Infection related stress adaptations in the secretome and wall proteome of Candida albicans
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Chapter 6

General discussion

This chapter is partially based on the two published reviews


Clemens J. Heilmann, Alice G. Sorgo, Frans M. Klis. “News from the fungal front: Wall proteome dynamics and host-pathogen interplay”, PLOS pathogens 8(12): e1003050
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1. General introduction

*Candida albicans* infections and fungal diseases in general are nowadays a serious problem. Recurrent fungal infections can affect even healthy individuals, but for patients that have a severely weakened immune defense (e.g. due to disease or immunosuppressive therapy), they may become life-threatening. Despite the advances in the health sector, a better understanding of the causative agent is needed and rapid diagnosis and effective antifungal therapy are still major concerns.

The wall proteome and secretome of *C. albicans* is indispensable for fungal fitness and virulence. In this thesis I analyzed the adaptations of these two subproteomes to different environmental challenges, like antifungal drugs, iron restriction and thermal stress. By adapting mass spectrometric techniques we could identify major protein changes as a necessary consequence to cope with the stress conditions. First the advantages of proteomics over transcriptomics data will be highlighted, followed by a discussion over secreted proteins and then over wall proteins of the clinical relevant fungus *C. albicans*.

2. Transcriptomics versus proteomics

While nowadays, advanced genomic techniques can relatively quickly sequence the genome of an entire organism, transcriptomic analysis gives much further insight in the complex regulation of cellular processes. Nevertheless it can only provide limited information about protein levels or protein location. RNA levels are subject to many alterations. After transcription splicing and also alternative splicing can occur, resulting in the translation of different proteins. In addition, transcript stability can vary greatly, and transcripts can form secondary structures, which are difficult to access thus providing the cell with another layer of controlling transcription. Fascinatingly, transcript binding by certain proteins such as She3 in *C. albicans*, which is involved in mRNA transport, can also influence the localization of protein formation and in this fashion result in asymmetric growth (19). Post-translational modifications frequently alter the
newly synthesized protein further and, depending on the type of modification
and the amino acid sequence, the half-life of a protein is also very variable.
Therefore, mRNA levels do not always correlate with their corresponding
protein levels, as was already shown for various organisms, from bacteria, to
yeast up to mammals (27, 29, 74). Since these large scale studies elucidated
major discrepancies between many protein levels and their encoding transcript
levels, the need for proteomics data became incontestable. Importantly,
proteomics can capture the final outcome of a cellular response to specific
conditions.

During our studies we often observed a correlation between previously
published transcriptional data and our proteomics results. However, despite
having the same direction of change as a consequence of certain stress
conditions, the measured transcriptome and proteome ratios varied
considerably. In addition, we could identify protein changes that had not been
described before on a transcriptional level. Our studies also revealed the
location of several proteins, i.e. either preferentially associated with the cell
walls or predominantly occurring in the culture solution, whereas other proteins
were found in both locations.

3. The secretome of Candida albicans
3.1. Identification of secreted proteins
To analyze the composition of the secretome we used a semi-quantitative
approach applying LC-MS/MS. This method is based upon the assumption that
peptides of a certain protein will be more frequently selected for MS/MS
analysis if the protein is abundant compared to conditions where it is only found
in low levels (79). We summed up all identified peptides per protein per
replicate and divided this number by the overall number of identified peptides in
this replicate. Afterwards we averaged this number over all the replicates per
condition, which allowed us to compare the relative abundance of each single
protein per condition. Since we measured for every growth condition sufficient
biological and technical replicates the use of this method is justified, although it is not a highly accurate method. Importantly, the abundance of different proteins cannot be compared with each other, since each protein may yield different amounts of identifiable peptides which differ in their likelihood of being identified, e.g. due to different ionization efficiency. However, the same is true for the highly accurate relative quantification techniques, as we used for the relative quantification of the wall proteome.

### 3.2. The size of fungal secretomes

As an opportunistic pathogen *C. albicans* can quickly adapt to the special needs required for the survival in different hosts and niches. Secreted proteins are crucial for pathogenicity and involved in many processes like nutrient acquisition, tissue invasion and evasion of the immune system.

