Review

Cell wall proteome of pathogenic fungi*

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A fast development of a wide variety of proteomic techniques supported by mass spectrometry coupled with high performance liquid chromatography has been observed in recent years. It significantly contributes to the progress in research on the cell wall, very important part of the cells of pathogenic fungi. This complicated structure composed of different polysaccharides, proteins, lipids and melanin, plays a key role in interactions with the host during infection. Changes in the set of the surface-exposed proteins under different environmental conditions provide an effective way for pathogens to respond, adapt and survive in the new niches of infection. This work summarizes the current state of knowledge on proteins, studied both qualitatively and quantitatively, and found within the cell wall of fungal pathogens for humans, including *Candida albicans*, *Candida glabrata*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and other medically important fungi. The described proteomic studies involved the isolation and fractionation of particular sets of proteins of interest with various techniques, often based on differences in their linkages to the polysaccharide scaffold. Furthermore, the proteinaceous contents of extracellular vesicles ("virulence bags") of *C. albicans*, *C. neoformans*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* are compared, because their production can partially explain the problem of non-classical protein secretion by fungi. The role assigned to surface-exposed proteins in pathogenesis of fungal infections is enormously high, thus justifying the need for further investigation of cell wall proteomes.

Key words: proteomics, fungal pathogens, cell wall, *Candida*, *Aspergillus*, *Cryptococcus*

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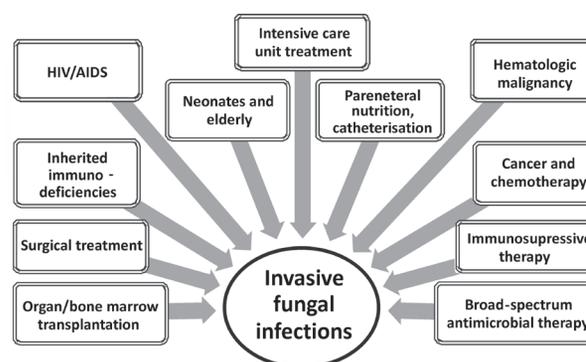
Figure 1. The main risk factors predisposing to invasive fungal infections (based on data of Rüping *et al.*, 2008; Hope *et al.*, 2013; Guinea, 2014; Köhler *et al.*; 2014; Quindós, 2014).

INTRODUCTION

The kingdom of fungi includes a wide diversity of organisms, some of which are dangerous pathogens for humans (Köhler *et al.*, 2014). Recent data indicate the increasing incidence of invasive fungal diseases, resulting in high mortality and morbidity, especially among people with serious and prolonged immunosuppression (Rüping *et al.*, 2008; Guinea, 2014; Quindós, 2014). The main risk factors of developing invasive fungal infections are schematically summarized in Fig.1.

Azie *et al.* (2012) showed that among over seven thousands of fungal species isolated from patients in different hospitals in the United States and Canada, 74.3% represented the genus *Candida* (of which *C. albicans* and *C. glabrata* constituted 47.8% and 25%, respectively), 13.3% were from the genus *Aspergillus*, with a predominance of *A. fumigatus*, and 6.2% belonged to other yeasts,

including *Cryptococcus* spp. A representative of the latter genus — *C. neoformans* — causes a fatal meningitis that results in the death of more than 600 000 people worldwide, mainly the patients suffering from AIDS (Park *et al.*, 2009). However, the importance of less common infections caused by other emerging fungal pathogens such as mucormycetes, hyalohyphomycetes, phaeohyphomycetes or endemic fungi should not be underestimated (Hope *et al.*, 2013; Köhler *et al.*, 2014).

Taking into consideration a limited susceptibility of some fungi, for instance *Candida* spp., *A. fumigatus* or *Mucorales* (Maroszynska *et al.*, 2013; Köhler *et al.*, 2014), to currently used antifungal drugs, the investigation of molecular mechanisms involved in the fungal pathogenicity is an urgent challenge. Fungal cell wall is that part of the cell that not only acts as a protective structural shield but also contributes to interactive contacts with the human host during the initiation of the infection and its further development. Thus, it is a potential target for new drugs, also because it is a structure essentially distinct from the surface and other elements of the host cells (Tada *et al.*, 2013).

Although the complex structure of fungal cell wall has been extensively studied in recent years, particularly in

the most commonly isolated pathogenic species (Chaffin, 2008; Klis *et al.*, 2011; Free, 2013; Beauvais *et al.*, 2014), still many issues remain to be elucidated. These unresolved problems include, in particular, the exact composition of polysaccharide or proteinaceous constituents of the cell wall as well as the nature of cross-links between these two major classes of cell wall components, the sophisticated regulation of the cell wall remodeling during mating, budding, hyphae formation or other morphological changes, the identification of numerous surface proteins the functions of which often remain unrecognized, unraveling the secret of the secretion and incorporation to the polysaccharide scaffold of several atypical proteins that lack a N-terminal signal peptide, directing them to the classical secretory pathway.

The aim of this review was to summarize the currently available knowledge of the cell wall proteome of certain species of human pathogenic fungi, since the development of advanced proteomics techniques actually allowed to obtain the insight into the detailed composition of this part of the pathogens' cells, which is an important contributor to the fungal survival in the physiological and pathological conditions.

THE COMPOSITION OF THE FUNGAL CELL WALL

In general, the fungal cell wall is composed of different types of linear and branched polysaccharides, proteins, often with various posttranslational modifications, lipids and other components such as melanins (Eisenman *et al.*, 2005; Klis *et al.*, 2006; Pihet *et al.*, 2009; Guimarães *et al.*, 2011; Puccia *et al.*, 2011; Free, 2013). However, between the particular species from this very diverse group of organisms considerable differences in cell wall composition have been observed even at the level of the polysaccharide scaffold. To mention only the most interesting examples, while *C. albicans* cell wall is composed mainly of β-1,3-glucan branched with β-1,6-glucan, N- and O-mannosylated proteins and a smaller amount of chitin (Kapteyn *et al.*, 1995), the cell wall of *A. fumigatus* is devoid of β-1,6-glucan but rich in α-1,3-glucan, β-1,3/1,4-glucan and proteins modified with N- and O-linked galactomannans (Bernard & Latgé, 2001). The cell wall of *C. neoformans* is built of β-1,3-glucan, β-1,6-glucan and α-1,3-glucan and the cell wall proteins are modified by asparagine-attached xylomannans (Park *et al.*, 2012). Additionally, this fungus possesses a complex, thick capsule composed of glucuronoxylomannan, galactoxylomannan and a smaller amount of mannoproteins, which jointly exert a great impact on the host immune system (Zaragoza *et al.*, 2009). The dimorphic fungus, *Histoplasma capsulatum,* possesses the cell wall composed of chitin, soluble galactomannan, β-1,3-glucan and α-1,3-glucan (Kanetsuna *et al.*, 1974), whereas the *Paracoccidioides brasiliensis* cell wall, apart from the above-mentioned polysaccharides, also contains β-1,6-glucan and α-1,4-glucose residues linked to α-1,3-glucan (Kanetsuna & Carbonell, 1970; Sorais *et al.*, 2010).

Polysaccharides that build up the pathogens' cell wall cannot be regarded as just a mechanical scaffold neutral to the host during the infection. Few of them act as the microbial associated molecular patterns (MAMPs) that are recognized by the pattern recognition receptors (PRRs) of the host; other are involved in evading the host immune response (Sukhithasri *et al.*, 2013). Some of the most important human PRRs that interact with the fungal cell wall include the Toll-like receptors (TLR2, TLR4), the macrophage mannose receptor, DC-SIGN,

as well as Dectin-1 and Dectin-2, the activation of which triggers the innate immune response, involving the respiratory burst, phagocytosis, opsonisation and complement activation (Levitz, 2010). Chitin coupled with β-glucan present in the *A. fumigatus* cell wall effectively induces the stimulation of host innate immunity and recruitment of neutrophils and eosinophils during infection (Dubey *et al.*, 2014). In general, fungal β-glucan is a highly stimulating molecule for host immune system due to the binding with Dectin-1, present at the surface of myeloid cells, the complement receptor 3 (CR3) and three receptors from scavenger receptor family (Levitz, 2010; Barreto-Bergter & Figueiredo, 2014). Thus, many fungi attempt to mask the glucan layer to avoid the recognition by immune cells. For this strategy, *C. albicans* and other fungi use the layer of mannan coupled with proteins, *H. capsulatum* probably uses α-glucans for the same purpose, and the thick capsule of *C. neoformans* and the layer of RodA protein of *A. fumigatus* provide protection for these pathogens (Aimanianda *et al.*, 2009). When the inner layers emerge from the cell wall surface layers during the cell division or filamentation, the host defense system can actively and efficiently counteract the pathogen. Admittedly, these other polysaccharides more exposed on the surface, such as mannans, can also be recognized by different host receptors, e.g., Dectin-2. Therefore the final host response to fungal attack is the result of various synergistic or antagonistic interactions some of which are not yet fully described (Levitz, 2010).

