The Structure and Function of *Candida albicans* Cell Wall

Maxwell G. SHEPHERD

Experimental Oral biology Unit, Faculty of Dentistry,
University of Otago, Dunedin, New Zealand

The wall and outer surface of pathogenic fungi is involved in adhesion and cell shape maintenance, and acts as a barrier to metabolites and drugs. In addition the cell wall contains immunogenic determinants, immunomodulators and secreted enzymes. The cell wall of *C. albicans* comprises some 30% of the dry weight of the cell and is composed predominantly of mannoproteins and β -glucans with small quantities of chitin, protein and lipid. Transmission electronmicroscopy of the wall reveals a number of different layers; the thickness and number of these layers varies during germ-tube formation and the cell cycle. The outer fuzzy layer of the wall contains receptors that bind plastic, laminin, fibrinogen, and C3 fragments of complement. The β 1, 3 and β 1, 6 glucans and chitin are the polymers which endow rigidity on the cell wall. These polymers form a microfibrillar scaffolding upon which other macromolecules, particularly mannoproteins are bound.

The wall mannoproteins are highly branched polysaccharides primarily composed of mannose units attached to protein through a GlcNac dimer bridge and asparagine. There are also smaller numbers of O-linked mannoproteins where the mannose units are attached to protein through serine and threonine residues. The mannoproteins have a backbone of α -1, 6 linked mannose units to which side chains of mannoses attach via α -1, 2- and occasionally α -1, 3 bonds. The side chains are the major antigenic determinants and the A and B serotypes are distinguished by differences between these side chains. A model for cell wall growth and development will be described.

Introduction

Cell wall studies of pathogenic fungi are important for a variety of reasons. Firstly, *Candida albicans* is a dimorphic organism and in infected tissues a mixture of yeast cells and hyphal elements is found. Since it is the rigid structure of the wall that confers mechanical stability on the cell maintaining its characteristic shape, *C. albicans* dimorphism is an example of wall morphogenesis. Secondly, the wall is the contact point for attachment and

subsequent invasion and contains specific adhesin molecules. Thirdly, the wall acts as a protective barrier and is an obstacle that must be considered in the construction and design of antifungal drugs that need to enter the cell for their effective action. In the wall are secreted hydrolytic enzymes and components that act as immunogenic determinants and immunomodulators. Finally, the β -glucans and chitin of the pathogen are not present in the host and hence these compounds and particularly the enzymes involved in their biosynthesis

and degradation and potentially safe targets for antifungal agents. It is not surprising therefore that the architecture of the fungal cell wall and the mechanisms of wall synthesis and degradation are currently areas of intensive research effort. The cell wall comprises a mosaic of components and it is the discussion of these components that is the subject of this paper. The cell envelope and cell wall of *Candida albicans* has recently been reviewed by Shepherd et al.¹⁾; Reiss²⁾; Shepherd³⁾; Odds⁴⁾; Shepherd & Gopal⁵⁾.

Dynamic nature of the cell wall

A major difficulty encountered in the investigation and description of the cell wall of *Candida albicans* is defining a typical wall. In addition to the qualitative and quantitative differences found between the yeast and mycelial cells^{6, 7)} we know that the wall of yeast cells alters during growth cycle and in different media⁸⁻¹⁰⁾. Tronchin *et al.*¹¹⁾ have described the dynamic changes that occur on the cell surface during germ-tube formation including rapid synthesis and degradation of surface mannoproteins. In a study on coaggregation of Streptococci with *C. albicans*¹²⁾ it was found that starving the yeast cells for a period of only 30 minutes promoted the bacterial/fungal attachment. The induction of the adhesin was inhibited by protein synthesis inhibitors implying new protein synthesis was occurring at the surface. Collectively, these studies highlight the subtle and rapid changes that occur in the cell wall under different environmental and physiological conditions.

