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Characterization of Lipids and Proteins Associated to the Cell Wall of the Acapsular Mutant *Cryptococcus neoformans* Cap 67

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Abstract

Cryptococcus neoformans is an opportunistic human pathogen that causes life-threatening meningitis. In this fungus, the cell wall is exceptionally not the outermost structure due to the presence of a surrounding polysaccharide capsule, which has been highly studied. Considering that there is little information about *C. neoformans* cell wall composition, we aimed at describing proteins and lipids extractable from this organelle, using as model the acapsular mutant *C. neoformans* cap 67. Purified cell wall preparations were extracted with either chloroform/methanol or hot SDS. Total lipids fractionated in silica gel 60 were analyzed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS), while trypsin digested proteins were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). We detected 25 phospholipid species among phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and phosphatidic acid. Two glycolipid species were identified as monohexosyl ceramides. We identified 192 non-covalently linked proteins belonging to different metabolic processes. Most proteins were classified as secretory, mainly via nonclassical mechanisms, suggesting a role for extracellular vesicles in transwall transportation. In concert with that, orthologs from 86% of these proteins have previously been reported both in fungal cell wall and/or in extracellular vesicles. The possible role of the presently described structures in fungal-host relationship is discussed.

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Keywords

glycolipid; monohexosyl ceramide; pathogenic fungus; phospholipid; proteome

CRYPTOCOCCUS neoformans is a ubiquitous basidiomycete that can be found in diverse types of environments, such as the soil, trees, and bird feces. It is an important opportunistic human pathogen that causes life-threatening meningitis and meningoencephalitis in immunocompromised hosts (Srikanta et al. 2014). Infection occurs when desiccated yeasts or spores are inhaled. Once they reach the pulmonary alveoli, they can rapidly be eliminated by the immune system or remain quiescent until an immunological misbalance leads to central nervous system tropism and disease development. In the last decades, the number of cryptococcosis cases has risen dramatically due to the association with AIDS and patients under immunosuppressive treatment (Srikanta et al. 2014).

A number of factors have been related to *C. neoformans* virulence, such as melanin production, the ability to grow at 37 °C, phospholipase and urease extracellular activities, induction of the Rim101 transcription factor and the virulence-associated protein VAD1. However, the best studied and most important fungal virulence factor is a polysaccharide capsule that surrounds the fungal cell wall (Vecchiarelli et al. 2013). Strains with larger capsule show higher virulence, primarily attributed to prevention of phagocytosis by host macrophages (Feldmesser et al. 2000). Therefore, investigators have concentrated their studies in capsule components, especially in the major glucuronoxylomannan (GXM). Despite the importance of the fungal cell wall in the relationship with the environment and the host, there is little information about it in *Cryptococcus* (Perfect and Casadevall 2002).

Fungal cell wall is mainly composed of structural glucans and chitin, with an outer layer of mannan and glycoproteins (Latge 2007). A proportionally minor lipid and protein total content has also been described (San-Blas and San-Blas 1984). In *Candida albicans* and *Saccharomyces cerevisiae*, cell wall structural proteins are covalently linked either to β-1,6-glucan via a remnant glycosylphosphatidylinositol (GPI) anchor or directly to the long β-1,3-glucan chain (e.g., proteins with internal repeats – Pir) (De Groot et al. 2005). Additionally, non-covalently linked proteins, associated to the cell wall by disulfide bonds or other unclear ways, have been described (Ruiz-Herrera et al. 2006, Guimaraes et al. 2011, Puccia et al. 2011).

The *C. neoformans* cell wall consists predominantly of chitin and a mixture of branched and linear glucopyrans (James et al. 1990). As opposed to *S. cerevisiae*, *C. neoformans* cell wall lacks structural mannan (James et al. 1990) and mannoproteins (Vartivarian et al. 1989). The literature on cell wall lipids focuses on a glucosylceramide (GlcCer) species localized preferentially to the *C. neoformans* cell wall, with a role in budding, growth, and pathogenicity (Rodrigues et al. 2000, Rittershaus et al. 2006). There are few studies on cell wall molecules in *C. neoformans* (Foster et al. 2007).

Considering the scarce literature about cell wall composition, the present work aimed to describe lipid and protein cell wall components using as model an acapsular *C. neoformans*

mutant (cap 67) in order to avoid contamination with capsule components. The role of the presently described structures in fungal-host relationship is speculated.

MATERIALS AND METHODS

Reagents and solvents

Otherwise stated, all reagents and solvents used in this study were HPLC-grade or higher, and mostly from Sigma-Aldrich Co. (St. Louis, MO).

C. neoformans cap 67 growth conditions

Acapsular mutant *C. neoformans* cap 67 was maintained in slants of modified YPD medium (0.5% yeast extract, 0.5% casein peptone, 1.5% glucose, pH 6.5) at 36 °C. Yeast cells were then seeded to grow at 36 °C for 48 h in 100 ml pre-inoculum flasks containing a defined medium (15 mM glucose, 10 mM $MgSO_4$, 29.5 mM KH_2PO_4, 13 mM glycine, and 3 μM thiamine, pH 5.5), transferred to fresh medium (200 ml) and cultivated for three more days before cell wall purification.

Cell wall purification

Cell wall purification was performed as previously described for *Paracoccidioides brasiliensis* (Longo et al. 2013b). Briefly, yeast cell pellets were washed three times in phosphate-buffered saline solution (PBS) and mechanically broken in a cell disruptor (B. Braun Biotech International GmBH, Melsungen, Germany) in the presence of glass beads (425–600 μm, Sigma Aldrich). Mechanical disruption was achieved by six turns of 10-min agitation alternated with a 10-min rest on ice. For proteomic analysis, self-proteolysis was prevented with the use of protease inhibitors (100 mM ethylenediamine tetraacetic acid, EDTA; 10 mM 1,10-phenanthroline; 1 mM phenylmethylsulfonyl fluoride, PMSF; 1 μM pepstatin A; and 15 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane, E-64). The cell wall-containing fraction was pelleted by centrifugation (5,000 × *g* for 10 min at 4 °C) and washed with deionized water. Cytoplasmic and membranous contents were eliminated by three sequential centrifugations (8,000 × *g* for 45 min at 4 °C) in 85% sucrose (Kanetsuna et al. 1969). The cell wall enriched fraction was then washed differently for proteomic and lipidomic analysis. Before lipid extraction (detailed below), cell wall fractions were washed 50 times in ultrapure water (Previato JO 1979). In preparations destined for protein extraction (detailed below), cell pellets were first washed five times with ultrapure water. We then sequentially carried out five washes with each NaCl solution (5% NaCl, 2% NaCl and 1% NaCl), to remove non-specifically bound components that could eventually remain in the cell wall preparation, followed by five washes with 1 mM PMSF (Pitarch et al. 2002).

