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Mass spectrometry-based proteomics of fungal wall glycoproteins

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The manifold functions of fungal wall glycoproteins include maintenance of cell wall integrity, homotypic and heterotypic adhesion, biofilm formation, acquisition of iron and sterols, protein degradation and coping with oxidative stress. Transcriptome studies indicate that the expression levels of most cell wall glycoproteins can vary widely and are tightly controlled. However, owing to their complex and variable glycosylation, fungal wall glycoproteins are difficult to analyze using traditional proteomics approaches. Recent advances in mass spectrometry-based proteomics have enabled rapid and sensitive identification and quantitation of fungal wall glycoproteins; this will be particularly useful for studying the dynamics of the subproteome of fungal wall glycoproteins, and for the development of novel vaccines and diagnostic tools.

Fungal wall glycoproteins and cell wall organization

Many fungi have a layered cell wall. For example, the wall of the ascomycetous yeast *Saccharomyces cerevisiae* consists of a skeletal inner layer, which is surrounded by a fibrillar outer layer consisting of glycoproteins that emanate outwards (Figure 1). In *S. cerevisiae*, cell wall glycoproteins account for up to 10% of the total biomass per cell. Most cell wall glycoproteins in this organism are glycosylphosphatidylinositol-modified cell wall proteins (GPI–CWPs), which are covalently linked to β-1,6-glucan through a trimmed form of their original GPI-anchor (GPI_t), forming the protein–polysaccharide complex: CWP–GPI_t–β-1,6-glucan–β-1,3-glucan and extending their effector domain away from the cell surface [1]. Protein with internal repeats (Pir)-CWPs belong to a second class of proteins and are believed to crosslink β-1,3-glucan chains through ester linkages, forming the putative protein–polysaccharide complex β-1,3-glucan–Pir-CWP–β-1,3-glucan. Consistent with their predicted β-1,3-glucan crosslinking function, Pir-CWPs are found throughout the skeletal inner layer. Evidence for a similar molecular organization of the wall has been obtained in other (dimorphic) ascomycetous yeasts, including various human pathogens such as *Candida* spp. and *Exophiala dermatitidis,* in the fission yeast *Schizosaccharomyces pombe,* and also in mycelial ascomycetous fungi such as *Aspergillus spp.* and *Fusarium oxysporum* [2]. The walls of the ascomycetous fungi *Sporothrix schenckii* and *Neurospora crassa* and, probably, many other ascomycetous species, and of the yeast form of the basidiomycetous fungus *Ustilago maydis* also show a bilayered organization, suggesting that they might have a molecular structure similar to that of *S. cerevisiae* [2].

The physiological importance of fungal wall glycoproteins

There are several categories of CWPs. CWPs are either covalently linked to cell wall polysaccharides through a glycosidic linkage or an ester bond, or to other cell wall proteins through disulfide bonds. They can also be non-covalently associated with cell wall polysaccharides through, for example, a glycan-binding domain; they can also be ionically bound to the many negative charges due to, for example, phosphodiester groups present in the *O*- and *N*-linked carbohydrate side chains of cell wall glycoproteins [1,3]. Ionically bound wall proteins often include nonglycosylated proteins of cytosolic origin [4,5], which raises the fascinating question of whether in fungi a non-classical protein export pathway might exist, as in mammalian cells. Unfortunately, many studies of non-canonical, cell wall-associated proteins in fungi are compromised by the use of extraction procedures, which perturb the plasma membrane, causing leakage of cytosolic proteins [6], and which thus complicate the interpretation of the results. Similar problems have been encountered in

