Infection related stress adaptations in the secretome and wall proteome of Candida albicans

Sorgo, A.G.

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Chapter 3

The effects of fluconazole on the secretome, the wall proteome and wall integrity of the clinical fungus *Candida albicans*

This chapter has been published

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Fluconazole is a commonly used antifungal drug that inhibits Erg11, a protein responsible for 14α-demethylation during ergosterol synthesis. Consequently, ergosterol is depleted from cellular membranes and replaced by toxic 14α-methylated sterols, which causes increased membrane fluidity and drug permeability. Surface-grown and planktonic cultures of Candida albicans responded similarly to fluconazole at 0.5 mg/L, showing reduced biomass formation, severely reduced ergosterol levels, and almost complete inhibition of hyphal growth. There was no evidence of cell leakage. Mass spectrometric analysis of the secretome showed that its composition was strongly affected and included seventeen fluconazole-specific secretory proteins. Relative quantification of 14N-query walls relative to a reference standard mixture of 15N-labeled yeast and hyphal walls in combination with immunological analysis revealed considerable fluconazole-induced changes in the wall proteome as well. They were however similar for both surface-grown and planktonic cultures. Two major trends emerged. (i) Decreased incorporation of hypha-associated wall proteins (Als3, Hwp1 and Plb5), consistent with inhibition of hyphal growth. (ii) Increased incorporation of putative wall repair-related proteins (Crh11, Pga4, Phr1, Phr2, Pir1 and Sap9). As exposure to the wall-perturbing drug Congo red led to a similar response, these observations suggested that fluconazole affects the wall. In keeping with this, the resistance of fluconazole-treated cells to wall-perturbing compounds decreased. We propose that fluconazole affects the integrity of both the cellular membranes and the fungal wall, and discuss its potential consequences for antifungal therapy. We also present candidate proteins from the secretome for clinical marker development.
Fluconazole-related phenotypes

Introduction

Although *Candida albicans* is normally dwelling in the human flora as a harmless commensal, it is also capable of causing disease, especially when the immune system is weakened. The resulting infections range from superficial infections of the mucosal layers to life-threatening systemic infections. A common way to treat this fungal overload is by azole administration – predominantly in the form of fluconazole (FCZ). Azoles execute their antifungal activity mainly by inhibiting the cytochrome P450 enzyme Erg11, which is required for 14α-demethylation of lanosterol during ergosterol biosynthesis (47). This results in the replacement of ergosterol by methylated sterols, such as 14α-methyl-3,6-diol. The presence of an additional 6-OH group disturbs membrane packing and decreases membrane rigidity, resulting in water penetration and increased drug uptake (1, 15). The azole-induced membrane changes evoke numerous other responses in the cell. For example, ergosterol depletion has also been found to inactivate V-ATPase function, impairing vacuolar acidification (71). Furthermore, hyphal development is affected (50). In addition, it has been suggested that azole treatment evokes oxidative and nitrosative stress (6). Finally, transcriptional data suggest that azoles could have an impact on the wall proteome and secretome of *C. albicans* (13, 39).

Secreted proteins, like hydrolytic enzymes, are mainly involved in the destruction of tissue and nutrient uptake (55). Together with the wall proteins they are acting at the interface between the host and the fungus. Wall proteins are also required for the adherence to plastic surfaces and tissues, formation of biofilms as well as tissue invasion. Furthermore, they promote nutrient uptake and defense against the host immune system and play a pivotal role in maintaining the integrity of the cell wall (29). Cell wall integrity is crucial for cell survival, since it acts as an opposing force to the intracellular turgor pressure preventing the cell from bursting under hypo-osmotic conditions. It is also fundamental for maintaining cell shape, bud formation and proper cell division. Since a strong wall is essential, adequate maintenance and regulation
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are required. External factors that generate cell wall stress as well as cell cycle progression lead to (local) wall weakening which in turn activates the cell wall integrity (CWI) pathway [reviewed in (37)]. For instance, stretching of the plasma membrane, hypo-osmotic shock, exposure to the wall-degrading enzyme preparation Zymolyase or to wall-perturbing compounds such as Calcofluor white, which is binding chitin, and Congo red, which preferentially perturbs wall construction in the neck region (30), are leading to the activation of the CWI pathway (17, 27, 43, 48). Another trigger is oxidative stress (4, 48). Upon alterations in the fungal wall, plasma membrane-bound sensor proteins get activated and trigger mitogen-activated protein kinase (MAPK) signaling cascades, that end in the activation of transcription factors involved in wall maintenance. Consequently, the transcription of wall biosynthetic and wall repair genes increases and the wall gets reinforced by higher chitin levels (45). In *C. albicans* the MAPKs Mkc1 and Cek1 are involved in the response to various forms of wall stress (32, 48, 53).

In this study we used mass spectrometry to systematically identify and quantify the effects of fluconazole on the wall proteome and secretome of *Candida albicans*. Surface-grown and planktonic cultures showed similar major fluconazole-induced changes in their wall proteomes. The secretome of planktonic cultures also showed considerable changes, including the appearance of many fluconazole-specific proteins. Interestingly, the incorporation levels of four wall proteins in surface-grown cultures were considerably higher than in planktonic cultures, both in the absence and presence of fluconazole, suggesting a role for these proteins in surface growth. Considerable increases in the relative abundance of proteins associated with the cell wall integrity response were observed. This was accompanied by decreased resistance against wall-perturbing agents. Additionally, the abundances of a similar set of wall proteins increased upon exposure to the wall-perturbing compound Congo red. In summary, our data show that fluconazole compromises fungal wall integrity and causes major changes in the proteomes of the wall and the culture medium.

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Materials and methods

Strains and growth conditions

Chemicals were obtained from Sigma-Aldrich, unless stated otherwise. *C. albicans* SC5314 (22) was precultured in liquid YPD (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) and incubated overnight at 30°C and 200 rpm in a rotary shaker. The preculture was spun down and washed in PBS buffer to remove the medium and either used to inoculate liquid cultures or to inoculate Petri dishes containing semi-solid low-agarose medium. For experiments with liquid cultures, 50 ml YNB-S (6.7 g/L YNB, 20 g/L sucrose, 75 mM MOPSO [3-(N-morpholino)-2-hydroxypropanesulfonic acid] set to pH 7.4) were inoculated with the preculture to an OD$_{600}$= 0.05. For drug-treated cultures 0.5 mg/L (1.6 $\mu$M) fluconazole from a 1 g/L stock solution or 2 mg/L (2.9 $\mu$M) Congo red were added. The liquid cultures were incubated for 18 h at 37°C and 200 rpm. For experiments with agarose-grown cultures, about 6x10$^6$ cells of the preculture were plated on low-agarose medium containing 1.7 g/L YNB without ammonium sulfate, 5 g/L porcine stomach mucin as sole nitrogen source, 3 g/L agarose, 5 mM glucose and 75 mM MOPSO set to pH 7.4, and with or without 0.5 mg/L FCZ. The Petri dishes were incubated for 18 h at 37°C.