Lipid extraction and fractionation

A lyophilized cell wall preparation (100 mg) from *C. neoformans* cap 67 was sequentially extracted three times, under shaking with glass beads, with 1 ml chloroform:methanol (2:1, v:v) and chloroform:methanol:water (2:1:0.8, v:v:v), as described (Yichoy et al. 2009). Both extracts were combined, dried under a N_2 stream and dissolved in 2 ml chloroform. Lipid fractionation was performed in 500 mg of silica gel 60 spheres (60Å, 200–400 mm, Sigma-

Aldrich, St. Louis, MO) packed in a Pasteur pipette. The silica was sequentially washed in 5 ml HPLC-grade methanol, acetone, and chloroform before loading with extracted total lipids. Neutral glycolipids and phospholipids were sequentially eluted with 5 ml of acetone and methanol, and dried under N_2 stream.

Glycolipid permethylation

Glycolipids were permethylated as previously described (Ciucanu and Kerek 1984). Glycolipids were stored overnight at −20 °C in desiccant silica and redissolved in 150 μl anhydrous dimethyl sulfoxide (DMSO). A few milligrams of powdered, anhydrous NaOH were added and the mixture was vortexed. After the addition of 80 μl iodomethane, the sample was incubated under shaking for 1 h at room temperature. Two ml of both deionized water and dichloromethane were added, the mixture was vortexed and centrifuged, and the aqueous upper layer was removed. The remaining organic phase was washed three times in 2 ml deionized water and dried under N_2 stream.

ESI-MS/MS analysis of phospholipids and glycolipids

In order to analyze phospholipids (negative- and positive-ion modes) and glycolipids, we used electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a linear ion-trap mass spectrometer (LTQ XL, ThermoFisher Scientific Inc., San Jose, CA) coupled with an automated nanospray source (TriVersa NanoMate System, Advion, Ithaca, NY), as described previously (Vallejo et al. 2012b). For the negative-ion mode analysis, phospholipid samples were redissolved in methanol containing 0.05% formic acid (FA) and 0.05% NH_4OH; 2.5 μM phosphatidylglycerol (C12:0/C12:0-PG) (Avanti Polar Lipids, Alabaster, AL) was used as internal standard. For the positive-ion mode analysis, phospholipid samples were redissolved in 10 mM LiOH/methanol and 2.5 μM phosphatidylcholine (C11:0/C11:0-PC) (Avanti Polar Lipids) was used as internal standard. Full-scan spectra were collected at the 500–1000 *m/z* range, and samples were subjected to total-ion mapping (TIM) (2 a.m.u. isolation width; pulsed-Q dissociation (PQD) to 29% normalized collision energy; activation Q of 0.7; and activation time of 0.1 ms). We redissolved permethylated glycolipids in methanol and analyzed them as described for phospholipids, using the following parameters: full-scan spectra acquisition at the 500–2000 *m/z* range, TIM settings at the 700–900 *m/z* range, 2 a.m.u. isolation width; PQD to 32% normalized collision energy; activation Q of 0.7; and activation time of 0.1 ms. All MS/MS spectra were analyzed manually, as described (Pulfer and Murphy 2003).

SDS extraction of cell wall-associated proteins

Proteins were extracted according to Casanova and coworkers (Casanova et al. 1989), with modifications. Briefly, a lyophilized cell wall preparation (100 mg) was incubated twice for 5 min at 90 °C in a solution containing 100 mM Na-EDTA, 50 mM Tris-HCl, pH 7.8 and 2% SDS. The SDS extracts were collected by centrifugation and filtrated through a sterile 0.22-micron filter. The filtered extract was incubated in ice-cold acetone (1 h at −20 °C), centrifuged for 30 min (16,000 × *g* at 4 °C) and the protein pellet was removed, washed in acetone and dried.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Protein digestion was carried out as described by Russell and coworkers (Russell et al. 2001). After denaturation at 95 °C for 5 min in buffered methanol/50 mM NH_4HCO_3 (60:40, v/v), proteins were incubated in 5 mM dithiotreitol (DTT) for 30 min at 37 °C for disulfide bond reduction and cysteine residues were alkylated with 10 mM iodoacetamide (90 min at room temperature in the dark). Proteolytic digestion was achieved by overnight incubation at 37 °C with 1 μg sequencing-grade trypsin (Promega), and 0.05% of trifluoroacetic acid (TFA) was added to stop the reaction. In-house POROS R2 microcolumns (Nakayasu et al. 2012) were used for desalting the ensuing peptides, which were then dried in a vacuum concentrator until the LC-MS/MS analysis.

LC-MS/MS analysis and peptide validation

We dissolved tryptic peptides in 20 μL 0.1% formic acid (FA), and loaded 5 μL of the mixture onto a reversed-phase trap column (1 cm × 75 μm, Luna C18, 5 μm, Phenomenex). LC was performed in a capillary reversed-phase column (20 cm × 75 μm, Luna C18, 5 μm, Phenomenex) coupled to a nanoHPLC (1D Plus, Eksigent), exactly as previously described (Vallejo et al. 2012a). Elution of bound peptides was carried out in a linear gradient (5% to 40%) of solvent B [solvent A: 5% acetonitrile (ACN)/0.1% FA; solvent B: 80% ACN/0.1% FA] over 200 min and directly analyzed in a linear ion trap-mass spectrometer equipped at the front end with a TriVersa NanoMate nanospray source. The nanospray was set at 1.45 kV and 0.25 psi N_2 pressure using a chip A (Advion). MS spectra were collected in centroid mode at the 400–1700 *m/z* range and the ten most intense ions were subjected twice to collision-induced dissociation (CID) with 35% normalized collision energy, before being dynamically excluded for 60 s.

Using Bioworks v.3.3.1 (Thermo Fisher), we initially converted into DTA files all MS/MS spectra from peptides bearing 800 to 3,500 Da (over 10 counts) and at least 15 fragments. DTA files were subsequently submitted to database search using TurboSequest (Bioworks 3.3.1, Thermo Fisher Scientific). Sequence information from the *C. neoformans var. grubii* H99 genome (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html), human genome (International Protein Index), besides porcine trypsin sequences (GenBank), were used in the correct and reverse orientations. The database search parameters included: i) trypsin cleavage in both peptide termini with one missed cleavage site allowed; ii) carbamidomethylation of cysteine residues as a fixed modification; iii) oxidation of methionine residues as a variable modification; and iv) 2.0 Da and 1.0 Da for peptide and fragment mass tolerance, respectively. TurboSequest outputs were filtered with DCn ≥ 0.05, peptide probability ≤ 0.05, and Xcorr ≥ 1.5, 2.0, and 2.5 for singly-, doubly-, and triply-charged peptides, respectively. Files were then exported into XML formats and peptide sequences were assembled into proteins using an in house written script (Nakayasu et al. 2012). Redundant protein hits were assembled into protein groups and protein hits were re-filtered with the sum of peptide Xcorr ≥ 3.0. The false-discovery rate (FDR) was estimated as described previously (Rodrigues et al. 2008, Albuquerque et al. 2008). Proteins with shared peptides were assembled into groups to assess the redundancy issue. Only proteins detected by five or more spectral counts were considered for further analysis.

