Adaptations in the wall proteome of the clinical fungus Candida albicans in response to infection-related environmental conditions

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Chapter 2

Hypoxic conditions and iron restriction affect the cell wall proteome of *Candida albicans* grown under vagina-simulative conditions

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Abstract

Proteins that are covalently linked to the skeletal polysaccharides of the cell wall of *Candida albicans* play a major role in the colonization of the vaginal mucosal surface, which may result in vaginitis. Here we report on the variability of the cell wall proteome of *C. albicans* as a function of the ambient oxygen concentration and iron availability. For these studies, cells were cultured at 37°C in vagina simulative medium (VSM) and aerated with a gas mixture consisting of 6% (v/v) CO$_2$, 0.01-7% (v/v) O$_2$, and N$_2$, reflecting the gas composition in the vaginal environment. Under these conditions, the cells grew exclusively in the non-hyphal form, with the relative growth rate being halved at $\sim$0.02% (v/v) O$_2$. Using tandem mass spectrometry and immunoblot analysis, we identified 15 covalently linked glycosylphosphatidylinositol (GPI) proteins in isolated walls (Als1, Als3, Cht2, Crh11, Ecm33, Hwp1, Pga4, Pga10, Phr2, Rbt5, Rhd3, Sod4, Ssr1, Ywp1, Utr2) and 4 covalently linked non-GPI proteins (MP65, Pir1, Sim1/Sun42, Tos1). Five of them (Als3, Hwp1, Sim1, Tos1, and Utr2) were absent in cells grown in rich medium. Immunoblot analysis revealed that restricted O$_2$ availability resulted in higher levels of the non-GPI protein Pir1, a putative $\beta$-1,3-glucan cross-linking protein, and of the GPI-proteins Hwp1, an adhesion protein, and Pga10 and Rbt5, which are involved in iron acquisition. Addition of the iron chelator ferrozine at saturating levels of oxygen resulted in higher cell wall levels of Hwp1 and Rbt5, suggesting that the responses to hypoxic conditions and iron restriction are related.
Introduction

*C. albicans* is a pleomorphic pathogenic fungus that is responsible for many mucosal infections in humans, and may cause systemic, often fatal infections in immuno-compromised patients (Calderone 2002). It is also well equipped to form biofilms on abiotic surfaces such as dentures and medical devices (Blankenship and Mitchell 2006; Ramage, Martinez *et al.* 2006). Covalently linked cell wall proteins (CWPs) play an important role in initiating and maintaining mucosal infections and biofilms (De Groot, Brandt *et al.* 2007; Li, Svarovsky *et al.* 2007; Naglik, Fostira *et al.* 2006; Nobile, Nett *et al.* 2006; Richard and Plaine 2007; Ruiz-Herrera, Elorza *et al.* 2006; Sundstrom 1999; Zhao, Daniels *et al.* 2006; Zupancic and Cormack 2007). This is consistent with their cellular location because covalently linked CWPs form an external protein layer surrounding the internal skeletal polysaccharide layer of the wall, and thus come directly into contact with biotic and abiotic surfaces. As in *Saccharomyces cerevisiae*, the cell wall proteome of *C. albicans* consists of 15 or more covalently linked CWPs and includes structural proteins, adhesion proteins, carbohydrate-active proteins, a proteases, superoxide dismutases, and iron acquisition proteins (Albrecht, Felk *et al.* 2006; De Groot, De Boer *et al.* 2004; Garcia, Castillo *et al.* 2005; Mao, Zhang *et al.* 2003; Weissman and Kornitzer 2004). In agreement with the outcome of similar studies carried out with *S. cerevisiae*, genomic transcript profiling studies of *C. albicans* indicate that the composition of the cell wall proteome may vary considerably with the growth conditions of the organism. For example, both yeast and hyphal walls seem to possess specific CWPs (Sohn, Urban *et al.* 2003; Staab, Bradway *et al.* 1999). The expression of several CWP-encoding genes changes when *Candida* cells become associated with different human epithelia or with abiotic surfaces (Garcia-Sanchez,
Aubert et al. 2004; Sohn, Senyurek et al. 2006) when transferred to blood (Fradin, De Groot et al. 2005), or when confronted with environmental stress conditions such as low or high pH (Bensen, Martin et al. 2004), the presence of azoles in the medium (De Backer, Ilyina et al. 2001; Liu, Lee et al. 2005); which interferes with sterol synthesis, or the presence of caspofungin, which inhibits the synthesis of the skeletal polysaccharide β-1,3-glucan and thereby causes cell wall stress (Liu, Lee et al. 2005). Iron deprivation and hypoxic conditions are also known to affect the expression of CWP-encoding genes (Lan, Rodarte et al. 2004; Setiadi, Doedt et al. 2006). Collectively, these observations strongly suggest that the composition of the cell wall proteome may vary considerably, both qualitatively and quantitatively, depending on environmental cues.

Many Candida infections involve colonization of the vaginal mucosal layer (Fidel and Sobel 2002; Sobel 2007). About three quarters of all women suffer from vaginitis at least once during their life time. Vaginal environmental conditions are characterized by a relatively low pH, a high partial pressure of CO$_2$ (approximately 6%, v/v) and low to vanishing partial pressures of O$_2$ (≤ 10%, v/v).

