RESEARCH ARTICLE

A study of the *Candida albicans* cell wall proteome

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Considering the importance of proteins in the structure and function of the cell wall of *Candida albicans*, we analyzed the cell wall subproteome of this important human pathogen by LC coupled to MS (LC-MS) using different protein extraction procedures. The analyzed samples included material extracted by hydrogen fluoride-pyridine (HF-pyridine), and whole SDS-extracted cell walls. The use of this latter innovative procedure gave similar data as compared to the analysis of HF-pyridine extracted proteins. A total of 21 cell wall proteins predicted to contain a signal peptide were identified, together with a high content of potentially glycosylated Ser/Thr residues, and the presence of a GPI motif in 19 of them. We also identified 66 "atypical" cell wall proteins that lack the above-mentioned characteristics. After tryptic removal of the most accessible proteins in the cell wall, several of the same expected GPI proteins and the most commonly found "atypical" wall proteins were identified. This result suggests that proteins are located not only at the cell wall surface, but are embedded within the cell wall itself. These results, which include new identified cell wall proteins, and comparison of proteins in blastospore and mycelial walls, will help to elucidate the *C. albicans* cell wall architecture.

Received: February 4, 2008
Revised: April 7, 2008
Accepted: May 20, 2008

Keywords:
Atypical wall proteins / *Candida albicans* / Deglycosylation of mannoproteins / Mycelial wall proteins / Typical wall proteins

1 Introduction

As occurs with all fungi, cells of the opportunistic pathogen *Candida albicans* are surrounded by a cell wall responsible for providing their shape, either as blastospores, or true or pseudo-hyphae. The wall also provides protection to the cell to withstand the difference in osmotic pressure with the medium, and against other physical and chemical aggressions. Nevertheless, the cell wall must not be considered as a static structure; its chemical composition and the assembly of the different macromolecules that make it up are modified during cell growth and morphogenesis.

In fungi, the cell wall makes up close to 30% of the cell dry weight [1], 80–90% of which corresponds to polysaccharides, while the rest is represented mainly by proteins. Despite their low amounts, the functional activities of these proteins make their role in the wall exceedingly important. Proteins enriched at the external surface of the cell wall play specific functions, such as adhesins and lectins that allow attachment of *C. albicans* to different surfaces of the host. In addition, different proteins showing enzymatic activities are found in the cell wall, some of them participating in the degradation of complex macromolecules used as nutrients, while others are involved in the cleavage of the protective superficial structures of host cells and tissues permitting their invasion. Additionally, other enzymes are involved in the assembly and synthesis of the cell wall itself including the assembly of β-1,3- and β-1,6 glucans, and chitin. Specific

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Abbreviations: β-ME, β-mercaptoethanol; **HF-pyridine,** hydrogen fluoride-pyridine; **SOD,** superoxide dismutase

cell wall components act as antigens or immunomodulators, whose effects can alter the balance between the fungus and its hosts or predators (for a recent review on *C. albicans* cell wall structure and synthesis, see ref. [2]).

It is known that in the fungal cell wall, some polymeric components are linked together by covalent bonds, whereas other components are associated through hydrogen bonds, hydrophobic interactions, or ionic associations. Proteins do not escape this generalization, and some of them are retained in the cell wall by physical means, whereas others are covalently bound to other proteins or to different polysaccharides [2, 3]. This type of organization gives rise to a scaffold with mechanical properties which are different in the nascent and the mature cell wall; the former is elastic, whereas the latter is viscoelastic [4], characteristics which are critical to the cell wall functions.

Considering the importance of proteins in the structure and function of the cell wall, it is not surprising that great efforts have been devoted to their identification, isolation, and characterization, since our early observations on the existence of covalently bound proteins in the cell walls of fungi, *C. albicans* included [5]. The identification procedures have included purification and sequencing by classical biochemical techniques, and more recently by MS and database searching [6, 7].

The aim of the present study was to carry out a systematic and exhaustive analysis of the cell wall proteins of *C. albicans* using LC-MS on samples obtained by different techniques aimed at a stepwise extraction of covalently bound proteins. Results demonstrate that in addition to the expected proteins containing a GPI domain and a predicted signal peptide, other proteins previously identified in the cytosol or cell organelles appear in the cell wall preparations. The biological meaning of these so-called "atypical" cell wall proteins is discussed. These data, in conjunction with previously reported results will help to provide an integrated view of *C. albicans* cell wall proteome.

2 Materials and methods

2.1 Strain and growth conditions

C. albicans CAI-4 cells were routinely grown in SD medium (0.7% yeast-nitrogen base without amino acids, 2% glucose) or Lee medium [8] supplemented with the appropriate nutrients in the amounts previously specified [9]. Inocula were prepared by transfer of loopfuls of cells from slants to SD liquid medium, depending on the experiment, and the cells were incubated with shaking overnight at the appropriate temperature. Mycelium was obtained as previously described [10].

