MINIREVIEW

Beyond the wall: *Candida albicans* secret(e)s to survive

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Abstract

The opportunistic fungal pathogen *Candida albicans* occupies various niches of the human body such as the skin and the mucosal surfaces of the gastrointestinal and urogenital tracts. It can also enter the blood stream and cause deadly, systemic infections, especially in immunocompromised patients, but also in immunocompetent individuals through inserted medical devices. To survive in these diverse host environments, *C. albicans* has developed specialized virulence attributes and rapidly adapts itself to local growth conditions and defense mechanisms. *Candida albicans* secretes a considerable number of proteins that are involved in biofilm formation, tissue invasion, immune evasion, and wall maintenance, as well as acquisition of nutrients including metal ions. The secretome of *C. albicans* is predicted to comprise 225 proteins. On a proteomic level, however, analysis of the secretome of *C. albicans* is incomplete as many secreted proteins are only produced under certain conditions. Interestingly, glycosylphosphatidylinositol proteins and known cytoplasmic proteins are also consistently detected in the growth medium. Importantly, a core set of seven wall polysaccharide-processing enzymes seems to be consistently present, including the diagnostic marker Mp65. Overall, we discuss the importance of the secretome for virulence and suggest potential targets for better and faster diagnostic methods.

Introduction

The fungus *Candida albicans* can thrive in humans and other warm-blooded animals as a benign commensal, but it can also cause deep-seated infections and systemic disease. Both lifestyles require a variety of molecular tools to ensure survival. The fungus needs to bypass the host immune defense and adapt to a changing environment in different host niches. Nutrient starvation, including limited iron availability, changes in carbon and nitrogen source, and antifungal drugs are frequently encountered challenges as well. Secreted proteins are important for coping with these challenges, as well as for virulence, nutrient acquisition, and evasion of the immune system. At the same time, many important secreted proteins also elicit a strong immune response. Only a subset of these highly regulated but crucial proteins is produced at any given time point.

In this minireview, we will discuss recent proteomic results and insights obtained from the secretome of *C. albicans* and other fungi. We focus on the importance of carbohydrate-active enzymes acting on the cell wall leading to wall remodeling, changes in stress resistance, and the accumulation of extracellular matrix. We also briefly examine the variations in secretome size and the presence of covalently anchored wall proteins as well as presumably cytoplasmic proteins in the medium. Finally, we identify a core set of secreted proteins that has been encountered in all conditions examined, suggesting targets for early-stage diagnostics as well as potential points of intervention during the course of infection.

Classical protein secretion and the size of fungal secretomes

In eukaryotes like *C. albicans*, the presence of a hydrophobic N-terminal signal sequence determines whether a protein enters the endoplasmic reticulum (ER) and the secretory pathway (reviewed in Fonzi, 2009). Secretory proteins enter the ER lumen or, in case of transmembrane proteins, get inserted into the ER membrane. After proper folding and post-translational modifications,

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including *N*- and *O*-glycosylation and potential glycosylphosphatidylinositol (GPI) anchor addition, proteins are further modified in the Golgi and packed in transport vesicles to convey them to the cell surface. Upon arrival at the cell membrane, transmembrane proteins and also some of the GPI proteins are retained. Other GPI proteins move further and become covalently attached to the wall via a truncated GPI anchor (Klis *et al.*, 2002). Wall-bound GPI proteins are partially released into the medium especially during growth-related remodeling of the cell wall. The soluble secretory proteins are released into the periplasmic region, from where most of them, except for some exceptionally large proteins (De Nobel *et al.*, 1989), will diffuse into the environment. In this review, we define the predicted secretome as the set of secretory proteins that have an N-terminal signal sequence, including GPI proteins, but excepting proteins with internal transmembrane sequences, or an ER-targeting signal (Lum & Min, 2011). The measured secretome is then defined as the subset of proteins from the predicted secretome detected in the medium.