In this study, we have analyzed the cell wall proteome of C. albicans cells grown in VSM at 6% CO$_2$ (v/v) and at O$_2$ levels ranging from 7% to 0.01% (v/v). We also studied the effect of iron availability by growing the cells at 7% O$_2$ with an iron chelator. Our results show that under these conditions a new set of CWPs is incorporated into the walls compared to cells grown in rich medium at pH 5-5.6 (De Groot, De Boer et al. 2004). At restrictive oxygen concentrations the levels of at least four CWPs increase, i.e., the structural protein Pir1, the adhesion protein Hwp1, and, in particular, Pga10 and Rbt5, two iron acquisition proteins. These observations are
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consistent with the notion that hypoxic conditions may lead to a cellular response that involves an increased scavenging capacity for iron.

Materials and methods

Strains and growth conditions

The C. albicans strains used in this study are listed in Table 1. VSM was modified from (Owen and Katz 1999; Moosa, Sobel et al. 2004) and consisted of 58 mM NaCl, 18 mM KOH, 2 mM Ca(OH)$_2$, 1.75 mM glycerol, 6.7 mM urea, 33 mM glucose, and 6.7 g l$^{-1}$ YNB (Difco). In addition, lactic acid (22 mM; pKa = 3.85) and acetic acid (17 mM; pKa = 4.76), which are natural compounds in the vaginal fluid, were added to maintain the pH at 4.2. Strains were pre-cultured to saturation in YPD medium [1% (w/v) Bacto-yeast extract; 2% (w/v) Bacto-peptone; 2% dextrose (w/v)] at 30°C and 200 rpm. The cells were then inoculated in VSM in batch fermentors (working volume 0.5 liter) at 37°C with an aeration rate of 0.5 liter min$^{-1}$ and stirred at 200 rpm. The cells were cultured overnight under ambient air to an OD$_{600}$ of ~0.1; aeration was subsequently switched to gas mixtures, consisting of N$_2$, 0.01-7% (v/v) O$_2$, and 6% (v/v) CO$_2$, which leads to dissolved O$_2$ concentrations that correspond to values measured in the human vagina (Wagner and Ottesen 1982). The cells were harvested at an OD$_{600}$ of ~1. To induce iron restriction, 1 mM ferrozine (5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4',4''-disulfonic acid; Sigma-Aldrich Co) was added to overnight cultures at OD$_{600}$ ~0.1. Culturing was continued until an OD$_{600}$ ~1 was reached, and the cells were collected for cell wall analysis. The OD$_{600}$ of cultures was measured using a Shimadzu model UV mini 1240 spectrophotometer (OD$_{600} = 1$ corresponds to 1.5 x 10$^7$ cells ml$^{-1}$ of culture). To determine the percentage of budded cells (the budding index), 200 cells were taken from two independent
cultures for each condition and counted. Relative growth rates were determined by following the OD\textsubscript{600} of three separate cultures in the range of OD\textsubscript{600} ∼0.1 to ∼1. A reference cell culture grown in rich medium (pH 5.5) (Table 2) was obtained as follows. The medium consisted of 20 g l\textsuperscript{-1} glucose, 10 g l\textsuperscript{-1} Casamino Acids (Becton Dickinson and Company, Sparks, Md.), 6.7 g l\textsuperscript{-1} YNB (Difco), 110 µg of leucine ml\textsuperscript{-1}, 55 µg of tyrosine ml\textsuperscript{-1}, 55 µg of tryptophan ml\textsuperscript{-1}, and 55 µg of adenine sulfate ml\textsuperscript{-1}, at pH 5.5, as described by (De Groot, De Boer et al. 2004). The cells were cultured overnight in a batch fermentor aerated with atmospheric air with stirring at 200 rpm at 30ºC and were harvested at mid-log phase.

Table 1 C. albicans strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314</td>
<td>Clinical isolate</td>
<td>(Fonzi and Irwin 1993)</td>
</tr>
<tr>
<td>CAF 2-1</td>
<td>\textit{ura3\textunderscore \textDelta::imm434/URA3}</td>
<td>(Fonzi and Irwin 1993)</td>
</tr>
<tr>
<td>BCa18-2</td>
<td>\textit{ura3\textunderscore \textDelta::imm434/ura3\textunderscore \textDelta::imm434 rbt5\textunderscore \textDelta::hisG/rbt5\textunderscore \textDelta::hisG-URA3-hisG}</td>
<td>(Braun, Head et al. 2000)</td>
</tr>
<tr>
<td>KC85</td>
<td>\textit{ura3\textunderscore \textDelta::imm434/ura3\textunderscore \textDelta::imm434 pga10\textunderscore \textDelta::hisG/pga10\textunderscore \textDelta:: hisG-URA3-hisG}</td>
<td>(Weissman and Kornitzer 2004)</td>
</tr>
<tr>
<td>KC100</td>
<td>\textit{ura3\textunderscore \textDelta::imm434/ura3\textunderscore \textDelta::imm434 pga10\textunderscore \textDelta::hisG/pga10\textunderscore \textDelta:: hisG-URA3-hisG}</td>
<td>(Weissman and Kornitzer 2004)</td>
</tr>
</tbody>
</table>

**Cell wall isolation**

Cell walls were isolated as described previously (De Groot, De Boer et al. 2004). Cultures were harvested in the exponential phase of growth, washed with cold de-mineralized water, and with 10 mM Tris-HCl buffer, pH 7.5, and disintegrated in a Bio-Savant Fast Prep 120 machine (Qbiogene, Carlsbad, CA), using 0.25–0.50-mm (diameter) glass beads (Emergo BV). A protease inhibitor mixture (Sigma-Aldrich Co) was added to protect CWPs from degradation by intracellular proteases. To remove noncovalently-linked proteins associated with the wall preparation, crude cell
walls were washed with 1 M NaCl, and boiled twice for 5 minutes in 2% (w/v) SDS, 150 mM NaCl, 100 mM Na-EDTA, 100 mM β-mercaptoethanol, 50 mM Tris-HCl, at pH 7.8. SDS-extracted walls were washed three times with de-mineralized water and freeze-dried.

