

# Extracellular glycosylphosphatidylinositol-anchored mannoproteins and proteases of *Cryptococcus neoformans*

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## Abstract

Extracellular proteins of *Cryptococcus neoformans* are involved in the pathogenesis of cryptococcosis, and some are immunoreactive antigens that may potentially serve as candidates for vaccine development. To further study the extracellular proteome of the human fungal pathogen *Cry. neoformans*, we conducted a proteomic analysis of secreted and cell wall-bound proteins with an acapsular strain of *Cry. neoformans*. Proteins were identified from both intact cells and cell walls. In both cases, extracellular proteins were removed with trypsin or  $\beta$ -glucanase, and then all proteins/peptides were purified by solid-phase extraction, spin dialysis, and HPLC, and identified by liquid chromatography–mass spectrometry. This study identified 29 extracellular proteins with a predicted N-terminal signal sequence and also a predicted glycosylphosphatidylinositol anchor motif in more than half. Among the novel proteins identified were five glycosylphosphatidylinositol-anchored proteins with extensive Ser/Thr-rich regions but no apparent functional domains, a glycosylphosphatidylinositol-anchored aspartic protease, and a metalloprotease with structural similarity to an elastinolytic metalloprotease of *Aspergillus fumigatus*. This study suggests that *Cry. neoformans* has the machinery required to target glycosylphosphatidylinositol-anchored proteins to the cell wall, and it confirms the extracellular proteolytic ability of *Cry. neoformans*.

## Introduction

*Cryptococcus neoformans* is responsible for life-threatening meningitis in immunocompromised individuals (Casadevall & Perfect, 1998). Dissemination of *Cry. neoformans* from the lung to the brain requires the activity of a secreted phospholipase and possibly proteases, thus supporting the notion that the extracellular proteome of *Cry. neoformans* probably plays a significant role in cryptococcal pathogenicity (Goldman *et al.*, 1994; Chen *et al.*, 1996, 1997; Cox *et al.*, 2001; Rodrigues *et al.*, 2003; Ganendren *et al.*, 2006). The extracellular proteome consists of cell wall-bound and secreted proteins, and many of these, such as the mannoproteins, share certain structural features; the presence of an N-terminal signal sequence, Ser/Thr-rich regions that are O-glycosylated and N-glycosylated, and a C-terminal glycosylphosphatidylinositol (glycosylphosphatidylinositol) anchor motif (Levitz *et al.*, 2001; Biondo *et al.*, 2002, 2006; Huang *et al.*, 2002; Levitz & Specht, 2006). The glycosylphosphatidylinositol anchor serves to covalently attach proteins to the cell

membrane or to  $\beta$ -1,6-glucans in the cell wall, and secretion of the proteins results if the C-terminal glycosylphosphatidylinositol anchor consensus motif is deleted (Djordjevic *et al.*, 2005; de Groot *et al.*, 2005). Cell wall proteins that are retained by noncovalent bonds generally do not have a glycosylphosphatidylinositol anchor motif (de Groot *et al.*, 2005; Ruiz-Herrera *et al.*, 2005). Several secreted mannoproteins of *Cry. neoformans* stimulate cell-mediated immune responses, and this immunogenicity is dependent on the presence of mannosylation (Levitz *et al.*, 2001; Biondo *et al.*, 2002; Huang *et al.*, 2002). However, fewer than 25% of glycosylphosphatidylinositol-anchored proteins lack O-linked and N-linked glycosylation; consequently, these proteins may localize to the inner cell wall (Frieman *et al.*, 2002; Levitz & Specht, 2006).

The extracellular proteolytic activity of *Cry. neoformans* has been reported in several studies (Muller & Sethi, 1972; Brueske, 1986; Aoki *et al.*, 1994; Chen *et al.*, 1996, 1997; Ruma-Haynes *et al.*, 2000; Rodrigues *et al.*, 2003; Yoo *et al.*, 2004). The activity of a cryptococcal serine protease resulted

in the cleavage of host basement membrane-associated proteins including collagen, laminin, and fibronectin (Rodrigues *et al.*, 2003; Yoo *et al.*, 2004). A further indication that cryptococcal proteases may play a role in host tissue degradation is the ability of *Cry. neoformans* to penetrate lung parenchyma from the alveolar space (Goldman *et al.*, 1994). Complement factors and immunoglobulins are also degraded by cryptococcal proteases, suggesting that some may also disrupt host immunity (Yoo *et al.*, 2004). In view of these studies, proteases might serve as virulence mechanisms in *Cry. neoformans*. In addition, some cryptococcal proteases may be immunogenic. Tarcha *et al.* (2006) demonstrated that a recombinant aspartic protease of the soil-borne fungal pathogen *Cry. posadasii* induced protection against pulmonary coccidioidomycosis in mice. Despite the numerous studies demonstrating the proteolytic activity of *Cry. neoformans*, very few extracellular proteases have been directly isolated and identified.

The aim of this study was to expand upon the relatively few confirmed extracellular proteins of *Cry. neoformans* by releasing cell wall-bound proteins from cell walls and intact cells treated with trypsin and  $\beta$ -glucanase. More than half of the proteins identified in this study contained a glycosylphosphatidylinositol anchor motif, suggesting that, like *Saccharomyces cerevisiae*, *Cry. neoformans* is capable of directing glycosylphosphatidylinositol-anchored proteins to the cell wall. Some of these glycosylphosphatidylinositol-anchored proteins share key structural features with other known immunogenic deacetylases, but because they lack an apparent functional domain, it is not clear what the function of these novel proteins might be. However, the presence of extensive sites for both O-linked and N-linked glycosylation suggests that they may have immunogenic properties. The results presented here also demonstrate that *Cry. neoformans* has at least seven extracellular proteases, including a glycosylphosphatidylinositol-anchored aspartic protease and an elastinolytic-like metalloprotease. The role of similar proteases in the virulence mechanisms of other fungal pathogens suggests that both proteases may be involved in cryptococcal pathogenesis.

