Mass spectrometric quantification of the adaptations in the wall proteome of Candida albicans in response to ambient pH

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The mucosal layers colonized by the pathogenic fungus Candida albicans differ widely in ambient pH. Because the properties and functions of wall proteins are probably pH dependent, we hypothesized that C. albicans adapts its wall proteome to the external pH. We developed an in vitro system that mimics colonization of mucosal surfaces by growing biomats at pH 7 and 4 on semi-solid agarose containing mucin as the sole nitrogen source. The biomats expanded radially for at least 8 days at a rate of ~30 μm h⁻¹. At pH 7, hyphal growth predominated and growth was invasive, whereas at pH 4 only yeast and pseudohyphal cells were present and growth was noninvasive. Both qualitative mass spectrometric analysis of the wall proteome by tandem mass spectrometry and relative quantification of individual wall proteins (pH 7/pH 4), using Fourier transform mass spectrometry (FT-MS) and a reference mixture of ¹⁵N-labelled yeast and hyphal walls, identified similar sets of ~20 covalently linked wall proteins. The adhesion proteins Als1 and Als3, Hyr1, the transglucosidase Phr1, the detoxification enzyme Sod5 and the mammalian transglutaminase substrate Hwp1 (immunological detection) were only present at pH 7, whereas at pH 4 the level of the transglucosidase Phr2 was ~35-fold higher than at pH 7. Sixteen out of the 22 proteins identified by FT-MS showed a greater than twofold change. These results demonstrate that ambient pH strongly affects the wall proteome of C. albicans, show that our quantitative approach can give detailed insights into the dynamics of the wall proteome, and point to potential vaccine targets.

INTRODUCTION

The pathogenic fungus Candida albicans preferentially colonizes mucosal surfaces of warm-blooded animals. Its wall has a bi-layered structure, with an internal layer consisting of load-bearing polysaccharides surrounded by a fibrillar protein coat. The external protein coat mainly consists of glycosylphosphatidylinositol (GPI)-modified glycoproteins, which are covalently linked to the polysaccharides of the skeletal layer and extend away from the cell surface in a perpendicular manner (reviewed by Klis et al., 2009). In addition, C. albicans walls contain some non-GPI proteins that can be released from the wall by mild alkali. This group of wall proteins includes Pir1, which is found in the inner wall layer and is believed to interconnect β-1,3-glucan chains (Ecker et al., 2006). The external protein coat plays an important role in the colonization of mucosal layers and, together with the proteins of the secretome, its proteins are in the front line of the continuous confrontation between the fungus and host defence mechanisms (Butler et al., 2009; Chaffin, 2008; Blankenship & Mitchell, 2006; Klis et al., 2009; Naglik et al., 2004; Sorgo et al., 2010). Coat proteins also directly interact with members of the bacterial communities found on mucosal surfaces, teeth and dental prostheses, and with the abiotic surfaces of indwelling medical devices. It is therefore not surprising that the external protein coat at any time consists of multiple protein species with a wide array of functions such as adhesion to epithelial or endothelial cells, covalent attachment to host proteins, biofilm formation, cell aggregation, recognition of host ligands and host proteins, detoxification of oxygen radicals, iron acquisition, masking the internal skeletal layer, and

Abbreviations: FT, Fourier transform; ; GPI, glycosylphosphatidylinositol.
A supplementary table is available with the online version of this paper.

Received 2 August 2010
Revised 16 September 2010
Accepted 18 September 2010
biofilm formation (Chaffin, 2008; Gantner et al., 2005; Klis et al., 2009; Martchenko et al., 2004; Nobile et al., 2006, 2008a; Otoo et al., 2008; Ramsook et al., 2010; Sosinska et al., 2008; Staab et al., 1999).

We have chosen to study the effect of pH on the wall proteome for several reasons. (1) pH 7 and pH 4 are representative of the pH in the oral cavity and of the vaginal pH, respectively, and the mucosal layers of both the host sites are regularly colonized and infected by *C. albicans* (Cannon & Chaffin, 1999; Sobel, 2007). (2) The conformation and activity of many proteins are strongly pH dependent, and as wall proteins have to function at the external pH, one may expect *C. albicans* to use different isoforms for important activities, depending on pH. The two transglucosidases Phr1 and Phr2 are a classical example of the use of isoforms (Fonzi, 1999; Mühlschlegel & Fonzi, 1997). Phr1 is used at pH 5.5 and higher whereas Phr2 is the preferred protein at more acidic pHs and, interestingly, is required for virulence in a vaginal infection model (De Bernardis et al., 1998). (3) The composition of the secretome of *C. albicans* is pH dependent (Sorgo et al., 2010), suggesting that the wall proteome might be equally responsive to variations in extracellular pH. (4) The relative gene expression under alkaline and acidic pH has been thoroughly studied, thus providing important clues of expected changes in the wall proteome (Bensen et al., 2004; Martchenko et al., 2004; Nobile et al., 2008b).

In this study, in an attempt to mimic in *vitro* the conditions encountered by *C. albicans* while colonizing the mucosal surfaces of the oral cavity and the vagina, we grew *C. albicans* as flat colonies (also designated biomats; Reynolds & Fink, 2001) on semi-solid surfaces, using mucin as the sole nitrogen source. In addition, we developed a mass spectrometric method for accurately monitoring the relative changes in wall protein levels. Quantification was performed using ESI-LC-MS/MS (electrospray ionization-liquid chromatography-tandem mass spectrometry) analysis.

