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Klis, F.M.; Brul, S.

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Adaptations of the Secretome of Candida albicans in Response to Host-Related Environmental Conditions

Frans M. Klis, Stanley Brul
Department of Molecular Biology and Microbial Food Safety, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands

The wall proteome and the secretome of the fungal pathogen Candida albicans help it to thrive in multiple niches of the human body. Mass spectrometry has allowed researchers to study the dynamics of both subproteomes. Here, we discuss some major responses of the secretome to host-related environmental conditions. Three β-1,3-glucan-modifying enzymes, Mp65, Sun41, and Tos1, are consistently found in large amounts in culture supernatants, suggesting that they are needed for construction and expansion of the cell wall β-1,3-glucan layer and thus correlate with growth and might serve as diagnostic biomarkers. The genes ENG1, CHT3, and SCW11, which encode an endoglucanase, the major chitinase, and a β-1,3-glucan-modifying enzyme, respectively, are periodically expressed and peak in M/G1. The corresponding protein abundances in the medium correlate with the degree of cell separation during single-yeast-cell, pseudohyphal, and hyphal growth. We also discuss the observation that cells treated with fluconazole, or other agents causing cell surface stress, form pseudohyphal aggregates. Fluconazole-treated cells secrete abundant amounts of the transglucosylase Phr1, which is involved in the accumulation of β-1,3-glucan in biofilms, raising the question whether this is a general response to cell surface stress. Other abundant secretome proteins also contribute to biofilm formation, emphasizing the important role of secretome proteins in this mode of growth. Finally, we discuss the relevance of these observations to therapeutic intervention. Together, these data illustrate that C. albicans actively adapts its secretome to environmental conditions, thus promoting its survival in widely divergent niches of the human body.

The fungal pathogen Candida albicans is a highly specialized inhabitant of warm-blooded animals (mammals and birds). It preferentially colonizes mucosal surfaces and the skin but can also invade deeper-lying tissues and cause systemic infections that are difficult to treat and frequently lethal (1). To survive under the challenging and divergent conditions associated with the various mucosal surfaces in the human body, C. albicans disposes of a wide arsenal of virulence traits that help it to cope with antimicrobial peptides, the complement system, engulfment by macrophages, antibodies, hypoxic conditions, iron restriction, etc. A fascinating trait is its ability to switch reversibly between various growth forms, including among others the single-cell yeast form, which is especially suitable for dispersion of the fungus; the hyphal form, which facilitates adhesion to host tissues and promotes invasive growth and escape from engulfment by immune cells; and an intermediate, pseudohyphal growth form. C. albicans also forms biofilms (surface-associated microbial communities), which clinically speaking represent a highly relevant mode of growth and in which yeast, pseudohyphal, and hyphal cells cooccur and become encapsulated by substantial amounts of extracellular, macromolecular material. Biofilm formation on abiotic surfaces of medical devices and prostheses and on teeth has therefore been extensively studied (2, 3). The first contacts between C. albicans and host cells occur predominantly at the cell surface, and this presumably explains why the external protein coat of C. albicans cell walls consists of a wide variety of glycoproteins with specialized functions, many of which are under tight control, thus promoting survival under diverse stress conditions (4, 5). Equally important, C. albicans secretes a variety of glycoproteins that help to forage for nutrients by degrading host proteins, lipids, and glycogen, while others acquire iron and zinc ions and provide protection against antimicrobial peptides. Other glycoproteins help to form and strengthen biofilms and to accumulate extracellular matrix material. Together, we designate these secreted proteins as the secretome sensu stricto (see below). The introduction of mass spectrometry in protein research has made it possible to study the protein assortment of entire cells or tissues and also well-defined subsets of proteins (subproteomes such as the cell wall proteome and the secretome), not only qualitatively but also quantitatively. This review discusses recent mass spectrometric explorations of the dynamics of the secretome of C. albicans depending, for example, on growth form and pH or in response to cell surface stress. For complementary reviews, the reader is referred to reference 4, which includes an extensive section about secretome proteins, and to a more recent review (6). In addition, the Candida albicans PeptideAtlas and the Candida Genome Database are recommended for detailed information about mass spectrometrically identified peptides (7, 8).

