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Review

Features and functions of covalently linked proteins in fungal cell walls

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Abstract

The cell walls of many ascomycetous yeasts consist of an internal network of stress-bearing polysaccharides, which serve as a scaffold for a dense external layer of glycoproteins. GPI-modified proteins are the most abundant cell wall proteins and often display a common organization. Their C-terminus can link them covalently to the polysaccharide network, they possess an internal serine- and threonine-rich spacer domain, and the N-terminal region contains a functional domain. Other proteins bind to the polysaccharide network through a mild-alkali-sensitive linkage. Many cell wall proteins are carbohydrate/glycan-modifying enzymes; adhesion proteins are prominent; proteins involved in iron uptake are present, and also specialized proteins that probably help the fungus to survive in its natural environment. The protein composition of the cell wall depends on environmental conditions and developmental stage. We present evidence that the cell wall of mycelial species of the Ascomycotina is similarly organized and contains glycoproteins with comparable functions.

Keywords: GPI-proteins; PIR-proteins; Proteomics; Cell wall porosity; Iron uptake; Antigenicity; Hydrophobicity; Biofilms; Host–pathogen interactions

1. Introduction

The fungal cell wall is essential for maintaining the osmotic balance of the cell, for creating and maintaining the shape of the cell, and for morphogenesis. Its mechanical strength and its role in protecting the cell against injury has led in the past to extensive studying of the stress-bearing glycans in the wall such as chitin and 1,3-β-glucan. Questions as to how, where, and when these are synthesized, which genes are involved, and how their activity is controlled have received much attention (Cabib et al., 1998; Orlean, 1997; Wessels, 1994). Other work revealed the presence of glycoproteins in the cell wall that are tightly associated with the structural polysaccharides in the wall. We know now, at least for ascomycetous yeasts, how these proteins may be linked to the skeletal network (Klis et al., 2002, 2004; Lipke and Ovalle, 1998). Genomic and proteomic approaches have made it clear that ascomycetous yeasts incorporate a large number of different proteins in their cell walls and that protein incorporation is tightly controlled. The population of cell wall proteins may vary in composition depending on the phase of the cell cycle, environmental conditions, and developmental stage. The genomes of both *Saccharomyces cerevisiae* and *Candida albicans* contain dozens of predicted cell wall protein-encoding genes, and mass spectrometric analysis of the walls of cells that are growing exponentially in rich medium has identified 15–20 different cell wall proteins in each organism (De Groot et al., 2004; Yin et al., 2005). This raises important questions with respect to the function

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of cell wall proteins. Why does the cell need so many different cell wall proteins and why is their incorporation in the cell wall so strongly regulated? This review focuses on the covalently linked proteins in fungal walls. We will therefore not discuss hydrophobins, an extremely interesting class of cell wall proteins in itself (Wösten, 2001; Wösten et al., 1999). Although most of our knowledge still comes from the ascomycetous yeasts, it is becoming clear that much of it may be extrapolated to mycelial Ascomycetes and to a lesser extent to Basidiomycetes as well. Importantly, with the rapid unraveling of more fungal genomes and the development of new analysis techniques, mycelial fungi are beginning to release their own secrets. Cell wall biology of fungi is at the dawn of a new era and we hope that this review may offer some guidance to researchers planning to enter or already working in this exciting field.

2. Morphological features of the fungal cell wall

The cell walls of known ascomycetous yeasts are bilayered. When viewed by scanning electron microscopy, the walls of *S. cerevisiae* and *C. albicans* reveal a fibrillar outer layer emanating from an underlying skeletal layer (Chaffin et al., 1998; Tokunaga et al., 1986). The cell wall fibrils of *C. albicans* seem to mediate adhesion to buccal epithelial cells (Tokunaga et al., 1990). Freeze-substitution techniques are also suitable to visualize the outer fibrillar layer of the yeast cell wall (Fig. 1A) (Baba et al., 1989; Hagen et al., 2004; Osumi, 1998). When transmission electron microscopy is used in combination with, for example, permanganate staining, a dark, electron-dense outer layer is observed surrounding a more transparent inner layer (Osumi, 1998). Zlotnik et al. (1984) have shown for *S. cerevisiae* that protease treatment

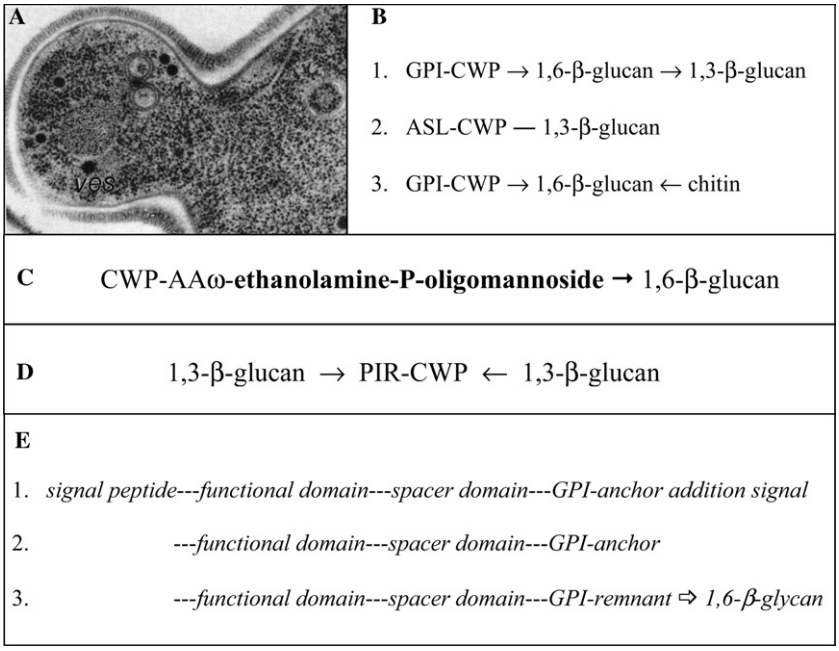


Fig. 1. Structural features of cell wall proteins in *S. cerevisiae* and *C. albicans*. (A) A freeze-substituted exponentially growing cell of *S. cerevisiae* (Baba et al., 1989). Permission granted by The Company of Biologists Ltd. Note the hair-like structures emanating from the transparent inner layer of the cell wall; ves, membrane vesicle. (B) The three most common CWP-polysaccharide complexes in the cell wall. The arrows represent glycosidic linkages and they point to a non-reducing end of the acceptor polysaccharide. The nature of the linkage between ASL-CWPs and 1,3-β-glucan is still unresolved. Complex 3 becomes much more abundant in cells subjected to cell wall stress (Kapteyn et al., 1997; Sestak et al., 2004). The branched 1,3-β-glucan chains aggregate laterally and form a continuous, hydrogen-bonded network (reviewed in Klis et al., 2002, 2004). Note that chitin chains may become linked to the 1,3-β-glucan meshwork of the lateral walls as chitin → 1,3-β-glucan (Cabib and Duran, 2005; Kollar et al., 1995). GPI-CWP, GPI-modified cell wall protein; ASL-CWPs, a group of 'alkali-sensitive linkage' cell wall proteins including the PIR family. (C) The carboxy-terminal amino acid of a mature GPI-modified cell wall protein is linked through a GPI-remnant to 1,6-β-glucan. The GPI-remnant (in bold) lacks GlcN-phosphatidylinositol. GPI-CWP, GPI-modified cell wall protein; AA_{ω}, the carboxy-terminal amino acid of the mature protein; the oligomannoside consists of 4 or 5 mannose residues and may be substituted with additional ethanolamine phosphate groups (Imhof et al., 2004; Kollar et al., 1997). The ethanolamine phosphate group that interconnects the protein with the oligomannoside is linked to the third mannose residue. The phosphodiester bridge is sensitive to HF. (D) Hypothetical scheme of how PIR-proteins may interconnect 1,3-β-glucan chains. The linkage between PIR-CWPs and 1,3-β-glucan is sensitive to mild alkali. Note that PIR-CWP-encoding genes of *S. cerevisiae* are upregulated during early G1, when the cells are growing isotropically, and in response to cell wall stress. The arrows represent glycosidic linkages and indicate that the reducing end of 1,3-β-glucan is presumably involved in the linkage to PIR-CWP. The precise nature of the linkage between PIR-CWPs and 1,3-β-glucan is still unresolved. (E) Regular domain organization of GPI-modified cell wall proteins. 1. Domain organization of the primary sequence of a GPI-protein. 2. The membrane-bound form. 3. The mature wall-bound form. The functional domain may be involved in ligand binding and adhesion, or it may have a catalytic function. The serine- and threonine-rich spacer domain often contains internal repeats.

removes the dark outer layer, but not the transparent inner layer. The early work by Kopecka et al. (1974) also demonstrates convincingly that the external layer of *S. cerevisiae* walls can be proteolytically removed without affecting the inner wall and that the internal layer can be digested using β-glucanases. Electron microscopic pictures of ascomycetous yeasts such as *Candida tropicalis*, *Hanseniaspora osmophila*, *Kloeckera*, *Kluyveromyces polysporus*, *Schizosaccharomyces pombe*, *Torulopsis glabrata*, and *Exophila dermatitidis* show a similar bi-layered cell wall (Baba and Osumi, 1987; Garrison, 1981; Yamaguchi et al., 2002). Collectively, these and many more additional data show that the walls of ascomycetous yeasts consist of an external protein layer strongly associated with and emanating from an inner polysaccharide layer. Depending on nutrient conditions, an inner dark layer close to the plasma membrane is observed as well. This layer consists of soluble, high-molecular-weight, cell surface proteins such as invertase and acid phosphatase (De Nobel et al., 1989; Tanner and Lehle, 1987).