Cell composition and ultrastructure

The cell wall makes up approximately 30% of the total weight of the cell and a number of studies have shown that the wall is composed of mannoproteins, (20-30%); β 1, 3 linked glucans (25-35%); β 1, 6 linked glucans (35-45%) and small quantities of chitin (0.6-2.7%), protein (5-15%) and lipid (2-5%)^{13-16, 7)}. The glucan and mannoprotein components of yeast and hyphal cells are similar although hyphal cells contain at least 3 times as much chitin as yeast cells^{7, 14, 17)}. Transmission electron

microscopy studies have shown the wall to be composed of a number of different layers and the thickness and number of these layers varies depending upon the morphology and the stage of growth¹⁸⁻²²⁾. Although the striated appearance of the wall in cross section indicates layers of different chemical composition it is likely that the changes in electron density reflect quantitative rather than complete qualitative differences. Fig. 1 is a schematic diagram showing the architecture, composition and organisation of the *C. albicans* cell wall. The outermost layer is composed of mannoprotein although mannoprotein is also distributed throughout the entire wall. This has been shown with lectin binding studies and extraction of the cell wall with different solvents^{23, 24)}, cytochemical staining²⁵⁾ and cross reaction of ferritin-conjugated antibodies²⁶⁾. The remainder of the wall is made up of a network of the structural microfibrillar components β glucans and chitin. These molecules form a scaffold which is enmeshed in a matrix of amorphous components including mannoproteins and proteins. Tronchin *et al.*²⁷⁾ used wheat agglutinin to show that one layer of chitin was distributed in the inner wall layer near the plasma membrane but that there were smaller amounts found disbursed throughout the outer wall layers. In this organisation the fibrils would be resistant to stretching while the matrix would resist compression and act to trap soluble components such as wall enzymes. It should be noted however, that the interpretation of electronmicrographs must be made with caution as artifacts can arise from differential staining, staining of minor components, drying and fixation procedures.

Outer surface of *C. albicans*

A number of workers have demonstrated an outer cell wall coat variously called slime layer, mucous layer or fuzzy layer^{19, 22, 28, 29)} and this layer is believed to play an important role in pathogenicity, phagocytosis and adherence. In addition the mannoproteins on the outer surface are the main immunogenic determinants in a *Candida* cell. It is known that this

Fig. 1. Schematic diagram of the architecture of the *C. albicans* cell wall. The molecules depicted are : chitin (—); β glucan microfibrils, (—); mannoprotein, (—); extracellular enzymes, (—); disulphide bridges, (—S—S—); phosphoester linkages, (—P—).

outer layer undergoes considerable alteration depending on the mode of growth^{18, 20, 22}). Fig. 2 shows that in the developing germ-tube there are distinct fibrils arranged radially and perpendicular to the cell but these are absent from the mother cells. McCourtie & Douglas⁸)

have demonstrated that the formation of this outer fibrillar/floccular layer is promoted by growing yeast cells of *C. albicans* in the presence of high concentrations of galactose and sucrose. The study of Howlett & Squiers³⁰) demonstrated the importance of the floccular

Fig. 2. Germ-tube fibrils. Ultra-thin section through a germ-tube-forming cell which was fixed in paraformaldehyde plus glutaraldehyde and postfixed with osmium. The cell wall is predominantly electron lucent with a thin electron-dense outer layer. An electron-dense outer layer of the germ-tube wall is shown (arrows in inset). In the cross-sectioned germ tube, the fibrils are seen to be arranged radially and perpendicular to the cell wall. Stained with lead citrate for 45 min. Marker bar=0.6 μ m. (From Hubbard MJ, Sullivan PA and Shepherd MG, *Can J Microbiol*, **31**:696, 1985. With permission).

layer in mediating adhesion of *Candida* to oral mucosal cells. Horisberger and Clerc³¹⁾ have described an apical concentration of anionic sites on germ-tubes and developing hyphae. The anionic sites are associated with the outer fuzzy coat and are believed to be derived from the phosphate groups on mannoproteins. These authors suggest that the anionic sites play an important role in tissue colonization.

Finally, the outer surface of the *C. albicans* wall contains receptors. The receptor for the C3 fragments of human complement C3d and iC3b has been characterized by several groups³²⁻³⁴⁾. In mammalian cells the iC3b interacts with the CR3 receptor on phagocytic cells promoting phagocytosis and stimulating the release of intracellular microbiocidal agents

such as superoxide or myeloperoxidase. Therefore, the receptor may allow *Candida* cells to bind the iC3b non covalently and avert recognition by neutrophil CR3. In this way, the *C. albicans* surface receptor for the C3 fragment promotes pathogenicity by inhibiting phagocytosis³⁴⁾. Immunofluorescence studies have shown that plasma and matrix host proteins bind specifically to the surface of germ-tubes³⁵⁾ and this adherence is mediated by adhesins with molecular weights of 60, 68 and 200 kDa. Tronchin *et al*¹¹⁾ have shown with Western blotting experiments that these adhesins interact with the host proteins laminin, fibrinogen and C3d and speculate that the same fungal components possess a variety of biological functions. Laminin has been impli-

cated in both adhesion and pathogenesis of bacterial infections and therefore the germ tube specific surface receptors for laminin could mediate attachment of these cells to basement membranes and so contribute to the establishment of candidiasis.

