MicroReview

The cell wall: a carbohydrate armour for the fungal cell

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Summary
The cell wall is composed of a polysaccharide-based three-dimensional network. Considered for a long time as an inert exoskeleton, the cell wall is now seen as a dynamic structure that is continuously changing as a result of the modification of culture conditions and environmental stresses. Although the cell wall composition varies among fungal species, chemogenomic comparative analysis have led to a better understanding of the genes and mechanisms involved in the construction of the common central core composed of branched β1,3 glucan-chitin. Because of its essential biological role, unique biochemistry and structural organization and the absence in mammalian cells of most of its constituent components, the cell wall is an attractive target for the development of new antifungal agents. Genomic as well as drug studies have shown that the death of the fungus can result from inhibition of cell wall polysaccharide synthases. To date, only β1,3 glucan synthase inhibitors have been launched clinically and many more targets remain to be explored.

Introduction
The cell wall of fungi provides both protective and aggressive functions. It is protective, as it acts as an initial barrier that is in contact with hostile environments encountered by the fungus. If removed or weakened, the fungi die unless they are osmotically protected. It also provides an aggressive function, as it harbours many hydrolytic and toxic molecules, most of them being in transit in the cell wall and required for a fungus to invade its ecological niche. Furthermore, its rigid structure is useful as a force for the penetration of insoluble substrates that it colonizes or invades.

In spite of its essential role, the cell wall of most fungi remains insufficiently studied and its biosynthesis incompletely understood, especially among filamentous fungi (Latgé and Calderone, 2005; Lesage and Bussey, 2006). Further difficulty in the analysis of the cell wall comes from the accumulation of data showing that the cell wall can no longer be considered as an inert exoskeleton, as its structure continuously changes over time. The role of the environment and signal transduction cascades regulating cell wall synthesis will not be discussed here; instead, the focus of this review will be the downstream events and enzymes responsible for the establishment of the specific three-dimensional (3D) polysaccharide network of the cell wall. Rather than summarizing all published literature on the cell wall, the aim of this review is to present new ideas and hypotheses on cell wall polysaccharide biosynthesis and remodelling that emerged from a comparative analysis of the cell wall of yeast and moulds, especially Aspergillus fumigatus.

Structure of the cell wall polysaccharides
Polysaccharides account for >90% of the cell wall. The cell wall is an insoluble structure that must be solubilized to be precisely analysed. Hot alkali is the reference chemical treatment used to solubilize the cell wall polysaccharides that can be then analysed using recombinant glycosylhydrolases and liquid chromatography, nuclear magnetic resonance and mass spectrometry methodologies. For almost all fungi, the central core of the cell wall is a branched β1,3, 1,6 glucan that is linked to chitin via a β1,4 linkage. Interchain, β1,6 glucosidic linkages account for 3% and 4% of the total glucan linkages, respectively, in Saccharomyces cerevisiae and A. fumigatus (Fleet, 1991; Kollar et al., 1995; Fontaine et al., 2000; Perez and Ribas, 2004), the only two fungi whose cell wall structure has been investigated in detail. This structural core, which is differently decorated depending on the fungal species (Fig. 1), is generally thought to be fibrillar and embedded in an amorphous cement (usually removed by alkali treatment) (Fig. 2).

Our understanding of the overall organization of all these different polysaccharides in the cell wall remains quite vague, especially for the alkali-soluble fraction. For example, it is unknown to date how mannan is integrated

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into the fungal cell wall. In yeast, simple calculations suggested that mannans cannot remain exclusively associated to proteins bound covalently to the cell wall. If we imagine that an yeast cell wall glycoprotein has four chains of mannan with 150 mannose residue per chain, mannan would account for 100 kDa per glycoprotein; if the average polypeptide moiety of the yeast proteins is 40 kDa, the cell wall should then contain > 10% polypeptides as mannans account for 40% of the yeast cell wall carbohydrates. The amount of protein detected is actually much lower and never exceed 2–3% of the cell wall (Klis et al., 2002; de Groot et al., 2007). Moreover, analysis of the sequences of many of the covalently bound cell wall proteins (such as the TIR, TIP or DAN families) shows that they do not have potential N-glycosylation sites. In addition, mannans are removed by boiling entire cells at neutral pH which suggests they are not covalently bound to other cell wall polysaccharides. In moulds, mannan structure is completely different: in A. fumigatus, for example, the mannan chains are shorter and seems to be bound covalently to the glucans without intermediary peptide moiety. A similar mystery holds true for α1,3 glucan, the major mould alkali-soluble polysaccharides. Decorating molecules and even polysaccharides belonging to the structural core have constantly evolved since the appearance of fungi almost a billion years ago. There is some correlation between the appearance or the loss of a polysaccharide over time and fungal taxonomy, but this correlation is not absolute (Latgé and Calderone, 2005). Differences also have been noticed among fungal morphotypes in the same species, in agreement with a tight regulation of cell wall synthesis throughout the cell cycle. Many studies using fluorescent markers or radiolabelled precursors (Wessels, 1986; Hummel et al., 2001; Cortes et al., 2007) suggest that septa and apices have different structures to the lateral, older cell wall regions (Fig. 3). These differences have never been fully characterized chemically as septa and apices represent a small proportion of the total cell wall (e.g. in A. fumigatus, septa account for around 2% of the lateral cell wall) and cannot