## Materials and methods

### Cell wall preparation

An acapsular serotype D strain (*cap59* gene disruption strain) (Chang & Kwon-Chung, 1994) of *Cry. neoformans* was grown in standard YPD medium/2% glucose to mid-log phase in 300-mL flat-bottomed flasks, rotating at 175 r.p.m. in a 37 °C incubator (New Brunswick Scientific, Innova 4230). The cells and medium were then divided into 50-mL capped polypropylene tubes (Sarstedt) and centrifuged at 1000 g on a tabletop centrifuge (Sorvall, Legend RT) at 4 °C.

The supernatants were removed, and the cells were combined in one 50-mL tube (10-mL wet pellet) with ice-cold water containing 500  $\mu$ L of yeast-specific protease inhibitor cocktail (Sigma), to a total volume of 20 mL. The suspension was then transferred to a glass bead grinder (Biospec Products, Bead Beater) along with 15 mL of glass beads (425–600  $\mu$ m, Sigma), and cells were broken by successive iterations while being maintained at 0 °C over ice. The extent of cell breakage was monitored by microscopic analysis ( $\times 40$  magnification, Nikon, Eclipse E200). The broken cells, beads and solution were transferred to a 50-mL tube, 20 mL of dichloromethane was then added, and the contents were centrifuged at 1000 g at 4 °C. To the pellet, 30 mL of dichloromethane was added, and the tube and contents were vortexed and centrifuged, again at 1000 g. This step was repeated twice, and then 30 mL of acetonitrile (ACN) was added, and the cell walls were vortexed and centrifuged as before. This was repeated twice, after which 30 mL of 8 M urea was added to the pellet, which was vortexed several times over a 5-min period, and then centrifuged for 5 min at 1000 g. Two final washes were conducted with 30 mL of HPLC-grade water (Sigma).

### N-Deglycosylation of surface proteins

The intact cells were aliquoted into two 50-mL capped polypropylene tubes and centrifuged at 1500 g at 4 °C, yielding 7.5 mL of wet pellet in each, and then 30 mL of ice-cold 1 M NaCl was added to each tube; the tubes were then vortexed and centrifuged as before. Cells in each tube were washed twice with NaCl, and then once with 30 mL of ice-cold water, after which intact cells were deglycosylated *in vivo* with 2500 U of PNGase F (New England Biolabs) in 6 mL of 50 mM sodium phosphate buffer (pH 7.5) for 6 h at room temperature. After N-deglycosylation, the cells were centrifuged at 1500 g at 4 °C. The pelleted cells were washed twice with 40 mL of cold HPLC-grade water in each tube prior to trypsin or glucanase digestion. For isolated cell walls, the same deglycosylation was conducted after the last isolation and water wash step and before the enzymatic removal of proteins/peptides. No attempt was made to remove O-glycosylates.

### Enzymatic release of exterior proteins and/or peptides from intact cells and isolated cell walls

Cell surface proteins were released in one 50-mL tube by treating intact cells with 1850 U of sequencing-grade trypsin (Promega) in 6 mL of 50 mM sodium phosphate buffer (pH 7.5). The trypsin digest was incubated at room temperature (20–25 °C) for 8 h on a nutator, and centrifuged at 1500 g. The supernatant was removed and set aside in a 50-mL tube at 4 °C.

The remaining washed cell pellet (in the other tube) was treated with 1000 units of purified 1,3- $\beta$ -glucanase (Inter-spex) in 6 mL of 50 mM sodium phosphate buffer (pH 7.5) with 700 mM sodium tartrate (as an osmoticum) and nutated for 2 h. The glucanase-treated cells were centrifuged at 1500 g (at 4 °C) and the supernatant was retained. The isolated cell walls were enzymatically treated in the same manner, except the sodium tartrate was left out in the glucanase treatment of isolated walls.

### Delipidation

To each enzyme-released protein/peptide solution, 20 mL of dichloromethane (Sigma) was added; the mixture was vortexed for 1 min (vigorously), and then centrifuged for 5 min at 1500 g to separate the lipid-containing lower (organic) phase and emulsion at the phase interface from the upper aqueous phase. The aqueous phase was retained. The delipidation steps were repeated until no cloudiness was visible in the dichloromethane (lower) phase or at the interface.

### Protein purification

After lipid removal, each aqueous sample was loaded onto 5 mL of Amberchrom CG-71 (Rohm Haas) RP-SPE packing in a 15-mL polypropylene cartridge column (Alltech), using a vacuum manifold apparatus (Supelco) with luer fittings to move liquids through the column. Each column was pre-treated with 15 column volumes (75 mL) of 60% ACN, followed by 75 mL of 1% ACN/0.1% trifluoroacetic acid (TFA) in HPLC-grade water (Sigma, Chromasolv). Subsequently, 5 mg of bovine serum albumin (BSA) (Sigma) in 1% ACN/0.1% TFA was loaded onto each cartridge column (to prevent irreversible binding of the protein sample to the packing), and the SPE cartridges were washed with 50 mL of 1% ACN/0.1% TFA before elution of the BSA with 50 mL of 60% ACN. The columns were then equilibrated with 50 mL of 1% ACN/1% TFA before loading with the respective samples. After loading, the protein samples were washed free of salts and other small, hydrophilic contaminants with 75 mL of 1% ACN/0.1% TFA per column; each protein sample was then eluted from the column with 5 mL of 60% ACN into a 15-mL polypropylene tube (Nunc), and aliquoted (0.5 mL each) into Microcon YM-3 (Millipore) 3000-Da-cutoff spin tubes. The spin tubes were centrifuged for 45 min at 10 000 g on a microcentrifuge (Beckman Coulter, Microfuge 18), and the above-membrane retentate (> 3000 Da, *c.*) was kept and pooled to produce a 0.5-mL total volume for each sample. To this was added 5 mL of 0.1% TFA, and then the samples were loaded separately onto a 4.6  $\times$  250 mm C<sub>4</sub> Vydac reversed-phase column with a Hewlett Packard (HP) 1050 pumping system. The samples were loaded using a Rheodyne injector, and the proteins

were detected with a UV/visible detector (HP 1050) at 220 nm and 100 absorbance units full scale (AUFs). Each sample was separated at room temperature at 0.5 mL min<sup>-1</sup>, with a gradient of 0–30% ACN over 30 min, and a gradient of 30–60% ACN over another 30 min, with 0.1% TFA maintained throughout. The column was equilibrated with 0.1% TFA before each run. Fractions were collected every 2 min during the run before final processing and MS analysis.

