Glycoconjugate expression on the cell wall of \textit{tps1/tps1} trehalose-deficient \textit{Candida albicans} strain and implications for its interaction with macrophages

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The yeast \textit{Candida albicans} has developed a variety of strategies to resist macrophage killing. In yeasts, accumulation of trehalose is one of the principal defense mechanisms under stress conditions. The gene-encoding trehalose-6-phosphate synthase (\textit{TPS1}), which is responsible for trehalose synthesis, is induced in response to oxidative stress, as in phagolysosomes. Mutants unable to synthesize trehalose are sensitive to oxidative stress in vitro. In mice, the \textit{TPS1}-deficient strain, \textit{tps1/tps1}, displays a lower infection rate than its parental strain (CAI4). We have previously demonstrated the reduced binding capacity of \textit{tps1/tps1} and its lower resistance to macrophages. At the same time, its outer cell wall layer was seen to be altered. In this study, we show that depending on the culture conditions, the \textit{tps1/tps1} strain regulates the carbohydrate metabolism in a different way to CAI4, as reflected by the enhanced $\beta$-mannosylation of cell wall components, especially at the level of the 120 kDa glycoprotein species, accessible at the cell surface of \textit{tps1/tps1} when cultured in liquid medium, but not on solid medium. This leads to changes in its surface properties, as revealed by decreased hydrophobicity, and the lower levels of ERK1/2 phosphorylation and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) production in macrophages, thus increasing the resistance to these cells. In contrast, in solid medium, in which over-glycosylation was less evident, \textit{tps1/tps1} showed similar macrophage interaction properties to CAI4, but was less resistant to killing, confirming the protective role of trehalose. Thus, the lack of trehalose is compensated by an over-glycosylation of the cell wall components in the \textit{tps1/tps1} mutant, which reduces susceptibility to killing.

\textbf{Keywords:} \textit{Candida albicans} / carbohydrates / cell wall / macrophages / trehalose

Introduction

The opportunistic yeast \textit{Candida albicans} is part of the human commensal microflora. The high levels of morbidity and mortality induced by \textit{C. albicans} in immunocompromised conditions, such as acquired immune deficiency syndrome or transplanted patients, or after aggressive surgery or prolonged chemotherapy (Coleman et al. 1993) reveal that this yeast is one of the most prominent human pathogens. Despite the present day availability of effective drugs for the treatment of candidiasis, the increasing prevalence of resistant strains demands the development of new antifungal agents (Calderone 2002).

Macrophages are phagocytic cells of the immune system able to recognize and eliminate the microorganisms that cross the epithelial barrier. These cells play a key role in the defense against pathogens because they can engulf and destroy pathogens even in the absence of an adaptive immune response. Macrophages recognize microorganisms through receptors expressed on the cell membrane, referred to as pattern recognition receptors (PRRs) and trigger the innate proinflammatory response. This initial immune response subsequently enables the development of further adaptive responses.

PRRs bind to conserved microbial structures called microbe-associated molecular patterns (MAMPs). The recognition of yeasts by macrophages is based on the MAMPs present as components of the cell wall, a complex structure mainly composed of glycan (Jouault et al. 2009). The outer layers of the cell wall are made of phosphopeptidomannan, a polymer of $O$- and $N$-linked mannose residues commonly referred to as mannan. The $O$-linked mannose residues are composed of short chains of $\alpha$-$1,2$- and $\alpha$-$1,3$-linked mannoses, whereas the $N$-linked part consists of a backbone of $\alpha$-$1,6$-linked mannoxyranose residues with branches composed of $\alpha$-$1,2$- and $\alpha$-$1,3$-linked mannoxyranose units. $N$-Linked mannan can be resolved by mild acid hydrolysis into an acid-stable fraction, consisting of all proximal
mannose residues, and an acid-labile fraction, which, in the case of Candida, corresponds to phosphomannan made of β-1,2-linked mannose chains (Shibata et al. 1991). In C. albicans, additional linked β-1,2 mannosides are present as terminal mannosides of acid-stable fraction side chains, constituting the C. albicans serotype A (Shibata et al. 1985; Kobayashi et al. 1992). β-1,2 mannosides are also associated with other carrier molecules such as mannoproteins (Cantelli et al. 1995; Fradin et al. 2008) and phospholipomannan (PLM), a glycolipid which is specifically expressed by some species of Candida. PLM consists of a core of ceramide attached by a unique mannosylphosphate/mannino-inositol phosphate spacer to an unbranched β-1,2-oligomannoside chain of up to 19 residues (Trinel et al. 2002). PLM, which is present within the cell wall, where it participates in β-1,2-mannoside expression attributed to mannan (Poulin et al. 2002), is shed by C. albicans, interacting with host cells (Jouault et al. 1998; Dalle et al. 2003). Beside mannan, the yeast cell wall is also composed of β-1,3- and β-1,6-linked glucans contained deep in the cell wall (Kaptyn et al. 2000), usually masked on live cells, but accessible to the cell surface after heat treatment which removes the mannan (Martínez-Esparza et al. 2006; Wheeler and Fink 2006).