**METHODS**

**Growth of biomats.** *Candida albicans* SC5314 was precultured overnight in liquid YPD medium (10 g yeast extract l⁻¹, 20 g peptone 1⁻¹, 20 g glucose 1⁻¹) at 30 °C and 200 r.p.m. to an OD₆₀₀ of ~20 (an OD of 1 corresponds to 1.5 × 10⁷ cells ml⁻¹). Biomats were grown on semi-solid medium containing, per litre, 6.7 g yeast nitrogen base (Difco) without amino acids and ammonium sulfate, 5 g porcine stomach mucin (Sigma-Aldrich) as the sole nitrogen source, 3 g agarose, and 5, 33, or 100 mM glucose. The medium was buffered with 75 mM phthalic acid/NaOH (pH 4) or 50 mM MOPS/NaOH (pH 7). To grow biomats, precultured cells were harvested and washed with soluble biomat medium having the same pH and glucose concentration as used for the agarose plates, but without mucin. Two microliters of washed cells (~6 × 10⁶ cells) was spotted on the agarose surface. All components were sterilized separately at 110 °C for 20 min and the medium was poured into Petri dishes 1 day prior to inoculation. The Petri dishes were covered with Parafilm to prevent drying out and incubated at 37 °C. Radial extension rates were determined by measuring the biomat radius at 24 h intervals and presented as average values (mean ± s; n=10).

**CSLM imaging.** Three-day-old biomats were stained for 1 h at 30 °C in the dark with 1 ml tetramethylrhodamine conjugate of concanavalin A (C-860; Molecular Probes) diluted in PBS buffer at a concentration of 200 µg ml⁻¹. Stained biomats were transferred to a chambered borosilicate cover-glass system (155380; Lab-Tek). Confocal scanning laser microscopy (CSLM) images were prepared using a HeNe laser (543 nm; 560 nm long-pass emission filter) and a Zeiss LSM 510 microscope with a 20 × 0.75 long-distance water objective. Image analysis was carried out using the Zeiss LSM510 software.

**Harvesting of biomats and wall isolation.** Biomats were harvested after 3 days of growth. Biomats grown at pH 4 were washed off the plates with cold, sterile, demineralized water, collected by centrifugation, and stored at −20 °C. Because biomats cultured at pH 7 grow invasively, they were cut out of the plates together with the agarose below them and stored at −20 °C. To remove agarose and mucin and to facilitate breakage of the filamentous cells, pH 7-grown cells were boiled four times for 10 min in 1% SDS. Walls were isolated as described previously (de Groot et al., 2004). The cells were washed with cold demineralized water and with 10 mM Tris/HCl pH 7.5, and disrupted in a Bio-Savant Fast Prep 120 machine (Qbiogene), using 0.25–0.50 mm diameter glass beads (Emergo) in the presence of a protease inhibitor mixture (Sigma-Aldrich). Non-covalently linked proteins associated with the wall preparation were removed by washing crude walls with 1 M NaCl and incubating them twice for 5 min at 100 °C in a mixture containing 150 mM NaCl, 2% (w/v) SDS, 100 mM Na EDTA, 100 mM β-mercaptoethanol, 50 mM Tris/HCl pH 7.8. SDS-extracted walls were washed three times with demineralized water, freeze-dried, and stored at −20 °C. The polypeptide content of isolated walls was determined as described before (de Groot et al., 2004).

**ESI-LC-MS/MS (electrospray ionization-liquid chromatography-tandem mass spectrometry) analysis.** For each analysis, 4 mg (gravimetrically determined) of biomat walls was subjected to reduction, S-alkylation and trypsin digestion (Yin et al., 2005). Tryptic peptides were fractionated using a nano-LC system (PepMap C18; LC. Packings, Dionex) and ionized by electrospray in a Micromass quadrupole time-of-flight mass spectrometer (Q-TOF MS) (Waters). Each biological sample was run twice with an MS/MS selection time of 1.5 s. To exclude overflow between samples, each sample run was followed by two blank runs. Masslynx software was used to select ions from the survey spectrum for fragmentation in a collision chamber. The ion spectra generated were analysed using Biolynx and Masslynx Pepseq software. Proteins were identified using Mascot software by comparing the amino acid sequences of ions with *in silico* digests of the proteins translated from Assembly 21 of the *C. albicans* SC5314 genome sequence (Skrzypek et al., 2010). Mascot search parameters were as follows: one missed cleavage, fixed modification of carbamidomethylcysteine, and an error tolerance of 0.3 Da for peptides and MS/MS were allowed. The search was repeated with the same parameters but with semitryptsin as the enzyme. Probabilistic Mascot scoring was used to evaluate the data.
identified peptides and an individual peptide score of at least 30 \((P \leq 0.05)\) was considered significant for peptide identification. All peptides with a score close to 30 had their raw MS/MS data manually checked before inclusion in the overview list.

**15N-labelled reference cultures.** To obtain a wide representation of covalently linked 15N-labelled wall proteins, liquid cultures were metabolically labelled with [15N]ammonium sulfate under conditions promoting yeast (pH 4) and hyphal (pH 7) growth, respectively. SC5314 cells were precultured overnight at 30 °C and 200 r.p.m. in synthetic medium containing 20 g L\(^{-1}\) glucose and 1.7 g L\(^{-1}\) yeast nitrogen base (Difco) without amino acids and ammonium sulfate, and supplemented with 5 g L\(^{-1}\) 15N-labelled ammonium sulfate (Spectra Stable Isotopes; cat. no. 5301; 15N content >99%). Precultured cells were then inoculated in the same medium buffered with 75 mM phthalic acid/NaOH at pH 4 or 50 mM MOPS at pH 7. The pH 4 reference cultures were grown at 30 °C from OD\(_{600}\) 0.1 to early exponential phase (OD\(_{600}\) ~1). Cells isolated from the pH 4 reference cultures were directly stored at -20 °C. The pH 7 reference cultures were initiated at an OD\(_{600}\) of 0.1 and grown for 7 h at 37 °C, resulting in extensive formation of hyphae. To facilitate breakage of cells grown at pH 7, they were washed with demineralized water and boiled four times for 10 min in 1% (w/v) SDS prior to the breaking step. To minimize the effect of biological variation, for both pHs three independent biological replicates were combined.