Analysis of morphology, biomass, and ergosterol content

For analysis of the morphology after 18 h of fluconazole treatment, the cells were visualized by light microscopy and photographed. For determination of the biomass, liquid cultures were spun down after 18 h of growth and the pellet was dried at 60°C overnight and weighed. Since cells grew invasively on a semi-solid surface, the whole content of the plates was first solubilized in 6 M guanidine thiocyanate at 60°C. Subsequently, the cells were spun down and the pellet was dried and weighed. For analyzing the ergosterol content, the cells were cultivated as described above. Cells grown on low-agarose plates were washed off the surface and collected. The extraction procedure was performed as described previously (9). Two ml of the wash-off were quenched with 6 ml ice-cold methanol, the remaining part was dried and the biomass was determined. For liquid cultures, two ml were quenched directly with 6 ml of ice-cold methanol. The remaining part of the culture was used for biomass determination. Six ml of petroleum ether (boiling point 40-60°C) were added to the 2 ml of cells suspended in 6 ml of methanol. After vortexing for 1 minute and centrifuging (2 min at
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3,000 rpm, the upper petroleum ether phase was transferred into a N2-flushed tube. The whole procedure was repeated once again and the upper phases were combined. When the petroleum ether was completely evaporated, the sample was resuspended with a glass rod in 60 μl of ethanol and 10 μl were injected onto a LiChrosorb RP-18 HPLC column (Chrompack; Bergen op Zoom, The Netherlands). Methanol was used as the mobile phase and the flow rate was 2 mL/min at 50°C. Ergosterol was detected at 290 nm and an ergosterol standard was used to calculate the measured amounts. The ergosterol content was normalized to biomass dry weight.

Cell wall isolation

For cell wall isolation, cells were harvested in the same way as for the biomass determination. Walls were isolated as previously described (16). In short, cells were first washed five times with cold demineralized water. Subsequently, they were resuspended in a 10 mM Tris-HCl buffer, pH 7.5, together with a protease inhibitor cocktail, and disintegrated with the help of 0.25–0.50 mm diameter glass beads using a Bio-Savant Fast Prep 120 machine (Qbiogene, Montreal, Canada). After washing five times with 1 M NaCl and five times with demineralized water, crude walls were boiled four times for 10 min in SDS extraction buffer (150 mM NaCl, 2% (w/v) SDS, 100 mM Na-EDTA, 100 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 7.8). To remove the buffer, walls were washed with demineralized water repeatedly, freeze-dried, and stored at -80°C.

Analysis of fluconazole-induced changes of the C. albicans secretome

The supernatant of the liquid cultures was centrifuged once again at 5,000 rpm for 10 min to remove remaining cells and was concentrated by 10-kDa cut off filters (Amicon Ultra-15 Centrifugal filter units, Millipore, Billerica, Mass.). Using a BCA assay and bovine serum albumin as a standard (57), the amount of secreted proteins was quantified and normalized to biomass dry weight. The concentrated proteins were reduced and alkylated and digested with trypsin as described for wall proteins. The peptide concentration was determined at 205 nm by a NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands) and peptides were diluted to a final concentration of 75 ng/μl in 0.1% trifluoroacetic acid and 1% formic acid. The tryptic peptide mixtures were analyzed with LC-nano ESI-MSMS using a QTOF mass
spectrometer (Micromass, Whyttenshawe, UK) coupled to an Ultimate 2000 nano-HPLC system (LC Packings, Amsterdam, The Netherlands). Data acquisition and processing details have been described previously (58). Five independent biological samples with three technical replicates each were analyzed for the two conditions: the cell cultures with (FCZ) and without fluconazole (Control). Proteins were identified by matching the processed MSMS data with the complete *C. albicans* protein data base (8) using the MASCOT (Matrix Science, UK) search engine. For each identified protein the peptide count was summed over all five analyses of the FCZ and Control samples (38). The FCZ-Control ratio of the total number of tryptic peptide identifications for each individual protein (peptide count) was taken as an indication for the change in the corresponding protein level.

15N-labeled reference culture

*C. albicans* from a preculture was used to inoculate a second preculture in YNB-S with 15N-labeled ammonium sulfate (Spectra Stable Isotopes; 15N content >99%), buffered with 75 mM tartaric acid at pH 4, and incubated over night at 30°C. Tartaric acid was used because it has two pKa values close to pH 4 (4.37 and 3.02 at 37°C) and is not metabolized by *C. albicans* (own observations). The 15N-labeled preculture was used to inoculate two cultures of 600 ml of 15N-labeled YNBS, either buffered with 75 mM tartaric acid at pH4 to favor yeast growth or with 75 mM MOPSO at pH 7.4 for hyphal induction, to an OD600= 0.1. The cultures were incubated at 37°C and 200 rpm for 18 h. After 18 h the 15N-labeled cells were spun down, the pellets were combined and the walls were subsequently isolated, divided in aliquots, freeze-dried, and stored at -80°C.

Sample preparation for relative quantification

14N-walls from planktonic cultures or low-agarose plates were mixed with 15N-reference walls in a ratio of 1:1 based on their dry weight. The combined walls with their covalently attached proteins were reduced with 10 mM dithiothreitol in 100 mM NH4HCO3 (1 h at 55°C) followed by alkylation with 65 mM iodoacetamide in 100 mM NH4HCO3 for 45 min at room temperature in the dark. After quenching with 55 mM dithiothreitol in 100 mM NH4HCO3 for 5 min in the dark, samples were washed six times with 50 mM NH4HCO3. The walls were digested with trypsin gold (Promega, Madison, WI) at 37°C for 18 h. After 18 h the walls were spun down and the
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supernatant containing the tryptic peptides was transferred to a new tube. Subsequently, the peptides were desalted by a C18 tip column (Varian, Palo Alto, CA) and their concentration was measured at 205 nm with a NanoDrop ND-1000 spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands).

Wall proteome analysis of Congo red-treated cells
Cells from liquid cultures supplemented with and without 2 mg/L Congo red were harvested by centrifugation and walls were isolated, reduced, and alkylated as described above. Further sample processing, data acquisition and processing were carried out as described for secretome analysis except that the samples contained 25 ng/μl of tryptic peptides.

FTMS data acquisition, processing, and relative quantification
800 ng of the mixtures of 14N- and 15N labeled peptides in a solution of 0.1% trifluoroacetic acid were loaded on an Ultimate 3000 (Dionex, Sunnyvale, CA, USA) HPLC system with a PepMap100 C18 (5 μm, 100 Å, 300 μm i.d. x 5 mm) precolumn and a PepMap100 C18 (5μm, 100 Å, 300 μm i.d. x 250 mm) analytical column (Dionex, Sunnyvale, CA, USA) coupled to an ApexQ FTMS (Bruker Daltonik, Bremen, Germany) equipped with a 7T magnet and a CombiSource™. Experimental details on data acquisition and processing were previously described [(60),(24)]. Proteins were identified based on their 14N/15N peptide pairs, with the corresponding 14N/15N isotope abundance ratio as the protein level relative to the reference standard. Three or four independent biological replicates were analyzed per condition for semi-solid surface- and liquid-grown cultures, respectively. First, the peptide 14N/15N isotope abundance ratios were averaged over the corresponding peptides for each protein. Then, resulting protein 14N/15N isotope abundance ratios were averaged over the replicates. Finally, the Fluconazole/Control ratio was calculated for each protein as the FCZ 14N/15N protein isotope abundance ratio divided by the Control 14N/15N protein isotope abundance ratio.