CLASSIFICATION OF MAJOR SECRETOME PROTEINS (SENSU STRICTO) OF C. ALBICANS

In this review, we will restrict ourselves to the secretome in the narrow sense of the word (secretome sensu stricto), that is, we will discuss only those proteins that possess an N-terminal signal peptide for entering the classical secretory pathway and lack internal transmembrane sequences. The major advantages of analyzing...
Foraging enzymes
- Gca1/2, Hex1
- Lips, Plbs, Saps
- Pho100
- Csa2, Pra1

Unknown function
- Coil, Pry family

Biofilm formation
- Als3, Bgl2, Phr1, Xog1

Cell separation-related
- Enzymes: Chl1, Eng1, Scw11 + GPI-WPs

FIG 1 Major features of the secretome (sensu stricto) of *C. albicans*. The wall proteins are represented as short line segments perpendicular to the cell surface. M, mother cell; D, daughter cell; GPI-WP, GPI-modified wall protein. The role of Csa2 in heme binding is speculative (6, 40, 41). Note that Als3 and Phr1 are possibly directly released from the cell wall by Sap9/10 activity (47).

this protein category separately are that it is physiologically well defined, is limited in size, and is not affected by accidentally released proteins. This facilitates statistical analysis of the data (9), simplifies discussion of the results, and leads to physiologically relevant conclusions and testable hypotheses. For more information about the secretome in the wide sense of the word (secretome sensu lato), which includes a number of known cytosolic proteins, we refer to references 6 and 10.

The experimentally identified secretome proteins (sensu stricto) (currently, about 70 [9–15]) can be classified into two major groups: nonglycosylphosphatidylinositol (non-GPI) proteins, which lack a C-terminal signal sequence for the addition of a GPI anchor in the endoplasmic reticulum, and GPI proteins (Fig. 1). To avoid an excessively long list, this review focuses on the more abundant secretome proteins that are observed under more than one growth condition and/or have a known or predicted function.

NON-GPI PROTEINS
Modification of cell wall polysaccharides. Eleven enzymes (distributed over six families of glycosylhydrolases [GHs]) are involved in glycan chain elongation and branching and in glycan degradation (for example, during cell separation and emergence of a new bud or hyphal branch). Throughout this paper, we will follow the GH family classification according to the CAZy (for carbohydrate-active enzymes) database (16).

(i) β-1,3-Glucan-modifying enzymes. β-1,3-Glucan-modifying enzymes include Xog1 (GH5); Tos1 (GH16); Bgl2, Mp65, and Scw11 (GH17); Eng1 (GH81); Sim1 and Sun41 (GH132); and Dag7 (Barwin-like endoglucanase domain, PF03330) and GH (unspecified) (8).

(ii) Chitin-degrading enzymes. Chitin-degrading enzymes include Chl1 and Chl3 (GH18); they carry out degradation of the primary septum between mother and daughter cells, thus initiating cell separation. Note that the GH18 family member Chl2 is a GPI protein.

Three of the 11 corresponding genes (CHT3, which encodes the major chitinase; ENG1, which encodes an endo-β-1,3-glucanase; and SCW11, which encodes a β-1,3-glucan-modifying enzyme) are periodic genes with maximal expression in the M/G1 period of the cell cycle and are target genes of the transcription factor Ace2 (17–20), consistent with a role in cell separation. Agar-grown colonies, mucosal biofilms, and biofilms formed on abiotic surfaces produce abundant amounts of extracellular matrix material (3, 21–23). The transglucosylase Bgl2 and the exoglucanase Xog1 (together with the GPI-modified, putative β-1,3-glucan-elongating enzyme Phr1) seem to be directly involved in formation and modification of extracellular matrix material in biofilms (24).