Cell wall polymers

Glucan

Two lines of evidence indicate that the β -glucans impart the structural strength. Firstly, osmotically sensitive cells are generated after degradation of whole yeast cells with a purified β 1, 3 glucanase³⁶⁾. Secondly, protoplasts of *C. albicans* regenerated in simple medium of magnesium chloride and glucose give osmotically resistant cells that have not incorporated mannoprotein into the wall³⁷⁾. Fig. 3 shows the fibrils of glucan forming a mesh around the protoplast cell. Although mannoprotein was not incorporated into the walls of these cells it was secreted into the medium. The

classical protocol for obtaining fungal wall glucans (reviewed in 38) is fractionation on the basis of solubility in acid and alkali. Analysis of *C. albicans* glucans obtained in this manner by methylation, glc mass spectroscopy and ¹³C NMR showed that β 1, 6 glucan was the major polymer and that it was highly branched and soluble. There was also a branched β 1, 3 glucan and mixed β 1, 3/ β 1, 6 glucan linked to chitin. The ratio of β 1, 3- to β 1, 6- glucan was similar in yeast and hyphal cells indicating that the morphology of *C. albicans* is not determined by the ratio of these linkages. However, the insoluble glucan from germ-tube forming cells contained considerably more β 1, 3 linkages than that found in yeast and hyphal cells⁶⁾. If the β 1, 3- β 1, 6 ratio in the mother cell is taken into account it would appear that during the early stages of germ-tube formation there is almost exclusive synthesis of β 1, 3-linked glucans and this is analogous to the situation observed with regenerating

Fig. 3. Scanning electron microscopy of glucan nets forming on regenerating spheroplasts. The conditions for regeneration were 3h at 30°C in a medium of 0.6M MgCl₂ and 50 mM glucose.

spheroplasts^{37, 39)} which preferentially synthesize β 1, 3 glucans. Surarit *et al.*⁴⁰⁾ isolated the insoluble glucan chitin fraction from regenerating spheroplasts of *C. albicans* and mass spectrometry analysis confirmed a glycosidic linkage between the 6th position of GlcNAc on chitin and the 1 position of a β 1, 6 glucan. Chitin is dispersed around the entire wall²⁷⁾ and its association with glucan would provide a structural scaffold to which the remaining components attach. There are also reports of glucan mannoprotein complexes⁴¹⁾ but the nature of these linkages has not been established. A major difficulty in cell wall chemistry is obtaining molecules for analysis that are identical to those in the intact wall of the organism under study. Extremes of temperatures, and pH and extraction with solvents causes degradation and rearrangement. Gopal and Shepherd (unpublished results) have solubilized glucans from the wall by partial zymolyase degradation. The first glucan fraction to be solubilized was analysed by two dimensional ^1H and ^{13}C NMR and the heteronuclear spectrum confirmed (i) a pure glucan of glucose and (ii) a β linked polymer. While the first zymolyase treatment released a β 1, 3 glucan, the second zymolyase treatment gave a mixture of β 1, 3- β 1, 6 glucan and mannoproteins. High resolution solid state ^{13}C NMR has been performed on the insoluble glucan (Gopal & Shepherd, unpublished results) and the spectra analysed according to the assignments given by Saito⁴²⁾. Solid state ^{13}C NMR gives information on the structure of both non crystalline and crystalline insoluble polymers. The spectra showed¹⁾ typical features of a β 1, 3 glucan with the anomeric peak of C1 at 103.7 ppm and the C2 and C5 peaks at 74.2 ppm. The most noteworthy feature of the spectrum was the doublet signal due to the C3; Ca at 85.7 ppm and Cb at 83.5 ppm (Fig. 4). These two C3 signals correspond to the laminarin type and paramylon type conformation respectively⁴²⁾. Finally, from the relative proportions of the two C3 signals we conclude that the degree of polymerisation is greater than 400. From

Fig. 4. The 75.43 MHz ^{13}C -CP-MAS NMR spectrum of *C. albicans* insoluble wall glucan. Contact time was 500 μ sec.

these data and by analogy with other structural glucans such as cellulose and paramylon it is concluded that this molecule would indeed act as a major structural component of the wall. There is also evidence that these structural β -glucans form helical structures⁴³⁾.