The composition of the cell wall, even if considered only at the level of its polysaccharides and relative proportions of various linkages, vary not only between species and strains, but also between different morphological forms of fungi, thus greatly complicating the analysis and description of this structure. Nevertheless, the most extensive studies have been focused on the protein components of the cell wall, considered to play a key role on every stage of fungal infection.

The comparative analysis of proteinaceous components of the fungal cell wall has led to the commonly accepted classification into two essentially different groups of proteins that are covalently or non-covalently associated with the fungal cell wall (Fig. 2). The first class is often termed as "typical" or "classical" cell wall proteins. There is a still unresolved issue how the more loosely associated "atypical" proteins, without any signal peptide that could direct them to the classical secretory pathway, can reach the cell surface, especially when they are the cytoplasmic enzymes involved in basic metabolic routes, the elongation factors or the ribosomal proteins. There is always a risk, that simple proteomic identification of such proteins can be artifacts, resulting from contaminations during the cell wall isolation. Thus, additional functional and imaging techniques are necessary to confirm the presence of these proteins at the cell surface. However, increasing number of studies on the atypical secretory pathways in yeasts have been recently performed (Nickel, 2003; Nombela *et al.*, 2006; Nickel & Rabouille, 2009; Nickel, 2011) and the exposition of atypical proteins at the cell wall starts to be considered as a rule rather than an exception or artifact (Karkowska-Kuleta & Kozik, 2014). Nevertheless, also the classical cell wall proteins, involved in the cell wall building and remodeling that are equipped with a N-terminal signal peptide and exported outside the cell through the classical secretory pathway via the endoplasmic reticulum and Golgi apparatus, are sometimes more loosely associated with the polysaccharide scaffold of the cell wall. A notable
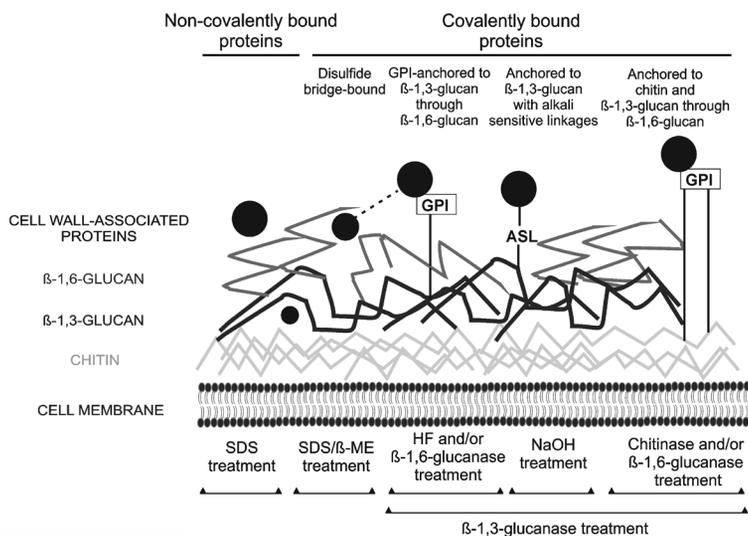
Figure 2. The simplified overview of the composition of fungal cell wall, with indication of the most abundant types of linkages between polysaccharides and proteins and the methods used for protein extraction (modified after Pitarch *et al.*, 2002; Pitarch *et al.*, 2008).

example is the *C. albicans* β-1,3-glucosyltransferase Bgl2 (Pitarch *et al.*, 2002).

The group of proteins covalently linked to the cell wall consists predominantly of proteins with conventional signal peptide and often equipped with glycosylphosphatidylinositol (GPI) anchor, for which the way of secretion followed by their incorporation into the cell wall is typical (Klis *et al.*, 2006). The sequence at the C-terminal end of protein that determines the coupling with GPI anchor in the endoplasmic reticulum has not been explicitly defined. The "ω" amino acid, to which GPI remnant is bound, can often be Gly, Ala, Ser, Asn, Asp, or Cys, preceded by about ten polar amino acids. The second amino acid after the "ω" residue is mainly Gly, Ala or Ser, followed by few moderately polar amino acids and further a stretch of hydrophobic residues (Orlean & Menon, 2007). The majority of the fungal cell wall proteins that possess GPI anchor are not directly connected to the cell membrane, but after the hydrolysis of the bond between glucosamine and mannose within the GPI remnant they are cross-linked to the β-1,6-glucan and then to the β-1,3-glucan or chitin (Pittet & Conzelmann, 2007). In *C. albicans*, about 90% or 10% of GPI-anchored proteins are connected to the first type or to the latter type of glucan, respectively (Marcilla *et al.*, 1991). The *C. albicans* proteins linked to the cell wall in this way and collectively designated with an abbreviation Pga (putative GPI-anchored protein) constitute a large family, many members of which possess various functions in the cell wall maintenance, fungal adhesion or iron acquisition (de Groot *et al.*, 2003; Weissman & Kornitzer, 2004; Chaudhuri *et al.*, 2011).

Some proteins might be directly bound to β-1,3-glucan via alkali sensitive linkages (ASL). These include the Pir proteins (proteins with internal repeats), highly modified with O-linked mannans that probably participate in the formation of ASL. Pir proteins are the common part of the cell wall for different ascomycetous fungi. Their construction comprises the signal peptide and the sequence sensitive for Kex2 endopeptidase action for the proprotein processing in the trans-Golgi network, 2-11 repetitive sequences and a C-terminal domain with four

Cys residues separated from each other with 66, 16 and 12 amino acids (Ruiz-Herrera *et al.*, 2006). Moreover, the Gln residue localized within the repetitive sequence of Pir proteins cross-links them through its γ-carboxyl group with hydroxyl group of glucose from β-1,3-glucan, thereby assigning the Pir proteins a structural role within the cell wall (Ecker *et al.*, 2006). The *C. albicans* Iff2/Hyr3 protein is covalently bound to the cell wall in a similar way, except that ASL is additionally cross-linked with other cell wall proteins through disulfide bridges. It possesses a highly glycosylated Ser/Thr-reach region which is probably responsible for the formation of two abovementioned types of covalent linkages and prevents its binding to the β-1,6-glucan through GPI anchor (Boisramé *et al.*, 2011).

These are the simplest examples of bonds that link the cell wall proteins to the fungal cell wall; however, in some proteins, the particular types of binding can act collectively, thus greatly complicating the description of the whole set of cell wall proteinaceous components (Pitarch *et al.*, 2008). A notable example is *C. albicans* Pga4 — β-1,3-glucanosyltransferase with a similarity to the *A. fumigatus* GEL family — which was found to be a GPI-anchored protein probably non-covalently and/or covalently bound to the cell wall via linkages with β-1,6-glucan linked to β-1,3-glucan or chitin network (Pitarch *et al.*, 2002).

METHODS USED FOR STUDYING THE PROTEOME OF FUNGAL CELL WALL

The procedures of isolation of proteins from the cell wall can involve the mechanical disruption of fungal cells, or can be carried out without the cell disintegration, as it was performed by Hernáez and coworkers (2010) and Vialás and coworkers (2012). In their studies, the surface of the *C. albicans* cells was "shaved" during short treatment with trypsin. However, a particular attention is needed to avoid the cells breakage and extracellular release of their content. The first method is more widely used, but also requires a stage of removal of

nonspecifically adsorbed cytoplasmic and membranous contaminants which can be done by extensive washing of the obtained cell walls with buffers with high ionic strength. In extracts from *C. albicans* cell walls, the presence of Sec14 protein was used as a marker of intracellular contaminations (Pitarch *et al.*, 2002).

The next step involves the incubation of the cell walls with denaturing agents (i.e., sodium dodecyl sulfate or n-octylglucoside) and reducing agents (i.e., β-mercaptoethanol or dithiothreitol) at high temperature. During this procedure a fraction of the non-classical proteins, as well as some typical proteins, non-covalently incorporated with the cell wall, can sometimes be incautiously removed. Thus, this step can be often an initial fractionation of the cell wall-associated proteins rather than just the isolation of the whole set of these cell wall constituents.

