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Chapter 4

The Cell Wall of *Candida albicans*: A Proteomics View

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Abstract

Candida species are a natural commensal of humans and when the immune system is compromised it can cause candidiasis. *C. albicans* is the main etiological agent of candidiasis, representing nearly 60% of the total cases worldwide. The cell wall provides protection against several physical and chemical aggressions and is responsible for the different shapes displayed by *C. albicans*. The cell wall is not a static structure, but a dynamic one, having great plasticity to allow different cell morphologies, molecular remodeling and changes in the cell wall composition as a result from adaptation to the surrounding environment. It is mainly composed of chitin, β-glucan and mannoproteins. Therefore, the cell wall components are putative targets for the discovery and development of new drugs. The cell wall reprogramming in response to several conditions, including a host carbon source, blood, serum, high temperature, acidic environment, and morphogenesis, have a direct impact on the mannoprotein content and might be involved in adherence, drug resistance and virulence of *C. albicans*. In this chapter, we performed an analysis of the proteins that have been identified in the *C. albicans* cell wall by our group and others, which allowed the identification of proteins from different intracellular compartments.

Keywords: *Candida albicans*, cell wall, proteomics, review

1. Introduction

Fungal infections are a serious health concern worldwide. There has been estimated that more than 13% of the total human population has a fungal infection on skin, 300 million people are suffering a severe fungal infection, and 25 million are in great danger of dying or blindness as a

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result of an infection by fungi [1]. Infections caused by fungi provoke over 1.5 million deceases worldwide, mainly in people carrying HIV infections or suffering two or more pathological conditions [2]. Even though there are more than 150 species of *Candida*, only nearly 20 species are known to cause human infections, being *C. albicans* the most frequent causative agent of candidiasis and the leading fungal infection [3]. *C. albicans* is part of the normal microflora in humans and can be found in normal mucosae or cutaneous microflora of healthy individuals [2]. Under certain conditions, as when patients are being treated with antibiotics, immunosuppressive drugs, or chemotherapeutic agents, or are in surgical intensive care units for prolonged time, the commensal *C. albicans* yeast cells turn into pathogenic cells implicated in life-threatening invasive candidiasis [3]. Moreover, there have been increasing reports of *C. albicans* strains exhibiting a multidrug resistance phenotype to antifungal drugs. Therefore, *Candida* infection is a clinical problem worldwide due to the difficulty of treating systemic candidiasis.

2. The cell wall of *Candida albicans*

C. albicans has developed several molecular tools for sensing and evading the host immune system, which include fungal virulence factors involved in adherence, cell damage, and invasion [4]. Many of these factors are localized in the fungal cell wall, which constitute the main structure to be in contact with host cells and is essential for the fungal cell integrity. The cell wall is a remarkable structure because of its key role in protection against environmental stress conditions, including osmotic changes, dehydration, heat, cold, immune system response, or attack by other microorganisms [5–7], and it is also responsible for the cell shape and adhesion to host cells through adhesins.

The cell wall is mainly composed of proteins, glycans, and chitin [5–7]. Cell wall proteins are heavily mannosylated through *O*- and *N*-chemical bonds and function as structural elements, adhesins, cross-linking enzymes, molecular sensors of environmental changes, as well as in protection from environmental changes [5–8]. There are two classes of covalently bound fungal cell wall proteins (CWPs), the glycosylphosphatidylinositol (GPI)-dependent CWPs and Pir-CWPs [5–7], which can be isolated from cell walls by treatment with mild alkali (alkali-sensitive linkage, ASL). In the case of *Saccharomyces cerevisiae*, Cwp2 is a GPI cell wall protein harboring a Pir repeat involved in linking Cwp2 to β-1,3-glycan for increasing cell wall integrity [6, 8]. Proteins lacking homology to Pir proteins have been designated alkali-sensitive linkage cell wall proteins (ASL-CWPs), and are covalently linked to the cell walls of *C. albicans* and *S. cerevisiae* by mild-alkali sensitive chemical bonds [5, 6, 8]. Other proteins are linked to CWPs through disulfide bonds [9].

Most of the cell wall components have covalent linkages established to enhance the cell wall stability and, consequently, protection against environmental changes. One type of proteins described as main cross linkers of cell wall components are the members of the gas family that only contains members of the fungal kingdom. *C. albicans* possesses five genes encoding members of this family, the *PHR1*, *PHR2*, *PHR3*, *PGA4*, and *PGA5* genes [10]. These proteins are β-1,3-glucanases that also exhibit transglucosidase activity and are needed for cross linking β-1,3-glucan and

β-1,6-glucan. *PHR1* and *PHR2* genes are the only members of the family that are responsive to changes in pH [10]. *PHR1* gene is able to complement the *S. cerevisiae GAS1*/*GGP1* null mutant, which has as main phenotypic characteristics, spherical shape, and affectation of normal budding pattern in the stationary phase of growth curve, indicating that the *PHR1* gene is the ortholog of *GAS1*/*GGP1* in *C. albicans* [11]. *PHR2* gene encodes a protein that shows 55.15 and 34.48% identity to Phr1 and Phr3, respectively, while Phr1 has 32.03% identity to Phr3, and interestingly, Phr3 does not have a GPI-anchoring site. *PGA4* and *PGA5* genes, the other members of the Gas family, encode proteins that share 25–35% identity to Phr1 and Phr2, and are not pH responsive.

Other proteins involved in the formation of covalent linkages are transglutaminases (TGases), a group of enzymes that are widely distributed in microorganisms, plants, and animals. They have been involved in a number of biological processes, such as differentiation, growth regulation, cellular adhesion, and tissue integrity. These proteins are enzymes with several functions, including posttranslational modifications, protein cross linking, incorporation of amine groups, and deamination. The best studied TGase function is cross linking, which involves a transamination reaction between the side chains of glutamine and lysine residues in target proteins to form N-ε-(γ-glutamyl) lysine amide bonds, rendering an increase in mechanical strength and resistance to proteolytic degradation [12]. Most of the transglutaminases are fully dependent of Ca^{2+} ions for their activity. However, there are transglutaminases from microorganisms that do not require Ca^{2+} for their function. Similarly, TGases from rodent intestinal mucosa are also independent of Ca^{2+} for their enzymatic activity [12, 13]. One of the best studied transglutaminases is the human TGase 2 that has a key role in the stabilization of the extracellular matrix and modulation of the interaction between fibronectin and integrin [14]. In addition, it has been involved in several human diseases of great impact in health worldwide, such as Alzheimer's, Huntington, and Crohn's diseases, as well as in fibrosis, cancer and other diseases [15]. In the case of *Chlamydomonas reinhardtii*, TGase has a key role in the assembly of cell wall through the cross linking of the cell wall components [16]. TGase activity has also been reported in *C. albicans* and *S. cerevisiae*, where it was found localized in the cell walls fraction of yeast cells and showed the capacity to establish covalent cross links between proteins. TGase activity from these microorganisms was inhibited by the specific inhibitor cystamine, which diminished the incorporation of several polypeptides into the cell wall, affected the regeneration of protoplasts, and inhibited the yeast-to-mycelium transition, revealing a role in the establishment of covalent cross links between cell wall proteins and the structural polymers chitin and/or glucan [17, 18]. In a recent work, the inhibition of TGase activity with cystamine inhibited the growth of yeast cells and induced autophagy. It also affected the yeast division pattern and the cell wall structure. Interestingly, the protein responsible for TGase activity in cell wall was identified as enolase by mass spectrometry [19].