**Sample preparation for quantitative Fourier transform (FT) data analysis.** Four milligrams (gravimetrically determined) of 14N-walls obtained from 30 biomats grown at pH 4 or pH 7 (query walls) were combined with 4 mg of 15N-walls (reference walls), i.e. 2 mg walls isolated from three independent pH 4 reference cultures and 2 mg walls from three independent pH 7 reference cultures, and subjected to reduction and alkylation followed by digestion with trypsin (Yin et al., 2005) (Fig. 1). The digest contained ~1.2 μg peptides μL\(^{-1}\) as measured by the bicinchoninic acid protein assay (Pierce). The peptides were desalted and concentrated using a Hilic tip (Top Tip Hydrophilic TTI1HIL96).

**MS data processing and analysis.** Accurate mass data were acquired using an ApexQ Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonic) equipped with a 7 T magnet and a CombiSource coupled to an Ultimate 2000 ( Dionex) HPLC system with a 100 μm i.d., 150 mm-long monolithic reverse-phase column (Omny C18; Phenomenex). Samples containing up to 2 μg of trypptic peptides were injected as a 3 μl 0.1% trifluoroacetic acid aqueous solution and directly loaded onto the analytical column. Following injection, a linear gradient (from 0.1% formic acid/100% H\(_2\)O to 0.1% formic acid/40% CH\(_3\)CN/60% H\(_2\)O) was applied over a period of 120 min at a flow rate of 2 μl min\(^{-1}\). During elution a chromatogram of up to 1850 high-resolution ESI-FT-MS spectra was recorded using an MS duty cycle of about 3 s.

The data were processed using the Data Analysis 3.4 software program (Bruker Daltonic). The 1850 mass spectra were batch-wise extracted from the chromatogram and the monoisotopic masses of the peptides were determined using Bruker’s SNAP II peak recognition technology. Mass calibration was achieved by selective extraction and subsequent summation of about six mass spectra from the chromatogram corresponding to trypptic peptides originating from autodigested trypsin. With the theoretical masses of these calibrate peptides the summed spectrum was mass calibrated and the resulting calibration parameters were applied to all spectra in the chromatogram. This resulted in a mass calibration of better than 1.5 p.p.m. over the entire chromatogram for all analyses. For each FT-MS analysis the resulting array of up to 1850 monoisotopic mass lists was exported as a Mascot generic file (mgf). Ion abundances in the exported array of monoisotopic mass lists were the sum of abundances of all isotopes over all charge states for each peptide.

Exported mgf files were imported in the in-house developed CoooolToolBox software program (Müller et al., 2009). From the imported array of up to 1850 monoisotopic mass spectra the CoooolToolBox program constructed up to 1000 peptide ion chromatograms. For each peptide ion chromatogram the mass and retention time were determined at the apex of the chromatogram profile and the abundance was summed over the ion chromatogram profile. The final LC-MS data processing resulted in a peptide monoisotopic mass list with corresponding abundance and LC retention time. Peptide assignments of the ion masses were obtained by matching the processed LC-FT-MS data from the trypptic peptides within a mass window of 1.5 p.p.m. with the masses of trypptic peptides from corresponding 15N- and 13N-labelled C. albicans proteins, obtained from an in silico digestion of a short list consisting of 140 putative wall proteins, thus diminishing the risk of false positives. The peptide elution profiles were shorter than 30 s. As expected, the retention behaviour on the monolithic column of the 14N- and 13N-peptides was alike, with the 15N-peptides consistently eluting only a few seconds before the corresponding 14N-peptides. Using this criterion together with the accurate masses of both 14N- and 15N-peptides, the CoooolToolBox program automatically picked out all 1850/1850-peptide pairs using a peptide pair retention time window of only 10 s over the total gradient of 120 min. This resulted in unique series of unambiguously assigned trypptic peptide pairs for all identified wall proteins. The within-sample variability based on the ratios for different peptides from the same protein, expressed as CV% (coefficient of variation), had an average value of ~5% and ranged from about 2 to 11% (see Tables 2 and 3). This method, which is now routinely used in our lab, shows minor variations between technical replicates, whereas the between-sample variability has a CV% of ~40%. Because processing enough biomats to obtain three separate biological samples at both pHs for FT-MS analysis would have required a disproportionate amount of effort, we decided to combine

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**Fig. 1.** Experimental strategy for the relative quantification of query wall proteomes by FT-MS versus a mixed 14N-labelled reference wall proteome. Reference 15N-labelled walls were prepared by mixing equal amounts of walls (gravimetrically determined) isolated from a liquid yeast culture grown at pH 4 and a hyphal culture grown at pH 7 to obtain a wide representation of yeast- and hyphal-specific wall proteins. Reference walls were mixed in a 1 : 1 ratio on the basis of dry weight with pH 7- or pH 4-biomat walls. These samples were processed in parallel for subsequent FT-MS analysis.