Transmission electron microscopy studies of ascomycetous mycelial fungi such as *Aspergillus* (*Emericella*) *nidulans*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium oxysporum*, and *Neurospora crassa* also reveal a bi-layered structure (Chiou et al., 2001; Kurtz et al., 1994; Mahadevan and Tatum, 1967; Polacheck and Rosenberger, 1977; Schoffelmeer et al., 1999). The outer layer of the walls of *N. crassa*, which is alkali-extractable, consists of coarse fibrils and contains glycoproteins, whereas the internal skeletal layer consists of 1,3-β-glucan and chitin (Mahadevan and Tatum, 1967). The electron-dense outer layer of the wall of *F. oxysporum* is resistant to extraction with hot SDS, but sensitive to pronase (Schoffelmeer et al., 1999). In addition, an electron-dense inner layer was observed between the electron-transparent wall layer and the plasma membrane. This layer was SDS-soluble and sensitive to pronase digestion, indicating that this layer consists of soluble cell surface proteins similar to ascomycetous yeasts. Taken together, these data indicate that the cell walls of ascomycetous mycelial fungi generally have an external protein layer tightly associated with an inner skeletal layer.

Compared to ascomycetous fungi, much less is known about covalently linked cell wall proteins in basidiomycetous fungi. Transmission electron microscopy studies of permanganate-stained walls of *Ustilago maydis* growing in the yeast form revealed an inner transparent layer surrounded by a loose electron-dense layer, which seems similar to what has been observed in ascomycetous yeasts. Mycelial walls had a diffuse inner layer, in which multiple layers of different electron density could be discerned, and a loose, electron-dense outer layer (Ruiz-Herrera et al., 1996). Electron microscopy of the cell wall of *Pisolithus tinctorius*, an ectomycorrhizal basidiomycete, revealed an inner transparent layer surrounded by a thin electron-dense layer (Martin et al., 1999). In contrast, electron micrographs of cleaned isolated walls of an acapsular mutant of *Cryptococcus neoformans* revealed an electron-dense inner layer and a less dense outer layer; in addition, chemical analysis of isolated walls showed that the walls contained only glucose and hexosamine, but lacked mannose, galactose, and xylose, indicating the (near) absence of covalently linked cell wall proteins in this organism (James et al., 1990). These data suggest that in the walls of at least some basidiomycetous fungi an outer protein layer may occur, but also warn against hasty generalizations about the cell wall organization of basidiomycetous fungi. In the next section, we will discuss how the proteins of the external protein layer may be linked to the skeletal layer.

3. Cell wall proteins in ascomycetous yeasts

Incubation of cell walls of *S. cerevisiae* with a purified endo-1,6-β-glucanase removes an amorphous outer layer, revealing an underlying layer of densely interwoven microfibrils sensitive to 1,3-β-glucanase (Kopecka et al., 1974). This elegant work foreshadowed later biochemical work, which established that a class of cell wall proteins (GPI-CWPs, GPI-modified cell wall proteins; GPI, glycosylphosphatidylinositol) is covalently linked to 1,6-β-glucan through a trimmed form of their original GPI-anchor. The 1,6-β-glucan can be further linked to 1,3-β-glucan or chitin resulting in a strong covalent attachment of GPI-CWPs to the cell wall (Fig. 1B) (Kapteyn et al., 1996, 1997; Kollar et al., 1997). A second class of proteins, which include the PIR-CWPs (protein with internal repeats) is directly linked to the cell wall 1,3-β-glucan network through an as yet unidentified mild-alkali-sensitive linkage (Kandasamy et al., 2000; Kapteyn et al., 1999, 2000; Mrsa et al., 1997; Toh-e et al., 1993). This general picture has later been confirmed for *C. albicans* and *Candida glabrata* and more indirectly for other ascomycetous yeasts such as *Yarrowia lipolytica* as well (Frieman et al., 2002; Jaafar and Zueco, 2004; Kandasamy et al., 2000; Kapteyn et al., 2000; Weig et al., 2004). Because the PIR-proteins were the first proteins found to be linked through a mild-alkali-sensitive linkage to cell wall glycans, this class of proteins has been designated as PIR-CWPs. Recently, De Groot et al. (2004) and Yin et al. (2005) have shown that the cell walls of both *C. albicans* and *S. cerevisiae* contain proteins that do not show homology to PIR-proteins, but are nevertheless covalently linked through an mild-alkali-sensitive bond. It is therefore appropriate to rename this class of cell wall proteins; we propose to designate them as 'alkali-sensitive linkage' cell wall proteins (ASL-CWPs). Table 1 presents an overview of known fungal GPI- and ASL-CWPs. Cell wall proteins may not only be linked to cell wall polysaccharides, but some of them may also be linked through disulfide bonds to

Table 1
Features and functions of proteins that are covalently bound to cell wall glycans in fungi

Protein name	Proposed role or properties	Method of release from cell wall matrix[a] (identification method)	Reference for covalent linkage[b]
Yeasts			
S. cerevisiae GPI-CWPs			
Awa1p (sake yeast)	Foam forming, S/T-rich, repetitive sequences	1,6-β-Glucanase (IB)	(1)
Ccw12p	Unknown function	Laminarinase (ED)	(2)
Ccw14p/Ssr1p	CFEM domain	Trypsin, zymolyase, laminarinase, HF (ED, IB, MS)	(2, 3, 4)
Cwp1p	Unknown function	Trypsin, laminarinase, HF, NaOH (ED, IB, MS)	(4, 5)
Cwp2p	Unknown function	Laminarinase (ED)	(5)
Crh1p	GH16,[c] involved in chitin incorporation	Trypsin, HF (MS)	(4)
Dan1p/Ccw13p	Member of Srp1p/Tip1p family	Laminarinase (ED)	(2)
Ecm33p	Unknown role in cell wall biosynthesis	Trypsin, HF (MS)	(4)
Gas1p	GH72, hydrolysis and extension of 1,3-β-glucan	Trypsin, HF (MS)	(4)
Gas3p	GH72, hydrolysis and extension of 1,3-β-glucan	Trypsin, HF (MS)	(4)
Gas5p	GH72, hydrolysis and extension of 1,3-β-glucan	Trypsin, HF (MS)	(4)
Plb2p	Phospholipase	Trypsin, HF (MS)	(4)
Pry3p	SCP-like extracell. domain, Swi5p regulated	Trypsin, HF (MS)	(4)
Sag1p	Sexual agglutinin, immunoglobulin-like domains	Laminarinase (IB)	(6)
Sed1p	Expressed during stationary phase	RPI, laminarinase (ED)	(7)
Tip1p	Member of Srp1p/Tip1p family	GluC, laminarinase (ED, MS)	(4, 5)
Tir1p	Member of Srp1p/Tip1p family	GluC (MS)	(4)
Utr2p/Crh2p	GH16, involved in chitin incorporation	Trypsin, HF (MS)	(4)
S. cerevisiae ASL-CWPs			
Cis3p/Pir4p	Conserved four-cysteine domain	Trypsin, NaOH (ED, MS)	(4, 8)
Hsp150p/Pir2p	Conserved four-cysteine domain	Trypsin, NaOH (ED, MS)	(4, 8)
Pir1p/Ccw6p	Conserved four-cysteine domain	Trypsin, NaOH (ED, MS)	(4, 8)
Pir3p/Ccw8p	Conserved four-cysteine domain	Trypsin (MS)	(4)
Scw4p	GH17, 1,3-β-glucanase	Trypsin, NaOH (MS)	(4)
Scw10p	GH17, 1,3-β-glucanase	Trypsin, NaOH (MS)	(4)
Tos1p	Target of SBF	Trypsin, NaOH (MS)	(4)
C. albicans GPI-CWPs			
Als1p	Adhesin	Quantazyme, HF (MS)	(9)
Als4p	Adhesin	Quantazyme (MS)	(9)
Cht2p	GH18, Chitinase	Trypsin, quantazyme, HF (MS)	(9, 10)
Crh11p	GH16, involved in chitin incorporation	Quantazyme, HF (MS)	(9)
Ecm33p/Ecm33.3p	Unknown role in cell wall biosynthesis	Quantazyme, HF (MS)	(9)
Gas1p/Pga4p	Hydrolysis and extension of 1,3-β-glucan	Quantazyme (MS)	(9)
Pga24p/Ywp1p	Internal repeats, downregulated in hyphae	Quantazyme, HF (MS)	(9)
Pga29p	Unknown function	Quantazyme, HF (MS)	(9)
Phr1p	GH72, hydrolysis and extension of 1,3-β-glucan	Quantazyme, HF (MS)	(9)
Rbt5p	Iron uptake, CFEM domain	Quantazyme, HF (MS)	(9)
Sod4p/Pga2p	Superoxide dismutase	HF (MS)	(9)
Sod5p/Pga3p	Superoxide dismutase	HF (MS)	(11)
Ssr1p	CFEM-domain	Quantazyme, HF (MS)	(9)
C. albicans ASL-CWPs			
MP65/Scw1p	GH17, 1,3-β-glucanase	Quantazyme, NaOH (MS)	(9)
Pir1p	Conserved four-cysteine domain	Quantazyme, NaOH (IB, MS)	(9, 12)
C. glabrata GPI-CWPs			
Crh1p	GH16, involved in chitin incorporation	HF (MS)	(13)
Cwp1.1p	Unknown function	HF (MS)	(13)
Cwp1.2p	Unknown function	HF (MS)	(13)
Epa1p	Adhesin	1,6-β-Glucanase, quantazyme (IB, IM)	(14)