In the subsequent steps (the "fractionation"), chemical reagents or various hydrolytic enzymes are used to obtain separate fractions of proteins that differ in a mode of the association with structural polysaccharides. Alternative approach used to identify the whole set of cell wall proteins is to subject the purified fungal cell walls to direct digestion with trypsin (Heilmann *et al.*, 2011). Unfortunately, this method does not give any information about the types of linkages between particular proteins and cell wall polysaccharides.

Proteins linked through ASL might be released from the cell wall by treatment with mild alkali or with β-1,3-glucanase. Similarly, this enzyme in a combination with β-1,6-glucanase is used for the isolation of GPI-linked proteins, although β-1,6-glucanase can also be used alone. Treating the cell walls with HF-pyridine allows to hydrolyze the phosphodiester bond between GPI remnant and proteins that are covalently attached through this anchor to glucans, whereas the digestion of the cell walls with exochitinase isolates a fraction of chitin-bound proteins. Trifluoromethanesulfonic acid (TFMS) can be used for the hydrolysis of polysaccharides and the network composed of chitin and glucans, resulting in the release of already deglycosylated proteins. For the detailed description of the fractionation methodology see Bernard & Latgé, 2001; Pitarch *et al.*, 2008; Maddi *et al.*, 2009 and Chauhan, 2009.

The typical proteomics work-flow presented in Fig. 3 was generally used to study the proteome of fungal cell wall (Yin *et al.*, 2008; Martínez-Gomariz *et al.*, 2009). Occasionally, the less common versions of analyses were performed, including isobaric tagging for relative and absolute quantitation (iTRAQ) (Cagas *et al.*, 2011a; 2011b), stable isotopic labeling by amino acids in cell culture (SILAC) (Heilmann *et al.*, 2011; 2013), or label-free quantitative proteomic techniques (MS[E]) (Champer *et al.*, 2012).

THE QUALITATIVE ANALYSES OF FUNGAL CELL WALL PROTEOME

The most extensive analyses of fungal cell wall proteome were performed on *C. albicans*. Occasionally, other medically important species were studied with this respect, such as some "non-albicans" *Candida* species, *C. neoformans* and *A. fumigatus*.

Candida albicans

It would appear that the simplest method to analyze the cell surface-exposed proteins is to directly hydrolyze them without cells disruption; however, the time and conditions of such treatment must be very carefully chosen in order to avoid the cell membrane permeation followed by the cell breakage. Hernáez and coworkers (2010) and Vialás and coworkers (2012) proposed such the technique for the analysis of the *C. albicans* cells existing in the unicellular forms or in the form of hyphae and biofilm. In the first approach, the appropriate time of digestion was tested and the 5-min incubation with addition of 5 mM dithiothreitol was applied for *C. albicans* cells from the logarithmic phase of growth, a procedure that allowed to identify with MALDI TOF/TOF instrument over 30 proteins with known function. Of these proteins, 40% were responsible for the cell wall organization and biosynthesis (e.g., glycoside hydrolase/cell wall mannoprotein Scw1, chitinase Cht3 or endo-1,3-β-glucanase Acf3), 24% for the cellular metabolism (e.g., glyceraldehyde-3-phosphate dehydrogenase Tdh3 or phosphoglycerate kinase Pgk1), 18% for the fungal defense and virulence (e.g., heat shock proteins Ssa1 and Ssb1) and 6% both for the transport and protein fate (Hernáez *et al.* 2010). Prolonged time of incubation with trypsin resulted in increasing number of proteins involved in the metabolism and protein synthesis.

The extension of these studies to other morphological forms of *C. albicans* allowed to indicate proteins shared under all tested conditions (27) and distinctive for yeast-like cells (38), hyphae (26) and cells that formed biofilms (3) (Vialás *et al.*, 2012). Five of the proteins with known functions were identified for all three morphological forms, including chitinase Cht3 responsible for cell wall maintenance, enolase Eno1, Tdh3 and heat shock proteins Hsp70 and Ssa2, which were described as non-covalently bound adhesin-like molecules, apart from their main role played in cytoplasm. Their involvement in fungal virulence is significant; however, the greater importance is generally admitted to the typical cell wall proteins, covalently linked to polysaccharides through GPI anchor, of which three phospholipases, Plb3, Plb4.5 and Plb5 were identified at the surface of yeast-like cells, and at least four typical or predicted adhesins (e.g., Als1, Als3, Hyr1 and Rbt1, similar to Hwp1) at the cell wall of hyphal forms. For 101 of found proteins, of which 22
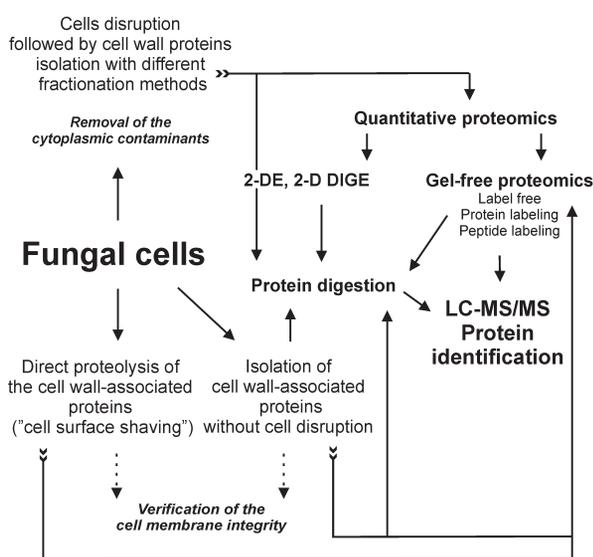

Figure 3. Procedures used for the isolation and identification of fungal cell wall-associated proteins (modified after Yin *et al.*, 2008; Kniemeyer *et al.*, 2011).

were common to all three morphological forms, assigning a specific role that can be played in the cell wall was impossible. Interestingly, this group included numerous ribosomal proteins.

The main disadvantage of these "cell-surface shaving" methods is that several proteins might be not sufficiently exposed at the surface of the cell, or not susceptible to digestion with proteolytic enzymes used in proteomics, until they are trapped within the polysaccharide network and highly glycosylated. Thus the disruption of fungal cells, leading to the isolation of purified cell walls and further fractionation of particular sets of proteins, have been more frequently adapted for the full analysis of the cell wall proteome.

Hence, using TFMS allowed to deglycosylate and purify the cell wall proteins of *C. albicans* yeast and hyphae through hydrolysis of chitin and glucan network, and was followed by their separation with SDS/PAGE and nano-LC/MS/MS analysis (Maddi *et al.*, 2009). In this study, 15 covalently linked cell wall proteins were identified; four of which, known to be involved mainly in cell wall organization, were common to both morphological forms (Mp65/Scw1, Pga29/Rhd3, glycosidase Phr2 and transglycosylase Crh11), five were typical for yeast-like cells (e.g., Ywp1, Pir1, Cht2, Als4 and superoxide dismutase Sod4) and six were unique for hyphae. The latter included abovementioned Rbt1, similar to Hwp1, and Hwp1 itself, both required for candidal full virulence (Plaine *et al.*, 2008). Hwp1, with its similarity to small proline-rich proteins of the host, might be a substrate for mammalian transglutaminases, facilitating the attachment of fungi to the surface of epithelial cells (Staab *et al.*, 1999).

Maddi *et al.* (2009) also evaluated the efficiency of the direct trypsin digestion of purified cell walls that allowed to identify only four cell wall proteins (Mp65/Scw1, Pga29/Rhd3, Phr2, and Pga2/Sod4), previously found in a group of 15 proteins isolated with TFMS. This comparison indicated the advantage of the latter method for the cell wall protein determination. Before treating the cell walls with TFMS, they were incubated with SDS to remove all non-covalently bound proteins, then the proteins linked to the cell surface through alkali-labile and disulfide bonds were removed by the action of the reducing agent (β-ME) in alkaline conditions. The remaining proteins were identified with TFMS and trypsin "shaving" as described above. Importantly, during this treatment numerous atypical proteins were identified. Within this group, 13 proteins were identified in the fractions obtained after using both β-ME and TFMS/trypsin, indicating their localization within the cell wall, despite the fact that they are lacking the signal of the canonical secretion pathway. These proteins included, among others, Tdh3, Eno1, pyruvate decarboxylase, pyruvate kinase Pyk1, mitochondrial malate dehydrogenase Mdh1, 6-phosphogluconate dehydrogenase Gnd1, transaldolase Tal1, heat shock protein Ssa1 and translation elongation factor-1 alpha Tef-1α (Maddi *et al.*, 2009). Similarly, Pitarch *et al.* (2002) found two first abovementioned glycolytic enzymes and, additionally, phosphoglycerate kinase Pgk1, in the fractions, obtained after cell wall treatment with SDS and β-ME, NaOH, β-1,3-glucanase and exochitinase. Whereas the vast majority of studies on *C. albicans* cell wall proteome were focused on the covalently bound proteins, they occasionally revealed a number of atypical proteins associated with the cell wall. However, the actual meaning of their presence within cell wall is recently a matter of extensive debate.

