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RESEARCH ARTICLE

The *Aspergillus nidulans pkcA* gene is involved in polarized growth, morphogenesis and maintenance of cell wall integrity

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Abstract The protein kinase C (PKC) family participates in maintaining integrity and growth of fungal cell walls. However, the precise molecular role of these proteins in the filamentous fungi remains unknown. In this work, *pkcA*, the gene encoding the PKC homolog in the filamentous fungus *Aspergillus nidulans*, was cloned and its function analyzed using a conditional *alcA-PKC* mutant strain. Repression of *pkcA* expression resulted in increased conidial swelling, decreased rates of hyphal growth, changes in the ultrastructure of the cell wall and increased sensitivity to antifungal agents. These results suggest that the protein encoded by *pkcA* is involved in key aspects of cell morphogenesis and cell wall integrity.

Keywords *Aspergillus nidulans* · *pkcA* · Fungal cell wall

Introduction

Enzymes of the protein kinase C (PKC) superfamily are serine/threonine kinases found exclusively in eukaryotic cells and are of central importance in signal-transduction processes (Mellor and Parker 1998). PKC homologs have been identified in both yeasts and filamentous fungi (Schmitz and Heinisch 2003). The prototypical fungal PKC contains an N-terminal regulatory region and a C-terminal kinase domain. The N-terminal region contains two cysteine-rich domains, C1 and C2. C1 is presumed to bind cellular membranes and the small GTPase Rho1p, but unlike mammalian homologs, does not bind diacylglycerol (Nonaka et al. 1995; Schmitz et al. 2001). The C2 domain mediates calcium-dependent activation in mammalian PKCs, but calcium does not seem to activate PKC in any fungus tested to date (Antonsson et al. 1994; Arpaia et al. 1999). The N-terminal region also contains a pseudosubstrate autoinhibitory site, a V5 region whose function in fungi has not been determined, and two ~55-amino-acid repeats HR1A and HR1B that present additional sites of interaction with Rho1p (Watanabe et al. 1994; Schmitz et al. 2001).

In yeast PKCs are pleiotropic, serving to integrate signals from diverse cellular processes. One of their main functions is to maintain cell wall integrity (Schmitz and Heinisch 2003). In *Saccharomyces cerevisiae* PKC signaling is activated during polarized growth such as budding and mating (Heinisch et al. 1999), and by environmental conditions that jeopardize cell wall stability, including high temperature (Kamada et al. 1995a), hypotonic shock (Davenport et al. 1995), and impaired cell wall synthesis (Ketela et al. 1999). *S. cerevisiae pkc1*null mutants display a cell-cycle arrest with small buds and lyse in the absence of osmotic stabilization (Levin et al. 1990). They are hypersensitive to cell wall destabilizing agents such as calcofluor white, congo red and SDS (Paravicini et al. 1992). The mechanisms responsible for this phenotype have been partially elucidated (Kamada et al. 1995b). *S. cerevisiae* Pkc1p phosphorylates and activates Bck1p, the first component in a MAPK signaling cascade resulting in the activation of the MAPK Slt2p. Slt2p phosphorylates and activates the transcription factor Rlm1p, which regulates the expression of genes whose products are involved in cell wall biosynthesis (Jung and Levin 1999).

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Deletion of the *Candida albicans* PKC homolog *CaPKC1* results in an osmotically remedial cell lysis defect of both the budding and hyphal growth forms and morphologically aberrant cells of the budding form. Despite these abnormalities, the transition between the two growth forms of *C. albicans* occurs normally, suggesting that *CaPKC1* is not involved in the dimorphic switch (Paravicini et al. 1996).

The exact function of PKCs in filamentous fungi has not yet been resolved. Attempts to delete the PKC gene in *Neurospora crassa* (Franchi et al. 2005), *Cochliobolus heterostrophus* (Oeser 1998) and *Aspergillus nidulans* (Herrmann et al. 2006) indicate that it is essential. In *N. crassa*, *NPKC* modulates light responses by regulating the blue light photoreceptor WC-1 (Franchi et al. 2005).