2.2 Cell-wall isolation and purification

Purified walls were obtained by a slight modification of the method described previously for *Saccharomyces cerevisiae* [11, 12]. Cells were broken with glass beads (1.5 g/mg of dry cells) by shaking in a vortex mixer at room temperature for eight periods of 1 min each with intermediate periods of 1 min on ice. Using this method, breakage of the whole cell population was obtained, as monitored under the phase-contrast microscope. After recovery of the cell walls by centrifugation at $1200 \times g$ for 5 min, they were repeatedly washed with cold 1 mM PMSF. Finally cell walls were spun down as above, washed four times with chilled distilled water, and boiled for 10 min (twice) with 2% SDS in distilled water. Alternatively the walls were suspended in 4% SDS and heated in a boiling bath for 2 h and further incubated overnight at 80°C. Finally, the walls were washed 15 times with chilled 1 mM PMSF in distilled water, freeze-dried and stored at −20°C until further use (extraction with β-mercaptoethanol (β-ME), hydrogen fluoride-pyridine (HF-pyridine) treatment, *etc.*).

2.3 β-ME treatment

The cell walls (100 mg dry weight) obtained as described in Section 2.2 were suspended in 2.5 mL of 1 mM ammonium acetate pH 6.3, containing 2% v/v of β-ME, and shaken for 3 h at 28°C. The suspension was then centrifuged at $1200 \times g$ for 10 min, the supernatant freeze-dried, the proteins separated by SDS-PAGE and detected by CBB staining.

2.4 HF-pyridine treatment

Dry cell walls (4 mg) were suspended in 300 μL of HF-pyridine (Sigma–Aldrich, Buchs, Switzerland) at 4°C and maintained for 3 h. The reaction was quenched by dilution of the reaction mixture with an equal amount of ice-cold H_2O. HF-pyridine was removed by dialysis overnight in water, and the nondialyzable material was freeze-dried.

To identify proteins that could be found within the wall mass, the HF-pyridine treatment was carried out after the superficial proteins had been exhaustively eliminated by overnight digestion with trypsin at 37°C.

2.5 Protein extraction by NaOH treatment (ALS-CWP)

Cell walls were kept in 30 mM NaOH at 4°C for 4 h. The reaction was stopped by adding neutralizing amounts of acetic acid, followed by centrifugation and dialysis of the released protein(s).

2.6 DTT/iodoacetamide treatment

Cell walls (4 mg dry weight) were suspended in 100 mM NH_4HCO_3 /10 mM DTT for 1 h at 56°C. The material was collected by centrifugation at $2500 \times g$ for 10 min and the cell walls were washed with 100 mM NH_4HCO_3. The material was suspended in 100 mM NH_4HCO_3/55 mM iodoacetamide and incubated at room temperature for 45 min in

darkness, washed three times with 50 mM NH_4HCO_3 and freeze-dried.

2.7 Deglycosylation of cell wall proteins

Freeze-dried cell wall HF-pyridine extracts (100 mg) were placed on ice and 200 μL trifluoromethanesulfonic acid (TFMS); (Sigma–Aldrich) and 100 μL anisole was added. The samples were kept for 5 h at 4°C and then neutralized with 400 μL *N*-ethylmorpholine, 5–10 volumes of acetone added, and the mixture set aside overnight at −20°C. Samples were then centrifuged for 10 min at 15 000 rpm, the pellet dried under vacuum for about 30 min, and retained until MS analysis. To ensure complete dryness of the samples and the anhydrous conditions during the acid treatment, all glass tubes and syringes used were vacuum-dried and all steps of the procedure performed in a chamber continually purged with N_2 gas.

2.8 Proteolytic digestion of cell-wall enriched preparations

The dried materials obtained by the different procedures described above, were suspended in 50 μL of 50 mM ammonium bicarbonate and pH 7.8. Modified trypsin (sequencing grade; Promega, Madison, WI, USA) was added at a final amount of 0.5 μg *per* sample. Digestion was performed under shaking conditions at 37°C for 2 h. Finally the tryptic peptides were dried and suspended in 5% ACN and 0.5% acetic acid.