The expression of MAMPs can be modulated by C. albicans depending on its environment (Wheeler et al. 2008; Klis et al. 2009). Thus, growth conditions, such as temperature, physical state or growth phase, affect the properties of the yeast cell surface (Colling et al. 2005a). It has been reported that the expression of carbohydrates on the fungal surface modifies its hydrophobicity and adherence to host tissues (Hazen et al. 1991; Lima-Neto et al. 2009).

Lectins and toll-like receptors (TLRs, mainly TLR-2 and TLR-4) are host PRRs that recognize sugars on the yeast cell wall, inducing the immune response: dectin-2 recognizes high mannosides; macrophage mannose receptor recognizes fungal mannan; dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, another C-type lectin, is able to internalize C. albicans by recognizing complex mannoside structures exposed on yeast cell walls; Minle C-lectin also binds to yeast cell wall components; galectin-3, a S-lectin originally described for its specific binding to galactose, binds Candida-specific β-1,2 mannosides; and finally, dectin-1 confers cellular responsiveness to microbial β-glucans (review by Jouault et al. 2009).

The interaction between host PRRs and C. albicans MAMPs results in intracellular signaling that leads, in most cases, to the proinflammatory response against the yeast that is necessary to acquire resistance to infection or to an anti-inflammatory response, which allow the yeast to establish the infection.

Once the yeasts have been recognized and engulfed by macrophages, they are destroyed by different enzymes and oxygen reactive species, including OH\(^{\bullet}\), O\(_2\) and H\(_2\)O\(_2\), among other oxidants. However, C. albicans has developed a variety of strategies to avoid being destroyed by macrophages, including the formation of hyphae, the synthesis of molecules and/or the activation of protective enzymes, and induction of apoptosis in macrophages through surface glycolipids (Ibata-Ombetta et al. 2003) or secreted proteins (Álvarez-Peral et al. 2002; Fernández-Arenas et al. 2009; Martínez-Esparza et al. 2009).

The accumulation of the disaccharide trehalose in yeast is one of the principal defense mechanisms developed under conditions of nutritive and environmental stress (oxidative stress, osmotic or heat shock; Argüelles 2000). The gene-encoding trehalose-6-phosphate synthase (TPS1), which is responsible for trehalose synthesis, is induced in response to oxidative stress. Mutants unable to synthesize trehalose are very sensitive to oxidative stress in vitro (Álvarez-Peral et al. 2002). Moreover, when inoculated in mice, the TPS1-deficient strain (tps1/tps1) displays a lower infection rate when compared with its parental strain (CAI4; Zaragoza et al. 1998), which is in accordance with its lower resistance to killing by macrophages in vitro (Martínez-Esparza et al. 2007).

The differences between the cell wall composition of the tps1/tps1 strain, which is unable to synthesize trehalose, and that of its congenic strain CAI4 have previously been postulated (Martínez-Esparza et al. 2007). The TPS1 mutant showed partial loss of material from the outer cell wall layer, higher fluorescein isothiocyanate (FITC) staining efficiency, a slight reduction in its capacity to bind to macrophages and slower kinetics of engulfment of the cells.

The aim of this work was to gain further insight into differences in the cell wall composition of CAI4 and tps1/tps1 strains and to evaluate the potential implication of these differences in the C. albicans–macrophage interaction.

**Results**

**CAI4 and tps1/tps1 yeast strains show similar sensitivity to chemical agents**

Disruption of the two chromosomal copies of TPS1 gene has pleiotropic effects, such as the impairment of hyphae formation and the inhibition of growth in glucose at certain temperatures (Zaragoza et al. 1998), although these effects can be avoided by replacing glucose by galactose as the source of carbon (YPGal medium). Other differences in the cell wall composition of tps1/tps1 strain have also been observed (Martínez-Esparza et al. 2007).