Fig. 1. Polysaccharides of the cell wall of the mould Aspergillus fumigatus and the yeast Saccharomyces cerevisiae. Components of the alkali-insoluble and alkali-soluble fractions are presented. α1,3 (○), α1,4 glucan (●); β1,3 (□) 1,4 (○) 1,6 (■) glucan; chitin (□); α1,2 (▲), 1,3 (▲) 1,6 (△) mannan; β1,5 galactofuran (□). The core that is common to the vast majority of fungi is in grey.
be isolated separately from the mycelial bulk. To take into account this cellular heterogeneity, chemical data should be complemented by immunocytochemical analysis that will require the development of a complete library of monoclonal antibodies specific of the different fungal glycosidic linkages encountered among cell wall polysaccharides. With such library, the dynamics and sequential events in the construction of the fungal cell wall will be dissected cytochemically, as it is done in plant science (Persson et al., 2007). Moreover, the localization of the polysaccharides on the cell wall has some direct implication for the fungal cell life (Netea et al., 2006).

### Polysaccharide biosynthesis

**Synthesis of the skeleton chitin and β1,3 glucan polysaccharides of the cell wall**

The chitin synthases that are responsible for the synthesis of linear chains of β1,4 N-acetylglucosamine from the substrate UDP-N-acetylglucosamine are a family of integral membrane proteins with molecular weight of 100–130 kDa (Roncero, 2002). Chitin biosynthesis is understood best in the model yeast *S. cerevisiae*. Three chitin synthases (*CHS1–3*) are responsible for the synthesis of the *S. cerevisiae* chitin (Cabib et al., 2001). The non-zymogenic Chs3p is responsible for the synthesis of the bulk chitin of the cell wall and for the increase in chitin synthesis as a response to cell wall stress. Chs2p is responsible for synthesis of septal chitin. Chs2p acts in the formation of the primary septum. Its function depends directly on the formation of the acto-myosin ring (Schmidt

![Fig. 2.](image)

**Fig. 2.** Cell wall polysaccharides have different structures. See as an example carbon-platinum replicas of the amorphous and fibrillar materials recovered from the cell wall of the zygomycete *Conidiobolus obscurus*: (A) insoluble material recovered after cold 1N NaOH treatment; (B) insoluble material recovered after successive hot treatments in 1N NaOH and 0.5N CH3COOH; (C) alkali-soluble material recovered after cold 1N NaOH treatment.

![Fig. 3.](image)

**Fig. 3.** Heterogeneity in the cellular localization of the different cell wall polysaccharides. Germ tubes of *Aspergillus fumigatus* stained with WGA-FITC for chitin (A) [note that the septum (arrow) and the conidium are intensively labelled whereas the germ tube is only poorly labelled], with aniline blue for β1,3 glucans (B) [only accessible at the apex of germ tubes] and with an anti-galactofuran monoclonal antibody (C) binding to the entire germ tube surface but not to the conidium.

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et al., 2002). Chs1p acts as a repair enzyme during cell separation. Chs1p and Chs2p activities in vitro were originally described as zymogenic as they require proteolytic activation, but direct evidence for this type of regulation in vivo is lacking. Among the CHSs, only the regulation of CHS3 has been investigated. The enzyme is transported in an inactive form to the plasma membrane, where it is activated by Chs4p (Trilla et al., 1997). The complex containing Chs3p/Chs4p is positioned at the myosin ring of the septum site through its interaction with the Bni4p/septin complex (Sanz et al., 2004). In Aspergillus, which lacks BNI4 orthologues (Roncero, 2002), localization of CHS at the septum might be regulated differently. At least one gene in the CHS families of Aspergillus and other moulds has a consensus domain that is homologous to kinesin or myosin motor-like domains (Takeshita et al., 2005). This myosin motor-like domain of class V localizes near actin structures at the hyphal tips and septation sites. It binds to actin and this binding is necessary for chitin synthase activity.

