológico nos Estados Unidos revelou que o número anual de sepses causadas por fungos aumentou 207% entre 1979 e 2000 (MARTIN *et al.*, 2003).

A incidência e a severidade das infecções fúngicas estão associadas a uma série de fatores, incluindo: patologias imunodepressoras, o amplo uso de drogas imunossupressoras, antineoplásicas e antibióticos de amplo espectro, e também do aumento de procedimentos médicos utilizando cateteres e próteses (PFALLER & DIEKEMA, 2007). Além disso, avanços tecnológicos na medicina e na farmacologia resultam em melhorias na sintomatologia de doenças crônicas de base e no aumento da sobrevida de pacientes criticamente doentes, porém os tornam mais vulneráveis às infecções fúngicas (LASS-FLORL, 2009). Assim, compõem a população de risco: indivíduos com a síndrome da imunodeficiência adquirida (AIDS, do inglês, *Acquired Immunodeficiency Syndrome*), pacientes com neoplasias e indivíduos transplantados de órgãos ou medula, além de neonatos prematuros, pacientes queimados, cirúrgicos ou sob longos cuidados em unidades de terapia intensiva (UTI) (HOLZHEIMER & DRALLE, 2002; DIMOPOULOS *et al.*, 2003; MAHFOUZ & ANAISSIE, 2003; PERES-BOTA *et al.*, 2004; MEERSSEMAN *et al.*, 2007).

Candida albicans, *Aspergillus fumigatus* e *Cryptococcus neoformans* são causadores de graves micoses oportunistas de alta incidência global e altas taxas de mortalidade (PFALLER & DIEKEMA, 2004; PROCOP & ROBERTS, 2004; PFALLER *et al.*, 2006; FISHMAN, GONZALEZ & BRANDA, 2008; TELLEZ *et al.*, 2008; ZILBERBERG, SHORR & KOLLEF, 2008). No Brasil, estudos apontam que as micoses sistêmicas são a décima causa de morte dentre as doenças infecciosas e parasitárias (PRADO *et al.*, 2009).

A lista de potenciais patógenos fúngicos está em clara expansão, bem como as taxas de morbidade e mortalidade associadas a infecções causadas por esses microrganismos. Nos últimos anos, infecções emergentes causadas por fungos antes considerados de baixa capacidade de invasão e colonização vêm sendo cada vez mais frequentes (NUCCI & MARR,

2005). Fazem parte dos fungos oportunistas emergentes: leveduras de espécies de *Candida* não-*albicans*, *Aspergillus* não-*fumigatus*, *Trichosporon* spp., *Rhodotorula* spp. e fungos filamentosos de espécies de *Fusarium*, *Acrenomium*, *Scedosporium*, entre outros (PROCOP & ROBERTS, 2004; RODEN *et al.*, 2005; NUCCI & ANAISSIE, 2007; PFALLER *et al.*, 2007; CORTEZ *et al.*, 2008; RICHARDSON & LASS-FLORL, 2008; ZARAGOZA *et al.*, 2008; PFALLER *et al.*, 2009).

Estudos recentes conduzidos em centros médicos americanos entre 2004 e 2008 revelaram que a distribuição de patógenos fúngicos varia de acordo com a doença de base ou com o procedimento clínico ao qual o paciente é submetido. Por exemplo, espécies de *Cryptococcus* têm uma maior incidência em pacientes com AIDS, enquanto espécies de *Aspergillus* infectam, em geral, indivíduos transplantados de medula. Fungos emergentes, como *Malassezia spp*, *Rhodotorula* spp., *Pneumocystis* spp. e *Trichosporon* spp., acometem principalmente pacientes com neoplasias hematológicas (FISHMAN, GONZALEZ & BRANDA, 2008; HORN *et al.,* , 2009; NEOFYTOS *et al.,* , 2009).

Desse modo, em função das altas incidência, morbidade e mortalidade decorrentes das infecções fúngicas, além da resistência aos agentes antifúngicos e da similaridade da apresentação clínica, as micoses vêm despertando grande interesse na comunidade científica. Nesse contexto, o entendimento dos mecanismos pelo quais os fungos interagem com o hospedeiro humano, bem como a relação entre estrutura e função de moléculas associadas à patogênese, pode revelar novas estratégias terapêuticas e de diagnóstico.

2 – *Cryptococcus* spp. e *Trichosporon* spp.: o que esses fungos têm em comum?

Tanto *Cryptococcus* spp. quanto *Trichosporon* spp. são patógenos fúngicos de grande importância médica, causadores da criptococose e da tricosporonose, respectivamente. Ambas as infecções são de incidência relativamente alta e apresentam altos índices de morbidade e mortalidade, principalmente em indivíduos imunocomprometidos. Além dessa semelhança, esses fungos têm uma importante característica em comum: são produtores de glucuronoxilomanana (GXM). A GXM é um polissacarídeo de alta atividade imunológica, composto de por manose, xilose e ácido glucurônico (CHERNIAK *et al.*, 1995; ICHIKAWA *et al.*, 2001). A molécula, bastante estudada em *Cryptococcus* spp., é o componente capsular majoritário nesse gênero, sendo considerada como o principal fator de virulência. Tal polissacarídeo também é produzido por *Trichosporon* spp. e foi caracterizado como um componente de superfície celular (MELCHER *et al.*, 1991). Entretanto, essa estrutura é pouco estudada nesse fungo, possivelmente em função de seu caráter ainda emergente.

O polissacarídeo em questão confere aos patógenos uma estratégia de escape das defesas do hospedeiro, aparentemente contribuindo para a patogênese fúngica (KOZEL & GOTSCHLICH, 1982; LYMAN & WALSH, 1994; FELDMESSER, TUCKER & CASADEVALL, 2001; KARASHIMA *et al.*, 2002; VECCHIARELLI, 2007; ZARAGOZA *et al.,* , 2008). Entretanto, os estudos com modelos de *Trichosporon* spp. ainda são muito prematuros, limitados e inconclusivos. No gênero *Cryptococcus*, ao contrário, diversos estudos sobre as funções do polissacarídeo estão disponíveis na literatura (ZARAGOZA *et al.*, 2009). No entanto, várias das funções do polissacarídeo ainda permanecem obscuras (RODRIGUES *et al.*, 2009).

Desse modo, tendo em vista a importância da GXM e as altas taxas de incidência e mortalidade nas infecções causadas por espécies de *Cryptococcus* e *Trichosporon*, investigações voltadas para a elucidação estrutural e funcional desse polissacarídeo podem gerar contribuições relevantes para o entendimento dos mecanismos de patogenicidade e para a descoberta de alternativas para o tratamento dessas micoses.

2.1 - *Cryptococcus* spp.: patógeno fúngico capsulado causador da criptococose.

Cryptococcus spp. é um basidiomiceto que, tradicionalmente, se apresenta como uma levedura haplóide, esférica e circundada por uma cápsula, característica única dentre os eucariotos (MCFADDEN, ZARAGOZA & CASADEVALL, 2006; LIN, 2009).

Embora mais de 30 espécies estejam incluídas no gênero *Cyptococcus*, apenas duas leveduras desse grupo são consideradas patogênicas, sendo capazes de causar criptococose em humanos: *C. neoformans* e *C. gattii* (CHAYAKULKEEREE & PERFECT, 2006).

De acordo com a classificação taxonômica atual, essas espécies compõem o complexo de espécies de *C. neoformans*, que, anteriormente, era dividido em duas variedades: *C. neoformans* var. *neoformans* e *C. neoformans* var. *gattii*. Entretanto, atualmente, *C. neoformans* var. *gattii* é considerado como uma espécie distinta (LIN, 2009). Essas espécies também são classificadas segundo a reação de aglutinação do seu polissacarídeo capsular em sorogrupos e ainda em variedades. Nessa divisão, *C. neoformans* var. *grubii* é representante do sorotipo A; *C. neoformans* var. *neoformans*, do sorotipo D e espécies de *C. gattii* estão incluídas nos sorotipos B e C, como esquematizado na **Figura 1**. (FRANZOT, SALKIN & CASADEVALL, 1999; BOEKHOUT *et al.*, 2001; KWON-CHUNG & VARMA, 2006; LIN & HEITMAN, 2006; LIN, 2009).

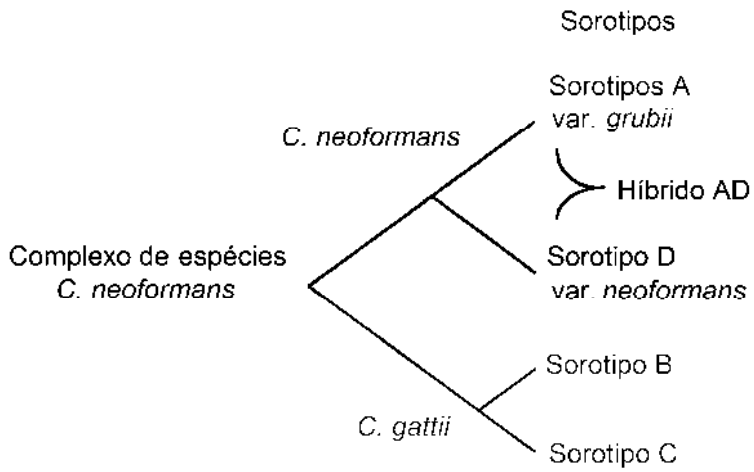

Figura 1. Classificação taxonômica atual do complexo *Cryptococcus*. O complexo *Cryptococcus* é dividido em duas espécies (*C. neoformans* e *C. gattii*) e 5 sorotipos (incluindo o sorotipo híbrido diplóide AD), de acordo com características de seus polissacarídeos capsulares. Adaptado a partir de LIN, 2009.

C. neoformans é um fungo cosmopolita e saprófito, que, apesar de colonizar humanos causando doença, não necessita desse hospedeiro para completar seu ciclo de vida (ELLIS & PFEIFFER, 1990). Sendo assim, os isolados clínicos encontrados são provenientes do ambiente, de acordo com a ampla sobreposição genotípica e fenotípica revelada por estudos de tipagem molecular (YAMAMOTO *et al.*, 1995; SORRELL *et al.*, 1996; NOSANCHUK *et al.*, 2000; DELGADO *et al.*, 2005). De fato, *C. neoformans* é encontrado em solo contaminado com excrementos de pássaros e em árvores (LIN, 2009). *C. gattii* (sorotipos B e C) é comumente encontrado em árvores de regiões tropicais e subtropicais, especialmente eucaliptos. Isolados de *C. neoformans*, pertencentes aos sorotipos A e D, por sua vez, estão associados a excrementos de pombos presentes no solo (LIN, 2009). Acredita-se, inclusive, que os pombos sejam responsáveis pela sua ampla distribuição, já que são capazes de carrear células de *C. neoformans* externamente ao seu corpo através do bico, penas e patas (PAL, 1989). Em ambas as espécies as condições naturais nas quais o fungo é encontrado são favoráveis ao seu crescimento como saprófito e propiciam a amplificação mitótica e fusão celular entre esses isolados (ABOU-GABAL & ATIA, 1978; STAIB, 1981; STAIB & BLISSE, 1982; GRANADOS & CASTANEDA, 2005; FILION, KIDD & AGUIRRE, 2006; NIELSEN, DE OBALDIA & HEITMAN, 2007).

2.1.1 – Incidência e patogênese da criptococose.

Apesar de *C. neoformans* ter sido descoberto como um patógeno há mais de um século (revisto por (MITCHELL & PERFECT, 1995), antes da pandemia da AIDS, a incidência da doença atingia níveis pouco relevantes de 0,2 a 0,8 em 100000 indivíduos (HAJJEH *et al.*, 1999; CHAYAKULKEEREE & PERFECT, 2006). Entretanto, há quase 30 anos, com a pandemia da AIDS, a incidência da criptococose aumentou drasticamente. De acordo com revisões de dados epidemiológicos da época, 80% dos casos de criptococose estavam associados a indivíduos HIV positivos, em virtude da imunodeficiência causada pela redução da função das células T CD4 nos mesmos (DROMER *et al.*, 1996; HAJJEH *et al.,* , 1999; CHAYAKULKEEREE & PERFECT, 2006).

Na década de 90, após a instituição da terapia anti-HIV, conhecida como HAART (do inglês, *Highly Active Antiretroviral Therapy*), a incidência da criptococose diminuiu nos países que instituíram esse tratamento. Entretanto, a infecção por *C. neoformans* ainda é um problema frequente e devastador, principalmente no continente africano (STANGEL, MULLER & MARX, 1998; CHAYAKULKEEREE & PERFECT, 2006; HUSTON & MODY, 2009; PARK *et al.*, 2009). Estudos recentes mostram que a incidência atual da criptococose varia de 0,04% a 12% por ano dentre indivíduos com AIDS (PARK *et al.,* , 2009). Segundo esse mesmo estudo, mundialmente, ocorrem quase 1 milhão de casos de meningite criptococócica por ano, resultando em cerca 600 mil mortes 3 meses após a infecção (PARK *et al.,* , 2009). Embora a região mais afetada mundialmente seja o sul da África, a criptococose também assume índices alarmantes de mortalidade em pacientes com AIDS no Brasil. Os valores de incidência associados a mortes em indivíduos com AIDS estão na faixa de 50%, fazendo que a criptococose seja a micose de maior importância nesses pacientes em nosso país (PARK *et al.,* , 2009; PRADO *et al.,* , 2009).

A infecção por *C. neoformans* pode afetar não só pacientes com AIDS, mas também aqueles que possuem algum grau de imunocomprometimento, seja associado ao uso de terapia imunossupressora, a doenças, como câncer, ou a procedimentos médicos, como transplante de órgãos ou medula. Essa chamada população de risco cresce progressivamente juntamente com a incidência da criptococose. De fato, a doença já é considerada a terceira maior infecção fúngica invasiva que acomete pacientes submetidos à transplante de órgãos (VILCHEZ, FUNG & KUSNE, 2002; CHAYAKULKEEREE & PERFECT, 2006). Dentre os casos de criptococose em indivíduos imunocomprometidos, *C. neoformans* var *grubii* (sorotipo A) é o isolado mais comumente encontrado em espécimes clínicos, contabilizando 95% de incidência (VILCHEZ, FUNG & KUSNE, 2002; CHAYAKULKEEREE & PERFECT, 2006).

É sabido que a criptococose causada por espécies de *C. neoformans* (sorotipos A, D e AD) tem uma ocorrência mundial e acomete majoritariamente imunocomprometidos,

enquanto que a criptococose causada por *C. gattii* (sorotipos B e C) é mais incidente em imunocompetentes e tem sua distribuição mais limitada a regiões tropicais e subtropicais (HUSTON & MODY, 2009). Uma notável exceção a essa proposta de distribuição global foi o surto ocorrido em Vancouver, Canadá, no final da década de 90. A incidência de infecções por *C. gattii* na ilha foi a maior já existente, sendo quase 40 vezes maior que a incidência na Austrália, onde a criptococose é considerada endêmica (KWON-CHUNG & BENNETT, 1984; KIDD *et al.*, 2004; CHAYAKULKEEREE & PERFECT, 2006). Além disso, foram observadas altas taxas de morbidade e mortalidade e os isolados clínicos foram considerados hipervirulentos, em virtude, provavelmente, de um evento de recombinação genética (CHAYAKULKEEREE & PERFECT, 2006).

Apesar de ser bem aceita pela comunidade científica a observação de que *C. gattii* e *C. neoformans* podem causar doenças com quadros de patogenicidade diferenciados, é fato que, independentemente da espécie fúngica causadora da criptococose, a terapia disponível para o tratamento é restrita, tóxica e pouco potente, o que torna a doença ainda incurável (LIN, 2009).

A similaridade entre isolados clínicos e ambientais caracterizada por estudos de tipagem molecular nos permite afirmar que o estabelecimento da infecção por *C. neoformans* se dá pela exposição humana a ambientes onde o fungo esteja presente, seja em árvores ou em solos contaminados por fezes de pássaros, como já dito anteriormente e ilustrado na **Figura 2** (CHAYAKULKEEREE & PERFECT, 2006). No entanto, o desenvolvimento ou não da doença estará associado ao balanço entre a resposta imune do hospedeiro e os fatores de virulência do fungo (CASADEVALL & PIROFSKI, 2003).

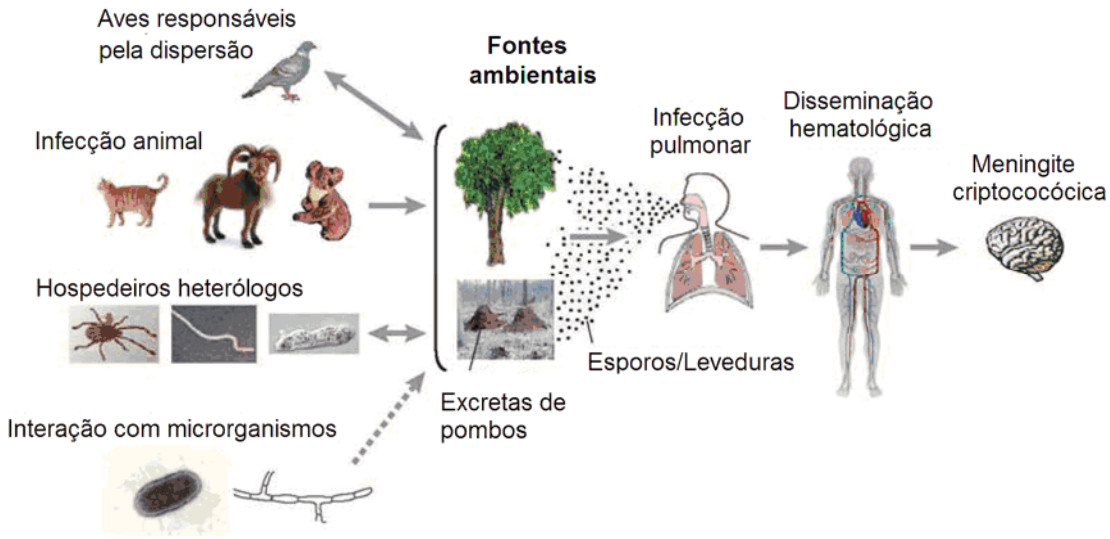

Figura 2. Ciclo de infecção de *Cryptococcus* spp. O fungo pode sobreviver em solos e em árvores. Pombos são considerados responsáveis pela ampla dispersão das células fúngicas. *C. neoformans*, as quais são capazes de infectar vários animais como gatos, cabras e coalas, além de sobreviverem ao ataque de predadores ambientais como insetos, vermes e amebas. Além disso, a levedura interage com outros microrganismos, como bactérias e outros fungos. *C. neoformans* causa, em humanos, uma infecção pulmonar através da inalação de esporos ou leveduras dessecadas presentes no ambiente, que pode evoluir para uma meningite de altas taxas de letalidade. Adaptado a partir de (LIN & HEITMAN, 2006).

Acredita-se que a criptococose humana é adquirida através da inalação de basidiosporos ou leveduras dessecadas, que são depositadas no espaço alveolar. Sendo assim, o sítio primário de infecção é o pulmão. Dependendo do estado imunológico do hospedeiro, *C. neoformans* e *C. gattii* podem colonizar o homem sem causar doença. Nesse caso, a infecção é assintomática e pode ser rapidamente debelada ou assumir uma forma latente. Por outro lado, quando o hospedeiro apresenta alguma falha na sua resposta imune, essa forma latente pode ser reativada e disseminada pela corrente sanguínea, causando uma infecção sistêmica, que pode envolver órgãos como pele, olhos, ossos, pulmão, próstata ou trato urinário (LIN & HEITMAN, 2006). Apesar da capacidade de disseminação para outros órgãos, as espécies de *Cryptococcus* têm uma predileção pelo sistema nervoso central, onde causam quadros de meningoencefalite. Essa é a doença mais frequente e grave, mostrando-se comumente fatal se não tratada em estágios iniciais. A natureza do neurotropismo desse fungo ainda é desconhecida (LIN & HEITMAN, 2006).

Como citado anteriormente, as partículas fúngicas inaladas são primeiramente depositadas no espaço alveolar e a partir daí podem alcançar o interstício pulmonar. Desse modo, as primeiras células de defesa a interagirem com *C. neoformans* são os macrófagos residentes e o epitélio alveolar. Portanto, uma interação bem sucedida de *C. neoformans* com essas células hospedeiras é crucial para o estabelecimento e desenvolvimento da infecção (BARBOSA *et al.*, 2006). No ambiente alveolar, os macrófagos residentes juntamente com as células epiteliais liberam quimiocinas pro-inflamatórias que estão associadas com o amplo recrutamento de leucócitos polimorfonucleares (PMN) da vasculatura pulmonar para dentro do alvéolo. Essas células de defesa recrutadas fornecem uma capacidade fagocítica extra, além de liberarem citocinas como interleucina (IL)-1β, IL-6, fator de necrose tumoral α (TNF-α), sendo essenciais para efetiva eliminação do fungo (ZHANG *et al.*, 2000; RODRIGUES *et al.,* , 2009).

Para disseminar-se e causar meningite, *C. neoformans* ainda deve ser capaz de atingir a corrente sanguínea e, então, interagir com e atravessar as células endoteliais microvasculares que formam a barreira hematoencefálica. Esse processo pode ocorrer por transcitose induzida pelo próprio fungo (CHEN *et al.*, 2003; CHANG *et al.*, 2004; FILLER & SHEPPARD, 2006) ou por um mecanismo conhecido como Cavalo de Tróia, no qual *C. neoformans* é internalizado por células monocluclereas circulantes, que são capazes de atravessar a barreira hematoencefálica e atingir o sistema nervoso central (CHRETIEN *et al.*, 2002; LUBERTO *et al.*, 2003; SANTANGELO *et al.*, 2004; LIN & HEITMAN, 2006).

Os mecanismos que auxiliam *C. neoformans* a burlar as defesas do hospedeiro, disseminar e gerar dano ao homem envolvem a múltipla expressão de fatores de virulência. A produção de melanina (WILLIAMSON, 1997) e enzimas extracelulares como urease (COX *et al.*, 2000), fosfolipase B (COX *et al.*, 2001) e superóxido dismutase (COX *et al.*, 2003), além da regulação de importantes vias sinalização para o fungo (LENGELER *et al.*, 2000) são fundamentais para o estabelecimento da infecção e disseminação do fungo durante o curso da doença. Entretanto, a produção de polissacarídeos capsulares é o mecanismo de patogenicidade mais bem estudado e talvez o mais importante utilizado por *C. neoformans*, conforme será mostrado mais a frente.

2.2 - *Trichosporon* spp.: um patógeno fúngico produtor de GXM.

Trichosporon spp. são basidiomicetos predominantemente leveduriformes que também são capazes de formar artroconídeos, blastoconídeos, hifas e pseudohifas (CHAGAS-NETO, CHAVES & COLOMBO, 2008). As espécies pertencentes ao gênero *Trichosporon* são

amplamente distribuídas na natureza e podem ser encontradas na água, no solo, em excretas de pássaros e em madeira em decomposição. Além disso, esses fungos podem ocasionalmente pertencer a microbiota gastrointestinal de humanos, bem como colonizar a pele e o trato respiratório. Entretanto, apesar de serem membros normais da flora microbiana, diferentes espécies de *Trichosporon* são causadoras de infecções superficiais e profundas em humanos (CHAGAS-NETO, CHAVES & COLOMBO, 2008). Atualmente, o gênero *Trichosporon* possui uma grande relevância médica, fato relacionado a sua capacidade de causar infecções invasivas em pacientes imunocomprometidos (PFALLER & DIEKEMA, 2004).

O gênero *Trichosporon* foi criado em 1890 para agrupar espécies causadoras de micoses superficiais em humanos que tinham características semelhantes. Entretanto, a taxonomia tradicional, baseada em aspectos morfológicos, ecológicos e fisiológicos, reuniu num mesmo *taxon*, isolados com comportamento heterogêneo, o que tornou urgente uma revisão taxonômica e uma nova classificação das espécies (GUEHO, DE HOOG & SMITH, 1992). Desse modo, pesquisadores propuseram uma nova classificação dentro do gênero, baseada em análises moleculares correlacionadas com características morfológicas, fisiológicas e bioquímicas (GUEHO, DE HOOG & SMITH, 1992). De acordo com esse estudo, *T. beigelli*, espécie patogênica mais incidente do gênero até o momento, foi reclassificada dando origem a 6 novas espécies: *T. asahii*, *T. cutaneous*, *T. asteroides*, *T. mucoides*, *T. inkin* e *T. ovoides*. Atualmente, existem mais de 35 espécies pertencentes ao gênero *Trichosporon* (CHAGAS-NETO, CHAVES & COLOMBO, 2008).

2.2.1 – Incidência e patogênese da tricosporonose.

A incidência das infecções causadas por *Trichosporon* spp., vem crescendo progressivamente, em função do aumento do número de indivíduos imunocomprometidos. Esse fato concedeu às espécies de *Trichosporon* o título de fungos patogênicos emergentes (PFALLER & DIEKEMA, 2004). A maioria dos casos é relatada em pacientes com câncer e com severo grau de neutropenia, seja por alguma doença subjacente ou por terapia imunossupressora. A tricosporonose sistêmica e invasiva, que podem ser causadas por *T. asahii* e *T. mucoides,* são as doenças mais graves associadas ao gênero, com altas taxas de morbidade e mortalidade. De acordo com dados epidemiológicos, a tricosporonose é considerada a segunda doença mais incidente em pacientes com tumores hematológicos, ficando atrás apenas da candidíase (PFALLER & DIEKEMA, 2004).

Mais de 90% das tricosporonoses é causada por *T. asahii* (ICHIKAWA *et al.*, 2004) e os índices de mortalidade associados a esta micose atingem até 80% (PFALLER & DIEKEMA, 2004). A infecção humana desencadeada por esse fungo pode ocasionar de simples problemas de pele a quadros respiratórios como a pneumonia de hipersensibilidade do verão (SHP, do inglês, *Summer-type Hypersensivity Pneumonitis*) (SANCHEZ & LARSEN, 2007). A SHP, bastante freqüente no Japão, é uma infecção associada a um quadro alérgico desencadeado pela inalação repetida de artroconídeos (SUGITA, IKEDA & NISHIKAWA, 2004; CHAGAS-NETO, CHAVES & COLOMBO, 2008). Essas partículas fúngicas contaminam o ambiente doméstico e no verão se transformam em aerossóis facilmente inaláveis. A época do ano de maior incidência, por ser quente e úmida, favorece ainda mais o crescimento do fungo (CHAGAS-NETO, CHAVES & COLOMBO, 2008).

Em condições de severo imunocomprometimento do hospedeiro, *T. asahii* pode se disseminar para vários órgãos como pele, olhos, cérebro e rins. Esse tipo de infecção disseminada é bastante grave, já que pode desencadear quadros de insuficiência respiratória e renal, além da síndrome da coagulação intravascular (KARASHIMA *et al.,* , 2002). Entretanto, o envolvimento pulmonar é mais comum e acomete pouco mais de 30% dos casos de tricosporonose disseminada (SANCHEZ & LARSEN, 2007).

Além do péssimo prognóstico da doença, a terapia é ineficiente, pois a tricosporonose não é responsiva a drogas clássicas como fluconazol e anfotericina B na maioria dos casos. A múltipla resistência a drogas de vários isolados de *T. asahii* também é um problema que reforça a grande complexidade no controle da tricosporonose (PFALLER & DIEKEMA, 2004).

Acredita-se que *T. asahii* penetra no hospedeiro pela vias respiratórias e/ou gastrointestinal, por lesões na pele, por alterações na microbiota intestinal ou através de dispositivos médicos inseridos no paciente (YAMAGATA *et al.*, 2000; PFALLER & DIEKEMA, 2004). Entretanto, é desconhecida a forma pela qual o fungo é capaz de se disseminar.

Apesar da complexidade da infecção, da resistência à terapia antifúngica e dos altos índices de morbidade e mortalidade, as informações disponíveis na literatura sobre os mecanismos patogenicidade de *T. asahii* são extremamente limitadas. Alguns autores sugerem que a GXM presente na superfície do fungo pode estar associada a mecanismos de escape frente a defesa do hospedeiro. Estudos comparativos realizados com isolados de *Cryptococcus* e *Trichosporon* revelaram que esses apresentaram menores índices de fagocitose quando comparados a cepas de *Candida*, que não apresentam esse polissacarídeo na sua estrutura (LYMAN & WALSH, 1994). Entretanto, nenhuma evidência direta do papel da GXM na proteção contra a fagocitose foi demonstrada no gênero *Trichosporon*. Além disso, também foi descrito que sucessivas passagens de isolados de *T. asahii* em camundongos resultaram numa mudança

fenotípica associada ao aumento da liberação do polissacarídeo no sobrenadante de cultura (KARASHIMA *et al.,* , 2002). Tal fato pode ter correlação com o aumento da capacidade de proteção do fungo contra a fagocitose em virtude da presença desse antígeno (KARASHIMA *et al.,* , 2002). Porém, não foi demonstrado nenhum mecanismo pelo qual a GXM de *Trichosporon* interage com células do hospedeiro.

Um estudo realizado por um grupo italiano demonstrou que o fungo é capaz de produzir biofilme similar ao formado por bactérias, fato que está associado a uma maior resistência a antifúngicos. A formação desse agrupamento de células envoltas por um substrato polimérico extracelular pode auxiliar na proteção do fungo contra o sistema imune do hospedeiro (DI BONAVENTURA *et al.*, 2006). Análises do biofilme formado utilizando a lectina concanavalina A (Con A) demonstraram a presença de polissacarídeos contendo manose, o que poderia indicar uma possível participação da GXM na matriz que une as células durante a formação do biofilme.

Diante do exposto, entendemos que é clara a necessidade de investigações sobre a fisiológica fúngica e sobre os mecanismos utilizados por *T. asahii* para interagir com células hospedeiras, escapar da defesa do sistema imune e desencadear a infecção. Com base na literatura disponível para *C. neoformans* e, de forma mais limitada, para *T. asahii*, acreditamos que a GXM pode desempenhar papéis fundamentais nesses processos.

3 – GXM: um importante polissacarídeo imunoativo.

A GXM é um polissacarídeo associado à superfície celular de espécies fúngicas como *Cryptococcus* e *Trichosporon*. Diferentemente de *T. asahii*, que não é um patógeno capsulado, *C. neoformans* tem a GXM como seu principal constituinte capsular. O polissacarídeo produzido por esses fungos, além de ser um componente estrutural importante, também pode ser secretado constitutivamente para o meio extracelular, seja na infecção humana ou mesmo no meio de cultura em condições laboratoriais. Essa liberação de GXM para o meio extracelular permite a purificação e o estudo de suas propriedades estruturais e funcionais. Entretanto, em *C. neoformans*, já foram descritas diferenças entre o polissacarídeo extraído diretamente da cápsula e aquele liberado no sobrenadante, o que pode inferir diferentes propriedades funcionais a essas GXMs (FRASES *et al.*, 2008).

Tanto a GXM secretada quanto aquela disposta na superfície do fungo são fundamentais na interação com o hospedeiro, constituindo um dos principais mecanismos de patogenicidade utilizados por *C. neoformans*. Além disso, a importância dessa molécula tem

motivado seu uso no estudo de vacinas e também como alvo para a produção de anticorpos monoclonais. Tais abordagens demonstram que a GXM pode ter impacto decisivo em alternativas terapêuticas contra a criptococose (ZARAGOZA *et al.,* , 2009).

3.1 – A cápsula de *C. neoformans* é composta majoritariamente de GXM.

A adaptação dos microrganismos a seus ambientes está freqüentemente associada à aquisição de certas estruturas que os ajudarão na sobrevivência em nichos específicos. Por exemplo, a cápsula polissacarídica, presente em várias bactérias e em um grupo seleto de fungos, como em *C. neoformans*, confere proteção ao microrganismo contra condições de estresse, como a desidratação. Além disso, tal estrutura tem um importante papel na interação com o ambiente onde ele está inserido (AKSENOV, BABYEVA & GOLUBEV, 1973; ZARAGOZA *et al.,* , 2009).

Em *C. neoformans*, a cápsula é considerada um importante fator de virulência, tendo em vista que mutantes acapsulares são incapazes de desenvolver criptococose em modelo murino (FROMTLING, SHADOMY & JACOBSON, 1982). Esses mutantes podem crescer e sobreviver em condições normais de laboratório, mas possuem sua virulência extremamente diminuída em camundongos, quando comparada com cepas selvagem e reconstituída (CHANG & KWON-CHUNG, 1994). Tal fato chama atenção para a possibilidade de que mecanismos de síntese de cápsula podem ser potenciais alvos no desenvolvimento de novas drogas para o tratamento da criptococose.

Uma característica importante do polissacarídeo capsular são os exacerbados efeitos imunomoduladores, que auxiliam no escape do sistema imune humano e, por conseguinte, na sobrevivência dentro do hospedeiro, seja em mamíferos ou mesmo em amebas, seu predador ambiental (VECCHIARELLI, 2000; STEENBERGEN, SHUMAN & CASADEVALL, 2001; MONARI, BISTONI & VECCHIARELLI, 2006).

C. neoformans possui a cápsula como sua estrutura mais externa, que circunda o corpo celular e conecta-se a parede celular por mecanismos não totalmente elucidados, mas que envolvem ligações carboidrato-carboidrato (REESE & DOERING, 2003; REESE *et al.*, 2007). Essa estrutura pode ser evidencia por algumas técnicas de microscopia, como ilustrado na **Figura 3**.

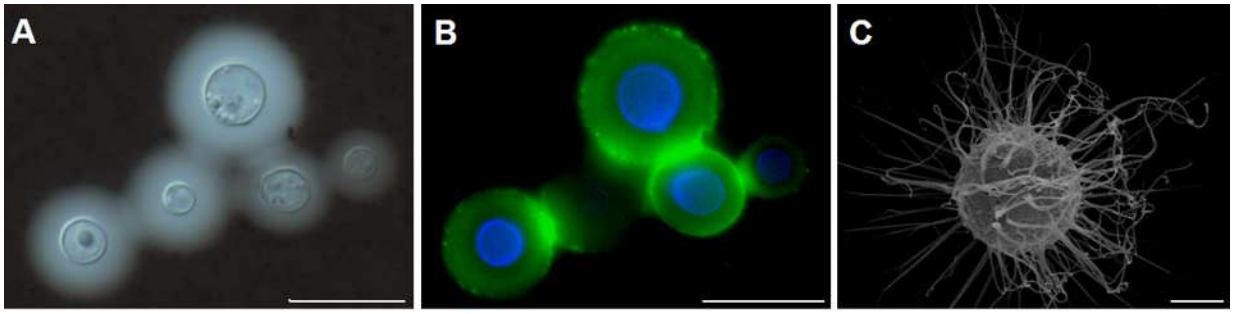

Figura 3. Diferentes técnicas de microscopia evidenciando a cápsula polissacarídica circundando o corpo celular de *C. neoformans*. Apesar de não ser possível a visualização da cápsula por microscopia óptica convencional, é possível observá-la com simples preparações utilizando-se tinta Nanquim (A), empregando anticorpos monoclonais contra GXM e calcofluor por microscopia óptica de fluorescência (em azul, a parede celular marcada com calcofluor e em verde a cápsula polissacarídica) (B) ou por microscopia eletrônica de varredura, que evidencia as fibras capsulares de diferentes tamanhos (C). Barras de escala: 10 µm (painéis A e B) e 1 µm (painel C). Imagens pertencentes ao acervo de nosso laboratório.

A cápsula de *C. neoformans* é altamente hidrofílica, apresentando um conteúdo de água que chega a 99% do peso total capsular (MAXSON *et al.*, 2007a). Além disso, possui uma forte carga negativa em virtude da presença de moléculas de ácido glucurônico ionizadas em pH neutro.

A cápsula é formada basicamente por dois polissacarídeos: a GXM e a galactoxilomanana (GalXM). Cerca de 90-95% da massa capsular é composta por GXM, enquanto de 5-8% correspondem a GalXM. Além disso, acredita-se ser possível que uma pequena fração de manoproteínas (MP, <1%) também componha a cápsula de *C. neoformans*. No entanto, alguns estudos sugeriram que essas moléculas estão principalmente no interior da parede celular e não associadas a GXM ou GalXM (VARTIVARIAN *et al.*, 1989). O papel estrutural e funcional das MP ainda permanece desconhecido.

A GXM é o componente capsular majoritário e por isso mais bem estudado de *C. neoformans*. De acordo com análises do polissacarídeo purificado a partir de sobrenadantes de cultura, a GXM apresenta alta massa molecular que pode variar de 1700 a 7000 kDa, dependendo da cepa (MCFADDEN, DE JESUS & CASADEVALL, 2006). Estruturalmente, a GXM é formada por um esqueleto linear de manose ligada de forma α-(1,3)- e apresenta unidades de ácido glucurônico ligadas β–(1,2)- à primeira manose, formando um cerne que se repete em todos os sorotipos. Unidades de manose também podem ser *O*-acetiladas no carbono 6 e

substituídas com unidades de xilose ligadas β–(1,2)- ou β-(1,4)-, o que varia dependendo do sorotipo, como mostra a **Figura 4** (CHERNIAK & SUNDSTROM, 1994; CHERNIAK *et al.,* , 1995; MCFADDEN, DE JESUS & CASADEVALL, 2006; MCFADDEN *et al.*, 2007; ZARAGOZA *et al.,* , 2009).

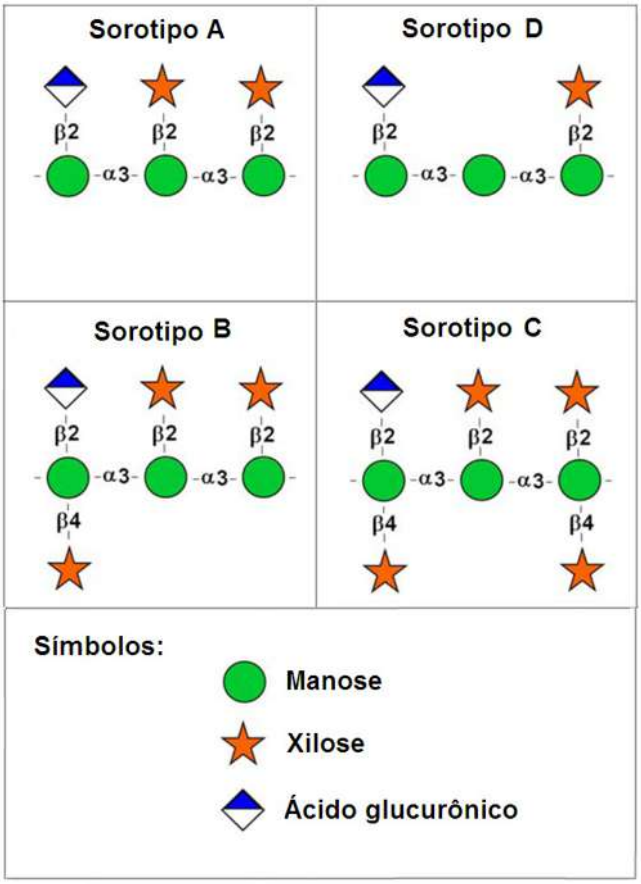

Figura 4. Estrutura da GXM em diferentes sorotipos de *C. neoformans*. A estrutura básica da molécula é a mesma, apenas ocorrem variações entre os sorotipos na posição das substituições de xilose e na *O*-acetilação das unidades de manose (não representada no esquema). Os sorotipos A e D correspondem às espécies de *C. neoformans* var *grubii* e var *neoformans*, respectivamente. Já os sorotipos B e C são espécies de *C. gattii*. Adaptado a partir de (ZARAGOZA *et al.,* , 2009).

Como já mencionado, *C. neoformans* apresenta diferenças estruturais em sua molécula de GXM dependendo do ambiente e da cepa em questão. Desse modo, as espécies desse fungo são classificadas em diferentes sorotipos de acordo com a reatividade da cápsula com soro policlonal de coelho (LIN, 2009).

O outro polissacarídeo componente da cápsula é a GalXM, que constitui aproximadamente 8% da massa capsular (VAISHNAV *et al.*, 1998; BOSE *et al.*, 2003) e possui peso molecular de cerca de 100 kDa (MCFADDEN, DE JESUS & CASADEVALL, 2006). A estrutura proposta por Vaishnav e colaboradores (1998), definiu a GalXM como um esqueleto de galactose ligada α-(1,6)- com cadeias laterais de galactomananas substituídas com unidades de xilose (VAISHNAV *et al.,* , 1998). Entretanto, a complexidade da estrutura desse polissacarídeo e a glicosilação não estequiométrica de alguns substituintes, tornam bastante complicada as análises estruturais dessa molécula. Um recente estudo na área de síntese capsular compararou a GalXM purificada a partir de uma cepa mutante deficiente em β-(1,2)-xilosiltranferase (Δ*ctx1*) e a cepa selvagem (HEISS *et al.*, 2009). Nesse trabalho, os autores notaram incoerências entre seus dados e a estrutura do polissacarídeo anteriormente elucidada (HEISS *et al.,* , 2009). Todas as análises de ressonância magnética nuclear (RMN), cromatografia gasosa acoplada à espectrometria de massas (GC-MS, do inglês, *gas chromatography-mass spectrometry*) e outras análises de composição monossacarídica e metilação corroboram com a presença de unidades de ácido glucurônico na molécula de GalXM. O uso de metodologias sem redução prévia do grupamento carboxil em estudos anteriores, impossibilitou a detecção de unidades de ácido glucurônico por cromatografia gasosa e por análises de metilação, acarretando também numa não identificação no espectro de RMN (HEISS *et al.,* , 2009). Assim, Heiss e colaboradores propuseram uma nova estrutura para esse polissacarídeo contendo galactose, como ilustrada na **Figura 5** (DOERING, 2009; HEISS *et al.,* , 2009). Uma nova nomenclatura baseada na elucidação estrutural também foi proposta nesse estudo. A GalXM passaria a ser chamada de glucuronoxilomananogalactana (GXMGal), em virtude da presença de unidades de manose, xilose, ácido glucurônico e galactose (DOERING, 2009; HEISS *et al.,* , 2009). Entretanto, a fim de evitar confusões relacionadas à nomenclatura, tal polissacarídeo será citado como GalXM, conforme utilizado classicamente na literatura.

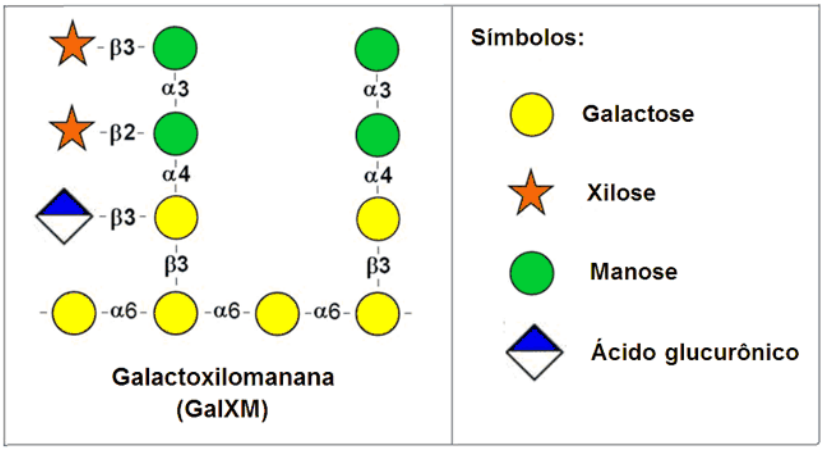

Figura 5. Estrutura da GalXM é composta por um esqueleto de galactose com substituições compostas por unidades de galactose, manose, ácido glucurônico e xilose, dispostos como mostra o esquema. Adaptado a partir de (DOERING, 2009).

Os dados acima descritos estão de acordo com outros estudos que também resultaram na detecção de ácido glucurônico na estrutura da GalXM, purificada a partir de 4 cepas mutantes acapsulares de *C. neoformans* (GRIJPSTRA *et al.*, 2009). Vale ressaltar que esses mutantes, apesar de não sintetizarem GXM, são conhecidamente, capazes de produzir e secretar GalXM. Além disso, análises físico-químicas recentes revelaram que a GalXM apresenta valor negativo de potencial zeta, o que corrobora com a presença do ácido glucurônico (DE JESUS *et al.*, ; NIMRICHTER *et al.*, 2007; HEISS *et al.*, , 2009).

Assim como a GXM, a GalXM também parece ser secretada em vesículas (DE JESUS *et al.*, 2009b). Esse polímero parece também desempenhar funções estruturais na capsula através da agregação de moléculas de GXM (FRASES *et al.*, , 2008). Análises por microscopia de fluorescência sugeriram que esse polissacarídeo está localizado na borda externa da cáspula (DE JESUS *et al.*, , 2009b). As funções da GalXM são ainda pouco conhecidas, mas é sabido que o polissacarídeo é capaz de inibir a proliferação de células T, além de induzir aumento da produção de IL-10 e interferon-γ (INF- γ) (PERICOLINI *et al.*, 2006; DE JESUS *et al.*, 2007). Esse polissacarídeo regula positivamente a expressão do receptor Fas e ativa a apoptose de linfócitos T (PERICOLINI *et al.*, , 2006; DE JESUS *et al.*, , 2007) e macrófagos (VILLENA *et al.*, 2008).

3.1.1 – Arquitetura capsular de *C. neoformans*.

O processo biossintético da GXM é complexo e envolve, dentre outros fatores, a disponibilidade de nucleotídeos fosfatados do polissacarídeo, a atividade de proteínas transportadoras e de glicosiltransferares, que serão responsáveis pela polimerização da molécula (JANBON, 2004).

Em geral, a síntese de polissacarídeos microbianos ocorre no lado externo da membrana plasmática ou mesmo no espaço extracelular. Entretanto, em *C. neoformans*, a síntese de GXM ocorre no ambiente intracelular, mais especificamente, no complexo de Golgi (FELDMESSER, KRESS & CASADEVALL, 2001; GARCIA-RIVERA *et al.*, 2004; YONEDA & DOERING, 2006). Alguns estudos demonstraram o reconhecimento de estruturas vesiculares citoplasmáticas por anticorpos monoclonais contra GXM (YONEDA & DOERING, 2006; RODRIGUES *et al.*, 2007; RODRIGUES *et al.*, 2008b). Além disso, corroborando esses achados, um grupo americano investigou o papel de uma GTPase homóloga a Sec4p em *C. neoformans*, que participa da regulação exocítica. Utilizando cepas mutantes no gene *SAV1*, que codifica essa proteína, foi possível detectar um acúmulo de vesículas exocíticas pós-Golgi no citoplasma. Essas vesículas eram reconhecidas pelo anticorpo anti-GXM, fato que confirma a síntese intracelular do polissacarídeo capsular e a participação da via de secreção derivada do complexo de Golgi (YONEDA & DOERING, 2006).

Desse modo, o tráfico de GXM para a superfície da célula, aparentemente, envolve a secreção de vesículas contendo esse polissacarídeo (FELDMESSER, KRESS & CASADEVALL, 2001; GARCIA-RIVERA *et al.,* , 2004; RODRIGUES *et al.,* , 2007). Entretanto, ainda são desconhecidos os mecanismos utilizados por essa levedura para que as vesículas sejam capazes de ultrapassar uma estrutura tão rígida como a parede celular, alcançar o meio extracelular e liberar a GXM, que será, posteriormente, incorporada à cápsula já existente. Especula-se que vesículas pós-Golgi contendo o polissacarídeo se integrem a compartimentos da via endossomal, resultando na liberação de estruturas semelhantes a exossomos contendo GXM. Essa hipótese é apoiada por diferentes estudos, que utilizaram análises morfológica e proteômica de vesículas extracelulares (RODRIGUES *et al.*, 2000; RODRIGUES *et al.,* , 2007; RODRIGUES *et al.,* , 2008b). Isso reforça ainda mais os indícios de que *C. neoformans* produz estruturas semelhantes a exossomos.

A biogênese de vesículas secretórias em *C. neoformans* ainda é um processo a ser esclarecido. No entanto, a ocorrência de vesículas contendo GXM é inteiramente indiscutível, uma vez que já foram localizadas no citoplasma, no periplasma, na parede celular, na borda da

cápsula e no meio extracelular em estudos *in vitro*. Além disso, foram detectadas vesículas em cortes histológicos de pulmão de camundongos infectados com *C. neoformans* (RODRIGUES *et al.,* , 2007). Dados do nosso grupo demonstraram que mutantes acapsulares foram capazes de reincorporar a GXM contida dentro de vesículas, o que sugere que o fungo possui aparato metabólico para extrair esse polissacarídeo de dentro das vesículas, ou mesmo que essas vesículas se rompam no meio extracelular (RODRIGUES *et al.,* , 2007). Dessa forma, o polissacarídeo transportado nessas estruturas para o meio extracelular é então conectado a parede celular ou incorporado na cápsula já existente. Tal processo de ancoramento capsular, apesar de ter um papel crucial na patogenicidade de *C. neoformans*, é pouco esclarecido.

A parede celular criptococócica é uma complexa malha composta de glucana, quitina, quitosana, proteínas glicosiladas, como manoproteínas, glicolipídeos, como glucosilceramida e pigmentos, como melanina (NIMRICHTER *et al.*, 2005). Tal estrutura é considerada fundamental para a manutenção da morfologia e integridade celular, além de conferir rigidez, proteção contra estresse ambiental e a lise osmótica (DOERING, 2009). Além disso, o fato dessa estrutura estar ausente em células animais torna a parede celular um alvo interessante para a terapia antifúngica.

