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Research Article

Deletion of *PDE2*, the gene encoding the high-affinity cAMP phosphodiesterase, results in changes of the cell wall and membrane in *Candida albicans*

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Abstract

A role for the cAMP-dependent pathway in regulation of the cell wall in the model yeast *Saccharomyces cerevisiae* has recently been demonstrated. In this study we report the results of a phenotypic analysis of a *Candida albicans* mutant, characterized by a constitutive activation of the cAMP pathway due to deletion of *PDE2*, the gene encoding the high cAMP-affinity phosphodiesterase. Unlike wild-type strains, this mutant has an increased sensitivity to cell wall and membrane perturbing agents such as SDS and CFW, and antifungals such as amphotericin B and flucytosine. Moreover, the mutant is characterized by an altered sensitivity and a significantly reduced tolerance to fluconazole. The mutant's membrane has around 30% higher ergosterol content and the cell wall glucan was 22% lower than in the wild-type. These cell wall and membrane changes are manifested by a considerable reduction in the thickness of the cell wall, which in the mutant is on average 60–65 nm, compared to 80–85 nm in the wild-type strains as revealed by electron microscopy. These results suggest that constitutive activation of the cAMP pathway affects cell wall and membrane structure, and biosynthesis, not only in the model yeast *S. cerevisiae* but also in the human fungal pathogen *C. albicans*. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: *Candida albicans*; cAMP-dependent pathway; *PDE2*; cell wall; antifungal susceptibility

Introduction

Candida albicans is the major human fungal pathogen and the aetiological agent of candidiasis (Odds, 1987). It exists as a commensal in healthy individuals but can become a serious medical problem, causing systemic candidiasis in immunocompromised patients. Fungicidal polyene antibiotics such as amphotericin B and nystatin and fungistatic azole drugs (imidazoles and triazoles) are currently used in the treatment of systemic candidiasis. The polyene antibiotics are known to bind preferentially to the primary fungal cell membrane sterol, ergosterol, disrupting the osmotic integrity of the membrane, and thereby compromising its barrier function. Use of amphotericin B has been associated with a number of side-effects: most notably nephrotoxicity, which can be reduced by using lipid formulations. The fungistatic effect of the azoles is achieved by inhibiting ergosterol biosynthesis, the major target being the product of *ERG11* gene, the P-450 enzyme 14α-demethylase. Depletion of ergosterol disrupts the fungal membrane structure, affects the activity of enzymes associated with nutrient transport and chitin synthesis and inhibits cell growth (Georgopapadakou, 1998).

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Because of its low toxicity, fluconazole is the preferred antifungal for the treatment of *Candida*; however, its prolonged use has increased the incidences of resistance amongst *Candida* and non-*Candida* species (Vanden Bossche *et al*., 1998).

Phosphodiesterase inhibitors are of increasing medical significance, as they form the basis of drugs useful in treating heart failure (Feldman and McNamara, 2002), asthma, depression, thrombosis and impotency. The last few years have seen major developments in characterizing the different types of mammalian phosphodiesterases capable of degrading cAMP (Houslay and Milligan, 1997). Around 30 forms of enzymes having cAMP phosphodiesterase activity could potentially be produced in mammalian cells, mostly as a result of alternative splicing. In the lower eukaryote *Saccharomyces cerevisiae*, however, there are only two phosphodiesterases — with low and high affinity for cAMP, encoded by *PDE1* (Sass *et al*., 1986) and *PDE2* (Nikawa *et al*., 1987), respectively. Under most conditions *pde1*Δ and *pde2*Δ mutants have wild-type phenotype; however, they are more sensitive to heat shock and nutrient starvation. Intracellular levels of cAMP dramatically increase upon addition of exogenous cAMP to *pde2* mutants, suggesting that Pde2p is responsible for breaking down exogenous cAMP (Wilson *et al*., 1993). Significant changes in the transcriptome have recently been described for the *S. cerevisiae pde2*Δ mutant (Jones *et al*., 2003). These changes which are representative of a constitutive activation of the cAMP pathway manifest themselves in a range of cell wall-related phenotypes, supporting the role for *PDE2* (and/or cAMP) in the maintenance of cell wall integrity in *S. cerevisiae*, as previously suggested (Heale *et al*., 1994; Tomlin *et al*., 2000).