The number of chitin synthase genes varies according to the fungal species, from one gene in the ancestral fungus Encephalitozon cuniculi to >20 genes in Rhizopus oryzae. BLAST analysis of amino acid sequences identifies six families of fungal chitin synthases. Three are specific for filamentous fungi (class III, V and VI) (Choquer et al., 2004). A QRRRW motif present in the cytosolic nucleotide sugar transferase domain of all CHS genes belong to the catalytic domain because mutations affecting this domain result in a loss of chitin synthase activity (Cos et al., 1998). These six families can be grouped into three major divisions (Fig. 4). Division 1, containing families I, II and III, has a Pfam domain 01644 at the N-terminal region of the catalytic domain. Division 2 (families IV and V, including Chs3p of S. cerevisiae) has the same catalytic domain preceded by a cytochrome b5-like domain (Pfam 00173, which has replaced Pfam 01644) and a myosin head-like domain (Pfam 00063) in moulds. A pfam domain 03142 is conserved at the C-terminus of all Chsps of division 2. Division 3 enzymes that contains only the family VI (i.e. CHS D of A. fumigatus) have the conserved catalytic sequences but do not display any of the characteristic Pfams encountered in the other Chsps (Choquer et al., 2004; http://www.cadre-genomes.org.uk/Aspergillus_fumigatus/). Interestingly, despite the fact that fungi are ancestors of animals, insect and nematode CHSs have homologous sequences that are quite different from those of fungi (E-value of 2e-14 between the Drosophila gene 72965213 and its closest fungal orthologue from E. cuniculi, ECU 01g1390). Interestingly, the insect and nematode CHSs, like the unique CHS of the ancestral fungus E. cuniculi, belong to division 2, suggesting that this division might be closest to the ancestral chitin synthase.

Although relatedness and divergent domain structures could be correlated with function, expression and mutagenesis studies in fungi have not been very informative in this area. It remains to be seen whether there is a relationship between a specific fungal CHS and the structure of the chitin polymer at a cellular location as well as their association with chitin deacetylases that modify chitin in the less rigid deacetylated chitosan (Baker et al., 2007). The significance of each of these six CHS classes is indeed not understood, as mutations affecting members of a common family do not always

![Fig. 4. Structure of the different chitin synthases of moulds and the contribution of the different members of each division to chitin synthase activity or chitin content of the cell wall deduced from the analysis of individual mutants (based on data from Mellado et al., 2003; Choquer et al., 2004; and the CADRE website). △: no difference with the wild type; and ▼: reduction of activity or chitin content of the cell wall of the mutant compared with wild type. The role of division 3 Chsp is unclear.](image-url)
result in a similar phenotype. Two groups of mutants can be identified, however: the first group (from division 2) has reduced chitin content but normal chitin synthase activity in vitro. In contrast, mutants from the other divisions are affected in enzyme activity but have a normal cell wall chitin content (Fig. 4). The requirement for specific chitin synthase genes also varies. In S. cerevisiae, none of the CHS genes is essential, although the triple mutant is very sick, and its survival results from the acquisition of suppressors (Schmidt, 2004). In contrast, Candida albicans has four CHS genes, of which the family II gene CHS1 is essential for cell viability (Munro et al., 2001). None of the CHS genes of A. fumigatus is essential (Mellado et al., 2003; C. Jimenez and C. Roncero, pers. comm.). These data suggest that the different fungal CHS perform distinct and specific functions in every fungus, even though they have homologous sequences. Overall regulation of chitin synthesis in fungi seems indeed quite complex as it is under the co-ordinated control of the PKC, HOG and Calcineurin pathways (Munro et al., 2007). A similar situation exits in insects, where functional analysis of the CHS genes revealed unique and complementary roles for each CHS. For example, one of the Chsps in Tribolium is specialized in the synthesis of the epidermal cuticle, whereas the other one is responsible for the midgut peritrophic matrix (Arakane et al., 2005). The amount of chitin produced per species is not directly correlated to the number of genes: although chitin is the most abundant component of insect cuticle, only one to two chitin synthases are encoded by these species (Merzendorfer, 2006).

