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The *Paracoccidioides* cell wall: past and present layers toward understanding interaction with the host

Rosana Puccia*, Milene Carmes Vallejo, Alisson Leonardo Matsuo and Larissa Valle Guilhen Longo

Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo – Escola Paulista de Medicina, São Paulo, Brazil

Edited by:
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****Correspondence:***
Rosana Puccia, Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo – Escola Paulista de Medicina, Rua Botucatu, 862, oitavo andar, 04023-062 São Paulo, SP, Brazil.
e-mail: rpuccia@unifesp.br; ropuccia@gmail.com

The cell wall of pathogenic fungi plays import roles in the interaction with the host, so that its composition and structure may determine the course of infection. Here we present an overview of the current and past knowledge on the cell wall constituents of *Paracoccidioides brasiliensis* and *P. lutzii*. These are temperature-dependent dimorphic fungi that cause paracoccidioidomycosis, a systemic granulomatous, and debilitating disease. Focus is given on cell wall carbohydrate and protein contents, their immune-stimulatory features, adhesion properties, drug target characteristics, and morphological phase specificity. We offer a journey toward the future understanding of the dynamic nature of the cell wall and of the changes that may occur when the fungus infects the human host.

Keywords: cell wall, *Paracoccidioides brasiliensis*, *P. lutzii*, polysaccharides, adhesins, enzymes, host interaction

The 60s and 70s were prolific decades in the study of the morphological and biochemical aspects of the *Paracoccidioides brasiliensis* cell wall (San Blas and San Blas, 1984, 1994). This fungus belongs to a group of pathogens whose morphology changes according to the temperature of growth: a mycelial phase develops under mild environmental temperatures up to 26°C, while multi-budding yeasts develop at temperatures near 37°C. The infectious particles are supposedly inhaled conidia produced by environmental mycelia (Bustamante-Simon et al., 1985; San Blas, 1986; Samsonoff et al., 1991); transition to yeasts in the lungs is mandatory for the establishment of paracoccidioidomycosis (McEwen et al., 1987), which is a granulomatous mycosis prevalent in Latin America (San Blas et al., 2002). Currently, *Paracoccidioides* isolates from Brazilian central–western areas are considered a new species – *P. lutzii* ("Pb01-like"; Teixeira et al., 2009), whereas *P. brasiliensis* represents a complex of three phylogenetic groups (Matute et al., 2006). There is free access to complete genomic information for isolates Pb18, Pb3, and Pb01 at the Dimorphic Fungal Database of the Broad Institute site (http://www.broadinstitute.org/annotation/genome/dimorph_collab/MultiHome.html), and genome comparative analyses have been reported (Desjardins et al., 2011). Transcriptome data have long been available for Pb18 and Pb01 and differentially expressed genes have been detected (Felipe et al., 2003, 2005; Goldman et al., 2003; Andrade et al., 2005; Nunes et al., 2005; Bailão et al., 2006; Borges et al., 2011). Yet, genetic manipulation in *Paracoccidioides* is only starting (Sturme et al., 2011), thus the association between translated proteins and the cell wall has generally been inferred by comparison with other fungal systems.

The relationship among *P. brasiliensis* cell wall, dimorphism, and virulence has intrigued pioneer researchers (San Blas, 1982). They used sophisticated high resolution microscopy and biochemical techniques available at the time to compare the surface structure of the yeast and budding forms (Carbonell, 1967), hyphae (Carbonell and Rodriguez, 1968), transition phases (Carbonell, 1969), isolated cell walls (Carbonell et al., 1970), and to identify the main cell wall components (Kanetsuna et al., 1969, 1972; Kanetsuna and Carbonell, 1970). They studied a Venezuelan fungal isolate whose cell wall was thicker in yeasts (0.2–0.6 μm) than in hyphae (80–150 nm). Both showed a wide translucent and homogeneous layer that lied over the plasma membrane. In yeasts, an electron dense outer layer decorated with fibrils covered this inner layer. Conidia cell walls (150 to 300-nm wide) were studied only later: they showed homogeneous appearance of medium electron density, being surrounded by a thick coat of long electron dense microfibrils (Edwards et al., 1991).