Table 1 (*continued*)

Protein name	Proposed role or properties	Method of release from cell wall matrix[a] (identification method)	Reference for covalent linkage[b]
Mycelial GPI-CWPs[d]			
AnCwpAp	Unknown function	HF (IB)	(15)
EnMnpAp	Unknown function	(IM)	(16)
FoFem1p	Unknown function	HF (ED, IB)	(17)
PmMp1p	Unknown function	Lyticase (intact cells) (IM)	(18)

[a] Quantazyme, zymolyase, laminarinase, 1,6-β-glucanase, lyticase, RPI (*Rarobacter faecitabidus* protease I), trypsin, and GluC are commercially available enzyme preparations. For conditions of HF and NaOH treatments, see De Groot et al. (2004). ED, Edman degradation; IB, immunoblotting; IM, immunoelectron microscopy; and MS, LC-MS/MS.

[b] 1, Shimoi et al. (2002); 2, Mrsa et al. (1999); 3, Moukadiri et al. (1997); 4, Yin et al. (2005); 5, Van der Vaart et al. (1995); 6, Lu et al. (1994); 7, Shimoi et al. (1998); 8, Mrsa et al. (1997); 9, De Groot et al. (2004); 10, Iranzo et al. (2002); 11, Fradin et al. (2005); 12, Martinez et al. (2004); 13, Weig et al. (2004); 14, Frieman et al. (2002); 15, Damveld et al. unpublished (acc nr. AT09020); 16, Jeong et al. (2004); 17, Schoffelmeer et al. (2001); and 18, Cao et al. (1998).

[c] GH, glycoside hydrolase, classification according to carbohydrate-active enzymes server at http://afmb.cnrs-mrs.fr/CAZY/.

[d] An, *Aspergillus niger*; En, *Aspergillus (Emericella) nidulans*; Fo, *Fusarium oxysporum*; Pm, *Penicillium marneffei*..

other CWPs (Cappellaro et al., 1998; Jaafar et al., 2003; Mrsa et al., 1997; Viudes et al., 2001). Finally, for separation and analysis of fungal cell wall proteins it is relevant to emphasize a technical point. Many cell wall proteins are heavily glycosylated and may have a very high and variable apparent molecular mass when run on gels; for optimal separation and analysis, gradient gels (commercially available) are recommended that allow separation up to at least 500 kDa.

4. GPI-modified cell wall proteins in mycelial fungi

Although GPI-CWPs have been identified in mycelial fungi, their precise linkage to the stress-bearing glycans has not been studied in detail. It is also not known how widespread the use of GPI-CWPs is in mycelial fungi. In *S. cerevisiae*, GPI-CWPs are linked to 1,6-β-glucan through a GPI-remnant, which includes a phosphodiester bridge connecting ethanolamine to the third mannosyl residue of the glycan core structure (Fig. 1C). This explains why GPI-CWPs can be specifically released using a phosphodiesterase, aqueous HF, or HF-pyridine (De Groot et al., 2004; Kapteyn et al., 1996). The first mycelial GPI-CWP was identified in *F. oxysporum* f. sp. *lycopersici*. When isolated, SDS-extracted cell walls of *F. oxysporum* were treated with aqueous HF, about 50% of all cell wall proteins were released, suggesting the retention of GPI-CWPs (Schoffelmeer, 1999). An amino acid sequence obtained from one of them led to the cloning of a gene called *Fusarium* extracellular matrix protein 1 (*FoFEM1*) (Schoffelmeer et al., 1999). FoFem1p has all the hallmarks of a GPI-CWP. It contains both an N-terminal ER targeting signal and a C-terminal GPI-anchor addition signal. In addition, the amino acid sequence upstream of the ω-site lacks basic amino acid residues consistent with its location in the cell wall (see below). Furthermore, when cell walls were digested with laminarinase, a 1,3-β-glucanase preparation, it could be shown immunologically that the proteins released were linked to both 1,6-β-glucan and 1,3-β-glucan and that this linkage was sensitive to aqueous HF, suggesting the presence of GPI-proteins linked through a GPI-remnant to a 1,6-β-glucan moiety, which in turn is linked to 1,3-β-glucan, similar to ascomycetous yeasts (Schoffelmeer et al., 1996).

Gibberella zeae (anamorph *Fusarium graminarium*) has a FoFem1p homolog that is 76% identical at the amino acid level. The GzFem1p contains further both an N- and a C-terminal hydrophobic sequence for targeting to the ER and for GPI-anchor addition, respectively. BLAST searches in other known fungal genomes revealed the presence of homologs in the genomes of *N. crassa*, *Magnaporthe grisea*, and *A. nidulans*. Analysis of the Fem1p homolog in *M. grisea*, Emp1, revealed that this putative cell wall protein is specifically transcribed during appressorium formation. Deletion of the gene resulted in reduced appressorium formation and pathogenicity, indicating that the protein helps to withstand the enormous turgor pressure in the appressorium during leaf penetration (Ahn et al., 2004). Comparison of the GPI-anchor addition signals of the different homologs further suggests that these proteins are localized to the cell wall because they all lack a dibasic motif upstream of their predicted ω-site (see also Section 9).

A second family of putative GPI-modified cell wall mannoproteins has been first discovered in *Penicillium marneffei* (Cao et al., 1998). PmMp1 is an abundant, glucanase-extractable mannoprotein, and immunogold labeling showed it to be present in the outer layer of the hyphal wall. Another member of the *MP1* family, produced by the GPI-protein-encoding gene *cwpA*, has been discovered in the walls of *Aspergillus niger*. This protein is HF-extractable and was immunologically shown to be abundantly present in the cell wall (Damveld, Ram, and Klis, unpublished). Using immunogold labeling, Jeong et al. (2003, 2004) discovered an Mp1-like protein in the walls of *A. nidulans*, which they

called MnpAp. Mp1 homologs have also been identified in *F. oxysporum*, *G. zeae*, *M. grisea*, and *N. crassa*. All members of this gene family possess a putative GPI-anchor addition signal.

Pneumocystis carinii is an ascomycetous fungus that infects the lungs of mammals and is a very common infection in immunocompromised people (Nakamura, 1998). Its cell wall consists primarily of glucose, mannose, and galactose. An abundant cell surface mannoprotein (MSG, major surface glycoprotein) is associated with the cell wall as shown by immunoferritin labeling and is believed to play an important role in the interaction with host cells. PcMSG from ferrets has the hallmarks of an authentic GPI protein (Guadiz et al., 1998), indicating that PcMSG from ferrets is a genuine GPI-modified cell wall protein. However, predicted MSG sequences form other organisms do not readily fulfill the sequence requirements of GPI-proteins, indicating the need for more (biochemical) research.