To refer to the covalently bound proteins, at least the results reported by de Groot *et al.* (2004) and Castillo *et al.* (2008) should be quoted. In their studies, HF-pyridine, NaOH or β-1,3-glucanase treatment were performed after removal of all non-covalently bound proteins. Ten GPI-anchored proteins were released from the purified cell walls with HF-pyridine (e.g., Als1, Cht2, structural protein Ecm33 and hem-binding protein Rbt5), additional two with β-1,3-glucanase (Als4 and Pga4) and the next two with mild alkali (Scw1 and structural protein Pir1) in a study of de Groot *et al.* (2004). Nineteen GPI-anchored proteins (e.g., Als1, Als2, Als3, Rbt1, Phr1 and Phr2, the latter two defined as β-1,3 glucanosyl transferases) and two other covalently linked proteins, Bgl2 and Scw1, devoid of GPI remnant but probably disulfide-linked to other cell wall proteins, were described by Castillo *et al.* (2008). Additionally, after deglycosylation of obtained protein sample with TFMS, one novel, adhesin-like protein Pga62/Flo1 was identified (Castillo *et al.*, 2008).

Except the proteins listed above, as might be expected, 66 atypical proteins were detected in all analyzed samples, including, just to mention the more abundant ones, Tdh3, Tef1, Eno1, Ssb1, pyruvate kinase Cdc19, translation elongation factor Eft2 and plasma membrane H⁺-transporting ATPase Pma1. Both atypical and typical cell wall proteins were detected not only at the outer and inner cell wall layers, but also were identified as embedded within cell wall structure, probably due to the extracellular transport and rearrangements, as it was shown after additional treatment of the cell walls with trypsin after all earlier fractionating procedures (Castillo *et al.*, 2008).

Admittedly, in silico analyses indicated the presence of at least 104 predicted GPI-anchored cell wall proteins (de Groot *et al.*, 2003), a number several fold greater than experimentally determined to date. The difficulties in the complete identifications might be caused not only by the problems with extraction, proteolytic digestion or ionization of highly glycosylated proteins, their low abundance or varied expression depending on changing environmental conditions, but also by the retention of many of them near the cell membrane, in contrast to the few detectable in described approaches, whose anchor was removed on the basis of special amino acids sequence near the ω fragment of GPI-anchored protein (Mao *et al.*, 2008).

"Non-albicans" *Candida* species

Apart from *C. albicans*, that is the common causative agent of fungal infections in humans, other species from genus *Candida* nowadays became emerging pathogens. The second most widespread *Candida* species that infects humans is *C. glabrata*, closely related to *S. cerevisiae* yeast, incapable to produce hyphal forms and frequently showing a reduced susceptibility to fluconazole (Quindós, 2014; Guinea, 2014). While the cell wall proteome of *C. albicans* has been widely studied, data on cell wall-associated proteins of "non-albicans" *Candida* species are limited, and broadening the knowledge on this subject can potentially lead to discovery of new drugs for treating the infections caused by these fungi.

Weig and coworkers (2004) isolated true cell wall proteins from *C. glabrata* strain ATCC 90876 and fractionated them into two groups, bound *via* ASL or GPI-anchored. After their separation by 2-DE, six protein spots were excised and three unique proteins were identified after HF-pyridine treatment, including two structural

proteins Cwp1.1, Cwp1.2 and putative glycoside hydrolase Crh1. Additionally, the Western Blotting technique allowed to identify a protein similar to *S. cerevisiae* Pir2. However, these authors also reported results from in silico analysis of *C. glabrata* genome, performed with the use of several bioinformatics algorithms, that revealed 106 predicted GPI-anchored proteins, at least five predicted Pir-like proteins and other five proteins from Bgl2-protein family linked via ASL to the cell wall. These findings suggested a need for further searches of covalently bound *C. glabrata* proteins. In the next work, the same group (de Groot *et al.*, 2008) presented results of a broader study in which two different *C. glabrata* strains, differing in relative adhesiveness were tested (ATCC 90876 and ATCC 2001) after growth at 30ºC or 37ºC to the mid-logarithmic or stationary phase. The subsequent digestion of obtained proteins with trypsin or proteinase Glu-C allowed to identify 18 covalently bound proteins, including Gas1, Gas2, Gas4, Gas5, Crh1, Utr2, Scw4, Ecm33 and Pst1, mainly involved in maintaining the cell wall integrity. Additional four identified proteins — Cwp1.1, Cwp1.2, Ssr1, and Tir1 — probably also play a structural role in *C. glabrata* cell wall. The last of thirteen identified GPI-anchored proteins was phospholipase Plb2. Further four proteins, covalently bound to the cell wall, were Pir proteins, linked to the β-1,3-glucan through ASL. Detailed analyses showed that Scw4 is probably linked with the cell wall in the same manner, whereas in the case of Cwp1.1 the ASL can be an additional element to couple with cell wall apart from binding *via* GPI remnant to β-1,6-glucan. The adhesin-like proteins Awp2-4, some of which are similar to *C. albicans* Iff/Hyr family and *C. glabrata* adhesin Epa6 (epithelial adhesin) were predominantly identified in the cell wall of strain ATCC 2001, while Awp2 in the cell wall at the stationary phase of strain ATCC 90876 after "cell-surface shaving" with trypsin (de Groot *et al.*, 2008). As these findings were correlated with functional analyses regarding the different adhesion capacity of two investigated strains, it was obvious, that ability to form biofilms and to adhere to artificial and biological surfaces is highly strain- and growth phase-dependent. The composition of the cell wall of *C. glabrata* strain ATCC 2001 cells was further investigated after the formation of biofilm on the polystyrene Petri-dishes (Kraneveld *et al.*, 2011). Twenty two covalently bound proteins were identified with nano-LC coupled with electrospray-Q-TOF mass spectrometer after the digestion of purified cell walls with trypsin. Eighteen proteins were described as predicted GPI-anchored, of which six were classified as adhesins, including Awp2, Awp4, Awp5, Awp6, Epa3 and Epa6. Two novel adhesins — Awp5 and Awp6 — were found only in cells from stationary phase or biofilm, not in YPD (Yeast extract Peptone Dextrose) planktonic cultures. Therefore, the medium- and morphology-dependent expression of *C. glabrata* surface-exposed proteins indicated their putative role in the different stages during host colonization (Kraneveld *et al.*, 2011).

Even less data about cell wall proteome are available for other important pathogenic *Candida* species, *C. tropicalis* and *C. parapsilosis*. Up to our knowledge, only immunoproteomic analyses were performed for these two species (Lee *et al.*, 2014a; 2014b). SDS/DTT-extractable cell wall-associated proteins were identified in yeast cells of these two "non-albicans" *Candida* species; more than 262 in *C. parapsilosis* and 325 in *C. tropicalis* protein spots were visualized after 2-D electrophoresis. Twelve distinct proteins from *C. parapsilosis* (Lee *et al.*, 2014a) and twelve proteins from *C. tropicalis* (Lee *et al.*, 2014b) that inter-

acted with anti-*Candida* mouse sera were identified with nanoACQUITY UPLC system coupled to quadrupole time-of-flight (Q-TOF) mass spectrometer with electrospray ionization (ESI) source. In the first species, translation initiation factor eIF4A subunit (Tif1), ATP synthase subunit alpha (Atp1), ATP synthase subunit beta (Atp2), Eno1, Tdh3, Ssb1, pyruvate decarboxylase (Pdc11), Pgk1, alcohol dehydrogenase (Adh1), fructose-bisphosphate aldolase (Fba1), isocitrate dehydrogenase (Idh2) and guanine nucleotide binding protein subunit beta-like protein (Bel1) were immunogenic. In *C. tropicalis*, in addition to proteins aforementioned for *C. parapsilosis* (Atp1, Atp2, Bel1, Eno1, Fba1, Tdh3, Idh2 and Tif1) also citrate synthase (Cit1), triosephosphate isomerase (Tpi1) and ketol-acid reductoisomerase (Ilv5) (also reported as immunogenic in *C. albicans*), were found to trigger the immune response of the host. Moreover, for the first time for any *Candida* species, dihydrolipoyllysine-residue succinyltransferase (Kgd2) was indicated as an antigen (Lee *et al.*, 2014b). Thus, the studies on "non-albicans" *Candida* species cell wall were just initiated, and definitely require further advancing.