Two phosphodiesterase-encoding genes *PDE1* and *PDE2* have been found in the fungal pathogen *C. albicans* (Bahn *et al*., 2003; Hoyer *et al*., 1994; Jung and Stateva, 2003). Mutants lacking *PDE1* are not yet available; however, hetero- and homozygous *pde2* null mutants have been generated and characterized (Bahn *et al*., 2003; Jung and Stateva, 2003). Deletion of *PDE2* elevates intracellular cAMP levels, increases responsiveness to exogenous cAMP and sensitivity to heat shock, causes growth defects at 42 °C and reduces levels of transcription of *EFG1* (Jung and Stateva, 2003). In liquid media, *PDE2* deletion inhibits normal hyphal, but not pseudohyphal, growth under most standard inducing conditions. On solid hypha-inducing media *pde2* null mutants form aberrant hyphae, with fewer branches and almost no lateral buds, which are also deficient in hypha-to-yeast reversion. The phenotypic defects of *pde2* mutants show that the cAMP-dependent pathway plays specific roles in hyphal and pseudohyphal development. Moreover, they suggest that Pde2p mediates a desensitization mechanism by lowering basal cAMP levels in response to environmental stimuli in *C. albicans*. *Capde2* null mutants have also recently been reported to be less virulent in an animal model (Bahn *et al*., 2003).

The present study reports the results of the characterization of *C. albicans* mutants with a constitutively activated cAMP pathway as a result of deletion of *PDE2*. The mutants are characterized by an increased sensitivity to a range of agents, including commonly used antifungals such as amphotericin B and flucytosine and a reduced tolerance to fluconazole, and changes in the levels of ergosterol and glucan levels in their membranes and cell walls, respectively. Moreover their cell walls have a reduced thickness, as revealed by electron microscopy. These results show that constitutive activation of the cAMP pathway shown previously to affect the synthesis and assembly of the cell wall and membrane in the yeast *S. cerevisiae* has a similar effect in *C. albicans* as well.

Materials and methods

Strains and media

Candida albicans strains used in this study are listed in Table 1. The standard minimal (SD) and rich (YPD) media were prepared as described (Sherman *et al*., 1986). RPMI1640 medium (with L-glutamine but without bicarbonate) was pre-buffered to pH 7.0 with 0.165 M MOPS. Chromagar from Becton-Dickenson, Oxford, UK, was used.

Susceptibility tests

For the plate spot assay, strains were grown in YPD at 37 °C overnight in a shaking incubator. Cell densities were determined using a haemocytometer and serial dilutions (10^7–10^2) were spotted onto plates.

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Table 1. *Candida albicans* strains used in this study

Strains	Genotype	Reference
CAF2-1	*URA3/ura3Δ::imm434*	Fonzi and Irwin, 1993
CAI4	*ura3Δ::imm434/ura3Δ::imm434*	Fonzi and Irwin, 1993
WH2-3U	*ura3Δ::imm434/ura3Δ::imm434 pde2Δ::hisG/pde2Δ::hisG-URA3-hisG*	Jung and Stateva, 2003
WH2-RU	*ura3Δ::imm434/ura3Δ::imm434 pde2Δ::hisG/pde2Δ::hisG::PDE2-URA3*	Jung and Stateva, 2003

Plates were incubated for up to 48 h at 37 °C. The microdilution method, according to the recommendations of the National Committee for Clinical Laboratory Standards (Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard NCCLS Document M27-A2), and the EUCAST method (European Committee for Antimicrobial susceptibility testing, Document 7.1, June 2002) were used for determination of minimal inhibitory concentration (MIC) of antifungal drugs. Briefly, strains were grown in YPD at 30 °C overnight. Antifungal drugs were diluted by serial two-fold dilutions in a total volume of 100 µl. Diluted cell suspensions of each strain, containing 2×10^5 or 2×10^4 cells (for EUCAST and NCCLS methods, respectively) were then added to the wells. The plates were incubated at 37 °C. The optical density (OD) of each well was read with a microtitre plate reader at 595 nm. MIC_{50}, the drug concentration needed to decrease the OD of the drug-free culture by 50% after 24 h incubation (EUCAST); MIC_{80}, the drug concentration needed to decrease the OD of the drug-free culture by 80%, (after 48 h in the case of amphotericin B and 24 h for fluconazole) and MIC_{100}, the drug concentration needed to decrease the OD of the drug-free culture by 100% (after 48 h in the case of flucytosine) were used in the antifungal assays.

Tolerance assay

Fluconazole tolerance was determined essentially as described by Sanglard *et al.*, (2003). Following the microtitre assay for MIC determination, 50 µl were withdrawn from each well, and diluted by stepwise 10-fold dilutions. Viable cell counts for each well were determined by counting colony-forming units (CFU) on solid YPD medium.

Ergosterol assay

Ergosterol content was determined as described (Arthington-Skaggs *et al.*, 1999).