**Table 1.** Comparison of relative quantification by FT-MS versus a mixed 15N-labelled reference cultures for selected wall proteins.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Relative quantification</th>
<th>FT-MS</th>
<th>Mixed reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide A</td>
<td>1.23</td>
<td>0.98</td>
<td>0.94</td>
</tr>
<tr>
<td>Peptide B</td>
<td>0.87</td>
<td>0.93</td>
<td>0.91</td>
</tr>
<tr>
<td>Peptide C</td>
<td>1.04</td>
<td>1.09</td>
<td>1.06</td>
</tr>
</tbody>
</table>

**Table 2.** Relative quantification by FT-MS versus a mixed 15N-labelled reference cultures for selected wall proteins.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Relative quantification</th>
<th>FT-MS</th>
<th>Mixed reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide A</td>
<td>1.15</td>
<td>1.07</td>
<td>1.03</td>
</tr>
<tr>
<td>Peptide B</td>
<td>0.98</td>
<td>0.94</td>
<td>0.92</td>
</tr>
<tr>
<td>Peptide C</td>
<td>1.02</td>
<td>1.07</td>
<td>1.04</td>
</tr>
</tbody>
</table>

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**Table 3.** Relative quantification by FT-MS versus a mixed 15N-labelled reference cultures for selected wall proteins.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Relative quantification</th>
<th>FT-MS</th>
<th>Mixed reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide A</td>
<td>1.09</td>
<td>1.03</td>
<td>1.01</td>
</tr>
<tr>
<td>Peptide B</td>
<td>0.95</td>
<td>0.92</td>
<td>0.90</td>
</tr>
<tr>
<td>Peptide C</td>
<td>1.03</td>
<td>1.06</td>
<td>1.04</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** Experimental strategy for the relative quantification of query wall proteomes by FT-MS versus a mixed 14N-labelled reference wall proteome. Reference 15N-labelled walls were prepared by mixing equal amounts of walls (gravimetrically determined) isolated from a liquid yeast culture grown at pH 4 and a hyphal culture grown at pH 7 to obtain a wide representation of yeast- and hyphal-specific wall proteins. Reference walls were mixed in a 1 : 1 ratio on the basis of dry weight with pH 7- or pH 4-biomat walls. These samples were processed in parallel for subsequent FT-MS analysis.
Table 1. Wall proteins from C. albicans biomats grown at pH 4 and pH 7 and identified by ESI-LC-MS/MS or immunologically

The MS/MS data from the proteins identified are presented in Supplementary Table S1.

<table>
<thead>
<tr>
<th></th>
<th>pH 4</th>
<th>pH 4 and pH 7</th>
<th>pH 7</th>
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</thead>
<tbody>
<tr>
<td>Phr2</td>
<td>Als4</td>
<td>Als1</td>
<td></td>
</tr>
<tr>
<td>Chl2</td>
<td>Als3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crh11</td>
<td>Hwp1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecm33</td>
<td>Hyr1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lhd1/Pga36</td>
<td>Pfr1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mp65/Scw1</td>
<td>Rbt1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pga4</td>
<td>Sod5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pir1</td>
<td>Tor1</td>
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<td>Rbt5</td>
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<td>Rhd3/Pga29</td>
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<td>Sod4</td>
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<tr>
<td>Sr1</td>
<td></td>
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<tr>
<td>Utr2</td>
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<tr>
<td>Ywp1/Pga24</td>
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</table>

*Immunological identification.

Table 1 shows the wall proteins found at pH 4, pH 4 and pH 7, and pH 7. The proteins are listed under their corresponding pH values.

RESULTS

Biomat culturing and biomat properties

When reconstituted human oral or vaginal epithelial layers are infected with C. albicans, a thick fungal layer appears above the epithelial cells (Cheng et al., 2005; Green et al., 2004). We attempted to mimic colonization of mucosal surfaces in vitro by culturing biomats on a semi-solid surface (Reynolds & Fink, 2001). Yeast cells were inoculated on a semi-solid growth medium containing 0.3% agarose at 37 °C. The growth medium for biomat culture consisted of 0.17% YNB without amino acids and ammonium sulfate. The medium was supplemented with 5 mM glucose as the carbon source and with 0.5% mucin as the sole nitrogen source. Based on a polypeptide content of 17% (Shepherd & Sullivan, 1984), a mucin concentration of 0.5% is estimated to correspond to ~10 mM nitrogen.

An important difference between pH 4- and pH 7-grown biomats was that biomats grown at pH 4 were friable and could be easily washed off whereas pH 7-grown biomats were strongly cohesive and resistant to washing, indicating invasive growth (Fig. 2). CSLM analysis showed that biomats growing on 5 mM glucose at pH 7 consisted largely of interwoven filaments, forming an approximately 20 μm thick structure, which grew above and below the surface of the agarose (Fig. 2 and data not shown). In contrast, biomats grown at pH 4 consisted exclusively of yeast and pseudohyphal cells including the few cells that remained behind in the upper agarase layer after gentle washing.

Biomats cultured on 5 mM glucose at pH 4 expanded with an average radial growth rate of 27±4 μm h⁻¹ over a period of 8 days (Fig. 2). As the length of an unbudded cell is ~6 μm (Kocková-Kratochvílová, 1990) and the generation time of exponentially growing C. albicans is >1 h, this strongly suggests that the observed radial growth rate is largely due to the expansive forces of the growing yeast cell population that push peripheral cells outwards over the agarose surface, especially because the agarose is semi-solid, which reduces friction. This form of colony spreading has also been observed in bacterial mats growing on low-agar plates and has been termed "sliding motility" (Henrichsen, 1972). Possibly, the presence of slimy extracellular matrix material as observed in yeast colonies (Joshi et al., 1973) reduces the friction between the cells and the agarose surface. We also tested higher glucose concentrations (33 and 100 mM) at pH 4. Although the radial growth rate of the biomats did not increase, their biomass increased considerably. Whereas the average biomass (dry weight) of a single biomat grown in the presence of 5 mM glucose after 8 days of growth was <1 mg, this increased to 8.9 mg