To explore the differences between CAI4 and tps1/tps1 strains at a cell wall level, we studied the behavior of these yeast strains cultured in the presence of several chemical agents known to affect the cell wall integrity: calcofluor white, a bipolar molecule which is inserted in the chitin chains inside the cell wall (Murgui et al. 1985) where it interferes with the cell wall biosynthesis; sodium dodecyl sulfate (SDS), a detergent that disturbs cell membranes and affects their stability, thereby hindering the final construction of the cell wall; NaCl or CaCl\(_2\), which cause variations in external osmotic concentrations, affecting cell integrity; and antifungal drugs, which act on different cell components.

The results shown in Figure 1 clearly demonstrate the absence of noticeable differences between the tps1 null mutant and its parental counterpart, CAI4, in the sensitivity they show to SDS, NaCl or CaCl\(_2\). However, high concentrations (50 μg/mL) of calcofluor white had a slightly higher inhibitory effect on the tps1/tps1 strain.
Next, we determined the minimum inhibitory concentration of several antifungal drugs that induced 90% of growth inhibition (MIC90) of both strains. The results pointed to the absence of differences between both strains in their sensitivity to amphotericin B (MIC90, 0.5 μg/mL), caspofungin (0.031 μg/mL), fluconazole (8 μg/mL) or voriconazole (0.062 μg/mL).

The tps1/tps1 yeast strain shows higher total expression levels of β-mannol conjugates

It should be noted that all the experimental work previously published with the trehalose-deficient mutant (tps1/tps1) and its congenic strain (CAI4) has been carried out with exponential yeast cultures grown in YPGal medium (Álvarez-Peral et al. 2002; González-Párraga et al. 2003; Martínez-Esparza et al. 2007). However, fungal culture conditions, like the physical state of medium, the temperature or growth phase also affect properties of the yeast cell surface (Colling et al. 2005a). Since glycoconjugates are the main components of the outer cell layer, we first analyzed their expression in CAI4 and tps1/tps1 strains under different culture conditions.

Whole extracts from CAI4 and tps1/tps1 strains cultured in liquid or solid medium were analyzed by western blot (Figure 2). Blotting with 5B2, a monoclonal antibody specific for β-1,2 mannoses, revealed the same expression levels of β-mannono conjugates and PLM when yeasts were cultured on solid medium. However, when yeasts were cultured in liquid medium, the expression levels of β-mannosylated protein species of ~120 kDa were higher in the tps1/tps1 than in CAI4. Moreover, the β-mannosylation of these protein species was more intense when both yeast strains were cultured on solid medium (Figure 2, 5B2 antibody, lanes 3 and 4 vs. lanes 1 and 2). The results obtained with S4.1 antibody, which recognizes immunogenic epitopes of C. albicans, showed expression profiles similar to those obtained with 5B2 antibody, although the difference in β-mannosylation of the 120 kDa glycoprotein species between CAI4 and tps1/tps1 was less pronounced in this case when yeasts were cultured in liquid medium. The β-mannosylation of these protein species was more intense and with apparent higher molecular weight when both yeast strains were cultured on solid medium (Figure 2, S4.1 antibody, lanes 3 and 4 vs. lanes 1 and 2). The results obtained with the antibody EB-CA1 (specific for α-1,2-linked mannoses) and concanavalin A (ConA lectin, specific for α-linked mannoses) showed that although the profile varies depending on the culture conditions, tps1/tps1 and CAI4 yeast strains exhibit similar α-mannoconjugate expression levels under both liquid and solid culture conditions. These results pointed to the
The tps1/tps1 strain induces lower levels of ERK1/2 and P90rsk phosphorylation

Although similar total levels of PLM and other β-1,2-mannosides recognized by 5B2 antibody were found between the CAI4 and tps1/tps1 strains (Figure 2), their accessibility on the external cell wall was seen to differ when yeasts were grown in liquid medium (Figure 3A). When the consequences of such differences on the first step of the interaction of yeast with macrophages was studied, endocytosis or the addition of PLM induced a disruption of the mitogen-activated protein kinase kinase-extracellular signal-regulated kinase (MEK–ERK) signal transduction pathway in macrophages, leading to decreased phosphorylation of the ERK1/2 and its downstream product, P90rsk (Ibata-Ombetta et al. 2001, 2003). This led us to examine the intracellular signaling induced in J774 macrophages by both yeast strains cultured at the exponential phase in both liquid and solid media.