Em *C. neoformans*, a parede celular é circundada por fibras capsulares, o que torna óbvia a existência de uma interação envolvendo componentes de parede celular e polissacarídeos capsulares. A participação de interações entre carboidratos presentes na parede celular e a cápsula de *C. neoformans* foi descrita por dois grupos, que evidenciaram a importância de moléculas de glucana (GILBERT *et al.*, ; REESE & DOERING, 2003; REESE *et al.,* , 2007) e quitosana (BAKER *et al.*, 2007) no ancoramento capsular.

Utilizando abordagens moleculares, pesquisadores demonstraram que a expressão diminuída do gene *AGS1*, que codifica a enzima α-glucana sintase, está associada a uma eliminação da cápsula de *C. neoformans* (REESE & DOERING, 2003). Além disso, esse mesmo grupo mostrou que mutantes incapazes de sintetizar α-1,3-glucana secretavam normalmente polissacarídeos capsulares. Essas células, entretanto, apresentavam defeitos na organização capsular, o que afetava sua virulência em modelo murino (REESE *et al.,* , 2007).

Recentemente, também foi descrito que a β-1,6 glucana, o componente mais abundante na parede celular de *C. neoformans*, está envolvida na conexão com a cápsula do fungo (GILBERT *et al.,*). Foi demonstrado que os genes *KRE5*, *KRE6* e *SKN1* participam da síntese de β-1,6 glucana e afetam o ancoramento capsular. A geração de cepas mutantes nesses genes está associada à perda da integridade da parede celular, defeitos na arquitetura capsular, diminuição na virulência em modelo murino, além de uma defectiva organização de parede celular, sobretudo na retenção de manoproteínas e distribuição de quitosana (GILBERT *et al.,*).

Não apenas as glucanas estão envolvidas no ancoramento de componentes capsulares a elementos da parede celular em *C. neoformans*. Mutantes acapsulares de *C. neoformans* tratados com glucanase foram capazes de incorporar GXM exógena (REESE & DOERING, 2003), o que indica que outras moléculas dispostas na parede celular também estão envolvidas no ancoramento capsular.

A quitina, polímero formado por unidades de *N*-acetilglucosamina em ligações β-1,4, é um componente essencial para rigidez e integridade da parede celular de fungos (ADAMS, 2004). A síntese desse polissacarídeo ocorre por polimerização de *N*-acetilglucosamina catalisada por quitina sintases (BANKS *et al.*, 2005). Em *C. neoformans*, a síntese de quitina parece preceder a geração de quitosana, forma de-*O*-acetilada de quitina, através de reações catalisadas por quitina deacetilases (BAKER *et al.,* , 2007). Mutações nos genes que codificam a enzima quitina sintase *Chs3* ou a proteína reguladora Csr2 ou enzimas deacetilases Cda1, Cda2, Cda3 afetam a viabilidade celular do fungo a 37^0C e aumentam a sensibilidade a inibidores de parede celular, além de reduzir a quantidade de quitosana (BANKS *et al.,* , 2005). Tal fato sugere que a quitosana seja um componente essencial na integridade da parede celular em *C. neoformans*. Células que apresentam quantidades reduzidas de quitosana possuem uma parede celular defectiva, problemas na manutenção de uma correta arquitetura capsular e na retenção de melanina (BANKS *et al.,* , 2005; BAKER *et al.,* , 2007).

Como descrito anteriormente, a síntese de quitina e moléculas correlatas requer estoques citoplasmáticos de UDP-GlcNAc para gerar polímeros de β1,4-ligados por ação enzimática (BANKS *et al.,* , 2005). Dessa forma, a inibição da biossíntese dessas unidades glicídicas acarreta em defeitos na parede celular de fungos, podendo ser considerada um bom alvo para drogas antifúngicas. O passo inicial para é a síntese de UDP-GlcNAc é a conjugação de glutamina com frutose 6-fosfato, através da enzima glucosamina 6-fosfato sintase. Essa reação gera glucosamina 6-fosfato, primeiro açúcar usado para a síntese de UDP-GlcNAc (MILEWSKI, GABRIEL & OLCHOWY, 2006). Dipeptídeos sintéticos contendo o análogo da glutamina N^3-(4-metoxifumaroil)-L-2,3-ácido diaminopropanóico (FMDP) foram descritos como inibidores da glucosamina 6-fosfato sintase em *Candida albicans* (MILEWSKI *et al*., 1999; WOJCIECHOWSKI *et al.*, 2005). Um desses dipeptídeos, o L-norvalil-FMDP (Nva-FMDP), demonstrou efeito antifúngico em modelo animal de candidíase sistêmica (MILEWSKI *et al.,* , 1999; WOJCIECHOWSKI *et al.,* , 2005). Esses dados revelam a importância do metabolismo de hexosamina na integridade e organização da parede celular em fungos (BANKS *et al.,* , 2005; MILEWSKI, GABRIEL & OLCHOWY, 2006; BAKER *et al.,* , 2007).

Uma vez corretamente ancorada a parede celular, a cápsula de *C. neoformans* pode variar em tamanho em resposta a vários estímulos tanto em condições laboratoriais quanto *in*

vivo (ZARAGOZA & CASADEVALL, 2004; ZARAGOZA *et al.,* , 2009). Os mecanismos pelos quais a cápsula de *C. neoformans* se expande são ainda pouco conhecidos, embora seja clara a necessidade de associação intermolecular de fibras de GXM mediada por cátions divalentes (NIMRICHTER *et al.,* , 2007) e síntese de fibras polissacarídicas de alto diâmetro molecular (FRASES *et al.*, 2009). O crescimento capsular se dá de forma apical e requer a incorporação de novas fibras de polissacarídeo que se ligarão àquelas já conectadas a parede celular, aumentando a densidade capsular (PIERINI & DOERING, 2001; GATES, THORKILDSON & KOZEL, 2004; ZARAGOZA *et al.*, 2006; MAXSON *et al.*, 2007b).

Um estudo recente que investigou a correlação entre o diâmetro das fibras de GXM e o tamanho capsular, utilizando técnicas de espalhamento de luz e microscopia de pinça óptica, propôs uma nova abordagem para explicar a arquitetura capsular do *C. neoformans* (FRASES *et al.,* , 2009). Foi demonstrado que o tamanho capsular está diretamente associado com o comprimento axial da molécula de GXM (FRASES *et al.,* , 2009). De acordo com os autores, longas moléculas de GXM determinariam o diâmetro capsular e serviriam como uma espécie de andaime para fibras menores. Na região capsular interna, fibras mais curtas estariam ligadas a outras mais longas através de ligações intermoleculares mediada por cátions divalentes. O modelo proposto nesse estudo divide a cápsula em três regiões em função das diferenças de densidade relativa do polissacarídeo, como mostra a **Figura 6**. A região 1 é a mais densa, junto ao corpo celular e é composta de uma malha compacta de moléculas de GXM. A região 2 apresentaria densidade intermediária e a região 3, de baixa densidade, teria menor contribuição para os aspectos morfológicos da cápsula observados em técnicas microscópicas (FRASES *et al.,* , 2009).

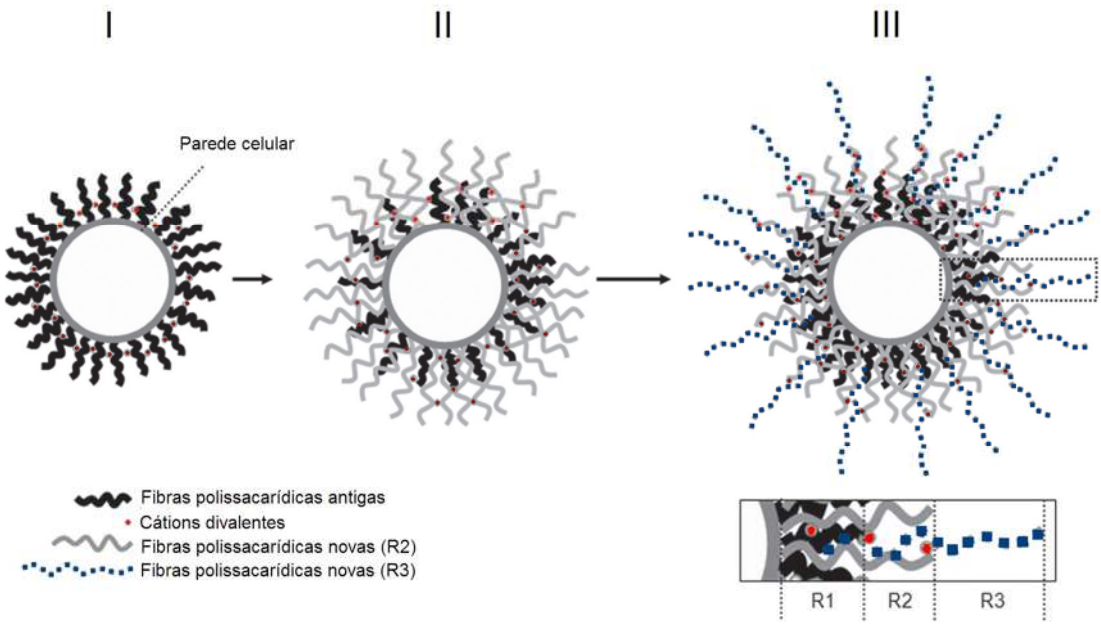

Figura 6. Representação esquemática do modelo atual proposto para a arquitetura capsular de *C. neoformans*. Células com pequenas cápsulas (I). Novas e longas fibras de GXM se intercalarão entre as fibras nascentes, formando uma região de alta densidade na parte interna da cápsula (II). Finalmente, a cápsula crescerá pela adição apical de longas fibras de polissacarídeos, formando uma região de alta densidade próxima a parede celular (R1), uma região de densidade intermediária (R2) e uma área de baixa densidade (R3) na parte externa da cápsula. Adaptado a partir de Rodrigues *et al*., 2010.

A agregação de fibras de GXM mediada por íons divalentes, além de promover crescimento capsular, permite o isolamento do polissacarídeo a partir de sobrenadantes de cultivo, através da remoção de água por um sistema de ultrafiltração (NIMRICHTER *et al.,* , 2007). Ao final do processo, é observada a formação de gel viscoso que, de acordo com análises estruturais GC-MS e RMN, correspondem a preparações de GXM com altos níveis de pureza (NIMRICHTER *et al.,* , 2007). Considerando que as frações purificadas por esse método não envolvem nenhuma modificação química, acredita-se que dessa forma seja possível obter o polissacarídeo secretado em sua forma nativa, diferente do que ocorre em metodologias classicamente usadas para isolamento do polissacarídeo, que envolvem precipitação com detergentes catiônicos (CHERNIAK *et al.*, 1991; NIMRICHTER *et al.,* , 2007). De fato, a realização de ensaios comparando frações de polissacarídeo obtidas pelo método de purificação por agregação com aquelas obtidas por precipitação com detergente mostrou diferenças físicas e estruturais importantes, como o reconhecimento por anticorpos monoclonais e o tamnho de

fibra Do polissacarídeo, que estariam diretamente associadas às funções descritas para a GXM (CHERNIAK *et al.,* , 1991; NIMRICHTER *et al.,* , 2007; FRASES *et al.,* , 2008).

A interação entre moléculas de GalXM e GXM também já foi descrita e parece ser relevante para a arquitetura capsular. Mutantes incapazes de sintetizar GalXM apresentaram tamanho capsular maior quando comparados a cepas selvagens (MOYRAND, FONTAINE & JANBON, 2007). Esse achado pode sugerir que na ausência de GalXM a célula responda com um aumento da síntese de moléculas de GXM, o que indicaria que o polissacarídeo minoritário de *C. neoformans* participa da manutenção do tamanho capsular e, por sua vez, na estrutura e densidade (MOYRAND, FONTAINE & JANBON, 2007). A ligação de GalXM a GXM, de fato, já foi sugerida, através de estudos que mostraram que a interação entre os dois polissacarídeos geram estruturas de massa molecular aumentada (FRASES *et al.,* , 2008).

Tendo em vista todas as características descritas acima, torna-se simples concluir que a cápsula de *C. neoformans* é uma estrutura bastante complexa e heterogênea, em aspectos que abrangem desde sua composição molecular até sua organização estrutural e espacial.

3.2 – Importância da cápsula e da GXM secretada na interação de *C. neoformans* com o hospedeiro.

Durante o curso da infecção, o tamanho da cápsula de *C. neoformans* varia em função das condições do seu microambiente, diferindo de acordo com o órgão infectado (RIVERA *et al.*, 1998). Alguns fatores já foram descritos como moduladores do tamanho de cápsula, seja em condições laboratoriais ou *in vivo* (GRANGER, PERFECT & DURACK, 1985; VARTIVARIAN *et al.*, 1993; RIVERA *et al.,* , 1998; ZARAGOZA & CASADEVALL, 2004). Não é claro se essa indução modulada por diferentes fatores ocorre por vias distintas ou se existe um elemento comum a todas as vias (ZARAGOZA *et al.,* , 2009).

Baixas concentrações de ferro e uma atmosfera enriquecida em CO_2 (5%) induzem o aumento da cápsula (GRANGER, PERFECT & DURACK, 1985; VARTIVARIAN *et al.,* , 1993). Essas condições mimetizam o ambiente que o fungo encontra ao colonizar o pulmão, local onde o tamanho capsular é aumentado (RIVERA *et al.,* , 1998). Acredita-se que o crescimento capsular é inversamente proporcional a taxa de crescimento do fungo, o que corrobora com o aumento da cápsula em condições de pouca oferta de nutriente (ZARAGOZA & CASADEVALL, 2004). Ambientes com alta osmolaridade, como em elevadas concentrações de glicose ou cloreto de sódio (NaCl), também são capazes de induzir uma redução capsular. Tal fato pode ser atribuído a uma diminuição do conteúdo de água da cápsula em função dessas condições, o que

resultaria em uma compactação das fibras polissacarídicas, levando a um menor tamanho capsular (DYKSTRA, FRIEDMAN & MURPHY, 1977; CLEARE & CASADEVALL, 1999).

Além de mudanças físicas relacionadas ao tamanho e densidade capsulares, alguns estudos indicam que a cápsula é um componente celular altamente dinâmico, já que sua estrutura pode ser alterada, inclusive durante o curso da infecção (GARCIA-HERMOSO, DROMER & JANBON, 2004). Alguns estudos já demonstraram a existência de estruturas capsulares diferentes dentro do mesmo sorotipo de *C. neformans* (TURNER *et al.*, 1992) com reatividades diferenciadas com anticorpos específicos (GARCIA-HERMOSO, DROMER & JANBON, 2004; MCFADDEN *et al.,* , 2007). Assume-se que essa variabilidade é importante tanto em processos fagocíticos quanto na disseminação de *C. neoformans* (SMALL & MITCHELL, 1989; CHARLIER *et al.*, 2005; ZARAGOZA *et al.,* , 2008).

Durante as primeiras horas da infecção, uma considerável quantidade de células inaladas são encontradas dentro de células fagocíticas no pulmão (FELDMESSER *et al.*, 2000). Tal fato está associado a mecanismos que se sobrepõem a inibição da fagocitose normalmente promovida pela cápsula. Esses fatores incluiriam a presença de anticorpos contra GXM e moléculas do sistema complemento, que atuariam como opsoninas (ZARAGOZA *et al.,* , 2009).

Apesar de o processo de fagocitose, na maioria das vezes, ser considerado como um mecanismo favorável ao hospedeiro, especula-se que esse processo possa ser benéfico para o *C. neoformans*. No interior dos fagócitos, o fungo é capaz de se replicar e possivelmente liberar GXM dentro de vesículas (FELDMESSER, TUCKER & CASADEVALL, 2001), o que modularia negativamente a resposta imune. Além disso, a cápsula pode atuar protegendo o fungo contra moléculas antimicrobianas produzidas durante a infecção, como espécies reativas de oxigênio (ZARAGOZA *et al.,* , 2008). Posteriormente, o patógeno pode ser liberado através da ruptura da célula hospedeira ou mesmo por meios que não afetam a integridade dos fagócitos (FELDMESSER, TUCKER & CASADEVALL, 2001; ALVAREZ & CASADEVALL, 2007; MA *et al.*, 2007). Vários mecanismos de extrusão de *C. neoformans* já foram descritos e, além disso, o fungo também é capaz atingir diretamente células adjacentes sem qualquer dano celular (ALVAREZ & CASADEVALL, 2006; MA *et al.*, 2006; ALVAREZ & CASADEVALL, 2007; MA *et al.,* , 2007). Dessa forma, esse microrganismo é considerado um patógeno intracelular facultativo. Essa característica parece ser um importante mecanismo de patogenicidade dependente de cápsula, já que mutantes acapsulares são incapazes de se replicar dentro de células fagocíticas (FELDMESSER *et al.,* , 2000).

Além de ser fundamental no escape contra a atuação antifúngica mediada pelo sistema imune quando associada à cápsula, a GXM secretada para o ambiente extracelular também contribui amplamente para subverter as defesas do hospedeiro e desencadear uma infecção

bem sucedida (ZARAGOZA *et al.,* , 2009). Esse polissacarídeo pode ser encontrado no sangue e em vários tecidos durante o desenvolvimento da criptococose (GOLDMAN, LEE & CASADEVALL, 1995; LEE & CASADEVALL, 1996; LEE, CASADEVALL & DICKSON, 1996; LENDVAI *et al.*, 1998; DE JESUS *et al.*, 2008), sendo considerado um marcador de desenvolvimento da doença. No hospedeiro, a GXM é a responsável por diversos efeitos imunomoduladores, conforme sumarizado na **Tabela 1**.

Tabela 1. Algumas atividades imunomodulatórias descritas para GXM de *C. neoformans*.

Atividade Imunomodulatória	Referências
Inibição da liberação de citocinas pró-inflamatórias por monócitos, como TNF-α e IL-1β	(VECCHIARELLI *et al.*, 1995; LENDVAI *et al.*, 2000);
Indução da produção de IL-10 por monócitos	(VECCHIARELLI *et al.*, 1996)
Supressão da proliferação de células T	(RETINI *et al.*, 1998; SYME *et al.*, 1999)
Regulação negativa da expressão de moléculas MHC de classe II e da expressão do co-receptor B7 em monócitos	(MONARI *et al.*, 1999)
Redução da resposta Th1 em função da inibição de IL-12	(RETINI *et al.*, 2001)
Redução da capacidade antimicrobiana de neutrófilos e macrófagos	(MONARI *et al.*, 2003; VECCHIARELLI *et al.*, 2003)
Redução da atividade quimiotática de neutrófilos devido à indução do "shedding" de L-selectina	(DONG & MURPHY, 1996; DONG & MURPHY, 1997; MONARI *et al.*, 2002)
Inibição da ativação e maturação de células dendríticas	(VECCHIARELLI *et al.,* , 2003)
Inibição da migração de leucócitos para os sítios de infecção	(ELLERBROEK *et al.*, 2004b)
Indução da apoptose via caspase em macrófagos e células T pelo acúmulo de Fas e FasL	(MONARI *et al.*, 2008; VILLENA *et al.,* , 2008)

A GXM é capaz de influenciar a resposta do hospedeiro durante a infecção através da ativação de diferentes receptores (LEVITZ, 2002; ELLERBROEK *et al.,* , 2004b; YAUCH *et al.*, 2004; MONARI *et al.*, 2005). Essas estruturas interagem com o polissacarídeo capsular e desencadeiam distintas respostas celulares, que incluem mecanismos descritos para células fagocíticas, epiteliais e endoteliais (LEVITZ, 2002; ELLERBROEK *et al.*, 2004a; YAUCH *et al.,* , 2004; MONARI *et al.,* , 2005; BARBOSA *et al.*, 2007; JONG *et al.*, 2008). O polissacarídeo pode interagir com CD18 (DONG & MURPHY, 1997), além de ligar-se a CD14 e receptores do tipo Toll (TLR, do inglês *Toll-like receptors*) (YAUCH *et al.,* , 2004). Tais receptores representam uma família de moléculas envolvidas no reconhecimento de uma série de compostos microbianos, incluindo lipopolissacarídeos (LPS) de bactérias Gram-negativas, peptidoglicanas de Gram-positivas (TAKEUCHI *et al.*, 1999), ácidos nucléicos virais (MOGENSEN & PALUDAN, 2005), glicolipídeos de protozoários (CAMPOS *et al.*, 2001) e polissacarídeos fúngicos (ROEDER *et al.*, 2004). A ligação aos TLR frequentemente leva à produção de citocinas pró-inflamatórias, incluindo TNF-α e IL-12, e o aumento da capacidade antimicrobiana e de apresentação antigênica de células fagocíticas (YAUCH *et al.,* , 2004). A sinalização mediada por TLR-2 e -4 é associada à ligação de moléculas microbianas ao receptor CD14 e, nesse tipo de interação, a participação da proteína adaptadora MyD88 é fundamental para a sinalização celular deflagrada pelos TLR (YAUCH *et al.,* , 2004). A GXM é reconhecida tanto por CD14 quanto pelos TLRs 2 e 4, num processo que parece determinante para o curso da infecção (YAUCH *et al.,* , 2004).

O envolvimento de CD14 na interação de GXM com células epiteliais alveolares foi descrito por nosso grupo (BARBOSA *et al.,* , 2006; BARBOSA *et al.,* , 2007). Nesses estudos, demonstramos que o polissacarídeo capsular é capaz de mediar a adesão do fungo às células epiteliais. As leveduras são posteriormente internalizadas, promovendo morte das células hospedeiras. O curso desse processo envolve a produção da quimiocina pró-inflamatória IL-8, num evento dependente da ligação da GXM ao receptor CD14 (BARBOSA *et al.,* , 2006; BARBOSA *et al.,* , 2007). Esses foram os primeiros relatos sobre um receptor epitelial para GXM associado a uma resposta biológica. Parece possível concluir, portanto, que células epiteliais também participariam na resposta imune contra *C. neoformans*.

3.3 – Similaridades entre GXMs de *Trichosporon* e *Cryptococcus*.

Como já dito anteriormente, apesar de *T. asahii* não ser considerado um patógeno encapsulado, o fungo sintetiza e secreta polissacarídeos de estrutura similar à GXM de *C.*

neoformans (LYMAN *et al.*, 1995; ICHIKAWA *et al.,* , 2001; KARASHIMA *et al.,* , 2002). Conforme determinado por degradação de Smith, RMN e eletroforese de carboidratos assistida por fluoróforo (FACE, do inglês *fluorescent-assisted carbohydrate electrophoresis*), a GXM produzida por *T. asahii* consiste de uma manana α-(1,3)- ligada apresentando substituições de ácido glucurônico e xilose, que formam ligações β-(1,2)-, β-(1,4)- e β-(1,6)- com as unidades de manose, conforme ilustrado na **Figura 7** (ICHIKAWA *et al.,* , 2001). Assim como a GXM de *C. neoformans,* o polissacarídeo de *T. asahii* apresenta unidades de manose *O*-acetiladas, que contribuem para a reatividade sorológica (ICHIKAWA *et al.,* , 2001).

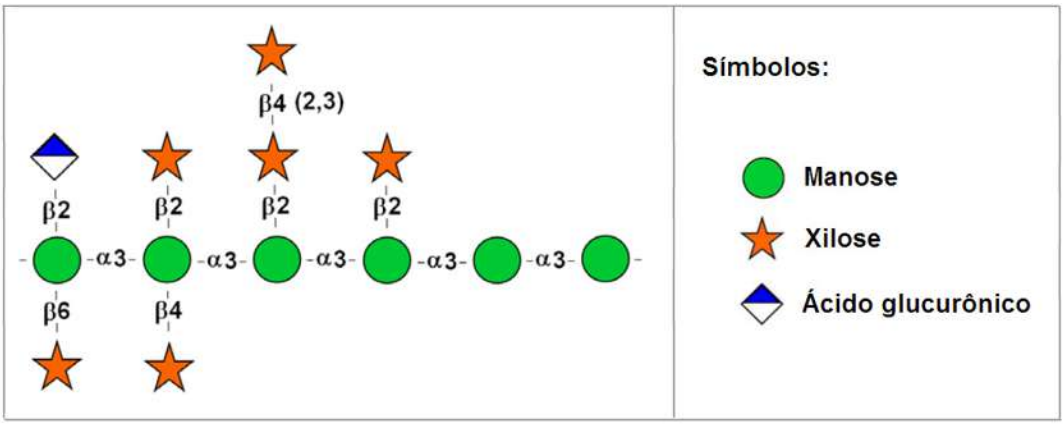

Figura 7. Estrutura da GXM de *T. asahii*. Esse polissacarídeo apresenta grandes semelhanças com a GXM de *C. neoformans*, sendo também formada por um esqueleto de manose e substituições de xilose e ácido glucurônico. Adaptado a partir de (ICHIKAWA *et al.,* , 2001).

A GXM de *T. asahii* está associada à parede celular (ICHIKAWA *et al.,* , 2001) e também é secretada para o meio extracelular (KARASHIMA *et al.,* , 2002). Considerando, portanto, a distribuição do polissacarídeo e a sua similaridade estrutural com a GXM de *C. neoformans*, pode-se especular que a molécula produzida por *T. asahii* apresente propriedades fisico-químicas e imunológicas relacionadas às já observadas para o polissacarídeo criptocócocico.

Correlações entre patogênese e expressão de GXM por *Trichosporon* spp já foram de fato sugeridas. Através de ensaios imunoenzimáticos, foi demonstrado que isolados oriundos de pacientes com tricosporonose invasiva produzem GXM em quantidades cerca de 8 a 9 vezes superiores a isolados ambientais ou obtidos de infecções superficiais (LYMAN *et al.,* , 1995). Além disso, pesquisadores demonstraram que a infecção de camundongos com *T. asahii* resulta na produção aumentada de GXM pelo patógeno e em mudanças morfológicas das

colônias, o que poderia contribuir com o escape do sistema imune do hospedeiro (KARASHIMA *et al.,* , 2002). Entretanto, apesar da clara relevância das infecções causadas por *T. asahii* e do potencial imunomodulador da GXM produzida por esse patógeno, não existem ainda na literatura informações sobre os mecanismos associados à fisiopatologia das infecções causadas por *Trichosporon* e sua relação com a produção de GXM.

II – OBJETIVOS GERAIS:

Patologias associadas a fungos se tornaram um grande problema de saúde pública a partir do final do século XX. O aumento descontrolado da incidência, os altos índices de morbidade e mortalidade e a ineficiência de controle e diagnóstico desse tipo de infecção faz urgente a busca pela elucidação de mecanismos de patogenicidade fúngicos e novos alvos terapêuticos. Tendo em vista a importância médica de fungos produtores de GXM, como espécies de *Trichosporon* e *Cryptococcus*, o presente estudo teve como objetivo a investigação do papel desse polissacarídeo na fisiologia fúngica e suas propriedades funcionais e estruturais em modelo de *C. neoformans*, *C. gattii* e *T. asahii*.

III – OBJETIVOS ESPECIFÍFICOS:

1- Investigar o papel da quitina e estruturas derivadas de quitina na arquitetura capsular;

2 - Avaliar as propriedades estruturais e físico-químicas de GXMs de diferentes sorotipos de *C. neoformans* e *C. gattii* purificadas a partir de agregação intermolecular. Correlacionar tais parâmetros com aspectos da resposta imune do hospedeiro;

3 - Analisar as propriedades da GXM de *T. asahii*, buscando características em comum que possam contribuir para a elucidação da função desse polissacarídeo na patogênese fúngica.

IV – TRABALHOS PUBLICADOS:

1- Resumo:

A ligação da lectina do germe de trigo a *C. neoformans* sugere uma associação de estruturas derivadas de quitina com a divisão celular e com a GXM presente na cápsula

A cáspula de *C. neoformans* é uma estrutura complexa cuja organização requer interações entre seus componentes. Nesse estudo, nós utilizados a lectina do germe de trigo (WGA) como ferramenta para demonstrar que esta se liga a oligômeros de β-1,4-*N*-acetilglucosamina (quito-oligômeros) que interagem com a estrutura capsular de *C. neoformans*. Análises por microscopia confocal demonstraram que os quito-oligômeros formam estruturas anelares ou em forma de gancho que se projetam da parede celular para a cápsula. Uma clara associação entre a detecção dessas estruturas e processos de brotamento foi observada. Análises de extratos capsulares por cromatografia gasosa acoplada à espectrometria de massas e cromatografia de troca iônica sugeriram que as moléculas reconhecidas pela WGA estão firmemente ligadas à parede celular. O tratamento de *C. neoformans* com quitinase resultou na liberação de GXM da superfície celular e reduziu o tamanho da cápsula. O tratamento de células acapsulares com quitinase foi capaz de modificar o perfil de reincorporação de GXM solúvel. Esses resultados indicam uma associação de estruturas derivadas de quitina com brotos celulares e com a GXM de *C. neoformans*, o que pode representar um novo mecanismo pelo qual polissacarídeos capsulares interagem com a parede celular e se organizam durante a replicação celular.

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Binding of the Wheat Germ Lectin to *Cryptococcus neoformans* Suggests an Association of Chitinlike Structures with Yeast Budding and Capsular Glucuronoxylomannan▽†

Marcio L. Rodrigues,[1]* Mauricio Alvarez,[2] Fernanda L. Fonseca,[1] and Arturo Casadevall[2,3]*

Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, Brazil,[1] and Department of Microbiology and Immunology[2] and Division of Infectious Diseases of the Department of Medicine,[3] Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461

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The capsule of *Cryptococcus neoformans* is a complex structure whose assembly requires intermolecular interactions to connect its components into an organized structure. In this study, we demonstrated that the wheat germ agglutinin (WGA), which binds to sialic acids and β-1,4-*N*-acetylglucosamine (GlcNAc) oligomers, can also bind to cryptococcal capsular structures. Confocal microscopy demonstrated that these structures form round or hooklike projections linking the capsule to the cell wall, as well as capsule-associated structures during yeast budding. Chemical analysis of capsular extracts by gas chromatography coupled to mass spectrometry and high-pH anion-exchange chromatography suggested that the molecules recognized by WGA were firmly associated with the cell wall. Enzymatic treatment, competition assays, and staining with chemically modified WGA revealed that GlcNAc oligomers, but not sialic acids, were the molecules recognized by the lectin. Accordingly, treatment of *C. neoformans* cells with chitinase released glucuronoxylomannan (GXM) from the cell surface and reduced the capsule size. Chitinase-treated acapsular cells bound soluble GXM in a modified pattern. These results indicate an association of chitin-derived structures with GXM and budding in *C. neoformans*, which may represent a new mechanism by which the capsular polysaccharide interacts with the cell wall and is rearranged during replication.

Cryptococcus neoformans is the etiological agent of cryptococcosis, a clinical syndrome associated with high indices of morbidity and mortality in immunosuppressed patients (41). The pathogenesis of *C. neoformans* involves the expression of several virulence factors, including pigment production (42), enzymatic activities (9, 10), regulation of signaling pathways (19), and synthesis of the capsular polysaccharides (15, 25).

The cell wall of *C. neoformans* is a complex molecular network comprising polysaccharides, proteins, lipids, pigments, and bioactive enzymes (reviewed in reference 28). Cell wall polysaccharides play key roles in the physiology and pathogenicity of *C. neoformans*. For instance, glucuronoxylomannan (GXM) anchoring to the cryptococcal wall requires α-1,3-glucan (33, 34). In addition, it has been demonstrated that chitosan, the de-O-acetylated form of chitin, is required for cell wall integrity and maintenance of the correct assembly of the pigment melanin and the cryptococcal capsule (2). The presence of cell wall chitinlike oligomers in *C. neoformans* was suggested by the reactivity of yeast cells with the wheat germ agglutinin (WGA) (12).

Capsule expression is probably the most-studied virulence factor of *C. neoformans* (15, 18, 24, 25). It is generally accepted that the cryptococcal capsule is a very complex structure comprising different polysaccharides (4, 23, 24, 27, 30) and mannoproteins (15, 20). The major capsular polysaccharide of *C. neoformans* is GXM, which represents around 88% of the capsular mass (15, 23, 24). GXM is synthesized intracellularly (11, 13, 43) and secreted to the extracellular space in vesicles (35). Secreted GXM is used for distal capsular growth (23, 44) in a process that apparently involves divalent cation-mediated polysaccharide aggregation (29). The molecular mechanisms by which GXM is attached to the cell wall are still obscure.

India ink staining is the simplest method for evaluating capsule expression in *C. neoformans*. In India ink preparations, the *C. neoformans* capsule presents a uniform aspect when examined by light microscopy. However, different regions of the *C. neoformans* capsule differ in density, sugar composition, and charge (45). Scanning electron microscopy of budding yeasts showed the existence of tunnel-like structures in the capsule at sites of nascent bud emergence (44). Further evidence for the existence of distinct structures within the capsule comes from the observation of capsular ringlike structures in India ink preparations (45). Although the molecular composition of the elements present at these capsular regions is still unknown, these findings strongly support the idea that the cryptococcal capsule is a highly complex structure that requires sophisticated mechanisms of assembly at the cell surface.

In the present study, we report the existence of external cell

* Corresponding author. Mailing address for Marcio L. Rodrigues: Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, 21941590, RJ, Brazil. Phone: 55 21 2562 6740. Fax: 55 21 2560 8344. E-mail: marcio@micro.ufrj.br. Mailing address for Arturo Casadevall: Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. Phone: (718) 430-2215. Fax: (718) 430-8968. E-mail: casadeva@aecom.yu.edu.

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wall structures protruding into and interacting with the capsule of *C. neoformans*. Several lines of evidence indicate that these structures contain chitinlike material that is expressed at the *C. neoformans* surface in close association with GXM and projected to the capsule during yeast budding.

MATERIALS AND METHODS

Fungal cells. The *Cryptococcus* cells used in this study included *C. neoformans* strains (strain H99, a serotype A clinical isolate, and strain Cap 67, an acapsular mutant of serotype D strain 3501) and one *C. gattii* isolate (strain NIH198, serotype B). Yeast cells were inoculated into 100-ml Erlenmeyer flasks containing 50 ml of a minimal medium composed of dextrose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM), and thiamine-HCl (3 μM) (pH 5.5). Fungal cells were cultivated for 2 days at 30°C with shaking. For the experiment whose results are shown in Fig. 2F and G, *C. neoformans* (strain H99) was cultivated in yeast extract peptone dextrose medium (YPD) for the same period. Yeast cells were obtained by centrifugation, washed in phosphate-buffered saline (PBS), and counted in a Neubauer chamber. The results presented in this report correspond to the results of experiments performed using strains H99 and Cap 67, but several analyses were also performed using the serotype D strain 24067, producing similar results. Data generated using the *C. gattii* isolate are presented in Fig. 2C.

Fluorescent probes. WGA is a 36,000-Da lectin with known affinity for β-1,4-*N*-acetylglucosamine (GlcNAc) oligomers, present in the fungal polysaccharide chitin, and for sialic acids (1, 32). If modified with a succinyl group, the lectin loses affinity for sialic acids (26). Early reports indicated that WGA also recognized hyaluronic acid, although it has not been clearly demonstrated (39). Calcofluor white is a relatively small (916-Da) fluorescent dye that has been extensively used to stain chitin in fungal cells, due to its ability to recognize the GlcNAc-β-1,4-GlcNAc linkage. Monoclonal antibody (MAb) 18B7 is a mouse immunoglobulin G1 (IgG1) with high affinity for GXM of different cryptococcal serotypes (6).

Fluorescence microscopy. Yeast cells (10^6) were suspended in 4% paraformaldehyde cacodylate buffer (0.1 M, pH 7.2) and incubated for 30 min at room temperature. Fixed yeast cells were washed twice in PBS and incubated in 1% bovine serum albumin in PBS for 1 h. The cells were then suspended in 100 μl of a 5-μg/ml solution of the Alexa Fluor 594 conjugate of WGA (Molecular Probes) and incubated for 30 min at 37°C. After being washed in PBS, the cells were incubated with 25 μM calcofluor white (Invitrogen) under the same conditions. The cells were washed again and incubated for 1 h in the presence of MAb 18B7 (1 μg/ml). After being washed in PBS, the cells were finally incubated with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Fc specific) antibody (Sigma). For a negative control, we used an isotype-matched irrelevant IgG at the same concentrations used for MAb 18B7. To eliminate the possibility that the fluorescence pattern was derived from a specific sequential use of reagents, the order of the reagents was changed, and the results were the same (data not shown). Three-dimensional (3D) images were obtained after placing *C. neoformans* cell suspensions in mounting medium (50% glycerol and 50 mM *N* propyl gallate in PBS) over glass slides. *Z* series (116 sections for each cell) were obtained by capturing images every 0.25 μm with a Leica AOBS laser scanning confocal microscope. The 3D images were finally processed using ImageJ (NIH; http://rsb.info.nih.gov/ij/) and Voxx (Indiana University; www.nephrology.iupui.edu/imaging/voxx/) software. Cell suspensions were mounted over glass slides as described above and analyzed under an Olympus AX70 microscope. Images were acquired by using a QImaging Retiga 1300 digital camera and processed using QCapture suite V2.46 software (QImaging, Burnaby, British Columbia, Canada).

Transmission electron microscopy. After being washed in PBS, *C. neoformans* cells were fixed with 4% formaldehyde–1% glutaraldehyde for 3 h. The cells were then treated with 1% osmium tetroxide and then 1% uranyl acetate, followed by dehydration through a graded series of ethanol solutions and, finally, embedded resin (Electron Microscopy Science, Fort Washington, PA). Ultrathin sections of 70 to 80 nm were prepared in nickel grids. The grids were sequentially incubated in 10% H_2O_2 and a saturated solution of sodium periodate for 10 min. After being washed in PBS, the grids were blocked in 5% goat serum and incubated overnight at 4°C with a 5-μg/ml solution of gold-labeled (30 nm) WGA (EY Laboratories). After sequential washes with PBS and distilled water and 10% uranyl staining, the sections were observed with a JEOL 100 CXII instrument at 80 kV.

Staining of infected macrophages with WGA. Mouse RAW 264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) supplemented with 2 mM L-glutamine and 10% complement-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD). *C. neoformans* cells were opsonized with MAb 18B7 (6), washed in PBS, and allowed to interact with phagocytes at a fungus/host cell ratio of 10:1 during 4 h at 37°C. Nonassociated fungi were then removed by washing, and the remaining cells fixed with cold methanol. After being washed in PBS, infected cells were incubated with fluorescent WGA as described above and observed with an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired by using a color view SX digital camera and processed with the software system analySIS (Soft Image System).

WGA targets in *C. neoformans*. As detailed above, the potential targets of WGA binding in *C. neoformans* include sialic acids, chitin, chitin oligomers, and hyaluronic acid, all of them previously described as surface components of cryptococci (2, 3, 12, 16, 37). To evaluate whether the binding of WGA to *C. neoformans* involved sialic acids, yeast cells were incubated in the presence of tetramethyl rhodamine isocyanate-labeled, succinylated WGA (EY Laboratories) under the same conditions described above for the Alexa 594 conjugate. Alternatively, cryptococci were treated with sialidase under conditions described previously (37) and then incubated with the Alexa 594 WGA conjugate. To evaluate the affinity of WGA for chitinlike molecules in cryptococci, fungal cells were incubated with fluorescent WGA in the presence of a chitin suspension (1 mg/ml, Sigma, extracted from crab shells) or a mixture of chitooligosaccharides (100 μg/ml, final carbohydrate content). The oligosaccharide mixture was prepared under the conditions described by Peumans and coworkers (31). Briefly, 9 mg of crab shell chitin was dissolved in 7 N HCl (3 ml) and partially hydrolyzed for 15 h at 40°C. HCl was then removed by evaporation; the residue was dissolved in PBS and diluted to a final concentration of 100 μg/ml. Chitin hydrolysis into chitooligomers was confirmed by thin-layer chromatography (not shown). Incubations of *C. neoformans* with fluorescent WGA were also performed in the presence of 100 μg/ml hyaluronic acid (purified from *Streptococcus* sp.; Calbiochem) or stachyose, a nonrelated tetrasaccharide (specificity control).

Sugar analysis of capsular extracts. To analyze capsular material for the presence of potential WGA ligands in *C. neoformans* capsular extracts, polysaccharides were extracted from washed cells by using dimethyl sulfoxide (DMSO) or gamma radiation from radioisotope ^{137}Cs as described previously (21). For DMSO extraction, *C. neoformans* cells (3×10^8) were suspended in DMSO (15 ml) and incubated for 15 min with shaking at room temperature. Supernatants containing released capsular polysaccharides were collected by centrifugation, and the pellet was again suspended in 15 ml DMSO for a second extraction under the same conditions. The supernatants were combined and extensively dialyzed against water for subsequent lyophilization and dry-weight determination. For the extraction of capsular polysaccharides by using gamma radiation, yeast cells (5×10^7) were suspended in water (15 ml) and irradiated for 90 min using a Shepherd mark I irradiator (J. L. Shepherd and Associates, San Fernando, CA) at 1,388 rads/min. The sugar composition was determined by gas chromatography-mass spectrometry (GC-MS) analysis of per-O-trimethylsilyl-derivatized monosaccharides from the polysaccharide film. Methyl glycosides were first prepared from the dry sample (0.3 mg) by methanolysis in methanol–1 M HCl at 80°C (18 to 22 h), followed by re-N-acetylation with pyridine and acetic anhydride in methanol for the detection of amino sugars. The sample was then per-O-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 h). GC-MS analysis of the per-O-trimethylsilyl derivatives was performed on an HP 5890 gas chromatograph interfaced to a 5970 MSD MS, using a Supelco DB-1 fused silica capillary column (30-m by 0.25-mm internal diameter). The carbohydrate standards used were arabinose, rhamnose, fucose, xylose, glucuronic acid (GlcA), galacturonic acid, mannose, galactose, glucose, mannitol, dulcitol, and sorbitol.

The presence of sialic acids in irradiated or DMSO extracts was also analyzed by high-pH anion-exchange chromatography (HPAEC) after the hydrolysis of samples with 2 M acetic acid at 80°C for 3 h. A mix of sialic acid standards (*N*-acetyl neuraminic acid and *N*-glycol neuraminic acid) with a known number of moles was hydrolyzed at the same time as the samples. The sialic acids were analyzed using a Dionex DX500 system equipped with a GP40 gradient pump, an ED40 electrochemical detector, and a thermo-separations AS3500 autosampler containing a stainless steel needle. The individual sialic acids were separated by using a Dionex CarboPac PA20 (3 by 150 mm) analytical column with an amino trap. All methods were based on protocols described previously (14).

GXM release after treatment of *C. neoformans* with trypsin and chitinase. After extensive washing with PBS, yeast cells (10^6) were suspended in 100 μl of 0.01 M phosphate buffer (pH 6.0) containing chitinase (0 to 100 μg/ml, purified from *Streptomyces griseus*; Sigma), followed by incubation at 37°C for 12 h. Alternatively, the cells were suspended in the same amount of phosphate buffer (pH 8.0) containing 500 μg/ml trypsin (cell culture grade; Sigma), followed by

similar conditions of incubation. The cell suspensions were incubated overnight at 37°C and centrifuged at 4,000 rpm for cell removal. The controls included cells treated in buffer containing no enzyme. The presence of GXM in the supernatants was determined by capture-enzyme-linked immunosorbent assay as previously described (5). Briefly, 96-well polystyrene plates were coated with a goat anti-mouse IgM. After the removal of unbound antibodies, a solution of MAb 12A1, an IgM MAb with specificity for GXM, was added to the plate, and this step was followed by blocking with 1% bovine serum albumin. Supernatants in different dilutions or purified GXM were added to the wells, and the plates were incubated for 1 h at 37°C. The plates were then washed five times with a solution of Tris-buffered saline supplemented with 0.1% Tween 20, followed by incubation with MAb 18B7 for 1 h. The plate was again washed and incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG1 for 1 h. Reactions were developed after the addition of *p*-nitrophenyl phosphate disodium hexahydrate, followed by reading at 405 nm with a Multiscan MS (Labsystem, Helsinki, Finland). The antibodies used in this assay were all at a concentration of 1 μg/ml. The effects of the enzyme treatments on surface-associated GXM were indirectly evaluated by measurements of capsule size in India ink-stained cells. Capsule sizes were defined as the distances between the cell wall and the outer border of the capsule. Capsule measurements were determined by using ImageJ software.

GXM binding by acapsular cells. Acapsular *C. neoformans* cells (strain Cap 67, 10^6 cells) were suspended in 100 μl of 0.01 M phosphate buffer (pH 6.0) supplemented or not with chitinase at 100 μg/ml and incubated for 24 h at 37°C. The cells were washed and suspended in the same volume of purified GXM at 10 μg/ml in PBS. The suspension was incubated for 12 h at 25°C and extensively washed with PBS, followed by fixation with 4% paraformaldehyde. The cells were further blocked by incubation for 1 h in PBS-bovine serum albumin and reacted with MAb 18B7 (1 μg/ml) for 1 h at room temperature, followed by an FITC-labeled goat anti-mouse IgG (Fc specific) to detect bound antibody (Sigma). Yeast cells were finally observed with an Axioplan 2 (Zeiss, Germany) fluorescence microscope.

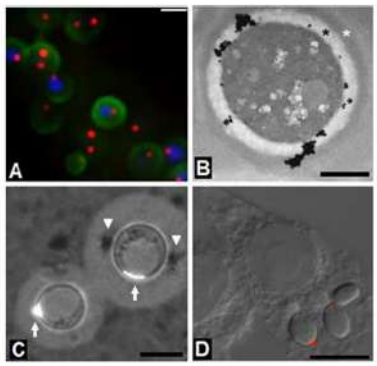

FIG. 1. Binding of WGA to surface structures of *C. neoformans*. Cells were treated with calcofluor (blue staining), MAb 18B7 (green staining), and WGA (red staining) and analyzed by fluorescence microscopy (A). WGA interacts with cryptococcal structures distributed in a polarized fashion, as demonstrated by fluorescence microscopy (A) and confirmed by TEM (B). The white asterisk in panel B denotes the capsule, and the black asterisk represents the cell wall. India ink staining of WGA-treated cells (C) reveals that the capsular channels of *C. neoformans* (arrowheads) do not correspond to the lectin-reactive structures (white arrows). (D) *C. neoformans* infecting macrophages is recognized by WGA (red staining) in similar patterns. Merged images from microscopic fields observed under differential interferential contrast are shown in panels C and D. Scale bars correspond to 10 μm in panel A, 500 nm in panel B, 2 μm in panel C and 10 μm in panel D.

RESULTS

WGA recognizes cryptococcal structures connecting the cell wall to the capsule and around nascent buds. Staining of *C. neoformans* cells with fluorescent WGA, calcofluor white, and MAb 18B7 revealed that structures recognized by the lectin were limited to certain regions of the cell surface (Fig. 1A). These regions are apparently in contact with the cell wall, but WGA-positive areas were also found separated from the cell wall, in a spatial location that would correspond to the capsule. Analysis of yeast cells by transmission electron microscopy after incubation with the gold-labeled lectin (Fig. 1B) indicated that WGA interacted with cell wall sites at opposite poles of the cell, confirming the data from fluorescence microscopy. Interestingly, lectin binding extended into the capsular polysaccharide of *C. neoformans*. Similar results were obtained in experiments using a serotype D strain of *C. neoformans* (data not shown).

The profile of *C. neoformans* reactivity with WGA shown in Fig. 1B resembled the capsular spots described by Zaragoza and coworkers after India ink staining (45). Hence, we investigated whether the areas of WGA staining corresponded to the India ink capsular spots. However, staining of *C. neoformans* with fluorescent WGA followed by India ink revealed that the regions recognized by the lectin and those described in the study by Zaragoza et al. did not colocalize (Fig. 1C). Actually, the regions of the cell surface recognized by the lectin formed 90° angles with the India ink-stained channels, supporting the idea that the capsule of *C. neoformans* manifests polarity in its architecture. To evaluate whether the profile of WGA staining of *C. neoformans* was also observed during infection of host cells, infected macrophages were incubated with the fluorescent lectin and analyzed by fluorescence microscopy. The polarized pattern of lectin staining was also detected in macrophage-associated *C. neoformans* (Fig. 1D), indicating that the fluorescence profile observed in Fig. 1A was not a culture artifact.

The structures recognized by WGA are associated with the cell wall but are visibly projected into the capsular network (see Movies S1 and S2 in the supplemental material). The structures recognized by the lectin appeared connected to the region stained by the anticapsular MAb 18B7 and formed round or hooklike projections. This purported association was supported by the 3D analysis of *C. neoformans* and *C. gattii* after sequential incubations with WGA, calcofluor, and MAb 18B7 (Fig. 2; see Movies S1 and S2 in the supplemental material). The cultivation of *C. neoformans* in YPD resulted in decreased capsule expression (Fig. 2F). Interestingly, the observation of WGA-reactive cell sites in this population was less frequent. For instance, WGA staining was observed only in 31% of the cryptococcal population grown in YPD, while 78% of the cells grown in minimal medium were stained after reaction with the lectin. These results suggest that the expression of the molecules recognized by the lectin is associated with capsule expression in *C. neoformans*.