Immunoblot analysis of Hwp1 and Rbt5
Glycosylphosphatidylinositol (GPI)–anchored proteins were released from freeze-dried walls as described previously (59). Four mg cell walls were incubated over night with 2.5 μl recombinant Trichoderma harzianum endo-β-1,6-glucanase (10) and 2 μl protease
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inhibitor cocktail (Sigma, St. Louis, Missouri) in 50 mM sodium phosphate buffer, pH 5.5, at 37 °C. The released wall proteins were first separated on a 3–8% Tris-acetate polyacrylamide gradient gel (Invitrogen, San Diego, California) and then transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Billerica, Massachusetts). Immunoblot analysis with polyclonal Hwp1 (61) or Pga10 (68) antiserum was performed in 5 % (w/v) milk powder in PBS buffer for 2 h. After thorough washing with PBS, the membrane was incubated with goat anti-rabbit antiserum that was conjugated to peroxidase (Thermo Fisher Scientific, Waltham, Massachusetts), in 5 % (w/v) milk powder in PBS. Proteins were visualized using ECL detection reagent (GE Healthcare, Waukesha, Wisconsin).

qRT-PCR
For qRT-PCR of orf19.7104 and ACT1, as a reference gene, liquid cultures were inoculated from a pre-culture to an OD_{600}= 0.05 and incubated at 37°C without and with 0.5 mg/L FCZ. After 4, 6, and 18 hours the cultures were harvested. For each time point and condition two independent biological replicates were analyzed. Total RNA was isolated using the RNeasy kit (Quiagen) and then treated with DNase (Ambion). cDNA was generated from 280 ng of RNA with Oligo(d)T12-18 primers using SuperScript First-Strand Synthesis System (Invitrogen). PCR reactions were carried out with 1x Power SYBR Green PCR Mastermix (Applied Biosystems), 8 μl of cDNA (derived from 10 ng of RNA), and 6 pmol of each primer (for orf19.7104 5’-3’ as forward primer: TTG TCC TTT CCT TAG CCG GTA TAG and 5’-3’ as reverse primer: TTG CTT GTG GAG GTG TAT CAA GA and for ACT1 5’-3’ as forward primer: AGC TTT GTT CAG ACC AGC TGA TT and 5’-3’ as reverse primer: AGT TGA AAG TGG TTT GGT CAA TAC C). PCR reactions were run in technical duplicates on a 7300 Real time PCR System (Applied Biosystems). Primer specificity was verified by determining a dissociation profile. The mRNA abundance of orf19.7104 was normalized with respect to ACT1 levels.

Analyzing the effects of cell wall-perturbing agents
C. albicans was grown in liquid YPD medium at 30ºC over night. Two μl of 1/10 serial dilutions were spotted onto low-agarose plates containing YNB-S at pH 7.4). The plates were supplemented either with 0.5 mg/L (1.6 μM) FCZ, Calcofluor white (100 mg/L),
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Congo red (200 mg/L) and sodium dodecyl sulfate (100 mg/L) or a combination of 0.5 mg/L FCZ with each stress agent. After incubation for 2 days at 37°C the plates were photographed.

Supplemental data can be found online: http://ec.asm.org/content/10/8/1071/suppl/DC1

Results

*FCZ influences surface-grown and planktonic cultures similarly*

The cell wall and its covalently attached proteins as well as the secreted proteins are of fundamental importance for fungal virulence and fitness. Since transcriptional studies have shown that azoles affect the transcript levels of several wall protein-encoding genes and also of some genes that code for proteins isolated from the culture medium (13, 39), we wanted to quantify the effect of fluconazole on the secretome and wall proteome of *C. albicans*. We chose two distinct ways of culturing *C. albicans* in the presence or absence of 0.5 mg/L FCZ. The first set-up is intended to mimic mucosal surfaces (60), where *C. albicans* frequently colonizes. It uses low-agarose plates, with mucin as the sole source of nitrogen and a low glucose concentration (60). After inoculation and incubation the plates were solubilized in order to harvest cells that grew invasively into the surface. The other set-up consisted of liquid cultures, using ammonium sulfate as nitrogen source, and 2% sucrose as carbon source to avoid glucose repression (58). We selected a concentration of 0.5 mg/L (1.6 μM) fluconazole, which severely inhibited the ergosterol synthesis (97 and 92% for planktonic and surface-grown cells, respectively; Table 1). Unexpectedly, growth of planktonic and especially surface-grown cultures was considerably less reduced (74 and 31%, respectively; Table 1). This suggests that nearly complete growth inhibition by fluconazole, as observed at higher fluconazole concentrations, is not only caused by inhibition of the cytochrome P450 enzyme Erg11 and hence abrogated demethylation, but is also due to an effect of fluconazole on other processes. Conceivably, at a higher concentration fluconazole could inhibit another cytochrome P450 enzyme, Erg5, which is...
responsible for a later step in ergosterol synthesis that involves Δ22-desaturation in the side-chain (36). It is further known that at higher azole concentrations desaturation and elongation of fatty acids are inhibited (19, 67), further perturbing cellular membranes and consequently growth. In both experimental set-ups, azole-treated cultures grew predominantly in the yeast form with clusters of non-dissociated yeast cells. Few pseudohyphae and hyphae were present compared to the control which comprised a mixture of yeast, pseudohyphal and hyphal cells (data not shown).

**Table 1.** Morphology, biomass, ergosterol content, and amount of medium proteins of *C. albicans* grown in planktonic and surface-grown cultures with or without 0.5 mg/L fluconazole (FCZ) for 18 h.

| Growth condition     | Morphology                  | Biomass mg/ml | Ergosterol conc. μg/mg biomass | Medium proteins μg/mg biomass |
|----------------------|-----------------------------|---------------|--------------------------------|------------------------------|
| **Planktonic culture** |                             |               |                                |                              |
| Control              | Yeast + hyphae              | 2.23 ± 0.09\(^a\) | 2.63 ± 0.93                    | 0.83 ± 0.33                  |
| + FCZ                | Mainly yeast, few           | 0.81 ± 0.28   | 0.08 ± 0.04                    | 1.00 ± 0.05                  |
|                      | (pseudo)hyphae              |               |                                |                              |
| **Surface-grown culture** |                             |               |                                |                              |
| Control              | Yeast + hyphae              | 0.59 ± 0.03   | 1.69 ± 0.56                    | -                            |
| + FCZ                | Mainly yeast, few           | 0.41 ± 0.04   | 0.14 ± 0.06                    | -                            |
|                      | (pseudo)hyphae              |               |                                |                              |

\(^a\) The values represent the means ± SD. All data are from at least three independent biological replicates, measured independently. The amount of secreted medium proteins was only determined for planktonic cultures.