As expected, western blot analysis (Figure 4) revealed a noticeable increase in ERK1/2 phosphorylation in J774 macrophages after 15 min of co-culture with CAI4 (lanes 2 and 4) or tps1/tps1 (lanes 3 and 5) yeast strains (1:10 cell/yeast ratio) compared with control cells (lane 1). After 60 min, the signal decreased to control cell levels. The results also showed that CAI4 induces higher levels of ERK1/2 and P90rsk phosphorylation than tps1/tps1 when cultured in liquid medium (Figure 4, lanes 2 and 3): ERK1/2 phosphorylation reached a 4- and a 2.5-fold increase over control levels, with CAI4 and tps1/tps1, respectively. These differences were not detected when yeasts were grown on solid medium (Figure 4, lanes 4 and 5).

tps1/tps1 induces a reduced proinflammatory response

ERK-phosphorylation has been seen to play a role in the activation of Nuclear Factor κ B and in the production of TNF-α (Netea et al. 2002). Bearing in mind the effect of TPS1 disruption on ERK phosphorylation, its consequences for TNF-α
release by macrophages incubated with CAI4 or tps1/tps1 yeast strains grown on solid or liquid medium was explored. The levels of TNF-α secreted by macrophages in response to tps1/tps1 were seen to be lower than those induced by CAI4, but, as above, this was only evident when yeasts were grown in liquid medium (Figure 5). Nonetheless, cultured in liquid medium, both strains induced higher TNF-α release than yeasts cultured on solid medium.
We found that after 2 h of co-culture at a 1:10 cell/yeast ratio, both strains showed an identical rate of phagocytosis (data not shown), allowing us to correlate the viability of phagocytosed yeast with the susceptibility to be killed by the macrophages. Both CAI4 and tps1/tps1 strains cultured in liquid medium showed the same sensitivity to macrophage killing (Figure 6). However, when cultured on solid medium, C. albicans tps1/tps1 strain was less resistant (1.5-fold) to macrophage killing than CAI4. Therefore, under conditions in which the cell wall defect associated with TPS1 disruption is less evident, only trehalose seems to be responsible for the resistance observed.

Discussion

A powerful approach to studying host-pathogen relationships is to analyze the in vitro interaction between a microorganism and purified populations of immune cells. We used this approach to study the resistance of C. albicans TPS1 null mutant (defective in trehalose synthesis) and its parental strain CAI4 to macrophage-mediated killing (Martínez-Esparza et al. 2007). We observed that the TPS1 null mutant showed lower resistance when interferon-γ and lipopolysaccharide were used to stimulate the J774 murine macrophage cell line, because of a deficient protection against oxidative stress inside the phagolysosome as a consequence of its inability to synthesize trehalose. Moreover, tps1/tps1 mutant showed a partial loss of material from the outer cell wall layer, a higher FITC staining efficiency, a slight reduction in macrophage bindings and slower ingestion kinetics, which suggests differences between the cell wall composition of tps1/tps1 mutant and its parental strain (Martínez-Esparza et al. 2007).

The expression of cell wall molecules can vary depending on the mode of growth and the combined inputs of several signaling pathways that sense environmental conditions (Klis et al. 2009). Therefore, in this work, we have examined the nature of such differences between CAI4 and tps1/tps1 strains at the cell wall level, the contribution of growth conditions to these differences and their implication for the interaction with macrophages.

Both strains show a similar sensitivity to antifungal treatments, which interfere with the yeast cell wall structure or the cellular membrane (amphotericin B, caspofungin, voriconazole and fluconazole). In contrast, tps1/tps1 exhibited increased sensitivity to calcofluor white, which binds chitin, thus competing with the interaction between chitin and β-1,3 glucans. This observation could be explained by a putative increment in the β-1,3-glucan content within the cell wall of tps1/tps1 mutant or their increased exposure, but this hypothesis was ruled out because both strains display similar sensitivities to caspofungin, which inhibits β-1,3-glucan synthesis. Moreover, β-1,3-glucan surface expression and TNF-α induction, for which β-1,3 glucans are known to play a major role (Brown et al. 2003), are similar for both strains arguing against a role for β-1,3 glucans in the increased sensitivity of tps1/tps1 to calcofluor.