2.9 Nano-LC and IT MS/MS analysis of tryptic peptides

The resulting tryptic peptide mixtures were injected onto a C-18 RP nano-column (Discovery® BIO Wide pore, Supelco) and analyzed in a continuous ACN gradient consisting of 0–50% B in 45 min, 50–90% B in 1 min (B = 95% ACN, 0.5% acetic acid). A flow rate of *ca.* 300 nL/min was used to elute peptides from the RP nanocolumn to a PicoTip™ emitter nanospray needle (New Objective, Woburn, MA, USA) for real-time ionization and peptide fragmentation on an Esquire HCT Ion Trap (Bruker-Daltoniks) mass spectrometer. Each second, the instrument cycled through acquisition of a full-scan mass spectrum and one MS/MS spectrum. A 3 Da window (precursor m/z ±1.5), MS/MS fragmentation amplitude of 0.90 V, and a dynamic exclusion time of 0.30 min were used for peptide fragmentation. Nano-LC was automatically performed on an advanced nanogradient generator (Ultimate nano-HPLC, LC Packings, Amsterdam, The Netherlands) coupled to an autosampler (Famos, LC Packings). The software Hystar 2.3 was used to control the whole analytical process. MS/MS spectra were batch-processed by using DataAnalysis 5.1 SR1 and BioTools 2.0 software packages and searched against the MSDB protein database using MASCOT software (Matrix Science).

2.10 Data analysis (MS/MS database search)

MS/MS data were searched against CandidaDB database from *C. albicans* (6165 entries; http://genolist.pasteur.fr/CandidaDB/help/flat-files.html) [13] using Mascot v2.2.1 (Matrixscience, UK). MASCOT search parameters were: trypsin as enzyme, up to one missed cleavage allowed, MS tolerance of 1 Da, and MS/MS tolerance of 0.8 Da. No fixed modifications were selected, and variable modifications included methionine oxidation. Under these conditions, only those peptides which showed a score higher than 32 were included in the previous dataset. Manual inspection of these spectra was carried out for validation of peak assignment and selection of high quality spectra to be used for protein identification. A minimum of two peptides was required for reliable protein identification, except for spectra with extremely high quality, in which case single-peptide identifications were permitted. For each single-peptide-based protein identification, properly annotated CID spectra, including the precursor m/z and charge state could be found in Figs. 1S–6S of Supporting Information. A shared sequence present in two proteins belonging to the same family (Table 2, proteins Pga30 and Pga31) was identified.

3 Results

3.1 Proteins identified in the *C. albicans* cell wall preparations

To selectively identify those proteins that were attached to *C. albicans* cell wall through covalent bonding, isolated cell walls were treated with different reagents and conditions (Table 1).

Identification of cell wall proteins containing a GPI motif was carried out initially following their extraction by HF as a saturated pyridine solution, a treatment that breaks the pyrophosphate bond of GPI proteins [7]. This approach led to the identification of a number of proteins both covalently- and noncovalently bound to the cell wall (Tables 2 and 3). Interestingly, we found that trypsin digestion of SDS-extracted cell walls led to the identification of the same proteins, plus some additional ones (Tables 2 and 3), suggesting that in the isolated cell walls, this set of proteins was accessible to direct trypsin digestion.

A total of 21 cell wall surface proteins predicted to contain a signal peptide in their immature form and a number of potentially glycosylated residues were identified (Table 2). Proteins Als1, Pga24, Pga30, Pga45, Rbt1, and superoxide dismutase (SOD) were identified with a single peptide. The corresponding CID spectra were annotated for assignation of the ions to the principal fragmentation series (Figs. 1S–6S of Supporting Information). Out of the 21 identified proteins, 19 contained a GPI domain, the exceptions being Bgl21 and Scw1. Some of these proteins had previously been docu-

Table 1. Different protocols used in the proteomic analysis of *C. albicans* cell walls

Sample number	Type of material	Treatment
1	Whole cell walls	Extracted with 2% SDS at 100°C for 10 min
2	Whole cell walls	Extracted with 4% SDS/PBS at 100°C during 2 h
3	Whole cell walls	Same as 2 + treatment with 4% SDS/PBS at 80°C overnight
4a	Whole cell walls	Extracted with SDS 4% SDS/PBS at 100°C during 2 h then β-ME
4b	β-ME supernatant from 4a	Same as 4a
5	Whole cell walls	Same as 2 + treatment with 100 mM NH4HCO3-55 mM iodoacetamide
6	HF-soluble material	Same as 5 + HF-pyridine
7	HF-soluble material	Same as 2 + HF-pyridine
8	Whole cell walls	Same as 2 from mycelium wall
9	HF-soluble material	Same as 8 + HF-pyridine
10	HF-soluble material	Same as 2 + trypsin treatment overnight at 37°C + HF-pyridine
11	Insoluble material from 10	Same as 10

mented by other research groups [7] but, interestingly, Als10, Bgl21, Pga30, Pga31, Pga45, Phr2, Rbt1, and Utr2 were identified in *C. albicans* cell wall for the first time (see below). Six proteins identified were thanks to a single peptide.