Nutrient acquisition. (i) Glucoamylases. Glucoamylases include Gca1 and Gca2 (GH31); the predicted substrates are glycogen and starch (4, 16, 25, 26). Interestingly, maltose, which is a degradation product of both polysaccharides, is a carbon source known to promote hyphal growth (27). It has been suggested previously that Gca1 and Gca2 are directly involved in promoting matrix production in biofilms by enzymatic release of soluble β-1,3-glucan fragments from insoluble β-1,3-glucan chains (28). However, Gca1 and Gca2 possess the hallmarks of an α-glucosidase/glucoamylase, cleaving alpha-1,4-glucosidic linkages (8), and hence, it seems unlikely that they might cleave beta-1,3-gluco- sidic linkages, which have a spatial structure highly different from that of alpha-1,4-glucosidic linkages.

(ii) N-Acetylhexosaminidase. HEX1 is a glucose-repressed gene (29). Hex1 (GH20) is found in the periplasm and in the culture supernatant (29). It is possibly involved in the release of GlcNAc residues from host tissues for use as a carbon or nitrogen source (29). Note that GlcNAc also acts as a signaling molecule and induces and maintains hyphal growth in glucose-repressed cells (30–33).

(iii) Lipid degradation by Lips and Plbs. Lipids are degraded by lipases (Lips) including Lip1 to Lip6 and Lip8 to Lip10 and by phospholipases (Plbs) including Plb1 and Plb2, reviewed in references 4 and 34.

(iv) Protein degradation by aspartyl proteases. Protein degradation by aspartyl proteases includes, for example, degradation of mucins and host immune proteins (4, 34–37), by aspartyl proteases such as Sap1 to Sap8. Note that Sap9 and Sap10 are cell surface-associated GPI proteins (38).

(v) Metal ion acquisition. Metal ion acquisition includes acquisition of zinc by Pra1 (39) and, probably, acquisition of heme (iron) by Csa2 (6, 12, 40, 41). Intriguingly, Pra1 is also involved in immune evasion (42, 43).

Pry family. The Pry family (11) consists of five members: Pry1, Rbl1, Rbt4, and two uncharacterized open reading frames (ORFs) (19.6200 and 19.2336). Rbl1 and Rbt4 have been often identified in culture supernatants (10–14). Interestingly, whereas Rbl1 is much more abundant in yeast culture supernatants, Rbt4 is much more abundant in hyphal culture supernatants (11–13) (see also Table 2). The virulence of an ∆rbl1 ∆rbt4 double deletion strain in the mouse model for systemic infection is strongly diminished (11), but the precise function of the Pry family proteins is still unknown.

Coil. Coil is a small protein of 191 amino acids that is relatively abundant and is consistently found in the medium under all
growth conditions tested (10–14). Homologous proteins are found in only a limited number of Candida spp.—Candida dubliensis, Candida orthopsilosis, Candida parapsilosis, and Candida tropicalis—and in Lodderomyces elongisporus (8). Its function is unknown.

Signaling protein Msb2. The signaling protein Msb2 is located in the plasma membrane and involved in signaling through activation of the Cek1 mitogen-activated protein (MAP) kinase (44). Although Msb2 has an internal transmembrane sequence, it is included in this classification and in the secretome sensu stricto, because it has a large extracellular, highly O-glycosylated domain that is shed into the medium and consistently identified in culture supernatants (10–14, 45). Interestingly, it also serves as a broad-range protectant against antimicrobial peptides (45, 46).

GPI PROTEINS

GPI proteins are targeted to the plasma membrane or become covalently linked to the β-glucan layer of the cell wall through their GPI anchor or are found at both locations. However, most GPI proteins are also identified in culture supernatants (10–14). There are several (possible) explanations for their presence.

Target proteins of Sap9 and Sap10. Some wall-bound GPI proteins are released by the surface-bound aspartyl proteases Sap9 and Sap10, such as Cht2 (47). Other candidates for active and controlled release, such as the adhesion protein Als3 and the transglucosylase Pfr1, will be discussed below.

