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Budding of melanized *Cryptococcus neoformans* in the presence or absence of L-dopa

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Cryptococcus neoformans is a pathogenic fungus that produces melanin when incubated in the presence of certain phenolic substrates such as L-3,4-dihydroxyphenylalanine (L-dopa). Melanin is an enigmatic polymer that is deposited in the cell wall and contributes to virulence. Substantial progress has been made in understanding the synthesis of melanin and the mechanisms by which it contributes to virulence, but relatively little is known about how melanin is rearranged during growth and budding. In this study we used transmission and scanning electron microscopy and immunofluorescence of melanized cells and melanin 'ghosts' to study the process of melanization during replication. Budding in melanized *C. neoformans* results in focal disruption of cell-wall melanin at the bud site. In the presence of L-dopa, bud-related melanin defects are repaired and daughter cells are melanized. However, in the absence of substrate, mother cells cannot repair their melanin defects and daughter cells are non-melanized. Hence, melanin in the parent cell is not carried to the daughter cells, but rather is synthesized *de novo* in buds. These results imply that melanin remodelling occurs during cell growth in a process that involves degradation and synthesis at sites of budding.

INTRODUCTION

Cryptococcus neoformans is an encapsulated, environmental fungus that can cause life-threatening meningitis, particularly in immunocompromised individuals. The ability of *C. neoformans* to make melanin was discovered in the early 1960s (Staib, 1962, 1963) and was associated with virulence in the 1980s (Kwon-Chung *et al.*, 1982; Rhodes *et al.*, 1982). Melanins are rigid, acid-resistant polymers of uncertain structure that are found in all biological kingdoms (Hill, 1992). The synthesis of melanin by *C. neoformans* occurs only in the presence of phenolic compounds, such as L-3,4-dihydroxyphenylalanine (L-dopa), and is catalysed by a laccase (Polacheck *et al.*, 1982; Williamson, 1994). The *C. neoformans* laccase has recently been shown to be tightly associated with the cell wall by a hydrolysable bond (Zhu *et al.*, 2001). When the fungus grows in the presence of phenolic compounds, melanin is deposited in the cell wall (Wang *et al.*, 1995) and comprises approximately 15 % of the dry weight of late-stationary-phase melanized *C. neoformans* (Wang & Casadevall, 1996).

C. neoformans is melanized in the environment (Nosanchuk *et al.*, 1999b) and during human infection (Nosanchuk *et al.*, 2000). In the environment melanin is thought to protect the fungus against extremes of temperature (Rosas & Casadevall, 1997), ultraviolet light (Wang & Casadevall, 1994a), heavy metals (Garcia-Rivera & Casadevall, 2001) and amoeboid predators (Steenbergen *et al.*, 2001). During infection melanin appears to function in virulence by protecting fungal cells against microbicidal oxidants (Wang & Casadevall, 1994b) and peptides (Doering *et al.*, 1999) produced by immune effector cells. Furthermore, melanin may interfere with the development of effective cell-mediated responses (Huffnagle *et al.*, 1995) and could affect the activation of complement (Rosas *et al.*, 2002) in infected hosts. Melanin can also contribute to the difficulty associated with the treatment of *C. neoformans* infections with antifungal drugs, since melanin can reduce the susceptibility of pigmented cells to amphotericin B and caspofungin (Van Duin *et al.*, 2002). Given that melanin appears to increase fungal fitness *in vivo*, it is a potential target for drug discovery. In this regard, treatment of infected mice with glyphosate, an inhibitor of melanization, was associated with a beneficial therapeutic effect (Nosanchuk *et al.*, 2001).