In approximately 40% of the dividing cells, the structures reacting with WGA appeared to form an interface between the capsule and bud necks (Fig. 2C; see Movie S2 in the supplemental material). The association of the capsule and molecules recognized by WGA in dividing cells was confirmed by the observation of septumlike structures extending from the bud neck to the capsular area (Fig. 2E). The septumlike structure, in fact, seemed to separate capsular structures from mother and daughter cells. To confirm this supposition, *z*-sections of a *C. neoformans* budding cell were obtained and analyzed sepa-

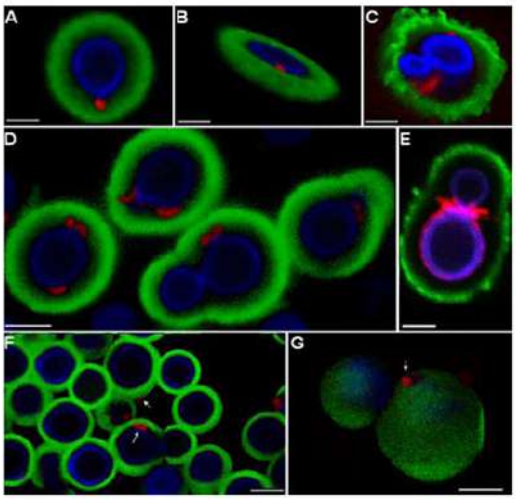

FIG. 2. Confocal analysis of cryptococci after treatment with calcofluor (blue staining), MAb 18B7 (green staining), and WGA (red staining). Fungal cells were cultivated in minimal medium (A to E) or YPD (F and G). The results reveal the existence of structural elements reactive with the lectin between the cell wall and the capsule in *C. neoformans* (A, B, D, and E) and *C. gattii* (C). The existence of lectin-reactive structures inside the capsular area, concentrated around the bud neck, was also observed (E). Cultivation of cryptococci in YPD resulted in decreased capsule expression and a reduced number of lectin-reactive cells (F). In these cells, the structures recognized by the lectin can localize to outer layers of the capsule (arrows in panels F and G). Scale bars correspond to 2 μm, except for panel F (4 μm).

rately (Fig. 3). This analysis demonstrated that the molecules recognized by the lectin form a ringlike structure around the bud neck. WGA was previously shown to interfere with fungal growth (8), but despite binding to cell wall and capsular regions at the budding sites of *C. neoformans*, the presence of WGA in a suspension of replicating yeast did not influence cryptococcal growth (data not shown).

Chemical analysis of released capsular polysaccharides. To evaluate whether the molecules recognized by WGA were covalently bound to the cell wall or associated with the capsule, *C. neoformans* cells were treated with DMSO or irradiated to release capsular polysaccharides. The various fractions were then analyzed by GC-MS and HPAEC for the presence of GlcNAc and sialic acids (Table 1). None of the monosaccharide components that are supposedly recognized by WGA were detected in the capsular extracts, despite efficient extraction of GXM from the capsule. On the other hand, WGA-stained, DMSO- and radiation-decapsulated cells were still recognized by the lectin, suggesting that the structures recognized by WGA were tightly associated to the cell wall and/or inner layers of the capsule (data not shown). Lectin binding to *C. neoformans* was unaffected by organic solvents (not shown), suggesting that lipids are not related to the structures reacting with WGA.

WGA binds to GlcNAc-containing structures in *C. neoformans*. Sialic acids and β-1,4-GlcNAc oligomers are recognized by WGA (1, 26, 32). We first investigated whether the WGA-binding molecules at the cryptococcal surface corresponded to

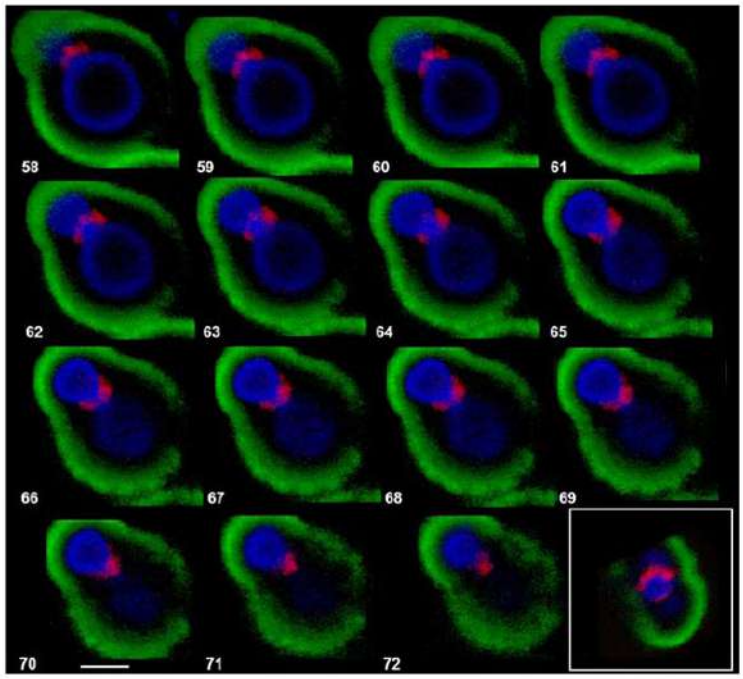

FIG. 3. Confocal microscopic analysis of *C. neoformans*. Sequential *z*-sections of *C. neoformans* after incubation with fluorescent WGA (red), calcofluor (blue), and MAb 18B7 (green) were taken, and the significant ones are shown here. WGA recognized a ringlike structure around the proximal bud periphery. This structure was most apparent after equatorial rotation of the nonsectioned image and *x*- and *y*-sectioning of budding cells (boxed image). Section numbers are shown for each image. Scale bar (presented in panel 70), 1 μm.

TABLE 1. Glycosyl composition analysis of polysaccharide extracts from *C. neoformans* cells[a]

Glycosyl residue	Irradiated sample		DMSO-treated sample	
	Mass (μg)	Mole (%)	Mass (μg)	Mole (%)
Arabinose	ND		ND	
Rhamnose	ND		ND	
Fucose	ND		ND	
Xylose	155.9	39.1	203.8	27.3
GlcA	23.8	4.6	63.0	6.5
Galacturonic acid	ND		ND	
Mannose	259.6	54.2	386.2	43.0
Galactose	6.9	1.4	5.0	0.6
Glucose	3.1	0.7	202.6	22.6
N-Acetyl galactosamine	ND		ND	
N-Acetyl mannosamine	ND		ND	
GlcNAc	ND		ND	
Sialic acid[b]	ND		ND	

[a] ND, not detected.
[b] Sialic acid analysis also included HPAEC.

sialic acids, since these sugars were previously reported as cell wall components of *C. neoformans* (37). Sialidase treatment did not affect WGA binding to *C. neoformans* (data not shown). Importantly, the pattern of binding of the succinylated lectin, which has no affinity for sialic acids, was similar to that observed after the incubation of *C. neoformans* with unmodified WGA (Fig. 4A). The identification of hyaluronic acid as the WGA target in *C. neoformans* was also discarded, since the affinity of WGA for *C. neoformans* cells was not influenced by the presence of the polysaccharide during the incubation of fungi with the lectin (not shown). These results were confirmed by the fact that only in a very small fraction of the cryptococcal population did we observe overlying fluorescence between the regions recognized by WGA and hyaluronic acid-binding protein (data not shown).

The levels of WGA binding to *C. neoformans* were similar when incubations of fungal cells with the lectin were performed under standard conditions or in the presence of a chitin suspension (Fig. 4B). This observation is in full agreement with the data presented in Fig. 2 demonstrating that calcofluor and WGA recognize clearly different cellular sites and with a previous demonstration that the lectin binds chitooligosaccharides with more affinity than chitin (1, 32). In this context, a partial hydrolysis of chitin was performed to generate a mixture of oligosaccharides (31). This mixture was used in competition assays. In the presence of the oligosaccharide mixture, lectin binding to *C. neoformans* was clearly inhibited. WGA binding to cryptococci was unaffected by the presence of the nonrelated carbohydrate stachyose, a tetraoligosaccharide consisting of β-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside. This observation supports the idea that the inhibition of lectin binding by the chitooligosaccharide mixture was specific. In combination, these results are interpreted as indicating that WGA interacts preferentially with GlcNAc-containing oligomers at the surface of *C. neoformans*, as described for bud scars in other fungal species (7, 8). Peptidase treatment did not affect WGA binding to cryptococci (not shown).

Chitinase, but not trypsin, releases GXM from *C. neoformans*. The finding that the WGA-binding structures in *C. neoformans* were in close association with capsular components suggested a role for GlcNAc oligomers in capsule anchoring. These molecules, in principle, could be components of glycoproteins or represent small branches of chitin. To discriminate between the two hypotheses, we treated *C. neoformans* cells with trypsin and chitinase and measured capsule size and concentrations of released GXM. Trypsin treatment did not affect capsule size (not shown). Supernatants of cells treated with trypsin, but not with buffer alone, contained polypeptides in the range of 5 to 10 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). The GXM levels in supernatants from trypsin-treated cells were similar to those detected in supernatants from cells incubated in buffer alone (Fig. 5). These results indicate that the structures recognized by WGA do not represent proteins and confirm the results of a previous report suggesting that structural proteins are not part of the capsular architecture (33). In contrast to the results with trypsin, the treatment of fungal cells with chitinase released GXM (Fig. 5A). A more-detailed analysis of chitinase-treated cells revealed a dose-dependent release of GXM from *C. neoformans*, which was accompanied by a decrease in capsule expression (Fig. 5B). The comparison of control cells with yeast cells treated with the highest chitinase concentration suggests that the enzyme can cause a decrease of approximately 70% in the capsule size.

Next, we examined the binding of WGA and GXM to acapsular *C. neoformans* treated with or without chitinase for 24 h. Chitinase treatment reduced the number of *C. neoformans* cells presenting the surface projections recognized by WGA by approximately 60% (Fig. 6). As expected, Cap 67 control cells incorporated GXM on their cell wall surfaces, resulting in binding of MAb 18B7 and WGA in overlying surface areas, as denoted by the green (MAb 18B7 alone) and orange (WGA and MAb 18B7) staining of fungal cells. Chitinase-treated cells were still able to bind GXM, although the pattern of polysaccharide binding differed from that observed in control yeast. After incubation with GXM, chitinase-treated cells presented a loose polysaccharide coat. The pattern of GXM binding

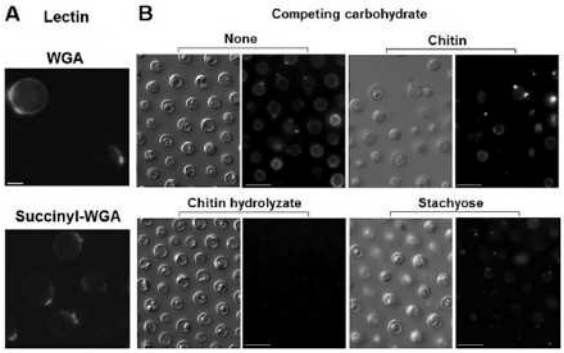

FIG. 4. WGA targets in *C. neoformans*. (A) WGA and its succinylated derivative bind to yeast cells in similar patterns. Scale bar, 1 μm. (B) Incubation of WGA with cryptococci in the presence of competing carbohydrates reveals that a chitin hydrolyzate, but not chitin or the nonrelated oligosaccharide stachyose, inhibits lectin binding, suggesting that WGA recognizes chitooligosaccharides at the fungal surface. Scale bars, 10 μm.

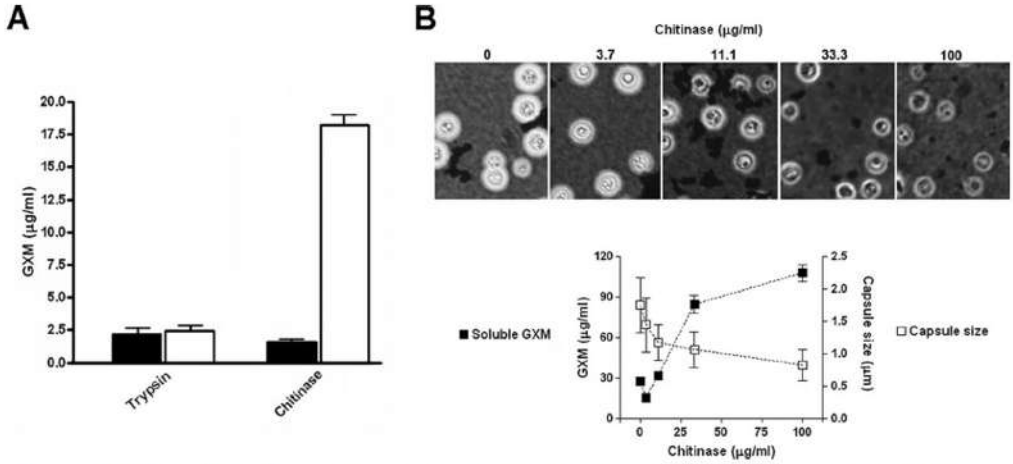

FIG. 5. Release of GXM from the cryptococcal surface after treatment with chitinase. (A) Yeast cells were incubated in the buffers indicated in Materials and Methods (control, black bars) or with the enzymes named on the *x* axis (100 μg/ml chitinase; 500 μg/ml trypsin). (B) Treatment of cryptococci with chitinase caused a dose-dependent decrease of capsule size and a correlated increase in the detection of soluble GXM.

following chitinase digestion resembled that observed for glucanase-treated cells in a previous study (33).

DISCUSSION

The cell surface of *C. neoformans* is unique. The diverse composition of the cryptococcal cell wall, which includes lipids (36), polysaccharides (2, 3, 34), pigments (42), structural proteins, and bioactive enzymes (28), makes evident its complexity. This dense layer is surrounded by a polysaccharide coat that forms the cryptococcal capsule, which confers to the cryptococcal cell surface high levels of complexity in terms of molecular architecture (4, 15, 21, 23, 24). The study of capsule structure and polysaccharide release is important because the capsular phenotype appears to make the largest relative contribution to the virulence of *C. neoformans* (22). Despite the importance of this remarkable structure, very little is known about the assembly process and the functions of capsular components other than GXM; the recent advances in understanding the structure and biophysical properties of GXM reinforce the idea that the capsular network of *C. neoformans* includes heterogeneous molecules (23, 29).

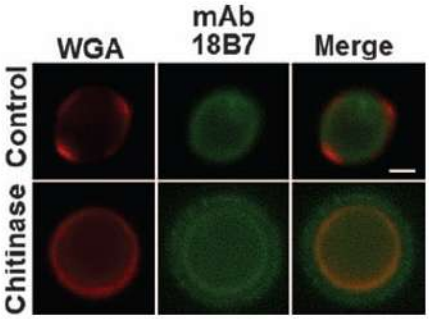

FIG. 6. Binding of GXM to control or chitinase-treated acapsular cells of *C. neoformans*. Yeast cells (Cap 67) were incubated in the presence of phosphate buffer (control) or chitinase and then GXM, followed by treatment with WGA and MAb 18B7. Labeling in red represents WGA binding, while green staining represents binding of the antibody to GXM. Orange staining denotes superposition between lectin and antibody binding to the cell wall. Scale bar, 1 μm.

The results of the present study, combined with the recent data of Zaragoza and coworkers (45), confirm that the *C. neoformans* capsule contains heterogeneous microenvironments despite a relatively homogenous appearance when visualized by India ink suspension and light microscopy. Our results establish that WGA staining molecules associated with the cell wall also project into the capsule. Furthermore, our results, in combination with the earlier report of India ink channels at equatorial locations in the capsule (45), imply that *C. neoformans* cells have a distinct geometry. If one considers the India ink channels to be at the equator of the cell, the WGA staining areas would then be at the cellular poles, such that the India ink-filled channels are at approximate right angles to the locations of nascent buds. The mechanisms by which this cellular geometry and polarity are maintained are not understood, but our ability to stain for these regions with WGA and India ink provides new tools for the study of capsular architecture.

The lectin WGA has been used extensively in the last three decades as a probe to study surface components of different organisms, including *C. neoformans* (12). The lectin has affinity for sialic acids and β-1,4-GlcNAc oligomers (1, 26, 32). On the basis of prior studies, we assumed that WGA would recognize sialic acid, which is produced by *C. neoformans* (37), or chitin, a β-1,4-GlcNAc polymer that interacts very efficiently with the fluorescent dye calcofluor white. Our current data, however, show that sialic acids are not the target of the WGA lectin in this fungus. Competition assays indicated that the lectin recognizes GlcNAc oligomers at the interface between the capsule and the cell wall, which is compatible with the previously described specificities of WGA and calcofluor (1, 26, 32) and the different cellular sites recognized by these fluorescent

probes (Fig. 2). More importantly, these results suggest that, besides the key roles played by chitosan and chitin in the architecture of the cryptococcal cell wall (2, 3), chitinlike oligomers can be important structures connecting cell wall to capsular components.

Treatment of *C. neoformans* cells with chitinase released a substantial amount of GXM and altered the pattern of WGA binding to the fungal cell, with an apparent release of the projected structures together with capsular polysaccharides. Interestingly, the cell wall of chitinase-treated cryptococci was uniformly recognized by the lectin, suggesting a preferential affinity for WGA in binding to cell wall structures exposed after the partial removal of chitin. Our current results and those of previous studies (1) indicate that, rather than binding to chitin, WGA recognizes β-GlcNAc oligomers. We therefore hypothesize that the uniform profile of lectin binding observed after chitinase treatment is a result of the generation of cell wall chitooligosaccharides after partial enzymatic hydrolysis of chitin, as described in other models (17). Based on the fact that GXM was released from the cryptococcal surface after the treatment of yeast cells with chitinase, but not peptidase, we believe that WGA is indeed interacting with outer chitin branches or chitinlike structures in *C. neoformans*. By "chitinlike" structures, we refer to *C. neoformans* components that manifest properties similar to those of chitin, such as GlcNAc composition and susceptibility to chitinases. Chitinlike material may include chitosan, the deacetylated form of chitin.

WGA has been previously described as recognizing bud scars in yeast cells (7, 12), whose content is supposedly enriched in chitin. In the present study, a relationship between yeast budding and WGA binding was also suggested. However, lectin staining was also detectable outside bud necks in dividing cells. This observation may suggest that chitinlike oligomers are only associated with bud necks at the final stages of cell division and then become components of bud scars. In this regard, cell wall chitooligomers could be formed from the chitinase-mediated hydrolysis of chitin during the cell wall rearrangement which is required for cell division.

The cryptococcal capsule is proposed to undergo local rearrangement during budding, possibly producing a tunnel for the bud to emerge (24, 44). In this context, the capsules of the mother and daughter cells have been proposed to be distinct, so that separation can occur, a finding demonstrated by scanning electron microscopy (44). The mechanisms involved in the physical separation of the capsules of dividing cells remain unknown. Given that the chitinlike projections identified here are found at the budding sites and that their stringy appearance resembles the types of structures that may be expected to form the type of tunnels visualized by electron microscopy (44), we propose that chitinlike oligomers form a septumlike structure that originates in the bud neck, extends to the capsular region, and helps to separate the capsules of budding cells. This observation suggests that the interaction between chitooligomers and capsular components is among the mechanisms involved in capsule separation during the replication of *C. neoformans*. This notion is consistent with the report that chitosan is involved not only with cell wall integrity and bud separation but also with the maintenance of normal capsule width (2). Since WGA was previously demonstrated to interact with chitosan (38), we cannot rule out the hypothesis that this polysaccharide, alone or in association with chitin, is also recognized by the lectin in our model.

α-1,3-Glucan is required for capsule anchoring at the surface of cryptococci (33, 34). Cryptococcal cells with disrupted alpha glucan synthase genes shed capsular material but lacked surface capsule (34). In addition, glucanase-treated acapsular cells bound to GXM in a defective manner (33). Indeed, cell wall glucans can anchor other polysaccharides at the fungal cell wall (28), which may explain the fact that chitinase treatment did not fully remove the cryptococcal capsule. In addition, the hypothesis that other components connect glucans and capsular components cannot be discarded, since loss of cell wall glucan would disturb cell wall assembly and, consequently, capsule anchoring. Our results indicate that chitinlike structures could also be relevant to direct GXM binding in *C. neoformans*. Chitinase-treated acapsular cells still bind soluble GXM, but they do so in a manner that forms a loose polysaccharide coat at the surface of *C. neoformans*.

Chitin synthesis and distribution and the relationship of chitins with other surface structures in *C. neoformans* remain poorly understood processes. Eight putative chitin synthase genes have been identified in cryptococci (3), and strains with any one chitin synthase deleted were viable at 30°C. Melanization in *C. neoformans* is regulated through the expression of several genes, including the chitin synthase-encoding gene *CHS3* (40). Although this observation suggests a link between virulence and chitin synthesis, an association between chitin and capsular polysaccharide has not been previously made. The association between chitin-derived material and capsular components could be due to ionic interactions, since chitosan, the most-prevalent form of chitin in cryptococci (2, 3), is a polycation at neutral-to-acid pHs that could interact with the polyanionic polysaccharide GXM. Alternatively, it has been recently reported that *C. neoformans* produces glycosyltransferases that link GlcNAc to GlcA residues (16), which are putatively used to form hyaluronic acid. In theory, the same enzymes could be used to bond GlcNAc residues in chitin to GlcA residues in GXM, providing *C. neoformans* with an additional mechanism for anchoring GXM to the fungal wall.

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2- Resumo:

Papel de quitina e quito-oligômeros na arquitetura capsular de *Cryptococcus neoformans*

Moléculas compostas por unidades de β-1,4-*N*-acetilglucosamina (β-1,4-GlcNAc) e glucosamina desempenham importantes funções estruturais como constituintes de superfície do fungo patogênico *C. neoformans*. Nesse estudo, nós avaliamos o papel de moléculas contendo GlcNAc na organização da cápsula criptococócica. A expressão *in vivo* de oligômeros de quitina variou em função do órgão infectado, como demonstrado pela reatividade diferencial de leveduras com a lectina do germe de trigo (WGA) em tecidos infectados. Análises cromatográficas e de dispersão de luz demonstraram que a GXM é capaz de se ligar a quitina e quito-oligômeros. A adição de quito-oligômeros a culturas de *C. neoformans* resultou na formação de complexos com a GXM que, aparentemente, estão associados à formação de cápsulas com propriedades alteradas. Essas alterações incluíram conexões defeituosas com a parede celular e perda da reatividade com um anticorpo monoclonal contra GXM. O cultivo de *C. neoformans* na presença de um inibidor da síntese de GlcNAc resultou na expressão alterada de quitina na parede celular. Essas células apresentaram cápsulas com alterações nas conexões com a parede criptococócica, além de conterem fibras com diâmetros reduzidos e composição monossacarídica alterada. Esses resultados contribuem para o entendimento do papel de quitina e oligômeros de quitina na estrutura capsular de *C. neoformans*.

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Role for Chitin and Chitooligomers in the Capsular Architecture of *Cryptococcus neoformans*▽

Fernanda L. Fonseca,[1] Leonardo Nimrichter,[1] Radames J. B. Cordero,[2] Susana Frases,[2] Jessica Rodrigues,[1] David L. Goldman,[2,3] Ryszard Andruszkiewicz,[4] Sławomir Milewski,[4] Luiz R. Travassos,[5] Arturo Casadevall,[2,6] and Marcio L. Rodrigues[1]*

Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-902, Brazil[1]; Department of Microbiology and Immunology,[2] Department of Pediatrics,[3] and Division of Infectious Diseases of the Department of Medicine,[6] Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461; Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, 11/12 Narutowicza Street, 80-952 Gdańsk, Poland[4]; and Disciplina de Biologia Celular, Universidade Federal de São Paulo, São Paulo, SP 04023-062, Brazil[5]

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Molecules composed of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and deacetylated glucosamine units play key roles as surface constituents of the human pathogenic fungus *Cryptococcus neoformans*. GlcNAc is the monomeric unit of chitin and chitooligomers, which participate in the connection of capsular polysaccharides to the cryptococcal cell wall. In the present study, we evaluated the role of GlcNAc-containing structures in the assembly of the cryptococcal capsule. The in vivo expression of chitooligomers in *C. neoformans* varied depending on the infected tissue, as inferred from the differential reactivity of yeast forms to the wheat germ agglutinin (WGA) in infected brain and lungs of rats. Chromatographic and dynamic light-scattering analyses demonstrated that glucuronoxylomannan (GXM), the major cryptococcal capsular component, interacts with chitin and chitooligomers. When added to *C. neoformans* cultures, chitooligomers formed soluble complexes with GXM and interfered in capsular assembly, as manifested by aberrant capsules with defective connections with the cell wall and no reactivity with a monoclonal antibody to GXM. Cultivation of *C. neoformans* in the presence of an inhibitor of glucosamine 6-phosphate synthase resulted in altered expression of cell wall chitin. These cells formed capsules that were loosely connected to the cryptococcal wall and contained fibers with decreased diameters and altered monosaccharide composition. These results contribute to our understanding of the role played by chitin and chitooligosaccharides on the cryptococcal capsular structure, broadening the functional activities attributed to GlcNAc-containing structures in this biological system.

Cryptococcus neoformans is the etiologic agent of cryptococcosis, a disease still characterized by high morbidity and mortality despite antifungal therapy (3). Pathogenic species belonging to the *Cryptococcus* genus also include *Cryptococcus gattii*, which causes disease mostly in immunocompetent individuals (24). A unique characteristic of *Cryptococcus* species is the presence of a polysaccharide capsule, which is essential for virulence (7–9, 19, 25, 33).

C. neoformans has a complex cell surface. The thick fungal cell wall is composed of polysaccharides (29), pigments (11), lipids (35), and proteins (36). External to the cryptococcal cell wall, capsular polysaccharides form a capsule (19). Seemingly, the assembly of the surface envelope of *C. neoformans* requires the interaction of cell wall components with capsular elements. Some of the cryptococcal cell wall-capsule connectors have been identified, including the structural polysaccharide α-1,3-glucan and chitooligomers (29, 30, 32).

Chitin-like molecules in fungi are polymerized by chitin synthases, which use cytoplasmic pools of UDP-GlcNAc (*N*-acetylglucosamine) to form β-1,4-linked oligosaccharides and large polymers. In *C. neoformans*, the final cellular site of chitin accumulation is the cell wall. The polysaccharide is also used for chitosan synthesis through enzymatic deacetylation (1). Eight putative cryptococcal chitin synthase genes and three regulator proteins have been identified (2). The chitin synthase Chs3 and regulator Csr2 may form a complex with chitin deacetylases for conversion of chitin to chitosan (1). Key early events in the synthesis of chitin/chitosan require the activity of glucosamine 6-phosphate synthase, which promotes the glutamine-dependent amination of fructose 6-phosphate to form glucosamine 6-phosphate, a substrate used for UDP-GlcNAc synthesis (23).

In a previous study, we demonstrated that β-1,4-linked GlcNAc oligomers, which are specifically recognized by the wheat germ agglutinin (WGA), form bridge-like connections between the cell wall and the capsule of *C. neoformans* (32). In fact, other reports indicate that molecules composed of GlcNAc or its deacetylated derivative play key roles in *C. neoformans* structural biology. For example, mutations in the genes responsible for the expression of chitin synthase 3 or of the biosynthetic regulator Csr2p caused the loss of the ability to retain the virulence-related pigment melanin in the cell wall (1, 2). These cells were also defective in the synthesis of chitosan, which has also been

* Corresponding author. Mailing address: Universidade Federal do Rio de Janeiro, Instituto de Microbiologia, Avenida Carlos Chagas Filho, 373, Cidade Universitária CCS, Bloco I, Rio de Janeiro, RJ 21941-902, Brazil. Phone: 55 21 2562 6740. Fax: 55 21 2560 8344. E-mail: marcio@micro.ufrj.br.

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demonstrated to regulate the retention of cell wall melanin (1). Treatment of *C. neoformans* acapsular mutants with chitinase affected the incorporation of capsular components into the cell wall (32). Considering that melanin and capsular components are crucial for virulence, these results strongly suggest that GlcNAc-derived molecules are key components of the *C. neoformans* cell surface. The expression of GlcNAc-containing molecules is likely to be modulated during infection since chitinase expression by host cells is induced during lung cryptococcosis (37).

In this study, we used β-1,4-linked GlcNAc oligomers and an inhibitor of UDP-GlcNAc synthesis to evaluate the role played by GlcNAc-containing molecules in the surface architecture of *C. neoformans*. The results point to a direct relationship between the expression of GlcNAc-containing molecules and capsular assembly, indicating that chitin and chitooligomers are required for capsule organization in *C. neoformans*.

MATERIALS AND METHODS

Inhibitors of capsule assembly. To study capsule assembly in *C. neoformans*, two different reagents related to the metabolism of GlcNAc-containing molecules were used. Chitin and chitooligomers were recently described as cryptococcal components linking the cell wall to the capsule (32). Chitin is not water soluble, but chitooligomers of two to six β-1,4-linked GlcNAc units are soluble in aqueous solvents. In our study, different chitooligomers were used for interactions with capsular components. The β-1,4-GlcNAc trimeric *N, N′,N″*-triacetylchitotriose $(GlcNAc)_3$ was the prototype in capsular assembly assays. Chitin-derived oligosaccharides were obtained from Dextra Laboratories.

Glucosamine 6-phosphate, the first aminosugar used for the synthesis of UDP-GlcNAc, is synthesized by amination of fructose 6-phosphate with glutamine by glucosamine 6-phosphate synthase (23). Synthetic dipeptides containing the glutamine analog N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP) were previously described to inhibit this enzyme in *Candida albicans* (21, 22). One of these FMDP dipeptides, L-norvalyl-FMDP (Nva-FMDP), was shown to inhibit enzyme activity and to mediate effective antifungal effects in a mouse model of systemic candidiasis (21, 22). A protein search in the *C. neoformans* database (http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans.2/Blast.html) revealed the existence of two hypothetical proteins with significant homology to the *C. albicans* protein (loci CNAG_01164.1 and CNAG_02853.1). Therefore, we hypothesized that Nva-FMDP might also inhibit GlcNAc synthesis in *C. neoformans* and monitored its action by chitin staining in fluorescence-based assays.

Fungal cells. The *C. neoformans* strain used in most experiments was the standard serotype A isolate H99. The serotype D isolate 24067 was used for animal infections. Yeast cells were inoculated into 100-ml Erlenmeyer flasks containing 50 ml of minimal medium composed of glucose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM), and thiamine-HCl (3 μM; pH 5.5). Fungal cells were cultivated for 2 days at 30°C, with shaking. Yeast cells were obtained by centrifugation, washed in phosphate-buffered saline (PBS), and counted in a Neubauer chamber. For morphological analyses, cryptococcal yeasts (10^4 cells) were suspended in 50 μl of minimal medium. Using U-shaped 96-well plates, this suspension was mixed with 50 μl of the same medium containing chitotriose $(GlcNAc)_3$ or Nva-FMDP in concentrations varying from 0 to 100 μg/ml. After 48 h of cultivation at room temperature, aliquots of the cultures were taken for microscopic analyses.

Fluorescence microscopy. The staining reagents used in fluorescence microscopy included the lectin WGA, with known affinity for β-1,4-linked GlcNAc oligomers, calcofluor white, which has been extensively used to stain chitin in fungal cells due to its ability to recognize the $(GlcNAc\text{-}\beta1,4\text{-}GlcNAc)_n$ polymer, and the monoclonal antibody (MAb) 18B7, a mouse immunoglobulin G1 (IgG1) with high affinity for glucuronoxylomannan (GXM) of different cryptococcal serotypes (5). Yeast cells (10^6) were suspended in 4% paraformaldehyde cacodylate buffer (0.1 M; pH 7.2) and incubated for 30 min at room temperature. Fixed yeast cells were washed twice in PBS and incubated in 1% bovine serum albumin in PBS (PBS-BSA) for 1 h. The cells were then suspended in 100 μl of a 5 μg/ml solution of the Alexa Fluor 594 conjugate of WGA (Molecular Probes) and incubated for 30 min at 37°C. After the cells were washed in PBS, they were incubated with 25 μM calcofluor white (Invitrogen) under the same conditions. The cells were washed again and incubated for 1 h in the presence of MAb 18B7 (1 μg/ml). After the cells were washed in PBS, they were finally incubated with a fluorescein isothiocyanate-labeled goat anti-mouse IgG (Fc specific) antibody (Sigma). For a negative control we used an isotype-matched irrelevant IgG at the same concentrations used for MAb 18B7. To eliminate the possibility that the fluorescence pattern was derived from a specific sequential use of reagents, the order of the reagents was changed, and the results were the same (data not shown). Cell suspensions were mounted over glass slides as described above and analyzed under an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired using a Color View SX digital camera and processed with the software system analySIS (Soft Image System). Images were finally processed using ImageJ (provided by NIH; http://rsb.info.nih.gov/ij/).

Capture of complexes containing GXM and chitin-like structures. The presence of complexes containing GXM and chitooligomers in supernatants was first determined by modification of conventional capture enzyme-linked immunosorbent assays (ELISAs). Ninety-six-well polystyrene plates were coated with 50 μl of a WGA solution at 1 μg/ml and incubated for 1 h at 37°C. After removal of unbound lectin molecules, the plate was blocked with PBS-BSA. Different dilutions of culture supernatants of *C. neoformans* after growth in concentrations of $(GlcNAc)_3$ varying from 0 to 100 μg/ml were added to the wells, and the plates were incubated for 1 h at 37°C. The plates were then washed five times with a solution of Tris-buffered saline supplemented with 0.1% Tween 20, blocked again, and incubated with MAb 18B7 for 1 h. The plate was again washed and incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG1 for 1 h. Reactions were developed after the addition of *p*-nitrophenyl phosphate disodium hexahydrate, followed by reading at 405 nm with a microplate reader (TP-reader; Thermo Plate). Controls included sterile culture medium or incubation of the complete reaction mixture in plates that were not initially coated with WGA.

The production of complexes containing GXM and chitooligomers in *C. neoformans* cultures was evaluated by affinity chromatography using an agarose conjugate of WGA (Sigma). The lectin-containing resin (1 ml) was packaged into a plastic chromatographic column (0.5-cm diameter) and extensively washed with sterile minimal medium. The column was loaded with a 10-ml sample of 48 h-culture supernatant of *C. neoformans*. After collection of unbound fractions and exhaustive washing with minimal medium, fractions that were retained by the lectin were eluted by passage of 10 ml of a 0.5 M GlcNAc solution through the column. Ten fractions (1 ml each) were collected and assessed for the presence of GXM by quantitative ELISA (6).

GXM binding to chitin and chitooligomers. To analyze the affinity of GXM for β-1,4-linked GlcNAc-containing structures, 25 mg of chitin obtained from crab shells (Sigma) was exhaustively washed with minimal medium and suspended in 0.5 mg/ml GXM solution (1 ml; in minimal medium). This suspension was incubated for 1 h at 25°C under gentle shaking, followed by removal of unbound GXM by extensive washing of the insoluble particles with minimal medium. The insoluble residue was then suspended in minimal medium (1 ml) and transferred to a plastic chromatographic column (0.5-cm diameter). This material was packaged into the column with minimal medium, and chitin-binding fractions of GXM were finally eluted with a step gradient of NaCl at concentrations ranging from 0.1 to 4 M. Three samples (1 ml each) were collected for each salt concentration, and the content of GXM in these fractions was determined by quantitative ELISA (6).

Binding of chitooligomers to GXM fractions was alternatively evaluated by the analysis of changes in the effective diameter of the polysaccharide after incubation in the presence of chitooligosaccharides. GXM extracellular fractions obtained by ultrafiltration (27) were dissolved in minimal medium to form 1 mg/ml solutions and incubated for 1 h at 25°C (control). Alternatively, the GXM solutions were supplemented with different GlcNAc-containing oligosaccharides (*N,N′*-diacetyl chitobiose; *N,N′,N″*-triacetyl chitotriose; *N,N′,N″,N‴,N⁗*-pentaacetyl chitopentaose; *N,N′,N″,N‴,N⁗,N⁗′*-hexaacetyl chitohexaose) to form 0.01 or 0.1 mg/ml solutions (final oligomer concentration) and then incubated as described above. Effective diameters of the GXM fractions incubated under control conditions or in the presence of the chitooligomers were determined by dynamic light scattering, following the methodology recently described by Frases and colleagues (14).

India ink counterstaining and morphological analysis. *C. neoformans* suspensions grown under control conditions or in the presence of the inhibitors of capsule assembly were placed onto glass slides and mixed with similar volumes of India ink. The suspensions were covered with glass coverslips and analyzed with an Axioplan 2 (Zeiss, Germany) microscope. Images were acquired as described for fluorescence microscopy. Capsule sizes were defined as the distances between the cell wall and the outer border of the capsule. Capsule measurements were determined by using the ImageJ software. For sonication experiments, *C. neo-*

formans suspensions were placed in microcentrifuge tubes and pulsed 10 times with an Ultraclear 800 (Unique) sonifier. The capsules were then visualized as described above.

Scanning electron microscopy. *C. neoformans* cells obtained after growth under control conditions or in the presence of Nva-FMDP were washed three times with PBS and fixed in 2.5% glutaraldehyde for 1 h at room temperature. Samples of fungal cells were serially dehydrated in alcohol, fixed in a critical-point drier (Samdri-790; Tousimis, Rockville, MD), coated with gold-palladium (Desk-1; Denton Vacuum, Inc., Cherry Hill, NJ), and analyzed with a JEOL (Tokyo, Japan) JSM-6400 scanning electron microscope.

Influence of Nva-FMDP on diameter and monosaccharide composition of capsular fibers. For capsule extraction, *C. neoformans* cells cultivated under control conditions or in the presence of Nva-FMDP (10 μg/ml; 3×10^8 cells) were suspended in dimethyl sulfoxide (DMSO; 15 ml) and incubated for 15 min with shaking at room temperature (18). Supernatants containing released capsular polysaccharides were collected by centrifugation, and the pellet was again suspended in 15 ml of DMSO for a second extraction under the same conditions. Supernatants were combined and extensively dialyzed against water for subsequent lyophilization and dry weight determination. GXM fractions obtained by DMSO extraction were analyzed by dynamic light scattering for effective diameter determination (14) or by gas chromatography-mass spectrometry analysis after per-O-trimethylsilylation of the products of acidic methanolysis (27).

In vivo analysis and WGA staining. To establish localized pulmonary infection, male Fischer rats weighing 200 to 250 g were inoculated intratracheally with 1×10^7 *C. neoformans* cells, ATCC strain 24067, using an otoscope. This strain has previously been shown to elicit lung chitinase activity in the rat model (37), and its staining pattern by WGA was similar to patterns observed for other cryptococcal strains (see Fig. 9 and reference 32). To establish brain infection, rats were infected intracisternally with 1×10^3 *C. neoformans* cells. In most cases, rats were sacrificed at 2 weeks, and organs were removed. In some instances, pulmonary infection was allowed to progress for as long as 1 year prior to organ harvesting. At the time of sacrifice, organs were removed, fixed in formalin, embedded in paraffin, and cut into approximately 6-μm sections. All animal work was carried out with the approval of the Animal Use Committee at the Albert Einstein College of Medicine.

After tissue sections were blocked with PBS-BSA, they were stained with a 5 μg/ml solution of the Alexa Fluor 594 conjugate of WGA. To characterize the staining patterns of *C. neoformans* in lung and tissue, approximately 70 yeast cells per slide were counted, and the average from 10 different tissue sections was determined. Statistical analysis was performed with the chi-square test, using the Prism for Windows software (version 4.02). The morphological features of WGA-stained *C. neoformans* from infected tissues were compared with those obtained in vitro, which included control or chitinase-treated cells. For chitinase treatment, PBS-washed yeast cells (10^6) cultivated in vitro were suspended in 100 μl of 0.01 M phosphate buffer (pH 6.0) containing chitinase (100 μg/ml; purified from *Streptomyces griseus* [Sigma]), followed by incubation at 37°C for 12 h. The cell suspensions were incubated overnight at 37°C and centrifuged at 4,000 rpm for cell removal. Controls included cells treated in buffer containing no enzyme.

RESULTS

Binding of GXM to chitin and chitooligomers. Chitooligomers were recently described by our group as candidate molecules that connect the cell wall to capsular GXM (32). The ability of GXM to bind to β-1,4-linked GlcNAc-containing structures was then explored by incubation of the cryptococcal polysaccharide with chitin and elution of bound fractions with increasing concentrations of NaCl. Analysis of eluted fractions by ELISA revealed one major GXM peak, in addition to several minor peaks corresponding to binding of the cryptococcal polysaccharide to chitin (Fig. 1A). This result suggests that GXM binding to chitin may occur at different levels of affinity.

The ability of GXM to bind chitin under controlled conditions led us to investigate whether such interaction would normally occur in *C. neoformans* under the growth conditions used. A capture ELISA was then used to detect chitooligomer (chitotriose)-GXM complexes (Fig. 1B), and the results confirmed that GXM-chitotriose complexes formed during fungal growth (Fig. 1C). Nonspecific binding of the polysaccharide to the surface of the ELISA plate, which might result in false-positive results, apparently did not influence serological reactions, as inferred from the results of running the reaction in the absence of WGA (plates coated with PBS-BSA only) (data not shown).

Although the efficacy of detection of complexes containing GXM and chitooligomers in culture fluids was directly influenced by the concentration of the exogenously added oligosaccharide in the medium, we observed positive ELISA reactions even when *C. neoformans* grew without supplementation of chitooligosaccharides. This observation suggests that a natural association of chitooligomers with GXM may occur during cryptococcal growth. To isolate these complexes, regular culture supernatants of *C. neoformans* were passed through an agarose-WGA column. Bound samples were eluted with 0.5 M GlcNAc, and the presence of GXM in these fractions was monitored in ELISAs with a MAb to GXM (MAb 18B7) (6). The chromatographic profile revealed two peaks of GXM (Fig. 1D), suggesting that WGA captured GlcNAc-containing molecules from culture supernatants in association with GXM. Absorbance values related to GXM detection were relatively low. The results shown in Fig. 1A may suggest that the association of GXM with chitin at pH 5.5 involves noncovalent bonds, possibly including some electrostatic and hydrophobic interactions. Therefore, the relatively low absorbance values probably reflect the fact that GXM-chitooligomer interactions in culture fluids are likely to be dynamic and transient, and consequently no quantitative information should be inferred from these results.

Growth in the presence of chitotriose led to modified cryptococcal capsules. The association of GXM with chitooligomers forming soluble hybrid complexes led us to hypothesize that high concentrations of the β-1,4-linked GlcNAc oligosaccharides could affect capsule assembly by interfering with the incorporation of capsular components by growing cells. Analysis of India ink-counterstained cells revealed that, after growth in minimal medium supplemented with $(GlcNAc)_3$ (6.25 μg/ml), the capsule of several cells of *C. neoformans* became permeable to India ink. The border of the capsule was apparently well preserved, but the interface of the cell wall with the capsule was impregnated with India ink, indicating the abnormal increase of capsular permeability (Fig. 2A and B). To explore the capsular structure that stained with India ink of cells grown in the presence of chitooligomers, cryptococci were stained with MAb 18B7, WGA, and calcofluor for detection, respectively, of GXM, β-1,4-linked chitooligomers, and chitin. Surprisingly, fluorescence microscopy revealed that yeast cells with India ink-permeable capsules stained neither with WGA nor with MAb 18B7 (Fig. 2C and D). In contrast, cryptococcal cells with a regular profile of India ink counterstaining exhibited normal reactivity with MAb 18B7. When *C. neoformans* was cultivated at higher (25 μg/ml) concentrations of $(GlcNAc)_3$, a highly distorted capsular morphology, with longer capsular projections, was observed (Fig. 2E and F).

Capsule assembly is not required for expression of β-1,4-linked GlcNAc oligomers. The partial sensitivity of the GXM-chitin linkage to increasing NaCl concentrations (Fig. 1A) is in line with data from previous studies showing that growth of *C. neoformans* in high-salt concentrations resulted in decreased

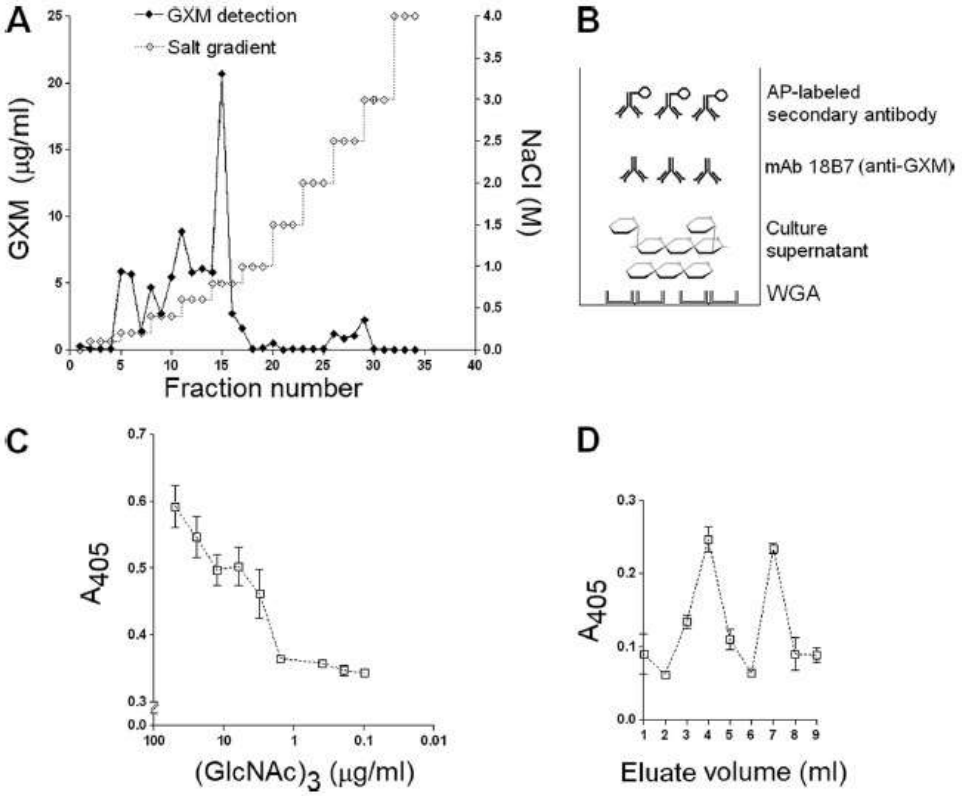


FIG. 1. Association of GXM and chitin-like molecules. (A) Binding of GXM to chitin followed by elution with a step gradient of NaCl resulted in different GXM peaks. (B) Experimental model used to detect β-1,4-linked GlcNAc oligomers in complex with GXM in culture supernatants. WGA was used to capture chitooligomers. Lectin-coated wells of ELISA microplates were incubated with culture supernatants for further detection of carbohydrate complexes using a MAb raised to GXM. (C) Chitotriose was added to *C. neoformans* cultures at different concentrations, and the chitooligomer-GXM complexes were captured by WGA on ELISA microplates and detected by anti-GXM monoclonal antibody. (D) Two peaks corresponding to chitotriose-GXM complexes in *C. neoformans* culture supernatant could be separated by affinity binding to an agarose-WGA column. AP, alkaline phosphatase.

expression of capsular polysaccharides (10, 34). We therefore evaluated whether cultivation of *C. neoformans* in minimal medium supplemented with 1 M NaCl would affect expression of surface chitooligomers. Control cells cultivated in minimal medium manifested relatively large capsules and patterns of antibody and lectin binding that were in agreement with previous observations by our group (32) (Fig. 3A and B). After cultivation in medium of high osmolarity, most of the yeast

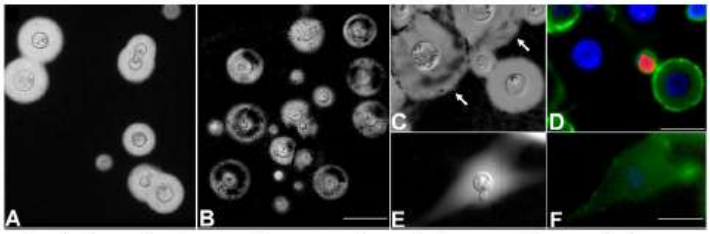


FIG. 2. Morphological and antibody-reacting features of cryptococcal capsules in yeast cells grown in the presence of $(GlcNAc)_3$. India ink counterstaining was used to analyze capsule morphology under control conditions (A) or after growth in the presence of the chitooligomer (6.5 μg/ml) (B). Scale bar, 10 μm. Yeast cells with defective capsules that became permeable to India ink (C; arrows) were not recognized by MAb 18B7 (D). Scale bar, 10 μm. Growth of *C. neoformans* in the presence of a higher $(GlcNAc)_3$ concentration (25 μg/ml) results in the formation of capsules with aberrant morphology that are not permeable to India ink penetration (E). These structures are still recognized by MAb 18B7 (F). Scale bar, 10 μm. Cellular structures stained in blue and green show, respectively, recognition sites of calcofluor and MAb 18B7.

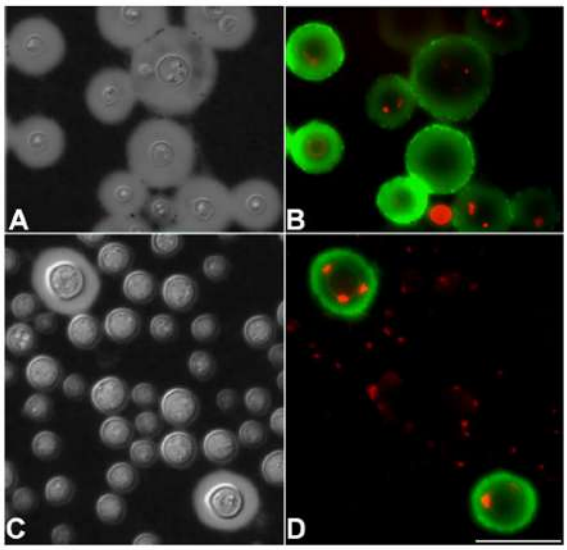


FIG. 3. Analysis of the *C. neoformans* capsule after growth in minimal medium (A and B) or in the same medium supplemented with 1 M NaCl (C and D). (A and B) India ink counterstaining and fluorescence analysis showing the regular profiles of capsule expression and staining of GXM and chitooligomers by MAb 18B7 (green fluorescence) and WGA (red fluorescence), respectively. (C) India ink counterstaining showing that the vast majority of the yeast cells have no visible capsule. (D) Only the yeast cells with normal capsules are recognized by the antibody to GXM. Most cells show the typical profile of staining of β-1,4-linked GlcNAc oligomers by WGA. Scale bar, 10 μm.

cells had no visible capsules and were not recognized by the MAb to GXM (Fig. 3C and D). The minor fraction of the fungal population that remained fully encapsulated was regularly recognized by the antibody. Growth in high-salt concentrations, however, did not affect the expression of surface β-1,4-linked oligomers, indicating that, in agreement with chromatographic analysis (Fig. 1A), high NaCl concentrations affect GXM-GlcNAc linkages without interfering with synthesis and surface exposure of chitooligomers in cryptococci.