Glossary

2-DE: 2D electrophoretic separation of proteins based on differences in their isoelectric point and mass.
Endo-H: endo-β-N-acetylglucosaminidase H, an endoglycosidase that cleaves the β-1,4-linked di-N-acetylchitobiose core of the asparagine-linked N-chains, leaving one N-acetylglucosamine residue remaining on the asparagine residue. In combination with MS, the cleavage reaction can be used to map the N-glycosylation sites of the glycoprotein.
ICAT: Isotope-coded affinity tag. Reagent that specifically labels the cysteine residues in proteins. The tag has an affinity group that can be used to separate the labeled peptides after proteolytic digestion.
iTRAQ: Isobaric tag for relative and absolute quantitation. Reagent that chemically labels primary amines, which are found at the N-terminus of peptides and in lysine residues. The tag is designed in such a way that peptides from differentially labeled samples have an identical mass (are isobaric).
LC/MS/MS: liquid chromatography-based separation of peptides coupled with tandem mass spectrometry.
PNGase F: peptide-N-glycosidase F, an amidase that hydrolyzes the glycosylamine linkage of asparagine-bound carbohydrate side chains, generating an aspartic acid residue at the site of hydrolysis and enabling mapping of the *N*-glycosylation sites of the glycoprotein.
qPCR: quantitative polymerase chain reaction.
SILAC: stable isotope labeling with amino acids in cell culture. A method to label cellular proteins by culturing the cells in growth medium containing stable isotope-labeled amino acids.

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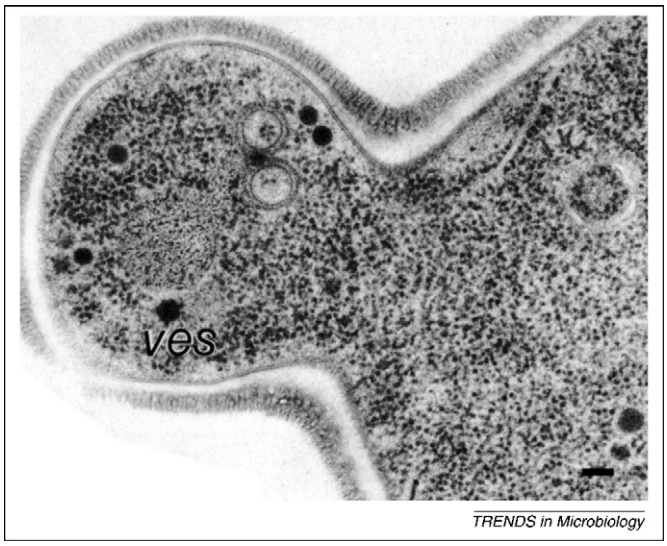

Figure 1. Electron micrograph showing the cell wall of *S. cerevisiae*. The fibrillar outer layer consists of glycoproteins emanating into the environment and is attached to the skeletal inner layer. The bar corresponds to 100 nm. Abbreviation: ves, vesicle. Adapted, with permission, from Ref. [56].

studies concerning non-canonical cell wall-associated proteins in plants [7]. Here, we focus on the subproteome represented by the fungal wall glycoproteins, which are the sole proteins found in hot detergent-extracted, isolated walls and which all possess an N-terminal signal peptide and thus are expected to follow the classical protein export pathway.

At any one time, there are over 20 different cell wall glycoproteins estimated to be present in the walls of *Candida albicans* and *S. cerevisiae*. Cell wall glycoproteins are involved in a wide diversity of functions [8–10] (Table 1). They contribute to cell wall integrity by crosslinking polysaccharides and by limiting access of the stress-bearing polysaccharides to glycanases from other organisms [1,11]. They also mask skeletal polysaccharides, such as β-glucans, from the immune system [12,13]. In addition, they mediate flocculation and mating [11,14], have a crucial role in biofilm formation on biotic and abiotic surfaces [15–19], promote adhesion to epithelial cells [16,20], mediate endocytosis and tissue invasion [21], cause proteolytic damage to the invaded tissues [22] and offer protection against oxidative stress [23]. Finally, they also facilitate iron acquisition [24,25]. Individually and collectively, they thus have a crucial role in fitness and virulence. The biosynthetic pathway of cell wall proteins therefore offers potential targets for the development of antifungal drugs. Cell wall proteins might also serve as the basis for novel vaccines and function as diagnostic tools for infectious diseases [26–29].