Melanization of *C. neoformans* results in the deposition of the polymer in the cell wall. Treatment of melanized cells with enzymes, detergents and hot acid results in the recovery of melanin 'ghosts' that retain the size and shape of the original fungal cells (Rosas *et al.*, 2000a). Although the molecular structure of melanin is unknown, it is presumed to be composed of a lattice of interlocking, covalently bonded aromatic and aliphatic structures (Bell & Wheeler, 1986; Wheeler & Bell, 1988). Hence, melanin is a dense, rigid

Abbreviations: L-dopa, L-3,4-dihydroxyphenylalanine; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

and amorphous material with significant material strength. In fact, melanization is believed to provide great rigidity to hyphal cells, which may aid in the penetration of plants by melanotic pathogenic fungi (Howard *et al.*, 1991; Money, 1997; Money *et al.*, 1998). Melanin synthesis may also provide a biomechanical advantage for invasive hyphal growth of the human-pathogenic fungus *Exophiala* (*Wangiella*) *dermatitidis* (Brush & Money, 1999; Dixon *et al.*, 1989, 1991).

Since melanin is located in the cell wall of pigmented *C. neoformans*, the fungus must bud through this dense polymer to replicate. Given the structural toughness of this pigment, budding in the presence of melanin is likely to be a complex process, requiring both degradation and synthesis of the polymer concurrently in the mother and daughter cells. Despite the biological importance of melanin and the widespread occurrence of melanin in the mycota, we are not aware of any published information as to the effect of budding on melanized cells. In this study we investigated the effect of budding on melanized and non-melanized *C. neoformans* cells in the presence and absence of substrate for melanin synthesis.

METHODS

C. neoformans. *C. neoformans* serotype D strains 24067 and 3501 were obtained from the American Type Culture Collection (Manassas, VA, USA). CAP67, an acapsular mutant of *C. neoformans* 3501, was a gift of E. Jacobson (Richmond, VA, USA). The *C. neoformans* strains were grown in a chemically defined minimal medium (15 mM glucose, 10 mM $MgSO_4$, 29·4 mM KH_2PO_4, 13 mM glycine and 3 μM thiamine; pH 5·5) with or without 1 mM L-dopa (Sigma) at 30 °C in a rotary shaker at 150 r.p.m. for 10 days. When grown with L-dopa, melanization of a culture is visibly apparent after 3–4 days and cells are heavily melanized by 10 days (Wang & Casadevall, 1996). The cells from the 10-day-old cultures were collected by centrifugation at 320 ***g*** for 10 min, washed 3 times in PBS and approximately 100 000 organisms were inoculated into 100 ml fresh minimal medium with or without L-dopa. Samples from the subcultures were removed at different intervals and cell counts were determined by haemocytometer.

Immunofluorescence. Cells from 10-day-old melanized and non-melanized *C. neoformans* cultures transferred to fresh medium with or without 1·0 mM L-dopa were collected after 36 h growth in fresh medium and washed. For immunofluorescence analyses, suspensions of *C. neoformans* cells were air-dried on poly-L-lysine slides (Sigma), then blocked for non-specific binding with Superblock (Pierce) for 1 h at 37 °C. The slides were then incubated with 10 μg melanin-binding mAb 6D2 ml^{-1} overnight at 4 °C. mAb 6D2 ($\mu\kappa$) was generated against melanin derived from *C. neoformans* and also binds other types of melanins, but does not bind *Candida albicans*, *Saccharomyces cerevisiae* or a laccase-deficient mutant of *C. neoformans* (Rosas *et al.*, 2000b). After washing, the sections were incubated with a 1 : 1000 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (Southern Biotechnologies Associates) for 1 h at 37 °C. The sections were washed, coverslips were mounted using a 50 % glycerol, 50 % PBS and 0·1 M *N*-propyl gallate solution, and viewed with an Olympus AX70 microscope equipped with a FITC filter. Negative controls consisted of cells incubated with mAb 5C11 ($\mu\kappa$), which binds mycobacterial lipoarabinomannan (Glatman-Freedman *et al.*, 1996), as the primary antibody or with FITC-labelled antibody alone. The non-melanized cells that were transferred to fresh medium without L-dopa also served as negative controls.