Aspergillus fumigatus

A. fumigatus is the most prevalent species from the genus *Aspergillus* comprising filamentous fungi, responsible for often fatal invasive aspergillosis in humans (Dagenais & Keller, 2009). The analysis of the cell wall proteins of *A. fumigatus* conidia and mycelium can be hindered by the presence of pigments and lipids localized within the thick cell wall, the factors that can interfere during the 2-D electrophoretic separation or gel-free LC-MS analysis and for these reasons necessary to be removed from the samples (Kniemeyer *et al.*, 2009). The majority of *A. fumigatus* surface-exposed proteins are probably non-covalently bound to the cell wall or are secretory proteins just passing through the cell wall, so they can be easily extracted with SDS/β-ME or β-1,3-glucanase treatment, as for example acid phosphatase PhoA (Bernard *et al.*, 2002).

One of the first analyses of GPI-anchored proteins, involved in the cell wall biogenesis and released from the cell membrane with endogenous GPI-phospholipase C after membrane solubilization with β-n-octylglucopyranoside, allowed to identify nine proteins (Bruneau *et al.*, 2001). The nano-ESI-MS/MS, MALDI-TOF MS or MS/MS analyses and internal amino acid sequencing were applied to reach the intended identification, although deficiencies in the databases resulted in frequent assigning only the homologues for *A. fumigatus* found proteins. They are mainly responsible for building and remodeling of cell wall, including proteins similar to *Candida* spp. or *S. cerevisiae* Crh1, Crh2, Csa1, Ecm33, Utr2, Gas1/Pga4 as well as *Aspergillus* acid phosphatase PhoA and two β-1,3-glucanosyltransferases Gel4 and Gel1 (Bruneau *et al.*, 2001).

As the genome of *A. fumigatus* was revealed in 2005 (Nierman *et al.*, 2005), further studies of cell wall proteome comprising identification of proteins isolated from conidia with β-1,3-glucanase in mild alkaline conditions allowed to properly identify 26 surface-exposed proteins (Asif *et al.*, 2006). All of them were considered as potential vaccine candidates or thought to act as allergens. After completing 2-DE protein separation and trypsin digestion of selected spots (51), the obtained peptides were transferred to LC-coupled nanoflow ESI Z-spray source Q-TOF mass spectrometer. From all identified 26 proteins, 12 were equipped with a signal peptide.

The major protein was spore coat hydrophobin Hyp1/RodA (rodlet A) protein, but also other proteins were described, including extracellular lipase, disulfide isomerase, secreted aspartic protease Pep2 probably involved in development of allergic reactions in response to *A. fumigatus* conidia, and a protein from family of phosphoglycerate mutases, interestingly found to possess a signal peptide. Hydrophobin RodA that builds the rodlet layer at the surface of conidia and is bound via GPI anchor to cell wall polysaccharides, was recognized as an important virulence factor which masks the Dectin-1- and Dectin-2-dependent immune system activation during aspergillosis (Carrion Sde *et al.*, 2013). The group of 14 proteins without a signal peptide, detected at the surface of conidia, contained elongation factor 1, peroxisomal membrane protein and well-known allergen Aspf3, CipC, tropomyosin, co-chaperon of Hsp90 p21 protein, and one ribosomal protein. Additional seven proteins were not assigned any specific function (Asif *et al.*, 2006).

In order to search for an effective vaccine against *A. fumigatus* and other fungi with significant similarities at the level of surface-exposed proteins, Champer and coworkers (2012) investigated the intracellular and cell wall proteomes of *A. fumigatus* and *Coccidioides posadasii* via label-free quantitative MS^E mass spectrometry with a Q-TOF system (data independent acquisition (DIA) approach), in which the protein ranking in terms of their amount was generated on the basis of various search iterations by three different algorithms in ProteinLynx Global SERVER (PLGS) (Law & Lim, 2013). Approximately 90 proteins were identified in the cell wall fraction of *A. fumigatus*, grown in two different media; the most abundant ones were, among others, major allergen Aspf2, protein Ecm33, putative glycosidase Crf1, catalase B, L-amino acid oxidase LaoA, alkaline protease 2 Alp2, 1,3-β-glucanosyltransferases Gel1, Gel2 and Gel4 if cells were grown in Czapek Dox media, whereas for cells grown in Potato Dextrose media the most abundant identified included pheromone processing carboxypeptidase (Sxa2), also Crf1 and Ecm33, Gel1 and Gel4, chitinases ChiA1 and ChiB1, BYS1 domain protein, putative glucan endo-1,3-β-glucosidase EglC, putative 1,4-β-D-glucan cellobiohydrolase A and putative α-N-arabinofuranosidase B (Champer *et al.*, 2012). Additionally, in the latter conditions, two interesting proteins PhiA and lysophospholipase 1 were detected; however, other putative GPI-anchored proteins and many atypical proteins, such as NAD-dependent malate dehydrogenase, ubiquitin, spermidine synthase and cytochrome b5 were detected, but the last five were considered as minor cytoplasmic contaminants or proteins with unknown export pathways. Interestingly, the proteome analysis indicated significant similarities between *A. fumigatus* and *C. posadasii* surface-exposed proteins; close homologies were also found for other fungal species including *C. albicans*, *C. neoformans*, *Penicillium marneffei* or *Fusarium oxysporum*. Therefore, these authors were able to indicate Crf1, Ecm33, Alp2, Gel4, and Crf2 as the best potential cross-protective vaccine candidates (Champer *et al.*, 2012).

Cryptococcus neoformans

A thick polysaccharide capsule is believed to be the main virulence factor of *C. neoformans* (Karkowska-Kuleta *et al.*, 2009). It is mainly composed of glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), in addition to a smaller amount of mannoproteins (Zaragoza *et al.*, 2009). The greatest attention was focused on the studies of capsule polysaccharides, and the fraction containing proteins, although considered as highly immunogenic during infection, was not characterized in details in terms of its content and structure and function of particular components. Furthermore, the capsule masks the proper cell wall of *C. neoformans*, so that the investigation of cell wall proteome is more difficult and often conducted with acapsulated strains. However, it is possible that some proteins might be more or less evenly distributed between these two structures.

To identify the cell surface proteins of acapsulated *C. neoformans*, cell walls were obtained after cell disruption and extensive washing with dichloromethane, acetonitrile and water, subjected to deglycosylation with PNGase F and the proteins were digested first with trypsin and then isolated with β-1,3-glucanase (Eigenheer *et al.*, 2007). The acquisition of proteins was also performed with the use of intact cells treated with the same enzymes, while the viability of cells was tested. Prior to the separation of peptides, obtained after tryptic digestion of isolated proteins and their analysis with ion trap mass spectrometer used for microcapillary LC-MS/MS, the samples were delipidated with dichloromethane, which greatly facilitates their applying to the reverse-phase liquid chromatography (RPLC) columns. Twenty nine proteins, all with predicted N-terminal secretory signal peptide (except superoxide dismutase), were identified, of which nine only after treatment with β-1,3-glucanase (e.g., α-amylase, trehalase, endo-β-1,3-glucanase and aspartic protease), suggesting their deeper location within the cell wall. Seventeen proteins were identified as probably equipped with GPI anchor; this group included a-amylase, glyoxal oxidase 1, glyoxal oxidase 2, Gas1 (a β-1,3 glucosyltransferase), phosphatidylglycerol-phosphatidylinositol (PG-PI) transfer protein, CFEM domain and aspartyl protease. Interestingly, the high proteolytic activity in the close vicinity of the *C. neoformans* cell surface was suggested, in justification of the presence of at least six proteases more loosely associated with cell wall and isolated from intact cells and one aspartic protease isolated from purified cell walls, possessing GPI anchor (Eigenheer *et al.*, 2007). This phenomenon can significantly contribute to fungal pathogenicity, facilitating lysis of host tissues and degradation of host proteins.