β1,3 glucans are also synthesized by a plasma membrane-bound protein complex that uses UDP-glucose on its intracellular side as a substrate and extrudes linear β1,3 glucan chains through the membrane into the cell wall space (Douglas, 2001). This protein complex contains both catalytic and regulatory subunits. The putative catalytic subunit (Fks1p) is encoded by large proteins (> 200 kDa) with as many as 16 transmembrane helices. A central hydrophilic domain of about 580 amino acids displays a remarkable degree of identity (> 80%) among all known FKS protein sequences (Douglas, 2001). This region might be located on the cytoplasmic face of the plasma membrane and essential for function of the glucan synthase (Douglas, 2001). However, neither of two proposed consensus UDP-glucose binding sites (R/K)XGG or D,D,D35QXXRW is found in any of the Fks1p orthologues of the CAZy database (http://www.cazy.org/). In contrast, the product of the CrdS gene of Agrobacterium sp. ATCC 31749 that uses nucleotide sugars to produce also a linear β1,3 glucan contains a UDP glucose consensus binding site but the bacterial gene is not homolo-

gous to any eukaryotic FKS (Karnezis et al., 2003). The regulatory subunit of the β1,3 glucan synthase is the GTPase Rho1p that switches between a GDP-bound inactive state and a GTP-bound active state via conformational changes (Mazur and Baginsky, 1996; Qadota et al., 1996). After synthesis in the ER, Rho1p is geranylgeranylated, a modification required for Rho1p attachment to the membrane and transport. Geranylgeranylated Rho1p and Fks1p are transported to the plasma membrane as an inactive complex through the classical secretory pathway. Rho1p is activated on its arrival at the plasma membrane by Rom2p, the GDP/GTP exchange factor of Rho1p that is only localized at the plasma membrane. This activation and the movement of Fks1p on the plasma membrane are required for proper cell wall β1,3 glucan localization (Inoue et al., 1999; Abe et al., 2003). The role of Rho1-GTP in the regulation of β1,3 glucan synthesis has been shown in many fungi such as Schizosaccharomyces pombe and Aspergillus species (Lesage and Bussey, 2006). The role of GEFs might be interchangeable, however, as, in contrast to yeast, the unique orthologue of ROM2 is not essential in A. fumigatus (Hu et al., 2007).

Like chitin synthases, the number of genes encoding β1,3 glucan synthases and the essentiality of each individual gene vary with the fungal species. Three FKS genes have been identified in S. cerevisiae, but none is essential, although FKS1 and FKS2 are synthetically lethal (Ishihara et al., 2007). In S. pombe, four genes BGS1–4 sharing homology with β1,3 glucan synthase catalytic subunits have been identified, of which three (encoding Bgs1, 3 and 4p) are essential (Martin et al., 2003; Cortes et al., 2005; 2007). In contrast to yeasts, all filamentous ascomycetes sequenced to date have one FKS orthologue that is essential (Mouyna et al., 2004). β1,3 glucan synthase genes have also been found in plants, where they are responsible for callose synthesis. Although β1,3 glucan is a lot less abundant in plants than in fungi, the number of β1,3 glucan synthase genes is much higher in plants than in fungi. In the plants Arabidopsis thaliana or Oryza sativa, 12–18 callose synthase genes have been identified whereas the highest number of genes in fungi never exceeds 4. Plant callose is involved in several steps in pollen development (primary and secondary cell wall of the pollen, germination pore and tube, pollen tube plug) as well as wound repair after injury or disease (Enns et al., 2005). All steps in which callose participates should involve different callose synthase genes, which would explain the existence of multiple callose genes in plants. Despite the fact that plant and fungi belong to two different kingdoms, plant and fungal glucan synthases display high sequence similarities (E-value of 6e-80 between the A. thaliana gene At1g05570 and the S. pombe SPAC 19B12.03). However,
In contrast to chitin and Synthesis of alkali-soluble polysaccharides insoluble products into the cell wall space. soluble intracellular nucleotide sugars and extrude a perfect cellular localization for these enzymes that use activated after contact with resident activators (Fig. 5). This is where they are arranged as complexes and become activated in an inactive form to the plasma membrane, understood. major fibrillar polysaccharides of the cell wall is basically glucan and chitin synthases, the biosynthesis of the two different clusters. genes involved in the synthesis of are totally unknown. This is the case for the the substrate of the enzymes through genetic screens, the substrate of the enzymes extremely limited. For genes that have been identified embedded in the chitin-glucan fibrillar core remains synthesis of the other polysaccharides linked to or through PSI-BLAST comparisons align fungal and plant genes into two different clusters.