### Assessment of cell viability

To assess cell membrane integrity during the *in vivo* enzymatic treatment of cells, 10  $\mu$ L of cell suspensions were removed hourly, so that nonviable cells could be stained with 100  $\mu$ g mL<sup>-1</sup> methylene blue (Sigma-Aldrich) (Bonilla & Cunningham, 2003) and examined by microscopy. Three sets of 100 cells (in random areas of the slide) were counted to determine the percentage of dead cells (blue) vs. viable cells (nonblue) during this timed procedure.

### MS sequencing

For MS analysis and identification of partially purified proteins (glucanase released) or peptides (trypsin released), the samples were lyophilized and resolubilized in 8 M urea/0.2 M Tris-HCl buffer, reduced with 20 mM dithiothreitol, and carboxyamidomethylated in 40 mM iodoacetamide. Reduced and alkylated protein samples were digested overnight with modified trypsin (Promega) at an enzyme/protein ratio of 1:50 (w/w). An HPLC system (Paradigm MG4, Michrom Bio Resources, Auburn CA) directly coupled with an ion trap mass spectrometer (LCQ Deca XP plus, Finnigan, San Jose, CA) was used for microcapillary LC-MS/MS data acquisition. A fritless microcapillary column (0.1  $\times$  180 mm) was packed with C<sub>18</sub> (Michrom Bio Resources, Magic C<sub>18</sub>AQ: 5  $\mu$ m, 100 Å) reversed-phase material (Shimizu & Wariishi, 2005). Tryptic peptide mixtures were desalted and concentrated using a reversed-phase trap column (0.25  $\times$  30 mm), and then chromatographically separated with the microcapillary column at a flow rate of 300 nL min<sup>-1</sup>. Eluted peptides were sprayed directly into the mass spectrometer.

An MS survey scan and MS/MS spectra of the three most intense peptide ions from the previous MS scan were continuously acquired over the chromatographic gradient. Dynamic exclusion for 3 min was used to acquire MS/MS spectra from low-abundance peptides, when possible. The two buffers used for the chromatography were 5% ACN/0.1% FA (buffer A) and 80% ACN/0.1% formic acid (FA) (buffer B). A 2.5-h gradient (0–10% buffer B for 20 min, 10–45% buffer B for 110 min, 45–100% buffer B for 20 min) was used for the maximum separation of tryptic peptides. Typically, 3000 MS/MS spectra were acquired during an HPLC run, and SEQUEST searches (Qian *et al.*, 2005) were

performed for MS/MS data against the most current NCBI NR *Cryptococcus*-only protein database (Loftus & Hyman, 2005), with two allowable modifications to tryptic or half-tryptic peptides: carboxyamidomethylation on Cys (+57 Da), and oxidation on Met (+16 Da). DTASELECT software (Tabb *et al.*, 2002) was used to filter out low score matching. Filtering parameters were as follows. Correlation values (*X*-corr) of 1.9, 2.2 and 3.75 were used for singly, doubly and triply charged half-tryptic or fully tryptic peptide ions, respectively. Any *Cn* value (the difference in *X*-corr between one peptide and the next highest one) less than 0.08 was disregarded. Proteins yielding two or more peptide ions satisfying the above criteria were filtered in; at least one MS/MS spectrum was manually evaluated for proteins identified; these proteins were accepted only when at least two peptide spectra passed manual validation (Moore *et al.*, 2002).

### Prediction of N-terminal peptide and glycosylphosphatidylinositol anchor in identified proteins

Full CDS protein sequences were taken from the NCBI protein database, using assigned accession numbers from the *Cry. neoformans* genome. Each protein sequence was inspected for the presence of an N-terminal signal sequence by the SIGNALP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>), and for the presence of a putative glycosylphosphatidylinositol cleavage site (glycosylphosphatidylinositol anchor) by the DPGI prediction server ([http://129.194.185.165/dgpi/index\\_en.html](http://129.194.185.165/dgpi/index_en.html)), as shown in Table 1.

## Results

Unlike most other fungal pathogens, *Cry. neoformans* has a polysaccharide capsule that is exterior to the cell wall. Initial *in vivo* experiments with wild-type cells yielded few identified proteins until an acapsular mutant strain (*cap59*) of *Cry. neoformans* was employed, lacking the polysaccharide capsule but displaying no cell wall defects (Moyrand & Janbon, 2004). Identification of cell wall proteins was problematic until intact acapsular cells were deglycosylated *in vivo* with PNGase F before the enzyme treatments (Fig. 1). The PNGase F treatment resulted in the cleavage of asparagine-bound glycans (*N*-glycans) from surface proteins (Tarentino *et al.*, 1985), and when the enzyme treatments followed *in vivo* deglycosylation, the number of identified proteins improved significantly. This is consistent with a recent report demonstrating that *N*-linked glycosylation of phospholipase B1 promoted its stability by protecting it from proteolysis (Turner *et al.*, 2006).

In addition, the highly effective partition delipidation used in this study allowed the separation of entire (partially processed) extracts at the analytical HPLC scale without

major column overload. The removal of hydrophobic molecules, such as lipids, is crucial in general, as these may be more strongly retained on reverse-phase liquid chromatography (RPLC) columns than the less hydrophobic peptides. The spin dialysis with 3-kDa Microcon tubes were also essential to this procedure: whereas the initial SPE desalting procedure with the reverse-phase column removed salts and most small, hydrophilic molecules, very small proteolytic fragments, single amino acids and small hydrophobic molecules require removal to avoid overloading HPLC columns.