No differences were found in glycoprotein expression between CAI4 and tps1/tps1 strains cultured on solid medium, either in the whole content or in the surface expression. However, enhanced β-1,2 mannosylation, in particular at the level of the 120 kDa glycoprotein species in the TPS1-deficient strain, was observed when yeasts were grown in liquid medium. Overall, these data suggest different regulation pathways of carbohydrate metabolism in response to environmental conditions—at least, these related to liquid or
solid culture media. Experiments are currently in progress to examine the nature of the 120 kDa glycoproteins and the role of trehalose in the regulation of its β-mannosylation.

Liquid medium increased the hydrophobicity of both strains and diminished mannoconjugate expression on the cell surface. Although this observation contrasts with other studies (Colling et al. 2005a), it agrees with the results published by Masuoka and Hazen (1997), who reported that cell wall mannosylation determines C. albicans cell surface hydrophobicity. Nevertheless, tps1/tps1 exhibited lower hydrophobicity in both liquid and solid media compared with its parental strain. Modification of the acid-labile β-1,2-mannoside chain length of mannan, but not of the acid-stable region, is one common mechanism by which C. albicans strains alter their cell surface hydrophobicity (Masuoka and Hazen 2004). This is supported by the more evident differences in hydrophobicity observed in liquid medium, in which differences in β-1,2-mannoside expression between both strains were also observed. However, on solid medium, where the expression of β-1,2 mannoside did not vary, a difference of hydrophobicity was still evident, indicating that other molecules must be involved in yeast surface hydrophobicity. In this regard, Lima-Neto et al. (2009) found a correlation between the adherence of C. albicans and C. parapsilosis with 1-fucose residues on glycoconjugates on their surface. Additionally, by means of electron microscopy, Masuoka and Hazen (2004) showed that the mannoprotein fibrils of the outer cell layer are shorter and more compact in hydrophobic yeasts, which is consistent with our previous results in the tps1/tps1 strain (Martínez-Esparza et al. 2007).

The above-mentioned reduced capability of tps1/tps1 to bind to macrophages (Martínez-Esparza et al. 2007) could be explained by the reduced hydrophobicity observed in this study with respect to the CAI4 strain. This reduced binding activity of the mutant strain also suggests the existence of differences in antigenic components on the yeast cell surface which may be involved in recognition by macrophages. Such differences in adhesion and antigen recognition would have consequences on the intracellular signaling processes. In this regard, it has been described that the endocytosis of C. albicans, or the addition of PLM, induced a disruption of the MEK–ERK signal transduction pathway in macrophages, leading to the modulation of ERK1/2 phosphorylation and of its downstream product, P90rsk (Ibata-Ombetta et al. 2001, 2003). The increased levels of β-1,2 mannosides shown by tps1/tps1 mutant compared with its parental strain, when cultured in liquid medium (as revealed by both western blot and flow cytometry) could explain the modulation of ERK, and secondarily of P90rsk, phosphorylations that were observed after 15 min of co-culture with macrophages. Concomitantly, other possible explanation for the lower phosphorylation levels induced by the mutant strain might be its reduced hydrophobicity, leading to a lower capacity of yeasts to interact with macrophages. However, when the yeasts were cultured on solid medium, the hydrophobicity differences was attenuated, leading to the identical up-regulation of ERK and P90rsk phosphorylation in both strains. This supports the idea that β-1,2-mannosylated yeast cell wall components are involved in the regulation of these signaling molecules in macrophages.

Recent data point to a central role for ERK activation in the regulation of TNF-α production—either positive or negative, depending on the yeast burden and efficient killing of yeasts. The inhibition of MAPK-interacting kinase (MNK), an mitogen-activated protein kinase (MAPK) downstream of ERK, blocked the proinflammatory response, but allowed interleukin-10 production in response to TLR stimulation (Rowlett et al. 2008), an effect which has been observed when large amounts of C. albicans are in contact with macrophages (Sarazin et al. 2010). The control of TNF-α mRNA nuclear export has also been shown to operate specifically through ERK-1 (Skinner et al. 2008), so that TNF-α mRNA is trapped in the nucleus when ERK-1 is inactive (Dumitru et al. 2000). Our results showed that the TNF-α levels produced and released by macrophages correlated with the ERK1/2 and P90rsk phosphorylation levels. When yeasts were grown in liquid medium, the levels of TNF-α secreted by macrophages were lower in response to tps1/tps1 than in the presence of CAI4. Interestingly, previous studies indicated that structural alterations, especially those affecting the branching of mannan, might be responsible for variations in the biological activity of macrophages (Ataoglu et al. 2000).