Transcriptome analyses in *S. cerevisiae* and *C. albicans* show that transcript levels of CWP-encoding genes vary widely depending on the phase of the cell cycle in which the cell occurs, and on growth conditions such as nutrient availability, temperature, pH and hypoxic conditions, indicating that the cell can adapt the protein composition of the newly formed walls to cope better with environmental conditions [1,30,31].

Table 1. Diverse functions of covalently linked fungal cell wall proteins

Function or property	Organism	Genes or proteins	Refs
Cell wall integrity			
Crosslinking β-1,3-glucans	*S. cerevisiae*	Pir1, 2, 3 and 4	[58]
	C. albicans	Pir1	[59]
Crosslinking β-1,6-glucan and β-1,3-glucan	*S. cerevisiae*	Cwp1, Cwp2, Tip1, Tir1, Tir2[a]	[1]
Cell wall architecture	*C. albicans*	Ecm33, Sun41	[19,60]
Adhesins			
Adhesion to epithelial cells	*C. albicans*	Als1, Eap1	[16,61]
	Candida glabrata	Epa1	[62]
Biofilm formation	*S. cerevisiae*	Flo11	[63]
	C. albicans	Als3, Hwp1, Eap1	[16–18]
Flocculation	*S. cerevisiae*	Flo1, 5, 9, and 11	[14]
Host transglutaminase substrate	*C. albicans*	Hwp1	[20]
Mating	*S. cerevisiae*	Sag1	[1]
Mimicking cadherins	*C. albicans*	Als3	[21]
Carbohydrate-active enzymes			
Chitinase	*C. albicans*	Cht2	[9]
Endo-β-1,3-glucanase	*S. cerevisiae*	Scw4 and 10	[8,64]
	C. albicans	Scw1	[9]
(Trans)glycosylases	*S. cerevisiae*	Crh1, Utr2, Gas family	[8,65,66]
	C. albicans	Crh11, Pga4, Phr1, Utr2	[9,67]
	S. pombe	Gas1 and 5	[35]
	C. neoformans	CNBN2300	[37]
Other enzymatic activities			
Chitin deacetylase	*C. neoformans*	CNBD2840, CNBD2750, CNBF2910	[37,68]
Glyoxal oxidase	*C. neoformans*	CNBA3760, CNBE5040	[37,69]
Phospholipase B	*S. cerevisiae*	Plb2	[8]
Protease	*C. albicans*	Sap9 and 10	[22]
	C. neoformans	CNBH1590	[69]
Superoxide dismutase	*C. albicans*	Sod4 and 5	[70]
Iron acquisition			
	S. cerevisiae	Fit1, 2, and 3	[25]
Haem binding	*C. albicans*	Rbt5, Pga10	[24]

[a]These GPI proteins contain a Pir repeat and are believed to crosslink β-1,6-glucan and β-1,3-glucan.

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Here, we summarize the latest progress in mass spectrometry (MS)-based proteomics of fungal wall glycoproteins. This will enable monitoring temporal changes in their expression levels in response to altered growth conditions and changes in morphology, such as the yeast–hypha transition in *C. albicans* and other dimorphic fungi. The methods developed are robust and highly sensitive and enable a detailed study of cell surface proteins involved in the maintenance of cell wall integrity, adhesion, biofilm formation, cell–cell recognition, virulence and coping with various stress conditions. These methods will be widely applicable in fungal wall biology and in similar studies in bacteria, algae and higher plants. They will also provide quantitative data as input for modeling the regulatory circuits that control the expression of cell wall glycoproteins. Finally, we discuss potential applications of this line of research in the development of new vaccines and diagnostics.