Carefully isolated yeast cell wall preparations were composed of around 81% carbohydrates (38% hexoses; 43% amino sugars), 10% amino acids, and 11% lipids; for mycelia, these percentages were, respectively, 51% (38% hexoses; 13% amino sugars), 33%, and 8% (Kanetsuna et al., 1972). Isolated cell walls were biochemically fractionated into alkali-insoluble (F1) or alkali-soluble components precipitated (F2) or not (F3) upon neutralization with acetic acid. Striking structural differences were then observed (Kanetsuna et al., 1972): in yeasts, 95% of the glucans were α-linked (F2) and 5% were β-linked (F1), while only β-linked glucans (F1) were found in mycelia. In both anomeric forms, long 1,3-linked chains were prevalent, with few 1,6-linked side chains. Chitin (F1)

was more abundant in yeasts. Freeze-section-shadow techniques (Carbonell et al., 1970) revealed that F3, composed of galactomannan and amino acids, was amorphous. In yeasts, F2 contained only α-1,3-glucans and appeared as thick (100 nm) and short microfibrils that resembled the cell wall outer layer. F1, containing chitin, β-glucan, and amino acids, displayed delicate nets of irregularly disposed thin fibers (20 nm), similar to those seen in the cell wall inner layer. Successive treatments with NaOH, β-1,3-glucanase and pronase damaged the mycelium cell wall, while the round yeast shape was maintained (Kanetsuna, 1981). The importance of randomly orientated chitin microfibrils for rigidity was then stressed. The round yeast shape was also kept in a mutant strain where most α-glucan was replaced by 1,3-mannan, as well as in the parent strain that presented only reduced amounts of α-glucan due to the effect of multiple sub-culturing (San Blas et al., 1976, 1977b). Later analysis of cell wall polysaccharides proved that their composition, however, could vary not only with culture conditions, but also among isolates (San Blas and Vernet, 1977; San Blas and San Blas, 1982; San Blas et al., 1984), and especially in mutants with defects in phase transition (San Blas et al., 1977a; Hallak et al., 1982; San Blas and San Blas, 1992). At that time, these mutants were useful to review the models of dimorphism (Kanetsuna et al., 1972; Kanetsuna, 1981; San Blas and San Blas, 1984, 1994). It has recently been reported that a few single residues of α-1,4-linked glucose may be attached to the linear α-1,3-glucan chains (Sorais et al., 2010).

Although a role for α-glucan in the yeast shape was discarded, its relationship with fungal virulence has long been speculated, especially because decreased virulence and loss of externally arranged α-glucan have been observed to occur concomitantly (San Blas et al., 1976, 1977b; San Blas and Vernet, 1977; Goihman-Yahr et al., 1980; Hallak et al., 1982; Brummer et al., 1990; Kashino et al., 1990). The role of fungal cell wall polysaccharides as pathogen-associated molecular patterns (PAMPs) has been widely explored in *Candida albicans* (Netea et al., 2008). β-glucan is a cell wall PAMP that is mainly recognized by macrophage dectin-1 (Brown et al., 2002) and may interact with TLR2 (toll-like receptor 2) to activate NF-κB signaling (Gantner et al., 2003). The role of external α-glucan to prevent dectin-1 recognition and stimulus of protective immune response has been recently demonstrated in genetically manipulated *Histoplasma capsulatum* (Rappleye et al., 2004, 2007). Dectin-1 activation by wild type *P. brasiliensis* has been suggested (Diniz et al., 2004), as well as cellular immune-stimulatory responses by *P. brasiliensis* F1 cell wall fraction have been demonstrated (Silva and Fazioli, 1985; Alves et al., 1987; Silva et al., 1997, 2011; Anjos et al., 2002; de Pádua Queiroz et al., 2010). Cell wall F1 obtained from avirulent *P. brasiliensis* elicited in mice higher levels of tumor necrosis factor (TNF-α; Figueiredo et al., 1993), leukocyte recruitment, and granuloma formation (Silva and Fazioli, 1985) when compared with F1 from a virulent isolate. The effects correlated with higher β-glucan levels in the cell wall of the avirulent yeasts. Therefore, the amount of both α- and β-glucans might contribute to differential virulence among *Paracoccidioides* isolates.

B-cell activation by *P. brasiliensis* cell wall fractions has also been reported (de Oliveira et al., 1993; Benard et al., 1995; Silva and Silva, 1995; Silva et al., 1997). Complement activation (Crott et al., 1997) and neutrophil chemotaxis (Crott et al., 1993) stimulated by F1 have been observed. It has been demonstrated by immunofluorescence that C3, C3a, C3d, C3g, C4, C5b-9, and factors H and B are present on the cell surface of *P. brasiliensis* yeasts (Munk and Silva, 1992) and that complement 3 and mannose receptors are important for phagocytosis by macrophages (Jiménez et al., 2006). Also, an opsonized cell wall preparation was able to regulate B lymphocyte by complement receptors 1 and 2, which recognize activated C3 and C4 (de Agostino Biella et al., 2006). The results concerning stimulation with whole and fractionated cell wall preparations should be viewed critically, though, taking into consideration the purity of the preparations and the fact that F1 contains chitin, proteins, and β-glucans.