Several computational studies have produced *in silico* estimates of the size of fungal secretomes (Lee *et al.*, 2003; Liu *et al.*, 2007; Swaim *et al.*, 2008; Brustolini *et al.*, 2009; Choi *et al.*, 2010; Lum & Min, 2011). Here we use the estimates obtained by Lum & Min (2011). As expected, the size of the predicted secretome was found to be correlated with proteome size. The putative *C. albicans* secretome comprises *c.* 225 proteins (3.1% of the proteome), about 60 of which are predicted GPI proteins. 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 (Fitzpatrick *et al.*, 2006; *Candida dubliniensis* 184, 3.1%; *Candida guilliermondii* 159, 2.7%; *Candida lusitaniae* 169, 2.8%; *Candida tropicalis* 212, 3.4%; *Debaryomyces hansenii* 148, 2.3%; *Lodderomyces elongisporus* 139, 2.4%). The predicted secretomes of yeasts from the Whole-Genome Duplication (WGD) clade (Fitzpatrick *et al.*, 2006), like the pathogenic yeast *Candida glabrata*, and the nonpathogenic yeasts *Kluyveromyces lactis*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *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 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* vs. 225 (3.1%) proteins in *C. albicans* (Lum & Min, 2011). Possibly, saprophytic filamentous fungi need to secrete a large spectrum of specialized enzymes to degrade dead plant and animal material (De Vries & Visser, 2001). 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, for example, if a certain nutrient is not present or if the pH for effective activity is not optimal (Sorgo *et al.*, 2010; Buerth *et al.*, 2011; Ene *et al.*, 2012).

The protein content of the growth medium of *C. albicans* under various conditions is relatively low and comprises only 0.1–0.2% of the total dry biomass (Sorgo *et al.*, 2010). Besides the expected secreted proteins, about one-third does not possess a secretion signal. However, the majority of proteins in the secretome contain a signal peptide (SP; about two-thirds); in addition, a significant amount of GPI-modified SP proteins (>40%), that are meant to be covalently attached to the cell membrane or wall, have been found in the growth medium (Sorgo *et al.*, 2010, 2011; Ene *et al.*, 2012; Heilmann *et al.*, submitted; Fig. 1).

Nonclassical 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 (Sorgo *et al.*, 2010). Possibly, retention is incomplete, and some ER proteins are, nonetheless, delivered to the cell surface.

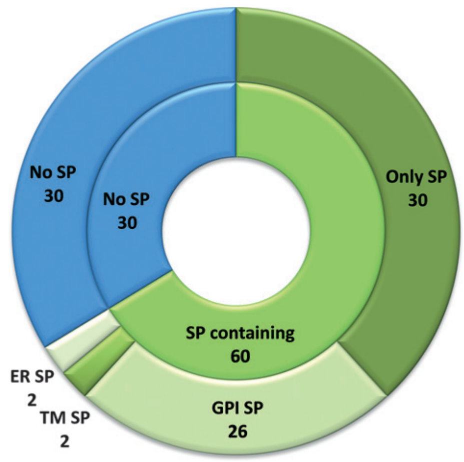

Fig. 1. Overview of protein classes present in the growth medium of *Candida albicans* based on previous proteomic studies (Sorgo *et al.*, 2010, 2011; Ene *et al.*, 2012; Heilmann *et al.*, submitted). While two-thirds of the 90 identified proteins possess a SP, one-third does not contain a signal for secretion. Among the SP-containing proteins, there are >40% GPI proteins, but also a few transmembrane proteins and proteins of the ER are found.