Immunoblot analysis. GPI-linked wall proteins were released from isolated walls using recombinant endo-β-1,6-glucanase (Kapteyn et al., 2000). Pir1 was released by mild alkali extraction (Mśra et al., 1997). Wall proteins (obtained from 150 μg or 10 μg of dried isolated walls as indicated) were separated by gel electrophoresis using 3–8% polyacrylamide gradient gels in Tris/acetate (Invitrogen) and transferred onto PVDF membranes (Millipore). Prior to the blocking step, immobilized proteins were treated with 50 mM periodic acid in 100 mM sodium acetate (pH 4.5) for 30 min at room temperature to increase the specificity of their reaction with antisera. Immunoblot analysis was carried out using polyclonal antisera directed against Hwp1 (Staab et al., 1996), Pga10 (Weissman & Kornitzer, 2004) or Pir1 (Russo et al., 1992) as described by Sosinska et al. (2008).
Table 2. Increased abundance at pH 7 vs pH 4 of covalently linked wall proteins in *C. albicans* biomats as determined by FT-MS analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tryptic peptides</th>
<th>Position</th>
<th>$^{14}$N/$^{15}$N</th>
<th>Fold-change† pH 7/pH 4</th>
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</thead>
<tbody>
<tr>
<td>Als1</td>
<td>YTTTSQTSVDLTADGVK</td>
<td>77–92</td>
<td>0.8 $^{15}$N</td>
<td>&gt;20</td>
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<tr>
<td></td>
<td>STVDPSGLYASR</td>
<td>176–188</td>
<td>1.0 $^{15}$N</td>
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<tr>
<td></td>
<td>GLNDWNYPVSSESFSYTK†</td>
<td>237–254</td>
<td>5.1† $^{15}$N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SQSKPFTRL</td>
<td>303–311</td>
<td>$^{14}$N $^{15}$N</td>
<td></td>
</tr>
<tr>
<td>Als3</td>
<td>FTTSQTSVDLTAHGV</td>
<td>77–92</td>
<td>3.5 $^{15}$N</td>
<td>&gt;20</td>
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<tr>
<td></td>
<td>ALGTVTPLAFNVTGGTSSVDLEDSDK</td>
<td>124–149</td>
<td>&gt;3 $^{15}$N</td>
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<td>CFTAGTNTVTNFDGGKK</td>
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<td>VIPSLLNK</td>
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<td>3.7 $^{15}$N</td>
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<td>TCSSNIGFITYK</td>
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<tr>
<td>Hyr1</td>
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<td>4.3</td>
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<td>&gt;3 $^{15}$N</td>
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<td>4.3 $^{15}$N</td>
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<td>265–277</td>
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<td>103–117</td>
<td>10.2 $^{15}$N</td>
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<tr>
<td></td>
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<td>103–140</td>
<td>$^{14}$N</td>
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<td>EYDDSYISLNK</td>
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<td>HGNIMGESYK</td>
<td>118–127</td>
<td>$^{14}$N</td>
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</tr>
<tr>
<td></td>
<td>HGNIMGESYKT                      TEYDDYSISLNK</td>
<td>118–140</td>
<td>10.3 $^{15}$N</td>
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<td></td>
<td>TEYDDYSISLNK</td>
<td>128–140</td>
<td>$^{14}$N</td>
<td></td>
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<td>19–34</td>
<td>8.2</td>
<td>14</td>
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<td></td>
<td>35–52</td>
<td>8.1</td>
<td>0.6</td>
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<tr>
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<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47–60</td>
<td>2.5 $^{0.24}$</td>
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<td></td>
<td></td>
<td>47–56</td>
<td>2.5 $^{0.25}$</td>
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<tr>
<td>Crh11</td>
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<td>29–45</td>
<td>&gt;2 $^{1.5}$</td>
<td>1.8</td>
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<td>170–182</td>
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<td>236–246</td>
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<td></td>
<td>QYSYSDQSGSWSIEK</td>
<td>247–261</td>
<td>2.6 $^{1.4}$</td>
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<tr>
<td>Sod4</td>
<td>TPAALELDLGSR</td>
<td>105–117</td>
<td>4.9</td>
<td>3.2</td>
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*The query/reference ratios ($^{14}$N/$^{15}$N) of individual tryptic peptides were determined by FT-MS analysis of $^{14}$N-query peptides, from biomats grown at the query pH, combined with $^{15}$N-labelled reference peptides. When only the $^{14}$N- or $^{15}$N-peptide was identified, the result is given as $^{14}$N or $^{15}$N.

†When no $^{15}$N-protein was detected at pH 4, an arbitrary value for the pH 7/pH 4 fold-change of >20 was assigned.

§Non-unique, present in Als1 and Als3.

$Non-unique, present in Phr1 and Phr2.

||Non-unique, present in Rbt5 and Pga10. However, Pga10 could not be identified immunologically (Fig. 3).
in the presence of 100 mM glucose. The strongly increased growth in the presence of 100 mM glucose indicates that the mucin concentration in our assay did not limit growth in the presence of 5 mM glucose. At pH 7, biomats seem to expand slightly faster (taking into account that for unknown reasons at pH 7 the initial spot size on day 1 tended to be smaller than at pH 4) with an average radial growth rate of $36 \pm 5 \text{ mm h}^{-1}$, which is similar to the values observed when *C. albicans* grows on serum agar at 37 °C (Gow & Gooday, 1982).