The almost complete inhibition of hyphal growth at the fluconazole concentration used suggests that it is not so much associated with the only moderately decreased growth rate but rather with ergosterol content (23, 41). Table 1 further shows that control cells and FCZ-treated cells release about 1 μg of protein per mg of biomass. This corresponds to about 0.25% of total cellular protein – based on the assumption that the protein content of yeast cells accounts for ~40% of the total biomass (29). These results indicate that fluconazole at the concentration used does not cause significant cell leakage and that trans-wall transfer of cytosolic proteins by vesicular transport (3), at least
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under our experimental conditions, does not strongly contribute to the protein content of the medium.

Importantly, the wall proteomes of surface-grown and planktonic cultures were largely similar (Figure 1; see for further discussion below). In view of the similarities between the results obtained with both approaches and the technical difficulties involved in processing agarose-grown cells, most experiments were carried out using planktonic cultures.

FCZ strongly influences the composition of the secretome

The tryptic digests of the medium proteins of planktonic C. albicans cultures grown with (FCZ) or without fluconazole (Control) were analyzed by LC-MS/MS. Five independent biological FCZ and Control replicates were each analyzed three times. For each identified protein the peptide count was summed over all analyses of the FCZ and Control samples. For all identified proteins the peptide counts are listed in Table 2, where the FCZ/Control peptide count ratio is an indication for the relative change in the corresponding protein level.

In total, fifty secretory proteins (Table 2) and thirty-four proteins with predicted intracellular localization (Supplementary Tables 1 and 2) were identified in the growth media under both conditions. The intracellular proteins probably originate from damaged cells, dying mother cells, apoptotic cells, non-conventional secretion (49) or vesicular trans-wall transport (3). Among the secretory proteins twenty-five contain just a signal peptide for secretion, five proteins possess a secretion signal followed by one or more transmembrane domains while twenty proteins have a secretion signal and a GPI-attachment signal. GPI-proteins are normally expected to be associated with the plasma membrane or the wall, but in agreement with our own and other previous studies (25, 58, 62) they were also detected in the growth media. Because the cultures are shaken to ensure sufficient aeration, some wall proteins possibly get accidentally released and washed out from the wall before being incorporated, or get released after their incorporation during isotropic growth and the
accompanying wall remodeling, or during local cell wall loosening at the incipient bud site. Loss of cell wall material at the site of cytokinesis, which requires severance of the wall between daughter and mother cell, seems likely. Another possibility is that shearing forces resulting from the use of shaking cultures might tear off wall proteins. In addition, similar to the yapsins in *C. glabrata* the yapsin-like CaSap9 could be involved in releasing wall proteins into the medium (28). The abundances of most GPI-proteins in the culture medium were influenced by the FCZ treatment and an elevated concentration in the growth medium correlated in most cases with an enrichment in the wall, and a lower concentration to the opposite (ranked correlation analysis R_p = 0.85, P = 0.00003; Figure 1), hence they will be discussed during the relative quantification of the wall proteome. Among the non-GPI secretory proteins the levels of several proteins were strongly affected by FCZ while the abundance of others seemed rather stable, including a previously identified core set of proteins consisting of 5 (trans)glycosylases (Cht3, Mp65, Scw11, Sim1, Sun41) as well as Coi1 and Tos1 (58). These proteins are consistently and abundantly present in the medium and neither FCZ treatment nor various other growth conditions seem to have a notable impact on their secretion.

Several proteins isolated from the culture medium might be involved in fluconazole-related stress responses. Op4, although described as an opaque cell-specific protein, was found among the FCZ-specific non-GPI proteins. As it has been identified before in the secretome of yeast cells grown at pH 4 (58), this might indicate that this protein is not opaque cell-specific but can be expressed in white-type cells depending on environmental conditions. In addition, we verified five predicted open reading frames on the protein level in FCZ-treated cultures (Table 2), like Orf19.1239, which is a predicted protein in Assemblies 19, 20 and 21 of the *Candida* genome and was found exclusively in the medium of FCZ-stressed cells. Orf19.3499 is regulated by Tsa1 (65), a protein required to cope with oxidative stress.
## Table 2. Changes in the secretome of planktonic cultures of *C. albicans* induced by fluconazole (FCZ), as indicated by the total number of identifications of tryptic peptides (peptide counts) from secretory proteins.

| Protein       | Peptide counts | Protein       | Peptide counts | Ratio |
|---------------|----------------|---------------|----------------|-------|
|               | FCZ Control    | FCZ Control   | FCZ/Control    |       |
| *Cfl2*        | 1 nd           | Phr1b         | 67 1           | 67    |
| *Chl1*        | 2 nd           | Crh11b        | 12 2           | 6.0   |
| *Ece1*        | 1 nd           | Sod4b         | 6 1            | 6.0   |
| *Op4*         | 3 nd           | Dag7          | 27 9           | 3.0   |
| *Orf19.1239*  | 6 nd           | Rbt4          | 11 4           | 2.8   |
| *Orf19.1765c* | 1 nd           | Ecm33b        | 25 10          | 2.5   |
| *Orf19.1766*  | 2 nd           | Gca1          | 5 2            | 2.5   |
| *Orf19.3499*  | 6 nd           | Tos1          | 56 30          | 1.9   |
| *Orf19.6553c* | 1 nd           | Bgl2          | 27 15          | 1.8   |
| *Pbr1c*       | 2 nd           | Rbe1          | 13 8           | 1.6   |
| *Pga7b*       | 2 nd           | Pga4b         | 8 5            | 1.6   |
| *Pga46b*      | 4 nd           | Ssr1b         | 9 6            | 1.5   |
| *Phr2b*       | 7 nd           | Utr2b         | 23 17          | 1.4   |
| *Plb3b*       | 2 nd           | Ywp1b         | 16 13          | 1.2   |
| *Sap9b*       | 5 nd           | Pir1          | 7 6            | 1.2   |
| *Sod5b*       | 1 nd           | Xog1          | 57 50          | 1.1   |
| *Sur7c*       | 3 nd           | Sun41         | 48 46          | 1.0   |
| *Als3b*       | nd 5           | Coi1          | 29 35          | 0.8   |
| *Fgr41b*      | nd 1           | Sim1          | 27 35          | 0.8   |
| *Hex1*        | nd 1           | Cht3          | 28 41          | 0.7   |
| *Pga45b*      | nd 2           | Sew11         | 36 53          | 0.7   |
| *Pra1*        | nd 1           | Msb2c         | 4 10           | 0.4   |
| *Rbt1b*       | nd 1           | Cht2b         | 1 4            | 0.2   |
| *Sap5*        | nd 1           | Eng1          | 2 19           | 0.1   |

*a* The numbers represent the peptide counts. Five independent biological samples with three technical replicates each were analyzed for both conditions. For more detailed information and information about proteins without secretion signal found in the control and in FCZ-treated cultures, see Supplemental Tables S2 and S3, respectively.

*b* GPI wall protein; Transmembrane protein; nd = not detected.