GPI proteins from the neck region. In single-cell yeast cultures, wall-bound GPI proteins are released from the neck region during cell separation. The wall between mother cell and growing bud is continuous, and complete cell separation therefore requires not only degradation of the primary septum by chitinase activity but also degradation of the lateral wall in the neck region.

Accidental release. Especially in shaken cultures, wall-bound GPI proteins might be released during emergence of a new bud or hyphal branch, which requires localized cell wall softening, or during periods of isotropic growth, which requires insertion of new cell wall polysaccharides and wall proteins into the existing wall.

Wall protein precursors may be washed out into the medium before they become covalently linked to the glucan-chitin network, especially in shaken cultures.

DYNAMICS OF THE SECRETOME OF C. ALBICANS

In this section, the term “apparent abundance” is introduced. It is defined as the number of spectral counts per protein divided by the total number of spectral counts of all secretome proteins (sensu stricto) and expressed as a percentage (48). This is a semi-quantitative measure that allows comparison of the individual contributions of the secretome proteins and, importantly, allows estimating and comparing the fold changes of individual secretome proteins upon changes in environmental conditions, including conditions that induce growth as single yeast, pseudohyphal, and hyphal culture supernatants and during pseudohypha-like growth induced by fluconazole (Table 1). Similar values for their apparent abundances have been obtained under diverse growth conditions (6, 9, 10, 12–14). Conceivably, they are involved in various ways in the construction and remodeling of the β-1,3-glucan layer in the cell wall during growth (8, 16, 51–55). Their combined apparent abundance accounts for one-fourth to one-third of all secretome proteins. Consistent with this, the gene sequences of Mp65, Sun41, and Tos1 have a relatively high codon bias index (MPI65, 0.71; SUN41, 0.64; TOs1, 0.59 [8]), suggesting that these genes are strongly expressed. Surprisingly, although Mp65 lacks a C-terminal GPI anchor to connect it covalently to the β-glucan network, it is usually also found in (hot-SDS-extracted) cell walls (10, 13, 14, 30, 37, 40). Scwl1 and Sim1 belong to the same families as Mp65 and Sun41, respectively, and both show high apparent abundances in single-cell yeast cultures (Tables 2 to 4). However, in hyphal cultures and to a lesser extent also in pseudohyphal-growth cultures, their apparent abundances are considerably lower, suggesting that Scw11 and Sim1 might play a direct role in cell separation.

Yeast state-enriched secretome proteins: yeast versus hyphal cultures. In single-cell yeast cultures, the daughter cells become separated from the mother cell, a process that requires chitinase activity to degrade the primary septum formed during cytokinesis and β-1,3-endoglucanase activity to degrade the β-1,3-glucan layer in the lateral wall of the neck region. Conceivably, also transglucosylase activity is needed for repair activity. This results in much higher apparent abundances of the cell separation enzymes Cht3 and Eng1 and the potential repair enzyme Scw11 in the culture solution of unicellular budding yeast than in hyphal cells (Table 2). Although the apparent abundances of Bgl2, Cht1, Dga1, Rbe1, Sim1, and Xog1 in the culture solution of hyphal cultures are also strongly reduced, the corresponding genes do not seem to be periodically expressed (8, 17).

Hyphal state-enriched secretome proteins: hyphal versus yeast cultures. Many host-related chemical and physical conditions, such as a neutral pH, a temperature of 37°C, low oxygen levels and high CO₂ concentrations, the presence of GlcNAc (de-
N-acetylglucosamine (GlcNAc) initiates and sustains hyphal growth (31, 32, 59). GlcNAc is, for the human body, GlcNAc and also GlcNAc derivatives strongly glucose-derepressing conditions, which occur in many niches in vivo. It is unknown whether Als3 is passively released from the cell wall or might become enriched with released Als3 and whether it might promote the cohesion of the extracellular matrix material.

Sun41, and Tos1 (Tables 1 and 2), namely, the aspartyl proteases Pir1, and apoptosis. Of interest, Als3 is a hypha-specific adhesive GPI protein with the GPI protein is in bold.