Essentially, *C. albicans* strains were incubated in 50 ml YPD medium at 37 °C for 16 h, the cells were harvested and the pellets washed once with distilled water. Ergosterol was extracted by the addition of 3 ml 25% alcoholic potassium hydroxide to each pellet and incubating at 85 °C for 1 h. After cooling to room temperature, a mixture of 1 ml distilled water and 3 ml *n*-heptane was added to each sample and mixed vigorously. The heptane layer was transferred to a clean glass tube and diluted five-fold with 100% ethanol. Its ergosterol content was analysed by measuring the absorbance at 240–300 nm with a scanning spectrophotometer.

Cell wall composition

The levels of alkali-insoluble and soluble glucans were measured as previously described (Popolo *et al.*, 1997). Cells grown in YPD at 30 °C were collected at mid-exponential phase and divided into five equal aliquots containing $OD_{600} = 25$. Two of these aliquots were used for determination of dry weight, the remaining three for glucan analysis. Chitin levels were measured as described by Bulawa (1992). Cells were pelleted ($OD_{600} = 200$), resuspended in 8 ml dH_2O and divided into four equal aliquots, two were used for the determination of the cell dry weight and the others processed as described (Carotti *et al.*, 2002). The conditions for serum hyphal induction prior to cell wall analysis of hyphae, were as described previously (Jung and Stateva, 2003).

Electron microscopy

Strains were grown overnight in YPD at 30 °C. Approximately $5.0–8.0 \times 10^6$ cells/ml were freshly

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diluted in the same medium and grown at 30 °C. Cultures were harvested at $OD_{600} = 1.2–1.6$ after 6 h incubation. Cells were washed twice with sterile distilled water and fixed in a 0.1 M sodium cacodylate buffer (pH 6.9) containing 3.2% (v/v) paraformaldehyde, 3.0% (v/v) glutaraldehyde, 4% (w/v) sucrose, 0.02% (w/v) calcium chloride overnight at 4 °C and processed as previously published (Lloyd *et al.*, 2002).

Results

pde2 mutants are more sensitive to cell wall- and membrane-perturbing agents

C. albicans pde2 mutants have been reported to be highly sensitive to heat shock at 50 °C and impaired for growth at 42 °C (Jung and Stateva, 2003). In the current investigation, their sensitivity to SDS and Calcofluor white (CFW), was tested. CFW binds to chitin, interfering with its ability to polymerize. Cell wall mutants defective in cell wall assembly, or in signal transduction pathways related to the cell wall, become more sensitive to CFW. CFW sensitivity test is therefore used for identifying mutants defective in cell wall assembly or in signal transduction pathways. SDS is a detergent with an indirect effect on the cell wall by reducing membrane stability, consequently any cell wall defects can be revealed if there is an increased accessibility to the plasma membrane. The *pde2* deletion mutant was more sensitive to CFW than the wild-type strains (Figure 1A). Lower concentrations of SDS were required for the *pde2* mutant to display a lytic phenotype, thus providing further evidence for defects related to its cell wall, unlike the reference wild-type CAF2-1 and the isogenic reconstituted wild-type strain WH2-RU (see Table 1