In order to ensure that acapsular cells of *Cry. neoformans* remained viable during the enzymatic treatments, aliquots of cells were periodically removed from the digestions, added to methylene blue, and counted. The percentage of cell death during treatments did not exceed 5%, and generally fell between 1% and 3% of cells counted (data not shown).

### Extracellular proteins were isolated by *in vivo* enzymatic digestion

Although this procedure had not been attempted with intact fungal cells prior to this study, we reasoned that enzymes would release peptides and proteins associated specifically with the cell surface (and secreted proteins), as most enzymes are unlikely to permeate the membranes of intact cells (Fig. 1). We also employed both trypsin and  $\beta$ -glucanase for the following reason: the trypsin would specifically release peptides from proteins exposed at the cell surface, but the  $\beta$ -glucanase allowed the release of proteins buried within the inner cell wall. The  $\beta$ -glucanase cleaves the covalent bridge of the glucan-anchored cell wall protein, thus releasing the wall-associated protein, whereas trypsin cleaves on the C-terminal side of arginine and lysine residues (Reese & Doering, 2003). One limitation of the trypsin procedure was the possibility that too many arginines and/or lysines in the surface proteins would yield very small peptides that would be lost during the final desalting steps, or be overshadowed in the noisier low molecular weight range of the MS/MS analysis. Alternatively, insufficient cleavage sites would result in peptides larger than the resolving capabilities of the MS/MS. These problems were not encountered.

### Cell wall-associated proteins were released from isolated cell walls

Isolated cell walls from acapsular mutant cells were treated with  $\beta$ -glucanase and trypsin to release cell wall-bound proteins. Extensive washes of isolated cell walls with both organic and high ionic strength reagents were performed to prevent cytosolic or membrane-associated proteins from sticking to the cell walls. Intact cells were not subjected to either the denaturing conditions of the urea washes or to

**Table 1.** Extracellular proteins of *Cryptococcus neoformans*

Accession number*	Protein function†	Protein size (amino acids)	T‡	N-terminal signal peptide§	GPI anchor¶	References
CNBE3490**	$\alpha$ -Amylase	532	+	+	+	Levitz & Specht (2006)
CNBG4090	Trehalase	756	+	+	–	
CNBC2400	Endo-1,3- $\beta$ -glucanase	452	+	+	–	
CNBM0110**	MP88-like	401	–	+	+	Huang <i>et al.</i> (2002)
CNBA7360**	MP88	388	–	+	+	Huang <i>et al.</i> (2002)
CNBD2840**	Chitin deacetylase 2	458	–	+	+	Levitz & Specht (2006)
CNBD2750**	Chitin deacetylase 3	410	–	+	+	Levitz & Specht (2006)
CNBF2910**	Chitin deactylase 1	470	–	+	+	Levitz & Specht (2006)
CNBN0250**	Polysaccharide deacetylase	253	+	+	–	
CNBC1420	Serine protease 1	553	–	+	–	
CNBF4600	Serine protease 2	561	–	+	–	
CNBH1590**	Aspartic protease	750	+	+	+	Levitz & Specht (2006)
CNBA5450	Aspartic protease	438	–	+	–	
CNBA1340	Serine protease 1 (subtilase family)	950	–	+	–	
CNB2870	Serine protease 2 (subtilase family)	518	–	+	–	
CNB1810	Metalloprotease	831	–	+	–	
CNB10550	Carboxylesterase 1	640	+	+	–	Biondo <i>et al.</i> (2006)
CNBC0510	Carboxylesterase 2	579	+	+	–	
CNBE5040**	Glyoxal oxidase 1	676	+	+	+	Levitz & Specht (2006)
CNBA3760**	Glyoxal oxidase 3	664	+	+	+	Levitz & Specht (2006)
CNBN2300**	Gas1	564	+	+	+	Levitz & Specht (2006)
CNBD4830	Superoxide dismutase	152	–	–	–	Biondo <i>et al.</i> (2006), Hamilton & Holdom (1997)
CNBD4370**	PG-PI transfer protein	204	+	+	+	
CNB12180**	CFEM domain	311	–	+	+	Levitz & Specht (2006)
CNBC3230**	Unknown	218	–	+	+	Levitz & Specht (2006)
CNBG1270**	Unknown	222	–	+	+	Levitz & Specht (2006)
CNBL2400**	Unknown	416	–	+	+	Biondo <i>et al.</i> (2006)
CNBG1100**	Unknown	202	–	+	+	
CNBE4750**	Unknown	314	–	+	+	Levitz & Specht (2006)
CNBC6500	Lipoprotein	317	–	+	–	

\*Accession number in this case refers to the locus tag assigned to each gene in the B-3501 *Cryptococcus neoformans* genome by Stanford Genome Technology Center. Protein sequences were identified by searching the protein database at NCBI (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein>).

†The function of each protein was assigned based on the lowest *E*-value in the NCBI protein – protein BLAST searches.

‡The proteins identified only by  $\beta$ -1,3-glucanase treatment (T) are indicated by (+).

§The presence of an N-terminal signal peptide was predicted by the SIGNALP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>) and PSORT II PREDICTION (<http://psort.nibb.ac.jp/>).

¶Prediction of a GPI cleavage site (GPI anchor) was performed by the DGPI prediction server ([http://129.194.185.165/dgpi/index\\_en.html](http://129.194.185.165/dgpi/index_en.html)).

||The listed references have previously reported the presence of the indicated proteins as extracellular in *Cryptococcus neoformans*.