Sample preparation for mass spectrometric analysis

For mass spectrometric analysis, CWPs were modified by reduction and S-alkylation as follows. Cell walls were treated with the reducing reagent 10 mM dithiothreitol in 100 mM NH₄HCO₃ for 1 h at 55 °C. The samples were then cooled and the walls were alkylated by incubating them for 45 minutes at room temperature in the dark in 65 mM iodoacetamide in 100 mM NH₄HCO₃. The reaction was quenched by incubating the walls in 55 mM dithiothreitol in 100 mM NH₄HCO₃ for 5 minutes. Prior to digestion with trypsin, the walls were washed three times with 50 mM NH₄HCO₃. Trypsin digestion of S-alkylated CWPs was carried out as described previously (Yin, de Groot et al. 2005). Cell walls were incubated overnight at 37°C in the presence of sequencing-grade trypsin (Roche Applied Science) using a cell wall protein/enzyme ratio of 50:1.

Mass spectrometric identification of covalently linked CWPs

Instrument and analysis settings were as described previously (Yin, de Groot et al. 2005). Tryptic peptides (5-10 µg) were desalted and concentrated on a C18-ZipTip pre-column (Millipore) and fractionated using a nano-LC system (PepMap C18; LC Packings, Dionex Corporation). Eluted peptides were directly ionized by electrospray in a Micromass quadrupole time-of-flight mass spectrometer (Waters, Manchester, UK). The ions from the survey spectrum were automatically selected for fragmentation in a collision chamber using the Masslynx software. Tandem mass
spectrometry spectra of ionized peptide fragments were analyzed with Biolynx and Masslynx Pepseq software. Proteins were identified by comparison of the identified amino acid peptide sequences with *in silico* digests of the proteins translated from Assembly 19 of the *C. albicans* SC5314 genome sequence (http://www.candidagenome.org/) using Mascot software. Several precautions were taken to optimize the reproducibility of the results over time and to monitor drift of the equipment. (1) The nano-LC column was cleaned daily by thoroughly washing it with 100% solvent B (acetonitrile + 0.1% formic acid). (2) To prevent carrying over from previous samples, at least one empty injection was run between each sample. (3) The amount of sample loaded in the liquid chromatography step of each run was measured through an A$_{214}$ chromatogram. (4) To control the efficiency of ionization and to verify that the run-to-run variation of the ion intensity was < 20%, the total ion count (TIC) profile of each LC/MS/MS run was determined. (5) To verify the accuracy of MS selection, the instrument was daily calibrated with 1 pmole cytochrome c (Dionex, Amsterdam, The Netherlands). (6) Each sample (containing ~1 pmole of protein) was run at least three times, and in two of them the excluding list was used to ensure complete coverage. (7) The general performance of the LC/MS/MS system was checked monthly with a cytochrome c digest to verify that a similar number of peptides of similar MASCOT score were identified.

**Isolation of CWPs for immunoblot analysis**

GPI-modified CWPs were released by treating cell wall material with recombinant *Trichoderma harzianum* endo-β-1,6-glucanase (Bom, Diebandhoesing *et al*. 1998) as previously described (Kapteyn, Ter Riet *et al*. 2001). Four mg of freeze-dried cell walls were incubated overnight with 2.5 µl (0.16 U mg$^{-1}$ of cell walls) of enzyme and 2 µl of a protease inhibitor mixture (Sigma-Aldrich) in 200 µl of 50 mM Na-phosphate
buffer, pH 5.5, at 37°C overnight. To release mild alkali-extractable CWPs, cell walls were incubated with 30 mM NaOH at 4°C for 17 hours with gentle shaking; the reaction was stopped by neutralization with 30 mM acetic acid (Mrša, Seidl et al. 1997). In each lane, the equivalent of 0.15 mg of dried walls (corresponding to ~ 1 mg of dried biomass) was applied.

**Immunoblot analysis of CWPs**

CWPs were separated by electrophoresis using linear 3-8% poly-acrylamide gradient gels in Tris-acetate (Invitrogen). The separated proteins were transferred onto an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore Corporation). To reduce non-specific staining by the antisera, the membrane-blotted proteins were first incubated with 50 mM periodic acid in 100 mM sodium acetate (pH 4.5) prior to the blocking step. Immunoblot analysis was performed with polyclonal Hwp1 (Staab, Ferrer et al. 1996) or Pga10 antiserum (Weissman and Kornitzer 2004), diluted 1:10 000 in 5% (w/v) milk powder in PBS buffer, pH 7.4, for 2 hours. *S. cerevisiae* Pir2 antiserum (Russo, Kalkkinen et al. 1992) was diluted 1:50 000 and incubated with the blots overnight to enhance interaction with the probed proteins. After washing with PBS the membranes were incubated with goat anti-rabbit antiserum, conjugated with peroxidase (GARPO) at a dilution of 1:10 000 in 5% (w/v) milk powder in PBS buffer, pH 7.4. Proteins were visualized using Enhanced Chemiluminescence (ECL) (Amersham Biosciences).

**Quantazyme sensitivity**

Quantazyme sensitivity of intact cells was measured as described (Kapteyn, Ter Riet et al. 2001). Exponentially growing cells were centrifuged and washed twice with 50 mM Tris-HCl, pH 7.5. The OD$_{600}$ of the cell suspensions was adjusted to 1 and
the cells were pre-incubated for 1 hour with 40 mM β-mercaptoethanol in 50 mM Tris-HCl buffer, pH 7.5. Quantzyme (from *Oerskovia xanthineolytica*, Quantum Biotechnologies) was added at 20 U ml\(^{-1}\) of cells suspension. Incubation was carried out at 30°C, and the decrease in optical density (OD\(_{600}\)) was measured at 5-minute intervals.