Bioinformatic analysis

Protein sequences were submitted to Blast2GO algorithm http://www.blast2go.de/ (Conesa et al. 2005) and classified by Gene Ontology (GO) according to their biological functions. Putative secretory proteins were classified according to the Fungal Secretome Database (FSD; http://fsd.snu.ac.kr/)(Choi et al. 2010), and were labeled as not classified, SP (containing signal peptide identified by SignaIP 3.0), SP3 (bearing signal peptide predicted by SigPred, SigCleave or RPSP), SL (subcellular localization predicted by PSort II and/or Target 1.1b), and NS (nonclassical secretion, predicted by SecretomeP 1.0f). Identification of orthologous proteins was performed manually or using the OrthoMCL software v2.0 (http://www.orthomcl.org/cgi-bin/OrthoMclWeb.cgi), with percentage match cutoff = 50 and E- value exponent cutoff = −5.

RESULTS

We carried out lipidomic and proteomic analysis of cell wall components from the acapsular mutant *C. neoformans* cap 67 by ESI-MS/MS and LC-MS/MS, respectively. Before specific extraction procedures, cell wall pellets were enriched and separated from membrane contaminants through sucrose density centrifugation (Kanetsuna et al. 1969), followed by exhaustive washes in water (for lipid extraction) (Previato JO 1979) or decreasing salt concentration (for protein extraction) (Pitarch et al. 2002), as detailed in Materials and Methods.

ESI-MS/MS analysis of cell wall phospholipids

Phospholipids from the acapsular mutant *C. neoformans* cap 67 cell wall preparations were analyzed by ESI-MS/MS in the positive- and negative-ion modes (Table 1). The full-scan spectra profile comprised a broad range of mass to charge ratio (*m/z*), suggesting the presence of phospholipid species from different classes (not shown). The identification of phospholipid species was performed exactly as in previous works from our group focusing on *P. brasiliensis* extracellular vesicles and cell wall lipids (Vallejo et al. 2012b, Longo et al. 2013a). Briefly, we first searched positive- and negative-ion modes for diagnostic fragment ions and neutral losses exclusive of phospholipids. Then, we looked for specific peaks and losses to identify phospholipid classes. In the negative-ion mode, phospholipid species were identified with chlorine (Cl^-) ($[M + Cl]^-$) or formate ($HCOO^-$) ($[M + HCOO]^-$) adducts. In the positive-ion mode, phosphatidylcholine (PC) species were identified with adduct of Li^+ ($[M + Li]^+$) or singly protonated ($[M + H]^+$). Fatty acid chains were also identified by neutral fragment losses (positive ion-mode, Fig. S1) or by the negatively charged carboxylate ions at *m/z* 255 (C16:0), 279 (C18:2), and 281 (C18:1) (Fig. S2–S6).

Phospholipid species of the PE (six), PC (two), PA (five), PS (four), and PI (two) classes were detected at *m/z* ranges between 477.3 and 836.5 in the negative-ion mode, and six PC species were identified between *m/z* 758.6 and 788.6 in the positive-ion mode (Table 1). Among the identified PE species, two of them showed only one fatty acid, *lyso*-C18:1-PE (*m/z* 478.4) and *lyso*-C18:2-PE (*m/z* 476.4 and *m/z* 534.4).

ESI-MS/MS analysis of cell wall glycolipids

Neutral glycolipids were extracted from cell wall preparations of the acapsular mutant *C. neoformans* cap 67, isolated in a silica-60 column, permethylated, and subjected to a positive-ion mode ESI-MS/MS analysis (Fig. 1). Fig. 1A shows peaks of two major glycolipid species detected in full-scan spectra at *m/z* 876.8 and 874.7, which were further characterized by the MS/MS analysis. Glycolipid species were identified as previously reported (Vallejo et al. 2012b, Longo et al. 2013a) by diagnostic fragment ions and specific neutral losses. A C18:0 [azirine-h18:0Me_2 – H_3COH + H]$^+$ or C18:1 [azirine-h18:1Me_2 – H_3COH + H]$^+$ fatty acid attached to a remnant of the sphingoid base could be identified at *m/z* 322.4 and 320.4 fragment ions, respectively in Hex-C18:0-OH/d19:2-Cer (at *m/z* 876.8) (Fig. 2B) and Hex-C18:1-OH/d19:2-Cer (at *m/z* 874.7) (Fig. 1B and S7). Although the MS/MS analysis is not able to indicate the unsaturation or the methyl and hydroxyl group positions in the sphingoid base, we believe that the main neutral glycolipid here identified is *N*-2'-hydroxyoctadecanoate (4*E*, 8*E*)-9-methyl-4,8-sphingadienine (*m/z* 876.8), previously described as the major cryptococcal glycosphingolipid (GSL) and abundantly localized at the fungal cell wall (Rodrigues et al. 2000).

LC-MS/MS analysis of *C. neoformans* cap 67 cell wall-associated proteins

Before protein extraction, cell wall preparations were extensively washed in solutions with decreasing concentrations of NaCl in order to remove proteins that might be non-specifically bound to the cell wall preparation during cell lysis. We used two sequential extractions with boiling SDS, which is supposed to release proteins loosely bound to the fungal surface (Casanova et al. 1989). In order to make our analysis more reliable, we used an arbitrary and stringent cut-off corresponding to proteins detected by five or more spectral counts. Using this criterion, a total of 192 proteins belonging to different metabolic processes were identified (Table S1). Proteins belonging to the most represented biological processes are listed in Table 2.

We analyzed the biological processes related to the identified proteins using the Blast2GO algorithm (Conesa et al. 2005). Proteins involved in diverse cellular processes were found (Fig. 2), but translation (22%) and carbohydrate metabolism (14%) were the most represented, followed by oxidation/reduction (9%), protein metabolism (9%), transport (4%), and response to stress (4%).

Translation was the most enriched process, in which ribosomal proteins from large and small ribosome subunits prevailed. In addition, two elongation factors (CNAG_00417 and CNAG_06105), a helicase (CNAG_00785), and an argonaute protein (CNAG_04609) were identified. The carbohydrate metabolism group comprised some metabolic enzymes, such as phosphomannomutase (CNAG_05351) and pyruvate kinase (CNAG_01820). We also point out the presence of a 1,3-beta-glucanosyltransferase (CNAG_06501), related to cell wall biosynthesis/remodeling. Some proteins were associated with response to stress and oxidation/reduction, which may potentially account for fungal virulence by playing specific roles in infection. In these groups we identified several heat shock proteins (CNAG_00334, CNAG_01727, CNAG_01750, CNAG_03891, CNAG_06150 and CNAG_06208), a peroxiredoxin (CNAG_03482), and a catalase (CNAG_04981).

Although many proteins identified in this work are typically cytoplasmic, such as ribosomal and carbohydrate metabolic proteins, almost 60% of them have previously been described at the cell surface of other fungal species as well (Table 2; Rodrigues et al. 2013).

Considering the importance of protein export to the extracellular environment in modulating the host-pathogen relationship, we used the Fungal Secretome Database (FSD) to classify proteins identified in this work according to their secretory features. FSD gathers information from nine secretion prediction programs for a number of fungal genomes, comprising classical and unconventional secretory mechanisms (Choi et al. 2010). The majority of identified proteins were classified as secretory (56%), being 44% via nonclassical (NS) mechanisms, therefore lacking secretory known signals (Fig. 3A).