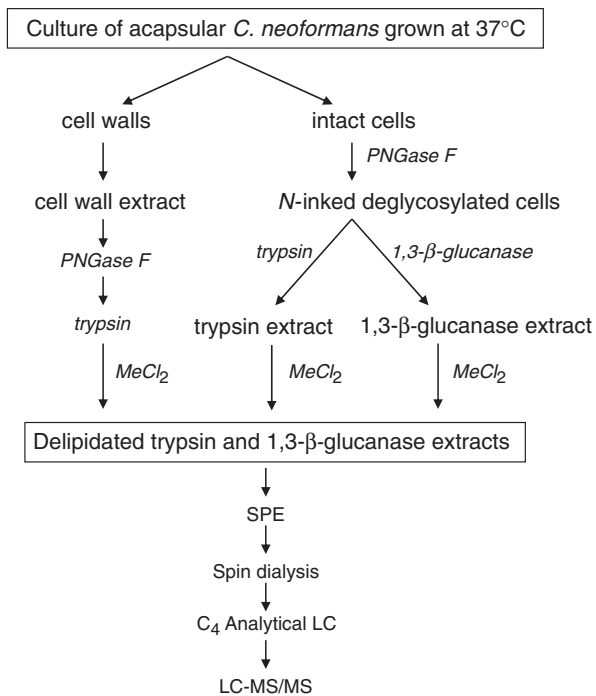
\*\*Proteins that were also identified in isolated cell walls.

organic solvents (ACN, MeCl<sub>2</sub>), which could partially account for the incomplete overlap between the *in vivo* and *in vitro* techniques (Table 1), especially with the trypsin treatment, as the denaturing of proteins would expose different areas of the proteins. Employment of both *in vitro* and *in vivo* methods ensured that secreted proteins and cell wall-bound proteins could be identified.

### Identification of extracellular proteins by MS

The protein sequences obtained in this study were a result of using full tryptic peptides in addition to half-tryptic pep-

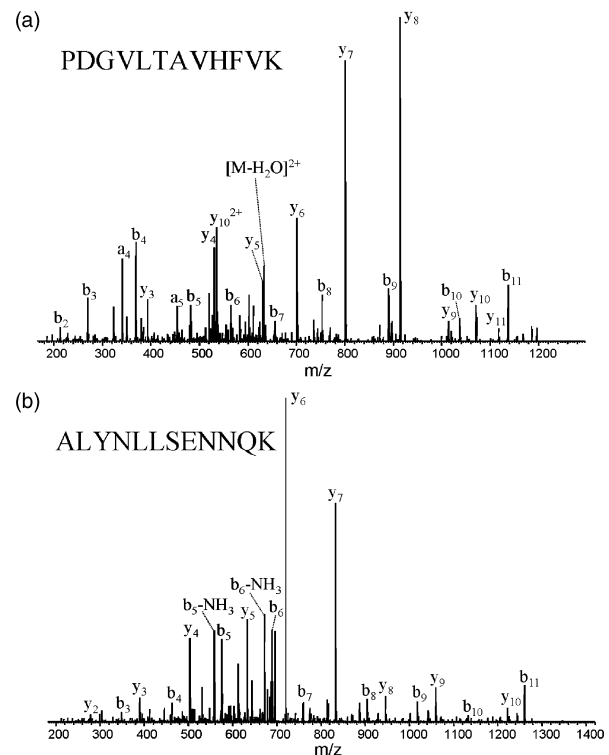
tides (Qian *et al.*, 2005). The inclusion of half-tryptic peptides in the SEQUEST search resulted in a subset of surface proteins that would have been otherwise undetected in the full tryptic peptide searches, as demonstrated in supplementary Table 1. Figure 2 shows two examples of MS/MS spectra identified in the current study; Fig. 2a is an MS/MS spectrum of [PDGVLAVHFVK+2H]<sup>2+</sup> from CNBA3760, and Fig. 2b is [ALYNLLSENNQK+2H]<sup>2+</sup> from CNBD2840. The X-corr values, 4.3 and 3.7 for Figs 2a and b, respectively, were well above the minimum X-corr cutoff value of 2.2 for a doubly charged ion, and as a result all the major peaks were successfully assigned as y, b and a series fragment ions



**Fig. 1.** Schematic representation of the protocol used to release extracellular proteins from acapsular cells and isolated cell walls of *Cryptococcus neoformans*. Details of the method are described in Materials and methods. SPE, solid-phase extraction; LC-MS/MS, liquid chromatography–mass spectrometry.

or as their neutral losses (such as water or ammonia loss). As shown in Fig. 2, adjacent amino acids on the N-terminal side of both peptides were not lysine or arginine (i.e. Ile and Asn for PDGVLTA VHFVK and ALYNLLSENNQK, respectively). Consequently, both spectra were not identified when only fully tryptic criteria were used in the SEQUEST search; however, by allowing half-tryptic peptides, we were able to identify those peptides. In the case of CNBD2840, all five peptide sequences identified were half-tryptic peptides (supplementary Table 1 and Fig. 3), which made the half-tryptic analysis an indispensable protocol improvement.

To eliminate the possibility of identifying false positives, we used a rigorous protocol to estimate the false-positive rate by searching selected datasets against a reversed-sequence database (Moore *et al.*, 2002). The false-positive rate was defined as the number of peptides identified with the reversed-sequence database divided by the number from the normal database. With this definition, our rate for tryptic peptide identification was 15.7% (91 peptides from a reversed-sequence database/579 peptides from a normal database). However, because we required at least two peptides for protein identification, the false-positive rate decreased to < 1% (0 proteins from reversed sequence/74 proteins from normal sequence). Furthermore, we manually validated all MS/MS spectra for proteins with fewer than



**Fig. 2.** MS/MS spectra of one representative protein sample from *Cryptococcus neoformans* identified by MS. (a) Spectra of [PDGVLTA VHFVK + 2H]<sup>2+</sup> from CNBA3760, and (b) [ALYNLLSENNQK + 2H]<sup>2+</sup> from CNBD2840, obtained in LC-MS/MS analysis of trypsin-released fractions. Details of the MS/MS analysis are described in the text.

four peptides. None of the false proteins from the reversed-sequence database passed this manual validation; hence, the final false-positive rate after manual validation was insignificant (< 1%, *c.*).