In other studies, focused mainly on host immune response to proteinaceous component of the *C. neoformans* cell surface, proteins were isolated from intact cells with the use of β-mercaptoethanol, so that only proteins linked to other components via disulfide bridges and non-covalently bound proteins were released, and after 2-DE separation subsequently identified with the capillary HPLC-electrospray ionization tandem mass spectrometer with the ion trap analyzer (Young *et al.*, 2009; Chaturvedi *et al.*, 2013). Among several immunodominant surface-exposed proteins that were identified, superoxide dismutase, heat shock proteins, enolase, transaldolase, glutamate dehydrogenase, translation initiation factor, aldehyde reductase, UMP-CMP kinase and dihydrolipoamide dehydrogenase should be mentioned as possible components of vaccines against cryptococcosis (Young *et al.*, 2009; Chaturvedi *et al.*, 2013). Obviously, many of identified surface-exposed proteins have their primary localization in the cytoplasm and thus the problem of their extracellular transport remained unresolved; however; this phenomenon is well known across the fungal kingdom and partially can be explained by the dis-

covery of so-called extracellular vesicles — "virulence bags" — described in more details below.

Other medically important fungi

In the field of interest there are also the cell walls of other medically important fungi, such as *Paracoccidioides brasiliensis* and *Histoplasma capsulatum*, although the knowledge of proteins exposed at their cell surface and localized within cell wall is still incomplete. *P. brasiliensis* is a dimorphic fungus, which exists in the mycelial form at room temperature and as budding yeast at higher temperatures, near 37°C. It is able to cause infections in both immunocompetent and immunocompromised individuals, after inhalation of air-born fungal cells. At the cell surface of this fungus, among others, a putative GPI-anchored membrane/cell wall β-1,3-glucan elongase and atypical proteins including heat shock proteins and other cytoplasm-derived enzymes were often identified with histochemical or immunological techniques (Castro Nda *et al.*, 2009; Puccia *et al.*, 2011). In *H. capsulatum* — the important endemic pulmonary fungal pathogen able to invade host phagocytes — the presence at its cell surface of several heat shock proteins, M- and H-antigens, histone 2B and protein YPS3, highly related to fungal virulence and linked to chitin, were reported (Guimarães *et al.*, 2011). However, to our current knowledge, there is no available description of cell wall proteome of these fungi, albeit in the last five years several reports about changes in intracellular proteome of *P. brasiliensis* and *H. capsulatum* have been presented. So, probably, in the near future the cell surface as the specific contact screen between host and pathogen, will be located in the center of attention of researchers.

PROTEOME OF EXTRACELLULAR VESICLES

Only briefly the recently discovered interesting structures, extracellular vesicles (EV), will be described herein as structures comprising a plurality of proteins, partially explaining the problem of non-conventional secretion of proteins lacking typical secretory signal peptide and their incorporation into cell wall or exposition at the cell surface (Rodrigues *et al.*, 2014). In different fungal species, these bilayer, spherical vesicles were identified, often differing at the molecular and structural level. However, it appeared that several proteins which are important components of EV are common to at least a few fungal pathogens as shown by proteomic studies (Fig. 4).

Unraveling the mechanisms of EV production is still the problem for future, but certainly this cellular process can be regarded as multifactorial and highly complex (Rodrigues *et al.*, 2014). Unquestionable is their role in pathogenicity of some fungal species in humans, as they transfer extracellularly a variety of virulence factors. One of the first reports describing the production of EV concerned *C. neoformans* (Rodrigues *et al.*, 2008). After EV isolation from fungal culture by ultracentrifugation, they were subjected to digestion with trypsin and the obtained peptides were identified with nanoHPLC system coupled with an electrospray linear ion trap mass spectrometer equipped with a nanospray source. Seventy six functionally diverse proteins were described as transferred within EV, among which there were enzymes responsible for cellular metabolism and signal transduction, histones, ribosomal proteins and at least 12 proteins related to *C. neoformans* pathogenicity, including enzymes that contribute to capsule synthesis, urease, lac-
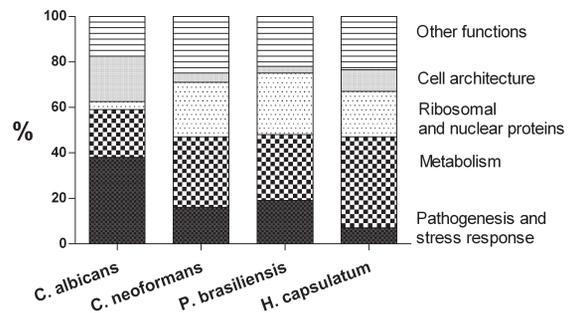

Figure 4. Distribution of extracellular vesicle proteins identified in *C. albicans*, *C. neoformans*, *P. brasiliensis* and *H. capsulatum* with the division into major functional groups according exclusively to their involvement in similar cellular processes and not merely their homology (based on data of Albuquerque *et al.*, 2008; Rodrigues *et al.*, 2008; Vallejo *et al.*, 2012; Vargas *et al.*, 2015).

case, acid phosphatase, heat shock proteins, superoxide dismutase, thioredoxin, thioredoxin reductase, thiol-specific antioxidant protein and catalase A (Rodrigues *et al.*, 2008). Also in *H. capsulatum* the set of proteins localized inside EV was characterized (Albuquerque *et al.*, 2008) with the same technique. 206 proteins were identified, of which several were functionally assigned as implicated in the interactions with the host and termed as immunogenic, e.g., heat shock proteins, superoxide dismutase and catalase B. Moreover, some enzymes such as enolase or glyceraldehyde-3-phosphodehydrogenase, well known "moonlighting proteins" involved in adhesion and interactions with host proteins in different species among Eukaryota (Karkowska-Kuleta & Kozik, 2014) were also detected in EV. This partly explains their increased presence at the cell surface. Additionally, several proteins responsible for cell wall building, growth and metabolism, as well as proteins from cytoskeleton, ribosomes, proteasomes and peroxisomes were listed as found in EV (Albuquerque *et al.*, 2008).

The protein transport inside EV is associated not only with their incorporation within the cell wall, but also in general with the extracellular secretion. The analysis of proteins secreted by *P. brasiliensis* showed 120 proteins common for protein fraction associated with EV-mediated transport and found in the EV-free culture supernatant, while 85 were identified only in the first, and 140 in the latter fraction (Vallejo *et al.*, 2012). Only about 10% of all found proteins were equipped with predicted typical secretory signal peptide; others were subjected to non-conventional secretory pathways. Similarly to findings in other fungal species, EV-related *P. brasiliensis* proteins are often involved in basic metabolic pathways, protein fate and expression, cell wall maintenance and fungal pathogenesis. Detailed analysis indicated that 72 proteins found in EV were common to at least two other fungal species, *C. neoformans* and *H. capsulatum*. These included superoxide dismutase, mitochondrial peroxiredoxin and thioredoxin, important for virulence and survival in the host organism (Vallejo *et al.*, 2012).

The most recent studies on composition of EV were related to the most widespread human fungal pathogen, *C. albicans* (Vargas *et al.*, 2015). From all identified proteins, 30% were assigned to the cell wall, 21% were derived from cytoplasm, two from nucleus, 17% were primarily found in extracellular environment and 16% in plasma membrane, and for 13% no specific localization was assigned. The functional classification was per-

formed, and 22% proteins were identified as related to fungal pathogenesis, 20% to cell wall architecture, 18% to carbohydrate and lipid metabolism, 12% to inducing host immunity, 4% to responses to stress, 3% to protein metabolism. The most interesting proteins associated with *C. albicans* virulence and found in EV included Eno1, Tdh3, Fba1, Pra1, Tpi1, Eft2, Als4 and Sap9 (Vargas *et al.*, 2015).

These interesting recent findings shed light on the problem of the presence of many atypical proteins at the fungal surface and their involvement in the interactions between the host and the pathogen. Since many of them are capable of evoking the response of immune system, they could be taken into consideration as potential components of vaccines. Albeit, still a lot of issues concerning EV release from cells need to be investigated in order to fully understand their specific structure, transition through the cell and function.