Laminarinase-extractable cell wall proteins carrying a 1,6-β-glucan epitope have also been identified in *A. niger* and *Paecilomyces variotii* (Brul et al., 1997). The cell walls of *Penicillium allahabadense* also seem to contain a 1,6-β-glucan polymer, but a possible linkage to cell wall proteins has not yet been investigated (Santos et al., 2000). HF treatment of SDS-extracted cell walls from *A. niger* released at least four distinct protein bands as detected by the lectin Concanavalin A, indicating the presence of mannosylated GPI-CWPs in *A. niger* (Damveld, Ram, and Klis, unpublished). Recently, a simple, qualitative 1,6-β-glucan assay has been developed for *S. cerevisiae* (Vink et al., 2004). This assay might help to identify 1,6-β-glucan synthesis in other fungi and to establish how widespread the use of this polysaccharide is in fungal cell wall construction. This is a relevant question because biochemical analysis of the alkali-insoluble part of the cell wall of *A. fumigatus* failed to detect 1,6-β-glucan molecules (Fontaine et al., 2000). This raises the question whether mycelial fungi might synthesize an additional CWP-polysaccharide complex (GPI-CWP → 1,3-β-glucan), in which a GPI-CWP is directly linked to 1,3-β-glucan without an intervening 1,6-β-glucan moiety. It is also conceivable that other carbohydrate polymers in the cell wall of mycelial fungi such as 1,3-1,4-β-glucan or 1,3-α-glucan might serve as alternative acceptor molecules for GPI-CWPs.

Melanization of the fungal wall might complicate the release and identification of covalently linked cell wall proteins. For example, the pathogenic black yeast *E. dermatitidis* is resistant to Zymolyase, a mixture of a protease and a 1,3-β-glucanase, whereas a melanin-deficient strain is highly sensitive (Montijn et al., 1997). The walls of *Rhizoctonia solanum*, which contain considerable amounts of melanin, are also highly resistant to cell wall lytic enzymes. In contrast, *Fusarium solani* walls, which contain little or no melanin, are rapidly digested (Potgieter and Alexander, 1966). Furthermore, whereas in exponentially growing cultures of *A. nidulans* melanization is negligible, melanin levels increase strongly in stationary phase cultures (Polacheck and Rosenberger, 1977).

Most information concerning covalently linked cell wall proteins comes from ascomycetous fungi, and data from basidiomycetous fungi are scarce. Ruiz-Herrera and co-workers found that SDS-extracted walls of both growth forms of the dimorphic basidiomycete *U. maydis* contain proteins, that could be specifically extracted with either chitinase or 1,3-β-glucanase, suggesting that they might be linked either directly or indirectly to chitin or 1,3-β-glucan, respectively (Ruiz-Herrera et al., 1996). However, it is unknown how exactly they are connected to the skeletal polysaccharides and whether GPI-proteins are involved or otherwise.

5. Mild-alkali-extractable cell wall proteins in mycelial fungi

PIR-CWPs of *S. cerevisiae* are directly linked to 1,3-β-glucan (Kapteyn et al., 1999). The repetitive sequences in PIR-CWPs seem important for cell wall anchoring. In contrast to the other PIR-CWPs, Pir4p has only a single repeat sequence and deletion of this sequence resulted in a failure to link Pir4p to 1,3-β-glucan (Castillo et al., 2003). If indeed the repeats are mediating the linkage between 1,3-β-glucan and PIR-proteins, this may have an interesting consequence in that PIR-proteins with multiple repeats, and possibly other ASL-CWPs as well, may interconnect two or even more 1,3-β-glucan chains, thereby strengthening the cell wall (Fig. 1D). This is consistent with the observations that PIR-CWPs in contrast to GPI-CWPs are uniformly distributed throughout the inner polysaccharide layer of the cell wall and that in case of cell wall damage the PIR-CWP-encoding genes are strongly upregulated (Boorsma et al., 2004; Garcia et al., 2004; Kapteyn et al., 2000; Lagorce et al., 2003).

Recently, ScPir2p has been successfully expressed in the cell wall of the mycelial fungus *F. oxysporum*, suggesting that the incorporation mechanism of ASL-CWPs is not only used in ascomycetous yeasts but also in ascomycetous mycelial fungi (Narasimhan et al., 2003). This is consistent with the presence of homologs of ScPIR-proteins in a wide range of Ascomycetes such as the yeasts *C. albicans*, *C. glabrata*, *Debaryomyces hansenii*, and *Kluyveromyces lactis*, and the mycelial fungi *N. crassa*, *M. grisea* (see Section 8). Possibly, mycelial fungi also incorporate PIR-proteins in their walls in response to cell wall stress or during periods of isotropic expansion as, for example, in conidia that are breaking dormancy (Momany, 2002). Mild alkali-extraction of SDS/β-mercaptoethanol-treated cells wall of *A. niger* revealed the presence of at least three proteins with a

molecular mass of 70, 110, and >200 kDa, indicating that mycelial fungi might contain ASL-CWPs (Damveld, Ram, and Klis, unpublished).

Although the putative cell wall protein PhiAp of *A. nidulans* shows (limited) homology to ScCwp1p, it lacks a GPI-anchor addition signal (Melin et al., 2003). This raises the question whether it might be linked to the cell wall by an alkali-sensitive linkage, or by an as yet unknown linkage. PhiAp is important for conidium development, and cells lacking PhiAp display reduced conidiation due to altered phialide development. Other mycelial Ascomycetes contain homologs of PhiAp, and they also lack a putative GPI-anchor addition signal (Melin et al., 2003).

6. Non-conventional cell wall-associated proteins

There is ample evidence that glycolytic enzymes and other abundant cytosolic proteins may be associated with the cell walls of *S. cerevisiae* and *C. albicans* (reviewed in Chaffin et al., 1998; Delgado et al., 2003; Edwards et al., 1999; Motshwene et al., 2003; Urban et al., 2003). Generally, they can be released by extracting the cells with reducing agents like mercaptoethanol, explaining why mass spectrometric analysis of isolated cell walls of *S. cerevisiae* and *C. albicans*, which beforehand are extracted with a mixture of SDS and mercaptoethanol, does not detect them (De Groot et al., 2004; Yin et al., 2005). Because they lack a canonical signal peptide and because their cell wall-associated forms do not seem to be glycosylated, it has been speculated that they are transported to the cell surface through a non-conventional export pathway. In itself, this is not unprecedented. For example, *C. albicans* can switch between two colony-forming phenotypes, called white and opaque, and only 'opaque' cells can mate (Lachke et al., 2003). Interestingly, 'opaque' cells possess pimples on their walls, which seem to be associated with channels through the wall, and from which small vesicles may emerge (Anderson et al., 1990). However, this cannot explain the occurrence of cell wall-associated glycolytic enzymes in 'white' *C. albicans* cells and in *S. cerevisiae* cells. One may further imagine that small amounts of cytosolic proteins 'hitch-hike' to the surface by leaking into the secretory pathway during the formation of transport vesicles or when transport vesicles fuse with their target organelles. This would agree with the observation that only abundant cytosolic proteins have been detected at the cell surface. Another explanation for the occurrence of cell wall-associated glycolytic enzymes might be the following. Cell walls of *S. cerevisiae* and *C. albicans* contain large numbers of negatively charged phosphate groups in the form of phosphodiester bridges in both N- and O-carbohydrate side-chains (Horisberger and Clerc, 1988; Jigami and Odani, 1999). These are negatively charged at pH 3 and higher and thus may bind positively charged proteins. Most glycolytic enzymes have a relatively high isoelectric point and are thus often positively charged depending on the pH of the culture medium. This raises the question whether cell wall-associated glycolytic enzymes may stem from aging cells or from cells damaged by shearing forces. In most studies up to now, such considerations have not been taken into account.

7. In silico identification of GPI-modified proteins

A genome-wide in silico survey of GPI-modified proteins in *S. cerevisiae* was already performed in 1997 (Caro et al., 1997). In their analysis, all proteins with a predicted N-terminal signal peptide were screened for the presence of a hydrophobic domain at the extreme C-terminus. Potential GPI-proteins were then further analyzed for the presence of a GPI-anchor attachment site according to the consensus rules of (Nuoffer et al., 1993), resulting in 58 putative GPI-proteins. More refined versions of this algorithm, based on additional sequence characteristics of known GPI-proteins from various Ascomycetes, later revealed 66, 104, 106, 33, and 97 putative GPI-proteins in *S. cerevisiae*, *C. albicans*, *C. glabrata*, *S. pombe*, and *N. crassa*, respectively (De Groot et al., 2003; Weig et al., 2004). Reliable Web-accessible algorithms for the identification of fungal GPI-proteins and prediction of their GPI-modification site have also become available (http://129.194.185.165/dgpi/index_en.html; http://mendel.imp.univie.ac.at/gpi/fungi_server.html; Eisenhaber et al., 2004).