Taking into account the number of its peptide identifications (27 vs. 9; Table 2), Dag7 levels were increased by the FCZ treatment. Relevantly, a mutation in this gene confers hypersensitivity to toxic ergosterol analogues (69), suggesting a
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protective function for this protein during azole stress. Sur7 was found exclusively in FCZ-stressed cells. It contains a signal peptide as well as four putative transmembrane domains and is located in the plasma membrane. Two of the peptides identified are derived from putative extracellular domains, while one originates from a predicted intracellular domain. It is probably involved in cell wall synthesis (5), suggesting that it could play a role in a cell wall stress response. In addition to Sur7 we found four other transmembrane proteins in the medium: Cfl2, Msb2, Orf19.1765, and Orf19.6553. Msb2 has an N-terminal signal peptide and is a glycosylation-sensor protein that transfers its signal through the Cek1 MAP kinase pathway (53). Upon activation the extracellular domain of Msb2 is cleaved off and released into the medium (58, 66). We only detected peptides from the extracellular domain (Supplementary Tables 1 and 2). Interestingly, the peptide count of Msb2 decreased from 10 to 4 in fluconazole-exposed cultures (Table 2), suggesting that the Msb2 MAP kinase pathway does not play a major role in the response to fluconazole. Except for Msb2 all other transmembrane proteins were identified only in the FCZ-treated cultures. The finding of several transmembrane proteins in the medium of FCZ-stressed cultures could be due to increased membrane fluidity caused by FCZ (1). Interestingly, the levels of the endo-1,3-\(\beta\)-glucanase Eng1 and the chitinase Cht2 were negatively influenced by FCZ. The transcript levels of the corresponding genes are also repressed by caspofungin (39). The lower abundances of Eng1 and Cht2 are probably related to diminished cell separation during FCZ treatment (see also next section).

FCZ-induced changes in wall protein incorporation

Quantitative changes of the wall proteome of C. albicans grown in surface-grown and planktonic cultures with (FCZ) or without fluconazole (Control) were analyzed by LC-FTMS. For this, the FCZ and Control cell walls were 1:1 mixed with the reference standard mixture of metabolically \(^{15}\text{N}\)-labeled yeast and hyphal walls and then digested.
### Table 3
Three representative examples of relative quantification of wall proteins of planktonic *C. albicans* cultures with (FCZ) and without fluconazole (Control). For full information see Supplemental Table 3.

| Function, protein length & FCZ | 14N/15N ratios | Control 14N/15N ratios |
|-------------------------------|----------------|-----------------------|
| tryptic peptides | R1 | R2 | R3 | R4 | R1 | R2 | R3 | R4 | R1 | R2 | R3 | R4 |
| **Cht2, Chitinase, GH18 domain, 583 aa** | | | | | | | | | | | | |
| TVLLSLGGVGVDYGFSDVASATK | 0.22 | 0.19 | 0.22 | 0.22 | 1.08 | 1.19 | 1.03 | 0.82 |
| FADTLWNK | 0.18 | 0.21 | 0.24 | 0.22 | 1.26 | 1.25 | 1.09 | 0.84 |
| NYLISAAPBPYPDAISLGDLLSK | 0.26 | 0.22 | 0.27 | 0.25 | 1.26 | 1.15 | nd | 0.91 |
| LFVGPATSNAGYVDTSK | 0.24 | 0.20 | 0.24 | 0.21 | 1.07 | 1.16 | 1.02 | 0.78 |
| LSSAIEIK | 0.26 | 0.20 | 0.23 | 0.24 | 1.06 | 1.13 | 0.81 | 0.88 |
| BGSFAGVSLWDASGAWLNDVEK | nd | nd | nd | nd | 1.20 | low | nd | 0.78 |
| GENFVQVK | 0.16 | 0.20 | 0.24 | 0.23 | 1.04 | 1.17 | 0.98 | 0.83 |
| **Average per replicate** | 0.21 | 0.20 | 0.24 | 0.23 | 1.14 | 1.17 | 0.98 | 0.83 |
| **Average per condition** | 0.22 ± 0.08 | 1.03 ± 0.01 |
| **FCZ/Control** | 0.22 ± 0.08b |
| **Crh11, Transglycosylase, 453 aa** | | | | | | | | | | | | |
| DTBNPLK | 1.38 | 8.16 | 6.97 | nd | nd | nd | nd | nd |
| SSDSVPVALGSSFLEKL | 3.87 | nd | 4.79 | 6.20 | 0.79 | 1.45 | 1.40 | 1.27 |
| FDNGLOPHPESLKL | 2.93 | 2.74 | 3.52 | 5.01 | 0.59 | 1.11 | 1.26 | 1.08 |
| FDNGLOPHESLKKK | 3.23 | 3.11 | 5.42 | 9.08 | low | 1.05 | nd | nd |
| QGTGDSGENGLSLTMK | 4.74 | nd | nd | low | nd | nd | nd | nd |
| RDFNPSFK | nd | 3.60 | 4.14 | 5.57 | 0.78 | 1.48 | 1.15 | low |
| FDNPVQKK | 3.48 | 3.86 | 4.92 | 5.95 | 0.74 | 1.45 | nd | 1.16 |
| SNFYMFGGR | 3.52 | 3.52 | 4.31 | 5.20 | 0.83 | 1.43 | 1.26 | 1.13 |
| GYHDIANPLK | 2.22 | 2.38 | 3.62 | nd | 0.25 | 0.49 | nd | nd |
| DVHTYVIDWTK | 3.51 | 3.52 | 4.50 | 5.62 | 0.85 | 1.39 | 1.30 | 1.15 |
| DAVTSVSDSVMIR | 4.82 | 3.43 | 4.09 | 6.02 | 1.36 | 1.37 | 1.36 | 1.22 |
| SVLVADYSGSNN | 3.59 | 3.64 | 4.53 | 5.31 | 0.84 | 1.43 | 1.34 | 1.17 |
| QYSYSQDSGWSVSNK | nd | low | 7.76 | 8.81 | nd | nd | nd | nd |
| YDQAOQDDIK | 6.82 | nd | nd | nd | nd | nd | nd | nd |
| **Average per replicate** | 3.44 | 3.59 | 4.75 | 6.15 | 0.72 | 1.21 | 1.29 | 1.17 |
| **Average per condition** | 4.48 ± 0.13 | 1.10 ± 0.03 |
| **FCZ/Control** | 4.05 ± 0.64 |
| **Ecm33, Role in wall integrity, 423 aa** | | | | | | | | | | | | |
| TGLTVGTSVAESVVISDCTLQSLTGL | | | | | | | | | | | | |
| TGLTVGTSVAESVVISDCTLQSLTGL | | | | | | | | | | | | |
| VELAELTSGSSTINK | 0.58 | 0.56 | 0.56 | 0.67 | 0.43 | 0.45 | 0.53 | 0.51 |
| NDDTELDPFK | 0.54 | 0.62 | 0.57 | 0.64 | 0.43 | 0.49 | 0.45 | 0.49 |
| TIGGALQISDNSLR | 0.86 | nd | 0.49 | 0.65 | 0.33 | 0.32 | 0.50 | 0.48 |
| SFSGFIPK | 0.67 | 0.67 | 0.67 | 0.61 | 0.49 | 0.50 | 0.48 | 0.51 |
| VSSGFILK | 0.33 | 0.59 | 0.58 | 0.61 | 0.43 | 0.45 | 0.48 | 0.46 |
| LSBASFK | 0.60 | 0.61 | 0.67 | 0.64 | 0.52 | 0.48 | 0.53 | 0.51 |
| **Average per replicate** | 0.57 | 0.60 | 0.57 | 0.64 | 0.43 | 0.44 | 0.49 | 0.51 |
| **Average per condition** | 0.60 ± 0.02 | 0.47 ± 0.01 |
| **FCZ/Control** | 1.26 ± 0.03 |