Water soluble galactomannan extracted in *P. brasiliensis* cell wall F3 (Kanetsuna et al., 1972) bears common antibody epitopes among dimorphs (Azuma et al., 1974). The galactomannan backbone has long α-1,6-Man*p* chains, which in hyphae are mostly decorated with α-1,6-Man*p*-α-1,2-Gal*f* ramifications (Ahrazem et al., 2003). In yeasts, these are minor structures, while β-1,6-Gal*f*-α-1,2-Man*p* and α-1,2-Man*p* prevail. Terminal β-galactofuranosyl residues are common fungal epitopes that can evoke cross-reactivity (Puccia and Travassos, 1991; Latge, 2009). Conversely, terminal α-galactofuranose has seldom been described in fungi (San Blas et al., 2005). We have recently characterized α-galactopyranosyl epitopes distributed along the cell wall and inside cytoplasmic vacuoles that were partially mapped in protein *O*-linked sugar chains (Vallejo et al., 2011). They are highly reactive with paracoccidioidomycosis patients' sera; however their role in the interaction with the host remains unknown. On the other hand, non-reducing units of terminal *N*-acetylneuraminic acid linked to sub-terminal β-galactopyranosyl units were observed on the yeast surface forming an anionic layer that possibly protects the fungus against phagocytosis (Soares et al., 1998). The nature of the glycoconjugates bearing these residues has not been described.

Cell wall-related sequences identified in the *Paracoccidioides* transcriptomes (Goldman et al., 2003; Felipe et al., 2005; Tomazett et al., 2005) and genomes (Desjardins et al., 2011) encode enzymes related to carbohydrate synthesis, remodeling, and degradation, as well as cell wall structural or associated molecules. A screening in the Pb01 transcriptome for GPI-anchored sequences (Castro et al., 2005) yielded six expression sequencing tags (ESTs) from possible cell wall structural proteins. A putative GPI-anchored membrane/cell wall β-1,3-glucan elongase capable of mediating fungal cell wall integrity (PbGel3p) was immunolocalized at the yeast surface (Castro et al., 2009).

The structural uniqueness and essentiality of fungal cell wall components indicate that proteins involved in their synthesis/degradation are potential drug targets, e.g., α-1,3-glucan synthase, glucanases [exo-β-1,3, endo-β-1,3(4)], α-1,2- and α-1,6-mannosyltransferases, chitinase, and dolichyl phosphate-D-mannose:protein *O*-D-mannosyltransferase (Desjardins et al., 2011). *Paracoccidioides* has a single β-1,3-glucan synthase gene that is overexpressed in Pb01 yeasts (Tomazett et al., 2010); however, echinocandin inhibitors of β-1,3-glucan synthesis have little effect against the yeast pathogenic phase due to the prevalence of α-glucan (San Blas and Niño-Vega, 2008; Rodríguez-Brito et al., 2010). Thus, a better target would be α-1,3-glucan synthase

and its regulatory molecules including GTPase proteins Rho1, Rho2, Rac1, and Cdc42 (Sorais et al., 2010). Not coincidentally, *P. brasiliensis* mutants knocked down for *CDC42* expression presented altered growth pattern and decreased virulence (Almeida et al., 2009).

Chitin synthases are broader targets for fungi. Genome analysis revealed the existence of seven chitin synthases in *Paracoccidioides*, one of each class, including a functional class III (Barreto et al., 2010), not described previously (Niño-Vega et al., 2000), and Pbrchs6 originally detected in transcriptome studies only in mycelia (Tomazett et al., 2005). Microarray hybridization analysis identified some chitin synthases up-regulated during the mycelium-to-yeast transition, while chitinases and endochitinases were downregulated (Nunes et al., 2005). A chitin deacetylase transcript (*CDA*), which encodes an enzyme that converts chitin to chitosan, was overexpressed in Pb01 yeasts (Felipe et al., 2005).