Herrmann et al. (2006) identified two putative PKC-encoding genes, *pkcA* (AN0106.3) and *pkcB* (AN5973.3) in the genome of *A. nidulans*. Comparative sequence analysis of the PKCB amino-acid sequence revealed that the similarity to other known PKCs is confined to the carboxy-terminal region of the protein, which harbors the catalytic region of all PKC proteins. However, only *pkcA* encodes a protein containing all the characteristic features of fungal PKCs, including an extended N-terminal regulatory domain containing C1, C2 and HR1 domains (Schmitz and Heinisch 2003; Herrmann et al. 2006). Knockdown of PKCA expression by antisense RNA leads to reduced growth, conidiation and penicillin production. However, using the antisense approach, expression of a given gene usually cannot be completely turned off but can only be reduced. Therefore, it is not possible to assume that the complete *pkcA* null phenotype was revealed.

In this work, we used an alternative strategy to generate a conditional *pkcA* mutant in *A. nidulans* in which the *alcA* promoter replaces the endogenous *pkcA* promoter to control gene expression. The *alcA* promoter activates gene expression in the presence of such carbon sources as ethanol, glycerol and threonine and represses it in the presence of glucose (Gwynne et al. 1987). This approach has been successfully used in the past to generate various conditional mutants in *A. nidulans*, including *myoA* (McGoldrick et al. 1995), *csmA* (Horiuchi et al. 1999), *hapB* (Steidl et al. 2001), *chsB* (Ichinomiya et al. 2002) and *hbrB* (Gatherar et al. 2004).

Our results indicate that like in yeast, *A. nidulans pkcA* is involved in multiple aspects of growth, cell-cycle control and cell wall integrity.

Materials and methods

Strains and culture conditions

Aspergillus nidulans strains R153 (*wA2;pyroA4*), GR5 (*wA2;pyroA4;pyrG89)* and *alcA-PKC* (*wA2;pyroA4; pyrG89::pyr4 alcA(p)::pkcAΔp*) were grown in defined minimal medium (MM) composed of 70 mM $NaNO_3$, 1% (w/v) glucose, 12 mM KPO_4 pH 6.8, 4 mM $MgSO_4$, 7 mM KCl, trace elements and 1.5% agarose (for solid plates). For minimal medium with glycerol (MMG) or threonine (MMT), glucose was replaced with 0.2% glycerol (w/v) or 100 mM threonine +0.1% fructose (w/v), respectively, as sole carbon source (Osherov et al. 1998; Ichinomiya et al. 2002). Conidia were harvested in 0.2% (w/v) Tween 80, resuspended in double-distilled water (DDW) and counted in a hemocytometer.

Nucleic acid preparation and manipulation

RNA was prepared from freshly harvested *A. nidulans* strains and conidia germinated in liquid MMG at 37°C for 24 h. Total RNA was prepared by the "hot SDS/phenol" method described previously (May and Morris 1988). RT-PCR was performed with PowerScript™ Reverse Transcriptase (BD Biosciences Clontech, NJ, USA) according to the manufacturer's instructions. PCR was performed with the ReddyMix PCR master mix (ABgene, Epsom, UK) using primers PKC1 and PKC2 to detect *pkcA* transcript, and *gpdA* 5′ and *gpdA* 3′ to detect the *A. nidulans gpdA* (glycerol-3-phosphate dehydrogenase) transcript used as a loading control (Table 1).