Occasionally, cytosolic proteins without secretion signal are also detected in the extracellular environment. As they do not possess an N-terminal SP, it is conceivable that they reach the cell surface via a nonconventional secretion route, as has been discussed (Chaffin *et al.*, 1998; Nombela *et al.*, 2006; Nickel, 2010). As the known functions of these proteins in *C. albicans* are directed toward 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* (Rodrigues *et al.*, 2007) 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 (Albuquerque *et al.*, 2008). Another possible explanation for cytosolic proteins in the extracellular environment is the presence of lysing cells or apoptotic cells, which can undergo membrane blebbing (Phillips *et al.*, 2003). Interestingly, after passing the culture supernatant through a 200-nm filter before protein preparation, significantly fewer proteins with normally intracellular localization are detected (Ene *et al.*, 2012; Heilmann *et al.,* submitted). In our studies, the majority of cytosolic proteins were found in the medium of hyphal- and fluconazole-treated cultures (Sorgo *et al.*, 2010, 2011), while in all other conditions, almost no proteins without an N-terminal SP 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.

GPI proteins in the growth medium

GPI proteins are consistently found in the growth medium of *C. albicans* and other yeasts (Hiller *et al.*, 2007; Madinger *et al.*, 2009; Stead *et al.*, 2009; Buerth *et al.*, 2011; Fig. 1). For detailed information on covalently attached cell wall proteins, the reader is referred to other reviews (Chaffin, 2008; Klis *et al.*, 2009). GPI proteins follow the secretory pathway but are either retained in the cell membrane or covalently attached to the cell wall (Pittet & Conzelmann, 2007). The presence of GPI proteins in the medium can be explained in various ways that do not exclude each other: (1) washing out of precursors of wall-bound GPI proteins. 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 (Lu *et al.*, 1994). (2) For full cell separation, not only the primary septum but also some wall material in the periphery of the neck region has to be degraded. (3) GPI proteins might also be released as a result of wall remodeling during isotropic growth, or when the wall is locally loosened to allow the formation of new buds or hyphal branches. Explanations (2) and (3) are consistent with the detection of β-1,3-glucan-associated Als3 and Hyr1 in the supernatant of *C. albicans* cultures (Torosantucci *et al.*, 2009). 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 (Heilmann *et al.*, 2011), Als3 was only found in the medium of hyphal cultures (Sorgo *et al.*, 2010).

Secreted proteins with roles in infection and nutrient acquisition

Numerous studies about the hydrolytic enzymes of *C. albicans* show the importance of this group of secreted proteins (Schaller *et al.*, 2005; Hruskova-Heidingsfeldova, 2008). The absence of some family members, from the lipases (Lips), phospholipases (Plbs), and aspartyl proteases (Saps) in the measured secretomes, is probably due to the tight regulation of secreted proteins. As laboratory conditions do not truly represent the host environment during infection, it is understandable that certain proteins (e.g. Lips, Saps) 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 examined, and more than 30% have only been detected under a single condition (Sorgo *et al.*, 2010, 2011; Ene *et al.*, 2012; Heilmann *et al.*, submitted).

The best studied secreted enzymes belong to the family of the secreted aspartic proteases, which comprises Sap1 through Sap10. Sap1 to Sap8 are secreted into the extracellular environment, while Sap9 and Sap10 are retained at the cell surface via a (modified) GPI anchor (Albrecht *et al.*, 2006). Saps are involved in multiple processes, like degradation of host tissues and proteins to facilitate invasion and nutrient uptake. Furthermore, they can degrade host immune proteins (Gropp *et al.*, 2009). While Sap1 to Sap3 activities are maximal at pH 3–5, Sap4 to Sap6 activities are optimal at pH 5–7, correlating with the fact that Sap4 to Sap6 are essential for systemic infections and were only present in the secretome of hypha-enriched cultures grown in the presence of GlcNAc at pH 7.4 (Felk *et al.*, 2002; Sorgo *et al.*, 2010). Accordingly, Sap2 and Sap3 were exclusively detected at pH 4. Also phospholipases are involved in tissue destruction and invasion. All five phospholipase B genes in *C. albicans* contain a signal sequence for secretion, while only *PLB3*, *PLB4.5*, and *PLB5* have a putative GPI attachment signal (De Groot *et al.*, 2003). Plb3 has been detected in fluconazole-stressed cultures but only at very low levels (Sorgo *et al.*, 2011), probably because the correct induction conditions were not met. Of the ten lipase genes encoded by *C. albicans,* all except *LIP7* contain an N-terminal signal for secretion. *LIP* genes were shown to be differentially expressed depending on the growth condition, and expression was independent of lipids (Hube *et al.*, 2000). Nevertheless, until now only Lip4 has been identified at very low levels in exponentially growing cultures with lactate as carbon source (Ene *et al.*, 2012).