*peptide position; a Mean ± SE; nd = not detected in the 14N cultures; low = peptides of too low intensity to be suitable for quantification.*
Proteins were identified based on their $^{14}$N/$^{15}$N peptide pairs, with the corresponding peptide $^{14}$N/$^{15}$N isotope abundance ratios. The protein $^{14}$N/$^{15}$N isotope abundance ratios were calculated as the averaged peptide $^{14}$N/$^{15}$N isotope abundance ratios and represent the protein levels relative to the reference standard [(60), (24)]. Relative standard errors for the individual $^{14}$N/$^{15}$N isotope abundance ratios per replica ranged from 0.5% to 54% with a mean of 7%. Next, the protein $^{14}$N/$^{15}$N isotope abundance ratios were averaged over all corresponding replicas. Relative standard errors for the averaging of the individual protein $^{14}$N/$^{15}$N isotope abundance ratios range from 2% to 74%, with a mean of 12%. These errors combine statistical experimental errors with the biological variation. Since both sets of standard errors are similar, it is concluded that the biological variation over the replicas is comparatively small. This justifies discussion of the biological basis of the protein quantification results. Finally, for the planktonic and semi-solid agarose-grown cultures the FCZ/Control ratio was calculated for each protein as the FCZ $^{14}$N/$^{15}$N protein isotope abundance ratio divided by the corresponding Control $^{14}$N/$^{15}$N protein isotope abundance ratio. The FCZ/Control ratios represent the changes in the protein levels induced by fluconazole. Representative examples for the calculation of the protein ratios (strongly decreased, strongly increased, and showing relatively minor changes) are presented in Table 3 while a comprehensive overview of all data is presented in Supplemental Table 3. The FCZ/Control ratios for all identified wall proteins are depicted in Figure 1. Figure 1 clearly shows that except for Orf19.7104 wall proteins induced by FCZ in surface-grown cultures are also induced in planktonic cultures and vice versa. The abundance of Plb5 in surface-grown cultures was also reduced by FCZ, but since there were too few peptides identified for reliable quantification the data were not included in Figure 1. Indeed, when performing a ranked correlation analysis we do find a correlation of $R_p = 0.85$ (P = 6.7 x $10^{-6}$) between the two data sets. Nevertheless, surface-grown and planktonic cultures show an important difference with respect to the incorporation levels of the wall proteins.
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Als4, Pir1, Rhd3 and Sod5. Although their FCZ/Control ratios were similar in both experimental set-ups (Figure 1), showing that their incorporation levels are affected by fluconazole independent of how the cells were grown, direct comparison of their wall incorporation levels to the corresponding levels in the \(^{15}\)N-reference culture showed that Als4, Pir1, Rhd3 and Sod5 were present at much higher levels in surface-grown cultures than in planktonic cultures (median value: 8-fold increase; range: 2.4- to 17-fold; Supplemental Table 4).

As the MAP kinase Mkc1 becomes phosphorylated in cells growing on a semi-solid surface (32), these wall proteins are possibly controlled by Mkc1 and could play a role in contact-dependent growth.

By relative quantification we could identify wall proteins that were increased by

Figure 1. Relative changes in the wall proteome of *C. albicans* induced by fluconazole, expressed as the protein ratio FCZ/Control. Grey bars, planktonic cultures; white bars, surface-grown cultures; striped bar, Orf19.7104 was exclusively identified in planktonic cultures of FCZ-treated cells and was given an arbitrary ratio of 10. Rations are plotted on a logarithmic scale. Values >1 represent increased protein levels and <1 decreased protein levels after 18 h of incubation with 0.5 mg/l FCZ compared to the control.
**Fluconazole-related phenotypes**

FCZ stress (Als4, Crh11, Pga4, Phr1, Phr2, Pir1, Sap9, Sod4, Sod5) or decreased (Als3, Cht2, Mp65, Rbt1, Rhd3, Plb5, Ywp1). Consistent with the observed changes in abundance, also peptide coverage tended to increase or decrease according to protein abundance (Table 3). Only few proteins showed less than a two-fold change upon FCZ treatment (Ecm33, Ssr1, Utr2) in both set-ups. Orf19.7104 was exclusively identified in walls from planktonic cultures of FCZ-treated cells and in walls from the reference culture, although only by one peptide pair. It is a predicted open reading frame in assemblies 19, 20 and 21 with sequence similarities to mucins (46). It contains an N-terminal secretion signal, but no putative GPI attachment site. Intriguingly, it contains an amino acid motif (DGQSQ, amino acid 160-164) that is similar to the one found in the Pir repeats in *C. albicans* and *S. cerevisiae* and that is believed to be involved in cross-linking wall proteins to β-glucans (18, 29). Unexpectedly, when measuring the relative transcript levels of ORF19.7104 by qRT-PCR with respect to *ACT1*, we found that in the presence of fluconazole they were systematically about 5- to 6-fold lower than in the control culture (0.20, 0.125, and 0.059 in the control cultures compared to 0.038, 0.021, and 0.012 in the fluconazole- treated cultures after 4, 6 and 18 h of culturing, respectively). Possibly, transcript stability, translation efficiency, or the efficiency of wall incorporation of Orf19.7104 changes during azole stress.

We found two proteins without secretion signal, Tdh3 and Ssa2, in the wall preparations of FCZ-treated and control cells. Although it remains elusive how these proteins reach the cell surface and are attached there, they were previously found associated with the fungal wall (21, 40). However, for Tdh3 and Ssa2 the observed peptide ratios varied more strongly compared to those for the other wall proteins (Supplemental Table 3). Since Thd3 and Ssa2 are very abundant intracellular proteins (both have a high codon bias index of 0.83 and 0.74, respectively; see CGD), it seems possible that they were not fully removed during wall isolation, possibly, because of incomplete cell breakage.

Hwp1 does not contain tryptic peptides of a suitable size detectable by mass
spectrometry, and Rbt5 shares a peptide with other proteins of the CFEM-family (Csa1, Csa2, Pga7, Pga10), making it unsuitable for quantification purposes. In addition, although one Rbt5-specific peptide was detected (Supplemental Table 1), it was very often of low intensity in the query or the reference culture. We therefore decided to use immunoblot analysis (Figure 2).

**Figure 2.** Immunoblot of Hwp1 (A) and Rbt5 (B). Cells were grown for 18 h without (control) or with 0.5 mg/L fluconazole. The GPI-wall proteins were released from isolated walls by β-1,6-glucanase. For detection of Hwp1 and Rbt5, proteins isolated from 40 or 160 μg of walls, respectively, were subjected to immunoblot analysis.