3. Glucan

Glucan is the main component of the yeast cell wall, and along with chitin, these are assembled into a three-dimensional and dynamic network responsible for cell shape that renders protection against environmental insults. In the dimorphic yeast *C. albicans*, chitin abundance

is approximately 1–2% of dry weight of the cell wall and is located adjacent to the cell membrane. β-1,3 and β-1,6-glucan represent 40% and 20% of dry weight of the cell wall, respectively, and are situated immediately after chitin. The outer layer of the cell wall is mainly composed by mannoproteins that define the cell surface properties [5, 20].

C. albicans glucan is a highly branched polymer. It is composed of linear D-glucose molecules bound through β-1,3- and β-1,6-glycosidic linkages. β-1,3-glucan forms a helical backbone as a single residue or a chain made of three strands attached by hydrogen bonds. β-1,6-glucan is responsible for the ramification due to cross linking with chitin and mannoproteins through a GPI-anchor bonded to the nonreducing end of β-1,3-glucan. Likewise, chitin and Pir proteins are attached to the nonreducing end of the β-1,3-glucan molecule [21]. It has been shown that during biofilm formation, the *C. albicans* glucan increases the resistance to several antifungal drugs, such as amphotericin B, fluconazol [22], and anidulafungin (an echinocandin) because of a mechanism of drug sequestration [23]. Biofilm lifestyle on medical devices is a high challenge on medicine because of the difficulty to treat patients with contaminated prosthetic materials.

The synthesis of β-1,3-glucan involves several steps and enzymes, and the major and best characterized is a plasma membrane complex named glucan synthase that uses UDP-glucose as universal donor and is stimulated by GTP; due to a transglycosylation reaction, the nascent linear chain is extruded into the cell wall where it can be further processed by several enzymes. The complex contains the catalytic subunit Fks/Gsc and the regulatory subunit Rho1 [24]. There has been identified three genes encoding the glucan synthase, *FKS*/*GSC*, *GSC1*, *GSL1* and *GSL2*. The main activity comes from *GSC1* gene and the incapability to obtain the null mutant highlight the importance of this gene [25]. Inhibition of glucan synthesis by blocking Fks1/Gsc1 activity to damage the cell wall is the main target of antifungal drugs. Echinocandins are the most important of these type of drugs. Most of the *C. albicans* clinical isolates are susceptible to these drugs; however, mutations in *FKS* genes result in echinocandin resistance [26], augmented chitin amount [27], and reduced expression of *FKS2* and *FKS3* genes [28]. *FKS2* and *FKS3* null mutants contain an increased amount of wall glucan and are more tolerant to echinocandin drugs indicating that both genes negatively regulate *FKS1* [29].

β-1,3-glucan undergoes some modifications after its synthesis, such as hydrolysis at the nonreducing end or within the glucan molecule, transference and bound to another chain or cross linking to β-1,6-glucan. Xog1/Exg1 is the major β-exoglucanase activity that is secreted to the cell wall and then to the milieu, and has a dual activity as it can also act as a glycosyltransferase [30, 31]. Xog1/Exg1 null mutant has no significant changes in viability, pathogenesis, or morphogenesis compared with the parental strain [32]. Bgl2 (GH17) is an endo-β-glucanase and 1,3-β-glucanosyltransferase in *C. albicans*. The null mutant of this enzyme has more sensitivity to chitin inhibitors and reduced virulence, but glucanosyltransferase activity remains in these null mutants suggesting the presence of other transferases [33]. *BGL2* is up-regulated during cell wall regeneration and when cells are challenged with fluconazole [34]. Interestingly, the absence of exoglucanases and glycosyltransferases augment the sensitivity to antifungal drugs during biofilm formation [22]. Another well-characterized glucanase is Eng1, an endoglucanase member of the family GH81; null mutants form cell chains because mother and daughter cells do not accomplish cytokinesis, indicating Eng1 has a key role in cell division [34].

The synthesis of β-1,6-glucan remains poorly understood. It requires Kre9, an *O*-glycosylated cell surface protein that belongs to the GH16 family. In the *KRE9* null mutant, the amount of β-1,6-glucan is undetectable and GPI-wall proteins are released to the extracellular medium and its virulence is attenuated [35]. Nevertheless, there have identified more genes involved in the synthesis of β-1,6-glucan [5].

4. Chitin

Chitin is a linear polymer of N-acetylglucosamine that corresponds to 1–2% of the dry weight of the yeast cell wall, and reaches up to 10% in hypha [36]. Chitin can also be deacetylated to produce chitosan by the enzymatic action of chitin deacetylases Cda1 and Cda2 [37], which has been reported in the formation of spores in *S. cerevisiae* under conditions of nitrogen starvation and absence of fermentable carbon sources [38]. The synthesis of chitin occurs as a transglycosylation reaction, where N-acetylglucosaminyl residues are transferred from UDP-N-acetylglucosamine (UDP-GlcNAc) to growing polysaccharide chains through β-(1–4) chemical bonds [39]. The *C. albicans* chitin is synthesized by chitin synthases organized into a family of four isoenzymes that are classified into three classes of chitin synthases, class I (Chs2 and Chs8), class II (Chs1), and class IV (Chs3) [39]. These enzymes synthesize chitin, which deposits at the tips during polarized growth of buds and hypha, and in septum. Chs1 protein is required for viability, cell shape, and septum formation. Under conditions of repression, the conditional mutants *Δchs1* can grow, but daughter buds are not separated from mother cells, forming yeast chains [40]. Class I chitin synthases participate in cell integrity during polarized growth in yeasts and hyphae, and contribute to the protection of nascent cell wall during polarized growth, as well as to the integrity of cells under stress affecting the cell wall [41]. Mutant *chs8* yeast cells showed the lack of long chitin microfibrils in septa of yeast and hyphae, while *chs3* mutants showed absence of short microfibrils [42].

The treatment of *C. albicans* with sub-minimum inhibitory concentration (MIC) levels of caspofungins has been reported to cause a compensatory increase in chitin content [43]. Walker et al. [44] showed an increase of chitin content in response to the use of echinocandins in several *Candida* species. The protein kinase C (PKC) and calcineurin signaling pathways were shown to be activated in isolates of *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. guilliermondii*, generating an increase in chitin and a reduced susceptibility to caspofungin. Thus, this is a mechanism of tolerance to caspofungin in *Candida* species [44].