**Composition of the wall proteome of biomats**

We preferred tryptic digestion of isolated, hot detergent-extracted walls (cell wall shaving; Yin *et al.*, 2008) to tryptic digestion of intact cells (cell shaving; Hernández *et al.*, 2010), because the first method results in a much more complete picture of the covalently linked wall proteins. To analyse the changes in the composition of the wall proteome we first used ESI-LC-MS/MS analysis of the tryptic peptides derived from the covalently bound wall proteins of biomat cells grown at neutral and acidic pH. Twenty-two wall proteins were identified in this way (Table 1; for full data see Supplementary Table S1, available with the online version of this paper). Except for Mp65, Pir1 and Tos1, all of them are GPI-modified wall proteins. Table 1 further suggests that biomat cells use different covalently linked wall proteins at the two pHs. As expected, the transglycosidase Phr2 was only found in pH 4-grown cells, whereas its isoform Phr1 was only observed at pH 7 (Mühlischlegel & Fonzi, 1997). In addition, under the growth conditions used here several wall proteins seem to be pH 7-specific, including the GPI-modified wall protein Hwp1 as determined by immunological analysis (Fig. 3; see below). This shows clearly that *C. albicans* adapts the composition of its wall proteome in response to a change in ambient pH.

**Relative quantification of wall proteins by LC-FT-MS and immunoblot analysis**

As the majority of the wall proteins were found at both pHs, we developed a quantitative mass spectrometric approach to determine if their relative abundance had changed. For this we used a mixture of $^{15}$N-labelled reference walls that were derived from metabolically labelled hyphal and yeast cultures grown in the presence of $[^{15}$N]ammonium sulfate. The $^{15}$N-labelled reference walls were combined in equal weight proportion to ensure the presence of both yeast and hyphal wall proteins. After determining the protein abundance ratios for both pHs with respect to the reference walls ($[^{14}$N/$^{15}$N]), the two ratios were divided by each other to obtain the ($[^{14}$N$_{\text{pH 7}}$])/$[^{14}$N$_{\text{pH 4}}$] ratio or vice versa for each protein (Tables 2 and 3). Using this approach, Als1, Als3, Hyr1, Phr1 and Sod5 were identified only at pH 7, consistent with the notion that the corresponding genes are hypha-coregulated, whereas Phr2 was highly enriched at pH 4. Tables 2 and 3 further show that the abundances of several proteins in the group present at both pHs were strongly affected by the ambient pH. For example, at pH 7 much more Ihd1 and Rbt5 were incorporated in the walls than at pH 4, whereas Als4, Ecm33, Pir1 and Rhd3 were much more abundant in pH 4 walls.

Immunological analysis of two wall proteins (Pir1 and Rbt5) confirmed that Pir1 is more abundant at pH 4, whereas Rbt5 is much more abundant at pH 7 (Fig. 3). We have no explanation for the observation that immunological analysis of Rbt5 did not detect Rbt5 in walls from pH 4-grown biomats, whereas mass spectrometric analysis...
did detect some Rbt5 (10% of the level observed in walls from pH 7-grown biomats). Note that Western analysis of Rbt5 was carried out with a cross-reactive antiserum, which also reacts with Pga10. To confirm that the protein detected at pH 7 was Rbt5, \( rbt5^{-/-} \) and \( pga10^{-/-} \) mutant strains were also analysed. This showed that only Rbt5 was detected at pH 7 (data not shown). Hwp1 was identified only by immunological means as no tryptic peptides for this protein were detected by mass spectrometry. Under our growth conditions, Hwp1 was only found at neutral pH.

### Table 3. Increased abundance at pH 4 vs pH 7 of covalently linked wall proteins in C. albicans biomats as determined by FT-MS analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tryptic peptides</th>
<th>Position</th>
<th>( ^14\text{N}/^{15}\text{N}^* )</th>
<th>Fold-change pH 4/pH 7</th>
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</thead>
<tbody>
<tr>
<td>Cht2</td>
<td>TCQSLGK</td>
<td>92–98</td>
<td>1.5 1.2</td>
<td>1.1</td>
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<td>FADTLWNK</td>
<td>122–129</td>
<td>1.5 1.3</td>
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<tr>
<td></td>
<td>NYFLSAAPCPYPDASLGDLLSK</td>
<td>178–200</td>
<td>1.8 1.8</td>
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</tr>
<tr>
<td></td>
<td>LFVGPATSNHGYVDTSK</td>
<td>240–258</td>
<td>1.7 1.5</td>
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</tr>
<tr>
<td></td>
<td>LSSAIEEIK</td>
<td>259–267</td>
<td>1.6 1.4</td>
<td></td>
</tr>
<tr>
<td>Ssr1</td>
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<td>41–54</td>
<td>2.3 1.8</td>
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</tr>
<tr>
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<td>EICPNGDADTAISAFK</td>
<td>64–79</td>
<td>2.5 14N</td>
<td></td>
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<tr>
<td>Tos1</td>
<td>SGEELYIFSGSK</td>
<td>288–299</td>
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<td>1.4</td>
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<td>TLQYGEATSCWGK</td>
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<td>1.1 0.72</td>
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<tr>
<td>Mp65</td>
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<td>SESQASEIAQLSFGDQVR</td>
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<td>1.8 0.72</td>
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<td>LYGVDCDQVSAVLK</td>
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<td>SNQQAASSIK</td>
<td>334–344</td>
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<td>ADGPYNAEKYGWIYSN†</td>
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<tr>
<td>Ywp1</td>
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<td>329–339</td>
<td>3.9 1.4</td>
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<tr>
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<tr>
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<td>7.1 2.3</td>
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<tr>
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<td>GLAEPHTGHFDAYVQGNCNAK</td>
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<td>4.3</td>
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<tr>
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<td>LSVIEFVNC†</td>
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<td>VSGGFLK</td>
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<td>ASGYESATNDYK</td>
<td>239–250</td>
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<td>NLGPIFFSEYGCNEVRPR</td>
<td>251–269</td>
<td>7.2 0.19</td>
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*The query/reference ratios \( ^{14}\text{N}/^{15}\text{N} \) of individual tryptic peptides were determined by FT-MS analysis of \( ^{14}\text{N}-\text{query} \) peptides, from biomats grown at the query pH, combined with \( ^{15}\text{N}-\text{labelled} \) reference peptides. When only the \( ^{15}\text{N}-\text{peptide} \) was identified, the result is given as \( ^{14}\text{N} \).