Several computational studies have produced *in silico* estimates of the size of fungal secretomes (10, 15, 41, 42, 44, 71). Here we use the estimates obtained by Lum and Min (44). Using different algorithms, like SignalP 3.0 (6), the entire fungal proteome was scanned for proteins with an N-terminal signal sequence, while proteins with predicted transmembrane domains (20) and subcellular localization were again excluded. As expected, the size of the predicted secretome was found to be correlated with proteome size. The putative *C. albicans* secretome comprises ca. 225 proteins (3.1% of the proteome), about 60 of which are predicted GPI-protein. Similar values (expressed as percentages) were obtained for the predicted secretomes of other species in the CTG clade (translating CTG as serine instead of leucine; (25) (*C. dublieniensis* 184, 3.1%; *C. guilliermondii* 159, 2.7%; *C. lusitaniae* 169, 2.8%; *C. tropicalis* 212, 3.4%; *Debaryomyces hansenii* 148, 2.3%; *Lodderomyces elongisporus* 139, 2.4%). The predicted secretomes of yeasts from the WGD (Whole Genome Duplication) clade (25) like the pathogenic yeast *Candida glabrata*, and the nonpathogenic yeasts *Kluyveromyces lactis*, *Pichia pastoris*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* tend to be slightly
smaller than in the CTG clade comprising 121 (2.3% of the proteome), 113 (2.1%), 105 (2.1%), 156 (2.7%), and 112 (2.2%) secreted proteins, respectively. The predicted secretomes of saprophytic filamentous fungi, which generally have larger genomes than yeasts, are considerably larger than in yeasts, not only in absolute numbers but also expressed as percentage of the proteome: for example, 832 proteins (5.9%) in *Aspergillus niger* versus 225 (3.1%) proteins in *C. albicans* (44). Possibly, saprophytic filamentous fungi need to secrete a large spectrum of specialized enzymes to degrade dead plant and animal material (16). These observations suggest that secretome size is not only correlated with genome size, but also with the complexity of the life cycle (resulting in more cell types), and also lifestyle. A common feature of all secretomes, including that of *C. albicans*, is the tightly controlled expression and secretion of the constituting proteins. Secreted proteins that are not required in specific niches are repressed, e.g. if a certain nutrient is not present or if the pH for effective activity is not optimal (11, 21, 65).

**Figure 1.** Overview of all protein classes encountered in the growth media of *C. albicans* based on previous proteomic studies (21, 31, 64, 65). While about two thirds of the 89 identified proteins possess a signal peptide [SP], about one third does not contain a signal for secretion. Among the SP-containing proteins several are GPI-proteins, but also few transmembrane [TM] proteins and proteins of the endoplasmatic reticulum [ER] are found.

We found a relatively low protein content of the growth medium of *C. albicans* under various conditions, since it comprised only 0.1-0.2% of the total dry
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biomass (65). Analyzing all our previous results [(22, 31, 64, 65); discussed in chapter 2-4] the large majority of proteins in the secretome contain a signal peptide (SP; about 66%); in addition, a significant amount of GPI-modified SP-proteins (about 24%), that are meant to be covalently attached to the cell membrane or wall, are regularly found in the growth medium (Figure 1). Besides the expected secreted proteins, about one third does not possess a secretion signal.