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Major changes in apparent abundance in the secretome of fluconazole-treated cells. Pseudohyphal (or pseudohypha-like) growth can be induced in various ways, for example, by treating single-cell yeast cultures with sublethal concentrations of the antifungal compound fluconazole (14). Fluconazole blocks an Erg11-mediated demethylation step of the planar ring structure during ergosterol synthesis, and this results in the formation of structurally suboptimal sterols and increased fluidity of the plasma membrane (66). The cells respond by suppression of cell separation and by increased phosphorylation of the MAP kinase Mkc1 (14), which mediates the cell wall integrity pathway. Pseudohypha-like cell aggregates are formed, which separate into single cells when treated with exogenous chitinase (14). These effects are not specific for fluconazole but also occur when the cells are treated with the detergent SDS, grown at the harmful temperature of 42°C, or treated with the cell wall construction-perturbing compound calcofluor white or Congo red (14, 51) (Fig. 2). This suggests that many forms of plasma membrane stress or cell wall stress, together here referred to as cell surface stress, will cause pseudohypha-like growth and constitutively activate the cell wall integrity pathway. Indeed, cell separation suppression is a common phenotype among all genetic mutants that have to cope with an ammonium salt as a rich nitrogen source in the culture medium at neutral pH does not seem to interfere with the accumulation of Sap4 and Sap6 at high levels in the medium (Table 2).

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tion of secretory glycoproteins (67–70) or lack proteins involved in cell wall formation, such as Bgl2 (71); Ecm33, which is a GPI protein required for cell wall integrity (72); the secretome enzyme Sun41 (51, 52); and the GPI-modified and cell-surface-located aspartyl proteases Sap9 and Sap10 (38), as well as Pir1, a putative cross-linker of β-glucan chains (73), and this list could be easily extended. Interestingly, CHT3 transcription is markedly down-regulated in micafungin-treated cells (74), suggesting that this inhibitor of β-1,3-glucan synthesis might also induce cell separation suppression. Similar observations have been described by O’Meara and coworkers, who treated wild-type cultures with serum in combination with various antifungal drugs at sublethal concentrations and found that under these conditions hyphal growth was suppressed and, instead, pseudohyphal cell clusters were formed (see Fig. 2c in reference 75). A relevant implication of these observations is that “cell separation suppression” as a phenotype does not necessarily prove that the mutated gene is directly involved in cell separation.

Consistent with the partial suppression of cell separation in fluconazole-treated cells, resulting in pseudohypha-like growth, the major chitinase Cht3 (GH18), the endo-β-1,3-glucanase Eng1 (GH81), and the β-1,3-glucan-modifying enzyme Scw11 are all decreased in the supernatants of fluconazole-treated cultures, although less so than in hyphal cultures (Tables 2 and 3). Other chitin- and β-1,3-glucan-modifying enzymes, namely, Cht1 (GH18) and Sim1 (GH132), are also decreased in fluconazole-treated and hyphal cells. How exactly suppression of cell separation is regulated in response to cell surface stress is currently unknown. As CHT3, ENGI, and SCW11 are known target genes of the transcription factor Ace2 (18–20), a regulator of cell separation that controls the expression of M/G1-specific genes, it seems likely that fluconazole treatment and many other forms of cell surface stress lead to suppression of Ace2 activity in cell separation. Possibly, cell separation is suppressed through phosphorylation of the transcription factor Efg1, which in turn represses Ace2 target genes and promotes pseudohypha-like growth (76).