**Determination of the polypeptide and chitin content of isolated cell walls**

Cell walls (4 mg) were suspended in 100 µl of 1M NaOH. The suspension was incubated at 100 °C for 10 minutes, cooled, and neutralized with 100 µl of 1M HCl. Insoluble material was pelleted by centrifugation and the supernatant was used for protein determination using the bicinchoninic acid protein assay (Pierce Chemical Co). A calibration curve was prepared by using BSA treated in the same way as the cell wall samples.

Chitin content was measured using the method described by (Kapteyn, Ter Riet *et al.* 2001). NaOH-extracted (4 mg; see ‘Protein determination’) cell walls were hydrolyzed for 17 hours in 1 ml of 6 M HCl at 100°C. Samples were evaporated under a stream of air and resuspended in 1 ml of de-mineralized water. To 0.1 ml of sample 0.1 ml of 1.5 M Na\(_2\)CO\(_3\) in 4% acetylacetone was added and the mixture was boiled for 20 minutes. After cooling, 0.7 ml of 96% ethanol was added and 0.1 ml of 1.6 g of p-dimethyl-aminobenzaldehyde taken up in 30 ml of concentrated HCl and 30 ml of 96% (v/v) ethanol, incubated 1 hour at room temperature. A calibration curve was prepared by measuring \(A_{520}\) in a concentration range of 0-40 µg glucosamine per ml.
Results

The effect of changes in the gas phase composition on the relative growth rate and cell wall composition of *C. albicans* grown in VSM

For the studies reported in this communication we used a growth medium consisting of yeast nitrogen base with modified concentrations of calcium, chloride, potassium and sodium ions, conform to the concentrations in the vaginal fluid of healthy pre-menopausal women during the non-menstrual phase (Owen and Katz 1999) (Materials and methods). The medium further contained urea and glycerol and was buffered at pH 4.2 by making use of the physiological buffers found in the vaginal fluid, *i.e.* 22 mM lactic acid (pKa 3.85) and 17 mM acetic acid (pKa 4.76) (Owen and Katz 1999). Glucose, which is present in the vaginal fluid at ∼33 mM, served as the main carbon source. The cells were grown at 37°C. The microenvironment of the human vagina is characterized by lower O$_2$ and higher CO$_2$ levels compared to atmospheric values; (Wagner and Ottesen 1982). The O$_2$ concentrations measured in the vaginal fluid vary from almost 0 to maximally 0.11 mM (equivalent to 10% (v/v) partial O$_2$ pressure), depending on the day of the menstrual cycle and the method used for measuring gas tensions. The vaginal CO$_2$ levels, however, vary only slightly during the menstrual cycle (6-8%, v/v) (Wagner and Ottesen 1982). Therefore, in this study we supplied O$_2$ to the cells in concentrations equivalent to a partial O$_2$ pressure between 0.01 and 7% (v/v), in combination with 6% (v/v) CO$_2$, using batch fermentors with controlled aeration.

The growth rate of *C. albicans* on the medium selected and under an atmosphere of 7% O$_2$/ 6% CO$_2$ equals 0.60 h$^{-1}$ (corresponding to a generation time of ∼70 min; see Figure 1), which is considerably faster than under atmospheric
conditions in the same medium (0.45 h\(^{-1}\), corresponding to a generation time of \(\sim 90\) min). As \textit{S. cerevisiae} can fix CO\(_2\) via pyruvate carboxylase to form the citric acid cycle intermediate oxaloacetate and as higher CO\(_2\) concentrations up to \(\sim 6\)% result in increased CO\(_2\) fixation (Cazzulo, Claisse \textit{et al.} 1968; Liener and Buchanan 1951), the increased growth rate might be caused by the elevated CO\(_2\) concentration. Figure 1 further shows that at 7\% (v/v) O\(_2\) is present at a saturating concentration with respect to the growth rate. The maximal growth rate of \textit{C. albicans} in batch culture decreases significantly, however, when the O\(_2\) level is lowered to less than 0.5\% O\(_2\) (v/v) and is halved at \(\sim 0.02\)% (v/v), which corresponds to a dissolved O\(_2\) concentration of approximately 0.23 µM. Further experiments were carried out using an O\(_2\) level of 0.02\% (v/v).

\textbf{Figure 1} The effect of lowering the O\(_2\) concentration on the relative growth rate of \textit{C. albicans} cells growing in VSM at 37°C and at 6\% CO\(_2\). The data shown represent the means of three cultures. The error bars represent SD.
Cells cultured in VSM at low O$_2$ levels in combination with elevated CO$_2$ levels grew exclusively in the yeast and pseudohyphal form, under all O$_2$ concentrations tested; a similar morphology was observed when the cells were grown in VSM and aerated with an atmospheric gas mixture (data not shown). The lower growth rates at low oxygen concentrations were accompanied by a decrease in the budding index, which dropped from 91% at 7% O$_2$ to 33% at 0.01% O$_2$, indicating that the G1 phase of the cell cycle is extended when cells are deprived of oxygen. Interestingly, lower O$_2$ levels resulted in increased resistance of intact cells to Quantazyme, a recombinant β-1,3-glucanase, indicating that the structure or composition of the cell wall had changed (Figure 2). This was not reflected in significant changes in the total polypeptide content of the walls, which was 2.4% at both 7% O$_2$ (v/v) and 0.02% O$_2$ (v/v). The respective chitin contents of these wall preparations were 3.5% and 2.9%. The small differences in these cell wall components may imply that the increased Quantazyme resistance of *C. albicans* is due to an altered protein composition of its wall (see next section).