In spite of the lack of structural data for transmembrane glucan and chitin synthases, the biosynthesis of the two major fibrillar polysaccharides of the cell wall is basically understood. β1,3 glucan and chitin synthases are transported in an inactive form to the plasma membrane, where they are arranged as complexes and become activated after contact with resident activators (Fig. 5). This is a perfect cellular localization for these enzymes that use soluble intracellular nucleotide sugars and extrude-insoluble products into the cell wall space.

Synthesis of alkali-soluble polysaccharides

In contrast to chitin and β1,3 glucan, our knowledge of the synthesis of the other polysaccharides linked to or embedded in the chitin-glucan fibrillar core remains extremely limited. For genes that have been identified through genetic screens, the substrate of the enzymes and/or the biochemical activity of the encoded protein(s) are totally unknown. This is the case for the KRE and AGS genes involved in the synthesis of β1,6 and α1,3 glucan synthesis respectively (Beauvais et al., 2005; Lesage and Bussey, 2006). For other enzymes, even though the nucleotide sugar substrate is known, the synthase activity and acceptor have not been shown in vitro. This is the case for UDP-galactofuranose, the substrate for the synthesis of galactofuran in A. fumigatus, as mutations in the UDP-galactose mutase gene lead to the production of a mutant devoid of galactofuran (C. Lamarre and J.P. Latgé, unpublished data).

The subcellular site where these amorphous polysaccharide are synthesized is also a matter of debate. Localization of synthases at the plasma membrane should favour the extrusion of polymers like α1,3 glucans, with a degree of polymerization > 200. In contrast, the yeast mannanoproteins or peptidomannan with N-mannan chains of around 150 mannose residues are synthesized in the Golgi compartment and then brought to the cell wall (Fig. 5). Although the organization of the mannans in the mould and yeast cell walls is different (namely long alkali-soluble chains in yeast versus short chains bound directly to glucan) in filamentous fungi, comparative genomic analysis suggests, however, that synthesis of mannan in moulds is via mannosyltransferases orthologous to the yeast genes such as OCH1 or the mannosyltransferase complex MN9/VAN1/ANP1 (Niernan et al., 2005). The subcellular site of cell wall mannan synthesis in moulds and its further transfer to the glucan chain remains unknown.

**Anchoring and cross-linking of cell wall polysaccharides**

Two of the major questions in cell wall studies that remain unresolved are the holy grail of all ‘cellwallogists’. First, how are polysaccharides anchored to the plasma membrane? In Mycobacterium, the cell wall is attached to fatty acylated phosphatidylinositolmannans that are inserted in the plasma membrane (Chatterjee, 1997). Do glycolipids play a similar role in fungi, as lipo(galacto)mannans anchored to the plasma membrane through phosphatidylinositol anchors have been identified recently in yeasts and moulds (Trinel et al., 2002; Costachel et al., 2005)? Second, how are the neo-synthesized polysaccharides branched and cross-linked (Fig. 6)? A major breakthrough in the analysis of cell wall that might answer this question is the finding that a major glucanosyltransferase (Gelp) that elongates β1,3 glucan chains is anchored to the plasma membrane by a glycosylphosphatidyl inositol (GPI) moiety. Anchoring of proteins by a GPI anchor would allow the protein to face the cell wall space, a perfect cellular location to fulfil the glycosyltransferase function of these proteins (Fig. 7). Another argument reinforcing the putative involvement of this enzyme activity in the establishment of a branched β1,3 glucan comes from the fact that no orthologue of Gelp has been found in the plant kingdom where callose, the plant β1,3 glucan, is a linear polysaccharide.
without side-chains. As the composition of the alkali-insoluble core of the cell wall of most ascomycetes sequenced to date is similar, transferases playing an essential role in branching and cross-linking of glucan with chitin should be common to all these species. Comparative genomic and proteomic analyses have indeed shown that six families of membrane-bound GPI proteins are present in all ascomycetes sequenced to date: SPS2, GAS/GEL, DFG, PLB, CRH, YPS (Bernard et al., 2002). Four of the GPI-bound proteins (SPS2, GAS/GEL, DFG, CRH) are involved in cell wall construction as SPS2 (ECM 33), DFG1/DCW5, CRH1/CRH2 and GEL/GAS mutants have defects in cell wall associated to reduced growth phenotype (Mouyna et al., 2000; Rodriguez-Pena et al., 2000; Kitagaki et al., 2002; Tougan et al., 2002; Cabib et al., 2007). However, the in vitro glycosyltransferase activity of all of them except Gelp/Gasp remains to be determined.