Aspergillus nidulans genomic DNA was prepared from freshly harvested flash-frozen mycelium using the hot SDS/phenol method as described previously (Jadoun et al. 2004). The *pyr4* probe was prepared by the random priming method, with [a-^{32}P] dCTP as described by Sambrook and

Table 1 Primers used in this study

PKC1	5′-ACTCCAGTGCAATCAGGTAAGACA-3′
PKC2	5′-GTTAAGTACTTGGTCCAAGATAGCG-3′
gpdA 5′	5′-CCACCGGTGTCTTCACTACC-3′
gpdA 3′	5′-CTTGACGGCATCCTTGATCT-3′
*Kpn*I-*PKC* 5′	5′-AT*GGTACC*ATGGACGGGGACGAGCTCATC-3′
*Kpn*I-*PKC* 3′	5′-AT*GGTACC*TTGCTGGGGAATATCTTCTTTCGC-3′
alcA-fwd	5′-TCAAGAGGTACGCGTATAGAGCC-3′
pkcA-kinase-rev	5′-CCTAGAACAGCAAGGAAGTTGAAG-3′
pkcA-prom-fwd	5′-CTCCTTCTTTACGTATTCTCTTAGG-3′

Russell (2001). Transfer and hybridization conditions were as described previously (Jadoun et al. 2004).

Generation of the *alcA-PKC* conditional mutant

A 2,552-bp DNA fragment flanking the regulatory region of the *A. nidulans pkcA* gene was generated by PCR, using the Expand high-fidelity PCR system (Roche Diagnostic, Penzberg, Germany), 1 μg of *A. nidulans* R153 genomic DNA as a template and primers *Kpn*I-*PKC* 5′ and *Kpn*I-*PKC* 3′ (Table 1). These primers were designed to contain a *Kpn*I restriction site at their 5′ end (Table 1, italics). The *pkcA* regulatory domain fragment was verified by sequencing, digested with *Kpn*I and ligated into the Pal3 plasmid (Waring et al. 1989) to generate Pal3-PKC (Fig. 1a). The orientation of the insert was determined by digestion with *Pst*I, which is located 180 bp downstream of the start codon. To produce the *alcA-PKC* strain, 10 μg of spin-purified Pal3-PKC plasmid was used to transform strain GR5. Transformation was performed as described by McGoldrick et al. (1995).

Primary transformants were selected for growth in MMG. Glycerol or threonine allows for transcription from the *alcA* promoter whereas glucose-containing medium (MM) represses *alcA* transcription (Fig. 1b). Transformants were then tested for growth on MM. Six strains that grew poorly on glucose, but grew on MMG were selected. Genomic DNA was prepared from these strains, digested with *Xba*I and analyzed by Southern blot using the *pyr4* sequence as a probe. All of the strains displayed the predicted Southern hybridization pattern (composed of a 7.5-kb band for the six strains, none for the control wild-type strain) expected for conditional disruption of *pkcA* (data not shown). Furthermore, the strains were analyzed by PCR using primer pair *alcA*-fwd/*pkcA*-kinase-rev to detect the *alcA*-driven *pkcA* gene, and primer pair *pkcA*-prom-fwd/*pkcA*-kinase-rev to detect the native *pkcA* gene (Table 1). In all six transformants, the expected *alcA*-driven *pkcA* gene product was detected, whereas the native *pkcA* gene product was only detected in the control wild-type strain (data not shown).

Microscopy

Mutant strains and R153 control *A. nidulans* conidia were incubated at concentrations of 10^4 conidia ml^{-1} for the indicated time at 37°C on glass cover slips in 24-well plates (Nunclon surface; Nunc, Roskilde, Denmark). For nuclear staining, cover slips were fixed in 10 mM KPO_4 pH 6.8, 4% (v/v) paraformaldehyde, 0.1% (w/v) Triton X-100 for 30 min at room temperature, stained with DAPI (4′,6-diamidino-2-phenylindole) (100 ng ml^{-1}) for 15 min, washed twice in PBS and analyzed. For calcofluor staining,