3.2 Functional characteristics of the identified surface proteins

Als1 and Als10 (Als2) are proteins with adhesion properties. They are probably involved in epithelial adhesion and invasiveness [14]. Bgl21 is a protein with a putative endo-β-l,3-glucanase motif belonging to the glycosyl hydrolases family 17, and shows homology to *S. cerevisiae* Bgl2. This protein is probably involved in the mechanism of assembly of β-l,3-glucan, as pointed down by the decreased virulence of the null mutant [15]. Cht2, an endochitinase 2 that belongs to the glycosyl hydrolases family 18, was identified in seven of the protocols used. This protein is located close to the crosswall that separates mother and daughter cells, suggesting that its role might be related with the process of cell separation after cytokinesis. Crhl1 is a protein belonging to the glycosyl hydrolases family 16. It probably plays an important role in cell wall organization, considering that the homologous *S. cerevisiae* protein (Crhl) has a transglycosidase activity involved in the crosslinking between yeast cell wall polymers [16–18].

Ecm33 is a typical GPI-anchored protein, predicted of having a signal peptide, a serine- and threonine-rich domain, and a GPI anchor signal, that was detected in all the experiments carried out. Though its function is unknown, it has been found to be important for cell wall integrity and required for normal cell wall architecture, as well as for the normal function and expression of some cell surface proteins in *C. albicans* [18, 19].

Pga2, Pga4, Pga24, Pga29, Pga30, Pga31, and Pga45 are predicted GPI proteins of the same family [20, 21]. The first four proteins were previously identified in the *C. albicans* cell wall [7], but Pga30, Pga31, and Pga45 were identified in this study for the first time. Pga2 is an SOD, homologous to Yjr104c from *S. cerevisiae*. These two proteins apparently act as scavengers that remove ROS formed during aerobic metabolism, including superoxide anions. It is relevant that increased levels of SOD activity have been observed in copper-treated cells, a situation that leads to alterations in the biosynthesis of the cell wall [22]. Pga4 is a homologue to the protein encoded by *S. cerevisiae GAS1* that has been described as having a cell wall-related phenotype [23], whilst Pga24 and Pga29 are proteins with unknown functions. Pga 30 and Pga45 have unknown functions, but Pga30 shows similarity to the *S. cerevisiae* abundant cell surface glycoprotein Sed1. Pga45 displays a low similarity to Yil169c, a glucan 1,4-α-glucosidase. Pga31 has been described as a putative membrane protein with a membrane-spanning domain, with low similarity to glucan 1,4-α- glucosidase and exo-α-sialidase related genes and may play an important role in the *de novo* construction of the cell wall [24].

Phr1, Phr2, and Utr2 were also identified in this study of *C. albicans* cell wall, the two latter ones for the first time. Phrl and Phr2 belong to the group of GPI proteins with putative glycosidase activity required for proper crosslinking of β-1,3- and β-l,6-glucans [25]. Both proteins are involved in the processing of β-1,3-glucans to make available acceptor sites for the attachment of β-1,6-glucans. These proteins are expressed at either alkaline or acidic pH, respectively. Deletion of the encoding genes results in pH-conditional defects in cell morphology and virulence [25, 26]. Utr2 seems to be a β-cell wall glucanase and the null mutant presents defects in filamentation, reduction in adherence, and less virulence [27].

Additional proteins identified in this study were Scw1 and Ssr1. Scw1 is a protein with glucanase activity. This protein lacks a GPI motif but had a predicted signal peptide in its immature form, and a high content of potentially glycosylated Ser/Thr residues. Ssr1 is a potential structural protein, since no catalytic function has being found for it [20].