3.3. Non-classical protein secretion

Some proteins of *C. albicans* that possess an ER retention signal or N-terminal transmembrane domain, are occasionally found in the culture medium (65). Possibly, retention is incomplete and some ER proteins are nonetheless delivered to the cell surface. Occasionally, cytosolic proteins without secretion signal are also detected in the extracellular environment. Since they do not possess an N-terminal signal peptide, it is conceivable that they reach the cell surface via a nonconventional secretion route, as has been discussed (13, 52, 53). Since the known functions of these proteins in *C. albicans* are directed towards intracellular targets, a designated export mechanism seems less likely. The active secretion of membranous vesicles containing cytoplasmic freight has been first described for *Cryptococcus neoformans* (58) and was later found in other fungi as well. In *Histoplasma capsulatum* the vesicle cargo mainly consisted of lipids and proteins, including important virulence factors, hinting at a function as “virulence bags”, most likely to increase the local concentration of an effector (1). Another possible explanation for cytosolic proteins in the extracellular environment is the presence of apoptotic cells, which can undergo membrane blebbing (55), or of lysing cells. Interestingly, after passing the culture supernatant through a 200-nm filter before protein preparation, significantly fewer proteins with normally intracellular localization are detected (21, 31). In our studies the majority of cytosolic proteins were found in the medium of hyphal and fluconazole-treated cultures (64, 65), while in all other
conditions, almost no proteins without an N-terminal signal peptide were detected. Possibly, stressed or hyphal cells tend to break easier than yeast cells, the porosity of the walls might increase under these growth conditions, or they might release more vesicles.

3.4. GPI-proteins in the growth medium
GPI-proteins are consistently found in the medium after growth of *C. albicans* and other yeasts (11, 33, 46, 70) (Figure 1). For detailed information on covalently attached cell wall proteins the reader is referred to other reviews (12, 38). GPI-proteins follow the secretory pathway but are either retained in the cell membrane or covalently attached to the cell wall (56). In the walls of *S. cerevisiae* a soluble periplasmic precursor of the wall-bound GPI-protein Sag1 has been identified, which had been cleaved off the plasma membrane but had not yet been attached to the wall and might thus be washed out into the medium (43). Alternatively, some wall material is released during cell separation or as a result of wall remodeling during isotropic growth or when the wall is locally degraded to allow the formation of new buds or hyphal branches. This is supported by the detection of β-1,3-glucan-associated Als3 and Hyr1 in the supernatant of *C. albicans* cultures (75). Finally, GPI-protein levels in the growth medium generally correlate with their relative abundance on the wall. For example, consistent with its association with hyphae (32), Als3 was only found in the medium of hyphal cultures (65).

3.5. Secreted proteins involved in infection and wall remodeling
Among the SP-proteins we identified about one third was involved in wall remodeling (primarily carbohydrate active enzymes), about another third was involved in nutrient acquisition (mainly hydrolytic enzymes), while the rest was important for metal ion acquisition, host interaction, biofilm formation or had a yet unknown function (Figure 2). Numerous studies about the hydrolytic enzymes of *C. albicans* show the importance of this group of secreted proteins
(34, 62). The absence of some family members, from the lipases, phospholipases and aspartyl proteases in the measured secretomes is probably due to the tight regulation of secreted proteins. Since laboratory conditions do not truly represent the host environment during infection, it is understandable that certain proteins are not encountered in vitro, but are abundant in vivo. This is supported by the fact that only 12% of the secreted proteins have been detected under all conditions tested, and more than 30% have only been detected under a single condition.

Figure 2. Functions of SP-containing proteins identified in the growth medium of C. albicans. The majority of the 59 secreted proteins identified are responsible for wall remodeling and nutrient acquisition. Others are required for the metal ion acquisition, the host interaction or biofilm formation or serve a yet to be determined function.

The majority of proteins detected under all growth conditions have a wall-related function. They are presumably very abundant, since multiple tryptic peptides were detected in almost every growth condition we tested (21, 31, 64, 65). All seven proteins belonging to the core set of proteins detected in all conditions tested in the secretome are glycosyl hydrolases (Table 1). They are generally responsible for maintaining cell wall integrity and wall remodeling.
and many of them are involved in cell separation, acting downstream of the RAM (Regulation of Ace2 and Morphogenesis) network (60).