MS identification of fungal wall glycoproteins

The term 'proteomics', which denotes analysis of the entire protein complement expressed by a genome, was initially coined in the context of 2D gel electrophoresis (2-DE; see Glossary). In a typical experiment, proteins are released from the insoluble polysaccharide network, and then separated by 2-DE by making use of differences in their isoelectric point and mass. The proteins are subsequently proteolytically fragmented and the resulting peptides are analyzed with MS to reveal their identities. However, there are two major disadvantages inherent to electrophoretic analysis of fungal cell wall glycoproteins. First, *N*- and *O*-linked carbohydrate side chains of fungal glycoproteins often carry a variable number of negative charges owing to the presence of phosphodiester bridges or uronic acids, or to pyruvylation [3], resulting in glycoforms with different isoelectric points. Second, *N*- and *O*-linked carbohydrate side chains of fungal glycoproteins can vary dramatically in length, and the degree of occupancy of individual glycosylation sites can vary [32], resulting in numerous glycoforms ranging widely in mass. Consequently, fungal wall glycoproteins often produce multiple, fuzzy spots, thus complicating mass finger printing and quantitation, and lowering the resolution and sensitivity of the method. Improved approaches to study fungal wall glycoproteins are thus desirable.

One such approach uses proteolysis and liquid chromatography (LC) in combination with tandem MS (LC/MS/MS), which enables the identification of multiple proteins from highly complex peptide mixtures [33]. In the so-called 'wall-shaving' method, stringently washed and hot detergent-extracted cell wall fragments are digested directly by a site-specific protease such as trypsin, followed by collection of the released wall peptides and separation by LC. Resolved peptides are fragmented in the mass spectrometer, and the MS/MS spectra are searched against a fungal genome database to identify the corresponding cell wall proteins [8] (Figure 2). An important advantage of this method is that it enables protein identification, irrespective of the nature of their covalent linkages to the cell wall lattice. Alternatively, CWPs can be first released by chemical or enzymatic means, thus providing information

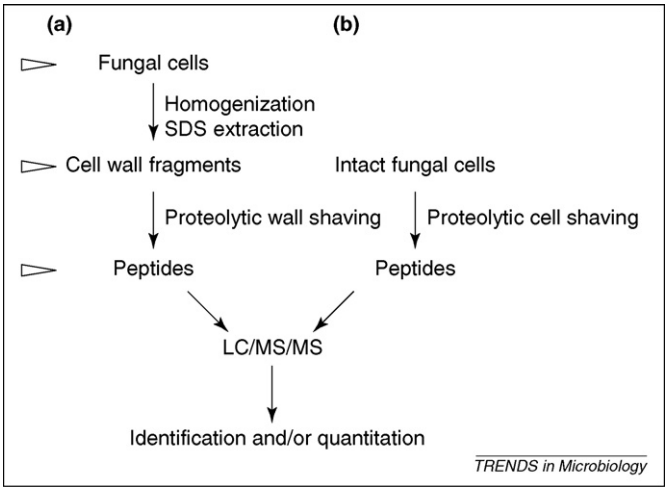

Figure 2. MS identification or quantitation of fungal wall glycoproteins. For identification, **(a)** stringently washed cell wall fragments or **(b)** intact fungal cells are digested by a protease (e.g. trypsin). Resulting peptides are subjected to LC/MS/MS and the spectra are searched against a fungal genome database to identify the corresponding cell wall proteins. For quantitation, stable isotope labels can be introduced at various stages of the experiment, such as during cell culturing and before or after a proteolytic 'shaving' step. The open arrow heads denote the stages where isotope-labeled samples can be combined with their non-labeled counterparts. Protein quantitation can be realized by comparing the corresponding peptide peak areas from differentially labeled samples.

about how these proteins are linked to the cell wall polysaccharides. For example, pyridine hydrofluoride specifically releases GPI-modified CWPs by cleaving the phosphodiester bridge in the GPI remnant that interconnects GPI-CWPs to β-glucan, whereas mild alkali releases Pir-CWPs and other CWPs linked through a mild alkali-sensitive linkage to cell wall polysaccharides [8,9,34]. Furthermore, identification of GPI proteins in a cell wall digest obtained with β-1,6-glucanase suggests the presence of the CWP–polysaccharide complex CWP-GPI$_t$–β-1,6-glucan–β-1,3-glucan and, possibly, also CWP-GPI$_t$–β-1,6-glucan–chitin [1,11]. A single peptide sequence is often sufficient for cell wall protein identification, although some known covalently linked cell wall proteins are not identified after tryptic digestion because of the absence of suitably sized peptides or because of abundant *N*- and *O*-glycosylation (Box 1). To obtain different sets of peptides, other proteases such as the endoprotease Glu-C or proteinase K can be used. Removal of *N*-chains can increase the accessibility of *N*-glycosylated proteins to endoproteases. This also helps to establish which potential *N*-glycosylation sites are used. *N*-chains can be removed with either endo-β-N-acetylglucosaminidase H (endo-H), which leaves a single *N*-acetylglucosamine residue attached to the asparaginyl residue, or peptide-N-glycosidase F (PNGase F), which completely removes the *N*-linked carbohydrate side chain, thereby converting the asparaginyl residue into an aspartyl residue. Unfortunately, no suitable fungal *O*-glycanases have yet been identified. The wall-shaving approach has been successfully applied for the comprehensive analysis of several fungal cell wall proteomes, including those of *C. albicans, Cryptococcus neoformans, S. cerevisiae, S. pombe* and *Phytophthora ramorum* [8,9,35–37].