The fact that after 2 h of co-culture the rate of phagocytosis was the same for both CAI4 and tps1/tps1 strains, allowed us to correlate the viability of phagocytosed yeasts and their susceptibility to macrophage killing. The results showed that the tps1/tps1 yeast strain is less resistant to macrophage killing than its parental strain CAI4, probably due to a deficient protection against oxidative stress within the phagolysosome as a consequence of its incapacity to synthesize trehalose. This effect was barely detected in liquid medium when yeasts were grown as single cells. The greater release of TNF-α in liquid-cultured CAI4 would increase the killing capacity of macrophages and lead to a similar resistance to macrophage killing as seen in the TPS1-deficient strain, masking the protective effect of trehalose accumulation by the yeasts. However, when the yeasts were cultured on solid medium, they displayed similar mannoconjugates on the surface and induce similar ERK1/2 phosphorylation levels and TNF-α release. The fact that the TPS1-deficient strain was more susceptible to macrophage killing under these conditions lends weight to the role of trehalose in yeast protection against the oxidative stress inside the phagolysosomes.

Overall, the results obtained in this study show that the tps1/tps1 strain regulates the carbohydrate metabolism differently to its parental counterpart, CAI4, in response to environmental conditions. Such differences are reflected in the enhanced β-mannosylation of the cell wall components, especially of the 120 kDa glycoprotein species, on the cell surface of TPS1 null strain when cultured in liquid medium, which leads to differences in its surface properties as revealed by decreased hydrophobicity. In this culture condition, tps1/tps1 induces lower levels of ERK1/2 phosphorylation and TNF-α production in macrophages, increasing the resistance to killing by these cells. In contrast, on solid medium, in which over-glycosylation was less evident, tps1/tps1 showed similar macrophage interaction properties to CAI4 but was less resistant to killing, underlining the protective role of trehalose. Thus, the lack of trehalose is compensated by over-glycosylation of the cell wall components in tps1/tps1 mutant, enhancing its resistance.
These data highlight the potential link between defective carbohydrate metabolism in \textit{C. albicans} and regulation of the glycosylation process of the cell wall components, especially \(\beta\)-1,2-mannosylated components, which are involved in yeast hydrophobicity, resistance to macrophage killing, and the induction of signaling and cytokine release by these cells.

**Materials and methods**

**Reagents and antibodies**

All reagents were obtained from Sigma-Aldrich (St Louis, MO). Horseradish peroxidase (HRP)-conjugated ConA that binds \(\alpha\)-mannosides was from Vector Laboratories (Burlingame, CA). The following monoclonal antibodies were used: EB-CA1 specific for \(\alpha\)-1,2-mannosides [rat immunoglobulin (Ig) M from Bio-Rad SA, Marnes la Coquette, France]; 5B2 specific for \(\beta\)-1,2 mannosides (rat IgM developed in our laboratory, Hopwood et al. 1986); antibody specific for \(\beta\)-1,3 glucans (mouse IgG provided by Biosupplies Australia Pty Ltd.). Polyclonal antibodies specific for the phosphorylated form of ERK1/2 and P90rsk (rabbit polyclonal IgGs) were from New England Biolabs (Beverly, MA). Total serum S 4.1, specific for all epitopes expressed by \textit{C. albicans} serotype A, was obtained from a human patient with deep-tissue \textit{C. albicans} invasion.

Specific HRP-conjugated secondary antibodies goat anti-rat IgM, goat anti-mouse IgG and goat anti-human IgG + M + A were from Zymed Laboratories (San Francisco, CA). HRP-conjugated anti-rabbit IgG, the streptavidin-HRP or fluorochrome complexes, and fluorescence-conjugated secondary antibodies were obtained from Southern Biotechnology Laboratories (Birmingham, AL).

**Cell culture**

The mouse macrophage-like cell line, J774 (ECACC 85011428), was derived from a tumor of a female BALB/c mouse. Adherent cells were cultured at 37°C in an atmosphere containing 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Invitrogen, Paisley, UK), 5 mM \(\alpha\)-glutamine, 100 \(\mu\)g/mL of streptomycin and 50 \(\mu\)g/mL of penicillin. Before use, cells were gently scraped off with a rubber policeman, counted in Neubauer hemocytometer chamber and, depending on the experiment, plated onto either 12- or 24-well tissue culture plates at a density of 10\(^6\) cells/mL for biochemical analysis or for TNF-\(\alpha\) detection in cell-free supernatants, respectively.