Fungal GPI-proteins may be either targeted to the plasma membrane or the cell wall. An intriguing question is how the final destination of a GPI-protein is determined. Vossen et al. (1997) observed that GPI-modified proteins that are predominantly found in the plasma membrane of *S. cerevisiae* generally contain two basic amino acids upstream of the GPI-anchor attachment site (ω-site). This seems also to be the case for other fungi. Many of the predicted GPI-proteins in the human pathogenic yeasts *C. albicans* and *C. glabrata* are believed to be involved in adhesion and are for that reason presumably exposed to the outer surface of the cell wall. Consistently, these proteins generally lack a dibasic motif in the ω-proximal region. Instead, hydrophobic amino acids such as valine, leucine or isoleucine upstream of the ω-site at the ω-2, ω-4, and ω-5 positions seem to act positively to localize the protein to the cell wall (Hamada et al., 1998, 1999). A systematic mutational analysis in *C. glabrata* of the amino acids upstream of the ω-site has confirmed the importance of the dibasic motif for retaining GPI-proteins in the plasma membrane (Frieman and Cormack, 2003). However, the final destination of GPI-proteins is not only

determined by the ω-proximal region. Apparently, the presence of long serine- and threonine-rich regions, which are characteristic of many GPI-proteins, may favor targeting of a GPI-protein to the cell wall and may even override the plasma membrane-retaining effect of a dibasic motif in the ω-proximal region (Frieman and Cormack, 2004).

Many of the predicted fungal GPI-proteins belong to protein families such as the family of lysophospholipases, the aspartic proteases, the Sps2/Ecm33 family, the Gas/Phr family, and the Crh family (Caro et al., 1997; Coutinho and Henrissat, 1999; De Groot et al., 2003; Naglik et al., 2003). In addition, many of them seem to be conserved among Ascomycetes since orthologs are present in all or most of the analyzed genomes. Interestingly, there are several families containing conserved putative carbohydrate-active enzymes such the Gas/Phr and Crh families. In vitro studies have demonstrated that members of the Gas family can indeed hydrolyze and elongate oligomers of 1,3-β-glucan (Hartland et al., 1996; Mouyna et al., 2000a,b); in addition, members of the Crh family seem to co-localize with the sites of chitin deposition in the cell wall (Rodriguez-Pena et al., 2000, 2002). Interestingly, orthologs, belonging to these families and predicted to be GPI-proteins, exist in the genome of the basidiomycetous yeast *C. neoformans* (De Groot and Klis, unpublished observations). In *A. fumigatus*, members of the Gas, Crh, and Ecm33 families have been identified in membrane extracts obtained with phosphatidylinositol-specific phospholipase C, leading to the hypothesis that their activity is plasma membrane-associated (Bruneau et al., 2001). On the other hand, analysis of cell wall preparations, pre-treated with hot SDS and β-mercaptoethanol, has demonstrated that their orthologs in *S. cerevisiae*, *C. albicans*, and *C. glabrata* are at least partially covalently incorporated into the cell wall, suggesting that they may also be active while being covalently bound to the cell wall polysaccharide network (De Groot et al., 2004; Weig et al., 2004; Yin et al., 2005).

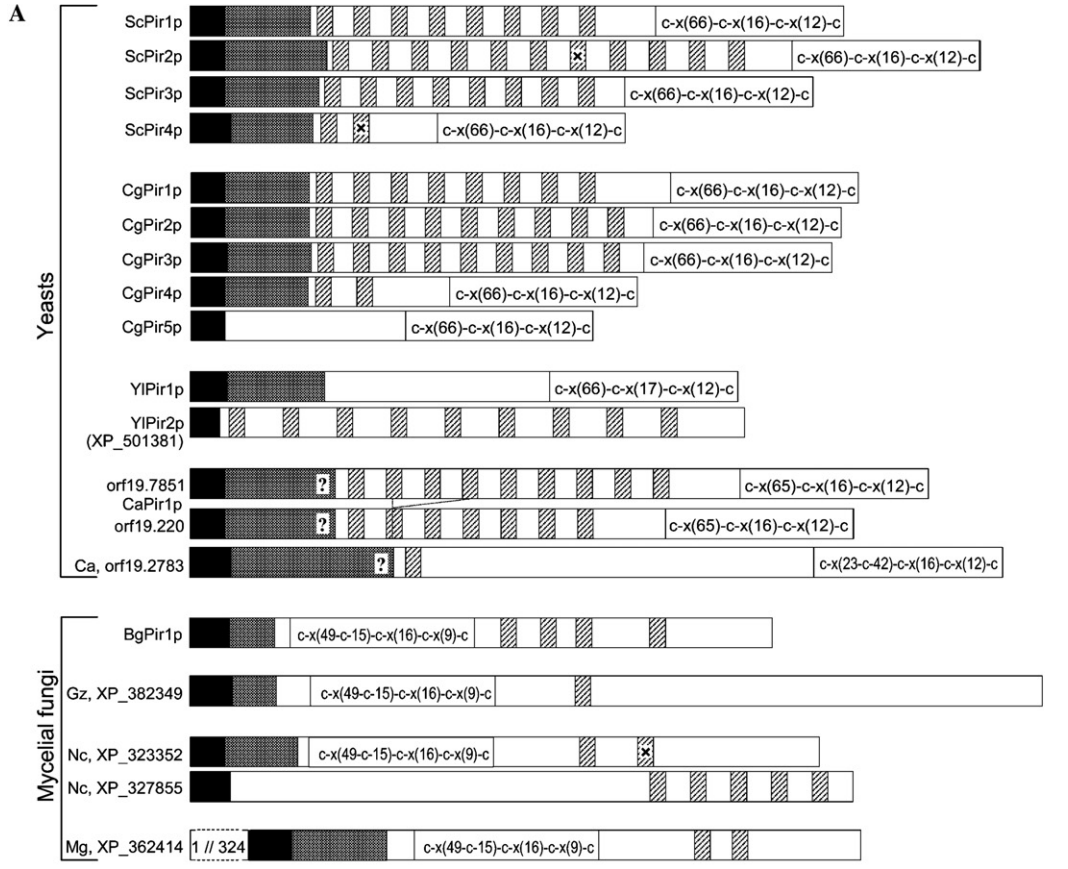
8. In silico identification of PIR-CWPs

As mentioned earlier, a second class of covalently linked cell wall mannoproteins are the ASL-CWPs, including the PIR-proteins. Typical features of PIR-proteins are: (i) they are synthesized as pre-pro-peptides, and the pro-part of the protein is cleaved off in the Golgi apparatus by the serine proteinase Kex2p; (ii) they contain a variable number of glutamine-containing internal repeats that conform to the consensus sequence Q[IV]XDGQ[IVP]Q (Prosite format); (iii) they have a conserved carboxy-terminal domain containing four cysteine residues with fixed spacing, called the four-cysteine domain (Fig. 2A). Based on these characteristics, PIR-proteins can be easily recognized and identified, for example, by performing pattern searches with the consensus repeat sequence or by BLAST searches using the entire four-cysteine domain as the query sequence. Such analyses convincingly show that the yeasts *D. hansenii* and *K. lactis*, like *S. cerevisiae*, *C. albicans*, and *C. glabrata*, contain a small family of PIR-proteins. PIR-proteins do not seem to be restricted to ascomycetous yeasts. For example, in the mycelial fungi *Blumeria graminis*, *G. zeae*, *N. crassa*, and *M. grisea* putative PIR-proteins are present, but they show some interesting new features (Fig. 2B): (i) they contain an additional cysteine residue in the conserved four-cysteine domain, and (ii) this domain is not at the carboxy-terminal part of the protein, but it is localized in the N-terminal region, before the repeat sequences, in contrast to yeast PIR-CWPs.

9. Posttranslational modifications of fungal glycoproteins

The synthesis of O- and N-linked carbohydrate side-chains of fungal glycoproteins begins in the ER and is completed in the Golgi. N- and O-glycosylation of glycoproteins of the ascomycetous yeasts *S. cerevisiae*, *C. albicans*, *Pichia pastoris*, and *S. pombe* has been studied in depth (Cutler, 2001; Dean, 1999; Gemmill and Trimble, 1999; Orlean, 1997; Strahl-Bolsinger et al., 1999). In these fungi, N-chains consist of a core structure common to all eukaryotic cells, which may be extended with a 1,6-α-mannosyl backbone heavily substituted with short mannosyl side-chains. Also, some degree of phosphorylation in the form of phosphodiester bonds is present in *N*-glycan side-chains, giving yeast its uniform anionic surface charge (Gemmill and Trimble, 1999; Horisberger and Clerc, 1988) and allowing the staining of yeast cells with Alcian blue (Conde et al., 2003). O-chains of these fungi form short oligomannosides linked to peptidylserine or threonine. In addition, O-chains of *S. cerevisiae* may contain a phosphodiester-linked mannose residue as well (Jigami and Odani, 1999; Nakayama et al., 1998). In *S. pombe*, O-chains may also contain galactosyl residues (Gemmill and Trimble, 1999).