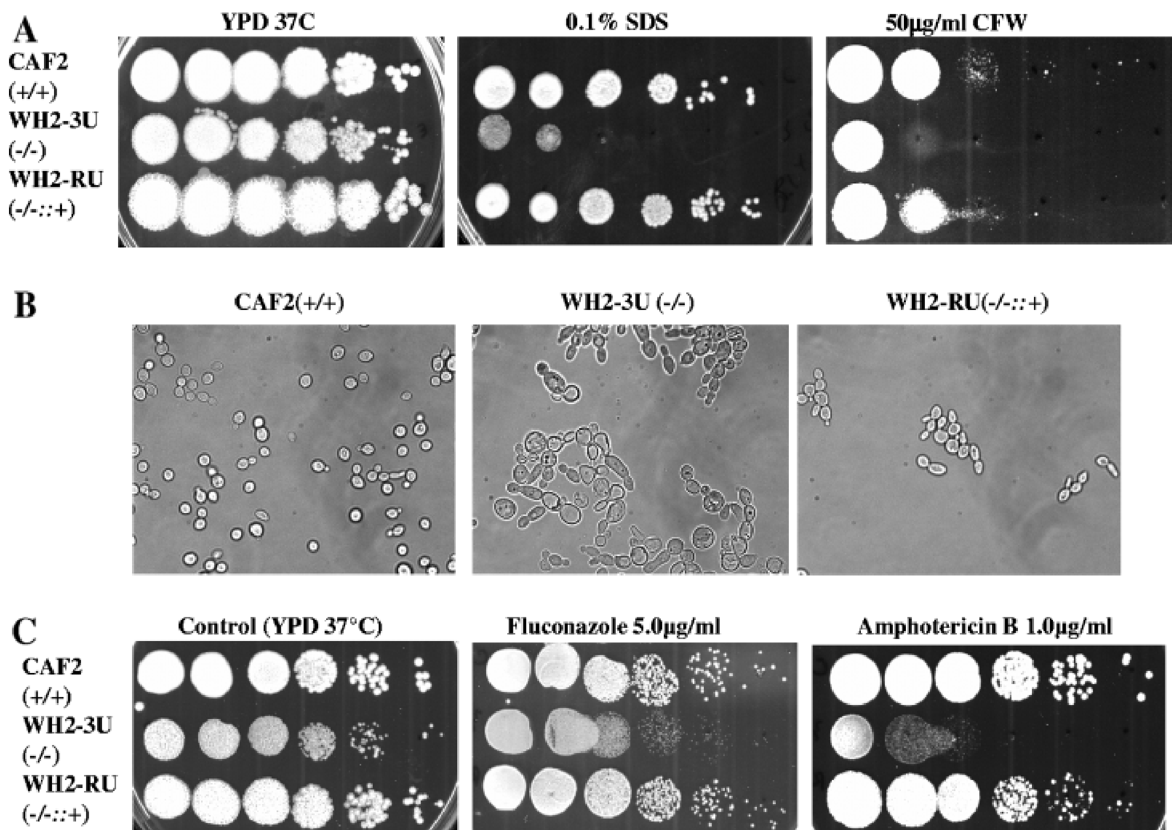

Figure 1. Susceptibility to cell wall and membrane-disrupting agents. (A) Cells were spotted at different cell densities ($10^7 – 10^2$) onto YPD medium without or with indicated chemicals. All strains were grown at 37 °C for 2 days. (B) Cells from SDS containing YPD medium were observed microscopically for changes in cell morphology. (C) Growth after 2 days on YPD medium without or with amphotericin B or fluconazole at 37 °C

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for respective strain genotypes). Furthermore, on SDS-containing YPD plates, only the *pde2* mutant formed smooth colonies (data not shown), which upon closer microscopic observation were found to contain abnormally large, elongated and highly swollen cells (Figure 1B), suggesting changes to the cell wall and membrane composition and/or structure.

pde2 mutants are characterized by an increased susceptibility to common antifungals

In a further attempt to characterize the *C. albicans pde2* mutants, susceptibility to several antifungals was tested. Under the conditions of the plate spot assay, the *pde2* mutant was more susceptible to amphotericin B compared to control strains (Figure 1C). This phenotype was further reinforced by a severe growth defect of the mutant in liquid medium at 37 °C, in the presence of 0.1 and 0.25 μg/ml amphotericin B (data not shown), and by the MIC_{80} values shown in Table 2. It is of note that the change in susceptibility of the *pde2* mutant was less in liquid medium than that demonstrated by the spot assay, but was consistent and reproducible upon several independent experiments. A similar pattern was observed in RPMI medium; however, in this case MIC_{80} for amphotericin B was considerably lower for all strains: 0.125 μg/ml for CAF2-1 (WT) and WH2-RU (*pde2−/−:PDE2*), and 0.06 μg/ml for the *pde2* mutant, respectively. The susceptibility to fluconazole was less consistent and varied slightly according to the methods applied. The mutant displayed increased susceptibility to fluconazole in the spot assay (Figure 1C) and by MIC_{80} (Table 2); however, MIC_{50} was almost identical for all tested strains in YPD, whereas in RPMI, the mutant displayed reduced susceptibility: $MIC_{50} = 0.5$ μg/ml for the *pde2* mutant compared to 0.25 μg/ml for the wild-type strains WH2-3U and WH2-RU (*pde2−/−:PDE2*). At fluconazole concentrations higher than MIC_{50}, however, the mutant's growth was greatly reduced in comparison to that of the control strains CAF2-1 (WT) and WH2-RU (*pde2−/−:PDE2*), a phenotype reminiscent of that of *C. albicans* mutant strains lacking the calcineurin A subunit, which displayed reduced tolerance to fluconazole (Sanglard *et al*., 2003). When the tolerance assay developed by Sanglard *et al*. (2003) was applied in the current investigation, the tolerance of the *pde2* mutant to fluconazole was found to be significantly reduced compared to the control strains (Figure 2). An attempt was made to determine susceptibility of the strains