Table 2. *Bona fide* surface proteins identified in the cell wall preparation

Protein name	Description	Sequence	Residues	Method[a)]	Score
Als1	Agglutinin-like protein	STVDPSGYLYASR	176–188	7, 8, 9	76
Als3	Agglutinin-like protein	ISINVDFER	167–175	8, 9	83
		SNVDPKGYLTDSR	176–188		
Als10	Agglutinin-like protein	ALGTVTLPISFNVGGTGSLVDLESSK	124–149	7	142
		TNEDASGYFIASR	176–188		
Bgl21	Endo-β-1,3-glucanase	IFLVGSEALYR	113–123	1,7	64
		EDLTASELASK	124–134		
Cht2	Chitinase 2	FADTLWNK	122–129	1, 2, 3, 5, 7, 10, 11	242
		LFVGVPATSNIAGYVDTSK	240–258		
		LSSAIEEIK	259–267		
		GENFVVQVK	291–199		
Crh11	Probable membrane protein	QGTIDSGSNGLSLTMK	60– 75	1, 2, 4a, 6, 7, 8	340
		FDNPSFK	78– 84		
		SNFYIMFGR	85– 93		
		VEVVLKGAEGK	94–104		
		SVLVADYSSGK	236–246		
		YDQAQDDIK	271–279		
Ecm33.3f	Cell wall biogenesis	TGLTAGITSAESVVISDTGLSSL	139–161	1, 2, 3, 4a, 4b, 5, 6, 7, 8, 9, 10, 11	200
		TGINVFK	162–168		
		NDDLTELDFPK	263–273		
		TIGGALQISDNSELR	276–290		
		VSGGFILK	322–329		
Pga2	Similar to SOD	TPAALELGDLSGR	105–117	1, 2, 3, 4a, 5, 6, 7, 8, 10, 11	105
Pga4	Protein related to phr-1,-2,-3and Gas1	YFQELGINTIR	69– 79	1, 2, 4, 5, 6, 7, 9, 10, 11	152
		TIPVGYSAASVDEYR	184–198		
		GDSVTTNDDFDNLK	302–315		
Pga24	Putative cell wall protein	NLYGAGAVPFFQVHLEK	93–109	7	78
Pga29	Unknown function	VDGLGLYSK	31– 39	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	324
		DGTLSFTGDDK	104–114		
		NVNDPYR	120–126		
		YSESEYAVSNK	127–137		
		TDDSAPITIVAK	139–150		
		HEGAAIDYLFLGK	40– 52		
		DGTLSFTGDDKVYASK	104–119		
Pga30	Unknown function	DSFAVVTNGGEGSIPFK	133–149	3, 7, 9, 10	117
		(NINDPYNYSK)	123–132		
Pga31	Unknown function	HEGAALNYLFLAAPGVAENLK	102–122	1, 5, 7	105
		QPLNVGNTVLQLGGSGDGTK	143–162		
		(NINDPYNYSK)	184–193		
Pga45	Unknown function	YSGALSQILQDLEK	351–364	3	75
Phr1	β-1,3-Glucanosyl transferase	LFQEIGTLYSDK	280–291	1, 8	117
		GIAYQQDAAGSVSSGYDADPNRK	49– 71		
Phr2	β-1,3-Glucanosyl transferase	DIPYLEAVDTNVIR	75– 88	1, 2, 3, 4, 5, 6, 7, 8, 10	244
		ASGYESATNDYK	239–250		
		SIPVGYSANDDSAIR	191–205		
Rbt1	Putative cell wall protein with similarity to Hwp1p	TLGLLLGLLK	262–271	8	59
Scw1	Glucosidase	SNQQAAISSIK	334–344	1, 2, 3, 7, 10	194
		SESQIASEIAQLSGFDVIR	142–160		
		IFAGIFDVSSITSGIESLAEAVK	182–204		
Ssr1	Secretory stress response protein 1	NSDVEKCLKEICPNGDADTAISAFK	55– 79	7, 10	62
		CLKEICPNGDADTAISAFK	61– 79		

Table 2. Continued

Protein name	Description	Sequence	Residues	Method[a)]	Score
Utr2	1,3-1,4-β Glucanase	MSTFQESFDSK	75– 85	1, 2, 3, 5, 6, 8	275
		YDYPQTPSR	245–253		
		IQFSLWPGGDSSNAK	254–268		
		EIYATAYDIPNDVK	297–310		

Sequence in parenthesis correspond to common sequence in the two proteins.
a) See Table 1.

3.3 Identification of "atypical" cell wall proteins

Besides the genuine surface proteins, a large number of probably noncovalently bound proteins, previously identified in the cytosol or cellular organelles, were detected in all the analyzed samples. Table 3 displays these proteins grouped according to the classification applied to the *S. cerevisiae* proteome [28]. Table 3 also shows the number of experiments in which each protein was detected. As expected only a few of them were consistently identified in most of the experiments carried out. Only nine proteins (Cdc19 (6), Eno1(8), Gap1/Tdh3 (10), Kar2 (6), Ssa4 (6), Ssb1 (7), Eft2 (8), Tef1 (10), Pma1 (6)) were specifically identified in at least 50% of the 12 experiments done, and only two proteins (Gap1 and Tef1) in 10 of them.

3.4 Identification of proteins extracted from the cell wall by reducing agents

Two catalytic proteins involved in the cell wall organization had been previously identified using extracts of β-ME fractionated by gel electrophoresis: chitinase2 (Cht2) and a glucanase (Scw1) (unpublished observations). In further experiments in which β-ME-extracted material was directly analyzed without previous SDS-PAGE separation, Ecm33 was also identified.