Another approach that is also independent of prior electrophoretic protein separation is the so-called

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Box 1. Cell wall GPI proteins

- The mature form of both CaSsr1 *and* ScCcw12 lacks the N-terminal signal peptide and the GPI-anchor addition signal peptide (Figure I). Both cleavage sites can be predicted using suitable algorithms such as SignalP (see: http://www.cbs.dtu.dk/services/SignalP) and big-PtdIns Fungal Predictor (see: http://mendel.imp.ac.at/gpi/fungi_server.html), respectively. To identify the N-terminal tryptic peptide of matured protein, partial tryptic digestion can be used as a searching criterion (see MASCOT as an example: http://www.matrixscience.com).
- The C-terminal peptide of the mature form of both proteins is covalently linked to β-1,6-glucan, which hampers its identification.
- The masses of some tryptic peptides can be altered dramatically by glycosylation of the potential *N*-glycosylation sites (e.g. in ScCcw12) [57] and of the serine and threonine residues within the sequence (e.g. in the C-terminal part of CaSsr1). Due to their increased mass, the resulting peptides might easily fall out of the detection range of an ordinary mass spectrometer. Furthermore, their corresponding MS/MS spectra might be difficult to interpret.
- Note that many GPI proteins, such as CaSsr1, are organized in a modular fashion, having a functional domain in the N-terminal part of the protein, whereas the C-terminal part is enriched in serine and/or threonine residues.

Figure I. Tryptic digests of two cell wall GPI-proteins. Key: *, potential N-glycosylation site; ↑, potential trypsin cleavage site; GPI, GPI-anchor addition signal, which is cleaved off in the endoplasmic reticulum; SP, signal peptide, which is removed in the endoplasmic reticulum. Highlighted: tryptic peptides identified by LC/MS/MS. A glycosylated form of peptide B of CaSsr1 with a 162-Da addition was also observed [9].

'cell-shaving' method. Intact fungal cells are incubated in the presence of proteases to digest accessible cell surface proteins. Because proteolytic enzymes are unlikely to permeate the plasma membrane of intact cells, this will release peptides that are associated specifically with the cell surface. Released peptides can be subsequently analyzed by MS, enabling the identification of cell surface proteins (Figure 2). This approach was introduced in fungal research by Eigenheer *et al.* [37]. By treating intact cells of the clinically important fungus *C. neoformans* with trypsin, 29 extracellular proteins with a predicted N-terminal signal sequence were found, more than half of which have a predicted C-terminal GPI-anchor addition signal. It should be noted that this method does not distinguish cell wall proteins from other potentially accessible proteins, such as plasma membrane-associated proteins.