A new vision for cell wall proteins

Most cell wall proteins are water- or detergent-soluble while in transit to the external milieu. A few of the cell wall proteins are covalently linked to polysaccharides. Two groups of proteins covalently bound to the cell wall have been identified in yeast. The first group of proteins (Ccwpds) are removed by a mild alkali treatment (30 mM NaOH overnight at 4°C) and are attached to β1,3 glucans through a glutamine residue following a transglutaminase reaction (Ecker et al., 2006). The second family of proteins extracted by β1,3 glucanases or β1,6 glucanases have defects in cell wall associated to reduced growth phenotype (Mouyna et al., 2000; Rodriguez-Pena et al., 2000; Kitagaki et al., 2002; Tougan et al., 2002; Cabib et al., 2007). However, the in vitro glycosyltransferase activity of all of them except Gelp/Gasp remains to be determined.

Studies in S. cerevisiae, mainly by the group of Klis and collaborators, suggest that the GPI proteins covalently linked to the polysaccharides play an essential structural role in cell wall organization. However, there are several arguments against an essential role of these proteins in the construction of the cell wall. (i) Proteins putatively covalently bound to the cell wall are only found in minute amounts (see above). (ii) Disruption of genes encoding the major GPI proteins isolated from the cell wall did not affect growth (van der Vaart et al., 1995). (iii) If these GPI-CWPs were essential in establishing the cell wall structure in yeast, they should also be found in other species. A careful comparative chemogenomic analysis of various yeast and moulds shows that this is not to be the case: orthologues of yeast GPI-CWPs have not been found in other ascomycetes such as S. pombe or

Fig. 6. Hypothetical successive steps that lead to the production of the alkali-insoluble core of the fungal cell wall.

Fig. 7. Putative functions of GPI-anchored proteins during the construction of the fungal cell wall. (i) GPI-anchored proteins (P1) that remain attached at the plasma membrane remodel cell wall polysaccharides (examples are Gel/Gas or Crh proteins). (ii) GPI-anchored proteins (P2) become covalently bound to β1,3 glucans through β1,6 glucans. This is a way for protein P2 to remain at the cell surface to fulfill its biological function, for example, in cell-to-cell interactions. See Fig. 1 for the legend of polysaccharide linkages.
A. *fumigatus*. (iv) Even after extensive boiling in a SDS-mercaptoethanol buffer, further incubation of the treated cell wall of *A. fumigatus* in the same buffer still releases soluble antigens after a few hours, indicating that soluble proteins can be tightly bound to cell wall through non-covalent linkages (Bernard et al., 2002). (v) Strikingly, some of the proteins with glycosyltransferase activity such as the Gasp, Ecm33p or Crhp are now claimed to be covalently linked to their product, the cell wall polysaccharides (Yin et al., 2005)! (vi) Soluble proteins such as the acid phosphatase PhoAp in *A. fumigatus* can be released by *β*1,3 glucanase treatment that loosens the polysaccharide net to which this protein is associated; these data show that the postulated ‘release by *β*1,3 glucanase equals covalent linkage to the cell wall polysaccharide’ is obviously not always true (Bernard et al., 2002). These comparative analyses suggest that proteins do not play any role in the establishment of the 3D polysaccharide cell wall network, even in yeast.

The majority of the polysaccharide-covalently bound proteins in yeast, such as Flop, Figp or Agap in *S. cerevisiae* or Alsp and Eap in *Candida*, are involved in cell–cell interactions such as flocculation, biofilm formation, mating, or adhesion to host cell surfaces (Verstrepen et al., 2005). Our current hypothesis on the role of covalently bound proteins is illustrated in Fig. 7. The covalent binding of proteins to polysaccharides is a way for the protein to remain at the surface of the cell wall, where it has to bind directly to its ligand to fulfil its biological function. Interestingly, many of the genes encoding these adhesins in yeast contained many intragenic tandem repeats with large (< 40 nt) repeats. Variation in intragenic repeat numbers results in gradual and reversible functional changes. This variability at the cell surface might permit evasion of the host immune response (Verstrepen et al., 2005) in a way similar to antigenic variation in parasites.