Apart from hydrolytic enzymes, *C. albicans* also secretes proteins to sequester metal ions. Zinc is an important trace metal required for microbial growth. Zinc uptake is facilitated by two proteins, the secreted protein Pra1 and the zinc transporter Zrt1 (Citiulo *et al.*, 2012). Pra1 (pH-regulated antigen) is highly expressed at neutral pH and shows negligible expression at acidic pH (Sentandreu *et al.*, 1998). Upon host cell penetration, *C. albicans* secretes Pra1 into the host cell cytosol, scavenges available zinc, and re-associates with the fungal cell, where it interacts with the zinc transporter Zrt1 to enable zinc uptake. Interestingly, Pra1 is recognized by a leukocyte receptor protein, and this probably explains why pra1 mutant cells are more resistant to leukocyte killing and more virulent in a murine model of systemic infection (Soloviev *et al.*, 2011). Freely available iron is also very scarce during infection, and iron is actively scavenged by *C. albicans* from its host. All five members of the *C. albicans* Rbt5 family, comprising Csa1, Csa2, Pga7, Pga10, and Rbt5, are CFEM proteins, which are characterized by the possession of one or more 8-cysteine-containing domains. Upon iron starvation, all genes of the *RBT5* family are directly activated by Sef1, a transcription factor for iron-uptake genes (Chen *et al.*, 2011). Additionally, Rbt5, Pga10, and Csa1 have been shown to be involved in heme binding (Weissman & Kornitzer, 2004). Csa2 is a small non-GPI protein (146 amino acids including its predicted 18 amino acid SP) and is only detected in the medium, while the others are GPI proteins that are covalently linked to the wall or plasma membrane. Although the function of Csa2 is unknown, these data suggest that it is involved in iron acquisition as well. Conceivably, it might function similar to Pra1.

Msb2 is a signaling mucin with a large, heavily glycosylated extracellular domain, a single transmembrane sequence, and a short cytoplasmic domain. It senses cell wall damage and activates the Cek1 MAP kinase pathway (Roman *et al.*, 2009). Despite its transmembrane sequence, Msb2 will be discussed here, because its extracellular domain is regularly found in the medium. It is cleaved off close to the plasma membrane and released into the extracellular environment (Szafranski-Schneider *et al.*, 2012). In contrast to the *S. cerevisiae* homolog ScMsb2, which is processed by the GPI-anchored Sap9 ortholog ScYps1 (Vadaie *et al.*, 2008), shedding in *C. albicans* is not dependent on Sap9 or Sap10 activity (Szafranski-Schneider *et al.*, 2012). Proteomic analysis has identified peptides originating from the cleavage region of Msb2 under almost every culture condition. This region is not glycosylated, which facilitates the identification of Msb2 (Sorgo *et al.*, 2010, 2011; Ene *et al.*, 2012; Szafranski-Schneider *et al.*, 2012; Heilmann *et al.*, submitted). Strikingly, the liberated extracellular part of Msb2 binds antimicrobial peptides, thus protecting *C. albicans* from the host immune response (Szafranski-Schneider *et al.*, 2012).