The identification of these three proteins is relevant. On the one side it should be stressed that Ecm33 and Cht2 have been previously identified as GPI proteins, and as expected, covalently attached to β-1,6-glucan. Accordingly, our results suggest that some GPI proteins can also be retained in the cell wall through the formation of disulfide bridges. On the other side, our data reveal that formation of disulfide bridges could be a mechanism of the attachment of wall proteins lacking a GPI motif (Scw1) to the cell wall.

3.5 Proteins resistant to trypsin treatment in the SDS-extracted cell walls

As indicated above, the digestion with trypsin of HF-pyridine treated SDS-extracted cell walls allowed the identification of "true" cell wall proteins exposed at the outer and inner surface of the walls. Taking into consideration the possibility that other proteins might be located within the wall structure itself, we proceeded to eliminate the proteins located on the surface by exhaustive trypsin digestion (overnight incubation at 37°C), followed by solubilization of the remaining proteins (if any) by treatment with HF-saturated pyridine.

Using this protocol, a number of both covalently bound, and noncovalently bound ("atypical") proteins were detected (Tables 4). This result suggests that proteins are present not only at the outer and inner surface of the cell wall, but also embedded in its structure.

3.6 Proteins detected following deglycosylation

It has been suggested that the glycan residues present in proteins could interfere in the identification of the proteins because they are less susceptible to proteases [29]. As a consequence it was thought that removal of mannose chains present in the wall proteins might help for identification additional proteins. Pga62 was identified for the first time and interesting enough, this protein presents six putative *O*-glycosylation sites. In addition we also recognized a new peptide in proteins Ssr1and Pga24 that had not been previously identified by other procedures.

3.7 Proteins present in the wall of mycelial cells

Since *C. albicans* can switch between the mycelial and blastospore morphologies, the cell wall proteomes of the two morphologies were compared, using both whole SDS-extracted cell walls and HF-pyridine extracted material. The results obtained revealed that in addition to several of the proteins previously detected in the blastospore wall, two additional wall proteins (Als3 and Rbt1) were present, and detected exclusively in the cell wall from the mycelial cells. The former protein is required for virulence and defined as an *N*-glycosylated α-agglutinin-like protein with a GPI motif and a predicted signal peptide. Rbt1 is a mannoprotein that is under the control of Tup1, a general transcription repressor.

Table 3. "Atypical" cell wall proteins identified in the cell wall preparation

Protein name and GO classification [a)]	Function	Frequency[b)]
Cell cycle		
Cdc25	CDC25 cell division cycle	2
Metabolism and energy		
Ach1	Acetyl-coenzyme-A hydrolase	1
Aco1	Aconitate hydratase	1
Adh1[c)]	Alcohol dehydrogenase	3
Ald5	Aldehyde dehydrogenase	1
Atp.exon2[c)]	F1F0-ATPase complex, F1 α subunit, exon 2	4
Atp1.exon3	F1F0-ATPase complex, F1 α subunit, exon 3	3
Atp2	F1F0-ATPase complex, F1 β subunit	4
Cdc19[c)]	Pyruvate kinase	6
Eno1[c)]	Enolase I (2-phosphoglycerate dehydratase)	8
Fas1	Fatty-acyl-CoA synthase, β chain.	2
Fba1[c)]	Fructose bisphosphate	1
Gap1	Glyceraldehyde-3-phosphate dehydrogenase	10
Gdh3	NADP-glutamate dehydrogenase	3
Glk1	Aldohexose specific glucokinase	2
Gnd1	6-Phosphogluconate dehydrogenase	2
Hxt5.5	Sugar transporter	2
Icl1	Isocitrate lyase	1
Ils1	Isoleucyl-tRNA synthetase	2
IPF8806	6-Phosphofructose-2-kinase	1
Lat1	Dihydrolipoamide S-acetyltransferase LAT1	2
Mdh1	Mitochondrial malate dehydrogenase precursor	1
Met6[c)]	5-Methyltetrahydropteroyltriglutamate methyltransferase	4
Pdc11[c)]	Pyruvate decarboxylase	5
Pgk1[c)]	Phosphoglycerate kinase	4
Pyc2.exon2	Pyruvate carboxylase 2, exon 2	4
Sah1	*S*-adenosyl-L-homocysteine hydrolase	2
Sdh12	Succinate dehydrogenase	2
Ura2	URA2 multifunctional pyrimidine biosynthesis protein	1
Others		
Act1	Actin	1
Bmh2[c)]	Bmh2p suppressor of clathrin deficiency	2
Cdc48[c)]	Microsomal ATPase	1
Cdc61.5f	Cytosolic leucyl-tRNA synthetase	1
IPF8321	Glycogenin glucosyltransferase activity	2
Kar2[c)]	Similar to chaperones of Hsp70p family	6
Mis11	Mitochondrial C1-tetrahydrofolate synthase precursor	2
Pet9	ADP/ATP carrier protein	6
Tkl1[c)]	Transketolase 1	2
Proteins synthesis		
IPF10301	Ribosomal protein L2/L12 homolog	1
Rpl3	60S Large subunit ribosomal protein	1
Rpl5	Ribosomal protein	1
Rpl7A.3	60S Ribosomal Protein	1
Rpl9B	RPL9B Ribosomal protein	1
Rpl12	Ribosomal protein	1
Rpl13	Ribosomal protein	1
Rpl18.exon2	Ribosomal protein	1
Rps6A[c)]	Ribosomal protein S6	1