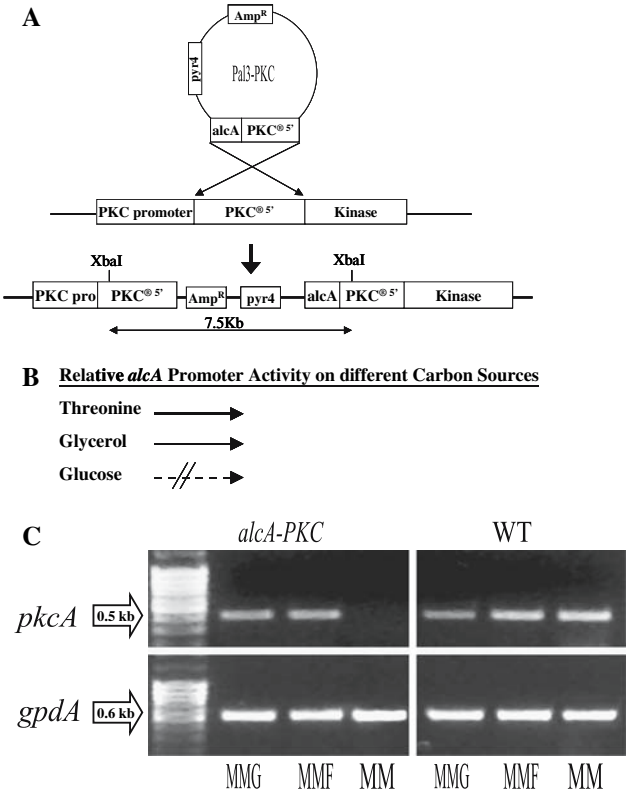

Fig. 1 Construction of a conditionally null *pkcA* mutant strain, *alcA-PKC*, containing an inducible *alcA* promoter. **a** The predicted integration and pattern of bands for *Xba*I-digested *pkcA* DNA and **b** effect of carbon source on *alcA* promotor activity. Transcription is induced on glycerol or threonine, and repressed when glucose is the carbon source provided. **c** Expression of *pkcA* mRNA in the *alcA-PKC* mutant is repressed in the presence of glucose (*MM*) and activated in the presence of glycerol (*MMG*) and threonine (*MMT*). RT-PCR was used to measure the levels of *pkcA* (*top panel*) and *gpdA* (*lower panel*) transcript. Expression of *gpdA* was used to evaluate RNA integrity and cDNA loading

cells were stained for 15 min at room temperature with calcofluor (0.5 mg ml^{-1} in PBS) and analyzed. Images were obtained by differential interference contrast (DIC) and fluorescence microscopy on an Olympus BX40 microscope (equipped for fluorescence with a fluorescein isothiocyanate filter for DAPI and calcofluor staining) at a total magnification of 200× and 400×. Images were recorded with a digital Olympus DP70 camera.

Hyphal growth rate and germination studies were performed by plating 10^3–10^4 freshly harvested spores ml^{-1} onto 96-well plates in 200 μl liquid MM or MMG at 37°C. At various time points, growth was observed under a grid-mounted Olympus CK inverted microscope at 200× magnification. The percentage of germinated conidia ($n = 60$) was assessed, and the lengths of the hyphae ($n = 60$) were measured in microns.

For transmission electron microscopy (TEM), conidia (0 h) and germinated conidia (16 h) were fixed in 2.5% (w/v)

glutaraldehyde in PBS. They were then washed, postfixed in 1% (w/v) OsO_4 in PBS and washed again. After dehydration in graded ethanol solutions the cells were embedded in glycid ether 100 (Serva Gmbh, Heidelberg, Germany). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined in a Jeol 1200 EX TEM.