**Lysis of structural polysaccharides is needed for fungal growth**

Glycosylhydrolases are essential for the separation of mother and daughter yeast cells (Fig. 8). The hydrolases identified reflect the chemical nature of the septum. In *S. cerevisiae*, a chitinase (Cts1p) and an endo *β*1,3 glucanase (Eng1p) present in the daughter cells are required for cell separation due to the presence of chitin in the primary septum and of *β*-glucan in the secondary septum (Kuranda and Robbins, 1991; Baladron et al., 2002). Separation in yeast is under the control of Ace2p, a transcription factor present in the daughter cells (King and Butler, 1998). Other glucanase-associated enzymes such as Scw11p, Dse1p and Egt2p are involved in cell separation but their role is unknown. In *S. pombe*, separation of the two yeast daughter cells requires the endo *β*1,3 glucanase Eng1p, which degrades the primary septum that is composed of *β*1,3 glucan and Agn1p, an endo *α*1,3 glucanase that degrades the septum edging composed of *α*1,3 glucan (Martin-Cuadrado et al., 2003; Alonso-Nunez et al., 2005). The secondary septum, mainly composed of linear or branched *β*1,6 glucans, seems to remain untouched. In filamentous fungi, cells do not separate during colony extension and orthologues of *ACE2* are absent in moulds. However, there are two stages, conidial swelling and hyphal branching, that require cell wall softening. In *A. fumigatus*, endo *α* and...
\( \beta 1,3 \) glucanase and chitinase activities are present in resting conidia (J.-P. Debeaupuis and J.-P. Latgé, unpublished). They could be responsible for cell wall hydrolysis once water has penetrated into the conidium. The comparison of these three different fungal situations suggests that correct localization of the glycosylhydrolases is essential to fulfill their biological role. This has been shown in yeast, where removal of the ‘carbohydrate-binding module’ in the Cts1p of \emph{S. cerevisiae} or Eng1p of \emph{S. pombe} abolishes their function in cell separation (A.B. Martin-Cuadrado \emph{et al.}, unpublished data).

**Cell wall polysaccharides are essential drug targets**

Three families of natural \( \beta 1,3 \) glucan synthase inhibitors have been identified: (i) the glycolipid papulacandins, which consist of a modified disaccharide linked to two fatty-acyl chains, (ii) acidic terpenoids and (iii) cyclic hexapeptides with an \( N \)-linked fatty-acyl side-chain (Onishi \emph{et al.}, 2000). Included in the later group are the non-competitive echinocandin inhibitors that are currently the only one of the three families used in clinical practice. Although these molecules are active in patients, mechanistic details of the non-competitive glucan inhibition by echinocandins still remain to be fully elucidated. Drugs inhibiting chitin synthesis, such as the peptide nucleoside antibiotics polyoxins and nikkomycins and their analogues, are substrate analogues of UDP-\( N \)-GlcNAc and are strong competitive inhibitors of chitin synthase \emph{in vitro}. These compounds are poorly active \emph{in vivo}, however. New strategies are presently being investigated to identify more efficient inhibitors (Behr, 2003; Yeager and Finney, 2004).

Although genetic data indicate that yeast glycosylhydrolase mutants are affected in growth, the search for antiglycosylhydrolases is in its infancy and focuses only antichitinase drugs. They are either substrate analogues, such as the pseudotrisaccharide allosamidin, which inhibit the oxazolinium reaction intermediate unique to chitinase, or new inhibitors that also bind to the chitinase active site. Only their role \emph{in vitro} has been analysed (Schuttelkopf \emph{et al.}, 2006). Inhibitors of glucanases have not been identified or proposed as antifungal drugs.

Combination therapy with drugs inhibiting synthesis and hydrolysis of the same polysaccharide or the synthesis of the two main polysaccharides has been recently proposed (Stevens, 2000). Such combinatorial strategies might be very efficient, as compensatory reactions leading to increased synthesis of a cell wall component occur when the synthesis of another component is perturbed (Lagorce \emph{et al.}, 2003). In yeast, an increase in chitin synthesis is a common compensatory reaction when the cell integrity pathway is affected. Other reported compensatory reactions might relate to the level of expression of different genes of the same family (Maubon \emph{et al.}, 2006). These compensatory modifications might target unrelated cell wall components; for example, an increase in melanin can be induced by mutation of chitin synthase gene in \emph{Wangiella dermatitidis} or \( \alpha 1,3 \) glucan synthase in \emph{A. fumigatus} (Liu \emph{et al.}, 2004; Maubon \emph{et al.}, 2006); an increase in \( \alpha 1,3 \) glucan follows the disruption of chitin synthase genes in \emph{A. fumigatus} (Mellado \emph{et al.}, 2003). Such compensatory reactions explain why antifungal therapy with cell wall inhibitors is difficult, but also reflect the dynamics of the synthesis of cell wall component.