**Figure 2** Increased resistance of cells, grown under low partial pressure of O$_2$, to the endo-β-1,3-glucanase Quantazyme. The cells were grown at 7% O$_2$/6% CO$_2$ (closed circles) or at 0.02% O$_2$/6% CO$_2$ (open circles). The values shown are the means of two replicates.
Effects of oxygen deprivation on the cell wall proteome

For a qualitative comparison of the covalently linked proteins in the cell wall of wild type cells grown at $O_2$ concentrations $\leq 7\%$ (v/v) and at 6% $CO_2$ (v/v) in VSM, we applied liquid chromatography followed by tandem mass spectrometry (LC/MS/MS) of CWP-derived peptides. In combination with immunoblot analysis we detected 19 proteins, including 15 known or predicted GPI proteins, and four non-GPI proteins (Table 2, Figure 3). For comparison, cell walls obtained from a fermentor-grown reference culture grown in rich medium were also analyzed. Importantly, the GPI-proteins Als3, Hwp1 and Utr2, and the non-GPI proteins Sim1 and Tos1 were only found in VSM-cultures. Additionally, in VSM cultures a specific peptide confirming the presence of Rbt5 was detected, which was not identified before (De Groot, De Boer et al. 2004). The transglucosylase Phr2 was identified in the walls at all tested $O_2$ concentrations, consistent with its function at acidic pHs (De Bernardis, Muhlschlegel et al. 1998; Fonzi 1999; Mühlschlegel and Fonzi 1997). Phr2 peptides were also identified in the reference culture, probably due to the fact
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that during culturing the pH of the reference culture dropped from 5.5 to 4.9. This is consistent with the known pH-dependency of PHR2 expression (Mühlschlegel & Fonzi, 1997). Als3, an adhesion protein that mimics mammalian cadherins (Phan, Myers et al. 2007), was also identified in all VSM samples (20 identifications of Als3 peptides, not shown), whereas no Als3 peptides were found in the reference culture. This is consistent with the frequent occurrence of ALS3 transcripts in vaginal samples during C. albicans infection (Cheng, Wozniak et al. 2005). Als3 has been described as a hypha-specific protein (Argimon, Wishart et al. 2007; Hoyer, Payne et al. 1998) however, as our samples did not contain hyphae, our observations demonstrate that the synthesis of Als3 may also occur in non-hyphal cells under selective environmental conditions. Table 2 also shows that the peptides released by trypsin from GPI-modified CWPs and identified by tandem mass spectrometry were predominantly found in the N-terminal region of these proteins, consistent with the observation that this region generally is less serine- and threonine-rich and therefore less glycosylated (Chen, Shen et al. 1995). No such tendency was observed for the non-GPI proteins.

Table 2 Proteins identified by LC/MS/MS or immunoblot analysis in cell walls isolated from cultures grown in VSM in the presence of 7 - 0.01% O2/6% CO2. For comparison, the CWP peptides identified in cells from a reference culture grown in rich medium and aerated with atmospheric air are also presented.

<table>
<thead>
<tr>
<th>Name</th>
<th>Function and features</th>
<th>Sequence of identified peptides</th>
<th>Position</th>
<th>VSM</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Als1</td>
<td>1260 aa; adhesion protein (Klotz, Gaur et al. 2007)</td>
<td>STVDPSGYLYASR GLNDWNYPVSSESFSYTK# SQSKPFTLR</td>
<td>176-188 237-254 303-311</td>
<td>+ + +</td>
<td>- - -</td>
</tr>
<tr>
<td>Als3</td>
<td>1155 aa; hypha-induced adhesion protein (Argimon, Wishart et al. 2007); highly</td>
<td>FTTSQTSVOLTAHGK CFTAGNTNTVTFNDGKK KISINVDFER</td>
<td>77-92 150-165 150-166 166-175 167-175</td>
<td>+ + + + +</td>
<td>- - - - -</td>
</tr>
<tr>
<td>Protein</td>
<td>Length</td>
<td>Description/Expression Conditions</td>
<td>Identifiers</td>
<td>Presence/Expression</td>
<td></td>
</tr>
<tr>
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<td>--------</td>
<td>-----------------------------------</td>
<td>-------------</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>Cht2</td>
<td>583 aa</td>
<td>Chitinase (McCreath, Specht et al. 1995)</td>
<td>SNQALYWQNGAGGQER&amp; FADLWNK FADSPAPN FADSPAPNKR LFVGVPATNGAYYDTSK FENFVQIK</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Crh11</td>
<td>453 aa</td>
<td>Putative transglucosidase (Pardini, De Groot et al. 2006)</td>
<td>SSDCSPVPAALGSSFLEK RFDNPSFK FDNPSFK DAVTWSVDGVSIR SVLVDYSSGK QYSYDOSGWSIK YDAOADDIK YDAOADDIKK</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ecm33</td>
<td>423 aa</td>
<td>Required for cell wall integrity (Martinez-Lopez, Park et al. 2006), induced by fluconazole (Copping, Barelle et al. 2005) and hypoxia (Setiadi, Doedt et al. 2006)</td>
<td>NDDLTELDFPK TIGALQISPNSER SFSGFPK VSGFILK VSGGFILKTLDK LSCAFNK</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Hwp1</td>
<td>634 aa</td>
<td>Hypha-specific protein (Staab, Bradway et al. 1999) hypoxia-induced (Setiadi, Doedt et al. 2006)</td>
<td>Immunological identification only</td>
<td>++</td>
<td>--</td>
</tr>
<tr>
<td>Pga4</td>
<td>451 aa</td>
<td>Transglucosidase</td>
<td>GVDYQPQGSSSELEDPLADTNVCER YQOEILGIINR LPSGLYFNCGDDDMAR GDSVTTNDDFNLK TNKPSGOGYLYK GALTPTGHGFDAYVQNGCNK</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Pga10</td>
<td>250 aa</td>
<td>Iron acquisition protein (Weissman and Kornitzer 2004), ketaconazole-induced (Liu, Lee et al. 2005) and hypoxia induced (this work)</td>
<td>IVDQLPECAC* IVDQLPECACEVK* Immunological identification (this work)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Phr2</td>
<td>544 aa</td>
<td>Transglucosidase expressed at pH&lt;5.5; fluconazole-induced (Copping, Barelle)</td>
<td>EDLPAIEIVGNK&amp; DIPYLEAVDTNVR PSDLDVDIFER KSNTDASAFVK SNTDASAFVK* SIPVGYSANDSAIR AGYESATNDYK NLGIPIFSEYYNEVRPR</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates immunological identification.
Hypoxic conditions and iron restriction affect the cell wall proteome of C. albicans