Sensitivity to reagents and antifungals

Aspergillus nidulans control wild-type strain R153 and the conditional *alcA-PKC* mutant were grown in 96-well plates at a concentration of 10^4 conidia ml^{-1} in MM or MMG supplemented with reagents and antifungals. MICs (minimal inhibitory concentration, i.e. the lowest drug concentration to completely arrest germination and growth) were evaluated after 24 h incubation at 37°C. Unless otherwise specified, all reagents were from Sigma–Aldrich. The concentration ranges of the reagents and antifungals were: congo red 1–160 μg ml^{-1}; caspofungin (Merck, NJ, USA) 0.06–160 μg ml^{-1}; calcofluor white 10–320 μg ml^{-1}; amphotericin B 0.6–80 μg ml^{-1}; voriconazole (Pfizer, NY, USA) 0.02–8 μg ml^{-1}; staurosporine 0.3–20 μg ml^{-1}). For sensitivity testing on agar plates, conidia from the wild-type and mutant strains (10^8 conidia ml^{-1}) were point- inoculated on MM and MMG plates containing either 15 μg ml^{-1} congo red, 0.25 μg ml^{-1} caspofungin, 1.25 μg ml^{-1} staurosporine or no drug as a control. The strains were grown for 48 h at 37°C.

Results

Generation of a conditional *pkcA* strain of *A. nidulans*

Previous efforts to disrupt or delete *pkcA* have been unsuccessful, which suggests that it is an essential gene (Herrmann et al. 2006). Therefore, an alternative strategy in which expression of the *pkcA* gene could be regulated was used to address the question of PKCA function in *A. nidulans.* A 2,552 bp *Kpn*I PCR fragment of *pkcA* spanning the first 748 amino acids of the PKCA protein sequence and encoding the regulatory domain was cloned downstream of the *alcA* alcohol dehydrogenase promoter in the vector pal3 to generate pal3-PKC (Waring et al. 1989). Integration of this plasmid by homologous recombination at the *pkcA* locus resulted in strains that have the *pkcA* gene situated immediately 3′ to the *alcA* promoter (Fig. 1a). These conditional *pkcA* null mutant strains are dependent on the activity of the *alcA* promoter for transcription of *pkcA*. Expression of the *alcA* promoter is regulated by the carbon source present in the medium. It is activated by ethanol, glycerol or threonine and repressed in the presence of glucose (Fig. 1b). We transformed strain GR5 with pal3-PKC and selected six transformants that grew normally on minimal media containing glycerol (MMG) but poorly in the presence of repressive glucose (MM). All six strains were verified by PCR and Southern hybridization for correct insertion of the plasmid as described in Materials and methods. They exhibited an identical mutant phenotype as assessed by growth in repressive MM and microscopic analysis. One of the strains, *alcA-PKC*, was chosen for further study.

To confirm that *pkcA* expression in the mutant strain *alcA-PKC* is regulated by the *alcA* promoter, we measured the level of *pkcA* mRNA in the mutant and wild-type strains following growth in MMG, MMT or MM (Fig. 1c). As expected, *pkcA* mRNA was present when *alcA-PKC* was grown in MMG or MMT, whereas none was detected after growth in MM (Fig. 1c, left panel). In contrast, *pkcA* mRNA was present in the wild-type strain under all growth conditions (Fig. 1c, right panel).

The *alcA-PKC* mutant strain exhibits reduced germination and hyphal growth on solid medium containing glucose

Radial growth analysis provides a sensitive assay for determining differences in growth rates of strains under various culture conditions. The radial growth of the *alcA-PKC* mutant strain was visually compared to that of the wild-type strain R153 after 48 h of growth at 37°C on solid MM or MMG (Fig. 2a). The *alcA-PKC* strain grew as well as R153 on the de-repressive glycerol-containing medium. On repressive MM, in which *alcA-PKC* is transcriptionally repressed, *alcA-PKC* did not grow visibly except for several aberrant colonies which probably arose due to plasmid eviction and heterokaryon formation. This suggests that *pkcA* is required for normal growth. Microscopic examination of the agar plate after 24 h of growth at 37°C revealed that 64 ± 4% of the conidia ($n = 400$) swelled but failed to initiate hyphal growth, 32 ± 3% produced a single, short hypha and only 4 ± 2% underwent branching and further growth. In contrast, 100% of the wild-type control conidia had undergone germination and hyphal growth by this time. Osmotic remediation with either 1 M sorbitol or 0.6 M KCl did not reverse the mutant phenotype (data not shown).