Isolation of melanin 'ghosts' from *C. neoformans*. Treatment of melanized *C. neoformans* cells with enzymes, denaturant and hot acid results in the isolation of purified melanin in the shape and size of the parental melanized cryptococcal cell, and these particles are referred to as melanin 'ghosts' (Wang & Casadevall, 1996). Briefly, *C. neoformans* from the subcultures of cells grown for 10 days and transferred to fresh medium with or without L-dopa for 36 h were collected by centrifugation at 2010 ***g*** for 30 min, washed with PBS and suspended in 1·0 M sorbitol/0·1 M sodium citrate (pH 5·5). Cell-wall-lysing enzymes (from *Trichoderma harzianum*; Sigma) were added at 10 mg ml^{-1} and the suspensions were incubated at 30 °C overnight. The resulting protoplasts were collected by centrifugation, washed with PBS and treated with 1 mg proteinase K ml^{-1} (Roche Laboratories) made up in a reaction buffer (10 mM Tris, 1 mM $CaCl_2$ and 0·5 % SDS; pH 7·8) at 37 °C overnight. The debris was collected, washed with PBS and then boiled in 6 M HCl for 1 h. If particles remained, they were collected, washed in PBS and dialysed extensively against distilled water. This procedure has been shown to solubilize non-melanized *C. neoformans*, *Paracoccidioides brasiliensis*, *S. cerevisiae* and *Candida albicans* yeast cells (Gómez *et al.*, 2001; Rosas *et al.*, 2000b).

Scanning electron microscopy (SEM). The particles isolated from the treated cells were studied by SEM as described previously (Nosanchuk *et al.*, 1999a). Samples were incubated in 2·5 % gluteraldehyde in 0·1 M cacodylate for 1 h at room temperature, applied to poly-L-lysine-coated coverslips and serially dehydrated in alcohol. The samples were dried (Samdri-790; Tousimis), coated with gold-palladium (Desk-1; Denton Vacuum) and viewed using a JEOL JAM-6400 electron microscope.

Transmission electron microscopy (TEM). Samples of melanized and non-melanized *C. neoformans* were high-pressure-frozen using a Leica EMpact High Pressure Freezer. Frozen samples were transferred to a Leica EM AFS Freeze Substitution Unit and freeze-substituted in 1 % osmium tetroxide in acetone. They were brought from −90 °C to room temperature over 2–3 days, rinsed in acetone and embedded in Spurrs epoxy resin (Polysciences). Samples of 'ghosts' were fixed in 2 % gluteraldehyde in 0·1 M cacodylate at room temperature for 2 h followed by an overnight incubation in 4 % formaldehyde/1 % gluteraldehyde/0·1 % PBS. The samples were subjected to a 1·5 h post fixation in 2 % osmium, serially dehydrated in ethanol and embedded in Spurrs epoxy resin. Ultrathin sections of 70–80 nm were cut on a Reichart Ultracut UCT and stained with uranyl acetate followed by lead citrate. Samples were viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

RESULTS

Growth rate

Since budding in melanized cells could conceivably pose a difficulty for the mother cell, we first considered the question of whether there were differences in the growth rate of melanized and non-melanized cells. Growth rates were similar for cells grown in minimal medium with or without substrate, whether or not they were initially melanized (data not shown). The growth curves observed for *C. neoformans* strain 24067, in both melanized and non-melanized states, closely paralleled previously reported replication studies of this strain where cell counts by

haemocytometer were shown to correlate with c.f.u. determinations (Chen *et al.*, 1996, 1997). Similar results were obtained with *C. neoformans* strains 3501 and CAP67. Hence, the melanized state did not slow the rate of growth of *C. neoformans*.

Immunofluorescence

Melanin-binding mAb only labelled cells from *C. neoformans* cultures grown for 10 days with L-dopa. All of the melanized cells transferred to fresh medium with L-dopa were labelled by the melanin-binding mAb and the buds on replicating cells were similarly labelled (Fig. 1a). In contrast, approximately 30 % of cells from the cultures without substrate initiated with previously melanized cells were labelled by the melanin-binding mAb. Additionally, only the parental portion of budding cells was labelled (Fig. 1b). Similar findings were seen with each of the *C. neoformans* strains examined, but the immunofluorescent staining by the melanin-binding mAb was superior with CAP67, which lacks a polysaccharide capsule that impedes antibody from reaching the cell wall.