Hwp1 was previously identified as a hyphal-enriched wall protein (61) and consistent with these findings its wall level was increased in our control cultures, where hyphae are more abundant. On the other hand, Rbt5 was more abundant after FCZ treatment, in agreement with a transcriptional study that found *RBT5* induced upon azole stress (39). Rbt5 is an iron acquisition protein and involved in hemoglobin or heme utilization (68). Several enzymes of the ergosterol biosynthesis pathway are induced during azole stress (39), like *ERG11*, which needs iron for its function. Hence, it seems likely that iron requirements during FCZ treatment increase explaining the induction of Rbt5. In spite of previous findings that indicated a membrane and not a wall localization of Rbt5 (68), we do consistently detect it in the wall of yeasts as well as hyphae under a variety of growth conditions. Moreover, Rbt5 was already shown to be wall-localized before (59). Taken together, these data indicate that Rbt5 is located both in the plasma membrane and in the cell wall.
**Fluconazole-related phenotypes**

*Fluconazole causes cell wall stress*

Since FCZ treatment results in increased fluidity of the plasma membrane (1), it seems possible that this could indirectly affect cell wall integrity - for example, by affecting the activity of plasma membrane-located cell wall construction enzymes or by affecting endocytosis. Fluconazole-treated *C. albicans* cells contain considerably less glucan in their wall, suggesting severe alterations (52). Chitin levels are probably increased to compensate for FCZ-induced wall perturbations (45).

For *Saccharomyces cerevisiae* the membrane-perturbing agents chlorpromazine and chitosan indeed trigger the CWI pathway (27, 70). In addition, protein kinase C (Pkc1), a key enzyme in the response to cell wall stress, plays a pivotal role in the tolerance of drugs that affect the cell membrane in *C. albicans* (34), and phosphorylation of the MAP kinase Mkc1 increases upon exposure to the membrane-perturbing agent chlorpromazine (32). We identified several wall proteins that were induced by fluconazole stress and that are required for cell wall cross-linking and integrity (Crh11, Pga4, Phr1, Phr2, Pir1 and Sap9). Additionally, we found that the changes in the transcript levels of several wall proteins were induced by fluconazole stress and that these changes were associated with the induction of the CWI pathway (27, 70).

**Figure 3.** *C. albicans* spot assays [1:10 serial dilution] on low-agarose plates incubated for two days at 37°C. Where indicated, plates were supplemented with 0.5 mg/L fluconazole [FCZ], 100 mg/L Calcofluor white [CFW], 200 mg/L Congo red [CR], 100 mg/L sodium dodecyl sulfate [SDS] or a combination of FCZ and one of the other drugs.
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protein-encoding genes when *C. albicans* is challenged with sublethal concentrations of caspofungin [Bruno et al., (12): increased transcript levels of *ALS1, CRH11, PGA13, PHR1, PHR2* and *SAP9*, and a decreased transcript level of *CHT2*] correctly predicted the directions of the changes in the corresponding wall protein levels upon treatment with FCZ (Figure 1). We therefore tested the wall-perturbing agents Calcofluor white, Congo red and SDS without and in combination with fluconazole. Figure 3 shows that fluconazole treatment renders *C. albicans* hypersensitive to all three agents, supporting the notion that fluconazole compromises cell wall integrity.

| Protein | Peptide counts a Ratio (CR/Control) |
|---------|------------------------------------|
| Pga31   | 14/14 nd                           |
| Sap9    | 6/6 nd                             |
| Pir1    | 6/1 1 6.9                          |
| Phr2    | 12/3 1 4.6                          |
| Sod5    | 18/9 2.3                           |
| Utr2    | 22/11 2.3                          |
| Phr1    | 33/17 2.2                          |
| Rhd3    | 21/11 2.2                          |
| Rbt5    | 7/4 2.0                            |
| Crh11   | 51/33 1.8                           |
| Ecm33   | 35/23 1.7                           |
| Mp65    | 19/14 1.6                           |
| Ywp1    | 7/8 1.0                            |
| Cht2    | 20/25 0.9                           |
| Pga4    | 9/12 0.9                            |
| Sod4    | 12/22 0.6                           |
| Ssr1    | 7/15 0.5                            |
| Als3    | nd/8                               |

We next analyzed the wall protein profile of Congo red-treated cells by QTOF mass spectrometry, using a Congo red concentration of 2 mg/L, resulting in a

Table 4. Changes in the wall proteome of planktonic cultures of *C. albicans* induced by Congo red (CR; 2mg/ml), as indicated by the total number of identifications of tryptic peptides (peptide counts) from secretory proteins

a Four biological replicates were analyzed for both conditions. Each biological replicate was analyzed twice, except for Congo red, where biological replicate 1 was analyzed only once. The ratios are normalized for the number of runs. For sequence information of the peptides identified in control and in FCZ-treated cultures, see Supplemental Tables S5 and S6, respectively. nd = not detected.
Flucnazole-related phenotypes

biomass decrease of 32% at the end of the culturing period. Like fluconazole, Congo red inhibited hyphal growth (data not shown).

Table 4 shows that exposure to Congo red increased the abundances of a set of wall proteins that are mainly involved in cell wall maintenance (Crh11, Phr1, Phr2, Pir1, Sap9 and Utr2) and furthermore of Rbt5, Rhd3, Pga31 and Sod5, whereas the incorporation level of the hyphal growth-associated wall protein Als3 strongly declined. Apart from Rhd3, the levels of which were reduced during fluconazole stress, and Pga31, which was not identified by relative quantification using FTMS, the incorporation levels of all these proteins were influenced in a similar way by fluconazole (Figure 1), offering further support to the notion that fluconazole causes cell wall stress.

Discussion

As the proteins of the secretome and the wall proteome act at the interface of the fungus and its host, we wanted to investigate the fluconazole-induced changes in protein abundances in both subproteomes. In steady-state-like (exponential-phase) cells of S. cerevisiae a statistically significant but only limited correlation between protein and transcript levels has been found (Spearman rank correlation coefficient $R_s = 0.57; R^2 = 0.32$) (20). This implies that in many cases and certainly in non-steady-state conditions there is no linear relationship between transcript levels and protein abundances and that transcript levels can only predict in which directions protein abundances are expected to move (14). Precise estimates of protein abundances are however important in themselves because they determine, for example, the output of metabolic pathways or the adhesive capacity of a cell. Precise estimates are also required for Systems Biology-based approaches (14).

Our proteomics data represent the first systematic overview of the changes induced by azole stress in the protein composition of the secretome and in the incorporation levels of wall proteins and greatly extend our current knowledge. The experimental strategy for relative quantification of the wall proteome of C.
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*albicans* was based on using $^{15}$N-ammonium sulfate as the sole nitrogen source to thoroughly label two large reference cultures grown under yeast- and hypha-promoting conditions, respectively. The walls from both cultures were then isolated, mixed, and stored. This combinatorial approach ensures a wide representation of differentially expressed wall proteins in the reference walls. The mixture of $^{15}$N-labeled reference walls can then be used to study the relative abundance of wall proteins in $^{14}$N-query walls obtained from cultures grown under various environmental conditions such as in the absence or presence of fluconazole (this paper) or different hyphal growth-inducing compounds ((24), own observations), obtained from infected tissues or organs, or from a time-course experiment. Importantly, using the same reference walls in a series of experiments makes it possible to construct a library of relative abundances of wall proteins and to calculate and analyze retrospectively relative abundances between all experimental conditions tested.