†C-terminal peptide.

‡Non-unique, present in Phr1 and Phr2.
DISCUSSION

Comparative analysis of the wall proteomes of pH 7- and pH 4-grown biomats

Colonization of the various mucosal surfaces in the human body requires adaptation of *C. albicans* to a broad range of pHs. We have compared the wall proteomes of *C. albicans* grown as biomats at 37 °C and at pH 7 or 4 on a semi-solid surface. As *C. albicans* cells can utilize proteins such as mucin and BSA as sole nitrogen source by secreting various aspartic proteases (Colina *et al.*, 1996; Hube *et al.*, 1994), mucin was chosen as the sole nitrogen source. In addition, we used a low glucose concentration of 5 mM. We selected these conditions because they reflect the conditions encountered by the fungus during colonization of the oral cavity and the vagina. Biomats grown at neutral and acidic pH have different properties. At neutral pH, hyphae developed that grew invasively and formed strong, cohesive biomats. We assume that under our culture conditions hyphal growth is due to the combined effect of neutral pH and the presence of mucin, because this protein, probably due the presence of N-acetylglucosamine residues in its carbohydrate side-chains, stimulates the formation of germ tubes (Shepherd & Sullivan, 1984). At pH 4, yeast and pseudohyphal cells were formed, which were easily washed off from the agarose surface, suggesting that low pH offers some protection against fungal infection by inhibiting invasive growth. CSLM did not reveal substantial amounts of extracellular matrix material in pH 7-grown biomats, indicating that biomats differ from biofilms in this respect.

Two different mass spectrometric methods were used for analysis of the wall proteome. ESI-LC-MS/MS allows sequence determination of the tryptic peptides and is primarily used for identification purposes. This approach identified 85 tryptic peptides corresponding to 22 wall proteins (Supplementary Table S1). LC-FT-MS in combination with stable isotope labelling can be employed for identification and quantification of tryptic peptides (Aebersold & Mann, 2003; Oda *et al.*, 1999; see also the Results section). By this method, 80 unique peptides were identified corresponding to 21 wall proteins, or on average almost four peptides per protein. These data yielded the pH/reference ratios of the individual wall proteins at the selected pHs and, after dividing out the reference, the pH 7/pH 4 ratios were obtained. Using 15N-labelled reference wall proteins allows the comparison of multiple query cultures with each other, not only in this work but also in future experiments. We expect that construction and future extension of a database containing quantitative estimates of wall proteins will be useful for exploring the regulation of this relatively simple subproteome, especially under infection-associated stress conditions such as iron depletion, nitrosative and oxidative stress, poor carbon sources, carbon and nitrogen deprivation, and the presence of antifungal drugs.

The 21 wall proteins identified by LC-FT-MS were also detected by ESI-LC-MS/MS, confirming that the two methods identified an almost identical set of proteins. Rbt1 was the only protein missed by LC-FT-MS; however, this protein is also often missed by ESI-LC-MS/MS (unpublished data) and in this study we detected only a single Rbt1 peptide (Supplementary Table S1), suggesting that it is a relatively rare wall protein. Both methods identified Als1, Als3, Hyr1, Phr1 and Sod5 as pH 7- or hypha-coexpressed wall proteins and Phr2 as preferentially or uniquely expressed at pH 4. In addition, by immunological means the GPI-modified wall protein Hwp1 was observed only at pH 7. Because covalently linked wall proteins are generally heavily glycosylated and numerous glycoforms of individual proteins can be present (Yin *et al.*, 2008), some wall proteins, such as Hwp1, might be missed by standard mass spectrometric means. Indeed, complete deglycosylation using trifluoromethanesulfonic acid has allowed mass spectrometric identification of three
additional proteins (CsA1, Hwp1 and Pga62) (Castillo et al., 2008; Maddi et al., 2009). Nevertheless, the results suggest that most wall proteins can be detected without prior deglycosylation.

**pH regulation of the wall proteome**

As our experiments were all carried out at 37 °C with the same growth medium, but at two different pHs, the pH difference is probably the major contributor to the compositional changes observed in the wall proteome. Rim101 is a major transcriptional regulator of pH-dependent gene expression including the expression of wall-protein-encoding genes (reviewed by Davis, 2009). Indeed, transcriptional studies show that the expression of at least 17 wall-protein-encoding genes (ALS1, ALS3, ALS4, ALS5, CHT2, CRH11, HWP1, HYR1, IHD1, PGA4, PFR1, PFR2, RBT1, RBT5, SIM1, SOD5 and YWP1) is Rim101 dependent (Bensen et al., 2004; Lotz et al., 2004; Martchenko et al., 2004; Nobile et al., 2008b). This is consistent with our results (Tables 2 and 3, Fig. 3), which show that the protein levels of eight of them (Als1, Als3, Hwp1, Hyr1, Ihd1, Pfr1, Rbt5 and Sod5) are increased at least 10-fold at pH 7 compared to pH 4, whereas the protein levels of Als4 and Pfr2 are ≥20-fold higher at pH 4. At neutral pH the solubility of ferric ions is much lower than at pH 4; this could explain why the levels of Als3 and Rbt5, which are both involved in iron acquisition (Almeida et al., 2008; Weissman & Kornitzer, 2004), are strongly increased. Interestingly, Als1 and Als3 on the one hand and Als4 on the other hand behave in an opposite way with respect to pH, similar to Pfr1 and Pfr2, raising the question whether they may also be isoforms with a comparable function but optimally active at a different pH range. Summarizing, our results clearly show that ambient pH strongly affects the wall proteome and indicate that Rim101 is a major regulator of the changes observed.