A number of intracellular proteins, particularly enzymes, have also been localized to the cell wall in *Paracoccidioides* and other fungi (Nombela et al., 2006). Their presumptive roles at the surface might involve essential activities like cell wall biosynthesis and remodeling, microorganism adaptation to different conditions, and also interaction with the host. We reported on a mitochondrial heat-shock chaperone Mdj1 labeled at the *P. brasiliensis* yeast cell wall (Batista et al., 2006, 2007), which is the preferential site in fully formed mycelia (Dourado et al., unpublished). Alkaline phosphatase activity on the cell wall was demonstrated by histochemical techniques in *P. brasiliensis* yeast phase (Campo-Aasen and Albornoz, 1994). A secreted 75-kDa protein with phosphatase activity was identified by confocal microscopy and flow cytometry on the cell surface, preferentially at the budding site of *P. brasiliensis* (Xander et al., 2007). Yeast cells opsonized with anti-75 kDa monoclonal antibodies showed growth inhibition, increasing phagocytic index, and *in vivo* protection against murine paracoccidioidomycosis. Formamidase, which participates in the nitrogen metabolism, was detected by immunoelectron microscopy on the cell wall of Pb01 yeast cells (Borges et al., 2009), as well as a 66-kDa secreted aspartyl protease (PbSAP) that was more abundantly expressed in the yeast phase (Tacco et al., 2009).

Adhesion of pathogens to host components is an important step in microorganism infection and dissemination. The dataset of a recently developed Fungal RV adhesin predictor algorithm (Chaudhuri et al., 2011) identified 27 proteins from *P. lutzii* (Pb01). Among them, only triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase (GADPH), two cytoplasmic glycolytic enzymes, have already been identified as cell wall adhesins (Barbosa et al., 2006; Pereira et al., 2007). Both can bind to cell culture pneumocytes and to proteins associated with the extracellular matrix (ECM). Similar results have been observed for glyoxylate pathway malate synthase (da Silva Neto et al., 2009) and for putative α-1,6-mannosidase PbDfg5p (defective for filamentous growth protein), both localizing mainly to budding yeast cell walls (da Silva et al., 2008). PbDfg5p transcripts were preferentially expressed in yeasts, where it was shown to be linked to β-glucan. Enolase is another glycolytic enzyme with fibronectin-binding features and double localization in *P. brasiliensis* (Donofrio et al., 2009; Nogueira et al., 2010). This protein is also capable of activating plasminogen conversion to plasmin, a serine protease that can facilitate pathogen dissemination. Host cell exposure to recombinant enolase had enhanced fungal adhesion, possibly due to an increased exposure of *N*-acetylglucosamine, which might be recognized by paracoccin, a TNF-α stimulatory lectin (Coltri et al., 2006). Paracoccin is a cell wall chitin-binding protein with *N*-acetyl-β-D-glucosaminidase activity that plays a role in fungal growth, supposedly by participating in cell wall remodeling and organization (Ganiko et al., 2007; dos Reis Almeida et al., 2010).

Secreted gp43 is a major diagnostic *P. brasiliensis* antigen (Puccia and Travassos, 1991) that partly resides on the cell wall (Straus et al., 1996). It presents vaccine features due to the presence of T-cell epitopes that stimulate Th-1 protective responses (Taborda et al., 1998; Travassos et al., 2008), but apparently it can also stimulate innate immunity (Nakaira-Takahagi et al., 2011). Gp43 has been appointed as a virulence factor considering its broadly demonstrated adhesive properties to ECM (Vicentini et al., 1994; Gesztesi et al., 1996; Hanna et al., 2000), while an internal peptide (NLGRDAKRHL) was able to compete in binding assays (Mendes-Giannini et al., 2006). A 32-kDa ligand to laminin, fibronectin, and fibrinogen was observed on the surface of yeasts, mycelia, and conidia, which could play a role in the initial attachment of the infectious particles to the lungs (González et al., 2005, 2008). In general, experimental data have positively correlated fungal