Analysis of melanin 'ghosts'

To gain insight into the budding process of melanized cells we analysed melanin 'ghosts' made from cells growing in the presence and absence of L-dopa. Pigmented cells transferred to medium with or without L-dopa for 36 h and then processed by the melanin isolation procedure yielded melanin 'ghosts' (Fig. 2) (Wang & Casadevall, 1996). As expected, no 'ghosts' were obtained from cells collected from samples grown initially without substrate, whether or not they were transferred to medium with L-dopa for 36 h. Non-melanized, stationary-phase cells are apparently unable to form a 'shell' of polymerized melanin over this period of time. The 'ghosts' isolated from melanized cells transferred to medium with L-dopa were a variety of sizes (diam. $4{\cdot}7 \pm 1{\cdot}5$ μm; range 1·5–7 μm), consistent with originating from a mixed population of melanized cells composed of older parental and recently budded cells. Budding cells demonstrated a connection of melanin between the parental and daughter cells (Fig. 2a). When melanized cells were grown in the absence of phenolic compounds, no budding-cell 'ghosts' were observed and the cells were more uniform in size (diam. $5{\cdot}3 \pm 0{\cdot}6$ μm; range 4·5–7 μm), consistent with the solubilization of all but the melanized parental cells. However, in these conditions the majority of 'ghosts' had defects, or 'holes' (Fig. 2b). These defects presumably represented locations of cell budding on the mother cell where non-melanized daughter cells were attached prior to being solubilized by treatment with enzymes and chemicals.

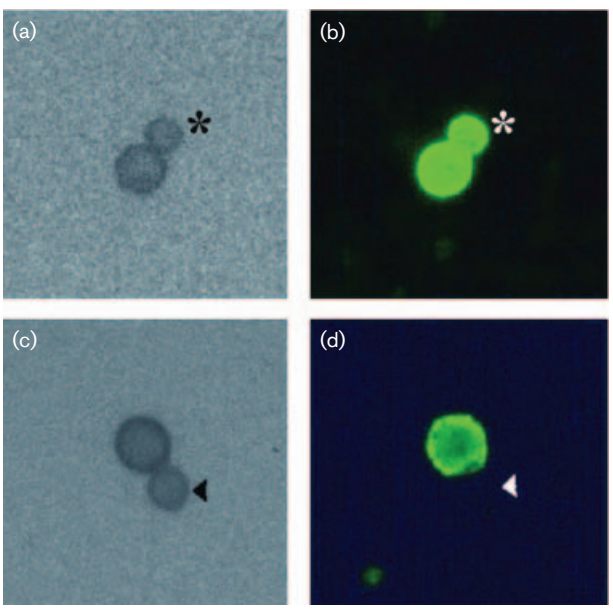

Fig. 1. Corresponding brightfield and immunofluorescent images of melanized *C. neoformans* strain CAP67 grown with (a, b) or without (c, d) L-dopa. The asterisk indicates a daughter cell on a replicating melanized cryptococcal cell grown with L-dopa that is labelled by melanin-binding mAb (a, b). The arrowhead indicates a daughter cell on a replicating melanized cryptococcal cell grown without L-dopa that is not labelled by melanin-binding mAb (c, d). The size of the larger portion of the replicating organism in each panel is about 5 μm.

A second related possibility is that these defects represent an inability of the parental cells to form melanin in the absence of exogenous phenolic substrate at the site of a recently attached daughter cell or to rearrange the melanin to cover the disrupted area. Similar findings were seen with particles isolated from each of the *C. neoformans* strains examined. TEM revealed the presence of a continuous electron-dense zone in the cell wall of melanized cells which was not present in non-melanized cells (Fig. 3a, b). In contrast, imaging of 'ghosts' generated from melanized cells showed defects in cross section at sites of apparent budding that are likely to correspond to the defects in the melanin layer observed by SEM (Fig. 3c). Hence, cell division for a melanized cell is accomplished by the formation of a break in the melanin layer where budding occurs.