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>MHC Peptides</th>
<th>MHC Peptides</th>
<th>MHC Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rbt5</td>
<td>241 aa; iron acquisition protein, expression induced by ketoconazole (Liu, Lee et al. 2005), and hypoxia (Setiadi, Doedt et al. 2006, this work)</td>
<td>IYDQLPECACK*</td>
<td>47-56</td>
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<td></td>
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<td>IYDQLPECACEVK*</td>
<td>47-60</td>
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<td></td>
<td></td>
<td>QSTSSTPCPYWDGCLCVMPQFAGA</td>
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<td>VGNCVAK</td>
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<td></td>
<td>Immunological identification (this work)</td>
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<tr>
<td>Rhd3</td>
<td>204 aa; unknown function</td>
<td>KTDDSAPITIVAK</td>
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<td></td>
<td>TDDSAPITIVAK</td>
<td>139-150</td>
<td>-</td>
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<td>Sod4</td>
<td>232 aa; putative Cu/Zn superoxide dismutase (Martchenko, Alarco et al. 2004)</td>
<td>GDSPISTDSK&amp;</td>
<td>16-25</td>
<td>+</td>
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<td></td>
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<td>GDSPISTDSK&amp;GK&amp;</td>
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<td>+</td>
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<td></td>
<td>GDSPISTDSK&amp;KAPLVAR&amp;</td>
<td>16-33</td>
<td>+</td>
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<td></td>
<td></td>
<td>TPAALELGDLSGR</td>
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<td>+</td>
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<tr>
<td>Ssr1</td>
<td>234 aa; structural cell wall protein (Garcera, Martinez et al. 2003)</td>
<td>APPACLLACVAK&amp;</td>
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<td>APPACLLACVAKVEK&amp;</td>
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<td>-</td>
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<td></td>
<td></td>
<td>CSQNLDSIICRTTK</td>
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<td>CSQNLDSLICRTKNSDVEK</td>
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<td>EICPNGDADTAISAFK</td>
<td>64-79</td>
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<td>Utr2</td>
<td>470 aa; possible role in cross-linking β-1,3-glucan and chitin (Pardini, De Groot et al. 2006)</td>
<td>MSTFOESFDSDKVK</td>
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<td>IQFSLWPGGDSSNAK</td>
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<td>GTIEWAGGLINWDSEIKK</td>
<td>269-287</td>
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<td>Ywp1</td>
<td>533 aa; unknown function, expression higher in yeast (Granger, Flenniken et al. 2005)</td>
<td>VITVVACDEHK</td>
<td>329-339</td>
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Non-GPI-proteins

<table>
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<th>Protein</th>
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<th>MHC Peptides</th>
<th>MHC Peptides</th>
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<tr>
<td>MP65</td>
<td>378 aa; endo-β-1,3-glucanase (Sandini, La Valle et al. 2007)</td>
<td>GITYSPYSDNGGCK</td>
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<td>SESQIAEIAQLSGFDVIR</td>
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<td>AYVDEGRK</td>
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<td>VWTACSNGK</td>
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<td>SNQQAIAISSIK</td>
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<tr>
<td>Pir1</td>
<td>346 aa; putative β-1,3-glucan cross-linking protein, fluconazole-induced (Copping, Barelle et al. 2005), hypoxia induced (this work)</td>
<td>ACSSANNLMTLHDSVLK</td>
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<td>Sim1</td>
<td>372 aa; unknown function; hypoxia-induced (Setiadi, Doedt et al.)</td>
<td>DGYVCSYACQAGMSK</td>
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<td></td>
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<td>APCSVVDGDTYFK</td>
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Table 2.1

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<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>O2 Level</th>
<th>Detected by MS/MS</th>
<th>Detected by Immunoblot</th>
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<tr>
<td>Tos1</td>
<td>468 aa</td>
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<td></td>
<td>fluconazole-induced (Copping, Barelle et al. 2005)</td>
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<td></td>
<td>SGEEYIIFSGSK</td>
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<td></td>
<td>TLQYGEATCSCWK</td>
<td>358-370</td>
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# Common peptide of Als1 and Als3, * common peptide of Pga10 and Rbt5, ‡ common peptide of Phr1 and Phr2, & predicted N-terminal peptide of the mature protein, + detected by MS/MS, - not detected by MS/MS, ++ detected by immunoblot, -- not detected by immunoblot