Another striking observation is the high apparent abundance of the GPI protein Pfr1 in the medium of fluconazole-treated cells, whereas it is not detectable in the medium of single-cell yeast cultures and hyphal cultures. This observation is consistent with the finding that the antifungal compounds ketoconazole and caspofungin stimulate the expression of PRHI (77). Pfr1 (GH72) is a pH-responsive transglucosylase and probably involved in expansion of the β-1,3-glucan layer (78, 79). It also plays an important role in the accumulation of β-1,3-glucan in the extracellular matrix of biofilms, possibly by extending the β-1,3-glucan chains there and thus increasing the cohesiveness of the biofilm (24). This raises the interesting question of whether biofilm-associated Phr1 in fluconazole-treated cells might contribute to protection against fluconazole. It is currently unknown how Phr1 is released from the cell surface. However, Phr1 contains two consecutive lysine residues (K451 and K452), suggesting that it might be a substrate of Sap9/10 (47).

Finally, the culture medium of fluconazole-treated yeast cultures contains several low-abundance secretory proteins that so far have not been observed under other growth conditions (13). This does not necessarily prove that this secretome response is specific for fluconazole-treated cells, because, as we have seen above, many other conditions lead to cell surface stress, and this might be of importance in glucose- derepressed cells (83). For example, the levels of three secretose proteins, glucoamylase(s) Gca1/2 (GH31), the aspartyl protease Sap7, and the exo-β-1,3-glucanase Xog1 (GH15), strongly increase when lactate, a nonfermentable carbon source and a much poorer carbon source than glucose, is used to support growth (9, 84). On the other end of the spectrum, there are also a number of secretose proteins whose apparent abundances strongly decrease in lactate-grown cultures (9). The induction of hyphal growth by GlcNac as discussed above is already blocked at a glucose concentration of 20 mM (33), and this represents another striking example of the importance of selecting the appropriate carbon source and/or glucose concentration in Candida research.

CONCLUDING REMARKS

Similarly to the wall proteome, the secretome of C. albicans operates at the fungus-host interface, and pronounced changes in abundance of individual proteins occur in distinct host niches. For example, the switch from single-cell yeast growth to hyphal growth results in the abundant secretion of the aspartyl proteases Sap4 and Sap6, which significantly contribute to virulence (85). As expected, the pH of the host niche also strongly affects the composition of the secretome. The ability to form biofilms, in which
yeast, pseudohyphal, and hyphal cells are found, with the concurrent accumulation of extracellular matrix material and the often strongly lowered susceptibility to antifungal agents, is another important virulence trait. Several secretome proteins, such as Als3, Bgl2, Mp65, Phr1, Xog1, and Sun41, are believed to be involved in biofilm formation (8). In view of their high apparent abundances under widely diverging growth conditions, the secretome proteins Mp65, Sun41, and Tos1 seem attractive candidates for diagnostic purposes. In addition, as Candida infections are usually associated with invasive, hyphal growth, and as Als3, Rbt4, Sap4, and Sap6 become abundant in the supernatants of hyphal cultures, these proteins also are potential diagnostic candidates. Two secretome proteins, Als3 (actually, a recombinant protein that covers the N-terminal immunoglobulin-like domain of Als3) and Sap2, are currently being actively pursued for vaccine development (86–88). Als3 is an abundant, multifunctional GPI-modified cell wall protein (86, 89, 90) and as such is a highly attractive vaccine candidate. The finding that Als3 also becomes an abundant secretome protein in hyphal cultures (12) with a proposed role in biofilm formation further increases its attractiveness. It has been argued that a multivalent vaccine might be more effective to combat the various types of Candida infections (91). Conceivably, a recombinant protein consisting of two or three of the most immunogenic epitopes from various cell wall proteins (92) and from secretome proteins such as the aspartyl proteases Sap4 and Sap6 (12, 85); the three secretome proteins involved in the formation of biofilm matrix beta-glucan (Bgl2, Phr1, and Xog1) (24); Sun41 and Sim1, which form a two-member family and are synthetically lethal (52); and the Pry proteins Rbe1 and Rbt4 (11) each separated from the other by a nonimmunogenic linker sequence, might be an option. Similar approaches might be considered for other pathogenic fungi. Finally, many regulatory pathways have been shown to be involved in controlling the abundance of individual secretome proteins, but more-systematic studies of how the secretome as a whole is regulated are still scarce.

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