5. Mannoproteins

The *C. albicans* cell wall mannoproteins correspond to approximately 40% of the cell wall content and are localized in the outermost layer of the cell wall, and consequently, they interact with host proteins and are the first line of defense against host response [45]. One of the main sugar residues in mannoproteins is mannose, which is linked to proteins during the *N*-, *O*-,

and *C*-glycosylation processes in *C. albicans*. The mechanisms leading to these modifications of proteins in *C. albicans* are quite similar to those present in *S. cerevisiae*. There are excellent reviews on this issue in both microorganisms. In this section, we will briefly describe the two main glycosylation processes.

Most of the cell surface glycoproteins are extensively glycosylated and harbor both *N*-linked and *O*-linked oligosaccharides. The *N*-glycosylation of proteins begins with the attachment of the Glc_3Man_9-$GlcNAc_2$ core oligosaccharide by the dolichyl-diphosphooligosaccharide-protein glycosyltransferase complex (OST), where Ost1 is essential for the catalytic activity [46]. This core is attached to the asparagine residue in Asn-X-Ser/Thr sequons of proteins in the lumen of the endoplasmic reticulum, where X is any amino acid but proline. The nature of the X amino acid has a key role in the extent of mannosylation of the outer chain. Hyperglycosylation is inhibited by negatively charged amino acids, while it is enhanced by positively charged residues [47].

The synthesis of the Glc_3Man_9-$GlcNAc_2$ core begins with the synthesis of dolichol-phosphate-mannose (Dol-P-Man) by the dolichyl-phosphate-β-mannosyltransferase or dolichyl-phosphate-mannose synthase (DPMS) using the isoprenoid lipid dolichol-phosphate (Dol-P) and GDP-mannose as substrates, while the dolichol-phosphate-glucose (Dol-P-Glc) is synthesized by the dolichyl-phosphate-β-glucosyltransferase or dolichyl-phosphate-glucose synthase (DPGS or Alg5p) using Dol-P and UDP-glucose as substrates [46]. Both Dol-P-Man and Dol-P-Glc are translocated from the cytosolic face to the lumen of the endoplasmic reticulum by not yet described flippases, where they will be used during the synthesis of the Glc_3Man_9-$GlcNAc_2$ core oligosaccharide. The synthesis of the lipid-linked oligosaccharide Glc_3Man_9-$GlcNAc_2$-P-P-dolichol begins with the transfer of N-acetylglucosamine-phosphate (GlcNAc-P) group to dolichol phosphate (Dol-P) from UDP-GlcNAc by the UDP-N-acetylglucosamine dolichyl-phosphate N-acetylglucosamine phosphotransferase (Alg7) to produce the dolichyl-pyrophosphate-GlcNAc (Dol-PP-GlcNAc), a chemical reaction inhibited by tunicamycin. The second N-acetylglucosamine residue is added by the Alg7, Alg13, and Alg14 protein complex from UDP-GlcNAc to generate Dol-PP-$GlcNAc_2$. The first mannose residue is then added to Dol-PP-$GlcNAc_2$ by the chitobiosyldiphosphodolichol β-(1,4)-mannosyltransferase (Alg1) using GDP-Man, followed by the sequential addition of two mannose residues by the α-1,3/1,6-mannosyltransferase (Alg2p) to generate Dol-PP-$GlcNAc_2Man_3$, being the first mannose linked through an α-1,3 chemical bond and the second through an α-1,6 linkage. These two enzymes are assembled into a protein complex as well. Two mannose residues are then further linked to the α-1,3-mannose of Dol-PP-$GlcNAc_2Man_3$ by the GDP-Man:$Man_3GlcNAc_2$-PP-Dol α-1,2-mannosyltransferase (Alg11) to assemble into Dol-PP-$GlcNAc_2Man_5$. Subsequent reactions occur in the lumen of the endoplasmic reticulum after the Dol-PP-$GlcNAc_2Man_5$ translocation by the oligosaccharide translocation protein (Rft1). The first mannose residue added in the lumen is linked through an α-1,3-chemical bond to the α-1,6-mannose of Dol-PP-$GlcNAc_2Man_5$ by the Dol-P-Man:$Man_5GlcNAc_2$-PP-Dol α-1,3-mannosyltransferase (Alg3). Then, a mannose residue is α-1,2 linked to the α-1,3-mannose of Dol-PP-$GlcNAc_2Man_6$ to generate Dol-PP-$GlcNAc_2Man_7$ by the Dol-P-Man:$Man_5GlcNAc_2$-PP-Dol α-1,3-mannosyltransferase (Alg9). The next mannose residue is linked to the α-1,6-mannose of Dol-PP-$GlcNAc_2Man_7$ by a Dol-P-Man:$Man_7GlcNAc_2$-PP-Dol α-1,6-mannosyltransferase (Alg12), which shows 37% identity and 56% similarity to *S. cerevisiae* Alg12. The last mannose is linked to the α-1,6-mannose of Dol-PP-$GlcNAc_2Man_8$ by the Dol-P-Man:$Man_8GlcNAc_2$-PP-Dol α-1,2-mannosyltransferase (Alg9p) to

get Dol-PP-$GlcNAc_2Man_9$. The final steps in the synthesis of the core oligosaccharide are the sequential additions of three glucose residues by the Dol-P-Glc:$Man_9GlcNAc_2$-PP-Dol α-1,3-glucosyltransferase (Alg6), Dol-P-Glc:$Glc_1Man_9GlcNAc_2$-PP-Dol α-1,3-glucosyltransferase (Alg8p) and Dol-P-Glc:$Glc_2Man_9GlcNAc_2$-PP-Dol α-1,2-glucosyltransferase (Alg10) [46].

The core oligosaccharide attached to proteins is further modified by the three α-glycosidases Cwh41, Rot2, and Mns1 in the endoplasmic reticulum before proteins are transported to Golgi apparatus. The mannosyl-oligosaccharide glucosidase Cwh41 cleaves the outer glucose residue bound through an α-1,2 chemical bond. Rot2 (glucan α-1,3-glucosidase subunit 2) removes the two glucose residues bound through α-1,3 linkages. Finally, Mns1 or mannosyl-oligosaccharide α-1,2-mannosidase removes the mannose residue bound through an α-1,2 linkage rendering the final mature Dol-PP-$GlcNAc_2Man_8$ oligosaccharide attached to proteins ready for further modifications in the Golgi apparatus [46].