Sun41 and Sim1/Sun42 belong to the SUN family since they both contain the so-called SUN domain. Like their orthologs in *S. cerevisiae*, mutations in *UTH1* and *SUN42*, *SIM1* and *SUN42* of *C. albicans* are synthetically lethal and their individual inactivation leads to a serious cell separation defect (24, 49). Both secreted proteins were detected consistently under all growth condition tested. Furthermore, they are required to maintain wall integrity of the mother cell after cell separation, which suggests them acting downstream of the RAM pathway (24).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
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<tbody>
<tr>
<td><strong>Core set of abundantly secreted proteins</strong></td>
<td></td>
</tr>
<tr>
<td>Cht3</td>
<td>Major chitinase; cell separation</td>
</tr>
<tr>
<td>Mp65</td>
<td>Abundant transglycosylase; biomarker</td>
</tr>
<tr>
<td>Scw11</td>
<td>Glycosyl hydrolase; cell separation</td>
</tr>
<tr>
<td>Sim1</td>
<td>Essential glycosidase; SUN domain</td>
</tr>
<tr>
<td>Sun41</td>
<td>Essential glycosidase; SUN domain</td>
</tr>
<tr>
<td>Tos1</td>
<td>Glycosidase</td>
</tr>
<tr>
<td>Xog1</td>
<td>Glycosidase; ECM formation</td>
</tr>
<tr>
<td><strong>Other secreted proteins with wall-related function</strong></td>
<td></td>
</tr>
<tr>
<td>Bgl2</td>
<td>Transglucosylase; ECM formation</td>
</tr>
<tr>
<td>Chl1</td>
<td>Chitinase</td>
</tr>
<tr>
<td>Eng1</td>
<td>Endoglucanase</td>
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<tr>
<td>Pir1</td>
<td>β-glucan crosslinking</td>
</tr>
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It is well known that wall stress leads to reinforcement of the cell wall with chitin, a minor but important component, through the activation of chitin synthases, resulting in increased stress resistance (40). Recently, it has been shown that reduced chitinase activity could also contribute to the increased chitin content of the walls, as cells subjected to wall or membrane stress became deficient in cell separation (31). Cht2 is a wall-bound GPI-modified chitinase, whereas Cht1 and Cht3 are both non-GPI-modified chitinases. Cht2 peptides were consistently identified in the cell wall and in the medium (32, 64, 65, 67).
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Cht1 and Cht3 peptides were only detected in the culture medium. Cht1 peptides were found under some growth conditions, while Cht3 was always present although it was much less abundant in a mainly hyphal culture (64, 65). Deletion of CHT3 in a yeast cell culture resulted in chains of cells that were not fully separated, underlining its importance during cytokinesis (18). Also the endoglucanase Eng1 and the glucanase Scw11 are involved in cell separation, since a mutation in ENG1 or SCW11 leads to the formation of cell clusters (23, 35). Expression of CHT3, ENG1 and SCW11 is regulated by the transcription factor Ace2 (35, 50). Ace2, which is involved in the RAM signaling network, acts specifically in daughter cells and is crucial for cell separation. Similar to any mutation of a gene involved in the RAM pathway, a mutation in ACE2 is causing a severe cell separation defect (35). Cultures grown at 42°C formed SDS-resistant cell aggregates and this was accompanied by decreased secretion of Cht3, Eng1 and Scw11, suggesting that the role of Ace2 in cytokinesis might be suppressed during thermal stress (31). A similar, but less pronounced effect was observed in cultures treated with fluconazole, an antifungal which also leads to decreased cell separation (64). Overall, the increase in chitin content leads to higher resistance to wall and membrane stressors, which, in combination with a decrease in cell separation enzymes, strongly reduces or abolishes cell separation.