Interaction with chitooligomers increased diameters of GXM fibers. The ability of GXM to bind structures related to chitin and previous studies showing that chitooligomers participate in GXM interactions with the cell wall (12, 32) led us to evaluate whether binding to β-1,4-linked GlcNAc-containing molecules could affect the diameter of GXM fibers. For this purpose we used the method described by Frases and coworkers (14), in which the dimensions of GXM fibers were analyzed by dynamic light scattering. Analysis of the distribution of polysaccharide molecules as a function of their effective diameter demonstrated that incubation in the presence of the β-1,4-linked GlcNAc oligomers caused a shift in the distribution curves to regions of enhanced diameters (Fig. 4). The increase in the GXM effective diameter was proportional to the chitooligomer concentration. The only exception was observed when the chitopentamer was used, when the increase in the effective diameter was apparently not influenced by oligomer concentration. Calculation of the molar ratios of chitooligomer and polysaccharide solutions using the values of GXM molecular mass proposed by McFadden and colleagues

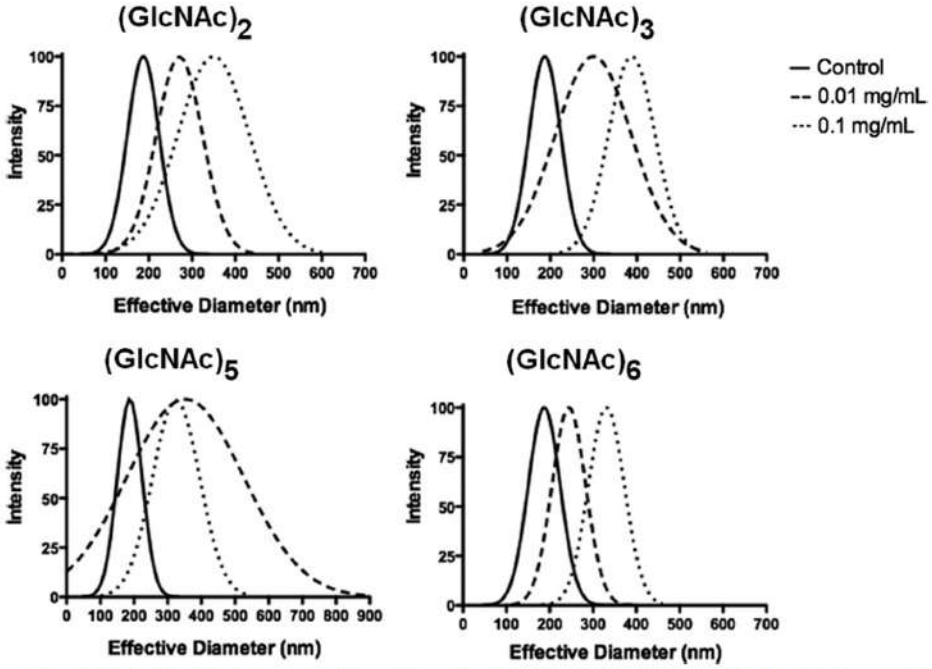


FIG. 4. Interaction of GXM with chitooligomers of differential lengths affects the effective diameter of the fungal polysaccharide. A shift of the distribution curves to areas of higher diameter was observed after incubation of GXM in the presence of all β-1,4-linked GlcNAc oligosaccharides in comparison to control systems in the absence of chitooligomers.

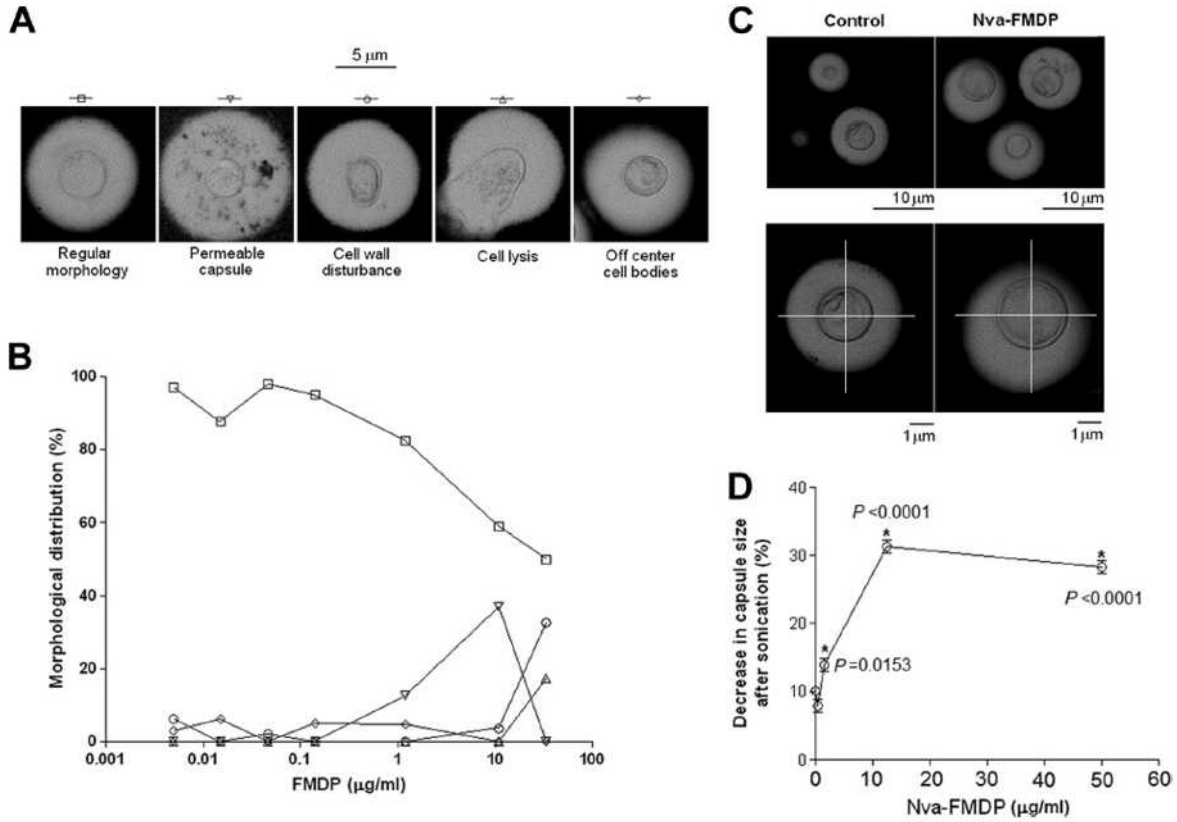


FIG. 5. Inhibition of glucosamine 6-phosphate synthesis by Nva-FMDP affects the morphology and cell wall-capsule connections in *C. neoformans*. (A) Major morphological features of control cells (regular morphology) or of yeast cells cultivated in the presence of Nva-FMDP. (B) Distribution of the morphological alterations shown in panel A after growth of *C. neoformans* with Nva-FMDP. (C) Off-center cell bodies suggesting a possible loose connection between the cell wall and the capsule as induced by the inhibitor. (D) Growth in the presence of increasing concentrations of Nva-FMDP rendered yeast cells more susceptible to capsule release by sonication. Asterisks denote inhibitor concentrations at which capsule release by sonication was significantly more efficient than under control conditions (no inhibitor).

(20) showed that the oligomer-GXM ratios in our model were in the range of 20 to 1. When these values increased up to 200, no correlation between oligosaccharide concentration and polysaccharide diameter was observed (data not shown).

An inhibitor of glucosamine 6-phosphate synthesis affects cell wall and capsule morphology in *C. neoformans*. Nva-FMDP is a dipeptide inhibitor that interferes with early steps of UDP-GlcNAc synthesis, resulting in defective chitin biosynthesis (21). The fact that growth of *C. neoformans* in the presence of chitooligomers affected capsular morphology led us to evaluate the effects of Nva-FMDP on *C. neoformans* morphology. Yeast growth was barely affected by the inhibitor (data not shown). In contrast, an overview of the cellular morphology after fungal cultivation in the presence of Nva-FMDP revealed several abnormal features (Fig. 5). Four major morphological alterations were observed for cells growing in the presence of inhibitor: increased capsule permeability, changed cell wall architecture, increased cell lysis, and dislocation of cell bodies from the central position in relation to the capsule. Quantification of these morphological cell types showed that as the number of *C. neoformans* cells with regular morphology decreased, the number of the other types fluctuated (off-center cell body position, cells with increased capsule permeability, apparent cell lysis, and cell wall with irregular border) (Fig. 5B). In all cases, the morphological changes induced by the inhibitor suggested that cell wall and capsular components were affected. The disturbances in the cell wall morphology (Fig. 5A) and, especially, the dislocation of the cell body in relation to the capsule center (Fig. 5C) suggested that connection of the capsule to the cell wall in *C. neoformans* had been affected after fungal growth in the presence of the inhibitor. In fact, capsules of cells grown with Nva-FMDP were more easily detached from the cell surface by sonication (Fig. 5D).

Lack of cell wall chitin is correlated with reduced capsular dimensions in *C. neoformans*. The cellular activity of Nva-FMDP in the *Cryptococcus* model was monitored by staining fungal cells with calcofluor white, based on the assumption that lack of calcofluor binding to cell walls implies a deficient chitin biosynthesis. Therefore, the effects of Nva-FMDP on the surface architecture of *C. neoformans* were evaluated by

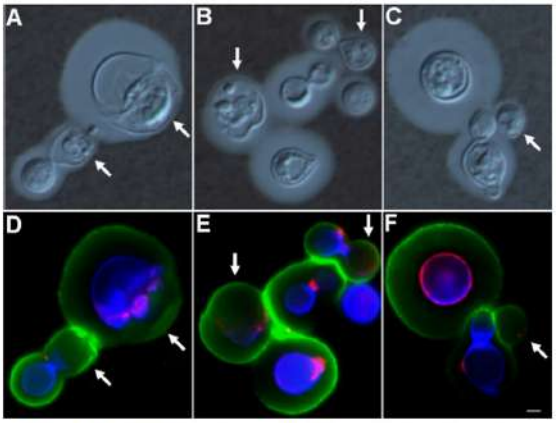


FIG. 6. Nva-FMDP interferes with chitin synthesis in *C. neoformans* cells. In this assay, calcofluor staining (blue) shows a regular chitin expression; lack of calcofluor reactivity indicates defective chitin biosynthesis. Staining in red and green indicates, respectively, detection of chitooligomers and GXM. Panels A to C show the morphological features of India ink-counterstained *C. neoformans* after growth with Nva-FMDP. Panels D to F show the corresponding fluorescence images. Regions of the cell surface with defective chitin biosynthesis were associated with reduced capsule size (arrows). Panels A and D, B and E, and C and F represent three different experiments prepared under the same conditions and producing similar results. Scale bar, 1 μm.

fluorescence microscopy in combination with India ink counterstaining.

Approximately 40% of the cells grown with 25 μg/ml of the inhibitor had altered chitin expression, as concluded from a comparison between the numbers of calcofluor-stained yeast cells grown under control conditions with the number of stained cells from inhibitor-containing cultures. Several cells from cultures supplemented with Nva-FMDP were regularly stained with calcofluor and WGA; these cells showed normal capsules that reacted with MAb 18B7 (Fig. 6). However, other cells showed no reactivity with calcofluor. In addition, some cells were stained for chitin in specific regions of the cell wall and lacked calcofluor reactivity at other surface sites. In all cases, negative calcofluor staining was correlated with the observation of capsular structures with noticeably reduced dimensions, as determined by India ink counterstaining in combination with tests of capsular reactivity with MAb 18B7. Negative calcofluor staining did not result in lack of MAb 18B7 reactivity, indicating that GXM was also associated with the cell wall of *C. neoformans* even in cells manifesting reduced capsules.

The fluorescence profile and related changes in capsule expression observed after growth of *C. neoformans* in the presence of Nva-FMDP led us to evaluate the morphological aspects of the capsule by scanning electron microscopy (SEM). The capsular fibrils of yeast cells exposed to the inhibitor were noticeably shorter than those in control cells (Fig. 7A, B, D, and E). In fact, extraction of wall-associated capsular material with DMSO followed by light-scattering analysis confirmed

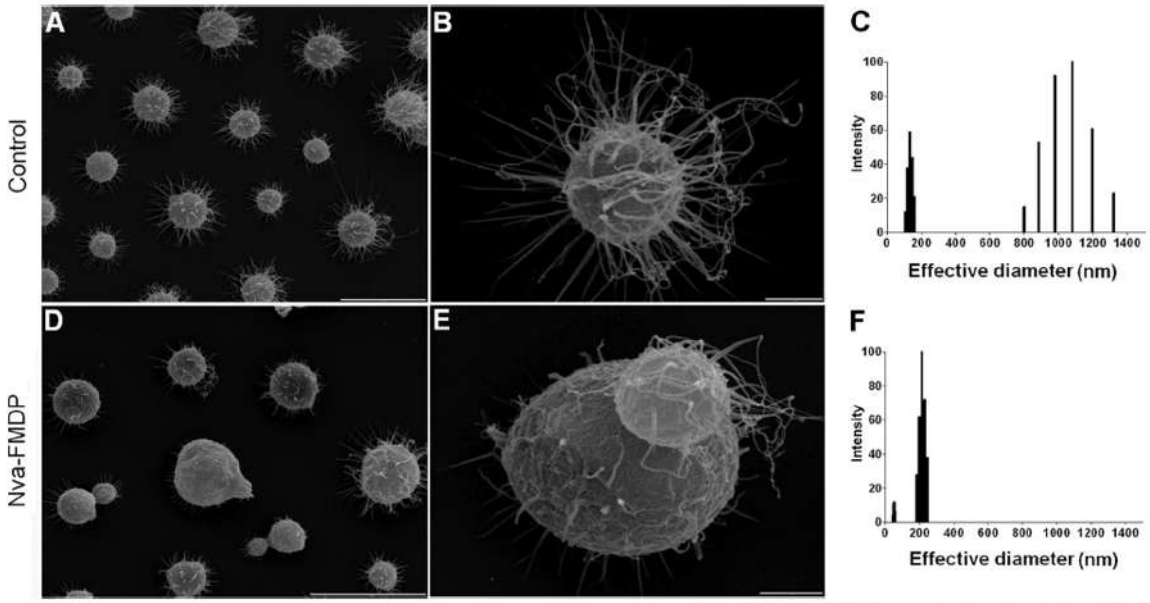


FIG. 7. Capsule fibrils are affected by growth of *C. neoformans* in the presence of Nva-FMDP. SEM of control cells at low (A) and high (B) magnifications show the regular aspects of capsule fibers. These structures were extracted from yeast cells with DMSO, and their effective diameters were determined by light scattering (C). SEM of fungal cells grown with Nva-FMDP (D and E) showed a reduction in the size of capsular fibers, which was confirmed by light-scattering analysis of DMSO extracts of capsule material (F). Scale bars, 10 μm (A and D) and 1 μm (B and E).

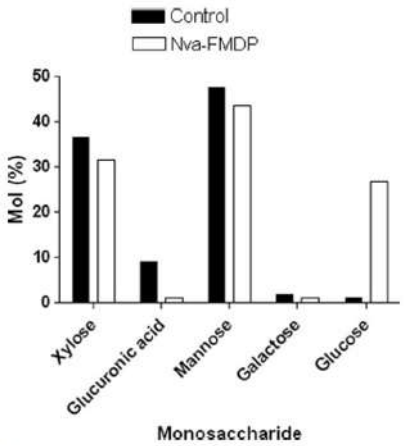
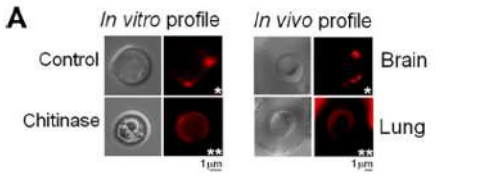


FIG. 8. Monosaccharide composition of capsular extracts of *C. neoformans* after growth under control conditions or in the presence of Nva-FMDP. Capsular extracts from *C. neoformans* cultivated in the presence of the inhibitor showed eightfold less glucuronic acid and a 10-fold increase in the content of glucose.



B

In vivo tissue distribution

WGA staining pattern	Lung	Brain
Localized *	21%	89%
Diffuse **	79%	11%

FIG. 9. WGA reactivity of *C. neoformans* cells in infected lungs and brains. (A) Tissue sections were stained with fluorescent WGA and analyzed microscopically. Results obtained in vivo were compared with the profiles of lectin reactivity observed in vitro. A limited staining of specific surface sites (localized pattern; single asterisks) was observed in untreated control cells compared to the diffuse pattern (double asterisks) of lectin reactivity in chitinase-treated cells. (B) Quantitative analysis revealed that the same fluorescence pattern observed for chitinase-treated cells in vitro is abundant in infected lungs, whereas the lectin binding profile of untreated control cells is more often detected in infected brains ($P < 0.0001$).

that the capsular fibers of cells grown in the presence of the inhibitor (30 μg/ml) were shorter than those extracted from control systems (Fig. 7C and F). Similar results were observed when *C. neoformans* cells were cultivated with 10 μg/ml Nva-FMDP (data not shown). These results strongly suggested that GlcNAc-containing molecules are required for extension of capsular fibers in *C. neoformans*, as initially inferred from the fact that chitooligomers can regulate GXM effective diameter (Fig. 4).

Capsule composition is influenced by the synthesis of GlcNAc-containing molecules. The altered dimensions of capsule fibers observed after cultivation of *C. neoformans* in medium supplemented with Nva-FMDP suggested that capsular structures were modified under these conditions. The monosaccharide composition of capsular extracts of control cells and of Nva-FMDP-treated cryptococci was analyzed by gas chromatography-mass spectrometry (Fig. 8). Mannose, xylose, and glucuronic acid were the main sugar constituents in control capsular extracts, which also showed trace amounts of glucose and galactose. Growth in the presence of Nva-FMDP, however, changed the molar carbohydrate content, with a reduced relative amount of glucuronic acid and increased glucose. Therefore, in addition to the morphological alterations caused by inhibition of GlcNAc synthesis in yeast cells, the carbohydrate content of *C. neoformans* capsule was also affected by Nva-FMDP.

Expression of chitooligomers in vivo. Intratracheal inoculation of *C. neoformans* induces chitinase activity in the lung and bronchoalveolar lavage fluid of infected rats (37). Since chitin and chitooligomers participate in the organization of *C. neoformans* cell envelope (1, 2, 32), we investigated the profile of WGA staining of yeast cells from experimental cryptococcosis in rats. Sections of lungs and brains from infected animals were stained with fluorescent WGA and analyzed microscopically. Two patterns of lectin staining were observed (Fig. 9A). The first staining profile corresponded to reactivity of the lectin with restricted areas of the cell surface, which was similar to the pattern observed in control *C. neoformans* cells (32). The second staining profile corresponded to a diffuse pattern of WGA binding. Diffuse lectin staining is usually observed after treatment of yeast cells with chitinase, which converts the localized profile of WGA binding to *C. neoformans* to a diffuse pattern of cell wall labeling (32). This effect is attributed to the enhanced generation of cell wall chitooligosaccharides after partial enzymatic hydrolysis of chitin (12, 32). Considering that WGA interacts with β-1,4-linked GlcNAc oligomers but not with chitin in its polymeric form, chitinase treatment generates an increased number of cellular sites for lectin binding (32). Therefore, we classified the two visible profiles of *C. neoformans* lectin staining described above as localized and diffuse patterns of WGA binding to the cell surface.

In infected lungs of rats, the diffuse pattern observed for chitinase-treated cells predominated in comparison with the number of fungal cells presenting the regular, localized profile of WGA staining ($P < 0.0001$) (Fig. 9B). As expected, due to the much reduced concentration of organ chitinase (15, 28, 37), the opposite pattern was observed in the infected rat brain.

DISCUSSION

Hexosamine metabolism in fungi is crucial for the biosynthesis of several macromolecules, including chitin, chitosan, and glycoproteins (23). In *C. neoformans*, synthesis of chitin and its deacetylated derivative, chitosan, is directly linked with cell wall assembly and fungal growth (1, 2). At least 14 different genes are associated with the synthesis of these polymers in cryptococci, including eight putative chitin synthases, three putative regulator proteins, and three deacetylases (1, 2). The relatively high gene redundancy observed for chitin synthases

may be responsible for the low susceptibility of *C. neoformans* to nikkomycin, an inhibitor of chitin synthesis (17).

Anchoring of the capsule or capsule precursors to the cell wall is crucial for the pathogenicity of *C. neoformans* since capsular components are required for virulence. The interactions between the components of the cell wall and the capsule responsible for attaching the capsule to the cell wall remain poorly understood, but various reports suggest that the surface connections are complex and could involve multiple cell wall components (29, 30, 32). α-1,3-Glucan was the first cell wall component associated to capsule anchoring. Cryptococcal cells with disrupted α-glucan synthase genes had normal biosynthesis of capsular material but lacked a surface capsule (30). Mutant cells lacking α-1,3-glucan also had modified cell walls (30), strengthening the notion that the lack of cell wall glucans could impair buildup of the cell wall and cell wall-capsule connections.

In a recent report, we demonstrated the existence of chitooligomers that form singular round or hook-like connections between the cell wall and the capsule of *C. neoformans* (32). Similar observations with *Trichosporon asahii* were described by our group (12). These results suggested that, in addition to its expected role in the synthesis of cell wall components, GlcNAc metabolism in fungi could be linked to GXM assembly at the cell surface. In this context, the role played by chitooligomers and chitin in the process of capsule assembly was further investigated.

In our model, growth of *C. neoformans* under conditions that hampered capsule expression (high salt concentration) resulted in normal expression of chitin oligomers (Fig. 3), suggesting that these structures are constitutively synthesized and assembled at the cell surface, even in cells lacking visible capsules. Using chromatographic and ELISA-based approaches in combination with dynamic light scattering (Fig. 1 and 4), we demonstrated that GXM can interact with chitin and chitooligomers. The reactivity of the captured complexes with MAb 18B7 depended on the concentration of the oligomers in the culture, and, interestingly, positive reactions were observed even when chitotriose was not added to the medium (data not shown). In fact, complexes containing GXM and β-1,4-linked GlcNAc-containing structures were isolated from culture supernatants (Fig. 1D), suggesting that such carbohydrate-carbohydrate interactions are common events in the cryptococcal physiology.

The ability of GXM to bind to insoluble chitin and the detection of complexes containing GXM and chitooligomers led us to hypothesize that soluble β-1,4-linked GlcNAc-containing structures could modify the molecular architecture of *C. neoformans* capsular components. Consistent with this hypothesis, yeast cells grown in the presence of chitotriose showed an altered capsular morphology that was most prominent at the cell wall-capsule interface (Fig. 2). Of particular interest we note that the permeabilization to India ink penetration observed after growth of *C. neoformans* in the presence of chitooligomers resulted in the loss of reactivity of capsular material with MAb 18B7, suggesting that the epitopes recognized by the antibody are somehow modified in these defective capsules. In fact, β-1,4-linked GlcNAc oligomers altered the effective diameter of GXM fibrils (Fig. 4), which suggests that aggregated structures containing larger GXM fibers and chitooligomers could form compact capsular regions that increase the permeability to India ink penetration. In addition, polysaccharide aggregation mediated by chitooligomers would likely promote hindrance of *O*-acetyl epitopes, which are required for MAb reactivity (16). Chitooligomers could also modify GXM structure and immunogenicity through the ionic association of glucuronic acid residues with GlcNAc units. The amino group on chitin has a pK_a of 6.1 (26), implying that chitooligomers would likely be charged at pHs between 5 and 6, which was the range used for fungal growth and also in most assays in this study.

To pursue the role of GlcNAc-containing molecules in capsule architecture, we explored the effect of interference with hexosamine metabolism in *C. neoformans* using an inhibitor of the synthesis of glucosamine 6-phosphate, a key step in the UDP-GlcNAc biosynthetic process (23). Nva-FMDP, the inhibitor used in this study, shows a high antifungal efficacy against *C. albicans* (21), but its anticryptococcal activity was limited to alterations in cellular morphology. Budding in the presence of the inhibitor was not affected (data not shown), which warranted its use to control the expression of GlcNAc-containing molecules in viable cryptococcal populations. Different analyses showed that the interference with the metabolism of GlcNAc resulted in loosened capsule-cell wall connections (Fig. 5) and suggested a direct relationship between a lack of chitin synthesis and reduced capsule size. Conversely, addition of chitooligomers to the culture medium at relatively high concentrations resulted in the formation of aberrant capsules with increased dimensions (Fig. 2E and F). Staining of fungal cells with MAb 18B7 and observation by fluorescence microscopy and SEM (Fig. 6 and 7) revealed that the major capsular component, GXM, was still associated to the cell wall in Nva-FMDP-treated cells. These analyses, in association with light-scattering determinations, revealed that capsular fibers showed reduced effective diameters and, apparently, were less abundant in the cell surface. The reduced effective diameters of GXM fibrils in cells that had altered hexosamine metabolism are in agreement with the observation that chitooligomers may regulate the size of GXM fibers (Fig. 4). Since inhibition of GlcNAc synthesis resulted in reduced capsule sizes but did not abolish capsule anchoring to the cell wall, we speculate that part of the capsular material in *C. neoformans* cells could be associated to α-1,3-glucans and related molecules at the cell wall (29, 30). The fact that cell wall components other than chitin-like structures are required for capsule anchoring was supported by the fact that *Saccharomyces cerevisiae* yeast cells, which also express WGA-binding structures, were unable to incorporate exogenous GXM (data not shown), indicating that chitin-like structures and chitooligomers are involved in the external organization of capsular components rather than in their attachment to the cell wall.

The changes in capsule morphology and physical properties induced by Nva-FMDP raised the question as to how the capsular composition would be affected after growth in the presence of the inhibitor. Under this condition the relative concentration of glucuronic acid in cell extracts was reduced eightfold (Fig. 8). We have shown previously that the negative charges of glucuronic acid are required for ion-mediated GXM aggregation and capsule enlargement (27). The reduced concentration of this acidic sugar could, therefore, account for the

formation of smaller capsules caused by the inhibitor. Conversely, polysaccharide fractions from Nva-FMDP-treated cells showed an increased content of glucose. The presence of glucose as a minor component of cryptococcal capsular extracts has been demonstrated before (13), but presently the amount of glucose was close to that of xylose, a major GXM constituent.

In the lungs of mice, but not in whole brain, acidic mammalian chitinase is the sole detectable endogenous chitinase (4). In rat tissues a comparable acidic chitinase was observed. The acidic chitinase in rodents is distinct from human chitotriosidase, and the protein could be identified in alveolar macrophages of silicotic rats (15), suggesting that in the rat lung this enzyme could be generated by macrophages. Since chitinase activity is induced in infected lungs by cryptococcal infections (37) and not in infected brain tissue (28), it was not surprising that the profiles of expression of chitooligomers in lung and brain from rats infected with *C. neoformans* were different. In this study, we demonstrate that most of the *C. neoformans* yeast cells in infected lungs of rats resemble the pattern of chitinase-treated cells in vitro while only 21% of the fungal cells infecting the brain showed the same profile of WGA staining (Fig. 9). These results suggest that the expression of chitin-derived molecules in fungi may be modulated by enzymatic activities derived from the host, which vary according to the infected anatomic site. Such variability could be related to the previously described organ-dependent variation of capsule thickness in *C. neoformans* during animal infection (31).

In summary, this study establishes a link between synthesis and surface distribution of chitin-like structures and the architecture of the capsule in *C. neoformans*. As suggested in prior reports (1, 2), understanding the different steps required for synthesis of chitin and chitin-like molecules could reveal the existence of promising antifungal targets. The fact that chitin-like molecules are both modulated in vivo and associated with capsule assembly reinforces this idea. In addition, cryptococcal virulence could be related to the synthesis of chitooligomers, which makes chitin metabolism and related pathways attractive areas to be explored within the field of fungal pathogenicity.

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3- Resumo:

Correlação entre efeitos imunomodulatórios da GXM de *C. gattii* (sorotipo B) com o diâmetro do polissacarídeo

A GXM de *Cryptococcus* interage com o sistema imune por várias maneiras, incluindo a ativação de receptores do tipo Toll (TLR) e modulação da produção de óxido nítrico (NO) por fagócitos. Nesse estudo, nós analisamos vários parâmetros estruturais de GXMs isoladas de *C. neoformans* (sorotipos A e D) e *C. gattii* (sorotipos B e C), tentando correlacioná-los com a produção de NO por macrófagos e a ativação de TLRs. As frações de GXM foram diferentemente reconhecidas por heterodímeros de TLR2/1 E TLR2/6 expressos em células epiteliais transfectadas para expressar esses receptores. Uma amostra de GXM (sorotipo B) de *C. gattii* foi a fração mais efetiva na ativação da resposta celular mediada por TLR. Essa amostra foi também a mais eficiente em ensaios de produção de NO por macrófagos. Essa amostra foi similar às outras testadas em composição monossacarídica, potencial zeta e mobilidade eletroforética. Entretanto, análises por imunofluorescência utilizando quatro anticorpos monoclonais contra GXM, entretanto, revelaram que o polissacarídeo acima citado apresenta peculiaridades sorológicas. Além disso, análises por dispersão de luz demonstraram que o polissacarídeo pertencente ao sorotipo B teve o menor diâmetro efetivo entre as amostras analisadas. O fracionamento da amostra acima citada e de outras GXMs de sorotipo B por ultrafiltração seguida da exposição dessas frações a macrófagos revelaram uma correlação entre a produção de NO e a redução no diâmetro efetivo dos polissacarídeos. Nossos resultados demonstraram uma grande diversidade funcional em amostras de GXM. Nós propomos que propriedades sorológicas, bem como o diâmetro de moléculas de polissacarídeo, podem influenciar a resposta imune contra *Cryptococcus* spp.

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Immunomodulatory Effects of Serotype B Glucuronoxylomannan from *Cryptococcus gattii* Correlate with Polysaccharide Diameter[▽]

Fernanda L. Fonseca,[1]† Lilian L. Nohara,[2]† Radames J. B. Cordero,[3] Susana Frases,[5] Arturo Casadevall,[3,4] Igor C. Almeida,[2]‡ Leonardo Nimrichter,[1]‡ and Marcio L. Rodrigues[1]*‡

Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Rio de Janeiro, RJ 21941-902,[1] and Laboratório de Biotecnologia, Instituto Nacional de Metrologia, Normalização e Qualidade Industrial, Rio de Janeiro, RJ 25250-020,[5] Brazil; Border Biomedical Research Center, Department of Biological Sciences, University of Texas at El Paso, El Paso, Texas 79968[2]; and Department of Medicine[3] and Department of Microbiology and Immunology,[4] Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

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Glucuronoxylomannan (GXM), the major capsular component in the *Cryptococcus* complex, interacts with the immune system in multiple ways, which include the activation of Toll-like receptors (TLRs) and the modulation of nitric oxide (NO) production by phagocytes. In this study, we analyzed several structural parameters of GXM samples from *Cryptococcus neoformans* (serotypes A and D) and *Cryptococcus gattii* (serotypes B and C) and correlated them with the production of NO by phagocytes and the activation of TLRs. GXM fractions were differentially recognized by TLR2/TLR1 (TLR2/1) and TLR2/6 heterodimers expressed on TLR-transfected HEK293A cells. Higher NF-κB luciferase reporter activity induced by GXM was observed in cells expressing TLR2/1 than in cells transfected with TLR2/6 constructs. A serotype B GXM from *C. gattii* was the most effective polysaccharide fraction activating the TLR-mediated response. This serotype B polysaccharide, which was also highly efficient at eliciting the production of NO by macrophages, was similar to the other GXM samples in monosaccharide composition, zeta potential, and electrophoretic mobility. However, immunofluorescence with four different monoclonal antibodies and dynamic light-scattering analysis revealed that the serotype B GXM showed particularities in serological reactivity and had the smallest effective diameter among the GXM samples analyzed in this study. Fractionation of additional serotype B GXMs, followed by exposure of these fractions to macrophages, revealed a correlation between NO production and reduced effective diameters. Our results demonstrate a great functional diversity in GXM samples from different isolates and establish their abilities to differentially activate cellular responses. We propose that serological properties as well as physical chemical parameters, such as the diameter of polysaccharide molecules, may potentially influence the inflammatory response against *Cryptococcus* spp. and may contribute to the differences in granulomatous inflammation between cryptococcal species.

Cryptococcus neoformans and *Crptococcus gattii* are the etiologic agents of the human and animal fungal disease cryptococcosis. Infection is usually acquired by inhalation of environmental basidiospores or desiccated yeasts. Cryptococcal disease in humans can involve every tissue, including cutaneous and pulmonary sites, but the most serious manifestation is central nervous system involvement with meningoencephalitis (43). Despite the similarities of the clinical syndromes in cryptococcosis caused by *C. neoformans* and *C. gattii*, these species differ in the types of hosts in which they cause disease. While *C. neoformans* preferentially causes disease in immunosuppressed patients, *C. gattii*-related disease is relatively common in immunocompetent individuals (33, 43, 48). Mortality rates are still high in different regions of the globe, and the current therapeutic options are inefficient (1). No vaccines for the prevention of cryptococcosis are available.

* Corresponding author. Mailing address: Avenida Carlos Chagas Filho 373, CCS, Bloco I. Instituto de Microbiologia, UFRJ, Rio de Janeiro, RJ, Brazil, 21941-902. Phone: 55 21 2598 3035. Fax: 55 21 2560 8344. E-mail: marcio@micro.ufrj.br.
† F.L.F. and L.L.N. contributed equally to this work.
‡ I.C.A., L.N., and M.L.R. share senior authorship of this article.
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Glucuronoxylomannan (GXM) is the major component of the polysaccharide capsule, which is the main virulence factor of *Cryptococcus* species (30). GXM is an anionic polysaccharide consisting of a α1-3-linked mannan that is O acetylated at carbon 6 of some of the mannosyl units and substituted with β1,2 glucuronyl and β1,2/β1,4 xylosyl residues (9). The polysaccharide is a capsular component of *Cryptococcus* species that is also abundant in its soluble form in culture fluids and infected tissues (31). Secreted and surface-associated forms of GXM are believed to modulate the immune response during cryptococcosis through multiple mechanisms (35). In addition, administration of monoclonal antibodies (MAbs) against GXM can modify the course of experimental cryptococcosis by prolonging host survival (3). Four serotypes of GXM (A to D) have been defined by serological reactions. This classification divides pathogenic *Cryptococcus* species into specific serotypes, such that *C. gattii* consists of serotypes B and C isolates, while *Cryptococcus neoformans* var. *grubii* and *Cryptococcus neoformans* var. *neoformans* correspond to serotypes A and D, respectively (23, 43). Most studies on the immunological functions of GXM have focused on the polysaccharide fractions from *C. neoformans* serotype A isolates. Although it is generally assumed that the immunological properties observed for

the serotype A polysaccharide are applicable to the other serological groups, this common assumption may not be correct, given the major structural differences among the four major serotypes.

The ability of GXM to activate the innate immune response has been reported in several studies (34, 46, 52, 53). Serotype A GXM has been reported to modulate the production of nitric oxide (NO) by phagocytes (5). In addition, GXM activates Toll-like receptor 4 (TLR4)-mediated intracellular signaling (46), but the contribution of this event to the global innate response against *C. neoformans* infections is uncertain (2, 39). GXM can also interact with TLR2 (46), which is believed to influence the response to cryptococcal infection (53). TLR2 recognizes a diverse set of pathogen-associated molecular patterns, and this recognition requires heterodimerization with TLR1 or TLR6 (14, 17, 22, 29, 50). The roles of TLR1 and TLR6 in the recognition of GXM by TLR2 have not been investigated yet.

In this study, we correlated the structural and physical chemical properties of five GXM samples with their abilities to stimulate NO production by macrophages and to activate nuclear factor κB (NF-κB) in cells expressing either TLR2/TLR1 (TLR2/1) or TLR2/TLR6 (TLR2/6). Our results demonstrate that a serotype B GXM sample is particularly efficient at activating these cellular responses. These immunomodulatory properties correlate with specific serological properties and with a reduced diameter of polysaccharide molecules.

MATERIALS AND METHODS

Fungal strains. The cryptococcal isolates used in this study were selected from the culture collection available in our laboratory. Strains that had previously been more extensively characterized according to their phenotypic characteristics, such as capsule expression, serotype, growth rate, and biochemical properties (6), were used for structural and immunological assays. These samples included *C. neoformans* strains $T_1$444, HEC2393 (serotype A; clinical isolates), and ATCC 28938 (serotype D; obtained from the American Type Culture Collection, Manassas, VA) and *C. gattii* strains CN23/10.993 (serotype B) and HEC40143 (serotype C) (both environmental isolates). Additional serotype B strains were included in this study based on the results obtained during structural/immunological investigations. These isolates comprised the well-characterized strain R265 (19) and strain ATCC 56990 (American Type Culture Collection). Stock cultures were maintained in Sabouraud dextrose agar under mineral oil and were kept at 4°C.

GXM purification. GXM was isolated as previously described by our group (46). Briefly, *C. neoformans* and *C. gattii* (4×10^9 cells) were suspended in 100 ml of a minimal medium composed of glucose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM), and thiamine-HCl (3 μM) (pH 5.5). For all experiments, we used lipopolysaccharide (LPS) free water and glassware. This suspension was then transferred to a 1,000-ml Erlenmeyer flask and was supplemented with 300 ml of the same medium. Fungal cells were cultivated for 4 days at room temperature, with shaking, and were separated from culture supernatants by centrifugation at 4,000 × *g* (15 min, 4°C). The supernatant fluids were collected and again centrifuged, at 15,000 × *g* (15 min, 4°C), to remove smaller debris. The pellets were discarded, and the resulting supernatant was concentrated approximately 20-fold using an Amicon (Millipore, Danvers, MA) ultrafiltration cell (cutoff, 100 kDa; total capacity, 200 ml) with stirring and Biomax polyethersulfone ultrafiltration discs (diameter, 63.5 mm). A nitrogen (N_2) stream was used as the pressure gas. After supernatant concentration, the viscous layer formed was collected with a cell scraper and was transferred to graduated plastic tubes for measurement of gel volumes. The procedure was repeated at least three times in order to ascertain average volumes. Alternatively, the supernatant fraction passed through the 100-kDa membrane was again concentrated using a 10-kDa filtration disc. The viscous layer was again collected and was used for structural and functional determinations.

ELISA for GXM quantification. The concentrations of GXM in supernatants and concentrated films were determined by capture enzyme-linked immunosorbent assays (ELISA), as previously described (4). Briefly, 96 well polystyrene plates were coated with a goat anti-mouse immunoglobulin M (IgM). After removal of unbound antibodies, a solution of MAb 12A1, an IgM MAb with specificity for GXM, was added to the plate, and this step was followed by blocking with 1% bovine serum albumin (BSA). Supernatants in different dilutions or purified GXM was added to the wells, and the plates were incubated for 1 h at 37°C. The plates were then washed five times with a solution of Tris-buffered saline (TBS) supplemented with 0.1% Tween 20, followed by incubation with MAb 18B7 for 1 h. This antibody is a well-characterized IgG1 that protects mice against lethal challenges with *C. neoformans* and binds to an epitope found in GXM from serotypes A, B, C, and D (3). The plate was again washed and was then incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG1 for 1 h. Reactions were developed after the addition of *p*-nitrophenyl phosphate disodium hexahydrate, and the absorbance at 405 nm was then measured with a microplate reader (TP reader; ThermoPlate). The antibodies for this assay were used at 1 μg/ml.

Monosaccharide analysis. Carbohydrate composition was determined by gas chromatography-mass spectrometry (GC-MS) analysis of the per-*O*-trimethylsilyl (per-*O*-TMS)-derivatized monosaccharides from the polysaccharide films, according to the methodology described by Merkle and Poppe (32). Methyl glycosides were first prepared from the dry sample (0.3 mg) by methanolysis in methanol–1 M HCl at 80°C (18 to 22 h). The sample was then per-O-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 h). GC-MS analysis of the per *O* TMS derivatives was performed on an HP 5890 gas chromatograph interfaced to a 5970 MSD mass spectrometer, using a Supelco DB-1 fused-silica capillary column (length, 30 m; inner diameter, 0.25 mm). The carbohydrate standards used were arabinose, rhamnose, fucose, xylose, glucuronic acid, galacturonic acid, mannose, galactose, glucose, mannitol, dulcitol, and sorbitol.

Transient transfection with TLRs. TLR constructs (16), as well as the β-actin–*Renilla* luciferase construct (49) and the ELAM 1 firefly luciferase reporter construct (45), were kindly provided by Richard Darveau (University of Washington, Seattle). All plasmids used in the transfections were purified using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. HEK293A cells (ATCC, Manassas, VA) were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), and the confluent monolayer was harvested by treatment with trypsin-EDTA (Sigma-Aldrich, St. Louis, MO). Cells were seeded in 12-well plates the day before transfection. HEK293A cells were transiently cotransfected with plasmids encoding either mouse TLR2 and TLR1 or TLR2 and TLR6 together with the ELAM-1–firefly luciferase reporter construct and β-actin–*Renilla* luciferase by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The amount of total DNA per well was normalized to 2 μg by adding an empty vector. On the following day, the transfected cells were plated in 96-well plates.

Luciferase reporter assays for NF-κB activation. Forty-eight hours after transfection, cells were stimulated with purified GXM (1 to 100 μg/ml) for 4 h in DMEM containing 10% FBS. Controls for TLR activation included stimulation of cells with ultrapure LPS from *Escherichia coli* strain 0111:B4 (Invivogen, San Diego, CA), Pam_3Cys-SKKKK (P3C), or FSL-1 (EMC Microcollections, Tübingen, Germany). Then cells were washed once in phosphate buffered saline (PBS) and were lysed in Passive Lysis Buffer (Promega, Madison, WI). The luciferase activity was measured using the Dual Luciferase Reporter Assay system (Promega, Madison, WI) according to the manufacturer's instructions. The relative light units (RLU) were quantitated using a Luminoskan luminometer. NF-κB activation is expressed as the ratio of NF-κB-dependent firefly luciferase activity to β-actin-dependent *Renilla* luciferase activity (16). The results are shown as the means and standard deviations of values for triplicate wells.

Nitric oxide production by phagocytes. The murine macrophage-like cell line RAW 264.7 (ATCC) was cultivated under LPS-free conditions in complete DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mg ml/ml gentamicin, MEM Non-Essential Amino Acids Solution (catalog no. 11360; Gibco-Invitrogen), 10 mM HEPES, and 50 mM 2-β-mercaptoethanol at 37°C under a 7.5% CO_2 atmosphere. Murine cells were washed twice in serum-free DMEM and were incubated in fresh medium supplemented with varying concentrations of GXM (1 to 100 μg/ml) for 16 h at 37°C (7.5% CO_2 atmosphere). As a positive control, macrophages were stimulated with 1 μg/ml LPS. Supernatants were then collected and assayed for NO production by the method of Griess (15). Negative controls consisted of supernatants of RAW cells cultivated in a medium containing no GXM. All experiments were performed in triplicate sets.

IF for GXM detection. Antibodies to GXM used in this assay included IgG and IgM. MAbs 12A1 and 13F1 are two clonally related IgMs that differ in fine

specificity and protective efficacy (37, 38). MAb 12A1 is protective and produces annular immunofluorescence (IF) on serotype D *C. neoformans*, while MAb 13F1 is not protective and produces punctate IF. MAb 2D10 (IgM) is also protective in a murine model of cryptococcosis. This antibody reacts with epitopes found through the cell wall and capsule of a serotype D strain of *C. neoformans* (13). MAb 18B7 is a protective IgG1 that has been tested as a therapeutic antibody in animals and humans (3, 24). This antibody reacts with all GXM serotypes. *C. neoformans* cells (10^6) were fixed with 4% paraformaldehyde. The cells were further blocked for 1 h in PBS-BSA and were incubated with the MAbs described above (1 μg/ml) for 1 h at room temperature, followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse (IgG or IgM) antibodies (Sigma). Yeast cells were finally observed with an Axioplan 2 fluorescence microscope (Zeiss, Germany). Images were acquired using a Color View SX digital camera and were processed with the analySIS software system (Soft Imaging System). Under control conditions, MAbs were replaced by irrelevant isotype-matched antibodies. Exposure times were similar for all conditions.

Biophysical studies. Particle sizes and negative charges of GXM samples were inferred from dynamic light-scattering and zeta potential (ζ) determinations, respectively, by following the methods described by Frases and colleagues (11, 12). For ζ determination, GXM solutions were adjusted to 1 mg/ml in water and were analyzed in a Zeta potential analyzer (ZetaPlus, Brookhaven Instruments Corp., Holtsville, NY). Final values were obtained from the equation $\zeta = (4\pi\eta n)/D$, where D is the dielectric constant of the medium, η is the viscosity, and m is the electrophoretic mobility of the particle. For determination of the effective diameters of GXM molecules, polysaccharide solutions were prepared as described above and were measured by quasi-elastic light scattering in a 90Plus/BI-MAS multiangle particle-sizing analyzer (Brookhaven Instruments Corp., Holtsville, NY). Particle sizes were calculated as described recently (12). Multimodal size distribution analysis of polysaccharides was performed with the values for intensity-weighted sizes obtained from the non-negativity-constrained least-squares (NNLS) algorithm.

Statistics. The existence of significant differences between the different systems analyzed in this study was ascertained using multiple statistical tests. The efficacies of TLR-mediated NF-κB activation and NO production, correlation tests, and biophysical tests were statistically evaluated using Student's *t* test for comparison of two different groups and analysis of variance for comparison of several groups. Statistical tests were performed with GraphPad Prism (version 5.0).

RESULTS

GXM samples from all strains manifest aggregation characteristics. GXM aggregation resulting in the production of purified gels of native polysaccharide was previously demonstrated for a serotype D strain of *C. neoformans* (11, 40). However, it was not clear whether the formation of the viscous polysaccharide films was a strain-specific phenomenon or a general property of cryptococcal strains. Therefore, we evaluated the abilities of polysaccharides from two strains, one *C. neoformans* var. *grubii* and one *C. gattii* strain (serotypes B and C), to form gels after concentration by ultrafiltration.

Supernatants were obtained from 400-ml cultures containing an initial inoculum of 4×10^9 cells. The final number of cells in each culture differed according to the growth rate of each strain (not shown). Supernatant concentration by ultrafiltration led to the deposition of viscous films on filters for all isolates tested. The volumes of the films were normalized to the final numbers of cells in each culture. This procedure was repeated at least three times for each strain, and different average polysaccharide volumes were generated (Fig. 1A). Next, we analyzed the ability of each isolate to produce extracellular GXM, normalizing the polysaccharide concentration found by ELISA to the final number of cells in the culture. The profile of GXM production by each strain, determined by ELISA (Fig. 1B), resembled very closely that observed for gel formation in the corresponding supernatant. In fact, GXM

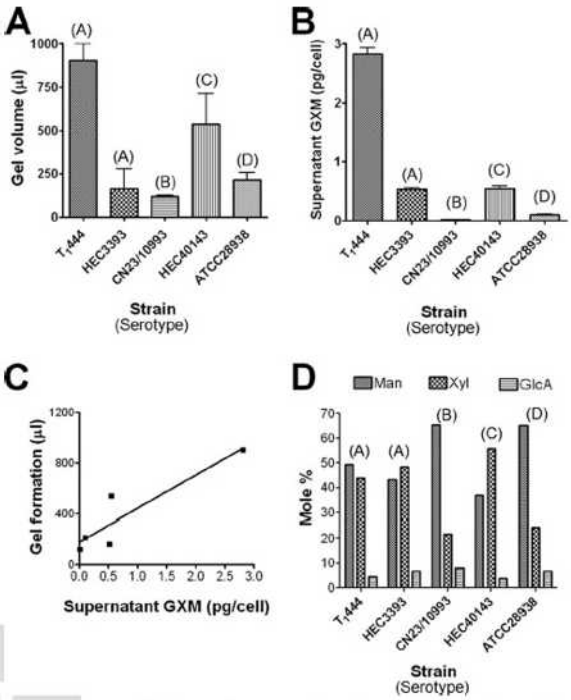

FIG. 1. Quantitative and structural analyses of GXMs from five *Cryptococcus* isolates. GXM was isolated by formation of polysaccharide gels after concentration of culture supernatants of five different isolates of *C. neoformans* and *C. gattii*. (A and B) The volume of gel formation in normalized cultures (A) apparently correlates with the ability of each strain to produce and secrete GXM to the extracellular medium (B). The results are expressed as means ± standard deviations for three different experiments. (C) Correlation properties. (D) Monosaccharide composition of polysaccharides obtained from the five different isolates of *C. neoformans* and *C. gattii*. Monosaccharides were identified by GC-MS; the relative amount of each sugar residue in the polysaccharides is shown as a molar percentage. The serotype of each strain is given in parentheses above the bars.

concentrations in supernatants and gel formation were correlated (R^2, 0.7390; P, 0.0014), as demonstrated in Fig. 1C.