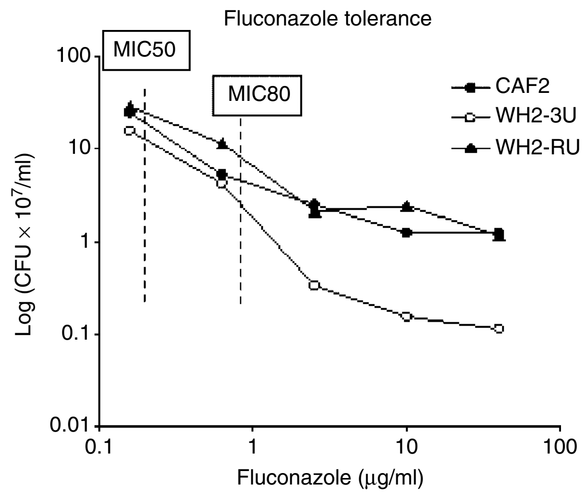

Figure 2. Fluconazole tolerance. Following the microtitre assay (MIC determination on YPD medium after 24 h at 37 °C), 50 μl were withdrawn from the cell suspensions in every well, diluted by stepwise 10-fold dilutions, and plated on YPD medium. The viable cell counts were determined by colony forming units (CFU) after 2 days incubation at 30 °C

Table 2. Determination of strains' susceptibility to the antifungal drugs amphotericin B and fluconazole as measured by respective MICs*

Strain (relevant genotype)	Amphotericin B MIC_{80} (μ g/ml)	Fluconazole MIC_{50} (μ g/ml)	Fluconazole MIC_{80} (μ g/ml)
CAF2-1 (+/+)	2.5 ± 0.00	0.25 ± 0.09	0.99 ± 0.34
WH2-3U (−/−)	1.2 ± 0.00	0.28 ± 0.07	0.55 ± 0.14
WH2-RU (−/−::+)	2.5 ± 0.00	0.32 ± 0.00	0.81 ± 0.42

* MIC_{80} and MIC_{50} were determined in YPD medium after 48 h and 24 h incubation at 37 °C for amphotericin B and fluconazole, respectively. Values are the average from five replicates (±SD).

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in the presence of exogenous cAMP (10 mM) in YPD medium; however, this almost totally inhibited the growth of the homozygous *pde2* mutant at 37 °C and made the test impossible. The MIC_{100} of flucytosine against CAF2-1 (WT) and WH2-RU (*pde2*−/−:*PDE2*) was identical (0.125 μg/ml) but WH2-3U (*pde2*−/−) was highly susceptible, with $MIC_{100} = 0.03$ μg/ml. Taken together, these results confirmed that deletion of *PDE2,* and higher cAMP levels, increase the susceptibility of *C. albicans* to cell wall and membrane-perturbing agents, including antifungals such as amphotericin B and flucytosine, and reduce tolerance to fluconazole at concentrations higher than the MIC_{50}.

Colony morphology and colour development on Chromagar demonstrated the typical *C. albicans* phenotype of green, rapidly growing, shiny colonies of the CAF2-1 strain. Both WH2-3U (*pde2*−/−) and WH2-RU (*pde2*−/−:*PDE2*) demonstrated turquoise colour development (indicating an alteration of the metabolism of the chromogenic substrates). WH2-3U (*pde2*−/−) also had poor growth on Chromagar.

pde2 mutants have altered cell wall and membrane composition

The phenotypes described above suggested that *pde2* mutants might have an altered membrane and cell wall composition. To test this, the levels of ergosterol, the major target for amphotericin B, and glucan and chitin, the main yeast cell wall components, were determined. The ergosterol content (Table 3) of the *pde2* mutant was increased by 25% and 54% in comparison to control strains WH2-RU (*pde2*−/−:*PDE2*) and CAF2-1 (reference WT), respectively, a result that could explain its increased sensitivity for amphotericin B. The levels of alkali-insoluble β-glucans were reduced in the *pde2* mutant and amounted to around 68% of those in the wild-type strains, whereas the alkali-soluble β-glucans were not as affected (Table 4). In total, the combined glucan levels of the mutant equalled 78% of those of the wild-type strains. No significant differences in the chitin levels of the tested strains were observed. The changes of the cell wall and membrane in the *pde2* mutant were further confirmed by TEM observations (Figure 3A), revealing a thinner cell wall (60–65 nm) compared to that of the control strains (80–85 nm). Moreover, an electron-dense inner layer that was largely visible in the rest of the strains was missing in the homozygous *pde2* null mutant (Figure 3B); however, at present we cannot be certain that this layer originates in the cell wall rather than the cytosol.