The synthesis of the mannan outer chain in the Golgi apparatus is initiated by the initiation-specific α-1,6-mannosyltransferase or Och1, which adds a mannose residue through an α-1,6 chemical bond to the inner α-1,3-mannose of the $GlcNAc_2Man_8$ core bound to the asparagine residue in proteins. The elongation of the outer chain is first carried out by the mannan polymerase I complex (M Pol I), which adds up to 10 mannose residues trough α-1,6 linkages. M Pol I has several subunits including the mannan polymerase I complex Van1 subunit and the mannan polymerase complex subunit Mnn9. The addition of further mannose residues (up to 50 in yeast) is performed by the α-1,6-mannosyltransferase activity of the mannan polymerase II complex (M Pol II), which contains Mnn9, Anp1, Mnn10, Mnn11, and Hoc1 [48, 49].

After the action of these enzymes, the α-1,6 mannose polymer that constitutes the backbone of the outer mannan chain, is ready for the formation of branches, mainly made of mannose residues linked through α-1,2 chemical bonds, both α-1,2/α-1,3 mannose units, or α-1,2/β-1,2 mannose residues. The addition of the first α-1,2-mannose is performed by the α-1,2-mannosyltransferase Mnn2, and the subsequent α-1,2-mannose residues is carried out by Mnn5 in yeast. *C. albicans* has an ortholog of Mnn2 protein, which shows α-1,2-mannosyltransferase activity and adds the fourth and fifth mannose residues during *O*-glycosylation [50]; however, the mannosylation role during *N*-glycosylation has been assigned to Mnt4 and Mnt5/Ktr2 [50, 51]. The addition of the α-1,3 mannose units is performed by Mnn1 in yeast, but the corresponding enzymatic activity has not yet been identified in *C. albicans*. The addition of β-1,2 mannose residues is performed by Bmt1 and Bmt3 in *C. albicans*. Another modification in oligosaccharide branches is the phosphomannosylation, which occurs in the α-1,2-mannose and is catalyzed by Mnn6 in yeast, while in *C. albicans* is done by Mnt3 and Mnt5/Ktr2. This phosphomannan can also be further modified by the addition of β-1,2 mannose units by the catalytic activity of Bmt2, Bmt3, and Btm4 [50, 51].

The initial step in the *O*-glycosylation process, also known as *O*-mannosylation, is the attachment of an α-1,2 mannose unit to serine or threonine residues on proteins in the endoplasmic reticulum by Pmt1, Pmt2, Pmt4, Pmt5, and Pmt6 enzymes using as substrate Dol-P-Man. The elongation of the *O*-oligosaccharide (up to six mannose units in length) is carried out in the Golgi apparatus. The second and third α-1,2 mannose units are added by Mnt1/Kre2 and Mnt2 enzymes. This *O*-Man_3 oligosaccharide can also be modified by the addition of β-1,2

mannose residues by Bmt1 and Bmt3 proteins. The addition of more α-1,2 mannose units is performed by Mnt1 [50, 52].

6. Proteomics of the *Candida albicans* cell wall

C. albicans sequencing genome project has revealed that it possess 6218 open reading frames (ORFs). However, only 1686 ORFs (~27%) have been characterized so far, and 4380 putative OFR's are of unknown function [53]. In order to determine which genes are expressed to proteins, it is necessary to determine the proteome through mass spectrometry, which has become the workhorse in mining proteomes. The cell wall is the first structure to make contact with host, and consequently, several studies have been performed to identify the proteins contained within the cell wall and those involved in the interaction with host human cells. One of the first works to elucidate the high complexity of the *C. albicans* cell wall was carried out by Pitarch et al. [54], who analyzed a series of fractionations from isolated cell walls of either blastospore or hyphae by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Unusual and conventional proteins were identified and grouped on classical CWPs, heat shock and chaperone, folding, elongation factors, glycosylation, fermentation enzymes, and miscellaneous with unknown functions. Proteins highly glycosylated were not successfully identified; nevertheless, identification was possible when PNGase F was used. Likewise, to minimize possible contamination with proteins from different cell compartments, biotinylation has been used to purify CWPs from yeasts and hyphae, prior to cell fractionation [55]. Proteins were purified by affinity chromatography, precipitated, separated by SDS-PAGE, and further identified by (MALDI-TOF-MS). Interestingly, some intracellular proteins were localized in the cell wall during blastospore formation. As an example, the thiol-specific antioxidant-like protein 1 (Tsa1) was found in the nucleus in blastospores, whereas during the hyphae formation, it was localized on the cell surface.

De Groot et al. [56] carried out a series of extractions using isolated cell walls, and the proteins in each fraction were submitted to anion-exchange chromatography. Proteins in fractions were then separated through SDS-PAGE and peptides, after proteinase digestion, were identified with a Quadrupole-TOF (Q-TOF) MS system. Covalently bound CWPs were identified and classified in several protein classes, including carbohydrate-active enzymes, adhesion, host defense, and proteins of unknown function. A total of 12 proteins were identified as GPI-linked proteins and two were classified as sensitive to mild-alkali extraction. In a similar way, peptides from proteins obtained through several chemical extractions of cell walls from blastospores and mycelium, separated through a C18 reverse phase column and identified with an ESI-quadrupole MS system allowed an increased number of identified proteins, both covalently and noncovalently bounded to the cell wall. In the former, most of the proteins were identified as GPI-anchored proteins. In the latter, they were classified in two groups, one named *bona fide* surface proteins, such as agglutinin-like sequence (Als) proteins, glucanases, chitinases, among others, and a group termed atypical cell wall proteins involved in cell cycle, metabolism and energy, protein synthesis, stress response, translation elongation, transport, and others, as well as those with unknown functions that had never been reported

as part of the cell wall. In addition, MS analysis of SDS extracts obtained from cell walls allowed the identification of most of these atypical cell wall proteins and some others, suggesting that it is possible to identify proteins directly form cell wall extracts [57]. Cell surface proteins that were nonbound to the glucan network were identified from *C. albicans* biofilms, blastospores, and hyphae using 2D-fluorescence difference in gel electrophoresis (2D-DIGE) and MALDI-TOF/TOF-MS, finding 108 differentially expressed proteins. Totally, 25 proteins were exclusively found in the biofilm and were classified in different classes, including metabolic process, protein fate and synthesis, cell surface rescue defense and virulence, biogenesis of cellular components, protein metabolism, and uncharacterized [58].

Reference cultures that were metabolically labeled with ^{15}N for relative quantification using an ESI-Fourier transform (FT) ion cyclotron resonance mass spectrometer (ESI FT-ICR MS) have been used to determine the difference among the yeast and hyphal cell wall proteomes, and cultures under different stimulus, including fetal calf serum, N-acetylglucosamine (GlcNAc), and Iscove's modified Dulbecco's medium (IMDM). The latter stimulus showed the strongest hyphal induction [59]. Cell wall fractions were obtained as described in [56] and quantified 21 proteins. Proteins involved in cell wall remodeling and maintenance showed no significant variations in all conditions tested. Several proteins that are specifically linked to hyphal growth were also identified, including Hyr1, Sod5, Hwp2, Plb5 and Als3, as well as to yeasts only, such as Rhd3, Ywp1, and Sod4. Similarly, the effects of fluconazole on the secretome and cell wall proteome and integrity were studied [60], revealing that fluconazole not only affects the cellular membranes, but the cell wall proteome as well, as was shown in both planktonic and sessile cultures, and confirming that fluconazole inhibits the morphogenesis to hyphae, lowering the amount of cell wall proteins distinctive from hyphae, and increasing the number of cell wall repair associated proteins.