Since β-1,3-glucan is the most abundant carbohydrate in the wall, several proteins are involved in its maintenance and remodeling. For example, Pir1, an essential gene, is an important structural protein of the wall and has been suggested to crosslink β-1,3-glucans (38, 48). In agreement with its involvement in cell wall cross-linking, heterozygous mutants display a cell wall defect accompanied by increased clumping. The roles of Mp65, a putative transglycosylase, and Tos1, yet of unknown function, which are both abundant secreted proteins under all conditions tested, remain unclear to date. Interestingly, both Bgl2 and Xog1 are less abundant in hyphal cultures. Xog1 is responsible for the major exoglucanase activity in C. albicans. The importance
of Xog1 for structural integrity is underlined by the fact that a mutation in XOG1 affects cell wall integrity (28), suggesting it might also possess transglucosylase activity. Similarly involved in cell wall integrity is the transglucosylase Bgl2 as the knockout mutant displays a wall defect and forms cell aggregates in stationary phase cultures (30, 61). In line with this, Bgl2 was only found at low levels at 42°C and during fluconazole exposure, both conditions displaying a severe cell-separation phenotype. In S. cerevisiae, ScBgl2 is strongly associated with β-1,3-glucan and is robust enough to stay functionally active after SDS boiling (36). Intriguingly, free Bgl2 in the medium was able to bind β-1,3-glucan as well as chitin. Both Bgl2 and Xog1, together with the GPI-anchored transglycosylase Phr1, have been recently suggested to function in a β-glucan delivery system to the extracellular matrix, contributing to biofilm formation and drug resistance (73). Individual knockout mutants formed less persistent biofilms that sequestered less fluconazole than the reference strain. Taken together, this suggests that extracellular matrix formation is a key function of the secretome, leading to increased resistance to different stresses e.g. antifungals.

3.6. Secreted proteins as diagnostic markers
Secretory proteins in the culture medium have multiple functions that are essential for fungal fitness and virulence. Secreted proteins with wall-related functions are required for the constant remodeling of the wall due to morphological adaptations, growth and cell separation, and cell wall repair. This correlates with the high number of peptide identifications in almost all growth conditions tested. Especially Cht3, Mp65, Scw11, Sim1, Sun41, Tos1 and Xog1 were found in every tested condition with ample peptide identifications (Table 1). In view of their accessibility and abundance, this set of proteins might offer new diagnostic markers, in addition to Mp65 (3). As highlighted in the introduction, rapid and correct diagnosis of fungal infections is a severe problem. If those proteins would be detected in the blood stream, they would
serve as direct indicators of infection. Therefore, they might be potential antigen-based diagnostic markers, where accessibility and abundance of the protein is a prerequisite. For the detection of antibodies infection-specific markers, like the hyphal-specific proteins Als3 or Sap3-6, would be preferable targets. By only using infection-associated targets, proteins that are constantly produced as a consequence of commensalism would not hamper the diagnosis of a disease.

Secreted hydrolytic enzymes generally serve tissue destruction and nutrient acquisition and are therefore closely linked to virulence. Conceivably, they could serve as suitable vaccine targets. Vaccines against Sap2 proved already to be effective against systemic and mucosal infections in mice (59, 77). In summary, the proteomic analysis of the secretome is still in its infancy. Nonetheless, the importance of the secretome for many functions, especially wall remodeling and nutrient acquisition is already clear. In addition to the proven targets in the secretome (Mp65, Sap2), other promising targets have been identified. Therefore, further inquiry into the nature of the secretome might lead to both a deeper understanding of its secrets as well as better diagnostic, prevention and treatment options for patients.

4. The wall proteome of C. albicans

4.1. The use of $^{15}$N-labeled reference walls for the relative quantification of wall proteins

Metabolic labeling (5) and subsequently using the $^{15}$N-labeled reference walls as internal standard allowed us to relatively quantify and compare the changes in the C. albicans wall proteome according to various stress conditions. The reference walls originated from two very large cultures that were subsequently mixed for cell wall isolation. Since these cultures were grown at different pH and temperature they contained different morphologies and differed in their wall proteome compositions, providing a sound basis for comparison with any query culture. The reference culture was grown repeatedly in minimal medium with
15N ammonium sulfate as sole nitrogen source to ensure maximal loading of the proteins with the heavy isotope. Using such an internal standard, as a comparison for different control and stress conditions, has the advantage that the query cultures can be grown in any medium, like the rich medium YPD, that contains many different nitrogen sources. It also allows comparing different experiments directly, irrespective of when or in which medium the cells were cultured.

Besides, if the proteome of the reference walls could be absolutely quantified, the absolute protein concentration could be calculated for any query condition. By e.g. using the AQUA approach for absolute quantification of proteins (5), labeled peptides could be spiked into the reference wall sample. Since the absolute quantities of the added peptides are known, the absolute wall protein amounts of the reference culture could be calculated. This would allow recalculating the data obtained from all the query cultures ultimately leading to the identification of absolute protein numbers.