N- and O-glycosylation in mycelial fungi are less well studied. Unfortunately, in many cases the data have been obtained using cell wall fractions such as galactomannan isolated by relatively harsh extraction methods without any attempt to determine if this material might represent protein-bound N-chains or perhaps otherwise (Ahrazem et al., 2002a,b; Domenech et al., 1999; Nakajima et al., 1984b). This has become especially relevant since the identification of a galactomannan in *A. fumigatus* cell walls directly linked to the 1,3-β-glucan network (Fontaine et al., 2000). The general structure of cell wall galactomannan in mycelial fungi is, however, similar to the structure of *N*-glycans in ascomycetous yeasts. They



B

ScPir1p/YKL164c	274	QFQFDGPPPQAGAIYAAGWSITPEGNLAIGDQDTFYQCLSGNFYNLYD	321
ScPir2p/YJL159w	345	QFQFDGPPPQAGAIYAAGWSITPDGNLAIGDNDVFYQCLSGTFYNLYD	392
ScPir3p/YKL163w	294	QFQFDGPPPQAGAIYAAGWSITPEGNLALGDQDTFYQCLSGDFYNLYD	341
ScPir4p/YJL158c	160	QFQFDGPPPQAGAIYAAGWSITEDGYLALGDSDVFYQCLSGNFYNLYD	207
CgPir1p/CAGL0I06204g	282	QFQFDGPPPQAGAIFAAGWSLTPEGNLAIGDNDVFYQCLSGNFYNLYD	329
CgPir2p/CAGL0I06182g	273	QFQFDGPPPQAGAIFAAGWSLTPQGNLALGDNDVFYQCLSGNFYNLYD	320
CgPir3p/CAGL0M08492g	268	QFQFDGPPPQAGAIYAAGWSLTPQGNLALGDSDVFYQCLSGNFYNLYD	315
CgPir4p/CAGL0I06160g	166	QFQFDGPPPQAGAIYAGGWSITEQGNLALGNSDVFYQCLSGNFYNLYD	213
CgPir5p/CAGL0M08514g	143	QFQFDGPPPQAGAIYAAGWSVTPEGNLALGDSDVFYQCSSGNFYNLYD	190
YlPir1p/XP_501130	218	QFQFDGPPPQAGAWYAAGWAISSDGNLAIGDNQVFWQCLSGTFYNLYD	265
CaPir1p/orf19.7851	319	QFQFDGPIPQAGTIYSAGWSI-KDGYLYLGDSNIFYQCLSGDFYNLYD	365
CaPir1p/orf19.220	280	QFQFDGPIPQAGTIYSAGWSI-KDGYLYLGDSNIFYQCLSGDFYNLYD	326
Ca, orf19.2783	357	QFQFDGPTPQHGAIYAAGWSVTKQGQLALGDSTKFYQCASGDFYNLYD	404
BgPir1p, AAK95385	82	QFQFDAP-PQINADSVGGYSSCSNGSLALAESSVFYQCLSGDFYNLYD	128
Gb, XP_382349	93	QFQFDKP-AQSGAIYTSGFSVCSNGTLALGPSAIFWQCKSGDFYNLYD	139
Nc, XP_323352	93	QFQFDGP-PQAGAIYTAGFSVCNNGSLALGGSTVFYQCRSGDFQNLYD	139
Mg, XP_362414	431	QFQFDKP-AQAGALYTAGFSLCPNGLMALGNSTQFWQCKSGDFWNLYD	477

Fig. 2. Fungal PIR-proteins have modular structures. (A) Domain organization of known and predicted PIR-proteins. Black bars indicate signal peptides for secretion (according to SignalP V3.0), grey areas denote (potential) pro-sequences, which can be cleaved off at the C-terminal end of the grey areas by the Kex2p endoproteinase. Hatched boxes indicate PIR-specific repeats consistent with the consensus sequence Q[IV]XDGQ[IVP]Q, and boxes marked with an × indicate imperfect repeat-like sequences. Conserved 'four-cysteine domains' are represented by open boxes in which the spacing between the conserved cysteins is indicated. The (putative) *C. albicans* PIR-proteins lack proper Kex2p substrate sites (KR or RR), instead they contain multiple lysine residues, indicated by a **?**, at the expected positions immediately preceding the first repeat sequence. The *M. grisea* PIR-protein homolog XP_362414 is annotated as a putative protein of 654 amino acids, which lacks an N-terminal signal peptide. However, we observed that translation of this ORF may actually start at Met_{325}, which would render a protein with typical PIR-protein characteristics. (B) Alignment showing the most conserved part, starting 30 residues behind the first cysteine, of the 'four-cysteine domains' of PIR-proteins. Amino acids that are identical in at least 14 of the aligned sequences (out of 17) are indicated by black shading and similar amino acids by grey shading. Sc, *Saccharomyces cerevisiae*; Cg, *Candida glabrata*; Yl, *Yarrowia lipolytica*; Ca, *Candida albicans*; Bg, *Blumeria graminis*; Gb, *Gibberella zeae*; Nc, *Neurospora crassa*; Mg, *Magnaporthe grisea*.

all have a 1,6-linked backbone of α-mannosyl residues heavily substituted with short α-mannosyl side-chains, which may be capped with galactosyl residues. Consistent with this, the *S. cerevisiae* genes *MNN9* and *OCH1*, which are involved in synthesizing the 1,6-linked backbone, have homologous genes in mycelial fungi such as *N. crassa*. *O*-glycans of mycelial glycoproteins also seem to have a structure that is closely related to that found in ascomycetous yeasts. Nakajima et al. (1984a) have identified putative *O*-glycans in *N. crassa*, which consist of a 1,2-α-linked mannobiosyl core that may be extended with one or two β-galactofuranosyl residues. Similar structures have been determined in *A. niger* (Wallis et al., 1999). Again, this is consistent with the presence of homologous genes in mycelial fungi that are related to the *PMT* genes of *S. cerevisiae*, which are responsible for attaching the first mannose residue to serine and threonine residues (Oka et al., 2004; Shaw and Momany, 2002; Strahl-Bolsinger et al., 1999; Zakrzewska et al., 2003). Mammalian homologs of *PMT* genes are also known (Willer et al., 2003).

GPI-proteins are characterized by the presence of an N-terminal hydrophobic signal sequence for translocation across the ER-membrane and a hydrophobic C-terminus. After translocation across the ER-membrane, the signal peptidase complex removes the N-terminal signal peptide, and a transamidase complex replaces the C-terminal GPI-addition signal by a pre-assembled GPI-anchor (Fig. 1E). The molecular mechanism for GPI-anchor biosynthesis and attachment is highly conserved among eukaryotes, including fungi (Fontaine et al., 2004). Proteins that receive a GPI-anchor in the ER are transported via the secretory pathway to the plasma membrane. As already discussed above, fungal GPI proteins can have two final destinations, either the plasma membrane or the cell wall. Cell wall attachment in *S. cerevisiae* has been shown to require the processing of the GPI-anchor resulting in attachment of the first mannose residue of the GPI-glycan core to a 1,6-β-glucan acceptor molecule (Kollar et al., 1997; Lu et al., 1994; Van der Vaart et al., 1996). The biogenesis of the sexual agglutinin Sag1p of *S. cerevisiae* has been studied in detail (Lu et al., 1994, 1995). Importantly, no evidence was obtained indicating that the linkage between 1,6-β-glucan and Sag1p is made intracellularly. This is consistent with the immunological observations by Montijn and co-workers, who could not detect intracellular 1,6-β-glucan formation (Montijn et al., 1999).

10. Modular structure of wall-bound GPI-proteins

Mature GPI-CWPs are linked to the internal glycan network of the cell wall through their C-terminus and extend their N-terminus into the medium (Kollar et al., 1997). Interestingly, most wall-bound GPI-proteins seem to have a similar modular structure in which the functional domain (for example, ligand binding or catalytic domain) is found in the N-terminal half of the protein (Fig. 1E). This is followed by a domain that is enriched in serine and threonine residues allowing it to become densely glycosylated, suggesting that this part may form a spacer domain that helps to extend the functional domain into the medium (Jentoft, 1990). It may also help to determine whether a GPI-protein is retained in the plasma membrane or incorporated in the cell wall (Frieman and Cormack, 2004). The notion of a densely glycosylated serine- and threonine-rich domain acting as a spacer domain is supported by the observation, that altering the length of this domain strongly affects the accessibility and activity of the N-terminal domain (Breinig and Schmitt, 2002; Frieman et al., 2002). Importantly, the spacer domain often contains a number of in tandem arranged similar amino acid sequences. Variation in the number of such repeats due to mistakes by the genetic machinery may possibly affect accessibility of the active domain and thus adhesion and virulence. Finally, the C-terminal end of the protein connects the protein to the cell wall glycan network.