Mannoproteins

Despite the large number of studies completed on *C. albicans* mannoprotein⁴⁴⁾ and reviewed in 2) and 3) detailed structures for either the polysaccharide portion or the protein portion are not known. In summary, the mannoprotein fraction makes up approximately 20 to 30% of the cell wall, they are the major antigenic components of intact and mycelial cells, and as shown in Fig. 5, their structures are similar to that of the mannoproteins found in *S. cerevisiae*. The major portion of the mannoprotein is an α 1, 6-linked polymannose joined to the protein through a chitobiose bridge and asparagine. In the outer chain of *C. albicans* mannoproteins 60-70% of the mannose units are substituted at the 2 and 3 position and oligosaccharides of mannose are joined by 1,2 linkages and occasionally 1,3 linkages. It is the heterogeneity in these outer regions which give rise to antigenic determinants and the two serotypes of *C. albicans*. Elorza *et al.*⁴⁵⁾ have estimated that 12% of the mannoprotein of the wall is O-linked. The molecular weight of the isolated mannoprotein depends on the method of preparation. The O-linked mannoproteins are not as complex as the N-linked molecules. Gopal & Shepherd (unpublished) carried out controlled β elimination on a highly purified cell wall mannoprotein fraction

Fig. 5. Proposed structure for *Candida albicans* mannoprotein.

and fractionated the products on a Biogel P2 sizing column. A mannobiose, mannotriose and mannotetraose was isolated from the column. The proton NMR of the oligosaccharides gave the linkage analysis shown in Fig. 5. These linkages are different from that found in *S. cerevisiae* and may be important in the taxonomy of the *Candida* species. In the *C. albicans* wall mannoproteins the phosphate content is between 0.2 and 1% whereas in *S. cerevisiae* mannoproteins the phosphate content is less than 0.1%. The presence of this highly charged phosphate group on the outer regions of the wall would affect the way these cells are attracted to and indeed attached to other surfaces. The anionic sites on developing germ-tubes³¹⁾ are believed to be from the negatively charged phosphate groups on the mannoproteins. Details on the mannophosphate linkage are not available as most of the published studies on the structures of *C. albicans* mannoproteins used isolation procedures that would have destroyed phosphoester linkages. Early studies used Fehling solution where acid was employed to dissociate the copper mannan complex. This procedure would destroy α 1, 3 mannosyl and phosphodiester linkages. The Cetavalon-borate mannoprotein complexes are larger but again separation of the complex must be done in the absence of strong acid. Molloy *et al.*⁴⁶⁾ carried out vectorial iodination on proteins of the cell surface and showed that a zymolyase degradation of a wall preparation released material that was not contaminated with membrane and cytosolic proteins but