We then questioned whether orthologs of the proteins identified in this work have been previously reported in fungal extracellular vesicles or cell wall. Fungal extracellular vesicles constitute an unconventional pathway for cytoplasmic components to reach the cell wall and the extracellular milieu, as already described for *C. neoformans* (Rodrigues et al. 2008, Wolf et al. 2014), *Histoplasma capsulatum* (Albuquerque et al. 2008), *S. cerevisiae* (Oliveira et al. 2010), *P. brasiliensis* (Vallejo et al. 2012a), and *Malassezia sympodialis* (Gehrmann et al. 2011). Using the OrthoMCL software, we found that 86% of the identified proteins in this work have been previously reported in fungal extracellular vesicles and/or cell wall (Fig. 3B). Proteins such as hsp90-like (CNAG_06150), ATP-dependent RNA helicase eIF4A (CNAG_00785), and enolase (CNAG_03072) have previously been described in extracellular vesicles and cell wall from several fungal species (Table 2).

DISCUSSION

In the present work we described the structure of phospholipids, neutral glycolipids, and the nature of non-covalently linked proteins extracted from purified cell wall preparations obtained from the acapsular *C. neoformans* mutant cap 67.

In our cell wall sample, PE and PC were the most represented *C. neoformans* cap 67 phospholipid classes, each one composed by six species, followed by PA (five species), PS (four species), and PI (two species). Recently, cell wall lipidome of the dimorphic pathogen *P. brasiliensis* also showed that PC and PE are the phospholipid classes comprising more species in the yeast form (Longo et al. 2013a). Overall, phospholipids from both pathogen cell walls were similar, except for phosphatidylglycerol (PG) that was identified only in *P. brasiliensis*. Previously, PC has been described as a major cryptococcal membrane phospholipid (Rawat et al. 1984) that has also been identified in the fungal extracellular vesicles (Oliveira et al. 2009). On the other hand, whole phospholipid analysis of extracellular vesicles identified PE, PS, and PC species in *H. capsulatum* (Albuquerque et al. 2008) and PC, PE, PS, PI, and PG in *P. brasiliensis* (Vallejo et al. 2012b).

We were able to identify two species of neutral GSLs, specifically Hex-C18:0-OH/d19:2-Cer (*m/z* 876.8), the most abundant, and Hex-C18:1-OH/d19:2-Cer (*m/z* 874.7). Considering that our strategy allows extraction of only neutral glycolipids, the presence of acidic GSLs at the cell wall cannot be discarded. Hex-C18:0-OH/d19:2-Cer (*m/z* 876.8) has previously been

identified as a major ceramide monohexoside (CMH) in whole *C. neoformans* lipid extracts (Rodrigues et al. 2000). Since glucose is the hexose identified in this CMH, we believe that the neutral glycolipids presently characterized are also β-glucosylceramide (GlcCer) species. GlcCer has already been detected in cell wall extracts from *P. brasiliensis* (Longo et al. 2013a) and *C. albicans* (Thevissen et al. 2012). It has also been localized by a monoclonal antibody at the surface of *P. brasiliensis*, *H. capsulatum*, *Sporothrix schenckii* yeasts, and mycelia from *P. brasiliensis*, and *Aspergillus fumigatus* (Toledo et al. 2001, Longo et al. 2013a). In *C. neoformans*, transmission electron microscopy images showed GlcCer abundantly distributed along the cell wall, and confocal microscopy revealed GlcCer accumulation in budding sites, correlating with thickened regions of the cell wall (Rodrigues et al. 2000). Identification of this CMH in the lipidome of our cell wall preparation was used as a marker for cell wall enrichment.

In *C. neoformans*, GlcCer is involved in fungal budding, growth, and pathogenicity (Rodrigues et al. 2000, Rittershaus et al. 2006). It also plays a role in *A. fumigatus* growth (Noble et al. 2010) and *C. albicans* virulence (Chattaway et al. 1968). In addition to cell wall localization, GlcCer species have been identified in extracellular vesicles from *P. brasiliensis* (Vallejo et al. 2012b) and *C. neoformans* (Rodrigues et al. 2007).

Comparison of the cell wall phospholipids, GSLs, and non-covalently bound proteins (discussed below) here identified in *C. neoformans* with those described in fungal extracellular vesicles (EVs) strongly reinforces the presumption that, while traversing the cell wall towards the extracellular environment, EVs may be entrapped and release their components, thus participating in the fungal cell wall composition either as transient or as permanent constituents (Longo et al. 2014). The role of EVs in the transportation of virulence factors through the cell wall to the external milieu has already been recognized (Casadevall et al. 2009) and in *C. neoformans* EVs also traverse the capsule and participate in its synthesis by releasing GXM that can then be incorporated to form capsule fibers (Rodrigues et al. 2007). Most cell wall-associated proteins identified here are typically cytoplasmic; however, it was interesting to find that only 14% of them have not been previously described in the surface or EVs from other fungal species. Additionally, 56% show secretory features, mostly of non-classical (NS) nature. A similar scenario has recently been reported for DTT-extractable cell wall-associated proteins in *P. brasiliensis* (Longo et al. 2014). Non-classical (NS) secretion includes exportation mechanisms using extracellular vesicles originated from plasma membrane blebbing, exosome release (Rodrigues et al. 2011, Nickel 2010), and the recently proposed biogenesis through cell membrane invagination (Rodrigues et al. 2013). These mechanisms result in membrane and cytoplasmic subtractions, thus helping to justify the finding of some components at the cell wall.

We identified 192 non-covalently bound cell wall proteins related to translation, carbohydrate and protein metabolism, and oxidation/reduction. Although *C. neoformans* cell wall is not the outermost fungal structure because it is surrounded by a polysaccharide capsule, its components probably play important roles in the host-pathogen relationship, as already reported in other species (Gow and Hube 2012). Proteins isolated from *C. neoformans* cell wall and membrane stimulated lymphocyte proliferation from both adults

and fetal cord blood, and that may account for cell-mediated immunity to this fungus (Mody et al. 1996). Some presently identified proteins may potentially contribute to fungal virulence by playing specific roles in infection, specially those related to response to stress and oxidation/reduction, as exemplified by heat shock proteins, a thioredoxin (CNAG_02801), and a catalase (CNAG_04981).

Tefsen and coworkers (Tefsen et al. 2014) showed that the *C. neoformans* inactivation of the *CAP10* genes, which impairs the polysaccharide capsule formation, leads to enlarged cells prone to aggregation and upregulation of various genes, mainly related to oxidative stress. Some resulting proteins are cell wall-associated according to our present analysis, for e.g., thiol-specific antioxidant protein 3 (CNAG_06917) and peroxiredoxin (alkyl hydroperoxide reductase subunit C) (CNAG_03482), also known as thiol-specific antioxidant protein 1 (Tsa1). Tsa1 has already been detected by confocal microscopy on *C. albicans* surface of the pathogenic hyphal phase and was shown to be related to oxidative stress resistance and correct cell wall composition (Urban et al. 2003). Tsa1 has been identified among proteins from *C. albicans* and *P. brasiliensis* cell wall preparations (Table 2) and we have localized it by confocal microscopy at the *P. brasiliensis* yeast surface (Longo et al. unpubl. data).