This domain organization has, for example, been found in adhesion proteins such as the flocculins Flo1p, Flo5p, Flo9p, Flo10p, and Flo11p, and the sexual agglutinin Sag1p of *S. cerevisiae* (Chen et al., 1995; Kobayashi et al., 1998), the Als proteins of *C. albicans*, *Candida dubliniensis*, and *C. tropicalis* (Hoyer, 2001; Hoyer et al., 2001), and the Epa family of *C. glabrata* (Frieman et al., 2002). In addition, the many wall-bound GPI-proteins with a functional domain predicted or shown to be involved in processing carbohydrates (glycosylase or transglycosylase activity) in *S. cerevisiae* and *C. albicans* show a similar domain organization (De Groot et al., 2004; Yin et al., 2005). Hwp1p, which is a wall-bound GPI-protein specific for the hyphal growth form of *C. albicans*, also has a similar modular structure. Its N-terminal functional domain contains glutamine-rich repeats, acting as a substrate for the extracellular transglutaminase activity of buccal epithelial cells, thus allowing the cross-linking of Hwp1p to proteins of the extracellular matrix of these cells. GPI-modified cell wall proteins with a so-called CFEM domain are organized in the same way. GPI-CWPs with a CFEM domain have been found in *S. cerevisiae* (Ssr1p/Ccw14p), *C. albicans* (Ssr1p, Rbt5p, Csa1p), and are also predicted to be present in the walls of filamentous fungi such as *M. grisea* and *N. crassa*. They are characterized by a conserved eight-cysteine pattern (De Groot et al., 2004; Kulkarni et al., 2003; Yin et al., 2005). The GPI-CWP MP1 in *A. fumigatus* has a similar domain structure (Chong et al., 2004).

Mild-alkali-extractable cell wall proteins such as the ScPIR-proteins seem to be attached through their

N-terminal region (Castillo et al., 2003). Consistent with this observation, these proteins possess a highly conserved C-terminal domain that is probably responsible for their as yet unknown function. The active domain (glycosyl hydrolase family 17 domain) of the mild-alkali-extractable proteins Scw10p and Scw4p in *S. cerevisiae*, and Scw1p in *C. albicans*, is also in the C-terminal region, suggesting that the N-terminal region may be involved in linking the protein to the cell wall glycan network.

11. Differential expression of fungal wall proteins

Growth conditions strongly affect the composition and structure of the cell wall of baker's yeast; also cell wall mass as a percentage of the total cell mass is quite variable (Aguilar-Uscanga and Francois, 2003; Kapteyn et al., 2001). Transcript analysis in *S. cerevisiae* and *C. albicans* has made it abundantly clear that transcript levels of cell wall proteins may vary hugely. The available evidence indicates that protein levels in the cell wall are correlated with transcript levels, implying that the composition of the protein population in the cell wall depends strongly on environmental conditions. Various factors affect the protein composition of the cell wall. (i) The phase of the cell cycle. Spatial and temporal control of incorporation of individual cell wall proteins has been observed (Caro et al., 1998; Ram et al., 1998; Rodriguez-Pena et al., 2000, 2002; Spellman et al., 1998). For example, in *S. cerevisiae* Pir1p, Pir2p/Hsp150p, and Pir3p are strongly expressed during early G1 when the immature daughter cells expand by isotropic growth (Colman-Lerner et al., 2001; Spellman et al., 1998). (ii) Usage of specific cell wall proteins depends on environmental conditions such as pH, oxygen availability, nutrient availability, and temperature (Abramova et al., 2001; Fradin et al., 2005; Kapteyn et al., 2001; Ter Linde et al., 1999). (iii) Cell wall stress activates a salvage mechanism that is dubbed the 'cell wall integrity' pathway (Boorsma et al., 2004; Garcia et al., 2004; Jung and Levin, 1999; Lagorce et al., 2003). When the mycelial fungus *A. flavus* is treated with an inhibitor of 1,3-β-glucan synthesis, the external protein layer becomes much thicker, consistent with the notion that in mycelial fungi a cell wall salvage pathway is active as well (Kurtz et al., 1994). In *A. niger* this is also evidenced by the increased deposition of chitin and the higher expression of a 1,3-α-D-glucan synthase-encoding gene in response to cell wall stress (Damveld et al., 2005; Ram et al., 2004). (iv) The protein composition in the cell wall also depends on the developmental stage. For example, the formation of a mating structure in *S. cerevisiae* cells in response to the addition of mating pheromone is accompanied by qualitative and quantitative changes in the protein population of the cell wall (Gomez-Esquer et al., 2004; Roberts et al., 2000). Because the developmental biology of mycelial fungi is more complicated than that of budding yeast, one may anticipate further discoveries. In this connection it is important to emphasize that turnover of cell wall proteins in *S. cerevisiae* is limited (Kratky et al., 1975; Pastor et al., 1982; Mol and Klis, unpublished data); in other words, changes in the protein population of the cell wall as measured in a cell culture are largely or entirely due to formation of new cells with a differently composed cell wall.

12. Functions of covalently linked fungal cell wall proteins

The functions of covalently linked fungal cell wall proteins are manifold (Table 1). The full picture is still far from complete, but some general features are beginning to emerge. Here we will discuss some known and putative functions of the fungal cell wall proteome.

12.1. Cell wall porosity

Earlier estimates of the porosity of isolated cell walls of *S. cerevisiae* and *N. crassa* point to a very low value of less than 1000 Da (Gerhardt and Judge, 1964; Scherrer et al., 1974; Trevithick and Metzenberg, 1966). These values are, however, a serious underestimation of the cell wall porosity in vivo, because the glycan network is highly flexible and under normal conditions considerably stretched due to osmotic pressure in living cells (De Nobel and Barnett, 1991; De Nobel et al., 1990a; Morris et al., 1986). It has been shown for bakers' yeast that small proteins such as cytochrome *c* (12 kDa) but even bovine serum albumin (67 kDa) can rapidly pass the cell wall of living cells (Svihla et al., 1969; Yphantis et al., 1967). It has further been shown that the external protein layer limits yeast cell wall porosity, and that in particular the extended N-chains of the cell wall proteins are responsible for this (De Nobel et al., 1990b; Zlotnik et al., 1984). Conceivably, this limited porosity may prevent soluble cell surface proteins such as invertase and acid phosphatase, which generally have a high molecular mass, from leaking out (De Nobel et al., 1989; Tanner and Lehle, 1987). In addition, it may reduce the loss of soluble precursor forms of cell wall proteins into the medium. This might explain why mutations in *S. cerevisiae* that prevent the extension of the core N-chain, such as *mnn9* and *och1*, result in fragile cells with strongly weakened walls (Ballou, 1990; Cherry et al., 1998; Nakayama et al., 1992). Limited porosity may also offer some protection against cell wall-degrading enzymes produced by host cells. Interestingly, the porosity of stationary phase cells of *S. cerevisiae* is much lower than in exponential phase cells and this is accompanied by increased resistance to cell wall lytic enzymes (De Nobel et al., 1990b). This is further accompanied by strongly increased levels of the GPI-CWP Sed1p, the presence of which is required for increased resistance to cell wall

lytic enzymes (Shimoi et al., 1998). Similarly, the apical tips of the mycelial fungus *A. nidulans* are much more sensitive to the enzyme preparation helicase than mature walls (Polacheck and Rosenberger, 1977). Interestingly, BLAST searches indicate the existence of homologs of Sed1p in ascomycetous mycelial fungi such as *N. crassa* and *M. grisea*. It has been further been shown that heterologous expression of the *S. cerevisiae* cell wall protein Pir2p in the cell wall of the mycelial fungus *F. oxysporum* increases its resistance to osmotin, a plant PR-5 protein with a broad spectrum of antifungal activity, and that it also increases its virulence (Narasimhan et al., 2003). A possible explanation is that incorporation of Pir2p in the wall of *Fusarium* causes cell wall porosity to decrease and thus limits the accessibility to host-defense proteins including osmotin.

12.2. Water retention

Cell wall glycoproteins are heavily glycosylated and may carry extended, heavily branched N-chains. In addition, the carbohydrate side-chains may contain phosphodiester bridges, which are negatively charged at physiological pHs. The external glycoprotein layer may thus offer protection against desiccation.