did release 93% of the iodine with a specific activity 45 fold higher than the original SDS extract. The requirement for β 1, 3 glucanase to release the ¹²⁵I labelled material is consistent with either covalent attachment or tight entrapment of the labelled proteins in the cell wall glucans. The major iodinated material moved as a diffuse band at 260 kDa but it is not clear whether this is one mannoprotein or a mixture. By analogy with the study of Frevert and Ballou⁴⁷⁾ on *S. cerevisiae* this 260 kDa mannoprotein may well be the major structural glycoprotein in the cell wall. The 260 kDa fraction was composed of 1.5% protein and 98.4% hexose and analysis of the peptide portion showed it contained 36-38% serine and threonine residues typical of mannoproteins enriched in O-linked oligosaccharides. Elorza *et al.*⁴⁵⁾ found *C. albicans* mannoproteins to contain 7% protein and 92% carbohydrate, predominantly mannose with a small amount of glucose. Saxena *et al.*⁴⁸⁾ analysed mannans from two relatively avirulent mutant strains of *C. albicans* and found that the mannan on the avirulent strains was more susceptible to acid hydrolysis indicating differences in phosphoester linkages. Cell surface mutants of *C. albicans* have also been analysed by Whelan *et al.*⁴⁹⁾ and these mutants exhibited a range of different phenotypes and chemotypes. The changes in the mannoprotein structure of the mutants implied that the immunodominant determinant of *C. albicans* are not directly involved in colonization ability. Again there was loss of a signal characteristic of the mannosyl α -phosphate linkage. The mannoproteins of *C. albicans* defines two serotypes; serotype A and serotype B^{50, 51)} and these two serotypes are distinguished by the side chain oligosaccharides attached to the backbone 1, 6 mannose. The mannoprotein immunochemistry and immunology has been reviewed by Reiss²⁾. Briefly in serotype A mannan the immunodominant haptan is believed to be a linear α -1, 2 linked mannohexaose with an α -1, 3 linkage at the penultimate sugar from the reducing end. The B serotype appears to contain a bran-

ched molecule. It should be noted, however, that the structures postulated do not take into account the important role of phosphate residues and antigenic responses⁵²⁾ and there is still some debate on the role of β -linked mannose residues the presence of which are indicated from proton NMR spectra.

Proteins

The number and quantity of proteins in the cell wall remains a matter of considerable debate. Some reports show as many as 40 discrete bands on electrophoresis. However, the study of Molloy *et al*⁴⁶⁾ highlights the contamination that occurs in walls obtained by traditional extraction methods. In that study⁴⁶⁾ there were only 12 wall proteins that could be resolved by electrophoresis and autoradiography that are accessible to vectorial iodination. The threadlike fibrils extending from *C. albicans* to epithelial cells are believed to be proteins. In addition the self aggregating clumps of germ-tubes can be dispersed by digestion with trypsin or sulphhydryl reagents but not with α -mannosidase⁵³⁾. Finally, it is known that the wall contains a number of

enzymes including N-acetylglucosaminidase, acid phosphatase, proteinase, glucanase and chitinase (reviewed in 4). There is evidence that the proteinase acts as an aggressin and is a determinant in the pathogenicity of *C. albicans*⁵⁴⁾.

Cell wall biosynthesis and model of wall formation

The essential elements of wall biosynthesis are shown in Fig. 6. It is proposed that vesicles originate in the endoplasmic reticulum in a membranous body equivalent to the Golgi apparatus. These vesicles contain new material for the plasma membrane as well as membrane bound enzymes, soluble enzymes, highly processed mannoproteins and perhaps a number of wall polymer primers. These vesicles proceed to the point of growth in the cell along actin fibrils and at the plasma membrane there is accretion of this new membrane from the vesicle into the plasma membrane. Yokoyama *et al*⁵⁵⁾ have demonstrated that actin microfilaments are important in apical cell elongation of *C. albicans* hyphae whereas the cytoplasmic microtubules are not essential. The plasma

Fig. 6. Cellular and biochemical mechanisms included in *C. albicans* wall formation.

membrane can be recycled through the process of endocytosis. After fusion of the vesicle with the plasma membrane the mannoproteins are released into the cell wall (Fig. 6, inset).

Chitin and glucan are synthesized by trans-membrane enzymes catalysing the vectorial synthesis of these polymers with the precursors UDP N-acetylglucosamine and UDP glucose inside the cell and the product extruded through into the wall. We do not understand the fine controls of these enzymes nor the mechanisms that activate them at the points of growth. When the products emerge from the cell membrane they undergo intussusception into the expanding wall. At this point the molecules are linear and many questions remain unanswered as to how the glucans are 1) branched 2) cross-linked with other polymers such as chitin and 3) complexed with mannoproteins. One proposal is that β -glucanases in the cell wall clip the existing glucan and allow additional glucan molecules to be incorporated. This is summarized in Fig. 7 which shows secondary wall formation. The secondary wall cross-linkages reinforce the wall structure and provide a network which could retain both the water soluble mannoprotein and the cell wall enzymes. The studies of Sonnenberg & Wessels⁵⁶⁾ with *Schizophyllum commune* showed that at the growing apex glucan and chitin are synthesized as unbranched polymers but in the subapical region modifications occur, particularly cross-linking.

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