### Extracellular proteins of *Cry. neoformans* are functionally diverse

In order to identify proteins that might more closely reflect the extracellular protein profile during infection, cells of *Cry. neoformans* were grown at the host temperature, 37 °C. This notion is in part supported by differences between transcription profiles of *Cry. neoformans* cells grown at 37 °C and those grown at 25 °C (Steen *et al.*, 2002; Kraus *et al.*, 2004). The *in vivo* and *in vitro* methods employed here resulted in the identification of 29 proteins with a predicted N-terminal signal sequence (Table 1). Several of the identified proteins, such as trehalase, endo-1,3-β-glucanase, polysaccharide deacetylase, both carboxylesterases, aspartic protease 1, glyoxal oxidase 1, and glyoxal oxidase 2, were only revealed by β-glucanase treatment, suggesting that these proteins may not be exposed to the cell surface (Table 1). Among the 29 proteins identified were 17 proteins

NCBI accession number	Sequence coverage	MW, Da	Description	Xcorr	DelCN	Charge
<b>gil50258542</b>	22.50%	48462	hypothetical protein CNBD2840 [ <i>Cryptococcus neoformans</i> var. <i>neoformans</i> B-3501A]gil13958034 gb AAK50770.1  chitin deacetylase-like mannoprotein MP98 [ <i>Cryptococcus neoformans</i> var. <i>neoformans</i> ] [MASS=48462]			
<b>gil57226794</b>	22.50%	48541	chitin deacetylase-like mannoprotein MP98 [ <i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21]gil58266810 ref XP_570561.1  chitin deacetylase-like mannoprotein MP98 [ <i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21] [MASS=48540]			
K.LPNDVPHGTPTGDWTGV.N				3.7966	0.453	2
K.LPNDVPHGTPTGDWTGVNY.N				4.1573	0.4246	2
V.NYNSSDPDCWWTNHNK.C				3.4752	0.3597	2
Y.NSSDPDCWWTNHNK.C				3.2132	0.3722	2
N.ALYNLLSENNQK.A				4.241	0.3169	2
K.ATMFFIGSNVMDWPLQAMR.A				4.4562	0.4264	2
V.LGVTPQCWRPPYGDVDNR.V				3.3936	0.133	2
T.PQCWRPPYGDVDNR.V				3.4451	0.1676	2
W.RPPYGDVDNR.V				2.7256	0.2863	2
W.AAGTDGVTEQDVTDNQAVIDK.A				5.0323	0.3971	2
D.GVTEQDVTDNQAVIDK.A				4.3016	0.4511	2
V.TEQDVTDNQAVIDK.A				2.5636	0.1328	2
Q.DVTDNYQAVIDK.A				3.5304	0.1971	2

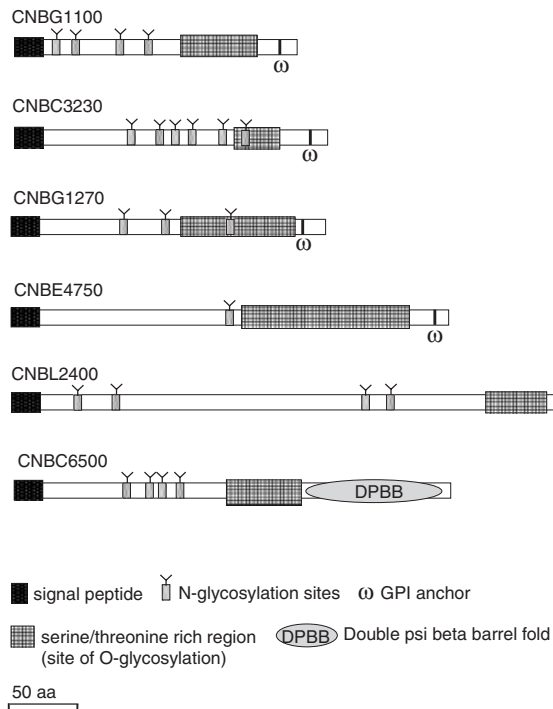
**Fig. 3.** A sample from the supplementary database of a protein identified in *Cryptococcus neoformans* by MS. The NCBI accession number, the molecular mass (in daltons), the peptide sequences identified and the description shown here were acquired for each protein identified. The sequence coverage represents the percentage of the total amino acids covered by the peptides identified. The MS properties of the identified peptides are also shown. Xcorr represents the cross-correlation score, and only peptides with Xcorr values > 2.2 were accepted for a doubly charged ion (charge = 2). DelCN represents the Delta Correlation value.

with a predicted glycosylphosphatidylinositol anchor, including:  $\alpha$ -amylase; glyoxal oxidase 1; glyoxal oxidase 2; Gas1 (a  $\beta$ -1,3 glucosyl-transferase); phosphatidylglycerol-phosphatidylinositol (PG-PI) transfer protein; CFEM domain; aspartyl protease; and five unknown proteins (Table 1).

Six of these 29 proteins were unknown, but the remaining 23 were assigned a function on the basis of conserved domains identified when each protein sequence was used to query the protein database at the NCBI (Table 1). Seven of the 29 proteins identified were proteases that varied in size from 438 amino acids to 950 amino acids. This group included: serine protease (two), aspartic protease (two), serine endoprotease (subtilase family) (two), and a metalloprotease (one). Only the glycosylphosphatidylinositol-anchored aspartic protease was identified using isolated cell walls (Table 1), whereas the six proteases that lacked a glycosylphosphatidylinositol anchor were identified with intact cells.

Several previously characterized mannoproteins were also identified in this study. This group included: MP88 (one); MP88-like (one); chitin deacetylase (three); and polysac-

charide deacetylase (one). A closer examination of the amino acid sequences of the unknown proteins revealed several structural features similar to those of the MP88-like immunoreactive mannoproteins (Fig. 4). All six (CNBG1100, CNBC3230, CNBG1270, CNBE4750, CNBL2400 and CNBC6500) of the unknown proteins had an N-terminal signal sequence, and five of the six proteins contained a glycosylphosphatidylinositol anchor motif. Each of the unknown proteins also had extensive Ser/Thr (S/T)-rich regions proximal to the C-terminus (Fig. 4). These S/T-rich regions provide sites for potential O-linked glycosylation. In addition, each of the unknown proteins contained several potential N-glycosylation sites, specified by the Asn-X-S/T consensus motif (Fig. 4). Interestingly, all of the unknown proteins lacked a functional domain. However, one of the unknown proteins (CNBC6500) contained a lipoprotein-like double psi  $\beta$ -barrel fold (DPBB-pfam03330) closest to its C-terminus, which could represent an enzymatic domain. One of the proteins identified here, CNBL2400, was previously shown to be among the major proteins secreted by *Cry. neoformans*; however, its function remains unknown (Biondo *et al.*, 2006).