Recognition of cell wall proteins by immune cells or antibodies can interfere in the host immune response (Gow et al. 2012). Many proteins presently identified at the *C. neoformans* cell wall proteins have also been identified in *P. brasiliensis* cell wall extracts from live cells (Table 2). Some are antigenic and recognized by paracoccidioidomycosis (PCM) patients' sera, such as hsp60-like protein (CNAG_03891) (Cunha et al. 2002), heat shock 70 kDa protein 4 (CNAG_06208) (Bisio et al. 2005), triose-phosphate isomerase (TPI) (CNAG_02035), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (CNAG_06699) (da Fonseca et al. 2001). Vaccination using Hsp60 induced protective cellular immune response against experimental pulmonary PCM (de Bastos Ascenco Soares et al. 2008), while in *H. capsulatum* it is involved in fungal attachment to macrophage CD11/CD18 receptors (Long et al. 2003). Importantly, TPI, GAPDH and Hsp60 are predicted adhesins in several fungal species (Chaudhuri et al. 2011) and may prompt fungal invasion. Enolase (CNAG_03072) can bind and activate plasminogen to plasmin, a serine protease involved in *P. brasiliensis* invasion to host tissues (Nogueira et al. 2010). In *C. albicans*, cell wall enolase and TPI can bind to human kininogen, a kinin precursor related to human host response to microbial infections (Karkowska-Kuleta et al. 2011).

Overall, the present work improves our knowledge about the poorly studied *C. neoformans* cell wall, opening doors to understanding its possible roles in fungal biology and pathogenesis. In addition, comparisons of the proteomic and lipidomic data with those from fungal EVs support the idea that these structures are at least partially responsible for adding components to the cell wall (Rodrigues et al. 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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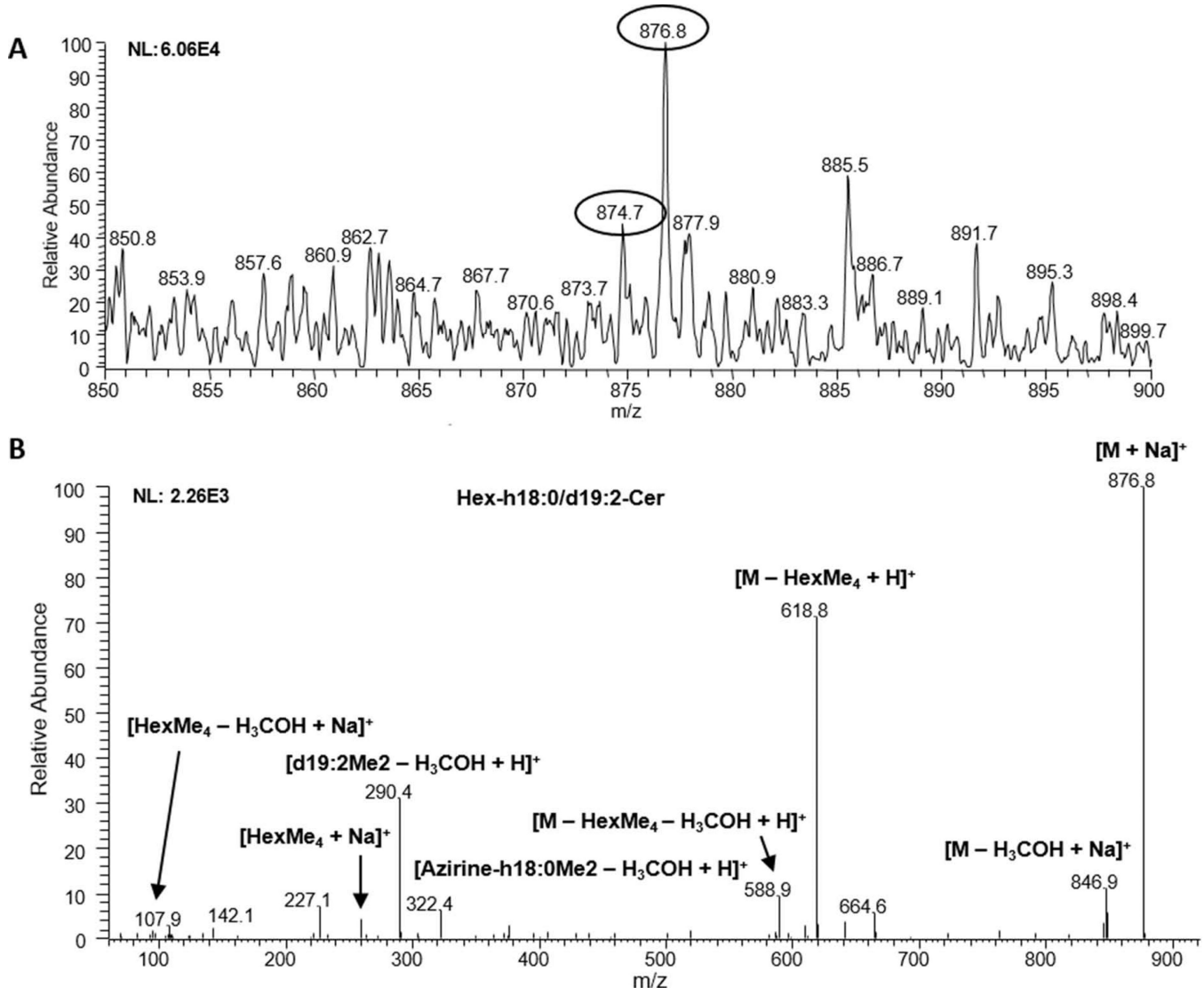

Fig. 1.

ESI-MS analysis of neutral glycolipids from *C. neoformans* cap 67 mutant cell wall. **A.** Full-scan spectrum in the positive-ion mode. The *m/z* of identified glycolipids Hex-C18:1-OH/d19:2-Cer (*m/z* 874.7) and Hex-C18:0-OH/d19:2-Cer (*m/z* 876.8) are circled. **B.** Tandem-MS spectrum of the major glycolipid species identified (*m/z* 876.8). *m/z*, mass to charge ratio.