Sosinska et al. [61] demonstrated that pH induces modifications of the *C. albicans* cell wall proteome using a system that resembles the mucosal surface. When cells were cultured at pH 7.0, they were mainly characterized by hyphal growth, while at pH 4.0, only yeasts and pseudohyphae were obtained. Relative quantitation of cell wall proteins labeled with ^{15}N using ESI-FT-MS allowed the identification of 21 proteins covalently bounded to the cell wall, being most of them GPI-linked proteins with the exception of Mp65, Pir1, and Tos1. Nine of these proteins were overexpressed at pH 7.0, while 12 were overexpressed at pH 4.0. Additionally, the transglucosidase Phr2 was detected at pH 4.0, while Als1, Als3, Hyr1, Phr1, Rbt1, Sod5, and Tos1 were found at pH 7.0. Moreover, it has been observed that the carbon source provokes changes in the cell wall proteome and secretome [62]. Glucose, lactate, and glucose plus lactate were utilized to induce changes in the cell wall architecture. There was found an upregulation of proteins implicated in cell wall remodeling when cells were grown in lactate in comparison with cells cultured in glucose, modifying the elasticity of the cell wall. Thus, the cell wall structure is modified in response to stress, antifungals, and osmotic changes. In the same way, the cell wall from cells grown as biofilms and lactate, incorporated proteins involved in adhesion, consequently, improving biofilm formation, and adhesion on plastic surfaces.

There has also been shown that incubation of *C. albicans* at 42°C triggers the expression of stress-related proteins [63] as previously described [60], increases both the phosphorylation the Mkc1 MAP kinase (involved in the cell wall integrity) and the chitin amount [64]; on the

contrary, the amount of Cht3 protein is diminished, impeding cell separation after cytokinesis and showing more sensitivity to Calcofluor white, Congo red, and SDS, suggesting that high temperature induces cell wall stress. Mannoproteins covalently linked to the cell wall either by disulfide or alkali-labile chemical bonds were determined after SDS-treatment, deglycosylation, SDS-PAGE, and reverse phase chromatography coupled to an ESI-LTQ-Orbitrap XL MS system [65]. A total of 10 proteins lacking a GPI-anchor were identified; nevertheless, these proteins possess a signal peptide and were classified as cell wall proteins by *in silico* tools. On the other hand, 16 proteins with a GPI-anchor were identified following extraction with β-mercaptoethanol, which were previously identified after HF-pyridine treatment [56]. Moreover, a group of proteins lacking a signal peptide and characteristic of other cell wall compartments, but not from the cell wall, were identified.

Hernández et al. [66] developed an in vitro model to study the yeast-to-hypha transition of *C. albicans* that allowed the determination of the cytoplasmic proteome of hypha that can be used as a reference. This study described the isolation of the cytoplasmic fraction from yeasts and hyphae for 2D-PAGE analysis and spot identification by MALDI-TOF/TOF. There were identified 66 proteins, but only 43 were found involved in diverse metabolic routes; 20 proteins belong to metabolic pathways of amino acids, carbohydrates, nucleotides, lipids and fatty acids, 23 corresponded to enzymes of glycolysis, gluconeogenesis, pentose and tricarboxylic acid pathways, 13 were involved in transcription and protein synthesis, 8 proteins have functions related to cell rescue, virulence and defense, and only 2 with unknown functions.

The cell wall proteome of *C. albicans* ATCC10231 cells cultured with 10% fetal bovine serum (FBS) has been recently published [67]. The morphogenesis induced with FBS at 30 and 37°C revealed that 285 proteins were differentially expressed from a total of 1177. At 30°C, 152 proteins were up-regulated and clustered in several processes, including signal transduction, cell wall biosynthesis, metabolism, stress response, DNA replication, transcription, RNA processing, among others, while 62 were down-regulated and grouped in cell wall proteins, metabolism, stress response, RNA processing, translation and posttranslational modification and transport. At 37°C, 18 proteins were up-regulated and found associated to cell wall biosynthesis, metabolism, translation and posttranslational modifications, and transport, whereas 53 were down-regulated and found with a role in signal transduction, cell wall, metabolism, stress response, transcription, RNA processing, and others. All these data indicate that morphogenesis induced with FBS at 30°C provokes more changes than those observed at 37°C. Thus, FBS induces morphogenesis through several signaling transduction pathways, the inhibition of glycolysis and enhancement of oxidative phosphorylation.

A gel-free method to identify cell surface proteins from alive *C. albicans* cells incubated for 5 min with trypsin and DTT, a technique named cell shaving or surfomics, has been reported [68]. Peptides were separated by RP-HPLC and analyzed in a MALDI-TOF/TOF system. Identified proteins are implicated in cell wall organization and biogenesis, cell rescue, defense and virulence, cytoskeleton organization, transport, metabolism, protein fate and those with unknown function. Using this approach, surfomics differences among yeasts, hyphae and biofilms were determined [69]. A total of 131 proteins covalently and noncovalently bound to the cell wall were identified and 35 of them had never been reported; 27 of these proteins were present in all tested conditions; however, 22 are of unknown functions. Three were found in the biofilm only, 26 on hyphae and 38 on yeast. Proteins were classified in GPI-anchored, cell wall biogenesis

and maintenance, ligand binding, unknown function, and noncell wall function or description. Under this similar method, proteins from planktonic and hyphae cells using an RP-ESI-LTQ-Orbitrap were determined [70]. A total of 943 proteins were found; 423 were shared between yeast and hyphae, 15 were solely detected in yeast and 505 in hyphae. Proteins were classified in cell wall organization and biogenesis, GPI-anchored, cell surface, pathogenesis, stress, plasma membrane, others, and unknown function, being the latter the largest group. About 14 out of 20 of the most abundant proteins were present in both growth styles, 6 were cell surface proteins, 3 involved in stress response, one from pathogenesis, and 4 from other classes. The normalized spectral abundance factor (NSAF) was utilized to calculate the relative protein abundance. Marín et al. [71] challenged *C. albicans* cells with normal human serum (NS) and heat inactivated serum (HIS); they were able to detect human serum proteins covering the cell surface and fungal cell wall proteins. A total of 214 human and 372 *C. albicans* unique proteins were identified, of which 371 proteins were found in NS, 134 in HIS, and 133 proteins were found in both conditions. Hyphae induction with human serum resulted in the identification of 12 proteins that were not found in any other morphogenesis induction. Moreover, 147 out of 372 proteins were classified as cell wall surface proteins, 60 possessed signal peptide, and 23 were GPI-anchored proteins, being glycerol 3-phosphate-dehydrogenase 2 (Gpd2) and pH-regulated antigen 1 (Pra1) the most relevant, both participating in complement evasion, and seven proteins that bind to plasminogen, resulting in immune protection and evasion.