Another way to interfere with the polysaccharide synthesis is to use killer antibodies instead of chemical inhibitors. A remarkable example of the direct antimicrobial effects of antibodies is provided by the broad-spectrum antimicrobial activities of anti-idiotypic antibodies to a neutralizing monoclonal antibody to \emph{Pichia anomala} killer toxin that binds to \( \beta 1,3 \) glucan (Magliani \emph{et al.}, 1997). These antibodies mediate antimicrobial activity by mimicking the internal image of the toxin in the toxin binding site and reproducing the antimicrobial effects of the killer toxin. Anti-\( \beta 1,3 \) glucan antibodies showed a direct antifungal effect on the fungus \emph{in vitro} and mediated protection against \emph{Candida} and \emph{Aspergillus} infection (Torosantucci \emph{et al.}, 2005). Potentiation of the fungicidal activity of the antibody is possible by radiolabelling the antibody. Treatment of \emph{Cryptococcus neoformans}-infected mice with a monoclonal antibody to the capsular polysaccharide, conjugated with either rhenium-188 or bismuth-213, was recently shown to prolong significantly the survival of lethally infected mice and to reduce organ fungal burden (Dadachova \emph{et al.}, 2003).

**Perspectives**

In spite of recent progress, the fungal cell wall remains a poorly understood structure in terms of both composition and biosynthesis. A major issue is technical and is related to the harshness of treating cell walls to analyse its composition. Does boiling it in NaOH reflect the true structure? Is carboxymethylation a better approach to solubilize cell wall polysaccharides? How are polysaccharides that appear after chemical treatment fibrillar or amorphous under electron microscopy really organized \emph{in vivo}? What are the linkages (non-covalent or alkali-labile covalent) and mechanisms governing the insertion of alkali-soluble material in the fibrillar core? Other issues are strictly biochemical. What transferases are responsible for establishing the polysaccharide 3D network of the cell wall? Are constitutive polysaccharides of the cell wall bound to specific membrane anchors? How can cell wall transferases work on insoluble substrates?

The structure of the cell wall has been at least partly chemically analysed. This remains insufficient, as it does not reflect the mechanical properties of the cell wall.
Atomic force microscopy has been introduced recently for such measurements (Zhao et al., 2005). This approach showed that the environment has a strong effect on cell wall elasticity; high osmotic conditions (0.6 M KCl) in the culture medium induce hyphae of Aspergillus nidulans to assemble a cell wall with a weaker molecular structure, resulting in lower elasticity and a reduction in the tensile strength of the apical cell wall. Associated with a better knowledge of the environmental sensors and the different signal transduction cascades activated under different environments, such biophysical approaches should lead to a better understanding of the dynamics of cell wall construction. A better analysis of the cell wall regeneration process might help this understanding and identify new pathways in cell wall synthesis. Several fungal orders include morphotypes without cell walls. A classical example is the motile zoospore of Chytridiomycota (one of the most ancient fungal phylum), which is required for survival of the organism in an aquatic environment. Other cell wall-less stages are produced by some fungi to avoid recognition by its host. For example, Pneumocystis carinii forms cell wall-less commensal trophozoites that associate with pneumocytes (de Souza and Benchimol, 2005). This is also the case for the entomopathogenic zygomycetous Entomophaga or Entomophthora protoplasts that, in contrast to their β1,3 glucan-rich cell-walled stage, do not induce a defence prophenoloxidase cascade in arthropods. Wall shading results from a reduction or even a shut off of polysaccharide synthase activity (Beauvais and Latgé, 1989). The association between cell wall synthesis inhibition and water or glycerol pumps regulating the internal osmotic pressure of the cytoplasm, which must play an essential role for these zoospores or protoplasts to withstand the external milieu in the absence of cell wall, has never been investigated. The genomic exploitation of such dimorphism between cell wall-less morphotypes and cells covered by a cell wall within a species might lead to the discovery of new regulatory pathways of cell wall biosynthetic enzymes.

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