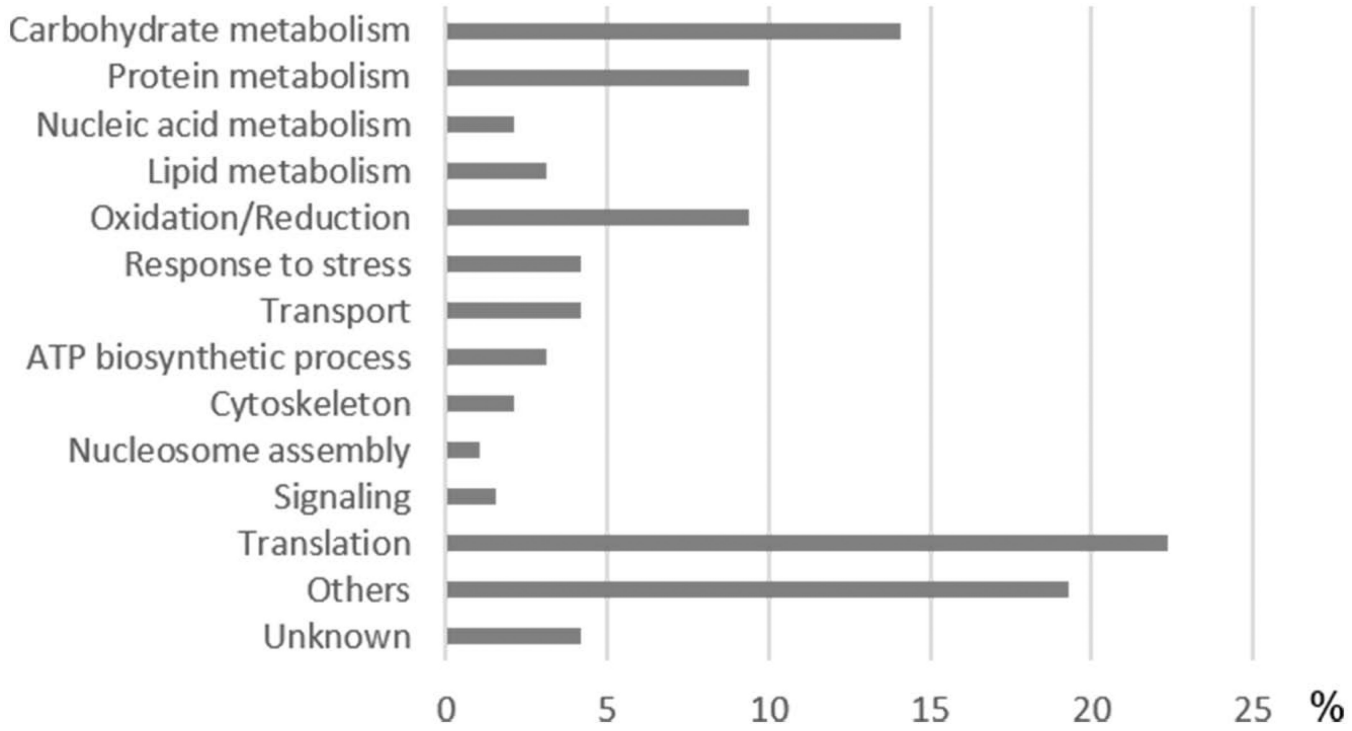

Fig. 2.

Functional categorization of cell wall-associated proteins from *C. neoformans* cap 67 mutant. Values represent the percentages relative to all identified proteins.

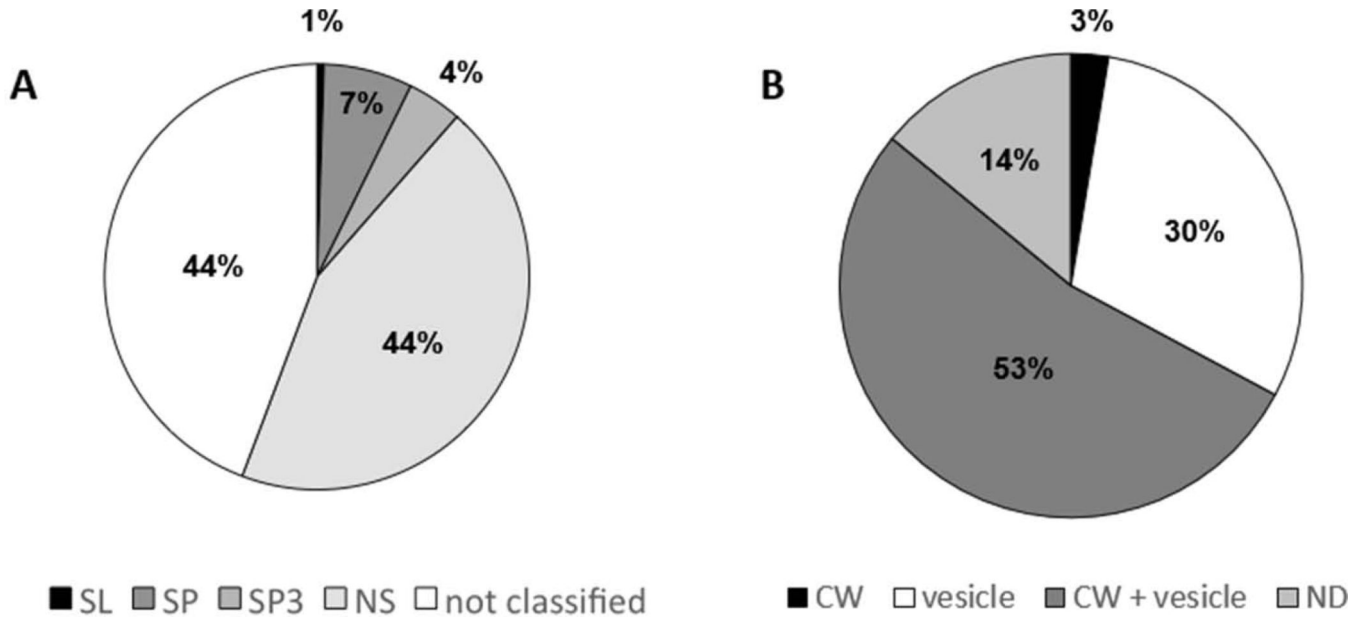

Fig. 3.

Characterization of cell wall-associated proteins from *C. neoformans* cap 67. **A.** Secretory potential classified by the Fungal Secretome Database (FSD): SP (presence of signal peptide as identified by SignaIP 3.0); SP3 (signal peptide predicted by SigPred, SigCleave or RPSP); SL (subcellular localization predicted by PSort II and/or Target 1.1b); NS (nonclassical secretion, predicted by SecretomeP 1.0f), and not classified (described as not secretory). **B.** Percentage of fungal orthologs previously reported in the cell wall (CW), extracellular vesicles (vesicle), both locations (CW+vesicle), or in neither of these compartments (ND).

Table 1

Composition of phospholipids extracted from *C. neoformans* cap67 cell wall preparations as identified by ESI-MS total-ion mapping

Phospholipid/Ionmode	Ionspecies	*m/z*	Proposed composition	Predicted mass (Da)
Phosphatidylethanolamine (PE)				
(−)	$[M-H]^-$	478.4	lyso C18:1	479.3
(−)	$[M-H]^-$	476.4	lyso C18:2	477.3
(−)	$[M + Cl]^-$	534.4		
(−)	$[M-H]^-$	716.6	C16:0/C18:1	717.5
(−)	$[M + Cl]^-$	774.7		
(−)	$[M-H]^-$	714.7	C16:0/C18:2	715.5
(−)	$[M-H]^-$	740.7	C18:1/C18:2	741.5
(−)	$[M + Cl]^-$	798.6		
(−)	$[M + HCOO]^-$	808.6		
(−)	$[M-H]^-$	742.6	C18:1/C18:1	743.5
(−)	$[M + Cl]^-$	800.6		
(−)	$[M + HCOO]^-$	810.6		
Phosphatidylcholine (PC)				
(−)	$[M + HCOO]^-$	802.6	C16:0/C18:2	758.6
(+)	$[M + Li]^+$	764.8		
(−)	$[M + Cl]^-$	821.7	C18:1/C18:1	786.6
(+)	$[M + Li]^+$	793.7		
(+)	$[M + Li]^+$	766.6	C16:0/C18:1	760.6
(+)	$[M + Li]^+$	768.7	C16:0/C18:0	762.6
(+)	$[M + Li]^+$	790.3	C18:1/C18:2	784.6
(+)	$[M + Li]^+$	794.7	C18:0/C18:1	788.6
PhosphatidicAcid (PA)				
(−)	$[M-H]^-$	671.5	C16:0/C18:2	672.5
(−)	$[M-H]^-$	673.5	C16:0/C18:1	674.5
(−)	$[M + Cl]^-$	731.6		
(−)	$[M-H]^-$	695.5	C18:2/C18:2	696.5