The sugar composition of each polysaccharide fraction was analyzed by GC-MS (Fig. 1D). After methanolysis of the polysaccharides and per-O-trimethylsilylation of the corresponding products, the resulting monosaccharides were initially identified by their retention times relative to those of standards, followed by structural authentication using MS-MS (not shown). All polysaccharide samples tested had xylose, mannose, and glucuronic acid as major constituents, consistent with the three sugar components of GXM. As previously reported (10, 11), galactose was a trace component of all samples (data not shown). The levels of each GXM building unit differed in polysaccharides from different isolates (Fig. 1D), as normally observed during analysis of different GXM samples. Strains HEC3393 (serotype A) and HEC40143 (serotype C) contained particularly high proportions of xylose, while strains T_1444 (serotype A), CN23/10993 (serotype B), and ATCC 28938 (serotype D) had mannose as their major monosaccharide constituent.

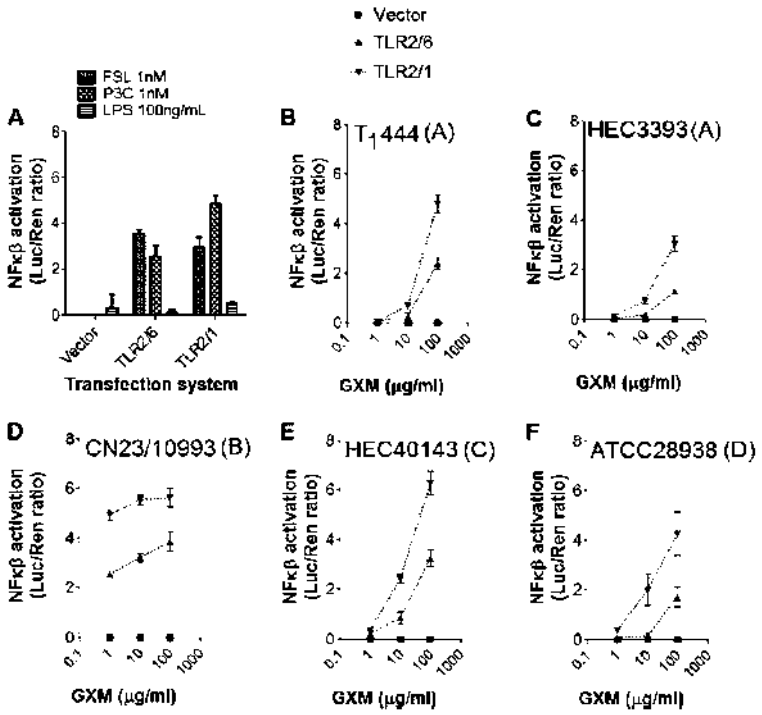

FIG. 2. NF-κB activation in cells expressing TLRs by GXM. (A) Control systems. Pam_3CSK_4 (P_3C) and FSL-1, but not LPS, activated NF-κB nuclear translocation in cells expressing either TLR2/1 or TLR2/6, as expected. Transfection of HEK293A cells with a plasmid containing no TLR-coding sequences (vector) resulted in unresponsiveness. (B to F) Stimulation of HEK293A cells expressing TLR2/1 (inverted triangles) or TLR2/6 (triangles) by GXM samples resulted in dose-dependent NF-κB activation. *Cryptococcus* strains (serotypes) from which each GXM sample was isolated are given at the top of each panel.

NF-κB activation in cells expressing the TLR2/1 or TLR2/6 heterodimer in response to GXM. HEK293A cells expressing either TLR2/1 or TLR2/6 were stimulated with one of the control molecules LPS, P3C, and FSL-1 or with GXM fractions obtained from one of five different *C. neoformans* or *C. gattii* strains (Fig. 2). Transfected cells showed efficient NF-κB activation in response to the synthetic triacylated lipopeptide P3C and to the synthetic diacylated lipopeptide FSL-1, which were used as positive controls for TLR2/1 and TLR2/6 activation, respectively (21). As expected, TLR2/6- or TLR2/1-transfected cells responded very poorly to LPS, the classic TLR4 ligand (18). Also, HEK293A cells transfected with the reporter construct and plasmids containing no TLR-coding sequences (vector) were unresponsive in all cases. All polysaccharide samples induced dose-dependent activation of NF-κB (Fig. 2). NF-κB activation by GXM was always more efficient in cells transfected with the TLR2/1 constructs. Translocation of NF-κB in GXM-treated cells was also more efficient in cells expressing TLR2/1 than in cells transfected with plasmids coding for TLR4/CD14 (data not shown), which were initially described as the receptors involved in GXM-mediated TLR activation (46).

A comparative analysis of the ability of each GXM sample to activate TLR-mediated cellular responses revealed unexpected differences. Although all polysaccharide fractions had the capacity to activate NF-κB in either TLR2/1- or TLR2/6-expressing cells at a concentration of 100 μg/ml, a *C. gattii* polysaccharide sample (serotype B) was significantly more efficient at activating NF-κB than all others ($P < 0.0001$), with strong signals apparent at 1 and 10 μg/ml (Fig. 3). At 1 μg/ml, NF-κB activation mediated by the serotype B GXM was at least 10-fold higher than that mediated by all other GXM samples for TLR2/1-expressing cells and 6-fold higher for TLR2/6-expressing cells. At 10 μg/ml, the serotype B sample was approximately 2-fold and 4-fold more effective than the other samples in TLR2/1- and TLR2/6-expressing cells, respectively.

NO production in response to GXM stimulation. The GXM samples used for TLR activation were also tested for their abilities to stimulate the production of NO by macrophage-like cells. Exposure of RAW 264.7 cells to GXM from *C. neoformans* cultures resulted in the production of NO at the background level (Fig. 4). Treatment of the phagocytes with *C. gattii* GXM, however, resulted in dose-dependent production of NO. As observed in TLR-based assays, the GXM sample from strain CN23/10993 was the most effective polysaccharide fraction at eliciting NO production.

Structural and serological properties of GXM from *C. neoformans* and *C. gattii*. The differences between the TLR-activating abilities of the various GXM samples led us to investigate the antigenic and physical properties of this polysaccharide set in more detail. The differences in monosaccharide composition between the different samples were not

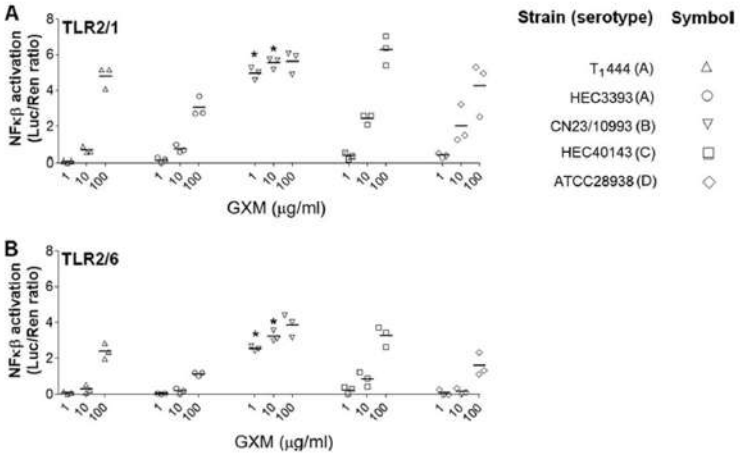

FIG. 3. Comparative analysis of the efficacies of GXM samples in the activation of TLR-mediated NF-κB nuclear translocation. Treatment of HEK293A cells expressing either TLR2/1 (A) or TLR2/6 (B) with GXM revealed that the polysaccharide fractions from strain CN23/10993 were significantly more efficient than all others ($P < 0.0001$) at 1 and 10 μg/ml (asterisks). No significant differences were observed at a higher concentration (100 μg/ml). Strains T_1444, HEC3393, HEC40143, and ATCC 28938 manifested similar efficacies in activating NF-κB nuclear translocation. Strain serotypes are given in the key.

correlated with the ability of GXM to activate cellular responses, since polysaccharide fractions with very similar compositions (strains CN23/10993 and ATCC 28938) manifested different efficacies at NO production and TLR2/1- and TLR2/6-mediated NF-κB activation (Fig. 1 to 4).

The negative charge of GXM is an important determinant of function for the capsular polysaccharide in *C. neoformans* (40, 41). Consequently, we determined the zeta potentials of the GXM samples from all the *C. neoformans* and *C. gattii* isolates, which were similar (Table 1). The electrophoretic mobilities of the various GXM preparations were also similar and correlated strictly with zeta potentials (r^2, 0.9992; P, <0.0001). These results therefore suggested that the polysaccharide charge did not affect the activation of NO production and TLR-mediated cellular responses by GXM.

Differences in GXM structure and functions can correlate with reactivity with monoclonal antibodies (42), which led us to evaluate whether the functional discrepancies observed in Fig. 2 to 4 were related to specific serological patterns (Fig. 5). Cells from each of the five *C. neoformans* strains were similarly recognized by MAb 18B7, as demonstrated by immunofluorescence analysis. For all strains, intensities were comparable and the binding pattern was annular. MAbs 2D10, 12A1, and 13F1 produced punctate patterns of reactivity with similar intensities after incubation with strains T_1444, HEC3393, HEC40143, and ATCC 28938. When strain CN23/10993 was used, however, very strong serological reactions were observed with MAb 12A1. In contrast, these cells were not recognized by MAb 13F1.

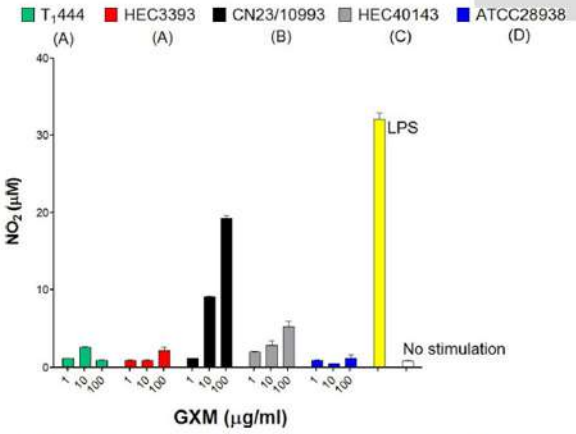

FIG. 4. Comparative analysis of the efficacies of GXM samples in the induction of NO production by macrophages. Polysaccharide fractions from strain CN23/10993 were significantly more efficient at inducing NO production than all others ($P < 0.0001$) at 1, 10, and 100 μg/ml. LPS was used as a positive control for NO production by macrophage-like cells; incubation of the phagocytes in the medium alone (no stimulation) was the negative control. Serotypes are given in parentheses for each strain.

Effective diameter of GXM. Epitope accessibility in GXM may differ according to the diameter of the molecule (11). In addition, polysaccharide size is a parameter known to influence the activation of TLR2-mediated innate responses (26). We therefore investigated the relationship between NF-κB activa-

TABLE 1. Electronegativities of polysaccharides from five different strains of *C. neoformans* and *C. gattii*

Strain	Serotype	Zeta potential (mV)	Mobility [(μ/s)/(V/cm)]
T_1444	A	−34.50 ± 0.32	−2.70 ± 0.02
HEC3393	A	−33.36 ± 0.51	−2.61 ± 0.04
CN23/10993	B	−33.34 ± 0.21	−2.60 ± 0.02
HEC40143	C	−38.15 ± 0.41	−2.98 ± 0.03
ATCC 28938	D	−34.62 ± 0.45	−2.71 ± 0.04

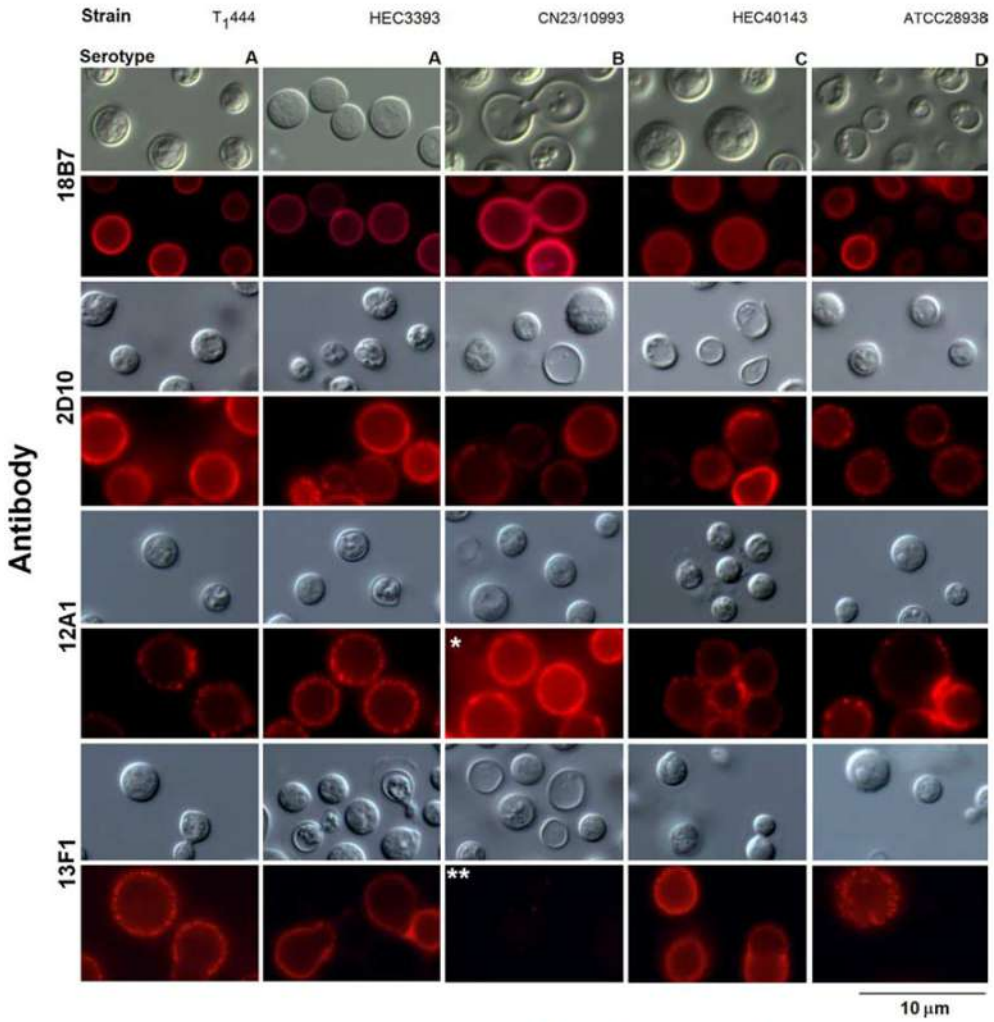

FIG. 5. Reactivities of *C. neoformans* and *C. gattii* isolates with four monoclonal antibodies to GXM. Fungal strains and serotypes are given at the top; antibodies are given on the left. Differential interferential contrast (gray) and fluorescence (red) images are shown. The overreaction of CN23/10993 cells with antibody 12A1 (single asterisk) and their lack of reactivity with antibody 13F1 (double asterisks) are highlighted.

tion and the effective diameter of GXM as measured by dynamic light scattering (Fig. 6). The polysaccharide molecules with the largest diameters came from isolates T_1444 and ATCC 28938 (serotypes A and D, respectively). GXM samples from strains HEC3393 and HEC40143 (serotypes A and C, respectively) showed smaller diameters, which were still larger than that for the polysaccharide isolated from strain CN23/10993 (serotype B). All strains produced polysaccharides with diameters greater than 2 μm, except for strain CN23/10993. Determination of effective diameters by 10 different analyses showed that GXM fractions from the CN23/10993 isolate were significantly shorter (P, <0.0001) than any other polysaccharide. Therefore, the GXM sample containing molecules of the smallest diameter was the most potent polysaccharide in activating the cellular responses in this study.

Smaller GXM fractions from *C. gattii* serotype B strains are more effective at eliciting NO production. In an attempt to establish a correlation between the effective diameters of GXM samples and their abilities to stimulate cellular responses, we fractionated culture supernatants from different cryptococcal isolates. GXM samples were isolated from different strains, including (i) T_1444, due to its ability to produce abundant extracellular polysaccharides with large diameters (Fig. 1 and 6), and (ii) CN23/10993, which was selected on the basis of its ability to produce GXM with apparently higher immunogenicity (Fig. 2 to 4). Two additional serotype B GXMs, from strains R265 and ATCC 56990, were included in this assay for comparative purposes. GXM fractions with molecular masses higher than 100 kDa and in the range of 10 to 100 kDa were obtained by supernatant filtration. The proto-

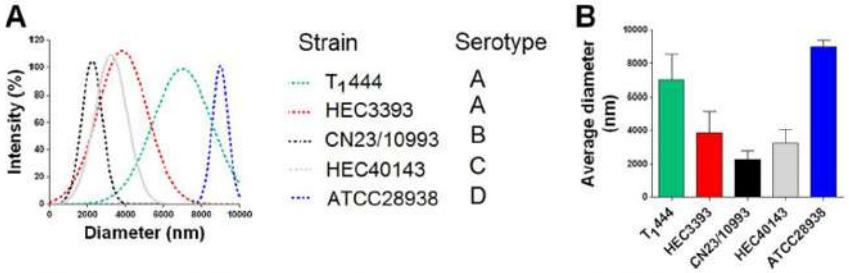

FIG. 6. Diameters of GXM fractions of different isolates of *C. neoformans* and *C. gattii*. (A) Distribution of effective diameters of GXM; (B) average diameters.

type assay used to analyze the relationship between the sizes of GXM samples and their abilities to stimulate cellular responses was NO production by macrophages, since it involves straightforward procedures and simple data interpretation.

Fractionation of the T_1444 supernatant revealed that the high-molecular-mass sample (>100 kDa) induced NO production by phagocytes more efficiently (P, <0.001) than the polysaccharide fraction in the 10- to 100-kDa mass range (Fig. 7A). The opposite pattern was observed for the *C. gattii* samples: all GXM fractions with lower molecular masses were significantly more effective at stimulating the production of NO than the high-molecular-weight polysaccharides (P, <0.0001 for all samples). Again, GXM fractions from strain CN23/10993 were the most effective samples at inducing NO production. The effective diameters of polysaccharides in these fractions were measured by dynamic light scattering, which confirmed that samples with higher molecular masses consisted of molecules of increased dimensions (Fig. 7B). Analysis of serotype B GXM samples (strains CN23/10993, R265, and ATCC 56990) in the 10- to 100-kDa molecular mass range revealed a direct correlation between their abilities to induce NO production and reduced effective diameters (Fig. 7C).

DISCUSSION

Recent studies indicate that the structure of GXM, and consequently its biological functions, differs according to parameters that include molecular mass and effective diameter (11, 12, 40). The functional diversity in cryptococcal polysaccharides is not exclusive to GXM. In fact, it has been reported recently that galactoxylomannan (GalXM) samples from *C. neoformans* are structurally and antigenically variable (8). Therefore, the task of establishing general functions for cryptococcal polysaccharides is complex, since very different characteristics of supposedly similar samples, which presumably reflect differences in polysaccharide structure, have been observed repeatedly in independent studies (8, 11, 40). GXM, for instance, has been classically defined as deleterious to the immune system (51), although it can also activate the host defense (46).

Fungal polysaccharides are potential candidates for activating TLR2-mediated cellular responses. The formation of lipid bodies (multifunctional organelles with critical roles in inflammation) induced by *Histoplasma capsulatum* β-glucan was inhibited in TLR2-deficient mice (47). Chitin, a cell wall structural polysaccharide, has been consistently characterized as a stimulator of TLR2-dependent production of interleukin 17 (IL-17) by macrophages, resulting in the induction of acute inflammation (7).

The ability of *C. neoformans* GXM to activate TLR-mediated innate responses was demonstrated in a number of pre-

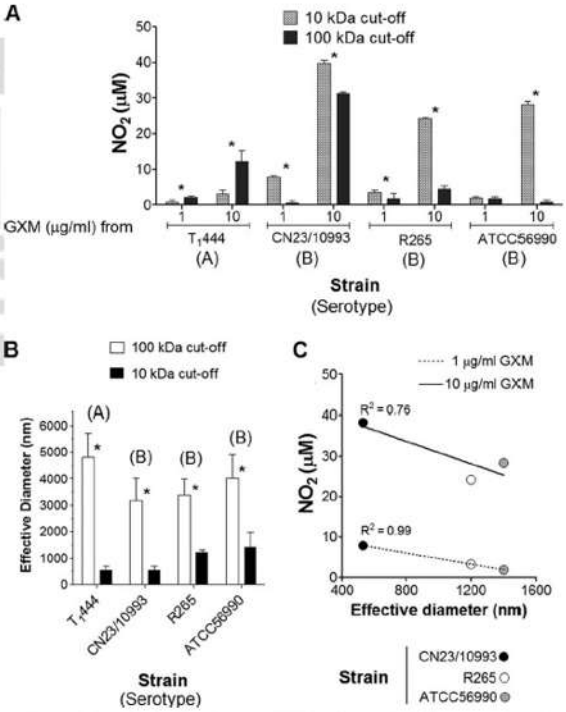

FIG. 7. NO induction by GXM fractions of different molecular masses and effective diameters. (A) Stimulation of macrophage-like cells with the GXM fractions results in differential production of NO. Asterisks indicate significant differences after stimulation of phagocytes with GXM fractions ($P < 0.0001$). (B) Determination of effective diameters of fractions obtained by sequential ultrafiltration through 100-kDa- and 10-kDa-cutoff filtration discs. Asterisks indicate that the differences in effective diameter are statistically significant ($P < 0.0001$). (C) Correlation analysis of effective diameters of serotype B GXM samples in the 10- to 100-kDa range and their abilities to induce NO.

vious studies (2, 27, 28, 36, 39, 46, 53), but comparable studies have not been carried out for *C. gattii* polysaccharides. TLRs and the CD14 receptor function as pattern recognition receptors for GXM (27, 28, 36, 44, 46, 53). The binding of GXM to TLR4 has been reported to result in the translocation of NF-κB to the nucleus in an incomplete process that does not induce the activation of mitogen-activated protein kinase pathways or the release of tumor necrosis factor alpha (TNF-α) (46). TLR4 was also implicated in the cellular uptake (34) and tissue distribution (52) of GXM. However, the roles of TLR2 and other TLRs in the immune response to GXM remain poorly understood. In the present study, we determined that the hydrodynamic size of GXM fractions is correlated with their ability to stimulate NO production by macrophages and to activate NF-κB in a TLR2-dependent manner. In fact, all GXM fractions stimulated the activation of NF-κB in HEK293A cells transiently transfected with TLR2/TLR6 or TLR2/TLR1 constructs. This response was always more intense in cells expressing the TLR2/1 construct than in those expressing the TLR2/6 construct. In all systems, the highest levels of NF-κB activation were obtained when transfected HEK293A cells were exposed to a serotype B GXM from a *C. gattii* strain.

To understand the structural characteristics responsible for NO and TLR activation, we evaluated several GXM parameters. The ability of GXM to induce NO production and the TLR-mediated response in transfected HEK293A cells was not an intraspecies property and did not depend on sugar composition, so other parameters were evaluated. Differences in antibody reactivity can imply differences in GXM structure (11), which also denote functional specificity (20). In this study, we found that the serological characteristics revealed by the binding of MAbs were similar in all strains, except for the *C. gattii* strain CN23/10993. Those cells showed strong reactivity with the protective IgM 12A1 but did not react with the clonally related nonprotective MAb 13F1. This observation is consistent with, and reflective of, the fact that MAbs 12A1 and 13F1 bind to different epitopes. In a recent study, it was suggested that antibody reactivity is influenced by the diameter of GXM (10), which led us to the inference that the effective diameters of the polysaccharide samples used in this study could also be related to the functionality of GXM.

Immunological studies with chitin have shown that large polysaccharide polymers are biologically inert, while their fragments are efficient regulators of TLR2-mediated innate immune responses (7, 25, 26). Human cryptococcosis caused by *C. gattii* is known to produce strong inflammatory responses in the lung, whereas the *C. neoformans* varieties often trigger little or no inflammation (49). Consequently, the result that serotype B GXM samples were more potent activators of cellular responses raises the tantalizing possibility that a correlation might exist between the activation of host cells and the type of granulomatous response made. In our model, the most effective GXM sample in activating cellular responses had the smallest effective diameter, a result that echoed previous findings with chitin (7, 25, 26). Using the model of NO production by macrophages after exposure to the serotype B GXM, we observed that polysaccharides with reduced dimensions induced a stronger cellular response, a property that was exclusive to serotype B GXM samples. NO production by macrophages is involved in both antimicrobial responses and the mediation of inflammation, illustrating the complex effects of GXM on host immune function. Given that our studies compared GXM preparations standardized by mass/volume and that smaller fibers have lower molecular masses, it is possible that the effects measured here reflect differences in the molarity of the GXM. Nevertheless, we urge caution in attributing these effects to simple differences in molarity, since interactions between polysaccharides and their receptors are likely to involve repeating structural motifs in polysaccharide molecules such that avidity considerations could be dominant. Furthermore, we note that immunological studies routinely measure effects using polysaccharide concentrations standardized by mass/volume, and consequently, this approach is experimentally relevant, especially for literature comparisons.

The cryptococcal capsule enlarges during infection, which is essential for virulence (54). A linear correlation between the effective diameter of GXM and the microscopic capsular diameter has recently been demonstrated (12), suggesting that the synthesis of large-diameter polysaccharides is essential for capsule enlargement. In our model, strain CN23/10993 produced GXM molecules with the lowest effective diameters and had the smallest capsular dimension (data not shown). The combination of the ability of GXM to modulate cellular responses and the capacity of *C. neoformans* to produce large GXM molecules and capsules may have a direct impact on fungal virulence. *C. neoformans* isolates producing large GXM molecules would be more efficient at producing capsules with increased dimensions, which are generally associated with pathogenic potential (54). On the other hand, isolates producing smaller GXM molecules, according to our current results, would manifest a potentially enhanced ability to activate some mechanisms of the immune response. Considering that the cryptococcal capsule also protects the fungus from a number of host antifungal mechanisms, a combination of smaller GXM molecules and the formation of a capsular network with reduced dimensions would favor the host defense by multiple mechanisms. We therefore suggest that the synthesis of capsular structures with reduced dimensions could have protean effects on the pathogenic capacities of cryptococcal strains, ranging from increased susceptibility to oxidative fluxes and phagocytosis to the production of molecules with an enhanced ability to activate host defenses. These observations suggest a mechanistic explanation for the consistent observation that strains with small capsules elicit more inflammation than those with large capsules (43). Furthermore, the higher NO-inducing activity associated with *C. gattii* polysaccharides, which correlates with smaller GXM diameters, suggests an explanation for the consistent observation of stronger granulomatous responses in cryptococcosis caused by this species (33, 43, 48).

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4- Resumo:

Propriedades estruturais e funcionais da GXM de *T. asahii*.

Os mecanismos de patogenicidade de *T. asahii* são praticamente desconhecidos, apesar de sua crescente relevância como agente causador de infecções fúngicas superficiais e invasivas em humanos. A GXM é um fator de virulência bastante investigado em espécies patogênicas do gênero *Cryptococcus*. Esse polissacarídeo é também produzido por *Trichosporon* spp. Ao contrário do observado para o polissacarídeo criptococócico, as propriedades da GXM de *T. asahii* são amplamente desconhecidas. Nesse estudo, nós analisamos aspectos estruturais e funcionais da GXM produzida por *T. asahii*, comparando suas propriedades com as do polissacarídeo criptococócico. Ambas GXMs compartilharam propriedades sorológicas, mas o polissacarídeo produzido por *T. asahii* apresentou diâmetro efetivo menor e maior carga negativa do que a GXM de *C. neoformans*. O ancoramento de GXM à parede celular foi rompido por dimetilsulfóxido e envolveu interações com oligômeros de quitina. Mutantes acapsulares de *C. neoformans* foram capazes de incorporar GXM de sobrenadantes de *T. asahii* o que conferiu a essas células maior resistência à fagocitose por macrófagos murinos. Nossos resultados demonstraram que apesar das similaridades sorológicas e de proteção contra a fagocitose, as GXMs produzidas por *T. asahii* e *C. neoformans* apresentaram diferenças estruturais que podem estar associadas a diferenças na arquitetura da superfície celular de ambos patógenos.

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Structural and functional properties of the *Trichosporon asahii* glucuronoxylomannan

Fernanda L. Fonseca [a], Susana Frases [b], Arturo Casadevall [b,c], Olga Fischman-Gompertz [d], Leonardo Nimrichter [a], Marcio L. Rodrigues [a,*]

[a] Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373, Cidade Universitária CCS, Bloco I, Rio de Janeiro – RJ 21941-902, Brazil
[b] Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Ave. Bronx, NY 10461, USA
[c] Division of Infectious Diseases of the Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Ave. Bronx, NY 10461, USA
[d] Disciplina de Biologia Celular, Universidade Federal de São Paulo, São Paulo, SP 04023-062, Brazil

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ABSTRACT

The virulence attributes of *Trichosporon asahii* are virtually unknown, despite its growing relevance as causative agent of superficial and invasive diseases in humans. Glucuronoxylomannan (GXM) is a well described virulence factor of pathogenic species in the *Cryptococcus* genus. GXM is also produced by species of the *Trichosporon* genus, and both polysaccharides share antigenic determinants, but unlike cryptococcal GXM, relatively little work has been done on trichosporal GXMs. In this study, we analyzed structural and functional aspects of GXM produced by *T. asahii* and compared them to the properties of the cryptococcal polysaccharide. Trichosporal and cryptococcal GXM shared antigenic reactivity, but the former polysaccharide had smaller effective diameter and negative charge. GXM anchoring to the cell wall was perturbed by dimethylsulfoxide and required interactions of chitin-derived oligomers with the polysaccharide. GXM from *T. asahii* supernatants are incorporated by acapsular mutants of *Cryptococcus neoformans*, which renders these cells more resistant to phagocytosis by mouse macrophages. In summary, our results establish that despite similarities in cell wall anchoring, antigenic and antiphagocytic properties, trichosporal and cryptococcal GXMs manifest major structural differences that may directly affect polysaccharide assembly at the fungal surface.

1. Introduction

Species belonging to the *Trichosporon* genus include basidiomycetes yeast found on human skin (Antachopoulos et al., 2007; Pfaller and Diekema, 2004). *Trichosporon* species have been reported to be the most common cause of non-candidal yeast-associated disease in patients with hematological malignancies, a condition that is associated with mortality rates in excess of 80% (Pfaller and Diekema, 2004). Up to 88% of deep-seated *Trichosporon* infections are caused by *Trichosporon asahii*, which is resistant to most of the clinically used antifungal drugs (Pfaller and Diekema, 2004). Clinical treatment failures with amphotericin B, fluconazole, and combinations of the two have been reported in human cases of trichosporonosis (reviewed in Pfaller and Diekema (2004)). Although *T. asahii* is an emerging pathogen resistant to most currently available antifungal therapies, its virulence factors and pathogenic mechanisms are largely unknown.

Glucuronoxylomannan (GXM) is a cell wall-associated and secreted polysaccharide produced by species of the genus *Trichosporon* (Ichikawa et al., 2001; Karashima et al., 2002) and *Cryptococcus* (reviewed in Bose et al. (2003)). In *T. asahii*, GXM building blocks consist of an α1,3-D-mannan-like hexasaccharide substituted with one β1,2-glucopyranosyluronic acid residue and six β-D-xylopyranosyl units (Fig. 1 and Ichikawa et al., 2001). Xylosylation includes *O*-2, *O*-4 and *O*-6 substitutions of the mannose residues.

In contrast to *Trichosporon* GXM, the structure and function of cryptococcal GXMs have been widely studied. In *Cryptococcus* spp., GXM is an extracellular/cell associated capsular polysaccharide that down modulates the immune response of infected individuals (Monari et al., 2006). Cryptococcal GXM is thought to have protean functions in virulence including protecting yeast cells against phagocytosis and oxidative burst (Kozel and Gotschlich, 1982; Zaragoza et al., 2008), impairing immune function through various mechanisms (Vecchiarelli, 2007) and promoting intracellular survival (Feldmesser et al., 2001). Cryptococcal GXM consists of a high-molecular mass polysaccharide (McFadden et al., 2006) that is synthesized in the Golgi apparatus and then packaged into vesicles destined to be released to the extracellular space (Panepinto

* Corresponding author. Fax: +55 21 25608344.
E-mail address: marciolr@pq.cnpq.br (M.L. Rodrigues).

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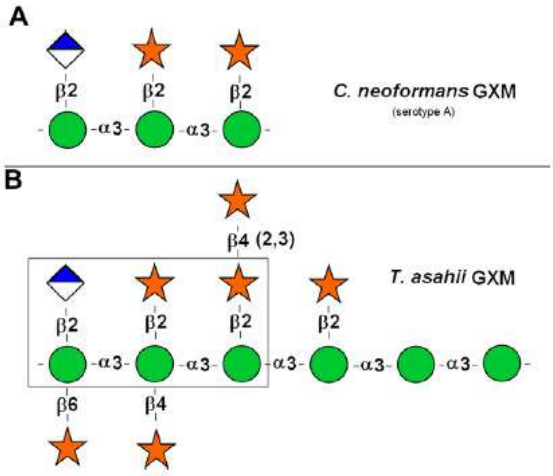

Fig. 1. Repeating motifs of *C. neoformans* (A) and *T. asahii* (B) GXMs. The boxed area in B is similar to the serotype A repeating motif of the cryptococcal GXM. Spheres represent mannosyl units; stars represent xylosyl units; diamond-shaped symbols represent glucuronyl units. Drawings of cryptococcal and *Trichosporon* GXMs were based on previous structural studies (Cherniak et al., 1998; Ichikawa et al., 2001). Structural differences include a higher variability in the type and positions of xylosyl substitutions for the trichosporal polysaccharide, which also shows a higher number of mannosyl units in its repeating motif.

et al., 2009; Rodrigues et al., 2007, 2008b; Yoneda and Doering, 2006). GXM is connected to cell wall through linkages to structural polysaccharides (Reese and Doering, 2003; Reese et al., 2007; Rodrigues et al., 2008a) and finally used for distal, cation-mediated capsular enlargement (Frases et al., 2009; Nimrichter et al., 2007; Zaragoza et al., 2006).

Although several structural aspects of *T. asahii* GXM have been described (Ichikawa et al., 2001), the functions of this polysaccharide for physiology and pathogenesis of *Trichosporon* spp. are virtually unknown. A comparative study revealed that *Cryptococcus* and *Trichosporon* isolates, which both express surface GXM, were less efficiently ingested by phagocytes than *Candida* strains (Lyman and Walsh, 1994). However, a direct role of GXM in protection of *Trichosporon* cells against phagocytosis was not demonstrated. A role for GXM in the pathogenicity of *T. asahii* was suggested by the observation that successive steps of inoculation and recovery of *T. asahii* from mice resulted in an increased release of the polysaccharide in culture supernatants (Karashima et al., 2002). Serological similarities of *Cryptococcus* and *Trichosporon* GXMs have also been reported. An antibody to cryptococcal capsular polysaccharides was demonstrated to cross-react with cell wall components of a *Trichosporon* isolate (Melcher et al., 1991). The mechanisms by which the *Trichosporon* GXM interacts with other cell wall components, however, are not known.

In this study, we analyzed several properties of GXM from *T. asahii*, including structural aspects, antibody reactivity, self-aggregation, surface expression, extracellular release, cell wall connections and antiphagocytic properties. Our results revealed both similarities and differences among cryptococcal and trichosporal GXMs. The potential impact of these findings on fungal pathogenesis and surface architecture is discussed.

2. Methods

2.1. Fungal strains

The *T. asahii* isolates used in this study included the standard strain CBS 2479 and the clinical isolate EPM21-05. The *Cryptococcus neoformans* isolates were the Brazilian clinical isolates HEC3393 and T_1444, which are serotype A strains expressing small and large capsules, respectively (Barbosa et al., 2006), the standard strain H99 (serotype A) and the acapsular mutant Cap67. Stock cultures were maintained in Sabouraud dextrose agar under mineral oil and kept at 4 °C. Yeast cells were grown in a chemically defined medium (pH 5.5) composed of glucose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM) and thiamine–HCl (3 μm) for 4 days (*T. asahii*), at 25 °C (*C. neoformans* and *T. asahii*) or 37 °C (*T. asahii*).

2.2. GXM purification

The basic protocol for polysaccharide purification was recently described by our group (Nimrichter et al., 2007). Fungal cells were cultivated with shaking for 4 days at room temperature in the minimal medium described above and separated from culture supernatants by centrifugation at 4000*g* (15 min, 4 °C). For both *C. neoformans* and *T. asahii*, cell densities were in the range of $2–8 \times 10^7$ cells/ml at day four of cultivation. The supernatant fluids were collected by centrifugation and again centrifuged at 15,000*g* (15 min, 4 °C), to remove smaller debris. The pellets were discarded and the resulting supernatant was concentrated approximately 20-fold using an Amicon (Millipore, Danvers, MA) ultrafiltration cell (cutoff = 100 kDa, total capacity of 200 ml) with stirring and Biomax polyethersulfone ultrafiltration discs (63.5 mm). Nitrogen (N_2) stream was used as the pressure gas. After supernatant concentration, the fluid phase was discarded, and the viscous layer was collected with a cell scraper and transferred to graduated plastic tubes for measurement of gel volumes. The procedure was repeated at least three times to ascertain average volumes.

2.3. Monosaccharide analysis

Carbohydrate composition analysis of the jellified GXM fractions from *T. asahii* and *C. neoformans* was performed by gas chromatography/mass spectrometry (GC/MS) analysis of the per-*O*-trimethylsilyl (TMS) derivatized monosaccharides from the polysaccharide films (Merkle and Poppe, 1994). Polysaccharide samples (0.3 mg) were methanolyzed in methanol/1 M HCl at 80 °C (18–22 h) for further per-*O*-trimethylsilylation with Tri-Sil (Pierce) at 80 °C (0.5 h). GC/MS analysis of the volatile per-*O*-TMS derivatives was performed on an HP 5890 gas chromatograph interfaced to a 5970 MSD mass spectrometer, using a Supelco DB-1 fused silica capillary column (30 m × 0.25 mm ID). Arabinose, rhamnose, fucose, xylose, glucuronic acid, galacturonic acid, mannose, galactose, glucose, mannitol, dulcitol and sorbitol were used as monosaccharide standards.

2.4. Zeta potential determinations in extracellular GXM fractions

Zeta potential (ξ), particle mobility and shift frequency of polysaccharide samples were calculated in a Zeta potential analyzer (ZetaPlus, Brookhaven Instruments Corp., Holtsville, NY). Polysaccharide samples were dissolved in water to generate 1 mg/ml solutions. Values of ξ were calculated using the equation $\xi = (4\pi\eta m)/D$, where D is the dielectric constant of the medium, η is the viscosity and m is the electrophoretic mobility of the particle.

2.5. GXM effective diameter

Effective diameter and size distribution of GXM preparations were measured by quasi-elastic light scattering in a 90Plus/BI-MAS Multi Angle Particle Sizing analyzer (Brookhaven Instruments Corp., Holtsville, NY), according with the method described by our group (Frases et al., 2009). GXM solutions were all used at 1 mg/ml. The autocorrelation function $C(t)$, where $C(t) = Ae^{2\Gamma t} + B$, was

used to process the fluctuating signal, originating from the random motion of particles in a liquid phase and the associated alterations in the intensity of the scattered light over time. In this equation, t is the time delay, A is an optical constant determined by the instrument design and Γ is related to the relaxation of the fluctuations by $\Gamma = Dq^2$. The value of q is calculated from the scattering angle θ, the wavelength of the laser light λ_0 and the index of refraction n of the suspended liquid, according to the equation $q = (2\pi n/\lambda_0)\,2\,\mathrm{Sin}(\theta/2)$. Particle size is related to the translational diffusion coefficient (D) for shapes including spheres, ellipsoids, cylinders and random coils. Assuming the spherical form as the most useful in the greatest number of cases, the equation $D = (K_B T)/(3\pi\eta(t)d)$, where K_B is Boltzmann's constant (1.38054×10^{-16} ergs/deg), T is the temperature in K (30 °C), $\eta(t)$ is the viscosity of the liquid in which the particles are moving and d is the particle diameter, was used. All GXM samples were analyzed under the same conditions. Multimodal size distribution analysis of polysaccharides was calculated from the values of intensity weighted sizes obtained from the non-negatively constrained least squared (NNLS) algorithm.

2.6. Enzyme-linked immunosorbent assays (ELISA) using fungal polysaccharides

The reactivity of *T. asahii* GXM with a monoclonal antibody (mAb) to the cryptococcal polysaccharide was determined by ELISA, using modifications of a previously described protocol for GXM detection (Casadevall et al., 1992). 96-Well polystyrene plates were coated with 5 μg/ml solutions of *T. asahii* (isolate CBS2479) GXM and incubated for 1 h at 37 °C. Alternatively, the plates were coated with a solution of *Saccharomyces cerevisiae* mannan (negative control) at the same concentration. Polysaccharide concentration was ascertained using the method described by Dubois (Dubois et al., 1951). After removal of unbound polysaccharide, the plates were blocked with 1% bovine serum albumin, followed by addition of a solution of mAb 18B7, an IgG1 with affinity for GXM of different serotypes (Casadevall et al., 1998). After incubation for 1 h at 37 °C, the plates were washed five times with tris-buffered saline (TBS) supplemented with 0.1% Tween 20, followed by incubation with an alkaline phosphatase-conjugated goat anti-mouse IgG1 for 1 h. Reactions were developed after the addition of *p*-nitrophenyl phosphate disodium hexahydrate, followed by measuring absorbance at 405 nm with a microplate reader (TP-reader, Thermo Plate). Antibody concentration in this assay corresponded to 1 μg/ml.

2.7. GXM release after treatment of T. asahii with chitinase

After extensive washing with PBS, yeast cells (10^6) were suspended in 100 μl of 0.01 M phosphate buffer (pH 6.0) containing chitinase (100 μg/ml, Sigma, purified from *Streptomyces griseus*), followed by incubation at 37 °C for 12 h. The cell suspensions were incubated overnight at 37 °C and centrifuged at 4000 rpm for cell removal. Controls included cells treated in buffer containing no enzyme. The presence of GXM in supernatants was determined by capture ELISA, as described previously (Casadevall et al., 1992). Control or chitinase-treated cells were analyzed by fluorescence microscopy as described further in this section.

2.8. DMSO extraction

The protocol used for GXM extraction from *T. asahii* was based on studies developed with *C. neoformans* (Bryan et al., 2005). Yeast cells (1.5×10^9) were suspended in 15 ml DMSO. The cells were then incubated for 30 min at room temperature with shaking and the supernatants were collected. Pellets were resuspended in 15 ml DMSO and again incubated for 30 min. The cells were extensively washed in PBS and prepared for immunofluorescence with mAb 18B7 as described below.

2.9. Fluorescence microscopy

The different systems tested in this assay included control cells of *C. neoformans* or *T. asahii*, as well as fungi treated with DMSO or chitinase. Yeast cells (10^6) were suspended in 4% paraformaldehyde cacodylate buffer (0.1 M, pH 7.2) and incubated for 30 min at room temperature. Fixed yeast cells were washed twice in PBS and incubated in 1% bovine serum albumin in PBS (PBS–BSA) for 1 h. For GXM staining, blocked cells were incubated with mAb 18B7 (1 μg/ml) for 1 h at room temperature, followed by a fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG (Fc specific) antibody (Sigma). For staining with fluorescent wheat germ agglutinin (WGA), yeast cells were suspended in 100 μl of a 5 μg/ml solution of the Alexa Fluor 594 conjugate of the lectin (Molecular Probes) and incubated for 30 min at 37 °C. After incubation with the fluorescent probes, the cells were washed with PBS and observed under fluorescence microscopy. Alternatively, WGA-stained cells were sequentially incubated with mAb 18B7 and secondary antibodies as described above. To eliminate the possibility that the fluorescence pattern was derived from a specific sequential use of reagents, the order of the reagents was changed and the results were the same (data not shown). Images were obtained after placing yeast cell suspensions in mounting medium (50% glycerol and 50 mM *N*-propyl gallate in PBS) over glass slides followed by observation under an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired using a Color View SX digital camera and processed with the software system analySIS (Soft Image System). Images were finally processed using ImageJ software (provided by NIH, http://rsb.info.nih.gov/ij/).

2.10. Flow cytometry analysis

Yeast cells of *T. asahii* or *C. neoformans* were washed in PBS and fixed in 4% paraformaldehyde cacodylate buffer 0.1 M, pH 7.2 for 1 h at room temperature. Fixed yeast cells were washed again in PBS and incubated in 1% bovine serum albumin in PBS (PBS–BSA) for 1 h at room temperature. The cells were then washed twice in PBS and sequentially incubated with mAb 18B7 (1 μg/ml) and fluorescein isothiocyanate (FITC) labeled anti-mouse IgG (1 μg/ml) for 1 h, at room temperature. Yeasts were again washed and 5000 cells were analyzed in a FACS Calibur (BD Biosciences, San Jose, CA) flow cytometer. Data were processed with CellQuest (BD Biosciences) or WinMDI (Salk Flow Cytometry) software. Control cells, in which mAb 18B7 was replaced by isotype-matched irrelevant antibodies, were analyzed first.

2.11. GXM binding by acapsular cells

Acapsular *C. neoformans* cells (strain Cap67, 10^6 cells) were suspended in 100 μl of culture supernatants (4 day cultures) from *C. neoformans* (H99 strain) or *T. asahii* (isolates CBS 2479 or EPM21-05). GXM in supernatants was normalized to 10 μg/ml. The suspension was incubated for 12 h at 25 °C and extensively washed with PBS. Control systems consisted of Cap67 cells incubated with sterile medium. For immunofluorescence with mAb 18B7, the cells were fixed with 4% paraformaldehyde and prepared as described above. For phagocytosis assays, yeast suspensions were prepared and incubated with macrophages, as described below.

2.12. Phagocytosis

The murine macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD) was grown to confluence in

25 cm^2 culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), at 37 °C in a 5% CO_2 atmosphere. For interaction with yeast cells, the macrophages were cultivated over sterile glass slides placed onto the wells of a 24-well plate, in the same conditions described above. Yeast suspensions were prepared in DMEM, to generate a ratio of 10 yeasts per macrophage-like cell. Interactions between fungal and host cells occurred at 37 °C at a 5% CO_2 atmosphere for 4 h. After removal of non-adherent fungi by washing, the cells were fixed with Bouin's solution and stained with Giemsa. In each system, 200 macrophages were counted and the index of association between *C. neoformans* and host cells was considered as the total number of yeasts per 100 phagocytes. All experiments were performed in triplicate sets and statistically analyzed by using Student's *t*-test.

3. Results

3.1. Isolation of Trichosporon GXM by ultrafiltration

GXM isolation by ultrafiltration is an efficient method for purification of the polysaccharide from *C. neoformans* (Nimrichter et al., 2007). Since *T. asahii* also releases GXM (Karashima et al., 2002), we evaluated whether the protocol previously used for isolation of GXM in *C. neoformans* supernatants was suitable for the recovery of trichosporal GXM. Gel formation with cryptococcal supernatants (strain $T_1$444) was observed when 400 ml fungal cultures containing at least 3×10^{10} cells were concentrated 20-fold in the ultrafiltration cell. Under the same conditions, gel formation was not observed after filtration of supernatants from *T. asahii* (data not shown). We therefore gradually increased culture volumes and kept constant the final volume of concentrated supernatants, generating, therefore, progressively more concentrated supernatants. Gel formation deriving from trichosporal supernatants were only observed after the culture fluids at a similar cell density were 300-fold concentrated. Carbohydrate determinations according to the method of Dubois (Dubois et al., 1951) revealed that GXM concentration in cryptococcal polysaccharide gels was approximately 13-fold higher than in the *Trichosporon* fractions. Therefore, we concluded that formation of GXM films is more efficient in *C. neoformans* than in *T. asahii*. This analysis was repeated at least three times showing similar profiles of GXM aggregation. A representative experiment is shown in Table 1.

3.2. Structural and serologic properties of GXM

The components of GXM, mannose, xylose and glucuronic acid, were detected in *T. asahii* ultrafiltration fractions by GC–MS (Fig. 2A). Glucose and galactose, which were already described as GXM contaminants in fractions purified by ultrafiltration (Frases et al., 2008), were also detected in the *T. asahii* preparations. The

Table 1
Formation of GXM films after ultrafiltration of *T. asahii* and *C. neoformans* culture supernatants.

Pathogen	Culture volume[a] (ml)	Ratio of supernatant concentration[a]	Cell density[a] (cells/ml)	Gel volume[b] (ml)	Carbohydrate concentration in GXM films[b] (mg/ml)
T. asahii	6000	300-fold	2×10^7	0.5	0.9
C. neoformans	400	20-fold	7.5×10^7	1.0	11.9

[a] Minimum values required for formation of GXM films.
[b] Values obtained after supernatant concentration and formation of GXM films.