12.3. Cell wall maintenance and protection against cell wall stress

Carbohydrate-processing proteins found in the cell wall of *S. cerevisiae* and *C. albicans* might be involved in remodeling the cell wall during bud formation, isotropic bud growth, cell separation, and during the formation of a mating structure, thus maintaining cell wall integrity. Interestingly, when the cell wall of *S. cerevisiae* is weakened, for example, by incubating the cells in the presence of 1,3-β-glucanase or genetically in deletion mutants, the transcript levels of several GPI-CWP-encoding genes such as *CWP1*, *SED1*, *CRH1*, *PST1*, and *CCW14*, and of all four PIR-CWP-encoding genes are upregulated through activation of the cell wall integrity pathway (Boorsma et al., 2004; De Nobel et al., 2000; Garcia et al., 2004; Jung and Levin, 1999; Lagorce et al., 2003). As discussed above, PIR-CWPs may conceivably interconnect 1,3-β-glucan chains to strengthen the 1,3-β-glucan network (Fig. 1).

12.4. Adhesive properties and cell–cell interactions

The outer protein layer is an obvious location for both sexual adhesion proteins such as the sexual agglutinins of *S. cerevisiae* and non-sexual adhesion proteins such as the flocculins in *S. cerevisiae*, the Als proteins in *C. albicans*, and the Epa proteins in *C. glabrata* (De Las Peñas et al., 2003; Hoyer, 2001; Klotz et al., 2004; Li and Palecek, 2003; Lipke and Kurjan, 1992; Sheppard et al., 2004; Verstrepen et al., 2003). Interestingly, all known (putative) adhesion proteins are GPI-CWPs. The GPI-CWP Hwp1p of *C. albicans*, the N-terminal domain of which mimics transglutaminase substrate proteins, is another fascinating example of a cell wall protein involved in adhesion (Staab et al., 1999, 2004). At the time of writing, no covalently linked cell–cell interacting proteins have yet been described in mycelial fungi.

12.5. Virulence

The outer protein layer of the wall is the primary site of interaction with other organisms. Adhesion proteins are expected to play an important role in virulence. In addition, proteins that confer protection against host-defense proteins are also expected to contribute to virulence. Overexpression of PIR-proteins in *S. cerevisiae* increased its resistance to the plant defense protein osmotin (Yun et al., 1997); equally, as mentioned earlier, expression of ScPir2p in *F. oxysporum* increased its resistance to osmotin and this was accompanied by increased virulence in plant infections (Narasimhan et al., 2003; Yun et al., 1997). Intriguingly, the cell wall proteome of *C. albicans* contains three more GPI-proteins that may contribute to virulence in its unique environment. One of them is a heme-binding protein that may help it to survive in an environment that is generally limited for iron (Weissman and Kornitzer, 2004), and the other two are superoxide dismutases that may help it to survive the oxidative attack by macrophages (De Groot et al., 2004; Fradin et al., 2005). Another virulence-related cell wall protein is a laccase found in the basidiomycete *C. neoformans*, a clinical fungus that may cause meningoencephalitis. It is believed to form melanin when this fungus is presented with exogenous phenolic substrates (Zhu et al., 2001). Laccase is a secretory protein, but it has no GPI-anchor addition signal, and can be extracted from isolated walls either by boiling in SDS or by reducing compounds. Immunogold labeling revealed that it is predominantly located in the outer layers of the cell wall.

12.6. Formation of interstitial material and biofilms

Mass spectrometric analysis of the cell wall proteomes of *S. cerevisiae* and *C. albicans* has shown the presence of an unexpectedly large number of proteins predicted or shown to have carbohydrate-processing activity (Coutinho and Henrissat, 1999; De Groot et al., 2004; Iranzo et al., 2002; Yin et al., 2005). This raises the question why they are located in the cell wall. One possibility is that they are involved in cell wall construction and remodeling (see above). Alternatively, they might be involved in the formation of interstitial material and biofilms on abiotic and biotic surfaces, allowing the creation of a micro-environment (Baillie and Douglas, 2000; Douglas, 2003; Iraqui et al., 2005; Reynolds and Fink, 2001). Biofilms of *C. albicans* are of medical importance,

because of the tendency of *C. albicans* to form biofilms on prostheses and because of the increased resistance of *C. albicans* to antifungal compounds when found in a biofilm (Andes et al., 2004; Douglas, 2003). Interestingly, the composition of the interstitial material in *C. albicans* biofilms is consistent with the notion that they are derived from cell wall macromolecules (Baillie and Douglas, 2000; Douglas, 2003).

12.7. Antigenicity

In clinical fungi, the outer protein layer may play an important role in the immune response (Cao et al., 1998; Chaffin et al., 1998; Gomez et al., 1996; Han and Cutler, 1995; Viudes et al., 2001; Woo et al., 2002, 2003). Further, protein glycosylation may modulate the immune response, allowing identification of various serotypes in *Candida* infections (Suzuki, 1997).

12.8. Iron uptake

Both in *S. cerevisiae* and in *C. albicans* GPI-modified cell wall proteins have been identified that may facilitate iron uptake and are strongly upregulated in response to iron starvation (Lan et al., 2004; Protchenko et al., 2001; Weissman and Kornitzer, 2004). This is probably a widespread phenomenon.

12.9. Hydrophobicity

Changes in hydrophobicity of the cell surface have been observed, for example, in *Candida* species (Hazen et al., 2001), but so far there is no evidence for the presence of hydrophobins in ascomycetous yeasts. Hydrophobicity may be regulated by incorporating proteins of increased or decreased hydropathicity in the cell wall and by controlling the formation of phosphodiester bonds in O- and N-side-chains (Jigami and Odani, 1999; Masuoka and Hazen, 1997). In *C. albicans* a GPI-protein has been identified that after being expressed in *S. cerevisiae* enhances adhesion to the hydrophobic material polystyrene (Li and Palecek, 2003).

12.10. Various enzymatic functions

In addition to the carbohydrate-active enzymes and virulence-related enzymes such as superoxide dismutases already discussed above, other enzyme activities have also been identified. For example, recently a predicted phospholipase B has been detected in the cell walls of *S. cerevisiae* by mass spectrometry (Yin et al., 2005). In *A. fumigatus* a potential GPI-CWP (PhoAp) with acid phosphatase activity has been characterized (Bernard et al., 2002). The majority of the protein was released by SDS-extraction of the wall, whereas glucanase digestion liberated only some PhoAp. This suggests that PhoAp is in fact a plasma membrane-bound GPI-protein and that only a small amount of the protein might be linked to the cell wall. Similar observations have also been made for plasma membrane-bound GPI-proteins in *S. cerevisiae* (De Sampaio et al., 1999; Hamada et al., 1999). Finally, it is worth mentioning that a number of bioengineering studies has appeared in which both GPI-CWPs and PIR-CWPs are used to target heterologous proteins to the cell wall of *S. cerevisiae* and other yeasts (Kondo and Ueda, 2004; Shimma and Jigami, 2004).

13. Perspectives

Cell wall proteins play a much larger role in cell wall biology than earlier recognized. An impressive number of powerful genomic tools has been developed for the yeast *S. cerevisiae*. As the number of fungal genomes that is becoming available in the public domain is steadily growing, this will allow development of similar tools for other fungi and comparative genomic analyses. Proteomics-based tools are becoming equally powerful. This will have an enormous impact on fungal biology and consequently on cell wall biology. On the other hand, development of well defined analysis techniques of cell wall composition, cell wall polymers, and interpolymer cross-linkages (Cabib and Duran, 2005; Grun et al., 2004; Kollar et al., 1995, 1997; Magnelli et al., 2002, 2005), more extensive use of molecular cytology (Hardham and Mitchell, 1998; Marshall et al., 1997; Momany et al., 2004; Rodriguez-Pena et al., 2000), and continued development of assays for glycan synthases and transglycosylases (Hartland et al., 1996; Mouyna et al., 2000a,b; Vink et al., 2004) are crucial to solve the many urgent questions and tasks that remain. For example, the identification of cell wall cross-linking enzymes, including those required for coupling proteins to the cell wall glycan network, and development of in vitro assays for them are still at their infancy. Because such enzymes are active outside the plasma membrane and are essential for cell wall formation and thus viability, they represent important potential targets for new antifungal compounds. Identification of the specificity of ligand-binding proteins is also an urgent task, especially in the case of clinical fungi. Signaling pathways that control cell wall formation and the composition of the protein population of the cell wall are just beginning to be explored. The role of cell wall construction and cell wall proteins in morphology and developmental processes is almost virgin territory. Systematic deletion studies have indicated that about 1200 of the 6000 genes of budding yeast affect normal cell wall construction (De Groot et al., 2001), demonstrating that cell wall biology is an integral part of cell metabolism. This raises the question how to integrate all the data coming from so many different fronts into a coherent picture, which will allow us to ask

and hopefully to predict how the cell wall will behave depending on the conditions imposed on the organism. Systems biology may come to the rescue here as recently proposed by Somerville for plant cell walls (Somerville et al., 2004). In short, cell wall biology is flourishing.

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