Our group recently performed the ^{14}C-lysine labeling of proteins of the cell wall using them as endogenous substrates by the TGase present in this cellular fraction. Labeled proteins were then sequentially extracted with 2% SDS, and chitinase, separated by 10% SDS-PAGE and the proteins in radioactive areas were identified by tandem mass spectrometry (MS/MS) [19]. Most of the radioactive labeled proteins were identified in the SDS-solubilized fraction (1048 proteins), 37 proteins in the fraction solubilized with zymolyase, and 41 proteins were released with chitinase. Only 24 proteins were extracted in common with the three treatments used. We found the S7A, S13, and S16A 40S ribosomal proteins; 60S ribosomal protein L30; the glycolytic enzymes enolase 1, glyceraldehyde-3-phosphate dehydrogenases 1 and 2, pyruvate kinase, fructose-bisphosphate aldolase, phosphoglycerate kinase 1, phosphoglycerate mutase, and pyruvate decarboxylase; the heat shock proteins of 70 kDa SSA1 and SSB1; the mitochondrial outer membrane protein porin 1 and ADP/ATP carrier protein; the plasma membrane ATPase PMA1, ADP/ATP carrier protein, and galactose transporter-related Hgt7; the elongation factor 1-α 1; the cell wall agglutinin-like protein 1 (Als1) and 2 (Als2), 1,3-β-glucanosyltransferase PGA4, and chitinase 2. About 16 of these proteins have evidence in the cell wall, being Eno1, Pgk1 and Als1 reported as immunodominant proteins [8]. Phosphoglycerate kinase 1 (Pgk1) was shown to confer poor immune protection against *C. albicans* in an infected mouse model [72]. Hsp70 protein is involved in protein folding and translocation, has antigenic properties, and could play a role in the biosynthesis, secretion, and assembly of other components of the cell wall [73]. The full list of cell wall proteins identified from the sequential extractions with 2% SDS, zymolyase, and chitinase has been reported [19].

Asc1, a protein component of the 40S ribosomal subunit and a signal transducer, was found in fractions extracted with both SDS and zymolyase, and has been described as a protein that has a key role in the process of cell-cell adhesion and cell adhesion to substrate. The deletion of *Asc1* showed that this gene could be related to the signaling pathway of the cAMP-dependent protein

kinase Tpk, which has a role in the processes of morphogenesis under differential environmental conditions [61]. Other identified proteins in the SDS cell wall extract were SOD1, SOD2, while SOD5 was found in the extract obtained with zymolyase. These proteins belongs to a family of at least six members in *C. albicans*, whose main function is the transformation of superoxide radicals into hydrogen peroxide that can be subsequently converted into water by catalase enzyme. SOD1 is cytosolic, SOD2 is localized in mitochondria, and SOD5 is a GPI-anchored protein localized in the cell wall [74]. SOD5 has been described as essential for the infection of *C. albicans* in a mouse model, although the deletion of *SOD5* gene showed no effect in *C. albicans* killing by macrophages. It has also been implicated in the degradation of reactive oxygen species generated by the host to evade surveillance of the innate immune system, resistance to miconazole through biofilm formation, and defense against host insults. Other protein that was also extracted with SDS and zymolyase was the extracellular glycosidase CRH11, a glycosylphosphatidylinositol-anchored protein involved in cell wall organization [75]. Tsa1-B was exclusively found in the fraction of proteins extracted with chitinase treatment. Tsa1-B is a peroxiredoxin, a thiol-specific peroxidase that plays a protective role against reactive oxygen species, organization of the cell wall [76], and is essential for the yeast-to-mycelium transition [77].

The analysis of all SDS-extracted proteins [19] in the PANTHER classification system according the cellular component revealed that 518 proteins (43.2%) correspond to cell part, two proteins (0.2%) belong to the extracellular region, 254 proteins (21.2%) are part of macromolecular complexes, 119 (9.9%) are membrane proteins, one (0.1%) belongs to the nucleoid, and

Nuclear proteins	U6 snRNA-associated SM-like protein LSM1 and LSM4; RuvB-like helicases 1 (RVB1) and 2 (RVB2); replication factor C subunit 3 RFC3; nuclear pore complex protein NUP50; histones H2AZ, H2A.1, H2A.2, H3.3, and H4; histone deacetylase complex subunit SAP30L; high mobility group protein DSP1; transcription elongation factors SPT5 and SPT6; mediator of RNA polymerase II transcription subunits 10 (NUT2) and 4 (MED4); DNA-directed RNA polymerase II subunits RPB2, RPB7 and RPB8; single-stranded telomeric DNA-binding/mRNA-binding protein GBP2; DNA-directed RNA polymerase I subunit RPA49; negative cofactor 2 transcription regulator complex subunit HFL2; DNA-directed RNA polymerase III core subunit RPC19 and RPC40; RNA polymerase-associated protein LEO1 homolog; transcription factor IWS1; snoRNP complex protein SIK1; nucleolar protein 58 NOP58; karyopherin β (importin subunit β) and karyopherin β-3 (importin subunit β); importin subunit α; restriction of telomere capping protein 3; U3 small nucleolar RNA-associated protein 22 UTP22; decapping protein 1, isoform a	U6 snRNA-associated SM-like protein LSM1 and LSM4; RuvB-like helicases 1 (RVB1) and 2 (RVB2); replication factor C subunit 3 RFC3; nuclear pore complex protein NUP50; histones H2AZ, H2A.1, H2A.2, H3.3 and H4; histone deacetylase complex subunit SAP30L; high mobility group protein DSP1; transcription elongation factors SPT5 and SPT6; mediator of RNA polymerase II transcription subunits 10 (NUT2) and 4 (MED4); DNA-directed RNA polymerase II subunits RPB2, RPB7 and RPB8; single-stranded telomeric DNA-binding/mRNA-binding protein GBP2; DNA-directed RNA polymerase I subunit RPA49; negative cofactor 2 transcription regulator complex subunit HFL2; DNA-directed RNA polymerase III core subunit RPC19 and RPC40; RNA polymerase-associated protein LEO1 homolog; transcription factor IWS1; snoRNP complex protein SIK1; nucleolar protein 58 NOP58; Karyopherin β (Importin subunit β) and Karyopherin β-3 (Importin subunit β); Importin subunit α; restriction of telomere capping protein 3; U3 small nucleolar RNA-associated protein 22 UTP22; decapping protein 1, isoform a