Phospholipid/Ionmode	Ionspecies	*m/z*	Proposed composition	Predicted mass (Da)
(−)	$[M-H]^-$	697.6	C18:1/C18:2	698.5
(−)	$[M + Cl]^-$	755.6		
(−)	$[M-H]^-$	699.5	C18:1/C18:1	701.0
(−)	$[M + HCOO]^-$	767.6		
Phosphatidylserine (PS)				
(−)	$[M-H]^-$	758.6	C16:0/C18:2	759.5
(−)	$[M-H]^-$	760.6	C16:0/C18:1	761.5
(−)	$[M-H]^-$	784.5	C18:1/C18:2	785.5
(−)	$[M-H]^-$	786.8	C18:1/C18:1	787.5
Phosphatidylinositol (PI)				
(−)	$[M-H]^-$	835.7	C16:0/C18:1	836.5
(−)	$[M-H]^-$	833.8	C16:0/C18:2	834.5

Table 2

Cell wall-associated proteins identified in *C. neoformans* cap67 cell wall preparations. The proteins areorganized according to GO functions. Minor and unknown functions are omitted, but can be seen in a complete Supplemental Table 1.

Codenumber*	Proteinname	CW**	EV**
	Transport		
CNAG_02817	GTP-binding protein ypt2	-	CnHcPbSc
CNAG_02974	voltage-dependent anion channel protein 2	Af	Cn
CNAG_04851	transitionalendoplasmicreticulumATPase	Ca Pb	HcSc
CNAG_04904	clathrin heavy chain	-	-
CNAG_06101	ADP, ATP carrier protein	Ca	CnHcPbSc
CNAG_06377	solute carrier family 25, member 3	-	Cn
CNAG_00499	solute carrier family, member 20/29	-	Hc
CNAG_06096	tricarboxylatecarrier	-	-
	Response to stress		
CNAG_00334	hsp75-like protein	Af Ca Sc	CnHcPbSc
CNAG_01727	hsp71-like protein	Af Ca Sc	CnHcPbSc
CNAG_01750	hsp72-like protein	Ca Sc	CnHcPbSc
CNAG_03891	hsp60-like protein	Ca Pb	CnHcPbSc
CNAG_05199	chaperoneDnaK	Af Ca	HcPbSc
CNAG_06150	hsp90-like protein	Af Ca PbSc	CnHcPbSc
CNAG_06208	heat shock 70kDa protein 4	Af Ca PbSc	HcPbSc
CNAG_06443	glucose-regulatedprotein	Ca Sc	CnHcPbSc
	ATP biosyntheticprocess		
CNAG_01004	ATP synthase F1, delta subunit	-	CnHcPb
CNAG_04439	V-type proton ATPase subunit B	-	CnSc
CNAG_05750	ATP synthase subunit alpha, mitochondrial	Ca Pb	CnHcPbSc
CNAG_05918	ATP synthasesubunit beta, mitochondrial	Ca Pb	CnHcPbSc
CNAG_06400	proton-efflux P-type ATPase	Ca Sc	CnHcPbSc
CNAG_02326	V-type proton ATPase catalytic subunit A	-	HcSc
	Translation		
CNAG_00034	largesubunitribosomalprotein L9e	-	CnPbSc
CNAG_00096	small subunit ribosomal protein S3	-	HcPbSc
CNAG_00640	small subunit ribosomal protein S4-A	Ca	CnHcSc
CNAG_00656	largesubunitribosomalprotein L7e	Ca Sc	CnPbSc
CNAG_00672	small subunit ribosomal protein S9	Sc	CnHc
CNAG_01628	small subunit ribosomal protein S20	Ca Sc	CnHc
CNAG_01024	large subunit ribosomal protein L18-A	-	Cn
CNAG_02928	largesubunitribosomalprotein L5e	Ca Sc	CnHcPbSc
CNAG_00779	largesubunitribosomalprotein L27e	Sc	CnHc
CNAG_00952	smallsubunitribosomalprotein S6e	Ca	HcSc
CNAG_00953	smallsubunitribosomalprotein S13e	Ca	CnPbSc

Codenumber*	Proteinname	CW**	EV**
CNAG_01884	large subunit ribosomal protein L3	Ca Sc	CnHcPb
CNAG_01486	large subunit ribosomal protein L15-A	Ca PbSc	CnSc
CNAG_01951	small subunit ribosomal protein S22-A	-	CnHcSc
CNAG_02144	large subunit ribosomal protein L1-A	-	CnHcPb
CNAG_02234	largesubunitribosomalprotein L6e	Ca Pb	Cn
CNAG_02331	small subunit ribosomal protein S9	Ca	Cn
CNAG_03000	smallsubunitribosomalprotein S19e	Ca	Cn
CNAG_03053	large subunit ribosomal protein L23	-	CnSc
CNAG_03198	smallsubunitribosomalprotein S8e	Ca	CnSc
CNAG_03510	largesubunitribosomalprotein L36e	-	HcPb
CNAG_03577	large subunit ribosomal protein LP0	AfPbSc	HcSc
CNAG_03739	large subunit ribosomal protein L10-like	Ca Sc	CnHcSc
CNAG_03780	small subunit ribosomal protein S16	Ca	CnSc
CNAG_04004	small subunit ribosomal protein S1	Ca Sc	CnHcPb
CNAG_00409	large subunit ribosomal protein L37a	-	Cn
CNAG_04021	large subunit ribosomal protein L24	-	Cn
CNAG_00494	small subunit ribosomal protein S0	Af Ca Sc	CnSc
CNAG_04445	smallsubunitribosomalprotein S7e	Ca Sc	CnHcPbSc
CNAG_04726	large subunit ribosomal protein L18Ae	Ca	Cn
CNAG_04762	largesubunitribosomalprotein L4e	Ca	CnHcPbSc
CNAG_04799	largesubunitribosomalprotein L14e	-	CnPbSc
CNAG_04883	small subunit ribosomal protein S18	-	CnHcPb
CNAG_00417	elongationfactor 1-gamma	Pb	CnPbSc
CNAG_00785	ATP-dependent RNA helicase eIF4A	Af Ca Sc	CnHcPbSc
CNAG_04609	argonaute	-	CnHc
CNAG_05555	large subunit ribosomal protein L7Ae	PbSc	CnSc
CNAG_06105	elongationfactor 1-alpha	Ca PbSc	CnHcPbSc
CNAG_06605	small subunit ribosomal protein S2	AfSc	CnHcPbSc
CNAG_00689	largesubunitribosomalprotein L22e	Pb	Pb
CNAG_05232	large subunit ribosomal protein L8	Ca PbSc	CnHcSc
CNAG_06231	large subunit ribosomal protein L13	-	-
CNAG_06447	large subunit ribosomal protein L22	Ca Sc	CnSc
	Proteinmetabolism		
CNAG_04009	aminopeptidase 2	-	HcPbSc
CNAG_00150	peptidase	-	Cn
CNAG_00919	carboxypeptidase D	Pb	CnSc
CNAG_01890	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	Ca PbSc	CnHcPbSc
CNAG_02500	calnexin	Pb	Cn
CNAG_02966	carboxypeptidase	-	Sc
CNAG_03507	mitochondrial peptidase subunit beta	-	CnHc
CNAG_04604	tryptophan-tRNAligase	-	-
CNAG_04625	cerevisin	Ca Pb	CnHcPbSc