Table 3. Continued

Protein name and GO classification [a]	Function	Frequency[b]
Rps17.3	Ribosomal protein 17.3	1
Rpl18	Ribosomal protein	1
Rps22A	Ribosomal protein S15a.e.c10	1
Rps31	Ubiquitin fusion protein	1
Stress Response		
Hsp104.5f[c]	Heat shock protein	1
Hsp90[c]	Heat shock protein	1
Ssa1[c]	Heat shock protein of HSP70 family	5
Ssa4[c]	Cahsp70 mRNA for heat shock	6
Ssb1[a]	Heat shock protein 70	7
Ssc1[c]	Mitochondrial heat shock protein 70-related protein	3
Translation elongation factor		
Eft2[c]	Translation elongation factor 2	8
Eft3[c]	Translation elongation factor 3	5
Sui2	Translation initiation factor	1
Tef1[c]	Translation elongation factor eEF1 α-A chain	10
Transport		
Hxt61	Sugar transporter	1
Hxt62	Sugar transporter	1
Mir1	Phosphate transport protein, mitochondrial	1
Por1	Mitochondrial outer membrane porin	2
Pma1	Plasma membrane H + -transporting ATPase1	6
Unknown function		
IPF17237	Unknown function	1

a) According to MIPS nomenclature (Mewes *et al* 1997).
b) Different experiments done (see Table 1).
c) Candida Genome Database: Cellular Component "cell surface" localizes to surface.

4 Discussion

The intrinsic cell wall proteins are expected to have either (or both) structural functions or catalytic activities related to: (i) assembly of macromolecules; (ii) remodeling of the cell wall during cell growth and morphogenesis; (iii) facilitation of nutrients uptake; or (iv) the adaptation of the cell to different types of stressing conditions. Three different types of covalently linked proteins exist in the cell wall: GPI proteins, ALS-CWP (alkaline soluble cell wall proteins), and RAE-CWP (reducing agents and extractable cell wall proteins) [30, 7, 2].

In this study we identified *C. albicans* cell wall proteins by using LC-MS. This technique is more sensitive and versatile than the others previously used, and allowed the identification of 21 genuine "true" cell wall proteins ("true" cell wall proteins according to our definition [31, 21]); in addition to several "atypical" proteins. The "true" cell wall proteins are characterized by having a predicted signal peptide, potential *O*- and *N*-glycosilation sites and frequently, but not always, a GPI signature or a series of internal repeats. Conversely, "atypical" cell wall proteins lack these characteristics. These proteins are probably retained in the cell wall by noncovalent bonds, and have been previously identified as members of different pathways occurring in the cell cytoplasm.

In the present study we included different protocols for protein extraction in order to identify the largest possible number of cell wall proteins. An important addition to the extraction protocols employed by other authors [7] was the proteomic analysis of tryptic peptides obtained by digestion of whole cell walls that had been thoroughly extracted by hot SDS. This approach had been previously employed for the analysis of the protein composition of bacterial cell walls [32, 33] but had not been previously utilized for fungal cell wall analyses. Interestingly, it proved to be as efficient for protein identification as for the analysis of HF-pyridine solubilized peptides, with the corresponding benefits in respect of speed and simplicity of analysis.

One important aspect to be taken into consideration is the low number (about 21) of "true" cell wall proteins identi-

Table 4. Identified proteins in the originally trypsin-resistant material of the cell wall

Fraction[a)]	"Atypical" proteins	True surface proteins
HF-supernatant	Gap1	Pga29
	Pet9	Ecm33.3
	Por1	Scw1
	Eno1	Pga30
	Tef1	Phr2
	Atp2	Cht2
	Atp1	Pga4
	Ssb1	Ssr1
	Rpl5	
HF-sediment	CdcC19	Pga29
	Tef1	Ecm33.3
	Pet9	Pga2
	Atp2	Cht2
	Gap1	Pga4
	Eno1	
	Eft2	

a) See treatments 10 and 11 from Table 1.