Many individual medium protein levels and wall protein levels were strongly affected by the FCZ treatment. The wall proteome changes were similar in both surface-grown and planktonic cultures (ranked correlation $R_p = 0.85**$), indicating that the results obtained with planktonic cultures are to a large extent representative for surface-grown cultures. However, the incorporation levels of Als4, Pir1, Rhd3 and Sod5 were specifically increased in surface-grown cultures both in the absence and presence of fluconazole, suggesting that surface growth could stimulate their wall incorporation levels. To the limited extent that they are available, the directional changes in protein levels predicted by transcriptional studies of azole-stressed cultures (13, 39) tend to be consistent with our results. In agreement with the transcriptional results of these studies, we found increased levels of Als4, Crh11, Phr1, Phr2, Pir1, Rht5 (using immunoblot analysis) and Sap9 in the presence of fluconazole and a decrease in abundance of Hwp1 (immunoblot analysis). However, we observed upon fluconazole exposure a decrease of the wall level for Ywp1 and an increase for Orf19.7104, both of which conflicting with the transcriptional data [(13), this...
Fluconazole-related phenotypes

paper], possibly because of differences in growth conditions or because of posttranscriptional control mechanisms, such as for example observed for the wall adhesin Epa3 of *C. glabrata*, which is released into the medium of post-exponential phase cells in a yapsin-dependent manner (28).

**FCZ affects the wall proteome by altering morphology and wall integrity**

Two major trends emerge from our study of the fluconazole-driven changes in the wall proteome both of which were also observed during Congo red-induced stress (Figure 4). First, fluconazole inhibits hyphal growth and strongly promotes growth in the yeast form [this study; (23)], raising the question whether this morphological change evokes some of the observed changes. We found that azole treatment strongly decreased the levels of Als3, Hwp1 and Plb5, all of them strongly associated with the hyphal morphotype (26, 61, 63). Also Mp65 and Rbt1 levels were decreased in the wall, the genes of which were previously shown to be induced upon the yeast-to-hypha transition (11, 33). Together, these data indicate that some of the FCZ-induced changes are morphotype-associated and result from the inhibition of hyphal growth and the promotion of yeast growth. Similar to fluconazole-treated cells, Congo red treatment also caused inhibition of hyphal growth and promoted growth in the yeast mode. Second, some of the observed changes in relative abundances seem to be correlated with cell wall stress (12). Consistent with this, cell wall perturbing agents sensitized *C. albicans* to the effects of fluconazole (Figure 3). As shown by our relative quantification data in Figure 1 and Table 3, the levels of several transglycosidases (Crh11, Pga4, Phr1, Phr2) and the putatively β-1,3-glucan cross-linking protein Pir1 were increased by FCZ stress. Congo red treatment also increased the levels of these proteins (Table 4). Although peptide levels of Crh11 are only 1.8-fold increased in the presence of Congo red, comparing the actual number of peptide identifications (58.3 from walls of Congo red-treated cultures vs. 33 from the control, normalized to 8 runs) indicates that this increase is significant and corroborates this observation.
Crh11, together with Crh12 and Utr2, belongs to a family of proteins that were found to serve a crucial role in cell wall biogenesis (51). Also in agreement with transcriptional data (39), it seems that within this family Crh11 is mainly responsible for counteracting stress elicited by azole treatment. Phr1 and Phr2 are two closely related transglycosidases and expressed oppositely with respect to pH (44, 54). Mutants in either \textit{phr1} or \textit{phr2} display an abnormal cell wall at elevated or low pH, respectively. Intriguingly, and consistent with the trends predicted by previous transcriptional studies (12, 13, 39), levels of both proteins were increased when \textit{C. albicans} was challenged with FCZ, although all our experiments were performed at pH 7.4 and also buffered to avoid acidification (64). It seems that under regular growth conditions either Phr1 or Phr2 – depending on the pH – is sufficient for a proper cell wall, but upon cell wall stress both proteins might be induced as part of a compensatory response to wall defects. Also \textit{PIR1} is required for cell wall maintenance (42) and we find it up-

**Figure 4.** Proposed scheme of the response of \textit{C. albicans} to fluconazole and Congo red.
regulated upon FCZ treatment. Both FCZ- and CR-induced stress resulted in increased levels of Sap9, a GPI-modified yapsin-like aspartyl protease that participates in maintaining cell wall integrity (2, 56).

Interestingly, in *S. cerevisiae* wall stress caused by Congo red and other wall-perturbing compounds leads to increased transcript levels of *YPS1*, which is the closest homolog in *S. cerevisiae* of *CaSAP9*, and this is mediated by the cell wall integrity pathway, whereas disruption of *YPS1* results in a much lower resistance to Congo red (31). These observations suggest that *CaSAP9* and *ScYPS1* share similar functions and might be similarly regulated. In addition to these two major trends, some FCZ-induced changes in the wall proteome are probably due to other effects of FCZ. For example, FCZ has been found to cause nitrosative and oxidative stress (6). This could explain the increased levels of the superoxide dismutases Sod4 and Sod5 in the walls of FCZ-treated cultures. Exposure to Congo red led to an increase in abundance of Sod5 as well (Table 4). Finally, phosphorylation of the MAP kinase Mkc1, which plays a role in the cell wall integrity pathway, has been shown to increase upon both membrane and cell wall stress (32, 48), suggesting a role for Mkc1 in the observed changes in the wall proteome and the secretome.

**Potential clinical markers from the secretome**

Providing immunocompromised patients with an early diagnosis of invasive *Candida* infections is an ongoing problem, since usually time-consuming blood cultures are first required, thus delaying life-saving therapies. Diagnosis based on the detection of antibodies against fungal antigens poses the problem how to distinguish between antibodies generated due to harmless colonization of the mucosa or due to a serious invasive infection. Additionally, immune-compromised patients might produce low levels of antibodies leading to false negative results [reviewed in (35)]. The detection of the actual antigens partially avoids these problems; nevertheless, due to relatively rapid clearance of some of them their levels need to be high enough. To serve as a potential diagnostic
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Marker candidate, several requirements should be fulfilled, including extracellular localization, and high abundance under different environmental conditions depending on the site of infection. We have already previously reported about a core set of proteins that seems to be abundantly secreted under a variety of growth conditions (58). Also treatment with the antifungal drug fluconazole did not seem to strongly influence the secretion of Cht3, Coi1, Mp65, Scw11, Sim1, Sun41 and Tos1. Indeed, Mp65 seems to be a promising marker (7). In addition, Xog1 also seems an abundant secretory protein (Table 2) and could thus function as a potential marker of early infections. Collectively, our findings provide valuable information for understanding the mode of action of azole drugs as well as for alternative therapeutic strategies and for potential infection markers.

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