Because our mass spectrometric approach identified the same set of covalently linked CWPs at all O2 levels tested (data not shown), we next used an immunological approach to detect quantitative changes in the cell wall proteome. Using a cross-reactive antiserum raised against *S. cerevisiae* Pir2 (Russo, Kalkkinen et al. 1992), we found increased levels of Pir1 protein in cell wall extracts obtained with mild alkali, when the O2 level decreased below 0.02% (v/v) (Figure 3a). As Pir1 protein is a potential β-1,3-glucan cross-linking protein and essential for cell wall integrity (Klis, Ram et al. 2007; Martinez, Castillo et al. 2004), this might explain the increased Quantazyme resistance of intact cells grown at low O2 levels.

As the expression of *HWP1* is increased in non-hyphal cells grown under hypoxic conditions (Setiadi, Doedt et al. 2006), we also quantitatively analyzed Hwp1 levels. Figure 3(b) shows that Hwp1 was indeed present in the walls isolated from cells grown under vagina-simulative conditions at all O2 concentrations tested and that its level increased at hypoxic O2 levels. Nevertheless, the highest levels observed under these conditions were still significantly lower than the levels found in hyphal walls; Hwp1 was not found in walls of cells grown in rich medium even after overexposure of the immunoblots (data not shown).

**Figure 3** Oxygen limitation results in increased cell wall levels of Pir1 (panel A), Hwp1 (panel B), Pga10 and Rbt5 (panel C). Cells were grown overnight in a batch fermentor under air until an OD600
Hypoxic conditions and iron restriction affect the cell wall proteome of C. albicans

~0.1. The cells were then cultured with controlled aeration using a gas mixture consisting of nitrogen, 6% CO$_2$ and 0.01-to 7 O$_2$ until an OD$_{600}$ ~1 was reached. Covalently linked CWPs were released from cell walls with mild alkali extraction (Pir1) or β-1,6-glucanase (Hwp1, Pga10, Rbt5). For each condition, proteins isolated from equal amounts of cell walls (~ 0.15 mg of dried walls, which corresponds to ~ 1 mg of dried biomass) were subjected to immunoblot analysis. Sizes of marker proteins are indicated on the left. The diffuse nature of the Pir1 band is probably due to allelic variability (CGD) and the high degree of glycosylation of Pir1.

The transcript levels of $RBT5$, which encodes a predicted GPI-protein involved in iron acquisition (De Groot, Hellingwerf et al. 2003; Weissman and Kornitzer 2004), also shows a considerable increase under hypoxic conditions (Setiadi, Doedt et al. 2006). Figure 3(c) shows that an antiserum raised against Pga10, a homolog of Rbt5 and also involved in iron acquisition (Weissman and Kornitzer 2004), identified two bands with molecular masses of about 100 and 130 kDa, at O$_2$ concentrations ≤0.5% (v/v). To investigate if these bands represented Rbt5 and Pga10 or other members of the family (Weissman and Kornitzer 2004), we analyzed the homozygous single-
deletion mutants and the double deletant. Figure 4 shows that the two bands incorporated in the cell wall of the control strain (CAF2-1) were not present in the double deletion strain and that only a single band is detected in each of the single mutant strains grown under low O$_2$ conditions. This is consistent with the notion that the 100 kDa band corresponds to Rbt5 and the 130 kDa band to Pga10. Interestingly, PGA10 and RBT5 belong to the same transcriptional module as HWP1 (level 16, module 16; see also CGD); (Ihmels, Bergmann et al. 2005), showing that these three genes are co-regulated under several other test conditions as well.

Figure 4 Immunoblot analysis of the Rbt5 family of proteins in rbt5Δ and pga10Δ mutant strains using a Pga10 antiserum. The cells were grown in VSM at 0.02% O$_2$/6%CO$_2$ (v/v). CWPs were released using β-1,6-glucanase.

Because the high-affinity iron uptake system of *C. albicans* requires molecular oxygen (Kosman 2003), hypoxic conditions are expected to lead to iron deprivation, raising the question whether iron deprivation alone might induce similar changes in the cell wall proteome as O$_2$ limitation. Figure 5 shows that the changes in the cell wall proteome of cells treated with the ferrous iron chelator ferrozine (1 mM, resulting
in a limited decrease in relative growth rate from 0.60 to 0.55 h\(^{-1}\)) partially overlapped with the changes observed in the cell wall proteome of cells grown under hypoxic conditions. The levels of Rbt5 and Hwp1 in cell walls increased in both ferrozine-treated cells and in cells grown under hypoxic conditions, but in ferrozine-treated cells the levels of Pir1 and Pga10 were not increased.

**Figure 5** Western blot analysis of CWPs from cells grown at 7\% O\(_2\)/6\% CO\(_2\) (control), 0.02\% O\(_2\)/6\% CO\(_2\) (low oxygen conditions) or 7\% O\(_2\)/6\% CO\(_2\) in the presence of 1 mM ferrozine (low iron conditions). Covalently linked CWPs were released from cell walls with β-1,6-glucanase (Pga10, Rbt5, and Hwp1) or by mild alkali extraction (Pir1) as described in Materials and methods.