4.2. The wall: first line of defense and first target

The fungus is surrounded by a robust wall that shields it against the many environmental attacks originating from the host or other microorganisms. This protective amour has to withstand also challenges from within, like during the morphological transition or during isotopic growth, when the wall needs to be partially loosened without dispensing too much stability. The wall is providing the opposing force to the intracellular turgor pressure and is required for maintenance of the cell shape. Understandably, if the integrity of the wall is compromised cells are more vulnerable to environmental stresses. To counteract cell wall weakening more chitin is incorporated to provide additional rigidity (51, 78). The cell wall is not only the first line of defense, but also a target for the immune system due to its immunogenic epitopes. For example, the receptor dectin-1, which is mainly expressed on dendritic cells and macrophages, recognizes the β-glucan of the wall and leads to the activation of pro-
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inflammatory cytokines (8). However, the mannoprotein coat largely prevents the detection of the underlying β-glucan layer. Additionally, the wall protein Hyr1 effectively reduces immune cell killing of C. albicans (45). In support of its protective role, heterologous expression of Hyr1 in Candida glabrata also mitigates immune cell killing, suggesting a direct function of the protein in immune evasion.

Antimicrobial peptides, like histatins, defensins, and cathelicidins, belong to the arsenal of host defense mechanisms as well. Recently, the shedding of the extracellular part of the plasma membrane-bound signaling mucin Msb2, which is involved in maintaining cell wall integrity, has been shown to convey resistance to histatin-5 and the cathelicidin LL-37 in a dose-dependent manner (72). This processing and shedding is likely mediated by wall-resident proteases like Sap9, since the S. cerevisiae homolog ScMsb2 is processed by the Sap9 homolog Yps1.

4.3. Membrane and wall stress both trigger the cell wall integrity pathway

The antifungal drug class of echinocandins inhibits fungal β-1,3-glucan synthases (17). It has been shown that treatment of C. albicans with echinocandins activates the cell wall integrity pathway (78), which was accompanied by a substantial increase of chitin in the wall (51). Furthermore the elevated chitin incorporation was to a certain degree conferring resistance to echinocandin stress (40). Also the azoles, like fluconazole, are very commonly used antifungals. We could show that besides membrane stress fluconazole also causes cell wall stress (64). In addition to the elevated chitin incorporation and hypersensitivity to cell wall perturbing agents we could show by relative quantification that mainly wall maintenance and repair proteins strongly increased in abundance on the wall (Crh11, Pga4, Phr1, Phr2, Pir1, and Sap9). These proteins are apparently necessary to compensate for the cell wall defects originating from theazole treatment. Besides, transcriptional studies suggest
that a similar set of proteins are required to cope with echinocandin-induced stress (9).

Cell wall stress is probably only a secondary effect of the increase in membrane fluidity due to fluconazole treatment, since many enzymes that are important for wall construction are localized in the plasma membrane. High temperatures are also known to affect membrane integrity. Intriguingly, prolonged thermal stress at 42°C led to a similar wall protein response. The β-glucan transglucosylases Phr1 and Phr2, the predicted chitin transglycosylases Crh11 and Utr2, the wall maintenance protein Ecm33, and the yapsin-like secreted aspartyl protease Sap9, which has been implicated in the processing and shedding of other wall proteins like Cht2 and Pir1 (63), were strongly increased in abundance as a consequence of thermal stress. Strikingly, when C. albicans is grown on a poor carbon source such as lactate, or on a mixture of lactate and glucose, the wall gets significantly thinner and more flexible. These alterations are accompanied by substantial changes in the wall proteome (21), including increased abundance of a similar set of wall remodeling enzymes. Conceivably, this protein set could also be important to survive other surface stresses, including membrane-perturbing antimicrobial peptides found in body fluids, epithelial
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layers, and immune cells. The functional domains of these proteins are conserved in the Ascomycotina, suggesting similar importance for other fungi as well.