**Yeast culture**

Two strains of \textit{C. albicans}, CAI4 (URA3::imm-434/ URA3:: imm-434; Fonzi and Irwin 1993), its congenic trehalose-deficient derivative, \textit{tps1/tps1} (Zaragoza et al. 1998), were used throughout this study. The cultures were grown in medium composed of 2% peptone, 1% yeast extract and 2% galactose (YPGal). The yeasts were maintained by periodic subculturing on solid YPGal (YPGal with 2% agar) or harvested, washed, aliquoted and stored at \(-80^\circ\)C in medium containing 25% of glycerol until used. Before the experiments, yeasts were transferred to a fresh liquid or solid YPGal medium and incubated for <14 h at 37°C, shaking only in the case of liquid medium. Growth was monitored by measuring the OD of cultures at 600 nm in a Shimadzu U/V spectrophotometer or by a direct cell counting in a Neubauer hemocytometer chamber.

**Sensitivity assay to chemical agents**

For the sensitivity assays, 5 \(\mu\)L of serial 1:10 dilutions of yeast exponential cultures were adjusted to OD\(_{600}\) = 1 and spotted on solid YPGal, containing either 1 or 1.5 M NaCl; 25 or 50 mM CaCl\(_2\); 10, 20 or 50 \(\mu\)g/mL of calcofluor white; or 0.001, 0.01 or 0.05% SDS, and incubated for 48 h.

**Sensitivity to anti-fungals**

Yeast exponential cultures were adjusted to OD\(_{600}\) = 1 and plated in 96-well plate with 2-fold serial dilutions of amphotericin B (4 \(\mu\)g/mL), fluconazole (64 \(\mu\)g/mL), voriconazole (32 \(\mu\)g/mL) or caspofungin (32 \(\mu\)g/mL) in RPMI 1640 medium (Gibco). After incubation for 24 h at 37°C, the OD was measured in each well and compared with control wells, and the concentration that induces 90% of growth inhibition was calculated for each treatment.

**Yeast cell surface hydrophobicity**

After culture, yeast cell hydrophobicity was analyzed by the microsphere-binding assay described previously (Colling et al. 2005a). Briefly, cells were washed and adjusted to OD\(_{600}\) = 1 in phosphate-buffered saline (PBS). The stock 10% solids suspension of deep blue-dyed styrene microspheres (0.8 \(\mu\)m diameter) was diluted one-fourth with PBS and microsphere suspensions were combined in Eppendorf tubes containing 580 \(\mu\)L of PBS, incubated at room temperature on a rotator at 14 rpm for 30 min and analyzed by fluorescence-activated cell sorter. The data presented show the FL4 associated with yeast, resulting from microsphere binding.

**Yeast cell surface staining**

Yeast staining was performed as described previously (Martinez-Esparza et al. 2009). Briefly, \(10^6\) cells were washed with PBS containing 2% FCS and then incubated for 15 min with 5B2 or EB-CA1 antibodies diluted 1:200, or anti-\(\beta\)-1,3 glucan antibody diluted 1:50. After washing, cells were incubated for 15 min with secondary FITC or phycoerythrin-labeled secondary antibodies diluted 1:100. The negative control was performed by adding labeled second antibody at the same concentration. The whole process was carried out at 4°C. After washing, cells were fixed in 0.4% paraformaldehyde and analyzed by fluorescence-activated cell sorter.

**Flow cytometry**

Flow cytometry analysis was performed using EPICS XL MCL4 (Beckman Coulter, High Wycombe, UK) equipped with an argon ion laser with an excitation power of 15 mW at 488 nm. Forward scatter (FSC) and side scatter (SSC) were analyzed on linear scales, whereas the analyses of green (FL1) and red FL (FL2 or FL4) were made on logarithmic scales. Analysis gates were set around debris and intact cells and unbound microspheres (for hydrophobicity assay) on an
FSC vs. SSC dot plot. The fluorescence histograms corresponding to 5000 cells were generated using the gated data. Data acquisition and analysis were performed using WINMDI software (available from http://facs.scripps.edu).