QUANTITATIVE ANALYSES OF THE CHANGES IN FUNGAL CELL WALL PROTEOME

The fungal cell wall is a dynamic structure responsible for pathogens' response to the environmental conditions in the various niches occupied within the host organism during infections. However, the changes in the cell wall proteome were extensively studied only in *C. albicans* and *A. fumigatus*. In the former species, the impact of morphological polymorphism on the proteins exposed at the cell surface was mostly investigated, because of the importance of this phenomenon in the pathogenicity of candidal infections (Jacobsen *et al.*, 2012). *C. albicans* is able to adapt to a wide variety of ambient conditions during spreading within human organism, including fairly wide pH range from acidic in some fragments of gastrointestinal tract and vagina to neutral in the bloodstream, hypoxia within tissues, different carbon sources and presence of antifungals; thus, the influences on cell wall proteome of these variables were also tested (Table 1). In *A. fumigatus*, the presence of human serum or antifungal drugs were tested to observe proteome changes under these conditions. Since quantitative data describing the proteome changes are very comprehensive and its analysis is quite complicated, for a detailed picture the reader should refer to the original literature, whereas in this review only overall view of the current state of this research is presented.

C. albicans

The relative quantification of ^{15}N-reference and ^{14}N-query cell walls isolated from *C. albicans* cells after their induction to form hyphae, considered as essential for tissue invasion during infection, were performed by Heilmann and coworkers (2011). For the metabolic ^{15}N-labelling of the cells, the cultures were grown with the stable nitrogen isotope (^{15}N) in the ammonium sulfate at the acidic (pH 4) and neutral (pH 7.4) media. The cell wall proteins were digested with trypsin after obtaining the purified cell walls. This approach allowed to collect and compare query cells — reference cells ratios of particular cell wall proteins for further analyses of changes of the genome during morphological changes (Heilmann *et al.*, 2011). Twenty one covalently bound proteins were identified in this study, of which five were upregulated in the hyphal forms (Als3, Hwp2, Hyr1, phospholipase Plb5 and superoxide dismutase Sod5), three in unicellular forms (Rhd3, Ywp1 and Sod4), two were related to cultures grown at low temperatures (Pir1 and Als4) and

eleven (e.g., Cht2, Ecm33, Rbt1 and secreted aspartic protease Sap 9) were morphotype independent. Moreover, two atypical, non-covalently bound proteins — Tdh3 and chaperone Ssa2 — were identified as present in yeast-like or both morphological forms, respectively.

The changes in composition of cell wall proteome were also frequently tested with a classic proteomics protocol that starts from 2-DE with the silver/Coomassie Brilliant Blue staining of separated protein spots (Pitarch *et al.*, 2002; Ebanks *et al.*, 2006). While in the latter work the quantitative analysis of the SDS/DTT-extractable proteins was performed, in the former the different fractions isolated from the cell wall as described in the previous section were tested. In the study of Ebanks and coworkers (2006), the yeast-to-hyphae transition was induced by adding fetal bovine serum and increasing the temperature of growth from 30°C to 37°C. At least two proteins detected in two replicates were down-regulated in the hyphae (Tef1 and enhancer of Gal4 DNA binding protein homolog Egd2), whereas seven proteins were up-regulated in hyphae (acetyl-CoA synthase Acs2, bifunctional purine biosynthesis protein Ade17, Cdc19, heat shock proteins Sse1, Hsp70, Hsp90 and long-chain base inhibitor of protein kinases Lsp1). None of covalently bound proteins was identified in this quantitative approach due to the chosen isolation method; however, during the qualitative analysis of proteins retained within the cell wall after treatment with DTT/SDS, 17 proteins were identified only in the hyphal forms (including Als3), 4 were identified only in the yeast forms, and 8 were identified in both morphological forms (e.g., Cht2 and Scw1). Thomas and coworkers (2006) reported the up-regulation of alcohol dehydrogenase 2 (Adh2) and malate dehydrogenase (Mdh2) among the β-ME-extractable cell wall-associated proteins isolated from planktonic cells, whereas Adh1, Eno1, Pgk1 and Ssa1 were up-regulated in cells that formed biofilms.

A broader quantitative analysis was proposed by Pitarch and coworkers (2002), with addition of the in-gel deglycosylation to facilitate the MS identification of proteins up-regulated after the yeast-to-hyphae transition at 37°C, pH 6.7, in Lee medium. Although minor differences were detected within the sets of SDS-, NaOH- and β-1,3-glucanase-extractable proteins (e.g., the up-regulation of Eno1 and Pgk1 in the latter extract), the significant up-regulation of 24 proteins in hyphal cells was observed after protein isolation with exochitinase (e.g., Eno1, Pgk1, Tdh3 and fructose-bisphosphate aldolase Fba1), probably due to a higher content of chitin in the hyphal cell walls and greater retention of chitin cross-linked proteins (Pitarch *et al.*, 2002).

The comparison between cell surface-associated, β-ME-extractable proteins of yeast cells, hyphae and biofilm was also performed with the use of spectrally resolvable fluorescent CyDyes™ (Cy2 for internal standard, Cy3 and Cy5 for sample pairs biofilms-yeast cells, biofilms-hyphae and hyphae-yeast cells) and two dimensional difference gel electrophoresis (2-DE DIGE) technique (Martínez-Gomariz *et al.*, 2009). Different excitation/emission wavelengths were used to distinguish the samples, 532/580 nm for Cy3, 633/670 nm for Cy5 and 488/520 nm for Cy2. This approach allowed to identify 38 and 32 proteins over- or under-expressed when biofilms and yeast cells were compared, 14 and 37 proteins between biofilm and hyphae and 27 and 13 proteins between hyphae and yeast cells, respectively. A quite detailed characteristics, although limited only to the non-covalently associated cell wall proteins, indicated that the planktonic cells, existing both in the unicellular or fila-

mentous forms, have the considerably different profile of surface proteins in comparison to the cells in biofilm (Martínez-Gomariz *et al.*, 2009).

A number of further analyses of the differences in proteome between at least three *C. albicans* morphological forms were performed, each adding important information that complement the overall view of the process of adaptation of this opportunistic pathogen to various conditions (Fig. 5). Although the influence of other factors on the cell wall proteome was also tested, the induced changes were generally associated with morphological transition (Sudbery *et al.*, 2004; Whiteway & Oberholzer, 2004).

Interesting example is a study of the differences in cell wall proteome obtained after digestion of proteinaceous components of purified cell walls with trypsin, as a consequence of growth in media with different carbon sources, mainly glucose and lactate, that in vivo are available for fungi mainly in the gastrointestinal and urogenital tracts, respectively (Ene *et al.*, 2012). Lactate-grown cells exhibited increased production of Cht1, Cht3, Pga4, Phr1, Phr1 and Pir1, whereas Cht2, Bgl2, Crh11 and Ecm33 were produced in greater amounts in glucose-grown cells. As is well known, all these proteins function as structural and building components within the cell wall; hence, the observed differences might lead to differences in the cross-links between polysaccharides, thus affecting the overall architecture of the cell wall. As regards the virulence factors, the up-regulation of Als1, Als2 and Als4 (involved in the adhesion and biofilm formation) as well as proteinase Sap9 in the lactate-grown cells, affecting fungal ability to bind to host tissues and further invasion, were observed (Ene *et al.*, 2012).

A very frequent type of infection caused by *C. albicans* is the vaginitis, since over 70% of women worldwide suffer from this disease at least once in lifetime (Mårdh *et al.*, 2002). The unique conditions in this niche (low pH and O_2 concentration, high CO_2 concentration) require special adaptations of the candidal cell wall composition during the invasion. Sosinska and coworkers (2008), within a group of 15 covalently and 4 non-covalently bound identified proteins (after treatment of SDS-purified cell walls with trypsin), indicated three (Als3, Hwp1 and putative GPI anchored cell wall glycosidase Utr2), and two (adhesin-like protein involved in cell wall maintenance Sim1 and protein similar to alpha agglutinin anchor subunit Tos1), respectively, present only in the medium mimicking the vaginal conditions and not in

the reference medium. All of them might be involved in increased adhesion and remodeling of the cell wall, thus facilitating the fungal attachment to vaginal epithelium.

Moreover, in further studies in the same laboratory (Sosinska *et al.*, 2011), the influence of pH 7.0 (in relation to conditions in oral cavity) and pH 4.0 (as reported in vagina) on cell wall proteome was tested. This time, a differential labeling was used in which the tested cultures were labeled with ^{14}N and the reference cells were cultured with ^{15}N-ammonium sulfate, and the quantitative analysis of the tryptic digest of covalently bound cell wall proteins was performed with Fourier transform mass spectrometry (FT-MS). The differences in the retention time on the chromatographic column of the ^{14}N- and ^{15}N-peptides were exploited, as the latter were slightly delayed in relation to corresponding ^{14}N-peptides. The appropriate peptide pairs together with the accurate masses of both peptides were analyzed and the variability within the set of identified proteins was determined. There were several proteins affected by ambient pH; Als1, Als3, Hyr1, Sod 5 and Phr1 were identified only at pH 7.0 whereas Phr2 only at pH 4.0, the result correlating with the pH-dependent gene expression of these two glycosidases. Moreover, the increased levels at higher pH were noticed for Rbt5, in contrast to Als4, Ecm33, Pir1 and Rhd3 that were more abundant in more acidic conditions, and the zinc-finger transcription factor Rim101 was indicated as a major regulator of these changes (Davis *et al.*, 2000; Sosinska *et al.*, 2011).