**Fig. 4.** The structural features of the amino acid sequences of the six unknown extracellular proteins identified in *Cryptococcus neoformans*. All six proteins share features common to mannoproteins, such as a signal peptide, a Ser/Thr (S/T)-rich region, predicted sites of *O*-glycosylation and *N*-glycosylation, and a glycosylphosphatidylinositol anchor motif. Unlike some of the mannoproteins previously characterized, the unknown proteins identified here do not contain a functional domain. However, one of the unknowns, CNBC6500, contains a rare lipoprotein-like double psi-barrel fold proximal to the C-terminus, which could be associated with enzymatic activity.

The protease CNBJ1810 contained a peptidase M36 domain (pfam 02128) closest to its C-terminal end, as well as an N-terminal signal peptide (Fig. 5a). These same structural features are present in an elastinolytic neutral metalloprotease of *Aspergillus fumigatus* (Fig. 5a). Sequence alignment analysis revealed that the two proteins shared 42% identity and 54% similarity at the amino acid level. Further inspection of the amino acid sequence revealed the presence of a conserved HEYXH motif within the peptidase M36 domain (Fig. 5b). This motif serves as the metal-binding site, and is characteristic of zinc-binding metalloproteinases of the zincins family (Miyoshi & Shinoda, 2000).

## Discussion

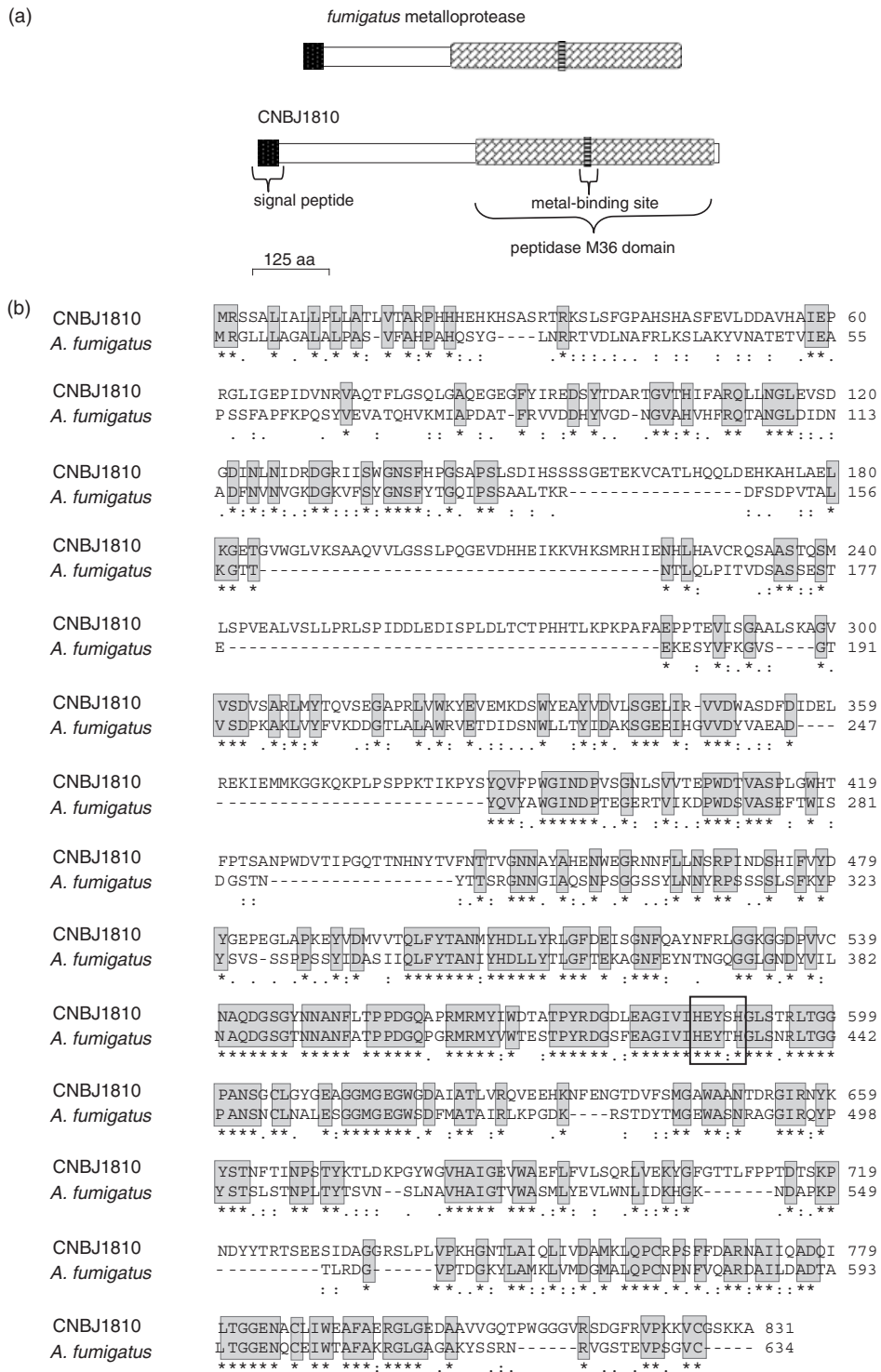
This study sought to identify extracellular proteins of *Cry. neoformans* by enzymatically releasing cell surface-associated proteins from both intact cells and isolated cell walls. Collectively, the two methods ensured that both cell wall-bound proteins and secreted proteins could be identified.