**Discussion**

CWPs of *C. albicans* play a major role in the infection of mucosal surfaces in the human body, including the vaginal mucosal surface. Vaginal environmental conditions are characterized among others by a growth temperature of 37°C, an acidic pH, and by an increased concentration of CO\(_2\) and low concentrations of O\(_2\) compared to atmospheric values. Here we have studied the effect of vaginal growth conditions on the cell wall proteome, and, in particular, the effect of low O\(_2\)*
concentrations. In the VSM that we developed for our studies, no hyphal growth was observed under any condition tested. It is important to realize that the medium used in this study and the way in which the cells were grown do not fully mimic vaginal conditions. For example, VSM contains yeast nitrogen base but no sterols or fatty acids, both of which may be expected to be present under *in vivo* conditions. In addition, *Candida* cells might grow in the vagina in the form of biomats or biofilms, whereas cells in our experiments were cultured in a rapidly stirred and fully aerated fermentor.

Using a combination of tandem mass spectrometry and immunoblot analysis we identified 19 CWPs, five of which (Als3, Hwp1, Sim1, Tos1, and Utr2) were not found in a reference culture grown in rich medium. This is in agreement with earlier results (De Groot, De Boer *et al.* 2004). This supports the notion that the composition of the cell wall proteome of *C. albicans* is tightly controlled. The presence of Phr2 in the cell walls is in agreement with earlier observations showing that *PHR2* is specifically expressed at pHs below pH 5.5 and that the resulting protein is directly involved in cell wall assembly and is essential for colonization of the vagina (De Bernardis, Muhlschlegel *et al.* 1998; Fonzi 1999; Mühlschlegel and Fonzi 1997). Immunoblot analysis identified the presence of an additional GPI-protein (Hwp1), which was missed by our mass spectrometric analysis due to the lack of suitable tryptic peptides (*i.e.*, not heavily glycosylated and with a mass that does not exceed the detection limit of the mass spectrometer). Both Als3 and Hwp1 have been described as hypha-specific adhesion proteins (Argimon, Wishart *et al.* 2007; Hoyer, Payne *et al.* 1998; Staab, Bradway *et al.* 1999). However, as our cultures did only contain yeast and pseudohyphal cells, our results show that both proteins can also be expressed in non-hyphal cells depending on the specific growth conditions. This is
consistent with the observation that \textit{HWP1} is moderately expressed in pseudohyphal cells (Snide and Sundstrom 2006). Interestingly, \textit{ALS3} transcripts have been frequently detected in clinical vaginal fluid specimens, suggesting that the adhesion protein Als3 is important for colonization of this environment (Cheng, Wozniak \textit{et al.} 2005).

Oxygen limitation affects multiple processes in eukaryotic cells, such as iron uptake, mitochondrial respiration, sterol synthesis, and the synthesis of unsaturated fatty acids, all of which at some stage require molecular oxygen (Berg, Tymoczko \textit{et al.} 2007; Kosman 2003; Schweizer 2004). There are several indications that the hypoxic conditions used in our study result in a molecular response that resembles the response observed when cells grow under iron-restricted conditions. First, when the O$_2$ concentrations were lowered, the levels of the iron acquisition proteins Pga10 and Rbt5 in the cell wall increased. Second, intact cells became more resistant to the endo-$\beta$-1,3-glucanase Quantazyme (Figure 2) and to the cell wall-degrading enzyme preparation Zymolyase (data not shown). This is in agreement with the observation that iron limitation in \textit{C. albicans} results in increased resistance of intact cells to Zymolyase (Sweet and Douglas 1991). Third, genomic transcript analyses have revealed that iron acquisition and iron uptake genes, including \textit{RBT5}, are not only up-regulated in cells grown at low-iron conditions (Lan, Rodarte \textit{et al.} 2004; Weissman and Kornitzer 2004), but also under hypoxic conditions (Setiadi, Doedt \textit{et al.} 2006). Finally, addition of the iron-chelating compound ferrozine to the medium, at saturating O$_2$-supply levels, resulted in an increased level of Rbt5 in the wall (Figure 5). This is in agreement with the observation that the antifungal agent ciclopirox, which is believed to possess iron-chelating properties as well, strongly increases the transcript level of \textit{RBT5} (Lee, Liu \textit{et al.} 2005; Sigle, Thewes \textit{et al.} 2005).
Interestingly, the presence of ferrozine in the medium also caused an increase in the cell wall of the adhesion protein Hwp1, suggesting an additional control mechanism for HWP1 expression. We propose that hypoxic conditions result in reduced iron uptake and competition for iron by iron-containing enzymes and that this might lead in various ways to increased expression of specific CWP-encoding genes. Another explanation for the response to hypoxic conditions may reside in the fact that numerous oxygen-dependent reactions in the cell are carried out by iron-containing enzymes (e.g. haems in respiration, and Fe-dependent sterol synthesis (Kaplan, McVey Ward et al. 2006)). Maintaining a sufficiently high rate of oxygen-dependent enzymatic reactions could therefore be achieved by an increased synthesis of iron-containing enzymes. To be able to do so, an increase in iron-scavenging proteins like PGA10 and RBT5 would indeed be expected. Our results do not exclude that processes such as sterol synthesis and the synthesis of unsaturated fatty acids, which are affected by the low O₂ concentrations used in our study, might also cause major changes in (plasma) membrane properties indirectly affecting the cell wall as well. Indeed, several CWPs (i.e., Ecm33, Phr2, Pga10, Pir1, and Rbt5; see Table 2) are induced when the cells are treated with azoles, which are known to affect ergosterol synthesis. We conclude that the cell wall proteome of C. albicans sensitively reflects the environmental conditions and helps the cell to adjust to stress conditions encountered during the infection process. Our results also show that the composition of the cell wall proteome is tightly controlled, and that multiple signaling pathways are involved in the regulation of its composition.
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Acknowledgments

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References


Chapter 2


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