The protein levels of Pir1, Ecm33 and Rhd3 are considerably higher (at least fourfold) at pH 4 than at pH 7. As the expression of the corresponding genes is not known to be under the control of Rim101, one may surmise that their expression is not so much controlled by the external pH, but is related to morphotype, in this case yeast and pseudohyphal growth forms. For example, it has been shown that the expression of Ecm33 and Rhd3 is strongly decreased upon serum and temperature induction (Kadosh & Johnson, 2005). Similarly, transcript levels of PIR1 and YWP1, which show an approximately three- to fourfold higher protein level at pH 4 compared to pH 7 (Table 3), rapidly decline upon transfer of YPD-grown yeast cells to hyphal-inducing conditions (Kadosh & Johnson, 2005; Sohn et al., 2003). Alternatively, as Pir1 has been postulated to cross-link β-1,3-glucan chains, this could imply that growth at low pHs involves cell wall stress leading to activation of a cell wall strengthening response. In this respect, it is noteworthy that in the absence of the genes encoding Ecm33 or Rhd3, the wall levels of which are almost fivefold higher in pH 4-grown biomats compared to pH 7-grown biomats, serious cell wall defects have been observed (de Boer et al., 2010; Martinez-Lopez et al., 2006).

**Correlation between transcript levels and protein levels**

The correlation between transcript levels and protein levels is often limited (Greenbaum et al., 2003). For example, because the turnover of wall proteins is slow (Ruiz-Herrera et al., 2002; Sorgo et al., 2010), this could obscure the correlation between transcript levels and protein levels. This raises the question how transcript levels of wall protein-encoding genes and wall protein abundances are related. As already discussed in the previous section, many wall protein levels seem to show similar trends as the corresponding transcript levels in response to ambient pH. Indeed, when the log2-transformed protein ratios obtained in this study [pH 7/pH 4: Pfr1 (6.889), Als1 (4.170), Ihd1 (3.764), Hyr1 (6.404), Tos1 (−0.457), Cht2 (−0.170), Sod4 (0.615), Crh11 (0.836); to allow calculation of a pH 7/pH 4 ratio for wall proteins not detected at pH 4, these were given an arbitrary pH 4/reference ratio of 0.05 corresponding to −4.322 after log2-transformation] were plotted against the available corresponding log2-transformed transcript levels as determined by Bensen et al. (2004) [pH 8/pH 4: Pfr1 (5.898), Als1 (4.446), Ihd1 (4.211), Hyr1 (3.499), Tos1 (1.747), Cht2 (1.667), Sod4 (1.042), Crh11 (1.018)], a reasonable value for the square of the correlation coefficient ($R^2=0.77$) was obtained. This suggests that transcript analyses can give relevant clues about how wall protein levels will behave in response to environmental changes in pH. It is furthermore tempting to extrapolate this relationship to other environmental challenges and to other fungal wall proteomes (Klis et al., 2010). Nevertheless, although transcript levels can predict trends, actual protein levels are required for a more quantitative analysis of cellular behaviour.

**Vaccine development**

Several covalently linked GPI-modified wall proteins (Als1, Als3, Hwp1, Hyr1) of *C. albicans* have been found to offer protection against systemic *Candida* infection in mice when tested as a vaccine candidate (Ibrahim et al., 2006; Luo et al., 2010; Spellberg et al., 2006; Xin et al., 2008). In addition, the non-GPI protein Mp65 has been shown to cause a cell-mediated host immune response (La Valle et al., 2000). Our results provide a solid base for these and other targets. Interestingly, Xin and co-workers have developed a fully synthetic vaccine consisting of β-1,2-mannotriose, an immunogenic structure found in the N-linked carbohydrate side-chains of *C. albicans*, tethered to an immunogenic peptide. Peptides from various proteins including the GPI-modified wall protein Hwp1 were...
successfully tested (Xin et al., 2008). Other wall proteins from which immunogenic peptides could be selected are Als1, Als3, Als4, Ecm33, Hyr1, Ihd1, Pfr1, Pfr2, Pir1, Rbt5, Rhd3 and Sod5 (Tables 2 and 3). In addition, a combinatorial vaccine consisting of various mannotriose-peptide conjugates could be conceived. Finally, absolute quantification of wall proteins in combination with determining their contribution to virulence in systemic infection models or phagocyte resistance assays will further help to select suitable candidates for such a combinatorial vaccine.

**ACKNOWLEDGEMENTS**

We cordially thank Drs D. Kornitzer, M. Makarow and P. Sundstrom for the CaPga10, ScPir2 and CaHwp1 antiserum, respectively. F. M. K. acknowledges the financial support by the EU Programs GALAR FUNGAIL II (MRTN-CT-2003-504148) and FINSysB (FP7-214004-2).

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Edited by: J. F. Ernst