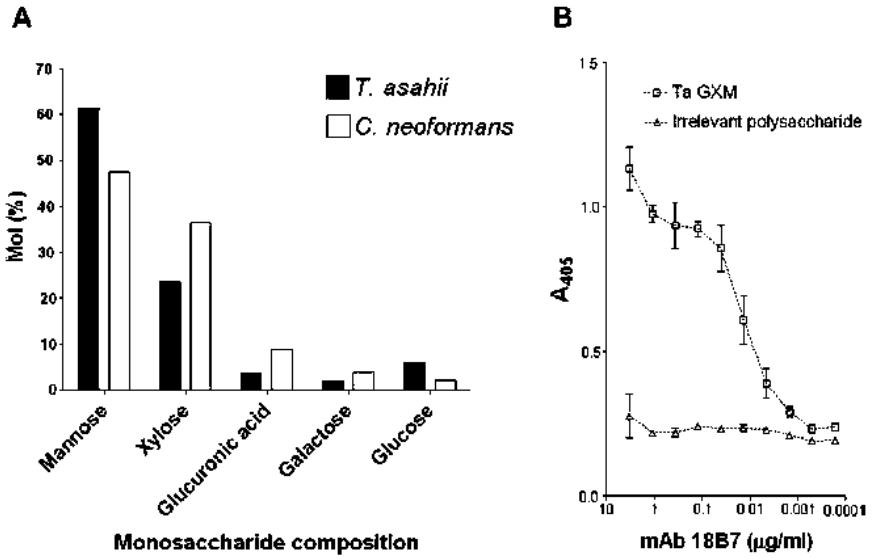

Fig. 2. Monosaccharide analysis and serologic properties of GXM fractions. (A) Monosaccharide composition of GXM fractions from *T. asahii* and *C. neoformans*, as determined by GC–MS. (B) Reactivity of the *T. asahii* GXM with a monoclonal antibody (mAb 18B7) raised against the cryptococcal polysaccharide. GXM is recognized by the antibody in a dose-dependent pattern. No significant reactions were observed when ELISA plates were coated with *S. cerevisiae* mannan (irrelevant polysaccharide). In all assays, ELISA plates were coated with 5 µg/ml solutions of fungal polysaccharides.

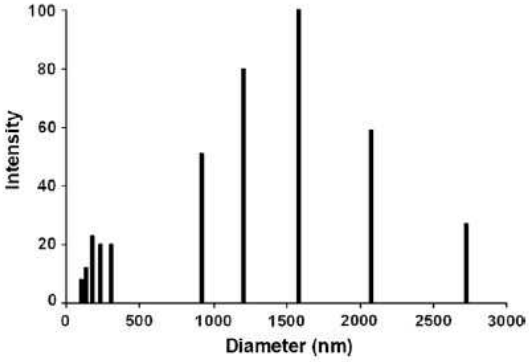

Fig. 3. Diameter distribution of GXM fibers isolated from *T. asahii*. An average value of 814.6 ± 35.3 nm was obtained.

C. neoformans (strain T_1444) ultrafiltration fraction was analyzed under the same conditions, revealing a similar compositional profile. Therefore, based on the currently presented data and the previous literature (Frases et al., 2008; Nimrichter et al., 2007), the *T. asahii* fraction obtained by ultrafiltration is likely to correspond to GXM, since it is a partially purified polysaccharide preparation containing the GXM building units. It is noteworthy to mention that the content of glucuronic acid in the samples described above is possibly underestimated, since this acidic sugar usually gives, after methanolysis and TMS-derivatization, minor peaks on the chromatogram that may be superposed on the peaks of galactose and glucose (Fenselau and Johnson, 1980), affecting its quantitative analysis.

Antibodies to cryptococcal GXM were previously demonstrated to cross-react with *Trichosporon* polysaccharides (Melcher et al., 1991). In addition, mAb 18B7 reacts with different GXM structures (Casadevall et al., 1998). The mAb to the cryptococcal polysaccharide, indeed, reacted with the *T. asahii* GXM (Fig. 2B). This result is consistent with the fact that *T. asahii* and *C. neoformans* GXMs share common domains (Fig. 1).

The size of extracellular GXM fibers from *T. asahii* was analyzed by dynamic light scattering. GXM fibers were in the range of 100–2700 nm (Fig. 3), with an average effective diameter corresponding to 814.6 ± 35.3 nm. Similar analyses were performed with ultrafiltration fractions from strains T_1444 and HEC3393 of *C. neoformans*, which had effective diameter values corresponding to 2635 ± 95.6 and 1342.7 ± 29.4, respectively. Median values corresponded to 770 nm for *T. asahii*, 2557.85 nm for the cryptococcal strain T_1444, and 1333.5 nm for the HEC3393 isolate of *C. neoformans*. Therefore, the GXM fibers secreted by *T. asahii* are smaller than those produced by *C. neoformans* cells. The analysis of the electronegativity of different GXM fractions revealed Zeta potential values of −29.17 ± 0.76 mV for *T. asahii* and −38.15 ± 0.41 mV for *C. neoformans* (strain T_1444). The higher Zeta potential value obtained for the *C. neoformans* GXM is consistent with its increased content of glucuronic acid, in comparison with the *Trichosporon* polysaccharide (Fig. 2A).

The expression of surface GXM in *T. asahii* and *C. neoformans* was also compared by fluorescence-based methods (Fig. 4). Controls consisting of acapsular *C. neoformans* cells (strain cap67) were prepared for immunofluorescence following the same steps used for the analysis of *T. asahii* and encapsulated *C. neoformans* cells. In microscopic analysis, the fluorescence levels for the controls were similar to those observed when the step of incubation with the antibody to GXM was omitted during preparation of *T. asahii*

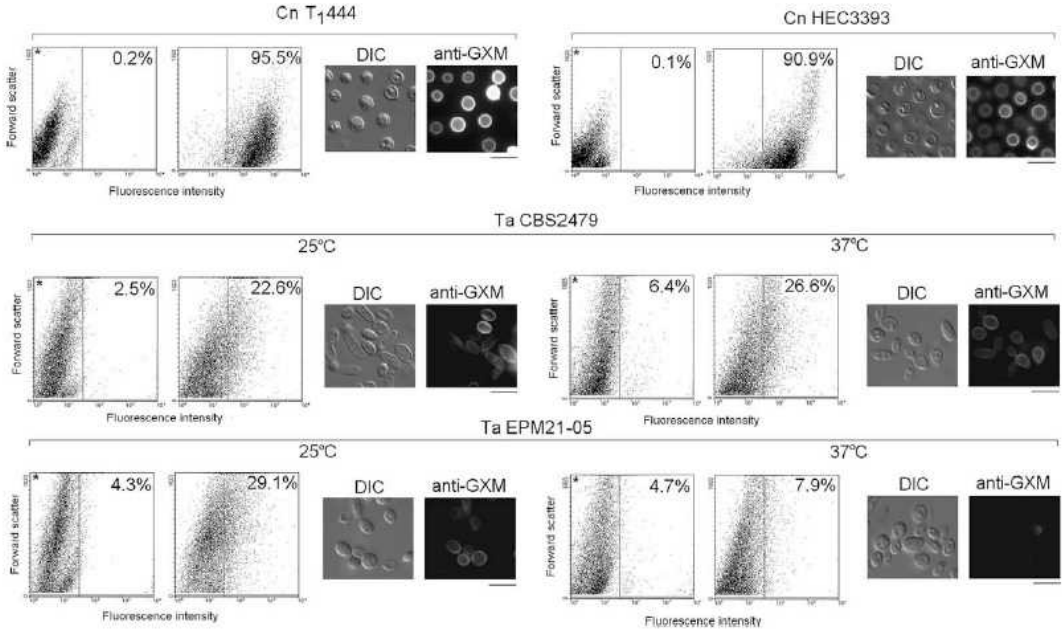

Fig. 4. Surface expression of GXM in *T. asahii* (Ta) and *C. neoformans* (Cn). Flow cytometry and fluorescence microscopy of fungal cells indicated that mAb 18B7 reacts with most of the *C. neoformans* cells, while the *T. asahii* population is only partially recognized by the antibody. The intensity of the fluorescent reactions with cryptococci is also higher. Polysaccharide expression by the Ta isolate EPM21-05 is apparently down modulated at 37 °C. In flow cytometry dot plots, asterisks denote control systems, in which fungal cells were not incubated with the anti-GXM antibody. The percentage of fluorescent cells is shown for each system. In fluorescence microscopy panels, fungal cells observed under differential interferential contrast (DIC) and fluorescence mode (anti-GXM) are shown. Scale bars, 5 μm.

and encapsulated *C. neoformans* cells (data not shown). In flow cytometry assays, the maximum percentage of fluorescent acapsular cells corresponded to 3% (not shown).

Microscopic observations of *T. asahii* revealed cell populations consisting mostly of round and ovoid yeast cells in the range of 3–7 μm (Figs. 4 and 5). The presence of differentiating forms resembling mycelial cells was observed occasionally. In fluorescence microscopy, images in all systems were acquired under the same conditions. Therefore, the results suggested that antibody reactivity with the *C. neoformans* surface was more intense than that observed in any other system. Interestingly, in one of the *T. asahii* isolates, fluorescent antibody–polysaccharide reactions were less intensive when the fungus was cultivated at 37 °C than when the cells were grown at 25 °C. The visual differences observed under fluorescence microscopy led us to quantify the reactivity of mAb 18B7 with fungal cells by flow cytometry.

As expected, the two different *C. neoformans* isolates (strains $T_1$444 and HEC3393) were efficiently recognized by the antibody (Fig. 4). In both strains, more than 90% of the population reacted with mAb 18B7. In contrast, the maximum percentage of fluorescent-positive cells in *T. asahii* was in the range of 29% (strain EPM21-05). As suggested by fluorescence microscopy, GXM expression in the EPM21-05 isolate was down modulated at 37 °C. In addition to a higher percentage of fluorescent cells, the intensity of the fluorescent reactions with cryptococcal cells was much higher than those with the *T. asahii* isolates.

3.3. GXM anchoring to the cell wall of T. asahii

In *C. neoformans*, oligosaccharides composed of *N*-acetylglucosamine (chitooligomers) are involved in GXM anchoring to the cell wall (Rodrigues et al., 2008a). To analyze GXM-cell wall connections in *T. asahii*, we evaluated the reactivity of fungal cells with the lectin WGA, which recognizes *N*-acetylglucosamine-containing structures that connect the cell wall to the capsule of *C. neoformans* (Rodrigues et al., 2008a). The profile of WGA staining in *T. asahii* (Fig. 5A) was very similar to that observed previously for *C. neoformans* (Rodrigues et al., 2008a), which includes a strong reactivity with bud-associated structures. Treatment of *T. asahii* with chitinase resulted in the release of surface GXM, as demonstrated by ELISA of extracellular fractions (Fig. 5B) and immunofluorescence of fungal cells (Fig. 5C). In control cells, WGA binding was concentrated at budding sites. Chitinase treatment, however, modified the punctate profile of lectin binding to the cell surface to a diffuse pattern, resulting in indiscriminate staining of the cell wall. Similar observations were described for *C. neoformans* (Rodrigues et al.,

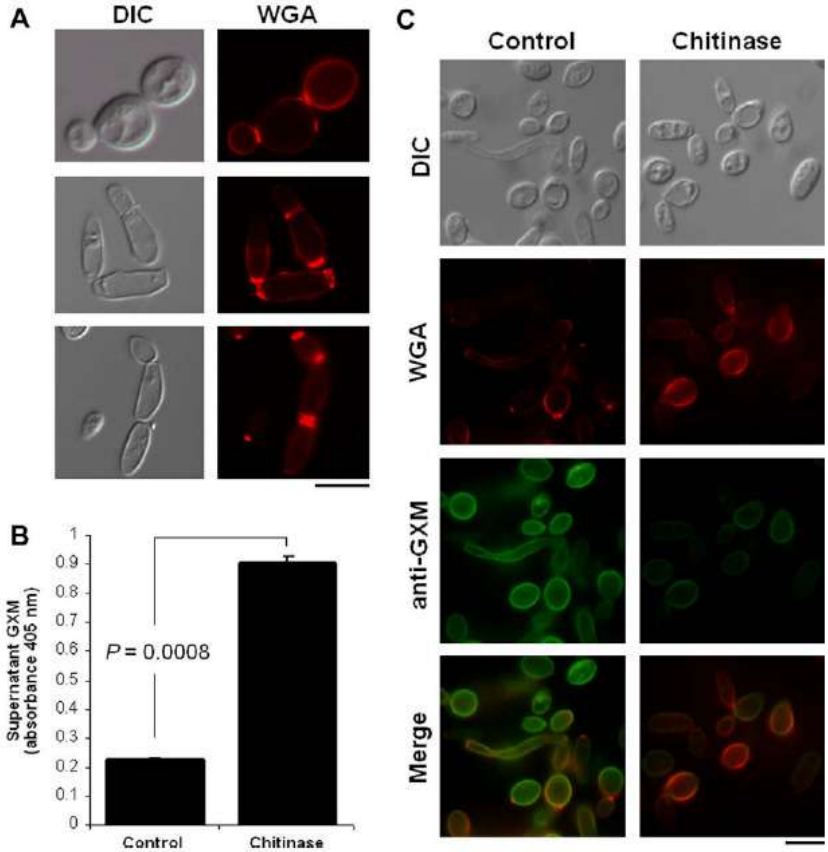

Fig. 5. GXM anchoring to the cell wall of *T. asahii* (isolate CBS2479) involves chitin oligomers. (A) Incubation of *T. asahii* cells with the lectin WGA reveals that external chitin-like structures are concentrated in cell division sites. Similar results of three different experiments prepared under the same conditions are shown. (B) ELISA of supernatants of *T. asahii* after incubation in PBS (control) or in the same buffer supplemented with chitinase reveals that GXM is released after exposure to the enzyme. (C) Incubation in the presence of the enzyme also changes the pattern of WGA binding to *T. asahii*. Analysis of the fungal cells after the conditions described in B confirms the reduction in the content of surface GXM after chitinase treatment. Fungal cells observed under differential interferential contrast (DIC) and fluorescence mode (anti-GXM and WGA) are shown. Scale bars, 3 μm.

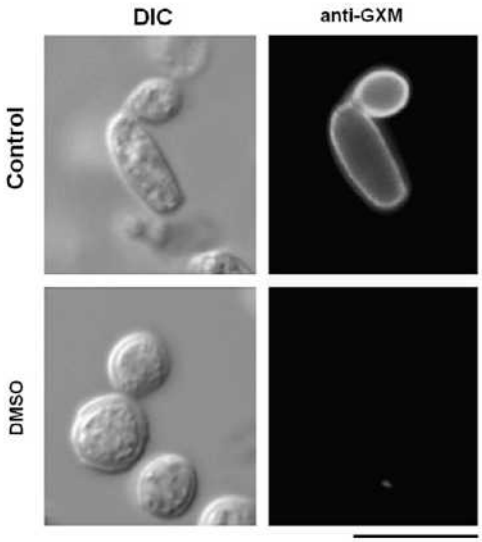

Fig. 6. Surface GXM is removed from the surface of *T. asahii* (isolate CBS2479) after treatment with DMSO. Fungal cells observed under differential interferential contrast (DIC) and fluorescence mode (anti-GXM) are shown. Scale bar, 3 μm.

2008a). Differentiating *T. asahii* cells were also recognized by the antibody to GXM and by WGA, suggesting that polysaccharide anchoring in mycelial forms follows the same pattern observed for yeast cells. GXM anchoring to the cell wall was demonstrated to be sensitive to DMSO treatment in *C. neoformans* (Bryan et al., 2005). In *T. asahii*, DMSO-treated cells were not recognized by the anti-GXM antibody (Fig. 6). We interpreted this result as a consequence of the release of surface GXM rather than loss of reactivity with the antibody, since supernatant fractions of DMSO treatments were still recognized by mAb 18B7 in ELISA after extensive dialysis (data not shown).

C. neoformans acapsular mutants that have defective GXM secretion are able to incorporate exogenously added GXM into the cell surface (Reese and Doering, 2003). The similarities in cell wall anchoring shared by cryptococcal and *Trichosporon* GXMs led us to evaluate the whether acapsular mutant of *C. neoformans* would incorporate heterologous GXM. The *C. neoformans* acapsular mutant became reactive with mAb 18B7 after incubation in the presence of different *T. asahii* culture supernatants (Fig. 7). The fact that the *C. neoformans* mutant became coated with *T. asahii* GXM allowed us to design an experimental system to evaluate whether the *Trichosporon* polysaccharide is antiphagocytic, as previously hypothesized in the literature (Lyman and Walsh, 1994).

3.4. The T. asahii GXM has antiphagocytic properties

After incubation with supernatants from an encapsulated strain of *C. neoformans* (strain H99), culture fluids of two *T. asahii* isolates or sterile medium, the cryptococcal acapsular mutant Cap67 was incubated with mouse macrophages for phagocytosis determination (Fig. 8). Yeast cells containing no detectable surface GXM were readily phagocytized by the macrophages. GXM-coated cells,

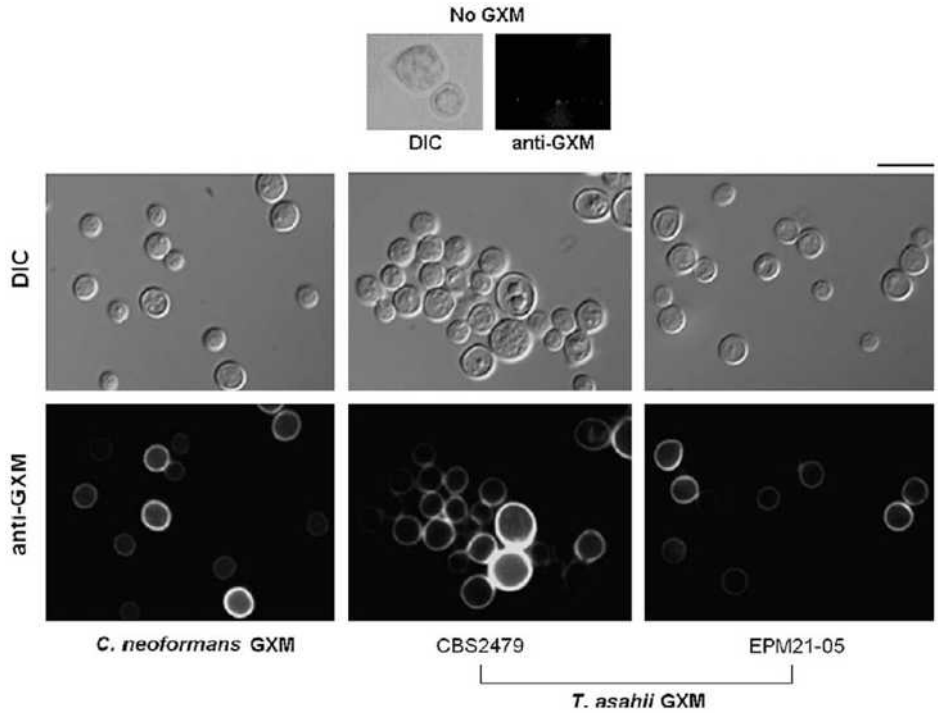

Fig. 7. Acapsular cells of *C. neoformans* incorporate GXM from culture supernatants of two *T. asahii* isolates. The culture supernatant of *C. neoformans* (strain $T_1$444) was used as a positive control. Upper panels (No GXM) show immunofluorescence results after incubation of the acapsular mutant in sterile culture medium. Fungal cells observed under differential interferential contrast (DIC) and fluorescence mode (anti-GXM) are shown. Scale bar, 3 μm. Images were acquired under identical conditions and processed similarly.

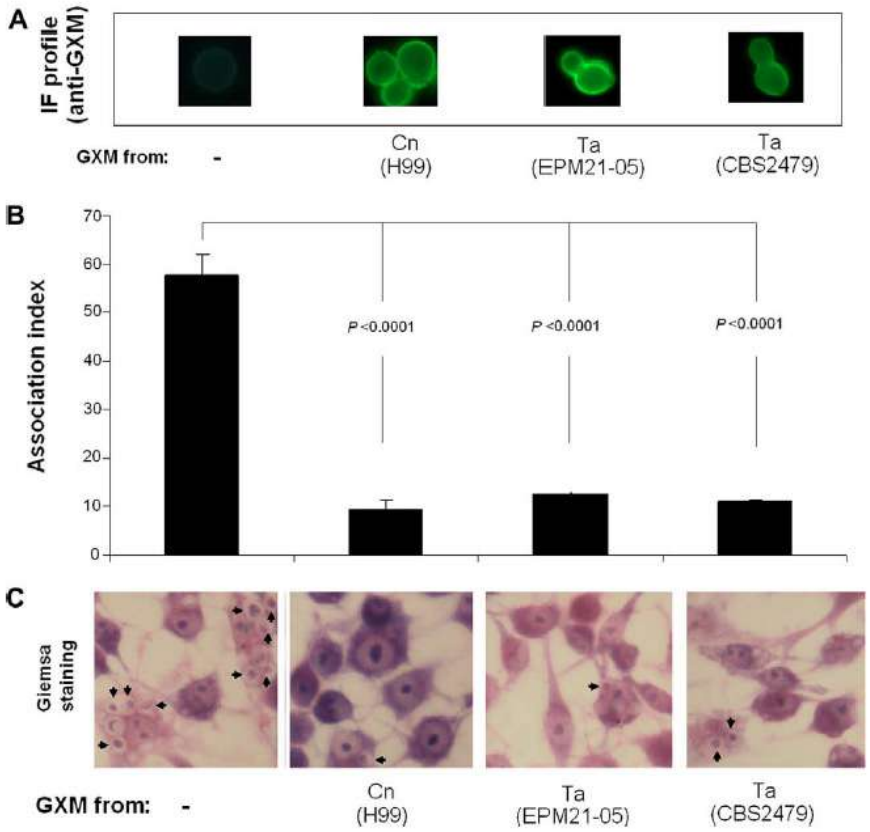

Fig. 8. GXM from *T. asahii* supernatants turns acapsular mutants of *C. neoformans* more resistant to phagocytosis by mouse macrophages. (A) Cap67 cells were incubated in sterile medium (control, no GXM) or in the presence of culture supernatants of *C. neoformans* (Cn) or *T. asahii* (Ta). After polysaccharide incorporation, yeast cells were incubated with macrophages for determination of phagocytosis. (B) The number of phagocytes containing intracellular *C. neoformans* was significantly higher when yeast cells were not incubated in the presence of GXM. (C) Microscopy of Giemsa-stained, infected macrophages confirms that GXM-coated cells are more resistant to phagocytosis. Arrows indicate intracellular Cap67 cells.

however, were much more resistant to phagocytosis. The phagocytic index for polysaccharide-coated yeast cells were around six-fold smaller than for cells containing no GXM. Acapsular mutants coated with GXM from *C. neoformans* or *T. asahii* were similarly resistant to phagocytosis.

4. Discussion

Structural and serological analyses have shown similarities and differences between the polysaccharides from *Cryptococcus* and *Trichosporon* (Ichikawa et al., 2001; Melcher et al., 1991). Depending on the species and related serotype, the building motifs of the cryptococcal GXM vary in complexity (Cherniak et al., 1998). The repeating motif of the *Trichosporon* GXM appears to be even more complex (Ichikawa et al., 2001). A comparison with the *Cryptococcus* motifs reveals that the *Trichosporon* polysaccharide manifested more variability in its structural features. These differences include a higher degree of xylosylation that forms disaccharyl branches, which are absent in *Cryptococcus* and different linkages connecting xylosyl units to the mannose backbone (Ichikawa et al., 2001). However, polysaccharides from both genera also share similar motifs, as the one highlighted in Fig. 1. Structural analysis of GXM fractions obtained from *T. asahii* and *C. neoformans* by ultrafiltration also revealed similarities in monosaccharide composition. The relative content of glucuronic acid in the *T. asahii* GXM, however, was approximately twofold smaller than in the cryptococcal polysaccharide, which probably accounts for its reduced value of Zeta potential. The *Trichosporon* GXM also showed a smaller effective diameter.

In *C. neoformans*, the ability of GXM to self-aggregate was determined based on the efficacy of formation of polysaccharide-containing gels after concentration of culture supernatants (Nimrichter et al., 2007). GXM self-aggregation, which requires the negative charges of glucuronic acid units (Nimrichter et al., 2007), was demonstrated to influence capsule assembly and enlargement in this species (Frases et al., 2008; Nimrichter et al., 2007). In *T. asahii*, the reduced negative charge is presumably an important parameter contributing to the lower efficacy of the trichosporal GXM to form aggregated gels during ultrafiltration of culture supernatants. In addition to influencing polysaccharide assembly, alterations in cell charge attributable to GXM may affect virulence, as suggested by Nosanchuk and Casadevall, (1997). Capsular enlargement in *C. neoformans* also depends on the size of polysaccharide fibers, given the linear correlation between GXM effective diameter and microscopic capsular size (Frases et al., 2009). Therefore, the reduced negative charge of the *Trichosporon* polysaccharide and its reduced effective diameter may be related with the observation that, although both genera can synthesize

GXM, only the polysaccharide components from *Cryptococcus* spp. form prominent India-ink visible capsules.

The structural similarities observed for the GXMs from *Cryptococcus* and *Trichosporon* may suggest similar functional aspects, including antigenicity, cell anchoring and influence on the interaction of the pathogens with host cells. In fact, a previous study demonstrated that an antibody raised against the cryptococcal polysaccharide reacts with the cell wall of *T. asahii* (Melcher et al., 1991). Our results confirmed that antibodies to cryptococcal GXM cross reacted with the *T. asahii* polysaccharide, which may be explained by the fact that both polysaccharides share common structural domains. Despite their similarities it is noteworthy that these polysaccharides appear to have very different structural functions in *Trichosporon* and *Cryptococcus* spp. The former genus does not have visible capsules and GXM appears to be a cell wall-associated polysaccharide that is also shed into culture media.

The *T. asahii* polysaccharide was detected in both cell-associated and extracellular fractions. Growth at 37 °C suggested that one of the *T. asahii* isolates down modulates GXM expression, raising questions as to whether the polysaccharide is relevant for *Trichosporon* during human or animal infections. Considering that GXM clearly works in favor of the fungus during infection of mammalian hosts by cryptococci (Monari et al., 2006), the variable ability of *T. asahii* to produce GXM under different conditions, including the host's temperature, may be related with its lower incidence as a human pathogen, in comparison with *C. neoformans*. This observation, however, contrasts with a previous study showing that production of extracellular GXM is increased after serial passage of *T. asahii* through mice (Karashima et al., 2002). Therefore, the possibility that the efficacy in GXM production is variable in different *Trichosporon* isolates cannot be ruled out. In addition, the lower detection of GXM by antibody-based tests in one of the *T. asahii* isolates could be a consequence of structural modifications that would result in altered serological properties, which could, in fact, represent a pathogenic strategy to escape the host defense.

GXM in *Trichosporon* species has been mainly characterized as a cell wall polymer (Ichikawa et al., 2001; Melcher et al., 1991). In this context, the similarities of cryptococcal and *Trichosporon* polysaccharides led us to evaluate if the connection of GXM with cell wall components would be similar in both genera. In *C. neoformans* and *C. gattii*, GXM anchoring to the cell wall was demonstrated to involve chitin-derived structures (Rodrigues et al., 2008a). These structures, which correspond to β1,4-*N*-acetylglucosamine oligomers that are recognized by the lectin WGA, were demonstrated to form molecular 'bridges' connecting the cell wall with the capsule in non-dividing and, more frequently, budding cells. Chitin and chitin-related molecules accumulate in sites of yeast budding (Bulawa, 1993; Cabib et al., 2008). In this study, a relationship between yeast budding and WGA binding to capsule-associated structures was also suggested. Our current results suggested a similar profile of binding of the lectin to budding regions of *T. asahii*, which led us to evaluate whether cell wall chitin-derived oligomers would be involved in GXM anchoring in this species. In fact, chitinase treatment resulted in the release of wall-associated GXM to the extracellular space accompanied by a modified pattern of binding of mAb 18B7 to the fungal surface. Interestingly, chitinase treatment changed the localized profile of lectin binding to a diffuse profile. As suggested before in *C. neoformans* (Rodrigues et al., 2008a), this effect is probably a result of the generation of cell wall chitooligosaccharides after partial enzymatic hydrolysis of chitin. Considering that WGA interacts with β1,4-*N*-acetylglucosamine oligomers, but not with chitin in its polymeric form (Rodrigues et al., 2008a), chitinase treatment would be expected to generate an increased number of cellular sites for lectin binding. Additional evidence that GXM anchoring to the cell wall is similar in the two species used in this study came from the fact that, as previously described for *C. neoformans* (Bryan et al., 2005), DMSO treatment extracted GXM from the cell surface of *T. asahii*. The ability of both *C. neoformans* and *T. asahii* to anchor GXM by similar molecular mechanisms suggests that other cell wall connectors, including the major structural polysaccharide α1,3-glucan, are required for anchoring of the trichosporal polysaccharide, as clearly described for cryptococcal cells (Reese and Doering, 2003; Reese et al., 2007).

Phagocytosis studies demonstrated that, in comparison with *Candida* strains, *Cryptococcus* and *Trichosporon* isolates are more resistant to phagocytosis (Lyman and Walsh, 1994). Acapsular *C. neoformans* mutants are known to incorporate exogenously added GXM into their cell surface (Reese and Doering, 2003). Considering that the process of GXM anchoring to the cell wall in *C. neoformans* and *T. asahii* showed similar elements, we hypothesized that the *C. neoformans* mutant would be able to bind the heterologous GXM produced by *T. asahii*. In fact, Cap67 cells became coated with the *Trichosporon* GXM after incubation in culture supernatants. Besides indicating that the motifs required for cell wall connection of GXM in *C. neoformans* and *T. asahii* could be similar, this result allowed the design of experimental models to evaluate the antiphagocytic capacity of the *T. asahii* GXM. Our results demonstrated that coating with the *Trichosporon* GXM protects the *C. neoformans* acapsular mutant against phagocytosis by murine macrophages. To our knowledge, this is the first demonstration that GXM from non-*Cryptococcus* species is antiphagocytic.

In the cryptococcal model, antibodies to GXM modify the course of animal infections prolonging the survival of lethally infected mice (Casadevall et al., 1998). In addition, a monoclonal antibody to GXM is in clinical trial in humans (Larsen et al., 2005). In addition to being the target of therapeutic antibodies, the polysaccharide is also represents a potential vaccine component that can elicit protective antibodies (Datta et al., 2008; Zhang et al., 1997). The similarities of the *Cryptococcus* and *Trichosporon* models described in this study suggest that antibodies raised against the cryptococcal polysaccharide could be therapeutic against *T. asahii* infections. The possibility that single therapeutic tools could be useful in different models of fungal infections highlights the importance of studies on the properties of GXM in *Trichosporon* species. In this context, detailed structural studies as those developed in the *Cryptococcus* model, as well as in vivo protection tests, are indispensible for validation of GXM as a therapeutic target in *Trichosporon* infections.

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VI - DISCUSSÃO:

Estruturas de superfície são fundamentais na patogênese microbiana, já que representam o primeiro contato do microrganismo com seu hospedeiro. Em alguns casos, tais moléculas conferem ao patógeno proteção física, bem como podem auxiliá-lo a camuflar-se e assim burlar as defesas do hospedeiro, determinando se o microrganismo invasor será ou não detectado (ZARAGOZA *et al.,* , 2009).

Capsídeos e envelopes virais, bem como cápsulas e paredes celulares de bactérias e fungos, exemplificam a complexidade e importância de estruturas de superfície de patógenos. Essas estruturas podem ser formadas por carboidratos, proteínas e lipídeos, compondo cadeias simples ou complexas. Algumas dessas estruturas medeiam a ligação de patógenos a células hospedeiras, evento esse que pode acarretar em modulação da resposta imune. Eventos dessa natureza podem ser decisivos para o curso da infecção e a sobrevivência do microrganismo.

A GXM, componente de superfície mais externo em espécies de *Cryptococcus* e *Trichosporon*, é um polissacarídeo de grande potencial imunogênico que parece ser fundamental na proteção desses fungos contra as defesas do hospedeiro. Além de ter um papel estrutural importante quando associada à superfície celular, a GXM também é secretada para o meio extracelular, sendo facilmente encontrada em fluidos corporais e tecidos infectados.

Análises da composição glicídica de GXM purificada a partir de sobrenadantes de cultura de *T. asahii* e de diferentes sorotipos de *C. neoformans* relevaram a presença típica dos 3 monossacarídeos principais: manose, xilose e ácido glucurônico. No entanto, apesar da mesma composição glicídica, são observadas diferentes proporções desses monossacarídeos, o que acarreta em diferenças estruturais tanto entre as GXMs de *Trichosporon* e *Cryptococcus*, como também dentro de uma mesma espécie fúngica (CHERNIAK *et al.*, 1998; ICHIKAWA *et al.,* , 2001; FONSECA *et al.*, 2009a).

Ao compararmos os polissacarídeos desses fungos produtores de GXM, é possível verificar alguns domínios conservados. É fato, entretanto, que a estrutura observada para *T. asahii* apresenta uma maior complexidade que a GXM de *C. neoformans* (**Figura 7**, apresentada na introdução). O polissacarídeo de *T. asahii* mostra maior grau de xilosilação, além disso, estão presentes cadeias laterais mais extensas, formando dissacarídeos de xilose ausentes na molécula criptococócica (ICHIKAWA *et al.,* , 2001; FONSECA *et al.,* , 2009a). O conteúdo de ácido glucurônico encontrado na GXM de *T. asahii* foi duas vezes menor do que o encontrado no polissacarídeo criptococócico (FONSECA *et al.,* , 2009a), o que acarreta em um valor reduzido do potencial zeta. Essa característica poderia explicar os baixos valores de diâmetro efetivo encontrado nas fibras do polissacarídeo de *T. asahii*, bem como o baixo rendimento da

purificação de GXM por agregação nos sobrenadantes de cultivo. Finalmente, essa observação poderia explicar a ausência de uma estrutura capsular convencional nesse fungo, já que a agregação intermolecular de GXM envolve a ligação entre cargas negativas oriundas do ácido glucurônico e metais divalentes para o crescimento e organização da cápsula (NIMRICHTER *et al.,* , 2007; FONSECA *et al.,* , 2009a; FRASES *et al.,* , 2009). Diferentemente de *T. asahii*, todos os sorotipos de *C. neoformans* são capsulados e a GXM produzida por tais cepas foi capaz de se agregar formando um gel após a concentração do sobrenadante de cultura em um sistema de ultrafiltração (FONSECA *et al.*).

A literatura atualmente disponível deixa clara a dificuldade de definição das funções da GXM em diferentes modelos. O polissacarídeo, aparentemente, apresenta várias funções contraditórias, exemplificadas a seguir. A GXM reconhecidamente protege o fungo contra fagocitose e inibe a migração leucocitária (ELLERBROEK *et al.,* , 2004b; MONARI, BISTONI & VECCHIARELLI, 2006), o que funcionaria a favor de *C. neoformans* no processo infeccioso. Entretanto, o polissacarídeo também é um indutor eficiente da ativação de vias alternadas do sistema complemento, que resultam na deposição de opsoninas na cápsula e em fagocitose aumentada, o que funcionaria em favor do hospedeiro. Outro exemplo de funções conflitantes de componentes capsulares de *C. neoformans* envolve as formas solúveis de GXM e GalXM. Ambos ativam a enzima óxido nítrico sintase, com consequente produção de NO (CHIAPELLO *et al.*, 2008; VILLENA *et al.,* , 2008). Entretanto, células de *C. neoformans* expressando cápsulas de grandes dimensões inibem a produção de NO em macrófagos ativados (XIAO *et al.*, 2008), o que, de fato, confirma dados prévios mostrando que *C. neoformans* é incapaz de induzir a óxido nítrico sintase em fagócitos murinos (NASLUND, MILLER & GRANGER, 1995). Esses resultados demonstram a grande complexidade dos eventos envolvidos na interação de *C. neoformans* com células hospedeiras e sugerem fortemente que preparações variadas de GXM podem apresentar uma alta multiplicidade funcional. Seguindo essa linha de raciocínio, investigamos determinados aspectos funcionais de amostras de GXM de *C. neoformans* e *C. gattii*, com diferentes propriedades estruturais, com o intuito de demonstrar a grande diversidade funcional do polissacarídeo.

A ocorrência de diferentes funções do polissacarídeo dentro do gênero *Cryptococcus* spp foi confirmada por ensaios de indução de NO em macrófagos e ativação de heterodímeros de receptores do tipo Toll em células transformadas (FONSECA *et al.,*). Todas as frações testadas foram capazes de induzir de forma dose-dependente a ativação do fator nuclear κB (NF-κB) em células epiteliais expressando dímeros TLR2/1 e TLR2/6. O processo foi sempre mais eficiente na presença de TLR2/1. A influência da interação de GXM de cepas de sorotipo A de *C. neoformans* com TLR2 na resposta imune contra a criptococose, incluindo a produção de

NO, já havia sido descrita (YAUCH *et al.,* , 2004). Entretanto, o envolvimento de heterodímeros desse receptor com TLR1 e TLR6 no reconhecimento da GXM ainda não havia sido demonstrado.

Tanto em sistemas transfectados com TLR2/1 ou TLR2/6, os maiores níveis de ativação de NF-κB foram obtidos quando as células foram expostas a GXM *C. gattii* (sorotipo B) Resultados similares foram obtidos em ensaios de produção de NO por macrófagos (FONSECA *et al.,*). Dentre todos os parâmetros avaliados em nosso estudo, propriedades sorológicas diferenciadas e diâmetro efetivo reduzido pareceram ser os fatores determinantes para a observação de respostas celulares mais intensas. O fato de fibras de GXM com dimensões reduzidas terem uma maior atividade imunológica corrobora estudos anteriores que demonstraram que longas fibras de quitina são inertes, enquanto fragmentos menores ativam eficientemente a resposta imune mediada por TLR2 (DA SILVA *et al.*, 2008; LEE *et al.*, 2008; LEE, 2009). No modelo de *Cryptococcus* spp, fibras curtas de GXM estão associadas a um reduzido tamanho capsular e indução de uma resposta imune mais eficiente, o que pode favorecer o sistema de defesa do hospedeiro. A observação de que polissacarídeos de sorotipo B de *C. gattii* induziram respostas inflamatórias mais intensas é condizente com a típica inflamação granulomatosa observada na criptococose causada por *C. gattii* (SWEETSER *et al.*, 1998). Em suma, especulamos que propriedades sorológicas aliadas ao diâmetro da molécula de GXM podem influenciar a resposta inflamatória contra *Cryptococcus* spp. Dessa forma, sugerimos que as propriedades verificadas para amostras únicas de GXM não devem ser generalizadas e atribuídas para todas as espécies de *Cryptococcus*. Essa hipótese está de acordo com dados na literatura que apontam uma diversidade funcional em moléculas de GXM (NIMRICHTER *et al.,* , 2007; FRASES *et al.,* , 2008; FRASES *et al.,* , 2009). Tal característica é compartilhada por amostras de GalXM, que também apresentam variações estruturais e antigênicas (DE JESUS *et al.*, 2009a).

É importante ressaltar que muitos dos estudos que avaliam a função imunológica da GXM utilizam frações de polissacarídeos purificados a partir de isolados de sorotipo A. Assumia-se, portanto, que as propriedades imunológicas observadas para esse sorotipo também são aplicadas para os demais grupos. Dessa forma, nossos dados também demonstram que a GXM pode responder de maneira diferente dependendo da cepa em questão. Nossos ensaios também sugerem que frações de polissacarídeo que combinem faixas amplas de massa molecular e diâmetro efetivo podem apresentar funções obscuras ou mesmo conflitantes. Os resultados obtidos também sugerem que existam peculiaridades na GXM de *C. gattii* (sorotipo B), motivando investigações futuras mais detalhadas.

Como já mencionado anteriormente, a capacidade de auto-agregação da GXM de *C. neoformans* e *C. gattii* não parece ser exclusiva para essas espécies fúngicas. Nossos estudos demonstraram que a GXM secretada por *T. asahii* também é capaz de formar um filme gelatinoso em presença de cátions divalentes. Entretanto, em virtude de alguns fatores como a menor quantidade de GXM secretada para o sobrenadante de cultivo, o pequeno diâmetro de fibra e possivelmente a menor proporção de ácido glurônico na molécula, a eficiência na formação de gel é extremamente menor quando comparada com a observada para *C. neoformans* (FONSECA *et al.,* , ; FONSECA *et al.,* , 2009a). O polissacarídeo de *T. asahii*, entretanto, foi reconhecido por anticorpos monoclonais contra GXM criptococócica, o que provavelmente é explicado pela ocorrência de domínios estruturais similares nos dois polissacarídeos. Dessa forma, levantamos a hipótese de que as GXMs produzidas pelos patógenos acima descritos compartilhariam também aspectos funcionais. Assim como é observado com o polissacarídeo criptocócico, a GXM purificada de *T. asahii* foi reincorporada por mutantes acapsulares de *C. neoformans* (FONSECA *et al.,* , 2009a). Os mutantes acapsulares de *C. neoformans* revestidos com a GXM de *T. asahii* foram protegidos contra fagocitose, sendo esse a primeira comprovação de que uma GXM não criptococócica é antifagocítica (LYMAN & WALSH, 1994; FONSECA *et al.,* , 2009a).

Sendo a GXM crucial em processos de patogenicidade fúngica, estudos sobre o ancoramento do polissacarídeo na parede celular podem ser reveladores no sentido de gerar ferramentas de controle da expressão da GXM em patógenos. A importância de alguns carboidratos nesse tipo de conexão foi demonstrada em alguns estudos (GILBERT *et al.,* , ; REESE & DOERING, 2003; BANKS *et al.,* , 2005; BAKER *et al.,* , 2007; REESE *et al.,* , 2007), mas a função de muitos dos componentes da parede celular em tais processos permaneceu obscura. Nesse sentido, investigamos a participação de estruturas derivadas de quitina no ancoramento de GXM à parede celular. A quitina é um polissacarídeo que desempenha funções vitais na arquitetura da parede celular de fungos (ADAMS, 2004), que também é reconhecida como o segundo polímero mais abundante na natureza.

Através de técnicas de microscopia óptica de fluorescência e microscopia eletrônica de transmissão utilizando a lectina do germe do trigo (WGA, do inglês, *wheat germ agglutinin*), nós demonstramos a existência de estruturas associadas à parede celular de *C. neoformans* que se projetam para dentro da cápsula. Por microscopia confocal, constatou-se uma marcação polarizada na célula, em interfaces entre a cápsula e a parede celular. Observamos em células em brotamento uma espécie de anel em torno da célula nascente, o que parece sugerir um papel das estruturas reconhecidas pela lectina no rearranjo capsular durante a divisão celular (RODRIGUES *et al.*, 2008a).

As moléculas reconhecidas pela WGA são compostas por estruturas oligoméricas de β-1,4-*N*-acetilglucosamina (β-1,4-GlcNAc) (PRIVAT *et al.*, 1974). Para confirmar a idéia de que essas moléculas participariam da conexão cápsula-parede de *C. neoformans,* as leveduras foram tratadas com quitinase, enzima capaz de hidrolisar a quitina e moléculas relacionadas com formação consequente de quito-oligômeros e unidades de *N*-acetilglucosamina. O tratamento enzimático resultou na liberação de GXM para o meio extracelular acompanhado de um decréscimo do tamanho da cápsula, sugerindo que a quitina está envolvida na arquitetura capsular. Esse resultado foi acompanhado de um perfil difuso de ligação da WGA à superfície celular, provavelmente em virtude do aumento do número de sítios disponíveis para a ligação da lectina (RODRIGUES *et al.,* , 2008a; FONSECA *et al.,* , 2009a). Curiosamente, o tratamento de leveduras acapsulares com quitinase fez com que as mesmas incorporassem GXM de forma pouco convencional, formando malhas polissacarídicas mais distanciadas da parede celular (RODRIGUES *et al.,* , 2008a). Esses resultados sugeriram que o aumento da oferta de quito-oligômeros na superfície celular facilitaria o crescimento da cápsula em *C. neoformans*.

A ligação da WGA à superfície celular, bem como a susceptibilidade ao tratamento com quitinase, foram similares em *T. asahii*. Nessa espécie, os sítios de reconhecimento pela lectina mais abundantes também estão dispostos na região de divisão celular (FONSECA *et al.,* , 2009a). Esses resultados evidenciam mais uma característica comum a ambos os fungos e reforça a idéia de que a GXM, independentemente do patógeno em questão, interage com oligômeros de quitina dispostos na parede celular.

A capacidade de ligação da GXM à quitina e estruturas similares foi de fato comprovada em modelos diversos, incluindo métodos cromatográficos, sorológicos e microscópicos. Complexos contendo GXM e β-1,4-GlcNAc foram detectados no sobrenadante de cultura, sugerindo que tal interação é um processo fisiológico normal do fungo (FONSECA *et al.*, 2009b). O cultivo do fungo na presença de concentrações aumentadas de oligômeros de quitina gerou cápsulas aberrantes. De fato, a interação de GXM com quitoligômeros solúveis acarretou em aumento do tamanho da fibra de GXM. Nós especulamos que esses dados estariam ligados a uma maior permeabilidade da cápsula à tinta Nanquim nesses sistemas, uma vez que o agregado composto por longas fibras de GXM e oligômeros de quitina formaria uma malha compacta na região capsular, tornando mais fácil a penetração do corante. Além disso, por microscopia de fluorescência, também foi observada nessa região uma perda de reatividade com o anticorpo anti-GXM, sugerindo uma possível mudança no epítopo ou um bloqueio dos epítopos *O*-acetil, fundamentais para o reconhecido pelo anticorpo (GATES-HOLLINGSWORTH & KOZEL, 2009). Unidades de β-1,4-GlcNAc são componentes de quitina e moléculas correlatas

e exercem um papel crucial tanto na fisiologia fúngica quanto na patogenicidade de *C. neoformans*. De fato, estudos revelaram que mutações de genes responsáveis pela expressão de quitinase sintase 3 ou do regulador biossintético Csr2p acarretaram na perda a capacidade de retenção de melanina, um importante fator de virulência situado na parede celular (BANKS *et al.,* , 2005; BAKER *et al.,* , 2007).

A quitina, componente essencial da parede celular de *C. neoformans (*ADAMS*,* 2004*),* é um polímero formado por unidades de GlcNAc, as quais são polimerizadas por quitina sintases. Essas enzimas utilizam *pools* de UDP-GlcNAc citoplasmática para formar oligômeros e polímeros de GlcNAc ligados β-1,4- e localizados prioritariamente na parede celular (BANKS *et al.,* , 2005). A forma de-*O*-acetilada da quitina, a quitosana, também é um polissacarídeo fundamental na integridade da parede celular, produzido enzimaticamente por quitina deacetilases (BAKER *et al.,* , 2007).

Um evento crucial na síntese de quitina e moléculas derivadas é a atividade da enzima glucosamina 6-fosfato sintase, responsável pela aminação da frutose 6-fosfato para formar glucosamina 6-fosfato, substrato para síntese de UDP-GlcNAc (MILEWSKI, GABRIEL & OLCHOWY, 2006). Tendo em vista a relação entre quitina/moléculas derivadas de quitina e a cápsula de *C. neoformans*, nossa abordagem seguinte foi focada na interferência na expressão de moléculas contendo *N*-acetiglucosamina em *C. neoformans*. Diante da multiplicidade de genes codantes para quitina sintase (BANKS *et al.,* , 2005; BAKER *et al.,* , 2007) e a conseqüente dificuldade de geração de mutantes com fenótipos claros, avaliamos em nosso modelo a ação de um dipeptídeo inibidor da glucosamina 6-fosfato sintase, o L-norvalil-FMDP (Nva-FMDP), na arquitetura capsular desse fungo (FONSECA *et al.,* , 2009b).

Ao contrário do que ocorre com *C. albicans*, Nva-FMDP não apresentou atividade antifúngica contra *C. neoformans*, restringindo sua ação a marcantes alterações morfológicas sem perda de viabilidade (MILEWSKI, JANIAK & WOJCIECHOWSKI, 2006; FONSECA *et al.,* , 2009b). De acordo com diversas técnicas microscópicas, sugerimos uma estreita relação entre a alteração do metabolismo de GlcNAc e interferências na arquitetura da cápsula. Todas as nossas abordagens indicaram que situações experimentais levando à inibição da síntese de *N*-acetilglucosamina são correlacionadas à diminuição das dimensões capsulares (FONSECA *et al.,* , 2009b). O fato de que, embora reduzidas, as estruturas capsulares se mantiveram na supefície celular sugere que a matriz capsular poderia estar ainda conectada a α-1,3-glucanas e a outras moléculas correlatas dispostas na parede celular. Assim, estruturas derivadas de quitina estariam muito mais envolvidas na organização capsular externa do que no ancoramento de componentes capsulares com a parede celular. Essa hipótese está de acordo com o fato de que *Saccharomyces cerevisiae*, foi incapaz de reincorporar GXM endógena, mesmo expressando em

sua superfície estruturas reconhecidas por WGA num mesmo padrão observado em *C. neoformans* (POWELL, QUAIN & SMART, 2003; FONSECA *et al.,* , 2009b). Sendo assim, concluímos que o metabolismo de GlcNAc é de extrema importância para a organização do principal fator de virulência de *C. neoformans* (FONSECA *et al.,* , 2009b), o que nos levou também a avaliar a expressão de estruturas quito-oligoméricas *in vivo*.