Taken together, these results indicated that the cell wall and membrane were significantly affected

Table 3. Determination of the sterol composition of the *pde2* mutant in comparison to relevant wild-type strains

Strain (relevant genotype)	Ergosterol (% total cell dry weight ±SD)*
CAF2-1 (+/+)	4.70 ± 0.36
WH2-3U (−/−)	7.28 ± 0.84
WH2-RU (−/−::+)	5.80 ± 0.10

* Results are from three independent experiments with standard deviations (SD).

Table 4. Analys is of cell wall composition during growth of strains in the yeast form

	Amount (μg glucose/mg dry weight of cells)[1]				
	Alk_i[2]				
Strain (relevant genotype)	Soluble zymolyase 1,3- + 1,6-β-glucan	Insoluble zymolyase 1,3- + 1,6-β-glucan	Alk_s[3] 1,3- + 1,6-β-glucan	Total (Alk_s + Alk_i) 1,3- + 1,6-β-glucan	Chitin (μg N-acetylglucosamine/mg dry weight of cells)
CAF2-1 (+/+)	188.0 ± 8.9	7.0 ± 1.2	119.8 ± 6.5	314.8 ± 14.2	11.0 ± 0.9
WH2-3U (−/−)	125.5 ± 5.6	8.0 ± 1.1	114.8 ± 8.2	248.3 ± 11.9	10.1 ± 0.8
WH2-RU (−/−::+)	182.3 ± 4.8	8.7 ± 1.1	120.6 ± 7.3	311.6 ± 9.9	11.3 ± 1.0

[1] Results are average (±SD) from two independent experiments at 30 °C in YPD.
[2] Alk_I, alkali-insoluble fraction.
[3] Alk_s, alkali-soluble fraction.

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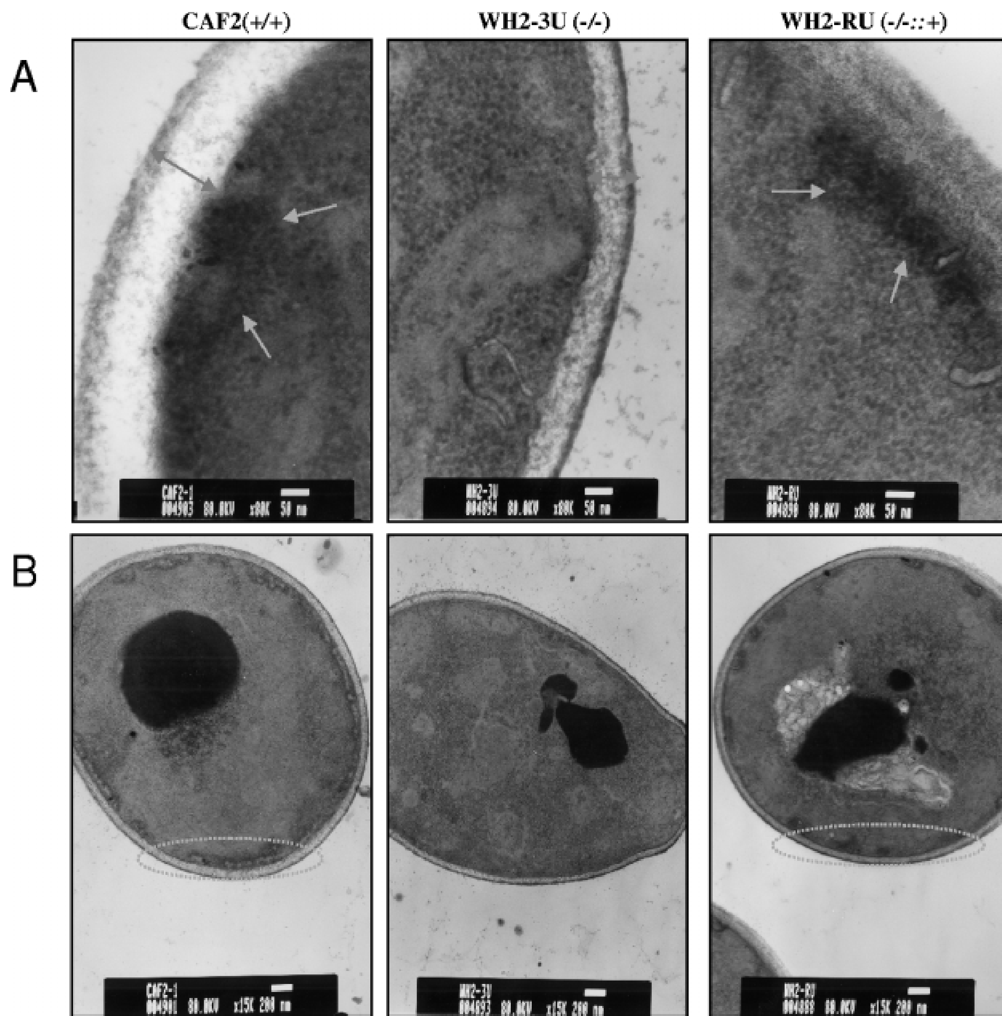