Proteasome	Proteasome endopeptidase complex subunits PRE8 (proteasome subunit α type 2) and PRE9 (proteasome subunit α type 4); proteasome subunit β, proteasome regulatory particle base subunit RPN2 (26S proteasome non-ATPase regulatory subunit 1); proteasome regulatory particle lid subunit (26S proteasome non-ATPase regulatory subunit 8); proteasome regulatory particle base subunit RPN10 (26S proteasome non-ATPase regulatory subunit 4); proteasome regulatory particle lid subunit RPN5 (26S proteasome non-ATPase regulatory subunit 12); proteasome core particle subunit beta 1 PRE3; proteasome endopeptidase complex PRE10 (proteasome subunit α type 3); proteasome endopeptidase complex PRE5 (proteasome subunit α type-related); proteasome assembly chaperone 2; proteasome core particle subunit β4; proteasome core particle subunit β6; proteasome regulatory particle base subunit RPN1; proteasome endopeptidase complex PRE6; proteasome endopeptidase complex PUP2; 26S proteasome non-ATPase regulatory subunit 13 (proteasome regulatory particle lid subunit); proteasome endopeptidase complex SCL1 (proteasome subunit α type-6).	Proteasome endopeptidase complex subunits PRE8 (proteasome subunit α type 2) and PRE9 (proteasome subunit α type 4); proteasome subunit β, proteasome regulatory particle base subunit RPN2 (26S proteasome non-ATPase regulatory subunit 1); proteasome regulatory particle lid subunit (26S proteasome non-ATPase regulatory subunit 8); proteasome regulatory particle base subunit RPN10 (26S proteasome non-ATPase regulatory subunit 4); proteasome regulatory particle lid subunit RPN5 (26S proteasome non-ATPase regulatory subunit 12); proteasome core particle subunit beta 1 PRE3; proteasome endopeptidase complex PRE10 (proteasome subunit α type 3); proteasome endopeptidase complex PRE5 (proteasome subunit α type-related); proteasome assembly chaperone 2; proteasome core particle subunit β4; proteasome core particle subunit β6; proteasome regulatory particle base subunit RPN1; proteasome endopeptidase complex PRE6; proteasome endopeptidase complex PUP2; 26S proteasome non-ATPase regulatory subunit 13 (proteasome regulatory particle lid subunit); proteasome endopeptidase complex SCL1 (proteasome subunit α type-6)
Endoplasmic Reticulum	α-1,2-Mannosidase (mannosyl-oligosaccharide 1,2-α-mannosidase) MNS1; endoplasmic reticulum vesicle protein 25 (ERV5); UDP-glucose:glycoprotein glucosyltransferase Kre5; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 OST1; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit WBP1; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2; dolichyl-phosphate-mannose-protein mannosyltransferase 2 PMT2; signal peptidase complex subunit SPC2; signal peptidase complex catalytic subunit Sec11.	α-1,2-Mannosidase (mannosyl-oligosaccharide 1,2-α-mannosidase) MNS1; endoplasmic reticulum vesicle protein 25 (ERV5); UDP-Gucose:glycoprotein glucosyltransferase Kre5; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 OST1; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit WBP1; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2; dolichyl-phosphate-mannose-protein mannosyltransferase 2 PMT2; signal peptidase complex subunit SPC2; signal peptidase complex catalytic subunit Sec11
Golgi Apparatus	SNAP receptor complex member 1; Golgi phosphoprotein 3 Sauron homolog; α-1,6-mannosyltransferase HOC1 α-1,2-mannosyltransferase MNT1	SNAP receptor complex member 1; Golgi phosphoprotein 3 Sauron homolog; α-1,6-mannosyltransferase HOC1 α-1,2-mannosyltransferase MNT1
Secretion	Protein transport proteins Sec61 subunit β, Sec31; translocation protein Sec63; coatomer subunit γ2 Sec21; Ras-related protein Sec4; signal peptidase complex catalytic subunit Sec11	Protein transport proteins Sec61 subunit β, Sec31; translocation protein Sec63; coatomer subunit γ2 Sec21; Ras-related protein Sec4; signal peptidase complex catalytic subunit Sec11
Vacuole	V-type proton ATPase subunit a VPH1; V-type proton ATPase catalytic subunit A TFP1; V-type proton ATPase subunit C VMA5; H(+)-transporting V0 sector ATPase subunit d	V-type proton ATPase subunit a VPH1; V-type proton ATPase catalytic subunit A TFP1; V-type proton ATPase subunit C VMA5; H(+)-transporting V0 sector ATPase subunit d

Cytoskeleton-related proteins	CDC10 cell-division-related protein; actin-related protein 2/3 complex subunit 3 (ARC18), subunit 4 (ARC19) and subunit 5 (ARC15); MLC1 (a protein related to cytoskeleton with function in the cellular bud neck contractile ring); septin CDC3 and septin CDC12; dynamin-like GTPase VSP1 (vacuolar protein sorting-associated protein 1); SLP2 (stomatin-like protein 2 mitochondrial); tubulin α chain TUB1; tubulin β chain TUB2; fimbrin SAC6; actin ACT1	CDC10 cell division related protein; Actin-related protein 2/3 complex subunit 3 (ARC18), subunit 4 (ARC19) and subunit 5 (ARC15); MLC1 (a protein related to cytoskeleton with function in the cellular bud neck contractile ring); Septin CDC3 and Septin CDC12; Dynamin-like GTPase VSP1 (vacuolar protein sorting-associated protein 1); SLP2 (Stomatin-like protein 2 mitochondrial); Tubulin α chain TUB1; Tubulin β chain TUB2; Fimbrin SAC6; Actin ACT1
Ribosomal and mitochondrial proteins	37S ribosomal proteins S9 and MRP51; 39S ribosomal protein L21; NAD-dependent malic enzyme; Protein FYV4 mitochondrial; 28S ribosomal proteins S7, S18C, S23, S29 and S36; aconitate hydratase; Mitochondrial 54S ribosomal protein YmL7/YmL5; 54S ribosomal protein L3; 39S ribosomal proteins L13, L15, L19, L24, L27, L40, L41, L43, and L49; aspartate transaminase; aspartate aminotransferase AAT22; cytochrome c peroxidase; D-lactate dehydrogenase; enoyl-(acyl-carrier-protein) reductase ETR mitochondrial; enoyl-(acyl-carrier-protein) reductase MRF1 mitochondrial; TOM20; COX assembly protein homolog (UniProtKB ID_Q5ALU3); 37S ribosomal proteins S8, S10, S16, and S17; 39S ribosomal proteins L11, L27, and L43; ATP synthase assembly factor FMC1; succinate dehydrogenase assembly factor 4; HSP60 mitochondrial; arginine-tRNA ligase; succinate dehydrogenase assembly factor 2; HSP10 mitochondrial; aconitate hydratase; 4-aminobutyrate transaminase UGA1; acetolactate synthase regulatory subunit ILV6; TOM40; mitochondrial outer membrane protein OM14; 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase mitochondrial COQ5; H/ACA ribonucleoprotein complex subunit 1 GAR1; cytochrome c CYC1; α-ketoglutarate dehydrogenase KGD1; mitochondrial fission 1 protein FIS1; altered inheritance of mitochondria protein 19.	37S ribosomal proteins S9 and MRP51; 39S ribosomal protein L21; NAD-dependent malic enzyme; protein FYV4 mitochondrial; 28S ribosomal proteins S7, S18C, S23, S29 and S36; aconitate hydratase; mitochondrial 54S ribosomal protein YmL7/YmL5; 54S ribosomal protein L3; 39S ribosomal proteins L13, L15, L19, L24, L27, L40, L41, L43, and L49; aspartate transaminase; aspartate aminotransferase AAT22; Cytochrome c peroxidase; ᴅ-lactate dehydrogenase; enoyl-(acyl-carrier-protein) reductase ETR mitochondrial; enoyl-(acyl-carrier-protein) reductase MRF1 mitochondrial; TOM20; COX assembly protein homolog (UniProtKB ID_Q5ALU3); 37S ribosomal proteins S8, S10, S16 and S17; 39S ribosomal proteins L11, L27 and L43; ATP synthase assembly factor FMC1; Succinate dehydrogenase assembly factor 4; HSP60 mitochondrial; Arginine-tRNA ligase; Succinate dehydrogenase assembly factor 2; HSP10 mitochondrial; aconitate hydratase; 4-aminobutyrate transaminase UGA1; acetolactate synthase regulatory subunit ILV6; TOM40; mitochondrial outer membrane protein OM14; 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase mitochondrial COQ5; H/ACA ribonucleoprotein complex subunit 1 GAR1; Cytochrome c CYC1; α-ketoglutarate dehydrogenase KGD1; mitochondrial fission 1 protein FIS1; altered inheritance of mitochondria protein 19