At neutral pH the solubility and accessibility of ferric ions is much lower than at pH 4.0; also the lower concentration of oxygen enforces a higher uptake of this element. As the accession to iron is essential for proper growth, metabolism and pathogenicity of microorganisms, they have to develop effective strategies to acquire iron from the host during infection. In the first stage of this process, mainly the proteins present at the fungal cell surface are involved in the adsorption and acquisition of iron-binding proteins such as ferritin, transferrin and hemoglobin (Almeida *et al.*, 2009). The detection method with protein metabolic labeling with ^{15}N was used for the quantitative analysis to investigate the influence of limited access to iron on cell wall proteome (Sorgo *et al.*, 2013). The increased amounts of GPI-anchored proteins involved in iron acquisition were observed, including Csa1, Pga7, Pga10 and Rbt5, all involved in hem/hemoglobin binding (Weissman & Kornitzer, 2004) that possibly takes place through CFEM domain (common in fungal extracellular membranes), containing conserved peptide sequence with at least three Cys residues (Sorgo *et al.*, 2013). Sod4 and hyphal-associated proteins, Hyr 1 and Als3, were also up-regulated in the iron-poor conditions, especially as the latter adhesive invasin-like protein was previously described as ferritin-binding protein (Almeida *et al.*, 2008). In contrary, Als2 and Tos1 were down-regulated under these conditions (Sorgo *et al.*, 2013).

In other study, the influence of thermal stress on the cell wall proteome was investigated (Heilmann *et al.*, 2013). The proteins from reference cultures were metabolically labeled with ^{15}N, as described above, and relative quantification of proteins induced after incubation at 42°C at different pH (pH 4.0 and pH 7.4) was performed. Although the changes were more significant at neutral pH, in both pH there was an up-regulation of Sap9, transglycosylases Phr1 and Phr2, chitin transglycosylases Crh11 and Utr2 as well as Ecm33, all involved in the cell wall maintenance. Interestingly, several GPI-anchored proteins, such as Als2, Als4, Cht2 and above-
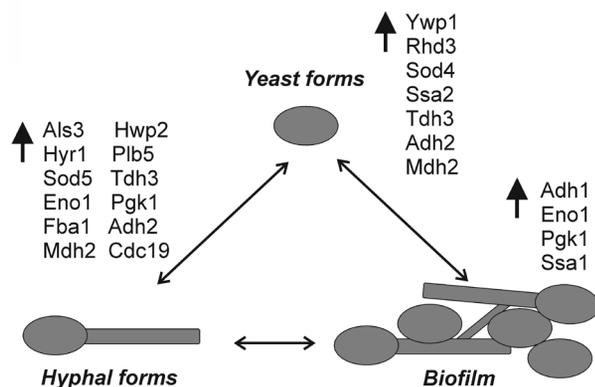

Figure 5. Differentiated up-regulation of particular *C. albicans* proteins exposed at the surface of various morphological forms of this fungus (based on data of Pitarch *et al.*, 2002; Thomas *et al.*, 2006; Martínez-Gomariz *et al.*, 2009; Heilmann *et al.*, 2011).

Table 1. Examples of *C. albicans* proteins that are up- or down-regulated under different environmental conditions.

Conditions	Changes in the protein level		Reference
Different carbon source	Up-regulated in the presence of lactose Als1, Als2, Als4, Cht1, Cht3, Pga4, Phr1, Phr2, Pir1, Sap9	Up-regulated in the presence of glucose Cht2, Bgl2, Crh11, Ecm33	Ene *et al.*, 2012
Vaginal conditions	Up-regulation Als3, Hwp1, Utr2, Sim1, Tos1		Sosinska *et al.*, 2008
Ambient pH	pH 7.0 Als1, Als3, Hyr1, Sod5, Phr1, Rbt5	pH 4.0 Als4, Phr2, Ecm33, Pir1, Rhd3	Sosinska *et al.*, 2011
Limited iron	Up-regulation Als3, Hyr3, Csa1, Pga7, Pga10, Sod4, Rbt5	Down-regulation Als2, Tos1	Sorgo *et al.*, 2013
Heat stress	Up-regulation Sap9, Phr1, Phr2, Crh11, Utr2, Ecm33	Down-regulation Als2, Als4, Cht2, Csa1, Pga10, Rbt5	Heilmann *et al.*, 2013

mentioned hem-binding proteins Csa1, Pga10, and Rbt5, were down-regulated in heat stress. Quite similar results were obtained for cells in the presence of fluconazole, antifungal drug affecting synthesis of ergosterol localized in the cell membrane thereby increasing its permeability (Sorgo *et al.*, 2011), suggesting that the response of the fungi to various stressors at the level of the cell wall as the primary protective barrier, might be quite similar, leading to the increase of the amounts of cell wall-repairing enzymes, participating in its strengthening.

Aspergillus fumigatus

The relative changes in the whole proteome of *A. fumgatus* were investigated during the morphological transition of this fungus from conidia to mycelium, which takes place at the first stage of the development of infection (Cagas *et al.*, 2011a). The iTRAQ system (the gel free system of isobaric tagging for relative and absolute quantitation), enabling the quantitative comparisons for up to eight samples, was used to label the peptides obtained after tryptic digestion. After separation by two dimensional liquid chromatography, the samples were analyzed with MALDI-TOF/TOF mass spectrometer. Except several intracellular proteins, the surface-exposed hydrophobin RodA were shown to be down-regulated during the morphological changes from conidia to mycelium (Cagas *et al.*, 2011a). Further fractionation of obtained proteins allowed to indicate, with the use of the same labeling and measuring method, changes of *A. fumigatus* proteome in the presence of antifungal drug, caspofungin. After 24 hours, 26 cell wall proteins were up-regulated and 30 down-regulated. In case of the first group, almost 81% (21) were identified as ribosomal proteins, and in the latter, the most significant decrease was noticed for chitinase ChiA1. After 48 hour-exposition to caspofungin, the levels of 9 proteins changed, including the down-regulation of two heat shock proteins Hsp98 and Sba1 (Cagas *et al.*, 2011b).

As the proteins that respond with a level change to the exposition on different antifungal drugs are potent and perspective targets for treatment and markers of infections, several further studies concerning changes in the whole protein set of fungal cells were carried out, in which proteins exposed at the cell surface were included. It was shown that after exposition to amphotericin B the levels of 4% of whole 48 identified proteins responsible for conidial cell wall maintenance were changed, including conidial hydrophobin B (RodB) — structural protein involved in the building of the conidial outer cell wall (Gautam *et al.*, 2008) — while after the exposure of mycelium to artemisinin, 3% of 65 proteins that were

described as cell wall-associated proteins were down-regulated, e.g., the abovementioned RodB and cell wall protein PhiA (Gautam *et al.*, 2011). However, it should be taken into account that if the isolation of total proteins from whole cells without fractionation is applied, only a functional classification of protein gives information about localization of proteins, so that many atypical proteins without classical export signal could be omitted from the further consideration.

THE BENEFITS FROM THE RECOGNITION OF FUNGAL CELL WALL PROTEOME

The genomes of several pathogenic fungi are nowadays reported and the investigations of transcription levels for particular genes involved in the pathogenesis mechanisms are still ongoing. Albeit these genomic studies are helpful to predict the main trends in protein involvement and functionality, the actual presence of proteins at the cell surface should be analyzed to appreciate the complexity of pathogen interactions with the environment (Sosinska *et al.*, 2011). Proteins exposed at the surface of fungal cell wall are the important part of dynamic response of pathogen to the stress, caused by the changes in the environmental conditions or interactions with the host during infection. Understanding the steady state and the changes that result from the adaptation of pathogens to these changes can help to develop new strategies of treatment, diagnosis and prevention of fungal infections, since the most abundant or specific proteins might act as the potent immune stimulants, plasma-derived markers of fungal infections or targets to inhibition (Yin *et al.*, 2008; Heilmann *et al.*, 2012).

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