Figure 3. Transmission electron microscopy observation of strains. (A) TEM pictures at magnification of ×80 000 (bar length 50 nm). Red and blue arrows indicate the thickness and the inner stained layer, respectively [cell wall thickness, WH2-3U (−/−), 60–65 nm; CAF2-1 (+/+) and WH2-RU (−/−::+), 80–85 nm]. (B) TEM pictures at magnification of ×15 000 (bar length 200 nm). Blue dotted areas indicate inner stained layer of each cell, which is largely absent in the *pde2* mutant cell

by deletion of *PDE2*, and the resultant constitutive activation of the cAMP pathway. Remarkably these cell wall changes persisted during hyphal growth and development, which was followed over a 5 h time-course after serum addition. As shown in Table 5, glucan levels in the mutant were 76% at time point 0, 73% and 77% at time point 3 h, and 76% and 81% at time point 5 h, in comparison to those of the wild-type reference (CAF2-1) and isogenic reconstituted (WH-RU) strains, respectively. Chitin levels increased during hypha formation in the control strains; 22% in CAF2-1 and 17% in WH-RU. No increase, however, was observed in the mutant, which has previously been shown to be unable to form normal hyphae in the presence of serum, and indeed, other standard *in vitro* hyphal inducers (Jung and Stateva, 2003).

Discussion

The present study reports that deletion of *PDE2*, and subsequent constitutive activation of the cAMP pathway, causes defects of the cell wall and

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Table 5. Analysis of cell wall composition during hyphal growth and development

Strain (relevant genotype)	Amount (µg glucose/mg dry weight of cells)*				Chitin (µg N-acetylglucosamine/mg dry weight of cells)*
	Alk_I				
	Soluble zymolyase 1,3- + 1,6-β-glucan	Insoluble zymolyase 1,3- + 1,6-β-glucan	Alk_s 1,3- + 1,6-β-glucan	Total (Alk_s+ Alk_i) 1,3- + 1,6-β-glucan	
CAF2-1 (+/+)					
0 h	200.2 ± 2.9	9.4 ± 0.8	129.6 ± 1.5	339.1 ± 8.1	12.7 ± 0.3
3 h	208.9 ± 4.5	11.1 ± 0.5	125.1 ± 6.4	345.1 ± 8.1	15.6 ± 0.5
5 h	206.1 ± 4.2	12.1 ± 0.6	126.1 ± 3.3	344.3 ± 6.7	15.6 ± 0.4
WH2-3U (−/−)					
0 h	123.4 ± 4.5	9.2 ± 0.4	126.8 ± 3.6	259.4 ± 7.3	13.3 ± 0.2
3 h	128.6 ± 3.3	9.7 ± 0.4	113.9 ± 3.2	252.2 ± 6.5	12.7 ± 0.4
5 h	140.6 ± 5.1	9.5 ± 0.5	112.6 ± 4.7	262.7 ± 4.3	13.6 ± 0.3
WH2-RU(−/−::+)					
0 h	196.0 ± 2.6	9.8 ± 0.5	131.5 ± 2.2	337.3 ± 4.8	14.1 ± 0.4
3 h	201.1 ± 4.4	10.8 ± 0.7	112.9 ± 2.9	325.3 ± 6.2	16.1 ± 0.3
5 h	194.0 ± 5.3	11.2 ± 0.5	115.2 ± 2.6	320.5 ± 3.9	16.5 ± 0.6

* Results are average (±SD) of two independent experiments in YPD + 10% serum.

membrane in *C. albicans*. These defects manifest themselves in the form of increased sensitivity to SDS, Calcofluor white, some antifungals, reduced thickness of the cell wall and alterations of the ergosterol and glucan composition. Changes in expression of cell wall-related genes and associated cell wall defects have previously been demonstrated in *S. cerevisiae pde2*Δ mutants (Jones *et al.*, 2003). Our results suggest that a similar effect might also exist in *C. albicans,* since recent reports have demonstrated, first, that *EFG1* is significantly downregulated in the *C.albicans pde2* mutant (Jung and Stateva, 2003) and second, that *EFG1* regulates transcription of several cell wall protein-encoding genes such as *HWP1, HWP2, YWP1* and *RBE1*, which are significantly downregulated in an *efg1* mutant (Sohn *et al*., 2003).