DISCUSSION

Melanin is an enigmatic polymer that is found in the cell wall of *C. neoformans* during human infection (Nosanchuk *et al.*, 2000) and in pigeon excreta (Nosanchuk *et al.*, 1999b). Relatively little is known about the mechanisms of budding in *C. neoformans* (Kopecka *et al.*, 2001) and there is no information available regarding budding in the presence of melanin or about the production of melanin during budding. Given that melanized cells grow and replicate, we postulated that rearrangement of melanin must occur despite the fact that this polymer is rigid, dense and tenacious. Although no specific melanin-degrading enzymes have been described, such enzymes must exist given the

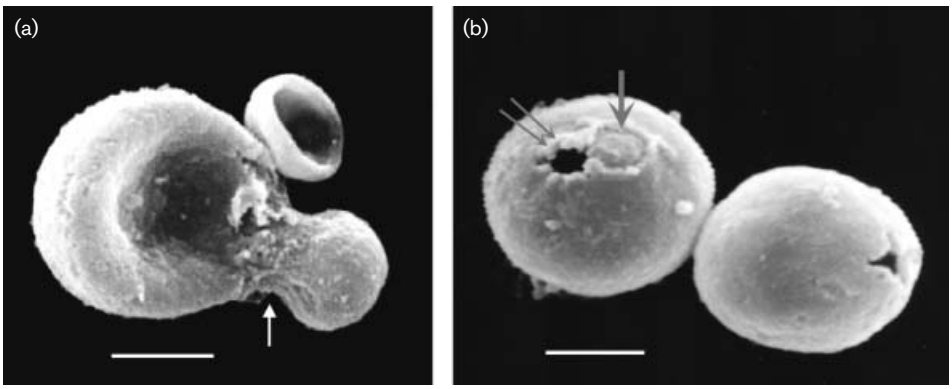

Fig. 2. SEM images of particles isolated from melanized *C. neoformans* 24067 grown with (a) or without (b) L-dopa. The white arrow (a) indicates the melanized connection between a mother and daughter cell. The grey single arrow in (b) indicates a melanized bud scar, whereas the double grey arrow in (b) shows where either a bud scar failed to melanize or where a non-melanized bud was attached prior to treatment with enzymes and chemicals. The second melanin 'ghost' has a similar defect. Bars, 3 μm.

abundance of this material in nature. Degradative processes have been identified, for the related polymer lignin and ligninases of *Phanerochaete chrysosporium* have been shown to destroy fungal melanins (Butler & Day, 1998). Interestingly, the white rot fungus, *Pycnoporus cinnabarinus*, produces a metabolite, 3-hydroxyanthranilate, that mediates the oxidation of non-phenolic substrates by laccase that results in the depolymerization of lignin (Eggert *et al.*, 1996b) and laccase is essential for the lignolytic ability of the fungus (Eggert *et al.*, 1996a, 1997). Decolourization of melanin by the fungi *Bjerkandera adusta*, *Galactomyces geotrichum*, *Trametes hirsuta* and *Trametes versicolor* is a phenomenon consistent with the destruction of the pigment, which provides further evidence for the presence of melanin-degrading enzymes (Ratto *et al.*, 2001). Additionally, *Aspergillus fumigatus*, an important human-pathogenic fungus that synthesizes melanin (Jahn *et al.*, 1997; Tsai *et al.*, 2001), can utilize environmental melanins as a sole carbon source for growth, indicating that the fungus is capable of degrading melanin (Luther & Lipke, 1980). Hence, there is considerable evidence that melanin-degrading enzymic mechanisms exist.