Marcações pontuadas e polarizadas depois de incubação com WGA também foram verificadas na interação do fungo com macrófagos murinos, comprovando que a presença de estruturas derivadas de quitina reconhecidas pela WGA não é um artefato oriundo de culturas *in vitro* (RODRIGUES *et al.,* , 2008a). Em cortes histológicos de cérebro e pulmão de ratos infectados, observamos padrões similares. Além disso, o ensaio *in vivo* demonstrou que a expressão dessas moléculas pode ser modulada de acordo com o órgão infectado (FONSECA *et al.,* , 2009b), o que parece estar relacionado a estudos recentes mostrando que a infecção pulmonar, mas não cerebral, induz a produção de quitinase no sítio colonizado (VICENCIO *et al.*, 2008). Esses dados parecem estar de acordo com nossos dados: nos cortes histológicos de pulmão evidenciamos um perfil anelar semelhante ao observado *in vitro* para a presença de quitinase. No cérebro, onde não ocorre a expressão de quitinase, a maior parte das células possui marcações pontuais (FONSECA *et al.,* , 2009b). Em conjunto, esses resultados sugerem que a expressão de moléculas derivadas de quitina no fungo pode ser modulada enzimaticamente pelo hospedeiro, de acordo com o órgão infectado (FONSECA *et al.,* , 2009b). Nós especulamos que tais observações poderiam estar relacionadas à produção de cápsula *in vivo*. A hipótese que propomos teria como base o aumento da exposição de quito-oligômeros por *C. neoformans* no pulmão, fato que seria estimulado pela atividade de quitinase no tecido pulmonar. A quantidade aumentada de quito-oligômeros, de acordo com nossos dados, facilitaria o crescimento capsular, o que não ocorreria no cérebro. De fato, é amplamente reconhecido que no pulmão são atingidas as maiores dimensões capsulares durante a infecção por *C. neoformans* (RIVERA *et al.,* , 1998).

Algumas de nossas imagens por microscopia confocal sugerem que estruturas reconhecidas por WGA também pode ser projetadas para a face externa da cápsula de *C. neoformans* (RODRIGUES *et al.,* , 2008a). Essa observação, aliada ao fato de essas moléculas serem expressas *in vivo,* pode representar um mecanismo de interação com o hospedeiro ainda não investigado. Os estudos atualmente disponíveis na literatura, em geral, estão focados na interação de *C. neoformans* com o hospedeiro mediada por GXM. Entretanto, seria razoável supor que uma resposta imune mediada por oligômeros de quitina ou híbridos de GXM-quitoligômeros possa ser desencadeada durante o curso da infecção. Estudos recentes demonstraram que a quitina é capaz de ativar a resposta imune inata e adaptativa e estimular

a produção de citocinas, num processo que ocorre via diversos receptores de superfície como TLR-2 e dectina-1 (DA SILVA *et al.,* , 2008; LEE *et al.,* , 2008). A presença de polissacarídeos híbridos contendo GXM e quito-oligômeros, portanto, poderia impactar expressivamente a resposta imune, considerando a imunogenicidade das duas estruturas.

Desse modo, o estudo da interação entre GXM e estruturas derivadas de quitina, além de ser fundamental na elucidação de um dos mecanismos de envolvidos na arquitetura capsular, também pode abrir novos campos de investigação na área de interação fungo/hospedeiro e de síntese de novos alvos terapêuticos.

VI- CONCLUSÕES:

1) A conexão da GXM com a parede celular de *C. neoformans*, *C. gattii* e *T. asahii* requer estruturas derivadas da quitina. Em *C. neoformans*, essas estruturas parecem fundamentais para o crescimento capsular.

2) A análise comparativa entre as GXMs de *C. neoformans*, *C. gattii* e *T. asahii* revelou várias propriedades comuns, assim como várias diferenças. Dentre as observações comuns, foi demonstrado que GXM de *T. asahii* é antifagocítica, conforme observado anteriormente para *C. neoformans* e *C. gattii*. Dentre as diferenças, foram observadas algumas características espécie-específicas, principalmente com relação a dimensões do polissacarídeo.

3) A ativação de respostas celulares mediada por uma amostra de GXM de *C. gattii*, sorotipo B, foi mais intensa do que a mediada por qualquer outro polissacarídeo testado. Nesse contexto, o determinante principal da ativação de respostas mediadas por TLR e produção de NO foi o diâmetro reduzido das fibras de polissacarídeo. Essas observações parecem estar associadas a propriedades sorológicas específicas da GXM obtida de um isolado classificado como sorotipo B de *C. gattii*.

4) Nossos resultados sugerem novas propriedades funcionais e estruturais para as GXMs de *C. neoformans*, *C. gattii* e *T. asahii*, o que pode estar diretamente associado a imunopatogênese observada nas infecções causadas por esses três patógenos.

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VII – ANEXOS:

Durante minha Tese de Doutorado também tive a oportunidade de participar como autora de trabalhos científicos envolvendo a interação de *C. neoformans* com células de epitélio alveolar, gerando a publicação de dois artigos que descreveram o envolvimento da GXM nesse processo (BARBOSA *et al.,* , 2006) e caracterização de receptor da célula hospedeira (BARBOSA *et al.,* , 2007). Isso acarretou no convite para a redação de uma revisão para a revista Medical Mycology (RODRIGUES *et al.,* , 2009). Além disso, tal experiência me possibilitou colaborar na publicação de um trabalho envolvendo a interação de uma enolase de *P. brasiliensis* com células hospedeiras (NOGUEIRA *et al.* 2010).

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Original article

Glucuronoxylomannan-mediated interaction of *Cryptococcus neoformans* with human alveolar cells results in fungal internalization and host cell damage

Fabiane M. Barbosa [a], Fernanda L. Fonseca [a], Carla Holandino [b], Celuta S. Alviano [c], Leonardo Nimrichter [a,d], Marcio L. Rodrigues [a,*]

[a] *Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Cidade Universitária, CCS, Bloco I, Ilha do Fundão, Rio de Janeiro, RJ 21941590, Brazil*
[b] *Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, RJ 21941590, Brazil*
[c] *Laboratório de Estruturas de Superfície de Microrganismos, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, RJ 21941590, Brazil*
[d] *Disciplina de Biologia Celular, Universidade Federal de São Paulo, São Paulo, SP 04023-062, Brazil*

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Abstract

Infection by *Cryptococcus neoformans* begins with inhalation of infectious propagules. Fungi reach the lung tissue and interact with epithelial cells in a crucial but poorly understood process. In this study, the interaction of *C. neoformans* with the human alveolar epithelial cell lineage A549 was investigated, focusing on the relevance of the capsular polysaccharide in this process. The association of encapsulated strains with A549 cells was significantly inhibited by a monoclonal antibody to glucuronoxylomannan (GXM), a major component of the cryptococcal capsule. A purified preparation of GXM produced similar results, suggesting the occurrence of surface receptors for this polysaccharide on the surface of alveolar cells. A549 cells were in fact able to bind soluble GXM, as confirmed by indirect immunofluorescence analysis using the anti-polysaccharide antibody. *C. neoformans* is internalized after GXM-mediated interaction with A549 cells in a process that culminates with death of host cells. Our results suggest that *C. neoformans* can use GXM for attachment to alveolar epithelia, allowing the fungus to reach the intracellular environment and damage host cells through still uncharacterized mechanisms.

Keywords: Glucuronoxylomannan; *Cryptococcus neoformans*; Alveolar epithelium

1. Introduction

Cryptococcus neoformans is an encapsulated fungal pathogen mainly infecting immunosuppressed patients. Infections are most probably acquired by inhalation of desiccated cells [1], which are available in the environment as basidiospores or poorly encapsulated yeasts. An effective interaction of *C. neoformans* with epithelial alveolar cells is crucial for disease establishment; once adhered to the pulmonary epithelia, *C. neoformans* can proliferate and induce primary lesions in the lung [2]. The infection is normally controlled by a cell mediated immunity in immunocompetent patients [1] who develop a Th1 response with $TCD4^+$ and $TCD8^+$ recruitment [3], accompanied by the production of inflammatory mediators such as IL-12, TNF, IFNγ and NO [4]. In individuals with impaired cell mediated immunity, however, cryptococci can disseminate to the central nervous system, which is consistently associated with a high level of mortality.

There is increasing evidence that *C. neoformans* uses the intracellular environment to replicate inside the host [5–12]. The presence of intracellular *C. neoformans* in lung tissue has been shown in early [7] and recent [8–10] reports. *C. neoformans* apparently uses intracellular parasitism as a mechanism of pathogenicity; for instance, the index of budding of intracellular yeasts is five times higher that that observed for extracellular cryptococci in vivo (reviewed in [11]). Inside macrophages, *C. neoformans* sheds its capsule components

* Corresponding author. Tel.: +55 21 2562 6711; fax: +55 21 2560 6344.
E-mail address: marcio@micro.ufrj.br (M.L. Rodrigues).

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[12], causing damage to the phagosomal membrane, followed by continuity with the cytoplasm and accumulation of vesicles filled with capsular polysaccharide.

The capsule of *C. neoformans* is a surface complex composed of glucuronoxylomannan (GXM), galactoxylomannan and mannoproteins (reviewed in Ref. [13]). Due to its surface cellular distribution, the cryptococcal capsule is expected to be a main fungal component modulating the interaction between host and yeast cells, which is in fact observed in a large variety of systems [1]. Additionally, large amounts of capsular material are detected in patient serum, which is related to disease progress [13]. The ability of cryptococcal capsule components to modulate the immune response has been described. GXM, the major cryptococcal polysaccharide, has an extensive deleterious effect, including inhibition of cytokine production and leukocyte migration [14].

The pathogenic mechanisms involved in the lung infection by *C. neoformans* are poorly known. Although several animal models of cryptococcal infections have been established, the functional differences between mouse and human lung cells [15] impair a complete understanding of how *C. neoformans* interacts with the pulmonary epithelium. In this context, the interaction of *C. neoformans* with the human type II alveolar epithelial lineage A549 has been studied by Merkel and Scofield [16,17]. Results by these authors suggested an adhesin-mediated attachment of *C. neoformans* to A549 cells, which was followed by internalization of yeasts. Since the alveolar epithelium is a key host site for a successful infection by *C. neoformans*, we investigated the mechanisms involved in the interaction of *C. neoformans* with A549 cells. We found that these cells express surface sites for GXM binding, which apparently mediate the attachment of *C. neoformans* to A549 monolayers. Cryptococcal adhesion is followed by fungal internalization, culminating with host cell death.

2. Material and methods

2.1. Microorganisms

C. neoformans strains T_1-444 and HEC3393 (serotype A, clinical isolates) are, respectively, largely and poorly encapsulated isolates, as previously characterized by our group [18]. Strain cap67 is a well characterized acapsular mutant of *C. neoformans* extensively used in previous studies [19]. Stock cultures were maintained on Sabouraud dextrose agar under mineral oil and kept at 4 °C. For interaction with host cells, *C. neoformans* was cultivated with shaking for 48 h, at 25 °C, in a chemically defined medium [20] containing dextrose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM), and thiamine-HCl (3 μM). Yeast cells were obtained by centrifugation and washed twice in 0.01 M phosphate buffered saline (PBS), pH 7.2. Cell growth was estimated by counting the number of yeasts in a Neubauer chamber. Capsule expression was determined by India ink staining and indirect immunofluorescence with a mouse monoclonal antibody produced against GXM (Mab18B7), kindly provided by Dr. Arturo Casadevall (Albert Einstein College of Medicine, New York, USA). This antibody is a mouse IgG1 with high affinity for GXM of different cryptococcal serotypes. It has been finely characterized in previous studies [21] and is currently in clinical trial for the therapy against cryptococcosis [22].

2.2. Human cells

A549 is a human type II alveolar epithelial-like lineage. It was provided by Dr. Sonia Rozental (Instituto de Biofisica Carlos Chagas Filho, UFRJ) and is derived from lung carcinomatous tissue of a human patient, being widely used as a model of infection of respiratory pathogens [23]. The cultures were maintained and grown to confluence in 25 cm^2 culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), at 37 °C in a 5% CO_2 atmosphere. For immunofluorescence experiments and interaction with cryptococci, A549 cells were cultivated on 96-well plates or over sterile glass slides placed onto the wells of a 24-well plate, in the same conditions described above.

2.3. GXM purification

GXM was purified from culture supernatants of *C. neoformans* [24]. Briefly, yeast cells were cultivated as described above and, after 5 days, the culture was autoclaved for 25 min at 121 °C. Cell suspensions were then centrifuged at 10,000 × *g* for 4 h. The supernatant was collected and gently mixed with three volumes of 95% ethanol. This mixture was then incubated overnight at 4 °C. The precipitate was sequentially washed with 95% ethanol, acetone and ether, followed by air drying. This crude polysaccharide mixture was then dissolved (10 mg/ml) in 0.2 M NaCl. GXM was separated from other polysaccharide components by the slow addition of cetyl trimethyl ammonium bromide (CTAB, 3 mg/mg of total polysaccharide). Insoluble material was removed by centrifugation (23 °C, 10,000 × *g*, 30 min) and the GXM-CTAB complex was precipitated through the slow addition, under gentle shaking, of a 0.05% CTAB solution. The CTAB-GXM complex was dissolved in 0.2 M NaCl, and for the selective recovery of GXM, two volumes of ethanol were added. The precipitate was then dissolved in 1 M NaCl and dialyzed against water for 5 days. Purity and molecular weight of GXM preparations were evaluated by SDS-PAGE followed by staining with the Schiff's reagent. Chemical composition was confirmed by acid hydrolysis followed by chromatographic analysis [24] and antigenicity was analyzed by ELISA [25] using Mab18B7. The maintenance of GXM biological properties was evaluated by the ability of *C. neoformans* acapsular mutants to incorporate the purified polysaccharide into their cell wall, as previously described [26]. In all these tests, results were indicative of an authentic and purified preparation of

GXM. Carbohydrate determinations were performed using the method of Dubois et al. [27].

2.4. Interaction of C. neoformans with host cells

A549 cells were suspended in DMEM and placed into the wells of a 24-well plate (10^5 cells per well). *C. neoformans* suspensions were also prepared in DMEM, to generate a ratio of 10 yeasts per host cell. Interactions between fungal and host cells occurred at 37 °C at a 5% CO_2 atmosphere for different periods which, depending on the specific aim of the experiment, varied from 1 to 18 h. After removal of non-adherent fungi by washing, the cells were fixed with Bouin's solution and stained with Giemsa. In each system, 200 epithelial cells were counted, and the index of association between *C. neoformans* and A549 cells was considered as the total number of yeasts per 100 epithelial cells. Alternatively, A549 cells were distributed in 96-well plates (10^4 cells per well) and interactions were performed at the same conditions. After washing for removal of unattached fungi, epithelial cells were lysed with sterile cold water and the resulting suspension plated onto Sabouraud agar plates. After 48 h, the number of colony-forming units (CFU) was determined. In the different conditions used, A549 cells were unable to kill cryptococci (data not shown), which was concluded from the observation that no significant differences were obtained between the number of viable yeasts before and after the exposure to human cells. In some systems, the monoclonal antibody to GXM (Mab18B7) and purified GXM were used as inhibitory tools. In these models, 10^6 yeast cells were treated with Mab18B7 (1–100 μg/ml) for 1 h at room temperature for further interaction with host cells. Alternatively, A549 cells were treated with the GXM solution (100 μg/ml) for 60 min before exposure to *C. neoformans*. Control systems were treated with no antibody or with an isotype matched irrelevant IgG at the same concentrations used for Mab18B7. The irrelevant antibody consisted of a mouse monoclonal IgG1 produced against human fibronectin (Sigma, catalog number F7387). All experiments were performed in triplicate sets and statistically analyzed by using Student's *t*-test.

2.5. Binding of GXM to A549 cells

A549 cells were cultivated in 24-well plates (10^6 cells per well) as described above. The medium was replaced by fresh DMEM supplemented with purified GXM (100 μg/ml), followed by incubation at 37 °C for periods varying from 1 to 18 h. After extensive washing for removal of unbound GXM, the cells were detached from the plastic support using 1 mM EDTA in PBS, fixed in 4% paraformaldehyde and blocked for 1 h (37 °C) in PBS supplemented with 10 mg/ml bovine serum albumine (PBS-BSA). Blocked cells were washed three times with PBS and then incubated with Mab18B7 (10 μg/ml) for 1 h at room temperature, followed by incubation with a fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG (Fc specific) antibody (Sigma). A549 cells were then analyzed in a FACSCalibur (BD Biosciences, San Jose, CA) flow cytometer and data were processed with CellQuest (BD Biosciences) or WinMDI (Salk Flow Cytometry) software. Control preparations were developed as described above, except for the incubation with the anti-GXM antibody. In these systems, the cells were treated with GXM for further incubation in the presence of PBS or mouse anti-human fibronectin antibody (100 μg/ml), followed by the FITC-labeled anti-mouse immunoglobulin.

Binding of GXM to A549 cells was also analyzed by fluorescence microscopy. In this analysis, human cells were incubated with purified GXM (100 μg/ml) for 2 h at 4 °C. After this period, the temperature was changed to 37 °C, and the incubations proceeded for 30 min, 1 and 18 h. After each period of incubation, the cells were fixed in paraformaldehyde, permeabilized with absolute methanol and processed for indirect immunofluorescence as described above. A549 cells were finally observed with an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired using a Color View SX digital camera and processed with the software system analySIS (Soft Image System).

2.6. Immunofluorescence analysis of the interaction between C. neoformans and A549 cells

The interaction of *C. neoformans* with alveolar epithelia was also evaluated by the reactivity of infected human cells with Mab18B7. Fungal and host cells were incubated as described above. Non-adherent fungi were removed by washing followed by fixation with paraformaldehyde. To evaluate whether *C. neoformans* was internalized by host cells, some systems were permeabilized with absolute methanol before exposure to paraformaldehyde. Complexes were further incubated with Mab18B7 followed by a FITC-labeled anti-mouse antibody, as described above. The cells were finally observed with an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Alternatively, A549 cells were incubated with fungi and, after extensive washing for removal of non-adherent yeasts, were detached from culture plates using 1 mM EDTA. Cell complexes were fixed as described above and processed for flow cytometry analysis, as detailed in "Binding of GXM to A549 cells".

2.7. Determination of internalization of C. neoformans by flow cytometry

To determine the levels of internalization of *C. neoformans* during interaction with A549 cells, the method of Chaka et al. [28] was followed. Briefly, yeast cells were incubated with FITC at 0.5 mg/ml in PBS (25 °C) for 10 min. After washing in PBS, *C. neoformans* yeasts were incubated with A549 cells for 1 h as described above, followed by extensive washing with PBS for removal of non-adherent fungi. Some of the fungi-host cell complexes were treated for 10 min at 25 °C with Trypan blue, adjusted to a final concentration of 200 μg/ml. Trypan blue is a quenching agent of FITC-

derived fluorescence and, since it is not capable to reach the intracellular compartment of viable cells, this dye is useful to discriminate surface-associated and intracellular *C. neoformans* [28], extinguishing the fluorescence of non-internalized cells. Unbound Trypan blue was removed by extensive washing with PBS and then the complexes were analyzed by flow cytometry as described above. The index of internalized fluorescent *C. neoformans* was derived from fluorescence decrease in infected A549 cells after Trypan blue treatment.

2.8. Analysis of host cell damage by the lactate dehydrogenase (LDH) assay

LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes, but it is released from cells with damaged membranes. To evaluate host cell damage induced by the presence of *C. neoformans*, A549 cells were incubated with cryptococci for periods varying from 1 to 18 h, as described above. After these periods, supernatants were collected, centrifuged for removal of yeast cells and assayed for the presence of released LDH. Supernatants were supplemented with NADH and pyruvate to the final concentrations of 0.3 and 4.7 mM, respectively, and incubated for 1 h at room temperature. Based on the property that NADH, and not NAD^+, absorbs strongly at 340 nm, the decrease in absorbance as a function of time ($\Delta_{340\ nm}$/h) was measured in Ultrospec 2000 (Pharmacia Biotech) spectrophotometer [29]. Positive and negative controls consisted, respectively, of an A549 Triton X-100 (10%) lysate and supernatants of non-infected human cells after 18 h of cultivation. All experiments were performed in triplicate sets and statistically analyzed by using Student's *t*-test.

3. Results

The association of *C. neoformans* (T_1-444 strain) with A549 cells was first measured after 1 h of interaction between fungi and human epithelial cells (Fig. 1). If compared to those obtained with other cell lines, the level of association between *C. neoformans* and alveolar cells observed here can be considered low. For instance, the determination of the association of cryptococci with macrophages by a similar method results in levels five to six times higher [30] than those observed in this study. However, macrophages are professional phagocytic cells and, in the phagocytosis model, the efficacy of the interaction between *C. neoformans* and host cells is dependent on opsonization of fungi. Therefore, we concluded that *C. neoformans* can efficiently interact with A549 cells in our experimental conditions, based on the fact that it is a non-phagocytic cell line exposed to cryptococci in the absence of positive modulators of adhesion.

A mouse antibody produced against human fibronectin (irrelevant antibody) presented no significant effect on the association between cryptococci and human cells, even at high concentrations (Fig. 1A). A monoclonal antibody to GXM, however, modulated negatively the interaction of *C. neoformans* with A549 cells, in a dose-dependent fashion. Using a different approach, our results supported the hypothesis that GXM influences the association of *C. neoformans* with alveolar cells. The interaction of fungi with host cells for 1 h followed by the determination of viable yeasts recovered after lysis of host cells revealed that pretreatment of yeast cells with Mab18B7 (10 μg/ml) or of A549 cells with soluble GXM (100 μg/ml) significantly reduced the recovery of viable cryptococci (Fig. 1B). Capsule size did not interfere in the interaction between fungi and human cells (Fig. 1C), since no significant differences ($P > 0.05$) were observed between association levels of strains T_1-444 (heavily encapsulated) and HEC3393 (small capsule) with epithelial cells even after 18 h of interaction. The acapsular strain cap67 tended to be less adherent to human cells, but there were no significant differences between the levels of association of encapsulated strains and the acapsular one. These results suggest that when GXM or their putative receptors are blocked, alveolar cells became less susceptible to the infection by *C. neoformans*, leading us to investigate whether A549 cells are able to recognize GXM.

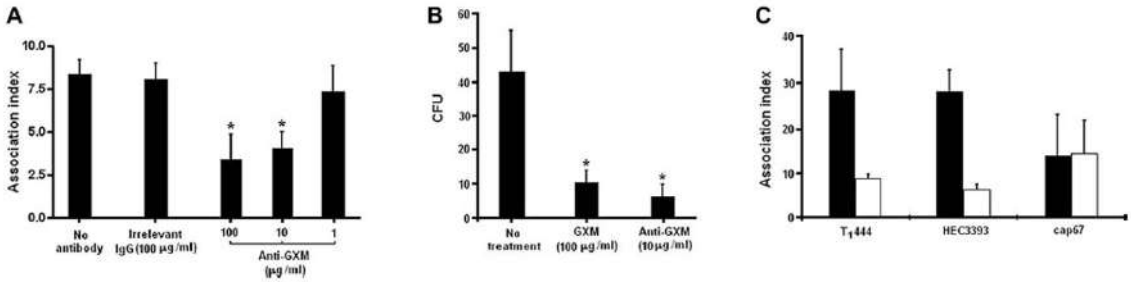

Fig. 1. GXM influences the interaction of *C. neoformans* with A549 cells. The interaction of *C. neoformans* with lung cells was determined after microscopic observations of Giemsa-stained preparations (A and C) or CFU counting of viable yeasts recovered after incubation with host cells (B). The analysis of Giemsa-stained preparations shows that *C. neoformans* attachment (A) is significantly inhibited (asterisks, $P < 0.05$) when yeast cells ($T_1$444 strain) are incubated in the presence of Mab18B7 at 10 or 100 μg/ml, but not by the same antibody at 1 μg/ml or by an irrelevant IgG1 at 100 μg/ml. Pretreatment of host cells with GXM or of $T_1$444 yeast cells with Mab18B7 (B) results in lower levels (asterisks, $P < 0.05$) of recovery of viable yeasts (CFU), suggesting the involvement of GXM in the interaction of *C. neoformans* with A549 cells. Capsule size did not influence adhesion (C), since no significant differences were observed when different encapsulated strains (T_1-444 and HEC3393) were incubated with human cells (black bars) for prolonged periods (18 h). After 18 h of interaction, Mab18B7 also inhibited association between cryptococci and host cells (C, white bars), except for an acapsular (cap67) strain of *C. neoformans*.

Human cells were incubated in the presence of purified GXM for 1 or 18 h and analyzed by flow cytometry with Mab18B7, as demonstrated in Fig. 2. After 1 h of incubation with GXM, the A549 population was strongly recognized by Mab18B7, suggesting the existence of polysaccharide-binding sites in these cells. A similar profile of reactivity was observed after 18 h of incubation, although the fluorescence levels of GXM-containing cells were less intensive. In this condition, A549 cells remained viable (data not shown). The cellular distribution of the polysaccharide as a function of time was analyzed by fluorescence microscopy using the same antibody. After 2 h of incubation at 4 °C in the presence of GXM, A549 cells were incubated for 30 min, 1 or 18 h at 37 °C. After 30 min, a diffuse pattern of surface binding of GXM was observed (Fig. 3A). Extended periods of incubation resulted in GXM internalization by A549 cells, as demonstrated in Fig. 3D (1 h) and F (18 h). In all the conditions described above, fluorescent reactions were negative when the anti-GXM antibody was not used (control systems).

These results were indicative that GXM binding to the surface of alveolar cells could result in the internalization of GXM-containing yeasts. Such observations led us to investigate whether *C. neoformans* is also internalized by A549 cells. To evaluate this hypothesis, we compared the reactivity of *C. neoformans* (T_1-444 strain) with Mab18B7 in infected lung cells before and after permeabilization of the complexes with absolute methanol (Fig. 4A). Very strong fluorescent reactions with the yeast-associated polysaccharide were detected in permeabilized complexes, while fluorescent reactions with *C. neoformans* in non-permeabilized cells were absent or very weak. Images shown in Fig. 4A are representative of several microscopic fields in each experimental condition. Alternatively, *C. neoformans* yeasts were labeled with FITC and then incubated with A549 cells for 1 h. After extensive washing for removal of FITC-labeled non-adherent fungi, some of the complexes were treated with Trypan blue, which extinguishes the fluorescence of extracellular yeasts [28]. In this method, internalized fungi will maintain their fluorescence intact, since this dye is not capable of crossing intact membranes. Therefore, the index of fluorescence decrease after Trypan blue treatment can be correlated with the percentage of yeasts that remained adhered to the surface of A549 cells. In our conditions, approximately 37% of A549 cells became fluorescent after incubation with FITC-labeled cryptococci (Fig. 4B). This result is comparable to that obtained by Chaka et al. [28], who demonstrated that 30–50% of monocytes became fluorescent after 1 h of interaction with opsonized *C. neoformans*. In the current model, the percentage of fluorescent cells was decreased to 17% after Trypan blue treatment. Therefore, considering only the A549 population infected by *C. neoformans*, we can assume that around 45% of initially adhered yeast cells reach the intracellular environment after 1 h of incubation.

The infection of A549 cells by *C. neoformans* was also analyzed by flow cytometry using Mab18B7. After 1 h of interaction between fungi and the model alveolar epithelium and extensive washing for removal of non-associated yeasts, the majority of the A549 population (65%) became reactive with Mab18B7 (Fig. 5). This index of association was higher that that observed after incubation of FITC-labeled cryptococci with A549 cells, which could be explained by a putative secretion of GXM during infection of human epithelium, resulting in increased antibody reactivity. Surprisingly, the percentage of fluorescent A549 cells was much less after 18 h of incubation. A more detailed cytometric analysis of these cells revealed that, after 18 h of incubation, a large fraction of the human cell population presented reduced levels of forward and side scatters, which were suggestive of the presence of cell debris. These results led us to investigate whether the infection by *C. neoformans* causes damage in A549 cells, as described below.

Lysis of alveolar cells in the presence of *C. neoformans* was evaluated by measurement of LDH activity in supernatants after interaction between fungi and epithelial cells. Oxidation of NADH and consequent decrease in absorbance at 340 nm was measured as a function of time ($\Delta_{340\ nm}$/h). As demonstrated in Fig. 6, no significant differences ($P > 0.05$) were observed between the levels of LDH released from non-infected A549 cells kept at the culture medium for 18 h and preparations in which living *C. neoformans* yeasts were present for 1 or 8 h. However, 18 h exposure of A549 cells to cryptococci resulted in levels of LDH activity comparable to

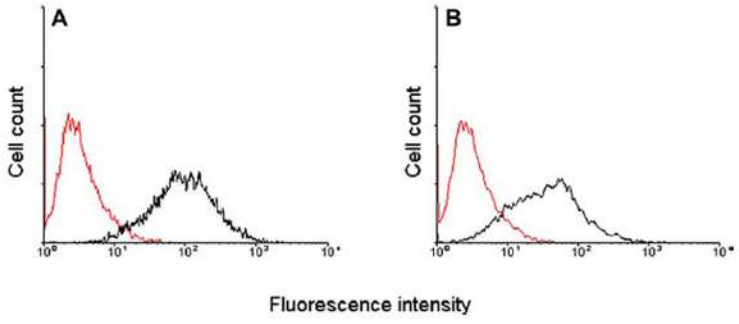

Fig. 2. Binding of purified GXM to A549 cells. Incubation of lung cells with soluble GXM for 1 (A) or 18 h (B), for further reaction with the anti-GXM antibody and a FITC-labeled anti-mouse antibody (black lines), shows that A549 cells are able to bind GXM. Control preparations were submitted to the same steps, but the anti-GXM antibody was replaced by PBS or an irrelevant mouse IgG1 (not shown). In both control systems, results were identical.

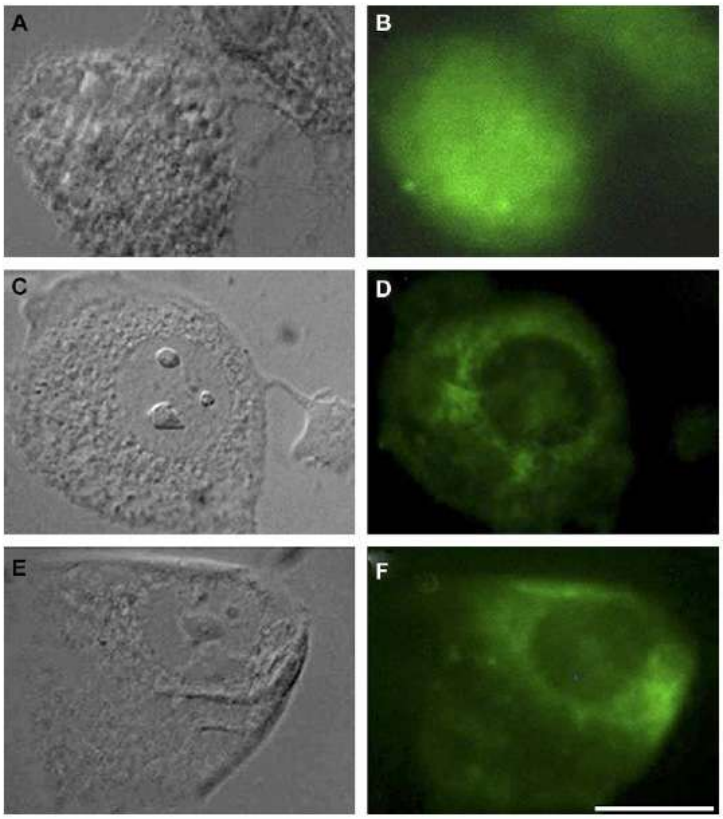

Fig. 3. Internalization of GXM by A549 cells. For GXM binding, lung cells were incubated for 2 h at 4 °C in the presence of the purified polysaccharide. The systems were then incubated for 30 min (A–B), 1 h (C–D) or 18 h (E–F) at 37 °C. After 30 min of incubation, a diffuse pattern of surface binding of GXM was observed (B). Extended periods of incubation resulted in GXM internalization, as demonstrated in D and F. Left panels show differential interferential contrast microscopy of GXM-containing lung cells; right panels represent the same images under the fluorescence mode. Control preparations were submitted to the same steps, but the anti-GXM antibody was replaced by PBS or an irrelevant mouse IgG1 (not shown). In both control systems, results were identical. Scale bar: 20 μm.

those obtained in a Triton lysate, indicating that *C. neoformans* causes lysis in this host cells. Paraformaldehyde-killed yeasts were unable to induce LDH release in A549 cells, since there were no significant differences between the enzyme activity observed in these systems and that observed after maintenance of non-infected alveolar cells for 18 h in culture medium. Purified GXM was not toxic to A549 cells (data not shown) and LDH activity from *C. neoformans* was not detected, even in mechanically-disrupted yeast cells (not shown).

4. Discussion

Infection with *C. neoformans* initiates in the lung, but cryptococci can surpass the local host defense and reach the central nervous system causing a fungal meningoencephalitis [1]. This observation implies that, in a successful infectious process, *C. neoformans* interacts with different host tissues, such as the lung epithelia [9,10], endothelial cells [31] and the blood brain barrier [32].

During the pulmonary infection by *C. neoformans*, inhaled yeasts are first deposited into the alveolar space, and then reach the lung interstitium [8]. Therefore, interaction of cryptococci with alveolar cells appears to be critical for establishment of infection, and the host elements involved in this process should include types 1 and 2 epithelial cells, macrophages and lung surfactant proteins. In this regard, the ability of cryptococci to interact with alveolar macrophages [12] and to bind surfactant proteins [33] has been well studied, and GXM has been shown to be involved in these different processes. However, the mechanisms involved in the fungal interaction with epithelial cells are largely unknown. Using A549 cells, Merkel and Scofield [16] demonstrated that simple carbohydrates such as sucrose, inositol, *N*-acetylglucosamine and *N*-acetylgalactosamine, as well as a monoclonal antibody produced against whole cryptococcal cells, inhibited adherence of *C. neoformans*, but host or fungal components involved in this process remained unknown. The inhibitory activity of purified capsular polysaccharides or capsule components, such as xylose and glucuronic acid, was not evaluated in this study.

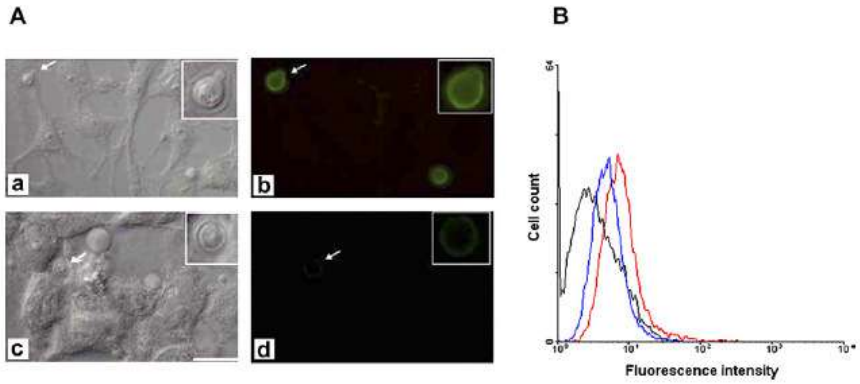

Fig. 4. Interaction of *C. neoformans* with A549 cells results in fungal internalization. Yeasts were incubated with epithelial cells and analyzed by indirect immunofluorescence with Mab18B7 (A). The strongest reactions between the anti-GXM antibody and yeast cells were observed after permeabilization of the complexes (a–b). In non-permeabilized cells (c–d), reactions were absent or very weak. Arrows indicate the presence of *C. neoformans* infecting A549 cells; these yeasts are also shown with higher magnification within insets. Scale bar: 10 μm. Alternatively (B), FITC-labeled cryptococci were incubated with A549 cells followed by flow cytometry analysis. After 1 h, approximately 37% of epithelial cells became fluorescent (red line), indicating their association with *C. neoformans*. When the complexes were treated with a fluorescence quenching agent (Trypan blue) after 1 h of interaction (blue line), the percentage of fluorescent cells was decreased (17%). Control systems (black line) consisted of non-infected A549 cells.

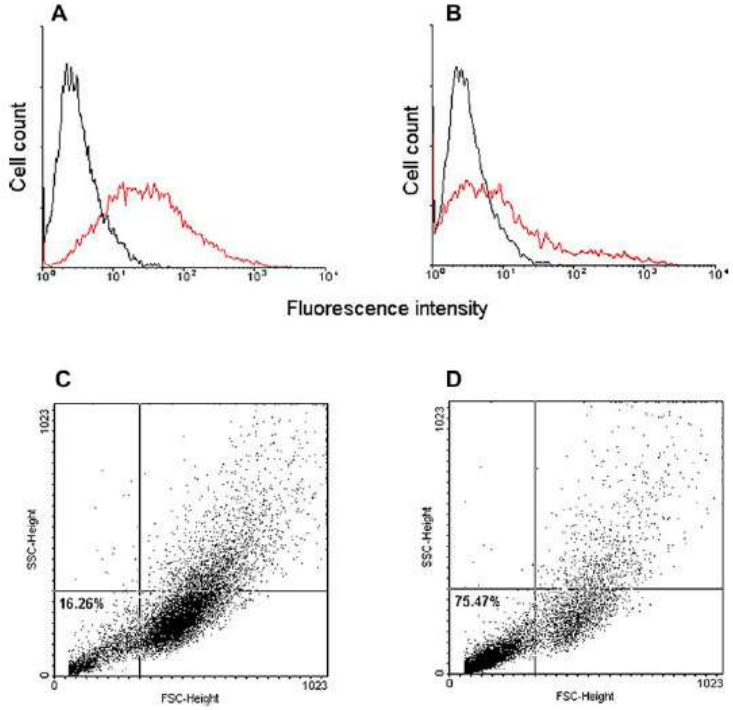

Fig. 5. Flow cytometry analysis of *C. neoformans* interaction with A549 cells. Fluorescence increase after Mab18B7 treatment (red lines) was the parameter used to identify A549 cells associated with cryptococci (A and B). In control preparations (black lines), *C. neoformans* was not present. After 1 h of interaction between fungi and host cells (A), the majority of the A549 population was fluorescent. After 18 h (B), the percentage of fluorescent host cells is markedly diminished. C and D show forward (FSC) and side (SSC) scatter analyses of the populations indicated by the red lines in A and B, respectively. These results are suggestive that A549 cells are damaged after 18 h of interaction with fungi.

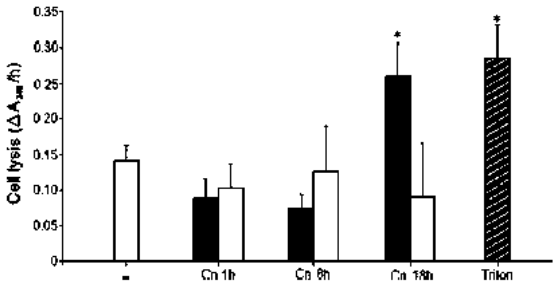

Fig. 6. *C. neoformans* (Cn) induces lysis in A549 cells. LDH activity was measured by the oxidation of NADH and consequent decrease in absorbance at 340 nm as a function of time ($\Delta_{340\ nm}$/h). Black bars represent LDH determinations in culture fluids of A549 cells incubated in the presence of living *C. neoformans* cells for the periods (h) indicated above, while white bars correspond to enzyme activity in supernatants of alveolar cells incubated with paraformaldehyde-killed cryptococci. The basal level of enzymatic activity (gray bar) was considered as the oxidation of NADH by culture supernatants of A549 cells kept in culture for 18 h, in the absence of *C. neoformans* (–). For a positive control of enzyme activity (hatched bar), a cell lysate was obtained by extraction with Triton. Levels of NADH oxidation in which statistical differences ($P < 0.05$) were obtained after comparison with the basal LDH activity are highlighted with an asterisk. There was no significant statistical difference between the levels of enzyme activity in the Triton lysate and that obtained from the culture supernatant of alveolar cells after 18 h of incubation in the presence of cryptococci. Levels of enzyme activity in the two later systems were significantly higher ($P < 0.05$) than any other measurement of NADH oxidation presented in this figure.

GXM has been largely reported as an inhibitory component during the interaction of *C. neoformans* with macrophages (reviewed in Ref. [1]). Recently, this polysaccharide has also been shown to negatively modulate the interaction of cryptococci with endothelial cells [31]. The effects of cryptococcal capsule components on epithelial infection are still unknown but, in other systems, microbial polysaccharides have been reported as key molecules modulating the association of bacteria with epithelia. For instance, Sharma and Qadri demonstrated that Vi capsular polysaccharide from *Salmonella typhi* modulates the bacterial interaction with the epithelial cell line Caco-2, involving lipid-raft associated host receptors [34]. In this context, our aim in the current study was to evaluate the influence of GXM on the interaction between *C. neoformans* and the human alveolar cell line A549.

Antibodies to capsular components are usually very effective in enhancing the association of *C. neoformans* with macrophages [21]. In our model, however, fungal adhesion to alveolar cells was inhibited by a monoclonal antibody to GXM. This observation could be explained by the fact that incubation of *C. neoformans* with monoclonal antibodies to GXM induces marked alterations in structural appearance of the polysaccharide capsule, as reviewed by Feldmesser and Casadevall [35]. However, the adhesion of cryptococci to alveolar cells was also inhibited by a purified preparation of GXM, suggesting that A549 cells express surface sites for capsule binding. In fact, our analyses by fluorescence microscopy and flow cytometry demonstrated that the alveolar cell line used in this study is able to bind soluble GXM, which may be related to the mechanism by which *C. neoformans* interacts with the alveolar epithelium.

Our results demonstrate that GXM is first associated with the surface of A549 cells and is then internalized producing an organized pattern of intracellular GXM distribution. This result contrasts with what has been shown in infected macrophages where GXM is randomly spread out the cytoplasm [12]. Such discrepancies may be explained by the different cellular compartments to which GXM is distributed, since the internalization of fungal cells or molecules can follow different mechanisms in phagocytic and non-phagocytic cells. In macrophages, the polysaccharide is shedded by ingested yeast cells, followed by damage to the phagosomal membrane and cytoplasmic accumulation of polysaccharide-containing vesicles. Secretion of GXM by *C. neoformans* infecting non-phagocytic cells could result in polysaccharide accumulation into different cellular compartments, but it is still unknown whether cryptococci is in fact able to shed capsule inside epithelial cells. Previous results by other groups [21] and those shown in Fig. 4A demonstrate that the reactivity of Mab18B7 in lung cells infected with *C. neoformans* in vivo or in vitro is limited to the wall-associated capsular polysaccharide. However, the fluorescence levels of A549 cells after incubation with *C. neoformans* followed by immunostaining with Mab18B7 are higher than those observed when epithelial cells are incubated with FITC-labeled cryptococci, suggesting that capsule secretion may occur. Another mechanism by which GXM could reach the intracellular space is the internalization of extracellularly secreted polysaccharides by alveolar cells, as we demonstrate in Fig. 3.

C. neoformans apparently uses GXM to adhere to A549 cells and this process also results in the passage of yeasts to the intracellular environment. It is still unknown what additional molecules are involved in this process. Surprisingly, preliminary results from our laboratory indicate that when incubations are performed with paraformaldehyde-killed yeasts, intracellular cryptococci are detected to a lesser extent (F.M. Barbosa, F.L. Fonseca, L. Nimrichter and M.L. Rodrigues, unpublished data). We believe therefore that the GXM-mediated adhesion of *C. neoformans* to host cells can be ensued by an active mechanism converging to invasion of the alveolar epithelium, possibly involving extracellular hydrolytic enzymes such as phospholipase B [8,36] and proteases [37].

After a relatively long (18 h) period in the presence of *C. neoformans*, a large number of A549 cells lost their reactivity with the anti-GXM antibody, compared to 1 h incubation. This observation, together with reduced levels of side and forward scatter measurements, indicated that *C. neoformans* escape from alveolar cells after an extended period of incubation, possibly through the lysis of host epithelia. This possibility was evaluated through the release of the intracellular enzyme LDH, which was considered as a parameter of host cell damage. Our results indicate that, after 18 h, LDH activity reaches maximum levels, which is in agreement with the fact that *C. neoformans* induces a critical injury in alveo-

lar cells [8]. Such pathogenic mechanism is in agreement with the observations that alveoli from infected mice present extensive epithelial lesions, accompanied by large amounts of extracellular budding yeasts [8]. GXM could be responsible for the toxic effects of *C. neoformans*, as observed in macrophages [12]. However, our results indicate that A549 cells keep their viability even after 24 h in the presence of a concentrated (1 mg/ml) solution of GXM (data not shown).

The potential receptors for GXM in A549 cells remain to be characterized. In other models, Toll-like receptors 2 and 4, CD14 and CD18 have been described as surface host molecules involved in GXM binding [38]. The expression of such receptors in epithelial cells is still controversial and is under investigation in our laboratory. Our most recent results indicate that the GXM-mediated adhesion of *C. neoformans* to A549 cells is dependent on the expression of CD14 receptor, whose activation by the major cryptococcal polysaccharide capsule results in cytokine production (F.M. Barbosa, F.L. Fonseca, R.T. Figueiredo, M. Bozza, L. Nimrichter and M.L. Rodrigues, unpublished data). Future studies about the interaction of fungal components with host receptors in alveolar cells could help elucidate the mechanisms of pathogenicity and local immune responses during respiratory and systemic mycoses.

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Binding of Glucuronoxylomannan to the CD14 Receptor in Human A549 Alveolar Cells Induces Interleukin-8 Production▽

Fabiane M. Barbosa,[1] Fernanda L. Fonseca,[1] Rodrigo T. Figueiredo,[2] Marcelo T. Bozza,[2] Arturo Casadevall,[3,4] Leonardo Nimrichter,[1]† and Marcio L. Rodrigues[1]†*

Laboratório de Estudos Integrados em Bioquímica Microbiana, Departamento de Microbiologia Geral,[1] and Departamento de Imunologia,[2] Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, 21941590 Rio de Janeiro, Brazil, and Department of Microbiology and Immunology[3] and Division of Infectious Diseases, Department of Medicine,[4] Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461

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Glucuronoxylomannan (GXM) is the major capsular polysaccharide of *Cryptococcus neoformans*. GXM receptors have been characterized in phagocytes and endothelial cells, but epithelial molecules recognizing the polysaccharide remain unknown. In the current study, we demonstrate that GXM binds to the CD14 receptor in human type II alveolar epithelial cells, resulting in the production of the proinflammatory chemokine interleukin-8.

Cryptococcus neoformans is an encapsulated fungal pathogen infecting mainly immunosuppressed patients. Infections are acquired by inhalation of desiccated cells, which are available in the environment as basidiospores or poorly encapsulated yeasts. Inhaled cells are deposited in the alveolar space, where they are ingested by macrophages (24) and interact with epithelial cells (2, 7, 8). An effective interaction of *C. neoformans* with epithelial alveolar cells is probably essential for the establishment of pulmonary infection. Microscopic studies of pulmonary cryptococcal infection reveal that yeast cells are in close apposition to lung epithelial cells (7). Once *C. neoformans* becomes established in the lung, it proliferates locally and causes a primary lesion that is usually contained by granuloma formation (10, 24). Two components of *C. neoformans* that are important for adhesion to human type II alveolar epithelial cells are phospholipase B (8) and glucuronoxylomannan (GXM) (2).

GXM is a virulence factor (4) that represents a potential vaccine component and is the target of therapeutic antibodies (3, 11, 17, 19). As the major constituent of the capsule, it is the primary component of a structure that is antiphagocytic and thus protects the fungal cell from immune cells. However, cryptococcal infections are also associated with the release of large amounts of GXM into host tissues, where they have many deleterious effects on the host immune response through multiple mechanisms (15). GXM has been reported to interact with numerous cellular receptors. Receptors for GXM in macrophages, neutrophils, and endothelial cells include Toll-like receptor 2 (TLR2), TLR4, CD14, and CD18 (12, 13, 16, 25). The receptors for GXM in epithelial cells, however, remain to be characterized.

* Corresponding author. Mailing address: Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde (CCS), bloco I, 21941590 Rio de Janeiro, Brazil. Phone: 55 21 2562 6740. Fax: 55 21 2560 8344. E-mail: marcio@micro.ufrj.br.

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GXM was purified from strain T_1-444 of *C. neoformans* (serotype A) by following standard methods (5) and incubated with A549 cells (100 μg/ml) for 1 h at 37°C (2). Binding of GXM to host cells was confirmed by immunofluorescence with the GXM-binding monoclonal antibody (MAb) 18B7 (3) (Fig. 1, inset). Lysates were obtained as described previously (21) and immunoprecipitated by sequential incubation with MAb 18B7 (3) and Sepharose-bound protein G. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the immunoprecipitated material indicated the presence of a major band with a molecular mass corresponding to 55 kDa (Fig. 1A). The same mixture analyzed by SDS-PAGE was boiled for 5 min to disintegrate protein-polysaccharide conjugates. GXM was then removed from this preparation by ultrafiltration (cutoff, 100 kDa), and the filtrate was incubated with Sepharose-bound protein G for antibody depletion. The purified fraction containing the GXM-binding protein, but no polysaccharide or antibodies, was concentrated to dryness and used to coat polystyrene 96-well plates (5 μg/ml, 100 μl/well). After the addition of GXM in serial dilutions, the plates were sequentially incubated for 1 h with MAb 12A1, a mouse immunoglobulin M (IgM) MAb with specificity for GXM (6), and a phosphatase-labeled goat anti-mouse antibody with specificity for IgM. Reactions were developed after the addition of *p*-nitrophenyl phosphate, followed by a reading at 405 nm. As demonstrated in Fig. 1B, the purified fraction binds GXM in a dose-dependent fashion.