The structural differences of the cell wall in the homozygous *pde2* mutant were demonstrated using biochemical analysis and transmission electron microscopy. The cell wall of the *pde2* null mutant cell appeared thinner than that of the control strains (Figure 3), most likely as a result of significantly reduced levels of alkali-insoluble β-glucans (Table 4). These results suggest a possible relationship between β-glucan synthesis and the cAMP pathway. These observations correlate well with findings of reduced β-glucan levels in the *S. cerevisiae pde2* null mutant and differential expression of genes such as *FKS1, GSC2, RHO1* and *ROM2*, responsible for β-glucan synthesis (Jones *et al*., 2003). However, unlike *S. cerevisiae pde2* null mutant, which showed slightly increased chitin levels (Jones *et al*., 2003), there was no significant difference in chitin levels between the *C. albicans pde2* null mutant and the control strains during growth in the yeast form (Table 4, last column). This suggests that the slightly higher sensitivity to Calcofluor white (Figure 1A) in the mutant could be due to other reasons, one being the Hog1 MAP-kinase pathway, which in *S. cerevisiae* has been demonstrated to be another target for Calcofluor white or, alternatively, to an increased permeability to the drug (Garcia-Rodriguez *et al*., 2000). Remarkably, the changes in the cell wall composition were demonstrated during hyphal growth as well. No increase in chitin level, which normally occurs in hyphal cell walls (Munro *et al*., 1998; Sullivan *et al*., 1983), was observed (Table 5). This is consistent with earlier observation of the inability of the *pde2* null mutant to form normal hyphae in liquid media in response to serum and, indeed, to other hyphal inducers (Jung and Stateva, 2003).

Deletion of *PDE2* increased the susceptibility to amphotericin B (Figure 1, Table 2). The primary

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mode of action of amphotericin B is well established. It binds to membrane sterols such as ergosterol, disrupting the osmotic integrity of cell membrane and its bound proteins, causing leakage of intracellular metabolites and ions (Bahmed *et al.*, 2003; Ghannoum and Rice, 1999). Our results suggest that the most likely reason for the increased *pde2* mutant's sensitivity to amphotericin B is the higher ergosterol content (Table 3), since reduction of ergosterol causes resistance to amphotericin B (Dick *et al.*, 1980). It is therefore reasonable to speculate that the ergosterol synthesis pathway is upregulated by activation of the cAMP pathway. Interestingly, in *S. cerevisiae*, deletion of *PDE2* increased transcription levels of *ERG3* and *ERG10*, which encode $\Delta^{5,6}$-sterol desaturase and acetyl-CoA C-acetylase, respectively (Jones *et al.*, 2004). So far, only *ERG3* homologue has been found in *C. albicans*, but its functions are still unknown (Geber *et al.*, 1995; Howell *et al.*, 1990).

Previously hyper-activated cAMP pathway has been shown to increase resistance to fluconazole in *S. cerevisiae* at 30 °C (Kontoyiannis and Rupp, 2000). Moreover, downregulation of the same pathway has recently been shown to modulate susceptibility to antifungal azoles and other sterol biosynthesis inhibitors (Pooja *et al.*, 1997). In the current study, the extent and level of sensitivity of the *pde2* mutant to fluconazole depended on the method of investigation and the medium used. The mutant showed slight resistance to fluconazole in RPMI medium, and no change in YPD, by the MIC_{50} assay; however, it displayed a higher susceptibility by the spot assay (Figure 1C), and also by MIC_{80} (Table 2). The fluconazole sensitive phenotype (Figure 1C, Table 2) correlates with the reduced tolerance of the mutant (Figure 2).

The cell wall defects of the homozygous *pde2* mutant and its increased susceptibility to cell wall- and membrane-disrupting agents and antifungals suggest the possibility to develop an antifungal therapy that combines antifungals with phosphodiesterase inhibitors. However, there is one additional phosphodiesterase in *Candida*, the low-affinity cAMP phosphodiesterase, encoded by *PDE1* (Hoyer *et al.*, 1994). At present there are no mutants available and the phenotypic consequences of its deletion are unknown. Further investigations are required to elucidate fully the effects of the two phosphodiesterases in *C. albicans* and identify specific inhibitors of their activity.

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