fied in this and previous studies [7]. This low value conflicts with results from *in silico* analysis of the *C. albicans* genome. These analyses have identified more than 100 ORFs encoding putative "surface," both plasmalemma- and wall-bound proteins, [20, 21]. The reasons for this discrepancy are unknown, but it can be suggested that: (i) The proteins that have been systematically identified are only the most abundant ones; (ii) Hydrolysis of the remaining cell wall proteins by trypsin is hindered by the high number of *O*-glycosilated residues or their location within the cell wall; (iii) Tryptic peptides of most of these proteins contain high amounts of sugar residues that interfere with their correct analysis; (iv) Different cell wall proteins are synthesized under different growth or stress conditions [supporting evidences are the analysis of gene expression under different conditions [34], and the observation that Als3 and Rbt1 were found only in hyphal cell walls; (v) Most GPI proteins are bound to the plasmalemma, and become separated during cell wall purification (previous data have suggested that the nature of amino acids around the ω site of the GPI-bound proteins may be indicative of their different locations in the plasma membrane or the cell wall; [35], but no systematic analysis exists in the matter); and (vi) some peptides are more difficult to be ionized than others.

Another important feature refers to the presence of numerous "atypical" wall proteins in the *C. albicans* cell wall. Our results agree with the numerous studies where this type of proteins have been found associated to the walls of *C. albicans* and *S. cerevisiae* [36, 37] and also in bacteria [32] and parasites [38], but in contrast with other analyses where these proteins failed to be detected or shown [7, 21]. In this regard, it is important to stress the reproducible and therefore *bona fide* occurrence of nine "atypical" proteins in at least 50% of the protocols used (see above). Accordingly, this discrepancy could be accounted for by methodological differences between laboratories.

Regarding the presence of these "atypical" proteins in the cell wall, it must be noted that they have been previously recognized to participate in different pathways occurring in the cytoplasm, and to lack the characteristics of *bona fide* cell wall or secreted proteins (see above). As indicated above, only a few of them were observed in a significant number of experiments, while most of them appeared only once or twice, indicating the randomness of their presence in the wall. Whether those proteins that appeared in most wall preparations are adventitiously trapped in the cell wall, as has been suggested by some authors [39], or somehow released by an alternative secretory process and associate to the wall polymers as suggested by others [35, 36] is still a matter of discussion (see below). One of these proteins, Tsa1p, for which a role in the cell wall has been proposed [40], was not detected in any of our preparations, including the mycelial cell walls. Accordingly, we consider that until a decisive experimental approach is worked out, it is advisable to treat this matter cautiously.

According to results from analysis of HF-extracted cell wall proteins that had been previously digested with trypsin, the observation that both "true" and "atypical" cell wall proteins were detected in samples theoretically devoid of proteins (which should have been previously digested by the protease) provide evidence that these proteins were embedded within the structure of the wall itself, at zones previously not accessible to the protease. Data in the literature indicate that the fungal cell wall is porous and that the threshold for the permeability of dextrans corresponds to an M_r of 4500 Da for *S. cerevisiae* [41], and 4750 Da for the wild-type strain of *Neurospora crassa* [42]. In contrast, a pore size allowing the entrance of dextrans up to an M_r of 30 600 or 2 70 000 Da for melanized or nonmelanized cell walls of *Cryptococcus neoformans*, respectively, was calculated by [43]. The corresponding Einstein–Stokes radii for these pores would be 4.0 or 10.6 nm. These data suggest that probably trypsin, with an M_r of around 23 300 Da and a compact molecular shape that contrasts with the linear shape of dextran molecules, might have access to most, but probably not all of the inner regions of the cell wall. This would explain the mechanism of retention of "atypical" proteins in the wall, and their resistance to extraction by detergents and chaotropic agents. Most likely they become physically entrapped within the structure of the growing wall, independently of the mechanisms that they employ to cross the permeability barrier of the cell. These data also suggest that the cell wall of *C. albicans* contains proteins not only at its outer surface, but also within its inner structure, as previously proposed [1]. The results presented and discussed here, reveal new insights on the structure of cell walls of *C. albicans*, and provide evidence that the strategy followed is extremely useful for our understanding of the building of such complex structure and may help to detect new targets to control deep infections.

The present work was partially supported by Ministerio de Educación y Ciencia, Spain (BFU2006-08684, BMC2003-01023), European Union (MRTN-CT- 2003-504148), Generalitat Valenciana (GVACOMP2006-255), CONACYT, México. The Centro Nacional de Investigaciones Cardiovasculares is supported by the Spanish Ministry of Health and Consumer Affairs and the ProCNIC Foundation.

The authors have declared no conflict of interest.

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