Several features of the identified proteins suggest that they belong to the extracellular proteome of *Cry. neoformans*. First, all of the proteins contain a secretory signal peptide, represented by an N-terminal hydrophobic stretch of amino acids. Second, many of the identified proteins are predicted to undergo posttranslational *N*-linked and *O*-linked glycosylation. Third, more than half of the proteins are glycosylphosphatidylinositol-anchored. The only protein that did not meet these criteria was superoxide dismutase, which we identified as a cell surface-associated protein. This is consistent with other studies that have found superoxide dismutases in concentrated supernatants from *Cry. neoformans*, suggesting that it is a secreted protein (Hamilton & Holdom, 1997; Biondo *et al.*, 2006).

Of the 29 cell surface-associated proteins identified here, 17 were glycosylphosphatidylinositol-anchored and most contained sites for both *O*-linked and *N*-linked glycosylation. Using an *in silico* approach, Levitz & Specht (2006) also predicted that 14 of these 17 proteins were glycosylphosphatidylinositol-anchored. Interestingly, fewer than 25% of the proteins that they identified did not have an S/T-rich region, suggesting a probable reduction in glycosylation. Similarly, of the glycosylphosphatidylinositol-anchored proteins identified in this study, most contained S/T-rich regions; however, a few, such as  $\alpha$ -amylase (CNBE3490), a deacetylase (CNBN0250) and glyoxal oxidase 2 (CNBA3760), did not. The absence of this potential S/T-mediated glycosylation and the fact that these proteins were released and identified only by  $\beta$ -glucanase treatment suggest that they are probably located within the inner layer of the cell wall and therefore not exposed to the surface. Taken together, the results presented here are in agreement with those of other studies that have shown that at least half of all extracellular proteins of *Cry. neoformans* are mannoproteins (Mansour *et al.*, 2002; Biondo *et al.*, 2006; Levitz & Specht, 2006). Although the function of several newly identified glycosylphosphatidylinositol-anchored proteins could not be predicted, the extensive S/T-rich regions suggest that, like other mannoproteins, these proteins may also represent immunodominant antigens and may prove useful in vaccine design (Levitz *et al.*, 2001; Biondo *et al.*, 2002, 2005; Huang *et al.*, 2002; Mansour *et al.*, 2002).

Several proteases were among the extracellular proteins identified in this study. Generally, fungal proteases promote the pathogenesis of infection by challenging the host response, including the cleavage of cytokines and complement components that are involved in resistance to antimicrobial attack, and the degradation of extracellular matrix to facilitate tissue invasion (Carruthers & Blackman, 2005). Indeed, strains of *Cry. neoformans* with extracellular serine protease activity were able to break down laminin and type IV collagen, suggesting that cell surface-associated proteases may promote tissue invasion by cleaving basement





**Fig. 5.** A metalloprotease in *Cryptococcus neoformans* is most similar to an elastinolytic neutral metalloprotease in *Aspergillus fumigatus*. (a) A schematic representation of the amino acids of the Cn metalloprotease (CnBJ1810) and of the Af elastinolytic metalloprotease reveals a common peptidase M36 domain with a metal-binding site in the C-terminal half of the protein and an N-terminal signal peptide. (b) Alignment of the amino acids of CnBJ1810 and Af elastinolytic metalloprotease revealed 54% similarity and 42% identity. The conserved zinc-binding motif (HEYxH) within the peptidase M36 domain is highlighted by a black box.

membrane components and/or degrading extracellular matrix (Naglik *et al.*, 2003; Rodrigues *et al.*, 2003). Also, the presence of *Cry. neoformans* within lung parenchyma *c.* 2 h after being deposited in the alveolar space of rats may be indicative of a secreted protease(s) capable of specifically disrupting and penetrating alveoli (Goldman *et al.*, 1994). One such protease may be the extracellular metalloprotease identified in this study. This metalloprotease is structurally similar to a neutral metalloprotease of *A. fumigatus* that is capable of breaking down elastin, a protein within connective tissue that is particularly abundant in lung and in arteries (Monod *et al.*, 1993; Markaryan *et al.*, 1994). Accordingly, immunogold localization studies demonstrated that *A. fumigatus* secretes an elastinolytic metalloprotease into invading lung tissue, and *Cry. neoformans* has been shown to have proteolytic activity against elastin (Muller & Sethi, 1972; Brueske, 1986; Monod *et al.*, 1993; Markaryan *et al.*, 1994). Although the mechanisms that allow *Cry. neoformans* to evade host pulmonary defenses and disseminate from the lung to other organs such as the brain are not clear, the metalloprotease, along with other proteases identified in this study, may potentially play a role.

Of the two aspartic proteases identified in this study, CNBH1590 was the only glycosylphosphatidylinositol-anchored protease; however, Levitz & Specht (2006) identified two additional glycosylphosphatidylinositol-anchored aspartic proteases. These proteases may be functionally similar to yapsins, a family of glycosylphosphatidylinositol-anchored aspartic proteases required for cell wall integrity in *S. cerevisiae* (Krysan *et al.*, 2005). Of the 10 aspartic proteases identified in *Candida albicans*, Sap1–6 are secreted and have been reported to hydrolyze host proteins and cause tissue damage (Naglik *et al.*, 2003). Sap9 and Sap10 are cell wall-bound and have been linked to the maintenance of the fungal cell surface (Albrecht *et al.*, 2006). Indeed, strains of *Can. albicans* lacking Sap9 and Sap10 displayed modified adhesion properties, thus linking both proteases to the infection process (Albrecht *et al.*, 2006). The glycosylphosphatidylinositol-anchored aspartic proteases in *Cry. neoformans* are predicted N-linked glycosylated proteins, like the cell surface-bound Sap9 and Sap10, and unlike the aspartic protease lacking the glycosylphosphatidylinositol anchor (CNBA5450). These structural differences suggest that the two subclasses of aspartic proteases may have fundamentally different roles in the pathogenicity of *Cry. neoformans*, as is the case for *Can. albicans*.

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### Supplementary material

The following supplementary material is available online:

**Table S1.** The data represent the proteins identified by SEQUEST searches. Each protein shown in the table is anno-

tated by: a description; an NCBI accession number; its molecular mass (in daltons); and the percentage of its total amino acids covered by the peptides identified (sequence coverage).

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