MS quantitation of fungal wall glycoproteins

The relative quantitation of biological samples using 2-DE is achieved by comparing the staining intensities of protein spots on the gel, whereas MS-based approaches use stable isotope labels that enable comparisons of corresponding peptide peak areas from different samples. Stable isotope labels can be incorporated at various stages of the experiment [38] (Figure 2). For example, they can be introduced by metabolic labeling with stable isotope-labeled amino acids [stable isotope labeling with amino acids in cell culture (SILAC); the amino acids contain ^{15}N, ^{13}C or both] [38,39]. Cells grown in so-called 'light' medium, containing the natural isotopes ^{14}N or ^{12}C, or in 'heavy' medium, containing the heavy isotopes ^{15}N and/or ^{13}C, will give rise to differentially labeled protein populations, enabling accurate quantitation. An advantage of metabolic incorporation is that the label is introduced at an early stage of the experiment. Cells from different experimental conditions can be mixed before cell lysis, fractionation, purification and subsequent protease digestion, meaning that these steps will not affect the accuracy of quantitation (Figure 2). A limitation of metabolic incorporation is that it does not enable direct *in situ* labeling of infectious fungi from clinical samples. To circumvent this problem, a heavy isotope-labeled culture can be used as the reference for relative quantitation of different samples of clinical origin. It is also important to use highly enriched isotopes to avoid complicated isotopic distributions resulting from partially labeled peptides [38,40].

Another way to introduce stable isotope labels is chemically to modify the fungal wall glycoproteins. Depending on the stage of introduction of stable isotopes, differentially labeled chemical reagents have been designed to target reactive sites on proteins or peptides. The first such labels to be described were isotope-coded affinity tags (ICATs) [41]. The first ICAT reagent described consists of a reactive group that is cysteine directed, a polyether linker with either eight deuterium or eight hydrogen atoms, and a biotin tag that enables recovery of labeled peptides. In a typical ICAT-labeling experiment, proteins from different samples are modified with the 'heavy' or 'light' ICAT reagent. Both samples are mixed, enzymatically digested, and the labeled peptides affinity purified and separated by liquid chromatography. Relative peptide abundances can then be determined by MS. This labeling method is relatively simple and the resulting peptide mixtures are considerably less complex than the complete peptide mixtures [38]. However, the method falls short because the label exclusively targets cysteine residues, a relatively rare amino acid in fungal wall proteins (e.g. ScCwp1 lacks cysteine residues; see *Saccharomyces* Genome Database at http://www.yeastgenome.org). In addition, the biotin group in the ICAT reagent significantly influences fragmentation spectra, complicating peptide identification and leading to low sensitivity. Therefore, cleavable ICAT reagents have been introduced to facilitate the removal of the biotin tag after the affinity-based enrichment of biotinylated peptides [42,43].

Labeling the cell wall proteome by targeting the primary amine groups in lysine residues and at the N-terminal end of peptides overcomes the limitations of ICAT labeling. This reaction is specific and largely complete. If the isotopic labeling is introduced after the protease digestion, peptides will generally possess at least one label at the N-terminus. A recently introduced reagent, called isobaric tag for relative

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and absolute quantitation (iTRAQ), has gained considerable popularity [44]. iTRAQ uses a set of four isobaric tags, each of which comprises an amine-specific reactive group, a carbonyl mass balance group ranging in mass from 31–28 Da, and a reporter group with a mass ranging from 114–117 Da. The latter two groups are combined in such a way that they always add up to the same mass (145 Da), so that the corresponding labeled peptides from different samples are isobaric (of the same mass). Hence, each peak observed in MS spectra represents a peptide from the combined four samples. Only after fragmentation, specific reporter ions will be observed, enabling relative quantitation of the peptide. iTRAQ enables quantitation of multiple peptides from a single protein, and analysis of four (or eight, with the latest product) separately labeled pools of protein in a single assay, increasing experimental accuracy and enabling, for example, quantitative studies on a time-resolved basis. Using this technique, Yin *et al.* [10] identified ten covalently attached cell wall glycoproteins that were differentially expressed in a *gas1*Δ mutant of *S. cerevisiae*, which has a weakened cell wall and in which the cell wall integrity pathway is constitutively activated. Furthermore, using unique synthetic peptides mimicking their natural counterparts in five selected cell wall proteins, the authors were able to estimate the absolute numbers per cell of these CWPs [10].