Wall remodeling, cell separation, and accumulation of extracellular matrix

Secreted proteins with wall-related functions are presumably very abundant, as multiple tryptic peptides were detected in almost every growth condition (Fig. 2). The core set of seven secreted proteins detected in all conditions examined are glycosyl hydrolases (Table 1). They are generally responsible for maintaining cell wall integ-

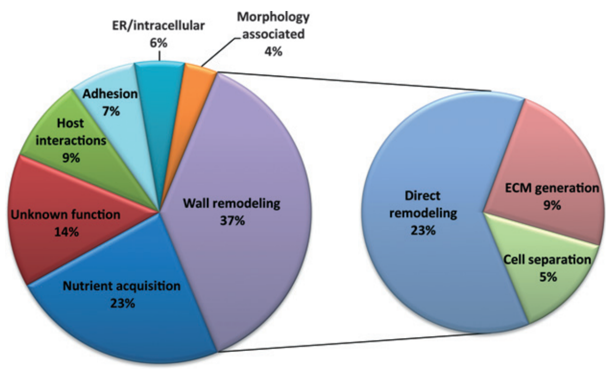

Fig. 2. Functions of SP-containing proteins identified in the growth medium of *Candida albicans.* The majority of the 60 secreted proteins identified are responsible for wall remodeling and nutrient acquisition. Others are required for the acquisition of metal ions, the interaction with the host or biofilm formation, or serve a yet to be determined function.

Table 1. Core set of abundantly secreted proteins of *Candida albicans*

Protein	Function
Cht3	Major chitinase; cell separation
Mp65	Abundant transglycosylase; biomarker
Scw11	Glycosyl hydrolase; cell separation
Sim1	Essential glycosidase; SUN domain
Sun41	Essential glycosidase; SUN domain
Tos1	Glycosidase
Xog1	Glycosidase; ECM formation

ECM, extracellular matrix.

rity and wall remodeling, and many of them are involved in cell separation, acting downstream of the regulation of Ace2 and morphogenesis (RAM) network (Saputo *et al.*, 2012).

Sun41 and Sim1/Sun42 belong to the SUN family as they both contain the so-called 'SUN' domain. Like their ortholog 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 (Mouassite *et al.*, 2000; Firon *et al.*, 2007). Both secreted proteins were detected consistently under all growth conditions examined. Furthermore, they are required to maintain wall integrity of the mother cell after cell separation, which suggests them acting downstream of the RAM pathway (Firon *et al.*, 2007).

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 (Lee *et al.*, 2012). 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 (Heilmann *et al.*, submitted). 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 (Sorgo *et al.*, 2010, 2011; Heilmann *et al.*, 2011; Sosinska *et al.*, 2011). 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 (Sorgo *et al.*, 2010, 2011). Deletion of *CHT3* in a yeast cell culture resulted in chains of cells that were not fully separated, underlining its importance during cytokinesis (Dünkler *et al.*, 2005). Also, the endoglucanase Eng1 and the glucanase Scw11 are involved in cell separation, as a mutation in *ENG1* or *SCW11* led to the formation of cell clusters (Kelly *et al.*, 2004; Esteban *et al.*, 2005). Expression of *CHT3, ENG1,* and *SCW11* is regulated by the transcription factor Ace2 (Kelly *et al.*, 2004; Mulhern *et al.*, 2006). 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 (Kelly *et al.*, 2004). Cultures grown at 42 °C formed SDS-resistant cell aggregates, accompanied by decreased secretion of Cht3, Eng1, and Scw11, suggesting that the role of Ace2 in cell separation might be suppressed during thermal stress (Heilmann *et al.*, submitted). Similar but less pronounced effects, including elevated chitin levels, were observed in cultures treated with the membrane-perturbing antifungal compound fluconazole, which, indirectly, also causes wall stress (Pfaller & Riley, 1992; Sorgo *et al.*, 2011).