Our results show that replication of pigmented *C. neoformans* cells is accomplished by the formation of a 'hole' in the melanin layer at the site of budding. The occurrence of a localized defect in conjunction with cell replication is consistent with an enzymic process linked to the cell cycle that results in the digestion of a section of the melanin layer. These findings establish that although *C. neoformans* can apparently rearrange melanin, buds do not take significant amounts of melanin from parental cells. After budding, the parental cell must form new melanin at the bud scar. The lack of phenolic substrate does not affect the growth of either melanized or non-melanized yeast cells.

The results obtained can be interpreted to synthesize a coherent model for the replication of melanized cells in the presence or absence of substrate for melanin (Fig. 4). Bud formation in melanized cells must initially be accompanied by a breakdown of cell-wall-associated melanin at the site

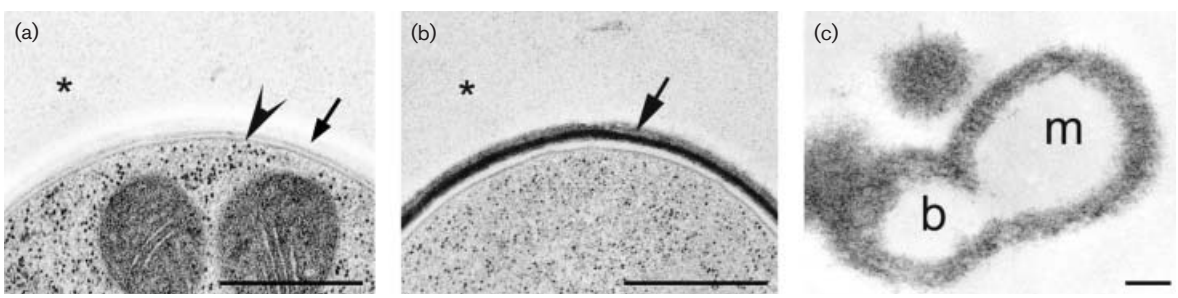

Fig. 3. TEM of non-melanized (a) and melanized (b) *C. neoformans* yeast cells and a melanin 'ghost' of a replicating yeast cell (c). The cytoplasmic membrane is indicated by the arrowhead, the cell wall of non-melanized yeast by the arrow, the cell-wall melanin of a pigmented yeast by the arrow and the fine fibrillar polysaccharide capsule by '*'. The melanin 'ghost' (c) depicts the open connection between what formerly was the mother cell (m) and the bud (b). Bars, 1 μm.

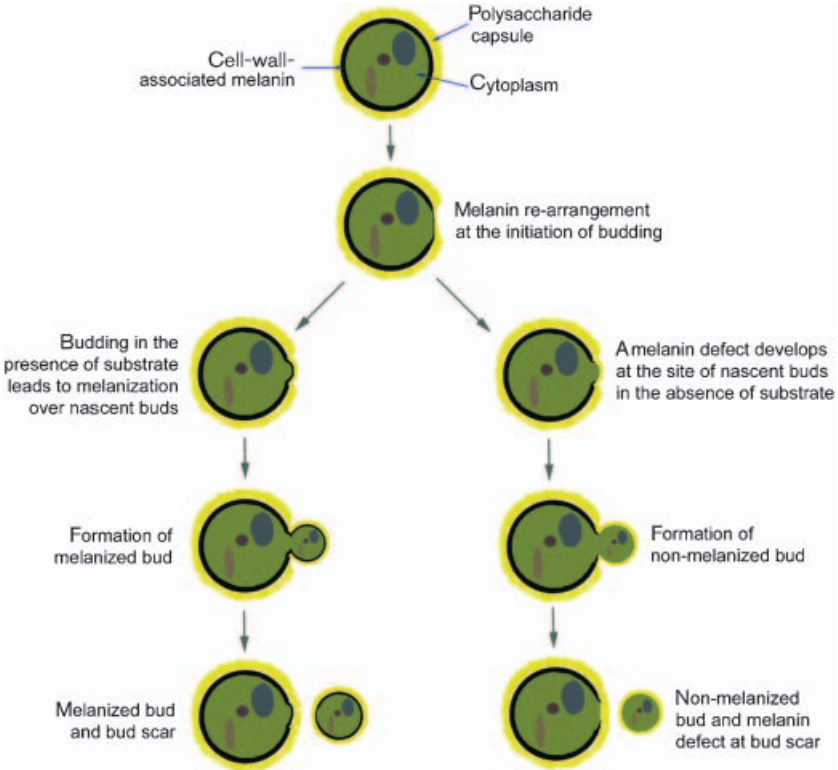

Fig. 4. Schematic representation of the proposed budding process of *C. neoformans* in the presence and absence of substrate for melanization.