Whereas until now we only discussed increases in wall protein abundances upon wall and plasma membrane stress, cell separation enzymes, like the chitinases Cht2 and Cht3 and the endoglucanase Eng1 showed a strong reduction in abundance. This presumably contributed to the incapability of full cell separation. This cell separation defect was also observed when C. albicans was subjected to other surface stresses. Also some cell wall mutants show a similar phenotype (26, 47, 54, 57, 68). It seems that as a general response to surface stress, the cell wall stress response is mounted (Figure 3). This causes an increase in wall chitin levels by increased chitin synthesis and decreased chitinase levels, both resulting in cell aggregates. In addition wall proteins involved in wall maintenance and repair increase in abundance.

4.4. Wall proteins involved in iron acquisition

One of the most restricted nutrients in the human body is iron. Because of its reactive nature but also in order to restrict growth of invading microorganisms, free iron is highly limited in the host and mainly found in association with proteins, either as a prosthetic group like in hemoglobin and myoglobin, stored inside ferritin, transported by transferrin, or liganded by lactoferrin. C. albicans has evolved a number of strategies to scavenge iron from these complexes. Of the five CFEM family proteins, characterized by an internal domain containing eight invariantly spaced cysteines (39), Csa1, Pga7, Pga10, and Rbt5 are found attached both to the plasma membrane and the wall, while Csa2 is secreted. It has been shown that Csa1, Pga10, and Rbt5 are involved in heme binding (80). Apart from the adhesin and ferritin receptor Als3 (2), the adhesin Als4, the hyphal-associated wall protein Hyr1 (4), and the superoxide dismutase Sod4, we found all members of the Rbt5 family highly enriched on the wall upon iron starvation. In addition we could demonstrate that certain peptides derived from
the CFEM domain of Csa1, Pga10, and Rbt5 can bind iron. Due to the possession of a CFEM domain, possibly also peptides derived from Pga7 and from the secreted protein Csa2 might be capable of forming iron adducts. As the expression of CSA1, CSA2, PGA7, PGA10, and RBT5 is co-regulated upon iron restriction (14), the question arises whether the CFEM family proteins might act as a relay system, similar to bacterial iron uptake systems (7) with Csa2 being secreted to scavenge heme and Csa1, Pga7, Pga10, and Rbt5 being wall- and membrane-associated working together to facilitate iron uptake.

4.5. Vaccine targets in the wall proteome

A vaccine that could be administered to risk groups, e.g. pre-surgery, or to women suffering from recurrent vaginitis, would be an important asset. As stated earlier, the functional domain of wall proteins is almost exclusively situated in the N-terminal region, while the C-terminal part is mainly of structural importance. This is reflected in the various vaccines that are currently being developed [reviewed in (37, 76)]. For example, mice immunized with the recombinantly expressed N-terminal domain of Als3 become resistant to infections by C. albicans as well as Staphylococcus aureus (69). The N-terminal domains of Als1 and Hyr1, and a short immunogenic peptide from the N-terminal domain of Hwp1 conjugated to a β-1,2-linked mannotrioside, have been used similarly as C. albicans vaccines (37, 45). Notably, these four vaccine targets are strongly associated with hyphae, suggesting that hyphal epitopes might be more easily recognized by the immune system as a threat, since they are associated with the breaching of host tissue.

Invasive growth in vivo is not only associated with hyphal growth, but probably also with iron restriction and thus with increased levels of the CFEM iron acquisition proteins in the wall (66). Relevantly, all five members of the CFEM family contain a conserved sequence (with Csa1 containing four copies) that could represent a prime target. Developing this approach further, it is conceivable to combine immunogenic epitopes from the N-terminal functional
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region of a selection of wall proteins in a single recombinant protein for use as a multi-component vaccine. In summary, the evolution of wall protein families in the human fungal pathogen *C. albicans* allows survival in diverse host niches and has resulted in an impressive plasticity of the wall proteome. The exposure of wall proteins on the surface together with their critical functions, and the use of single- or multi-component vaccines, makes them promising targets to combat fungal infections.

References

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Vesicular polysaccharide export in Cryptococcus neoformans is a eukaryotic solution to the problem of fungal trans-cell wall transport. Eukaryot Cell 6:48-59.


General discussion