The conidiospores of the *alcA-PKC* mutant strain swell excessively in liquid medium containing glucose

To examine the microscopic phenotype of the *alcA-PKC* mutant strain, *alcA-PKC* and control wild-type strain R153 conidia were germinated on coverslips in liquid MM containing glycerol or glucose. The *alcA-PKC* strain exhibited normal hyphal growth on MMG (Fig. 2b, lower panel). On MM (containing glucose), the growth of the *alcA-PKC* strain was apparently normal until 8 h post-germination (data not shown). At later time points, the spore bodies of

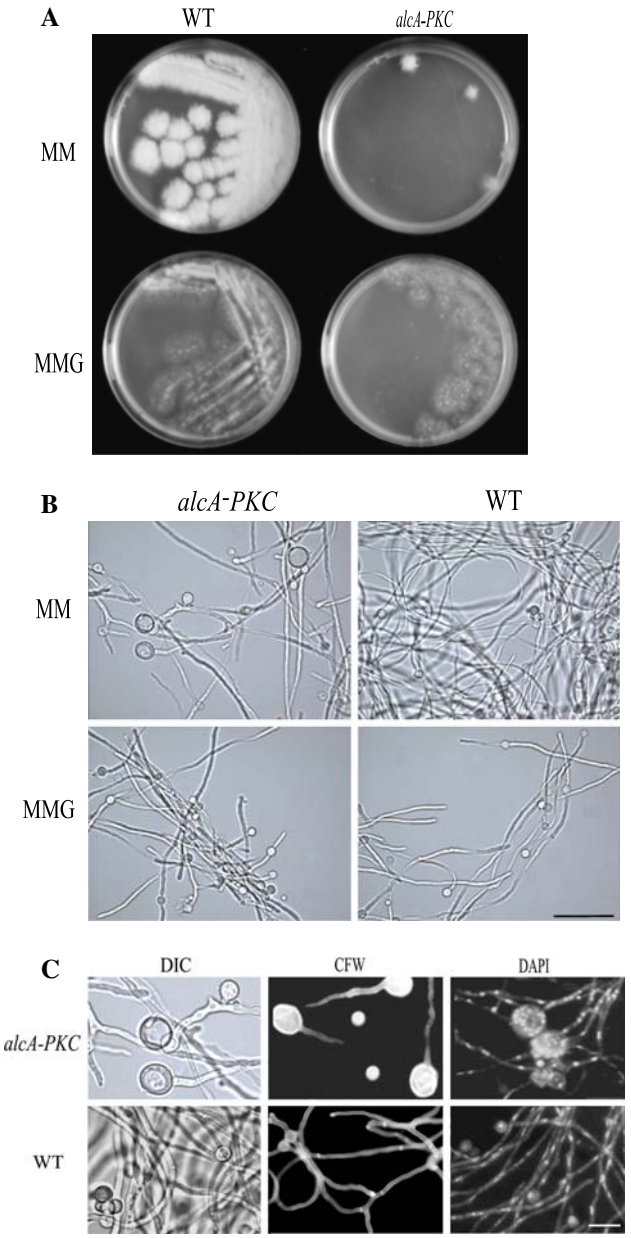

Fig. 2 Impaired growth of the *alcA-PKC* mutant in the presence of glucose. Freshly-harvested conidia from the wild-type R153 (*WT*) and *alcA-PKC* mutant strains were **a** streaked on minimal medium agar plates containing glucose (*MM*) or glycerol (*MMG*) as a sole carbon source, grown for 48 h at 37°C and photographed, **b** grown on cover slips in liquid MM and MMG for 16 h at 37°C, viewed by DIC microscopy and photographed. *Bar* 20 μm. **c** Enlarged view of the *alcA-PKC* mutant and WT strains after 16 h of growth. *Left image* DIC; *centre* calcofluor staining; *right* DAPI nuclear staining. *Bar* 10 μm

the mutant swelled considerably (10 ± 3 μm diameter; $n = 20$) compared to the control strain (3 ± 1 μm diameter; $n = 20$) and hyphal growth slowed in comparison to the wild type (Fig. 2b, top panel). The swollen spore bodies contained an enlarged vacuole (in $71 \pm 14\%$ of cells, $n = 50$), and multiple nuclei, and stained intensely with calcofluor (Fig. 2c).