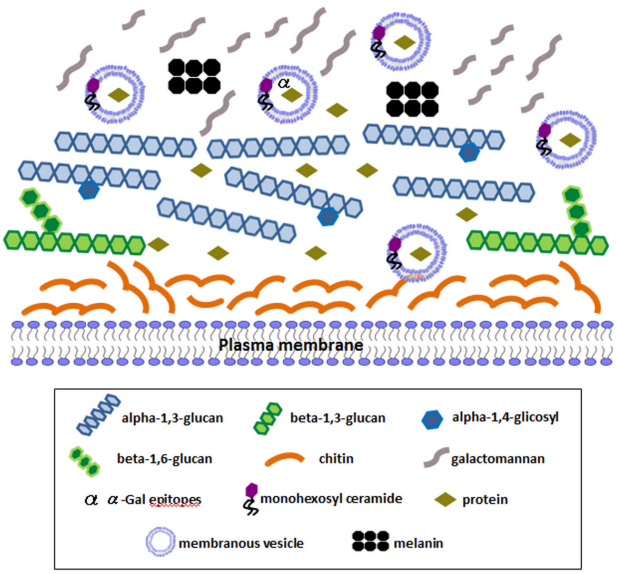

FIGURE 1 | Schematic representation of an updated view of *Paracoccidioides* yeast phase cell wall components. Structural polysaccharides are represented: an outer layer of alpha-1,3-glucan (with eventual single alpha-1,4-linked glucose residues) in higher amounts than beta-1,3-glucan, which lies in internal layers and is decorated with small beta-1,6-glucan side chains. Internal chitin fibers maintain the round shape and galactomannan composes an external fibrillar layer. Melanin is shown on the surface. Extracellular membranous vesicles containing monohexosyl ceramide transport components outside the plasma membrane, including numerous intracellular proteins. A number of intracellular proteins that have been immunolocalized to the cell wall in yeast cells using TEM or confocal microscopy (see text) are represented, as well as α-Gal epitopes. Some of them have also been detected in isolated extracellular vesicles, like enolase, GADPH, formamidase, and triosephosphate isomerase.

adhesion with the degree of virulence among isolates (Hanna et al., 2000), and also with *in vivo* passage (Mendes-Giannini et al., 2006): higher expression of the 30-kDa laminin-binding protein (Andreotti et al., 2005) and enolase (Donofrio et al., 2009) was observed in the latter situation, suggesting once again the induction of cell wall virulence factors within the host or in host-like culture conditions.

Apart from polysaccharides and (glyco)proteins, the negatively charged pigment melanin was found *in vitro* and *in vivo* in the *P. brasiliensis* conidia and yeast cell wall (Gómez et al., 2001), conferring resistance to phagocytosis, antifungal drugs, and antimicrobial oxidants (da Silva et al., 2006, 2009). Data on the composition of cell wall lipids are hard to find, although early works reported up to 80% of lipids in the cell wall F3 fraction of highly subcultured and mutant *P. brasiliensis* strains (San Blas et al., 1976, 1977a). Neutral and acidic glycosphingolipids, specifically glucosylceramide (GlcCer) and glycoinositol phosphoryl-ceramides (GIPCs), have later been fully characterized and found to probably interfere in dimorphism; they bear antigenic oligosaccharide epitopes and present surface localization, possibly residing on the cell wall (Toledo et al., 2001, 2010; Bertini et al., 2007; Takahashi et al., 2009). In *C. neoformans*, a similar GlcCer was localized to the cell wall and correlated with cell wall thickening; anti-GlcCer antibodies were able to inhibit fungal growth and budding (Rodrigues et al., 2000). Later, this component was observed in vesicle membranes that crossed the cell wall toward the extracellular compartment of *C. neoformans* (Rodrigues et al., 2007).

We have recently characterized extracellular membranous vesicles in the yeast phase of *P. brasiliensis* (Vallejo et al., 2011), where a monohexosyl ceramide has also been detected (Vallejo et al., unpublished). They carry antigens recognized by patients' sera, including components containing α-galactopyranosyl epitopes (Vallejo et al., 2011), macrophage stimulatory molecules (Vallejo et al., unpublished), and a large number of proteins recently identified using proteomic approaches (Vallejo et al., submitted). Among them are some of the proteins mentioned above to be localized to the cell wall and also surface proteins mildly extracted from *P. brasiliensis* cells (Longo et al., unpublished). Besides, over 40% of these proteins have orthologs among fungal vesicle proteins (Vallejo et al., submitted; Rodrigues et al., 2007; Albuquerque et al., 2008; Oliveira et al., 2010). In fungi, membranous extracellular vesicles seem to have several possible biogenesis routes; therefore they contain proteins of diverse nature (Casadevall et al., 2009; Oliveira et al., 2010). At least part of vesicle components could remain in the cell wall and be functional. An updated scheme of the *Paracoccidioides* cell wall, yeast phase, is presented in **Figure 1** that considers past and recent findings of cell wall components discussed in this review. It is a future challenge to understand the transport of components outside the plasma membrane using non-conventional pathways, how they interact with structural cell wall molecules and interfere in its biology. These findings will help to update our view of the dynamic nature of this essential organelle and of the changes that may occur when the fungus infects the human host.

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