As 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 (Martinez *et al.*, 2004; Klis *et al.*, 2009). In agreement with its involvement in cell wall cross-linking, heterozygous mutants display a cell wall defect accompanied by increased clumping. While interconnection of β-1,3-glucan is important for general structural integrity, remodeling is just as important for general plasticity of the wall and during growth. The roles of Mp65, a putative transglycosylase, and Tos1, which are both abundant secreted proteins under all conditions examined, 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 (Gonzalez *et al.*, 1997), 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 (Hartland *et al.*, 1991; Sarthy *et al.*, 1997). Bgl2 was only found in the medium at low levels at 42 °C and during fluconazole exposure. In *S. cerevisiae*, ScBgl2 is strongly associated with β-1,3-glucan and is robust enough to stay functionally active after SDS boiling (Klebl & Tanner, 1989). 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 (Taff *et al.*, 2012). Individual knockout mutants formed less persistent biofilms that sequestered less fluconazole than the reference strain. Intriguingly, this phenotype did not affect the overall composition of β-glucan in the wall itself. As *PHR1* and *PHR2* serve the same function but are expressed at a different pH, Phr2 might contribute to biofilm formation as well. 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).

Outlook

Secretory proteins in the culture medium have multiple functions that are essential for fungal fitness and virulence (Fig. 3). Secreted proteins with wall-related functions are required for the constant remodeling of the wall due to

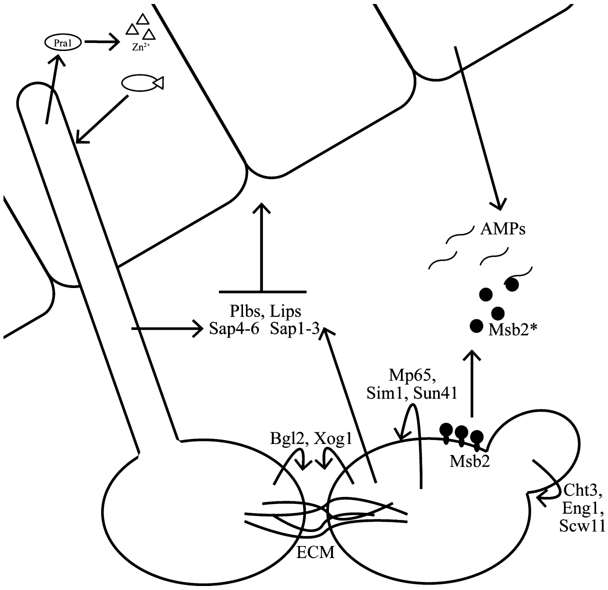

Fig. 3. Schematic representation of the most important functions of several secreted proteins (not to scale). Upon tissue penetration, Pra1 is secreted to scavenge zinc from the host. Plbs, Lips, and Saps are secreted into the environment to facilitate tissue destruction and nutrient acquisition. While Sap4–6 are produced by hyphae, Sap1–3 are yeast cell specific. Bgl2 and Xog1 are crucial for the formation of extracellular matrix (ECM). Mp65, Sim1, and Sun41 are involved in wall remodeling and possibly also biofilm formation (Hiller *et al.*, 2007; Sandini *et al.*, 2011b), while Cht3, Eng1, and Scw11 are important for cell separation. The extracellular part of Msb2 (Msb2*) is released into the environment where it can bind antimicrobial peptides (AMPs).

morphological adaptations, growth and cell separation, and cell wall repair. This correlates with the high number of peptide identifications in almost all growth conditions examined. Cht3, Mp65, Scw11, Sim1, Sun41, Tos1, and Xog1 were found in every condition tested with ample peptide identifications (Table 1). As they are accessible and abundant, this set of proteins might be used in serological detection of invasive candidiasis, both by direct detection of these proteins in host samples and by detection of antibodies elicited in the host against these proteins (Laín *et al.*, 2008; Ostrosky-Zeichner, 2012).

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 (Vilanova *et al.*, 2004; Sandini *et al.*, 2011a). 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.

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