Table 1. Proteins found in several intracellular compartments that were identified in the 2% SDS-extracted cell wall fraction by tandem mass spectrometry according to [29].

304 (25.4%) to organelles. Proteins related to nucleus, proteasome, endoplasmic reticulum, Golgi apparatus, secretion, vacuole, cytoskeleton, and mitochondria are shown in **Table 1**.

The zymolyase-extracted proteins were classified in cellular component as follows: 18 proteins (48.6%) to cell part, one (2.7%) to extracellular region, nine (24.3%) to macromolecular complex, one (2.7%) to membrane, and eight (21.6%) to organelles. The proteins solubilized

with chitinase were classified in cell part (23, 43.4%), macromolecular complex (14, 26.4%), membrane proteins (3, 5.7%), and from organelles (13, 24.5%). Three proteins were exclusively found in the fraction of proteins extracted with zymolyase, including the cell surface Cu-only superoxide dismutase 5 (SOD5), the extracellular glycosidase CRH11, and the covalently linked cell wall protein 14 (SSR1 or CCW14).

According to our results and those from other groups, the cell wall of *C. albicans* contains many intracellular proteins that are commonly found in nucleus, cytoplasm, endoplasmic reticulum, Golgi apparatus, and vacuole. Many of these proteins have no signal peptide for entry into the classic secretory pathway involving the endoplasmic reticulum and the Golgi apparatus. For these type of proteins, an unconventional secretory pathway has been reported [19, 55, 78–81]. Some of these proteins are glycolytic enzymes, which perform different functions inside and outside the cell [82], including enolase and phosphoglucose isomerase, which do not have enzymatic activity outside the cell.

The secretory pathway of glycolytic enzymes is yet to be characterized. This pathway seems to be nonconventional since glycolytic enzymes have no secretion signal. One of this nonconventional pathways has been revealed for the acyl coenzyme A-binding protein (AcbA) of *Dictyostelium discoideum*, which has full requirement of the Golgi reassembly stacking protein (GRASP), a protein attached to the cytosolic face of the Golgi apparatus membrane [83]. The Acb1 protein of *S. cerevisiae* also requires the yeast GRASP protein for its secretion, which is also mediated by proteins involved in autophagy and Sso1 protein, a t-SNARE localized in the plasma membrane [84]. The *S. cerevisiae* Eno2 protein does not possess a secretion signal sequence, since Eno2 and Pgi1 proteins were secreted in a *sec23–1* mutant at 37°C. These data provide evidence that the secretion of some glycolytic enzymes is independent of the conventional secretory pathway. Analysis of deletion mutants showed that the knock-in of the SNARE coding *TLG2* gene inhibits the enolase secretion. However, other proteins might be involved, since the inhibition of secretion is not complete [85]. One of the best studied nonconventional secretion pathways is the secretion of the a-factor mating pheromone, which has a full requirement of the Ste6 protein, a transport protein with homology to MDR proteins [86]. Other protein that does not follow the classical secretion pathway is galectin-1, a mammalian lactose-binding lectin that was expressed in yeast [87]. The galectin-1 expression was dependent on the yeast genes *NCE101*, *NCE102*, *NCE103*, while the corresponding *C. albicans* are XP_019330961.1(CaNCE1), XP_723171.1 (CaNCE2), and XP_721792.1(CaNCE3, also named carbonate dehydratase), and therefore, the existence of this protein export pathway is possible in *C. albicans*.

Results obtained by mass spectrometry of the cell walls of *C. albicans*, *C. glabrata*, *C. krusei,* and *C. parapsilosis* exposed to different concentrations of H_2O_2 have allowed the identification of wall proteins known as multifunctional or *moonlighting*. This class of proteins includes: (i) glycolytic enzymes, (ii) thermal shock, (iii) oxidative stress response proteins, (iv) general metabolic enzymes, and highly conserved proteins, being over- or subexpressed in the presence or absence of reactive species of oxygen (ROS). Some of the identified glycolytic enzymes are the triphosphate isomerase (Tpi1), glyceraldehyde 3-phosphate dehydrogenase (GADPH), fructose bisphosphate aldolase (Fba1), phosphoglycerate kinase (Pgk), phosphoglycerate mutase (Gpm1), pyruvate kinase (Pk), and enolase 1 (Eno1) [88].

Finally, we want to call the attention to the fact that ^{14}C-labeled proteins labeled by TGase activity and extracted with SDS showed a molecular mass lower than 50 kDa [19]. However, the theoretical molecular mass of many of them was in the range between 50 and 220 kDa. Therefore, fragments of these proteins are being transported to the extracellular milieu and can be cross linked each other, either to β-1,3- or β-1,6-glucan, or chitin to strengthen the protection properties of the cell wall. This observation was evidenced by the inhibition of TGase by cystamine that facilitated the action of zymolyase on *C. albicans* yeasts with the consequent reduction of the osmoprotective properties of the cell wall [19]. However, more studies are needed to support this hypothesis.

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