of a newly forming bud. In the mother cell, we postulate that enzymic processes digest the melanin and then reform the polymer at the bud scar upon completion of budding if substrate for melanin synthesis is available. Concurrently, the bud synthesizes melanin *de novo* and the daughter cell is melanized. When substrate is not available, a defect occurs in the mother cell's melanin layer and the daughter cell is not melanized.

Melanin and capsule synthesis in *C. neoformans* are of particular interest because they are critical to pathogenicity and represent potential targets for drug discovery (McFadden & Casadevall, 2001). The capsular polysaccharide of *C. neoformans* is thinner over budding cells (Cassone *et al.*, 1974) and qualitatively different from the parental polysaccharide (Feldmesser *et al.*, 2000). In fact, the capsule of buds results from new synthesis rather than from parasitism of capsule present on the parent cell (Pierini & Doering, 2001). The findings of *de novo* synthesis of melanin and capsule by buds of *C. neoformans* is consistent with prior data demonstrating localized synthesis of other cell wall constituents in buds of *Candida albicans* (Osumi, 1998) and *S. cerevisiae* (Cid *et al.*, 2002; Drees *et al.*, 2001; Sekiya-Kawasaki *et al.*, 2002).

The presence of melanin can significantly protect *C. neoformans* from the antifungal compounds amphotericin B and caspofungin by binding the antifungals, which reduces their availability and efficacy (Van Duin *et al.*, 2002). Disruption of melanin in the cell wall during budding may provide an avenue for drug entry into *C. neoformans*. Glyphosate, an inhibitor of the shikimate acid pathway, inhibits melanization of *C. neoformans in vitro* and prolongs the survival of mice with cryptococcosis (Nosanchuk *et al.*, 2001). This demonstrates that inhibition of melanin production in wild-type *C. neoformans* has important biological significance. Similarly, mAbs to melanin and melanin-binding peptides inhibit the growth of melanized cells and melanin-binding mAbs prolong survival in mice infected with *C. neoformans* (Rosas *et al.*, 2001). The mechanism for inhibition of cell growth by melanin-binding peptides and mAbs is unknown, but may be due to alterations in the properties of the melanin in the cell wall or by blocking of pores in melanin that permit the entry of essential nutrients for *C. neoformans*. The effects of these compounds on budding were not directly assessed; however, budding represents a period of heightened cellular activity during which reagents targeting melanin production or other synthetic processes may be most affected.

Our observations imply a very complex regulation of melanin metabolism and linkage of melanin degradation and synthesis to the cell cycle. Melanin is one of several virulence factors linked to cAMP-associated signalling pathways (Alspaugh *et al.*, 1997, 1998). Disruption of a gene encoding a cAMP-dependent protein kinase results in mutants that are avirulent, as they do not produce melanin or a polysaccharide capsule (D'Souza *et al.*, 2001). Melanin synthesis and pathogenicity of *C. neoformans* are significantly impaired by the down-regulation of the gene responsible for an inositol-phosphoryl ceramide synthase (Luberto *et al.*, 2001). Avirulent mutant strains that have reduced melanin synthesis, capsule production, urease expression

and growth at 37 °C have been generated by disruption of a gene encoding a vesicular proton pump (Erickson *et al.*, 2001). These findings show that the regulation of melanin synthesis is associated with several important virulence determinants and suggest that targeting enzymes involved in melanin production and rearrangement may have a broad impact on *C. neoformans* virulence.

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