How profiling of fungal wall glycoproteins could contribute to vaccine development and diagnostics

Some promising applications of cell wall proteomic research are related to vaccine development, diagnostics, biofilm studies and cell surface engineering. For example, CWP-profiling studies might help to identify suitable vaccine candidates, as also shown for bacterial cell surface proteins [45,46]. LC/MS/MS provides information for selecting the best candidate proteins and the most promising protein regions. Such information has wide-reaching implications. (i) Protein abundance. Abundant CWPs with high predicted immunogenicity and known to contribute significantly to fitness and virulence are preferred. (ii) Protein regions accessible to the immune system. The peptides released by both the cell- and the wall-shaving method and identified by LC/MS/MS are predominantly from the effector domains of GPI-CWPs [8,9]. Importantly, the effector domains of GPI-CWPs are generally found in their N-terminal half, which extends furthest away from the cell surface [11]. Together, these data suggest that the effector regions of GPI-CWPs are most accessible to the immune system. (iii) Specificity. The identified proteolytic peptides are also likely to originate from less glycosylated regions of the corresponding CWPs, which is important for the specificity of the antibodies raised. (iv) Finally, multivalent vaccines can be developed, consisting of cell wall proteins that are specifically and abundantly expressed under different infection-related environmental conditions [47]. For example, both the yeast and the hyphal forms of *C. albicans* are believed to contribute to virulence. A potential vaccine should therefore preferentially be active against both growth forms. However, some CWPs are specific for only the yeast or the hyphal form [17,27]. To combat fungal surface infections, a different approach is conceivable, which is based on the construction of fusion proteins consisting of a fungal CWP-specific antibody and an antifungal peptide, as earlier achieved for attacking fungal plant pathogens [48].

Most fungal wall glycoproteins are covalently bound to the stress-bearing polysaccharides of the wall but a minor fraction is often found in the medium, probably as a result of limited turnover in the wall or cell wall remodeling, or depending on the growth phase [1,27]. This suggests that fungal wall glycoproteins might also be useful as diagnostic agents [26,28,29]. Fungal cell wall proteins also have a crucial role in biofilm formation on biotic and abiotic surfaces [15–19] but at present their precise role is not known and the identification of biofilm-promoting CWPs is far from complete. Another challenge is the analysis of the cell wall proteomes of mixed-species biofilms, such as those found in the oral cavity and on other human mucosal layers, and which consist of fungi and bacteria [49]. This might help to identify additional cell wall proteins, the action of which is crucial in the development of such biofilms.

Identification of the targeting mechanisms of fungal cell wall proteins has enabled genetic engineering of the fungal cell surface and the introduction of heterologous proteins [50]. In combination with the quantitative techniques discussed here, this will enable the development of novel live vaccines [51] and fine tuning of cell surface properties to improve fungal productivity in bioreactors.

As mentioned earlier, fungi can synthesize different sets of cell wall glycoproteins, depending on the environmental conditions [1]. Because many of the identified cell wall glycoproteins are abundant proteins, studying their regulatory mechanisms and developing mathematical models to analyze these control mechanisms in more depth becomes an attractive proposition. In combination with measuring transcript levels of CWP-encoding genes using microarrays or real-time quantitative PCR, reliable relative and absolute quantitation of cell wall glycoproteins will generate essential data for such studies.

Concluding remarks and future perspectives

A key challenge for fungal wall biology is to investigate the role of fungal wall glycoproteins in infection and disease, in biofilm formation and in morphogenesis. It is equally important to study the dynamics of fungal wall glycoproteins in relation to environmental conditions in a reliable, sensitive and high-throughput way. Comparison between species will reveal how the subproteome of fungal wall glycoproteins of each species is adapted to its particular niche. Direct MS analysis of the fungal wall glycoproteins by using the 'shaving' methods discussed here, and without prior gel electrophoretic separation steps, is a powerful tool for solving such questions. Importantly, these approaches can also be applied to bacterial walls and to the walls of green algae and higher plants, and might be equally effective in other taxonomic groups [7,52–55]. Finally, many of the approaches presented here for fungal wall glycoproteins seem, at least in principle, to be equally applicable to fungal glycoproteins secreted into the growth medium and for membrane-associated glycoproteins, thus extending their application to a much larger group of proteins.

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