Extraction and western blot analysis of yeasts
Whole cell extracts of C. albicans exponential cultures grown on solid or liquid YPGal were obtained by alkaline extraction in reducing conditions as described previously (Trinel et al. 1992). Briefly, cells were treated for 15 min on ice with 0.5 mL of 1.86 mM NaOH–5% β-mercaptoethanol. After the addition of 0.5 mL of 50% trichloroacetic acid, cells were incubated for a further 15 min on ice, harvested, washed with 1 M Tris (pH 11), and then extracted for 5 min at 100°C with 62.5 mM Tris–HCl buffer–2% SDS (pH 6.8).

Extracts were adjusted to the same protein concentration and analyzed by SDS–Polyacrylamide gel electrophoresis on a 5–20% acrylamide gel slab. Electrottransfer was performed in a semi-dry transfer system for 1 h. After staining with 0.1% Ponceau S in 5% acetic acid to confirm the equivalence of loading and transfer, membranes were blocked with TNT [10 mM Tris, 100 mM NaCl (pH 7.5), 0.1% Tween]–1% nonfat milk or TNT–1% bovine serum albumin for blotting with lectins. Membranes were then probed for 1 h at 20°C with the corresponding antibodies or lectins: 5B2 antibody (1:5000), ConA lectin (1:4000), ConA lectin (1:5000) and S 4.1 antibody (1:400).

After washing, the binding was examined with the same dilution of the corresponding HRP-conjugated secondary antibodies and developed with the enhanced chemiluminescence (ECL) detection method (SuperSignal Chemiluminescent substrate, Pierce, Rockford, USA) on ECL hyperfilm.

Co-culture of yeast cells with mammalian cells
J774 were gently scraped with a rubber policeman and distributed into 12-well culture plates at 10^6 cells/well. After 18 h, the adherent cells were washed with culture medium. For co-culture studies, plated cells were incubated with yeasts at a concentration of 10 yeasts/J774 cells. After incubation, the cultures were washed with DMEM to remove the unbound yeasts and then prepared for either biochemical analysis or the fungicidal assays.

Extraction and western blot analysis for J774 cells
Cells were washed with 1 mL of ice-cold PBS containing 1 mM Na_3VO_4 and 10 mM NaF. The cultures were extracted with 250 μL of boiling 2× electrophoresis sample buffer (1× electrophoresis sample buffer: 125 mM Tris–HCl, pH 6.8, 2% SDS, 5% glycerol, 1% β-mercaptoethanol and bromophenol blue). Lysates were collected and clarified by centrifugation for 10 min at 12,000 × g at 4°C. Extracted proteins from macrophages were separated by 12% SDS–PAGE before blotting onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) for 1 h at 200 mA in a semi-dry transfer system. After staining with 0.1% Ponceau S in 5% acetic acid to confirm the equivalence of loading and transfer, the membrane was blocked by incubation with TNT containing 5% nonfat milk for 1 h at 20°C. Membranes were probed with antibodies diluted 1:1000 in TNT–5% nonfat milk overnight at 4°C. After incubation with secondary HRP-conjugated antibody and washing, the membrane was incubated with ECL detection reagents and exposed to hyperfilm ECL.

Fungicidal assays
Fungicidal assays were performed as described previously (Ibata-Ombetta et al. 2003). Briefly, J774 cells were incubated for 120 min at 37°C with yeast. The cultures were washed with DMEM, and endocytosed yeast cells were released by lysing the J774 cells with sterile distilled water. The yeast cells recovered were counted, and 100 individual yeast cells in 1 mL of PBS were plated onto solid YPGal. After incubation for 48 h, the number of colony-forming units was determined.

TNF-α production
J774 cells were incubated with medium alone or with different concentrations of yeasts. After different periods of time, 50 μL of cell-free supernatants were collected and stored at −80°C. The concentration of the cytokine in cell-free supernatants was determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, USA).

Statistical analysis
Data were represented as the mean ± SE. Data were analyzed by the unpaired Student’s t-test. The results shown were from 3–5 independent experiments.

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Conflict of interest
None declared.

Abbreviations
ConA, concanavalin A; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FL, fluorescence intensity; FSC, forward scatter; HRP, horseradish peroxidase; Ig, immunoglobulin; MAMP, microbe-associated molecular pattern; PBS, phosphate-buffered saline; PLM, phospholipomannan; PRR, pattern recognition receptor; SDS, sodium dodecyl sulfate; SSC, side scatter; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; TPS1, trehalose-6-phosphate synthase.

References