CD14 is a 55-kDa glycosylphosphatidylinositol-anchored membrane protein found mainly on cells derived from the monocyte/macrophage lineage, as well as neutrophils and B lymphocytes (8, 18). This molecule, which is a member of the heteromeric lipopolysaccharide (LPS) receptor complex that also contains TLR4 and MD2 (17), is a GXM receptor in endocytic cells (12, 16, 25). This information and the molecular mass of the purified GXM-binding protein presented in Fig. 1A led us to investigate whether CD14 could be the GXM receptor expressed by A549 cells. Human cells were detached from culture plates using 1 mM EDTA and fixed in 4% para-

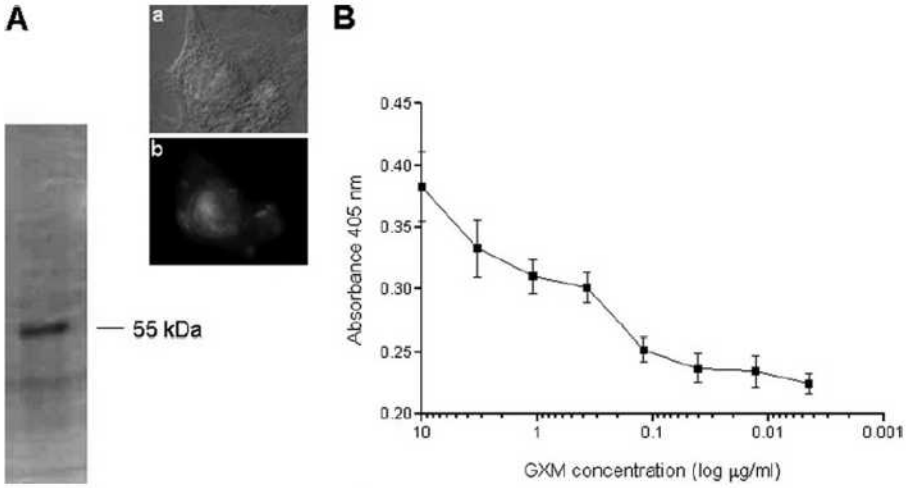

FIG. 1. Purification of a GXM-binding molecule from A549 cells by immunoprecipitation. (A) Protein extracts from GXM-treated human cells were sequentially incubated with MAb 18B7 and Sepharose-bound protein G for further analysis by SDS-PAGE. GXM binding to epithelial cells was confirmed by immunofluorescence with MAb 18B7 (inset). An A59 cell observed under differential interferential contrast (a) and fluorescence (b) modes is shown. A single major protein with a molecular mass corresponding to 55 kDa was detected. The purified fraction was used in GXM-binding assays in 96-well plates. (B) Dose-dependent binding of the 55-kDa protein to GXM detected by an IgM MAb to GXM.

formaldehyde. After blocking with Tris-buffered saline containing bovine serum albumin, A549 cells were incubated with a phycoerythrin (PE)-labeled mouse MAb to CD14 (BD Biosciences, San Jose, CA). Analysis in a FACSCalibur (BD Biosciences, San Jose, CA) flow cytometer revealed that A549 cells express CD14 (Fig. 2A), confirming previous reports (9, 20, 23). Control cells, incubated with an irrelevant PE-labeled antibody, presented low levels of fluorescence.

Given our recent observations that *C. neoformans* efficiently infects A549 cells (2), we evaluated whether a CD14-binding molecule would interfere with this process. Untreated human cells or an A549 population that was pretreated with LPS (10 μg/ml) for 1 h was incubated with *C. neoformans* under previously established conditions (2). The index of association between cryptococci and alveolar cells was measured as the reactivity of A549 cells with MAb 18B7 in flow cytometry assays (2), which showed that the efficacy of the interaction of cryptococci with untreated cells was higher than that observed with LPS-treated epithelia (Fig. 2B). Controls consisted of similar preparations that were not infected with *C. neoformans*. Pretreatment of A549 cells with anti-CD14 antibodies also resulted in a lower association between *C. neoformans* and host cells (data not shown).

To confirm that CD14 is indeed a GXM-binding molecule in human alveolar cells, a modification of classical immunoprecipitation methods was used. Lysates of GXM-treated cells, presumably containing polysaccharide-CD14 complexes, were added to the wells of a 96-well plate previously coated with the

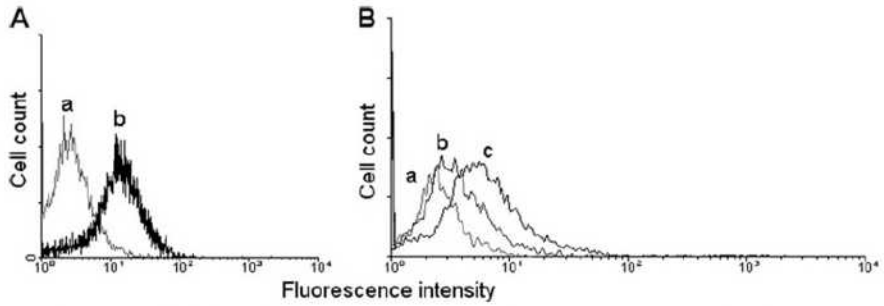

FIG. 2. Expression of CD14 by A549 cells. (A) Flow cytometric analysis of human cells incubated with a PE-labeled MAb to CD14 demonstrating that the receptor is expressed on the surface of A549 cells. Unstained (a) and antibody-treated (b) cells are shown. (B) Pretreatment of human cells with LPS, a CD14-binding compound, decreases their association with cryptococci. The increase in fluorescence after incubation of infected cells with MAb 18B7 was used to identify A549 cells in association with cryptococci. Infected cells that were pretreated with LPS (b) presented a reduced reaction with MAb 18B7, in comparison with untreated cells (c). The fluorescence levels of uninfected cells is shown in panel a.

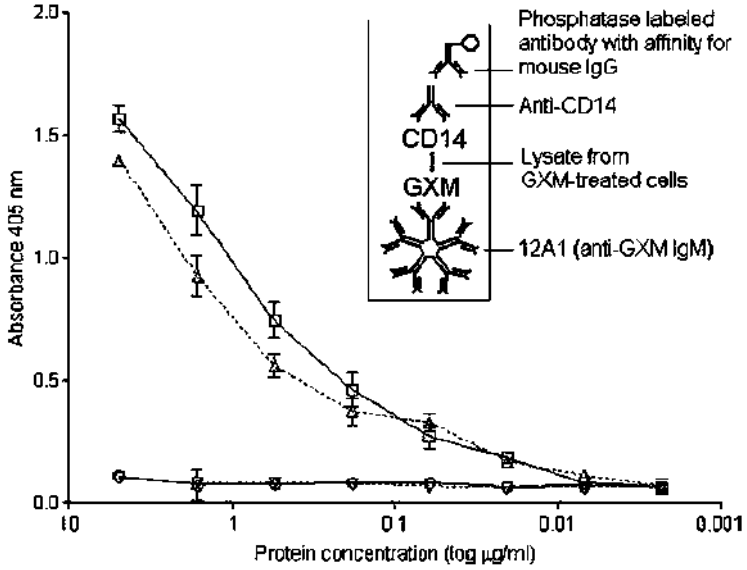

FIG. 3. Identification of CD14 as the epithelial receptor for GXM in A549 cells. CD14-GXM complexes (triangles) from polysaccharide-treated cells were captured by IgM antibodies in ELISA. An anti-CD14 was used as the detection probe, as demonstrated in the schematic presentation of the capture ELISA (inset). No significant reactions with the anti-CD14 antibody were observed when protein extracts from untreated cells (inverted triangles) were used. Additional controls included an ELISA in which A549 lysates (positive control; squares) and protein extracts from Chinese hamster ovary cells (negative control; circles) were used to coat the plates, followed by sequential incubation with the anti-CD14 MAb and phosphatase-labeled secondary antibodies.

anti-GXM IgM 12A1. After successive blocking and washing, the plate was incubated with a mouse MAb (IgG) to CD14 (BD Biosciences, San Jose, CA) and then with a phosphatase-labeled goat antibody with specificity for mouse IgG. CD14 was coprecipitated with GXM, as suggested by the dose-dependent recognition of complexes by the anti-CD14 antibody (Fig. 3). Controls consisted of similar assays in which lysates from GXM-treated cells were replaced by extracts from untreated alveolar cells. Additional controls included enzyme-linked immunosorbent assays (ELISAs) in which A549 lysates (positive control) and protein extracts from Chinese hamster ovary cells (negative control) were used to coat the plates, followed by blocking and sequential incubation with the anti-CD14 MAb and phosphatase-labeled secondary antibodies.

The association of microbial compounds with CD14 in alveolar cells can elicit the secretion of proinflammatory cytokines (20). We therefore speculated whether A549 cells could produce a cytokine response activated by GXM. For cytokine determinations, the culture medium was replaced by fresh medium containing no serum but supplemented with 10 μg/ml GXM. After 4 h at 37°C in a 5% CO_2 atmosphere, culture supernatants were collected and assayed for cytokines by using a RayBio human cytokine antibody array (RayBiotech, Inc.). Procedures followed the manufacturer's protocol. Cytokine production was quantified by using Scion Image 2000 software (Scion Corporation, NIH). Twenty cytokines were assayed, and under our experimental conditions, the production of interleukin-8 (IL-8) by GXM-treated cells was considered to be significantly different from that detected in nonstimulated supernatants (Fig. 4). GXM induced the secretion of growth-related oncogene α and IL-3 by human cells (data not shown), although the results obtained with the control and GXM-treated cells were not significantly different.

Since the CD14-LPS association resulted in IL-8 production by epithelial cells in previous studies (20), we investigated whether binding of GXM to CD14 molecules would have the same effect. Supernatants of nonstimulated A549 cells or of human cells after treatment for 1 h with LPS (positive control, 10 μg/ml), *C. neoformans* (2), or GXM (100 μg/ml) were col-

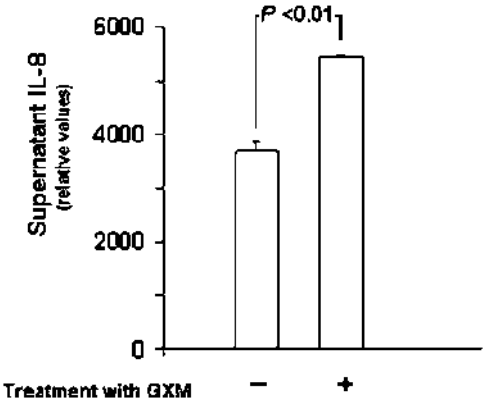

FIG. 4. Production of IL-8 by GXM-treated cells. Supernatants of untreated (–) or polysaccharide-treated (+) cells were collected for cytokine detection. An increased production of IL-8 was observed in supernatants of GXM-treated cells.

TABLE 1. Stimulation of A549 cells with GXM results in CD14-mediated production of IL-8[a]

Treatment of A549 cells[b]	Supernatant IL-8 (ng/ml) (mean ± SD)	P[c]	Statistical significance
None	0.82 ± 0.06		
LPS (10 μg/ml, 1 h)	1.33 ± 0.01	0.007	Yes
C. neoformans (10 yeast cells/host cell)	1.06 ± 0.01	0.030	Yes
GXM (100 μg/ml, 1 h)[d]	1.18 ± 0.03	0.017	Yes
Anti-CD14 (10 μg/ml), then GXM (100 μg/ml)	0.54 ± 0.12	0.085	No

[a] Results are representative of three different assays producing similar results. The error bars were generated from multiple trial wells ($n = 3$) of each single sample.

[b] A549 cells were treated with LPS, fungal cells, GXM, or anti-CD14 antibodies for 1 h at 37°C.

[c] P values were obtained by the use of Student's t test, after comparison with results of control systems (no stimulation). Differences between results with stimulated systems and control cells were considered statistically significant when P values were smaller than 0.05.

[d] Results obtained for A549 cells treated with GXM alone were statistically compared to the data obtained for alveolar cells sequentially treated with the antibody to CD14 and GXM. IL-8 levels detected in cells after treatment with GXM alone were significantly higher ($P < 0.01$) than those obtained in A549 cells that were first treated with the antibody to CD14.

lected, and the presence of IL-8 was assayed by ELISA-based techniques (human CXCL8/IL-8 detection kit; R&D Systems, Minneapolis, MN). Under any of the conditions used for chemokine determination, the viability of the A549 population was significantly affected (data not shown). A significant increase in IL-8 production was observed when fungal cells, GXM, or LPS was incubated with A549 cells (Table 1). The levels of IL-8 produced under our experimental conditions were very similar to those observed in a previous study using LPS-treated A549 cells (14). Supplementation of the polysaccharide solution with polymyxin B produced similar ($P = 0.18$) levels of IL-8 (data not shown), indicating that the results obtained were not due to LPS contamination. When human cells had been previously incubated with antibodies to CD14, however, GXM-treated and nonstimulated cells expressed similar levels of IL-8 (Table 1).

In microbial infections, a primary function of the airway epithelium is to act as a physical barrier for the exclusion of inhaled infectious propagules. However, there is increasing evidence that lung cells can induce a localized immune response through the production of a variety of mediators, including proinflammatory cytokines and chemokines (1). These mediators could act, for example, to recruit polymorphonuclear leukocytes from the pulmonary vasculature into the alveolar space. With fungi, it has been demonstrated that *Aspergillus fumigatus* proteases elicit IL-6 and IL-8 production in lung epithelial cells in vivo and in vitro (26). In cases of cryptococcosis, the production of these proinflammatory mediators is associated with the survival of human patients (22). The production of such molecules by the airway epithelium could therefore represent an effective mechanism for the establishment of a local immune response controlling microbial lung infections.

GXM receptors have been characterized in a number of cells (12, 13, 16, 25). This polysaccharide mediates the interaction of *C. neoformans* with different host cells and induces a deleterious effect on the immune system (15). Interactions of cryptococci with epithelial cells are influenced by GXM (2), but the host receptors involved in this process are unknown. The identification of CD14 as an epithelial molecule interacting with GXM with the consequent production of IL-8 brings to light the potential role of epithelial respiratory cells in immunity against *C. neoformans*.

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Paracoccidioides brasiliensis Enolase Is a Surface Protein That Binds Plasminogen and Mediates Interaction of Yeast Forms with Host Cells[▽]

Sarah Veloso Nogueira,[1] Fernanda L. Fonseca,[2] Marcio L. Rodrigues,[2] Vasanth Mundodi,[3] Erika A. Abi-Chacra,[2] Michael S. Winters,[4] John F. Alderete,[3] and Célia Maria de Almeida Soares[1]*

Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74001-970 Goiânia, Goiás, Brazil[1]; Laboratorio de Estudos Integrados em Bioquimica Microbiana, Instituto de Microbiologia Professor Paulo de Goes, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, Brazil[2]; School of Molecular Biosciences, Washington State University, Pullman, Washington 99163[3]; and Division of Infectious Diseases, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267[4]

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Paracoccidioidomycosis (PCM), caused by the dimorphic fungus *Paracoccidioides brasiliensis*, is a disseminated, systemic disorder that involves the 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 glutathione *S*-transferase (GST) fusion protein (recombinant *P. brasiliensis* enolase [r*Pb*Eno]). The *P. brasiliensis* native enolase (*Pb*Eno) was detected at the fungus surface and cytoplasm by immunofluorescence with an anti-r*Pb*Eno antibody. Immobilized purified r*Pb*Eno bound plasminogen in a specific, concentration-dependent fashion. Both native enolase 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 upregulated in yeast cells derived from mouse-infected tissues. These data indicate that surface-associated *Pb*Eno may contribute to the pathogenesis of *P. brasiliensis*.

A 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 abilities of different organisms (28, 40). 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 infection processes (47, 51).

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 lungs and the reticuloendothelial 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 *P. brasiliensis* is not traditionally considered 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 adhesiveness of the fungus *in vitro* as well as to an enhancement of pathogenic potential. We have been systematically searching for new adhesion proteins in *P. brasiliensis* that have the potential to play roles in the fungal virulence, and proteins such as *P. brasiliensis* malate synthase (*Pb*MLS) (34), *Pb*Dfg5p (defective for filamentous growth protein) (10), triosephosphate isomerase (*Pb*TPI) (41), and glyc-

* Corresponding author. Mailing address: Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, ICBII, Campus II, Universidade Federal de Goiás, 74001-970, Goiânia, Goiás, Brazil. Phone and fax: 55-62-35211110. E-mail: celia@icb.ufg.br.

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eraldehyde-3-phosphate dehydrogenase (*Pb*GAPDH) (4) were found to associate with ECM components. In particular, enolase from *P. brasiliensis* (*Pb*Eno) 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. Its 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) and tissue-type plasminogen activator (tPA) (9). 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). Microbe-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 now. In this study, 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, recombinant *Pb*Eno (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 *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 enolase coding region into an 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′; SalI restriction site underlined) and antisense (5′-GCG GCC GCT TAC ATA TTA ATA GCT GCC C-3′; NotI 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 at 200 rpm. Protein expression was induced by the 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 rabbits with Freund's adjuvant three times at 2-week 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 phenylmethylsulfonyl 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 12,000 × *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 10,000 × *g*. The pellet was washed twice with acetone and air dried prior to resuspension 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 in 1975 (37). The second dimension was performed as described by Laemmli in 1970 (27). Protein spots were excised from the gel and submitted to reduction, alquilation, and in-gel digestion with trypsin (Promega, Madison, WI). The resulting tryptic peptides were extracted and submitted to mass spectrometry (MS) analysis. The protein tryptic fragments were analyzed using a 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) by using the MASCOT search engine (Matrix Science).

Western blot and ligand binding analyses. 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 phosphate-buffered saline (PBS) and incubated o/n with human plasminogen (hPlg; Sigma) (35 μg/ml) in PBS–1% bovine serum albumin (BSA) (wt/vol). The blot was washed and incubated with mouse anti-human plasminogen monoclonal antibody (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. An 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 (i) rabbit anti-r*Pb*Eno antibodies or (ii) plasminogen followed by incubation with antiplasminogen 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 1:1,000. 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 and 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 to 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

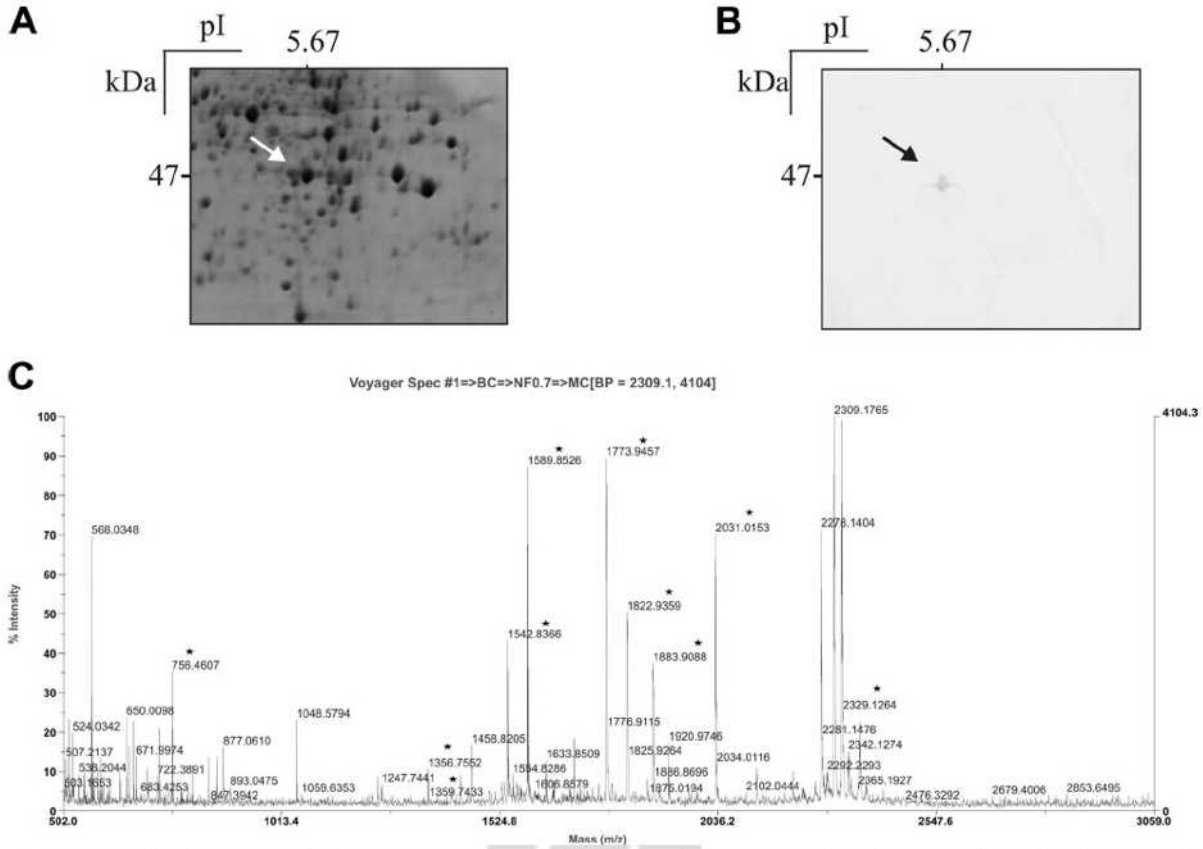

FIG. 1. Identification of enolase in the *P. brasiliensis* proteome by two-dimensional gel electrophoresis. (A) Protein staining with Coomassie blue. (B) Reactivity of the *P. brasiliensis* total extract with rabbit polyclonal antibodies to enolase raised to the recombinant protein. Numbers to the left of panels A and B refer to the molecular mass of the enolase. At the top is the isoelectric point of the protein. Arrows point to enolase. (C) Peptide mass spectrum generated from tryptic digestion of *Pb*Eno. The protein reacting with polyclonal antibodies was removed from the gel and submitted to mass spectrometry analysis after trypsin digestion. The black stars indicate the peaklist for enolase, and each peak corresponds to a peptide. Experiments represent three gels from independent protein preparations.

the addition of 1 μg of hPlg. Binding was determined by incubation with antiplasminogen 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} by 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 the 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 antiplasminogen monoclonal antibody. Following three washes, the wells were developed as described above. All final volumes for the enzyme-linked immunosorbent assay (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-lysyl-*p*-nitroaniline hydrochloride) (Sigma), and 15 ng of tissue plasminogen activator (tPA) (Sigma). Control experiments were performed by measuring the generation of plasmin either in 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 preincubated 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 (4 mg; Sigma) in a final volume of 2 ml. Controls consisted of untreated cells (no plasminogen incubation) or incubation systems where no cells were added. The jellified matrix was incubated in a humidified chamber at 37°C 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 [RAW] 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) supple-

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

Position	Identified amino acid sequence	Mass	
		Exptl (in-gel digestion)	Theoretical (*in silico* digestion)
16–32	R.GNPTVEVDVVTETGLHR.A	1,822.9489	1,822.9293
33–50	R.AIVPSGASTGQHEACELR.D	1,882.9548	1,825.8861
90–103	K.VDEFLNKLDGTPNK.S	1,589.8751	1,588.8678
106–120	K.LGANAILGVSLAIAK.A	1,410.9979	1,410.8678
164–184	R.LAFQEFMIVPTAAPSFSEALR.Q	2,325.2178	2,325.1947
243–254	K.IALDIASSEFYK.A	1,356.7701	1,356.7045
274–285	K.WLTYEQLADLYK.K	1,542.8513	1,542.7838
314–331	K.TCDLQVVADDLTVTNPIR.I	2,030.0239	1,973.0008
377–393	R.SGETEDVTIADIVVGLR.A	1,773.9489	1,773.9228
411–416	K.LNQILR.I	756.4464	756.4726

[a] Protein scores higher than 76 are significant ($P < 0.05$). 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.

mented with 10% (vol/vol) fetal bovine serum (FBS) at 37°C with 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 various 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 tetramethyl rhodamine isothiocyanate (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 with 5% CO_2 for 18 h. Cells were washed three times with PBS to remove nonadherent yeasts. Fungus-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 by using uninfected cells and nonstained 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 seventh day after infection; livers and spleens were removed from mice infected intraperitoneally, and lungs were removed from mice infected intranasally. One hundred milliliters of this suspension was plated onto brain heart infusion (BHI) agar (Becton-Dickinson, MD) 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 described above.

Quantitative analysis of RNA transcripts by qRT-PCR. Total RNAs were treated with DNase, and cDNA was prepared using Superscript II reverse transcriptase (Invitrogen) and oligo$(dT)_{15}$ primer. Quantitative real-time reverse transcription-PCR (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 picomoles 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 nontemplate 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 triplicate with samples in triplicates. Results are presented as means ± standard deviations. Statistical comparisons were performed using Student's *t* test. Statistical significance was accepted for *P* values of <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 *Pb*Eno (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 a pI of 5.67 was recognized by polyclonal antibodies raised to r*Pb*Eno (Fig. 1A and B). The protein was analyzed by mass spectrometry (Fig. 1C). Experimental masses were searched against data from public gene databases by using MASCOT. The peptides obtained (Table1) matched *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 the culture filtrate (Fig. 2A, lane 6). Bovine serum albumin (Fig. 2A, lane 1) and r*Pb*Eno (Fig. 2A, lane 2) were employed as negative and positive controls, respectively. Altogether, these results suggest that *Pb*Eno is associated with the cell wall and is secreted to the extracellular space in addition to its expected intracellular distribution in *P. brasiliensis*.

To further validate *Pb*Eno's association with the fungal surface, immunofluorescence was performed. As shown in Fig. 2B

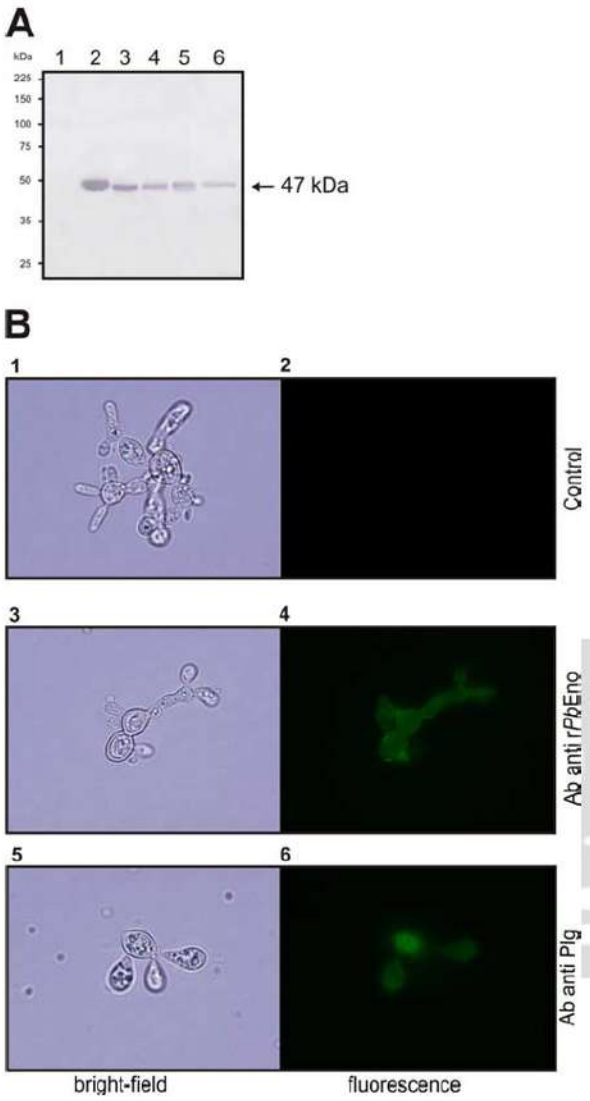

FIG. 2. Detection of *Pb*Eno and plasminogen binding at the cell surface of *P. brasiliensis*. Experiments were performed in triplicate. (A) Western blot analysis of bovine serum albumin (BSA; lane 1), r*Pb*Eno (lane 2), *P. brasiliensis* crude protein extract (lane 3), cell wall enriched fraction proteins (lane 4), the soluble cytoplasmic fraction (lane 5), and secreted proteins (lane 6) blotted onto a nylon membrane and detected with rabbit polyclonal anti-recombinant enolase antibodies. The arrow indicates enolase. (B) Paraformaldehyde-fixed, nonpermeabilized cells were incubated with r*Pb*Eno antibodies (Ab; panels 3 and 4) or treated with human plasminogen followed by incubation with an antibody raised to this protein (panels 5 and 6). Control systems were obtained with anti-rabbit immunoglobulin G (IgG) coupled to alkaline phosphatase antibody only (panels 1 and 2). Bright-field microscopy is shown in left panels. The same cells are shown under the fluorescence mode in the right panels.

(panel 4), rabbit polyclonal antibodies reacted with the surface of the organism. Plasminogen antibodies (Fig. 2B, 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 (Fig. 2B, panel 2).

***P. brasiliensis* and *Pb*Eno bind plasminogen.** By the immunofluorescence 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, the 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 for other organisms (15). Therefore, the ability of r*Pb*Eno to bind hPlg was tested in an 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 by using competitive antagonism with the lysine analog ε-ACA. The results shown in Fig. 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 had been seen to bind hPlg, we had expected that this interaction could also activate hPlg. For this reason, an ELISA was performed to determine the abilities 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 resulted 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 (1, 25). As demonstrated in Fig. 4C, the association of *P. brasiliensis* with plasminogen and tPA promoted increased fibrinolysis (Fig. 4C,

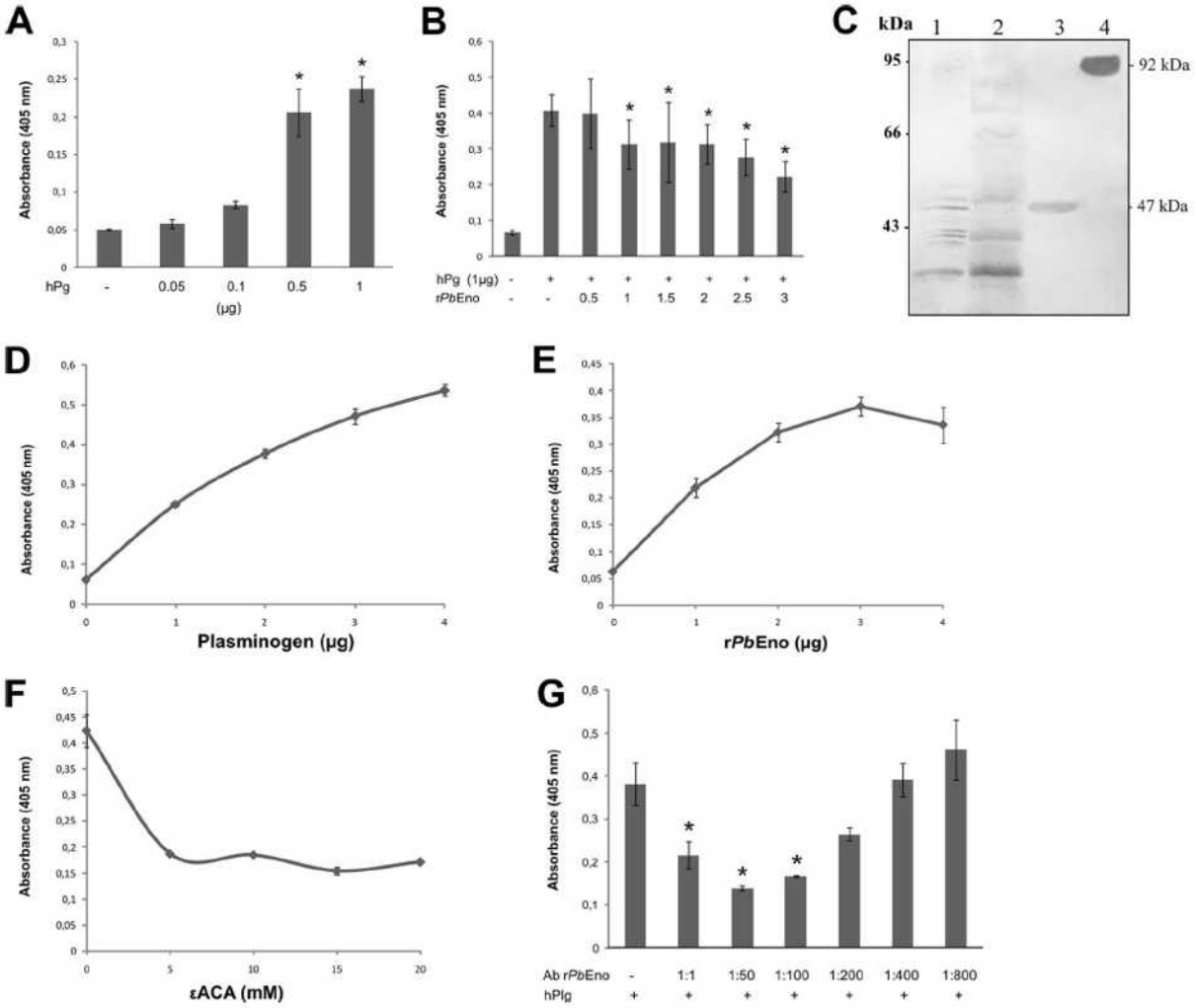

FIG. 3. Plasminogen binding assays. Microtiter plates were coated with fixed *P. brasiliensis* yeast cells as detailed in Materials and Methods. (A) Plasminogen (0.05 to 1.0 μg) binds to fixed *P. brasiliensis* in a concentration-dependent manner. (B) In a competition assay, binding of plasminogen is inhibited by increasing amounts of r*Pb*Eno (0.5 to 3.0 μg). (C) Binding of *P. brasiliensis* proteins to plasminogen. *P. brasiliensis* crude protein extract (lane 1), cell wall enriched fraction proteins (lane 2), r*Pb*Eno (lane 3), and hPlg (lane 4) were sequentially incubated with plasminogen and a mouse monoclonal anti-human plasminogen antibody. The numbers on the left side are molecular size markers. (D) Plasminogen (1 to 4 μg) binds to r*Pb*Eno (1 μg) immobilized on microtiter well plates in a concentration-dependent manner. ELISAs were developed at A_{405} with antiplasminogen antibody. (E) r*Pb*Eno (1 to 4 μg) binds to immobilized plasminogen (1 μg) in a similar fashion. The assay was developed at A_{405} with antibodies to r*Pb*Eno. (F) Effects of different ε-ACA concentrations (5 to 20 mM) on plasminogen binding. (G) Plasminogen binding to immobilized r*Pb*Eno is specifically inhibited by anti-r*Pb*Eno. Microtiter plates were coated by overnight incubation with 1 μg of r*Pb*Eno. After blocking, the wells were incubated with decreasing concentrations of rabbit polyclonal r*Pb*Eno antibodies. Reactions were developed after incubation with hPlg, the antiplasminogen antibody followed by secondary antibodies. Panels A, B, D, E, F, and G show the averages of three independent experiments performed in triplicates. The error bars indicate the standard deviations from three independent experiments performed in triplicate. *, significantly different from control, at a *P* value of <0.05.

lane 3). Aprotinin (lane 4) and PMSF (lane 5) inhibited proteolysis, indicating the specificity of the reaction. No proteolysis was observed either when *P. brasiliensis* yeast cells were used alone or in the presence of plasminogen (lanes 1 and 2, respectively). Figure 4C, lane 6, shows a 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% of adhesion to A549 epithelial cells. In this work, the participation of enolase in the infection of host cells by *P. brasiliensis* was evaluated by flow cytometry and fluorescence microscopy. Exposure of human epithelial cells (Fig. 5A, panel a) and murine phagocytes (Fig. 5A, panel b) to r*Pb*Eno resulted in an expressive increase in their reactivity with WGA, suggesting that the enzyme modifies the surface of host cells to

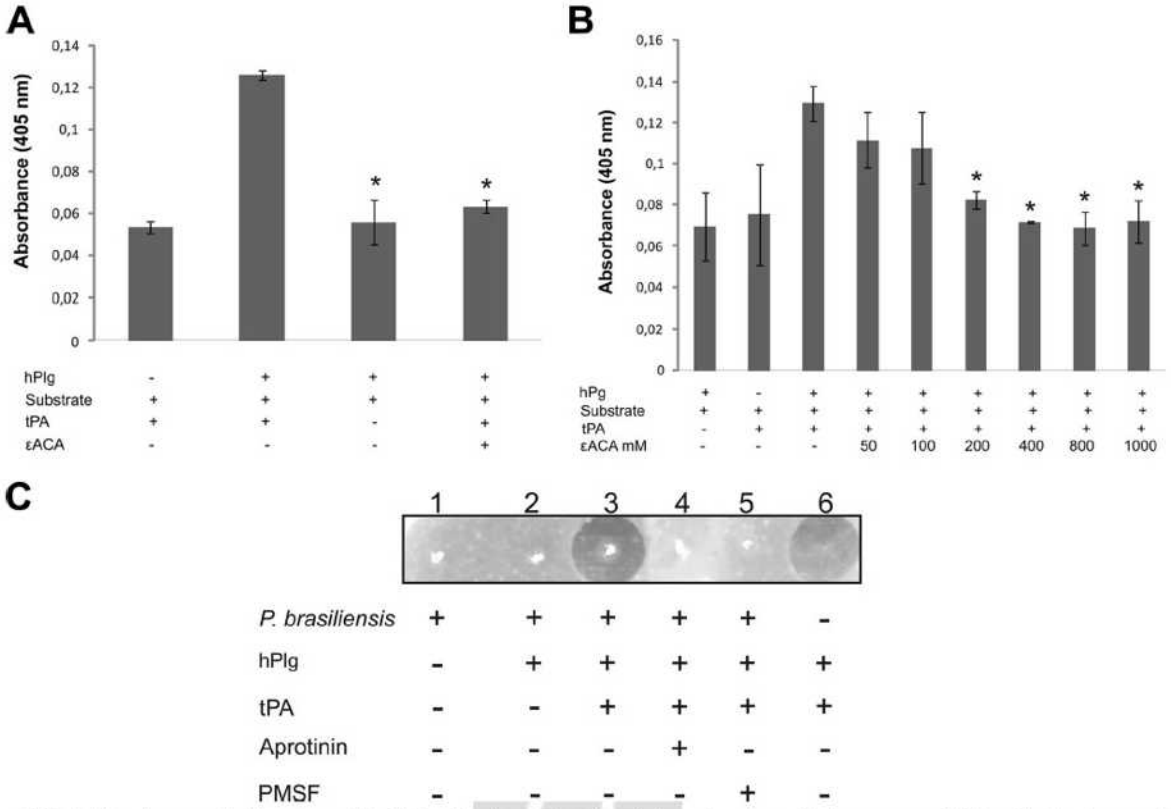

FIG. 4. Plasminogen activation assays. (A) r*Pb*Eno (1 μg) generates plasmin from plasminogen in the presence of tPA and in the absence of ε-ACA. (B) *P. brasiliensis* converts plasminogen into plasmin in the presence of tPA. Various concentrations of ε-ACA (50 mM to 1,000 mM) were added to wells containing fixed *P. brasiliensis*, followed by the addition of plasminogen, and an ELISA was performed as described in Materials and Methods. The error bars indicate the standard deviations from three independent experiments performed in triplicate. *, significantly different from control, at a *P* value of <0.05. (C) Fibrinolytic activity of plasminogen-bound *P. brasiliensis*. Lane 1, *P. brasiliensis* cells in the absence of plasminogen; lane 2, *P. brasiliensis* cells after binding to plasminogen. Lanes 3, 4, and 5 are similar to lane 2, except that they reflect the presence of tPA, tPA plus aprotinin, and tPA plus PMSF, respectively. Lane 6, controls consisting of plasminogen and tPA.

promote an enhanced exposure of GlcNAc residues, which are recognized by a *P. brasiliensis* adhesin (23). We therefore asked whether exposure to the enzyme would make mammalian cells more susceptible to infection by *P. brasiliensis*.

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

To evaluate if *P. brasiliensis* yeast cells were internalized by epithelial cells, the fungus was treated with trypan blue. Exposure to this dye caused an expressive decrease in the levels of fluorescence of infected A549 cells, suggesting that fungal cells adhered to but were not internalized by alveolar epithelial cells. In contrast, the fluorescence levels of infected macrophages were barely affected by exposure to trypan blue. This indicates that internalization of *P. brasiliensis* by the phagocytes, and 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 *Pb*Eno by real-time PCR in models of infection. We speculated that if *Pb*Eno was required for efficient

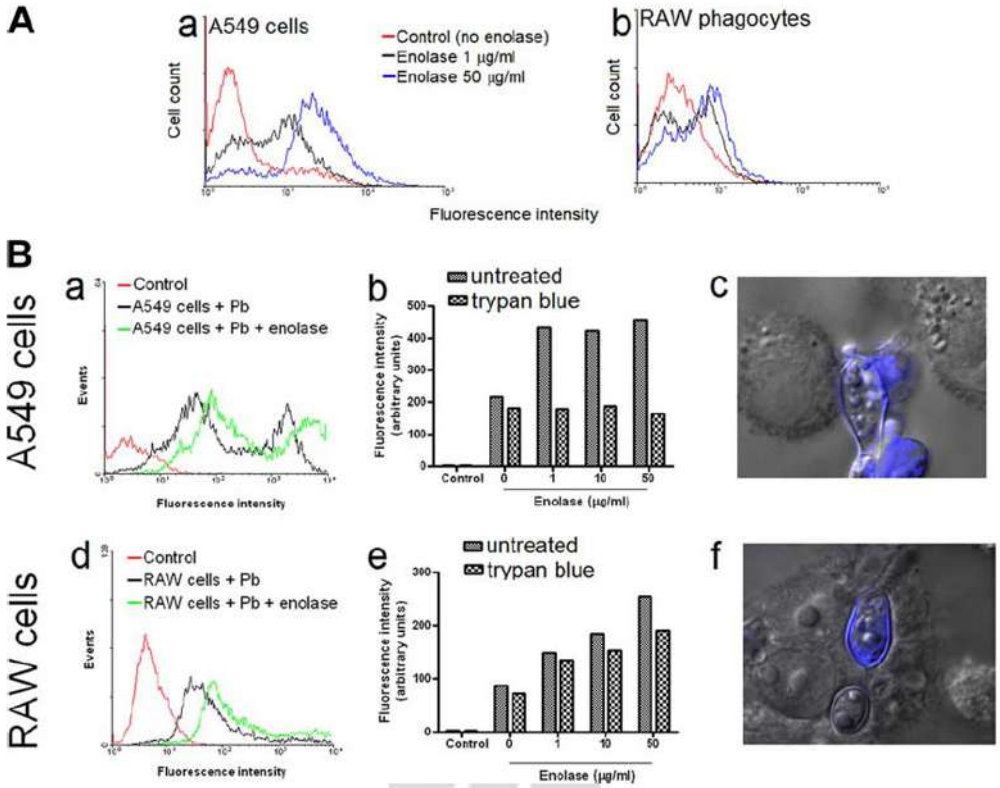

FIG. 5. Exposure of host cells to r*Pb*Eno enhances the efficacy of association of *P. brasiliensis* to host cells. (A) Treatment of A549 epithelial cells (panel a) or RAW phagocytes (panel b) resulted in increased reactivity with WGA, indicating enhanced exposure of GlcNAc residues. (B) Effects of r*Pb*Eno on the infection of host cells by *P. brasiliensis*. Panels a and d demonstrate that *P. brasiliensis* (Pb) efficiently infects epithelial (A549) and macrophage-like (RAW) cells. Histograms of control cells (noninfected) are shown in red. Exposure of host cells to r*Pb*Eno (10 μg/ml, green histogram) results in their increased association with fungi, as determined by the comparison with infection systems prepared in the absence of enolase (black histograms). Exposure of cells infected with FITC-*P. brasiliensis* to trypan blue (b and e) resulted in an accentuated reduction of fluorescence levels in A549 cells but not macrophages. The suggestive internalization of *P. brasiliensis* by macrophages, but not by epithelial cells, was supported by fluorescence microscopy (c and f). In this analysis, yeast fluorescence appears in blue.

fungal attachment and invasion of host cells, the upregulation of the gene during infection would be necessary. Relative quantification of gene transcripts was examined by real-time PCR in yeast cells of *P. brasiliensis* derived from lungs, spleens, and livers of infected mice (Fig. 6). Enolase expression was upregulated in yeast cells derived from tissues at 7 days postinoculation.

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 *Candida 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 the cell surface is unresolved. It is clear, however, that fungal cells express many molecules with apparently conflicting functions (35). The nuclear histone-like protein H2B, for instance, is also found at the cell wall of *Histoplasma capsulatum*, where it functions as a target for protective antibodies (36). In *P. brasiliensis*, the mitochondrial protein Mdj1p and the cytosolic enzymes GAPDH and TPI were also characterized as cell wall components (4, 5, 41). This multiplicity in cellular distribution and functions is also common to enolase because this protein functions in sugar metabolism but is also present at the cell surface (30) and in secretory vesicles that reach the extracellular space (45). Enolase has been also described as a component in bacterial cell walls (26), where it mediates the interaction of *Streptococcus pneumoniae* with human plasminogen (13). The dual location in the cytosol and on the cell surface indicated the pivotal roles of enolase in glycolysis and pathogenesis, respectively. However, an important and challenging important issue that needs to be addressed further is the discerning of the mechanism of its export to the cell surface.

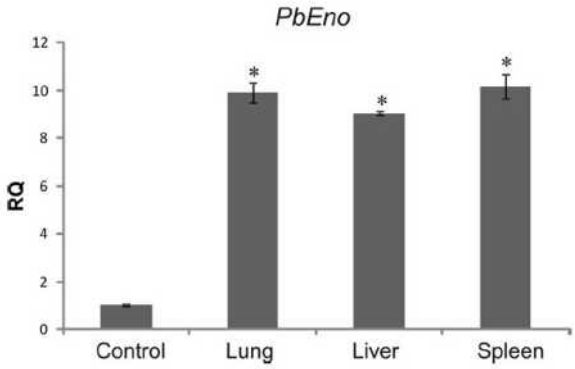

FIG. 6. Analysis of enolase transcripts by quantitative real time RT-PCR. qRT-PCR plot of *Pb*Eno expression levels of transcripts from yeast cells of *P. brasiliensis* derived from lungs, livers, and spleens of mice after 7 days of infection. Control systems consisted of yeast cells from cultures inoculated in BHI agar. The primers were as follows: sense, 5′-GATTTGCAGGTTGTCGCCGA-3′; antisense, 5′-TGGCTGCCTGGATGGATTCA-3′. The expression values were standardized using the expression values for the constitutive gene encoding the protein tubulin. The relative quantification (RQ) of the experiment was performed in triplicate. The error bars indicate the standard deviations from three independent experiments performed in triplicate. *, significantly different from the control, at a P value of <0.05.

*Pb*Eno has previously been characterized as a 54-kDa fibronectin binding protein (17). Differences in molecular mass related in the previous work could be related to the potential sites for glycosylation and myristoylation, present in the deduced sequence of the protein (data not shown). We demonstrated that *Pb*Eno was not the only adherence protein but it is involved in *P. brasiliensis* binding to plasminogen. Similarly, enolase is a predominant plasminogen binding and cell wall protein in *C. albicans*, *Aspergillus fumigatus*, and *Pneumocystis carinii* (19, 21, 25, 30). Plasminogen is abundant in the circulation, and its activation by invasive pathogens could increase the organism's potential of tissue invasion. The binding of plasminogen to mammalian and bacterial cells is mediated by its five kringle domains, which have affinity for lysine (43). Lysine-dependent binding is characteristic of the plasminogen-pathogen interaction (50). We observed that the lysine analogue ε-ACA inhibited plasminogen binding to both *P. brasiliensis* and r*Pb*Eno while it 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 (25, 33, 38). With these data taken together, we hypothesized that *P. brasiliensis* may take advantage of the plasminogen-clotting system during invasion of host tissues.

In addition to describing the localization and functional characterization of *Pb*Eno, we 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). There are remarkable studies showing that host susceptibility to invasive aspergillosis is strongly influenced by the plasminogen system and that plasminogen activation on the surfaces 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 (19, 21, 25, 30, 53). The capture of plasminogen by adhesins such as enolase and its conversion to plasmin has in fact been described for different pathogens (20).

In the current study, exposure of epithelial cells and phagocytes to r*Pb*Eno enhanced the efficacy of *P. brasiliensis* association with host components. Treatment of host cells with enolase caused an increase in exposure of surface *N*-acetylglucosamine. Although the mechanisms connecting exposure to enolase with changes in surface carbohydrates are unclear, this observation echoes previous findings showing that animal infection with *S. pneumoniae*, an enolase-producing pathogen (6), results in an increased surface exposure of *N*-acetylglucosamine residues by host tissues (29). Since *P. brasiliensis* uses *N*-acetylglucosamine as a surface site of adhesion in host cells (12, 18, 23), we hypothesized that treatment of host cells with enolase could result in increased infectivity.

Yeast cells preferentially adhered to epithelial cells and were internalized by phagocytes. The mechanisms explaining how an enzyme could alter surface interactions of a fungal pathogen with host cells are still obscure. Although the mechanisms by which enolase interferes with steps of the interaction of pathogens with host cells are unknown, it is evident from the current literature that this enzyme may be involved in adhesion to and microbial infection in host elements. Our analysis demonstrated that enolase is a surface-secreted protein in *P. brasiliensis* as it is in *C. neoformans* (45). In this way, release of the enzyme to the extracellular space, as demonstrated here, could somehow increase the availability of adhesion sites in host cells. This putative phenomenon would result in higher efficacy of association of fungi with host cells, as currently described in our manuscript.

The results described in this paper expand current knowledge on the adhesion and invasion processes of *P. brasiliensis*. The transcript encoding *Pb*Eno was upregulated in yeast cells derived from tissues from infected mice. Overall, the present work is the first study, to our knowledge, to demonstrate the plasminogen binding and activation activity of *P. brasiliensis* enolase and shows that, similar to its role in 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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