Codenumber*	Proteinname	CW**	EV**
CNAG_05725	ketol-acidreductoisomerase, mitochondrial	Ca	CnHcSc
CNAG_05932	peptidyl-prolylcis-transisomerase D	Pb	HcPb
CNAG_06026	aspartateaminotransferase	Pb	HcPbSc
CNAG_06088	aspartateaminotransferase, mitchondrial	-	-
CNAG_00136	ubiquitin-activatingenzyme E1	-	Sc
CNAG_05722	alanine-tRNAligase	Sc	Sc
CNAG_04906	26S protease regulatory subunit 10B	-	-
CNAG_05886	ubiquitin-conjugatingenzyme E2	Pb	-
CNAG_06755	threonine-tRNAligase	-	Sc
	Carbohydratemetabolism		
CNAG_00061	citratesynthase, mitochondrial	Ca	CnHcPbSc
CNAG_00937	aconitatehydratase, mitochondrial	Ca Pb	HcPbSc
CNAG_00964	glutamine-fructose-6-P transaminase	-	-
CNAG_01820	pyruvatekinase	Ca Sc	CnHcSc
CNAG_01896	alcoholdehydrogenase	Ca Pb	CnPbSc
CNAG_01984	transaldolase	Ca Sc	CnHcPbSc
CNAG_02035	triose-phosphateisomerase	Ca Sc	HcPbSc
CNAG_02818	glycine cleavage system T protein	-	CnHc
CNAG_03072	enolase	Af Ca PbSc	CnPbSc
CNAG_03225	malatedehydrogenase, NAD-dependent	Ca	CnHcPbSc
CNAG_03358	phosphoglyceratekinase	Af Ca Sc	CnHcPbSc
CNAG_03525	alpha, alpha-trehalase	-	-
CNAG_03674	oxoglutaratedehydrogenase, E1	-	Hc
CNAG_03725	dTDP-4-dehydrorhamnose reductase	Pb	Cn
CNAG_04640	ATP-citrate synthase subunit 1	-	CnHcSc
CNAG_04659	pyruvatedecarboxylase	Ca PbSc	CnSc
CNAG_04969	UDP-glucose 6-dehydrogenase	-	Cn
CNAG_05653	malatesynthase A	-	CnHcSc
CNAG_05907	pyruvatecarboxylase	-	CnHcSc
CNAG_06313	phosphoglucomutase	Ca Pb	HcPbSc
CNAG_06666	starchphosphorylase	-	Cn
CNAG_06699	glyceraldehyde-3-phosphate dehydrogenase	Af Ca PbSc	CnHcPbSc
CNAG_06770	fructose-bisphosphatealdolase 1	Ca PbSc	HcPbSc
CNAG_05351	phosphomannomutase	Sc	HcSc
CNAG_02748	UTP-glucose-1-Puridylyltransferase	Ca	CnSc
CNAG_04217	phosphoenolpyruvatecarboxykinase	Pb	HcSc
CNAG_06501	1,3-beta-glucanosyltransferase	Ca PbSc	CnHcSc
	Lipidmetabolism		
CNAG_02099	fatty acid synthase subunit beta, fungi type	-	HcSc
CNAG_02100	fatty acid synthase subunit alpha, fungi type	Sc	CnSc
CNAG_02562	acyl-CoAdehydrogenase	-	-
CNAG_04308	3-hydroxyacyl-CoA dehydrogenase	-	Hc

Codenumber*	Proteinname	CW**	EV**
CNAG_04869	carboxylesterase	-	Cn
CNAG_07004	dihydrolipoyldehydrogenase	-	CnPbSc
	Nucleicacidmetabolism		
CNAG_01395	ribose-5-phosphate isomerase	Ca	Pb
CNAG_00165	methylthioadenosinephosphorylase	Pb	Hc
CNAG_02285	nucleosidediphosphatekinase	-	CnHcPbSc
CNAG_04577	nucleoside-diphosphatekinase	-	CnHcPbSc
	Oxidation/Reduction		
CNAG_00452	isovaleryl-CoAdehydrogenase	-	Hc
CNAG_00938	cytochrome c peroxidase, mitochondrial	Af	Hc
CNAG_01492	hypotheticalprotein	-	-
CNAG_01594	glycinedehydrogenase	-	Hc
CNAG_01981	sulfide:quinoneoxidoreductase	-	-
CNAG_02399	glutathione-disulfidereductase	PbSc	Cn
CNAG_02801	thioredoxin	Ca Pb	CnSc
CNAG_03482	peroxiredoxin	Ca Sc	CnSc
CNAG_03618	NADPH2:quinone reductase	Ca Sc	HcSc
CNAG_04981	catalase	Pb	CnHcPbSc
CNAG_06628	aldehydededehydrogenase	PbSc	CnHcSc
CNAG_06917	thiol-specific antioxidant protein 3	Ca Pb	CnHcPb
CNAG_00162	alternative oxidase, mitochondrial	-	-
CNAG_03936	NAD(P)H:quinoneoxidoreductase, type IV	-	PbSc
CNAG_05753	N-acetyl-gamma-glutamyl-Preductase	-	-
CNAG_05721	multifunctional beta-oxidation protein	-	Hc
CNAG_05069	ubiquinol-cytochrome c reductase sub. 10	-	-
CNAG_06431	acyl-CoA oxidase	-	-
	Signaling		
CNAG_01523	CMGC/MAPK/P38 protein kinase	-	Sc
CNAG_05235	protein BMH2	Ca PbSc	CnHcPbSc
CNAG_05465	guanine nucleotide-binding protein	Af Ca PbSc	HcSc
	Nucleosomeassembly		
CNAG_06745	histone H3	-	-
CNAG_06746	histone H2B	Ca Pb	CnHcPbSc
	Cytoskeleton		
CNAG_00483	actin	Ca PbSc	CnHcPbSc
CNAG_01840	tubulin beta chain	-	CnPbSc
CNAG_03787	tubulin alpha-1A chain	Ca	Cn
CNAG_04948	tubulin beta	-	-

*Proteins were classified by the Fungal Secretome Database (FSD) into NS (nonclassical secretion, predicted by SecretomeP 1.0f) (dark gray code number), SP or SP3 (containing signal peptide identified by SignaIP 3.0, SigPred, SigCleave or RPSP) (light gray code number) and not classified as secretory (white code number).

** Orthologs previously reported in cell wall (CW) or extracellular vesicles (EV) from *H. capsulatum* (Hc), *P. brasiliensis* (Pb), *S. cerevisiae* (Sc), *C. albicans* (Ca), and *A. fumigatus* (Af) are indicated.