The *alcA-PKC* mutant strain exhibits delayed germination and reduced hyphal growth on liquid MM containing glucose

We next quantitatively examined the germination and growth rates of the *alcA-PKC* mutant strain compared to wild-type strain R153. Conidia were germinated for 0–16 h at 37°C in liquid MM with glycerol or glucose as carbon sources and examined microscopically at each time point to determine the percent of germinating conidia (i.e. those containing visible hyphae) and the lengths of the hyphae. The *alcA-PKC* strain germinated and grew as well as R153 on derepressive MMG (Table 2; Fig. 3b). However, in the presence of repressive MM, the *alcA-PKC* mutant strain showed a slight delay in germination (Table 2) and a significant decrease in hyphal growth after about 8 h of growth, relative to the wild-type strain (Fig. 3a). Osmotic remediation with either 1 M sorbitol or 0.6 M KCl did not reverse the mutant phenotype (data not shown).

The *alcA-PKC* mutant strain exhibits structural cell wall defects

PKC homologs participate in maintenance of the fungal cell wall. To determine the role of *A. nidulans pkcA* in this process, we analyzed the microscopic ultrastructure of the spore body cell wall in the *alcA-PKC* strain using TEM. The results indicated that in the presence of MM (which contains glucose and represses *pkcA* expression), the *alcA-PKC* strain exhibits defects in the ultrastructure of the cell wall (Fig. 4). Whereas the cell wall of the control wild-type strain contains an electron-dense outer layer (black arrow) and a lighter inner layer (white arrow), the mutant has an additional thick but irregular external deposit composed of a very dense outer coating and a very light inner layer (hatched arrow) (Fig. 4). The mutant strain also exhibits enlarged vacuoles relative to the wild-type (Fig. 4b, arrows).

The *alcA-PKC* mutant strain is hypersensitive to cell wall disrupting agents

The *alcA-PKC* strain was tested for sensitivity to antifungal drugs and cell wall perturbing agents when grown on solid (Fig. 5) and in liquid (Table 3) media. In the presence of MMG the *alcA-PKC* strain exhibited wild-type sensitivity towards all the agents tested (Table 3; Fig. 5, lower panel). However, the *alcA-PKC* strain showed hypersensitivity to sub-MIC concentrations of caspofungin, staurosporine and congo red on glucose-containing solid medium (Fig. 5, MM). In the presence of repressive liquid MM, the *alcA-PKC* strain exhibited hypersensitivity towards the cell wall perturbing agents congo red (CR), and calcofluor white

Table 2 Germination rates of wild-type control strain R153 (WT) and *alcA-PKC* mutant on minimal glucose (MM) and glycerol (MMG) liquid media

Time (h)	4	5	6	7	8	10	12	16
MM								
WT	0.22 ± 0.02[a]	0.53 ± 0.06	0.85 ± 0.06	0.96 ± 0.06	0.96 ± 0.03	1	1	1
alcA-PKC	0.22 ± 0.08	0.46 ± 0.11	0.55 ± 0.07	0.91 ± 0.11	0.94 ± 0.05	0.89 ± 0.05	0.83 ± 0.02	0.96 ± 0.04
MMG								
WT	0	0	0	0.12 ± 0.07	0.39 ± 0.04	0.56 ± 0.09	0.98 ± 0.02	1
alcA-PKC	0	0	0	0.17 ± 0.06	0.46 ± 0.17	0.69 ± 0.11	0.91 ± 0.04	1

[a] Fraction of conidia containing emerging hyphae ($n = 60$)

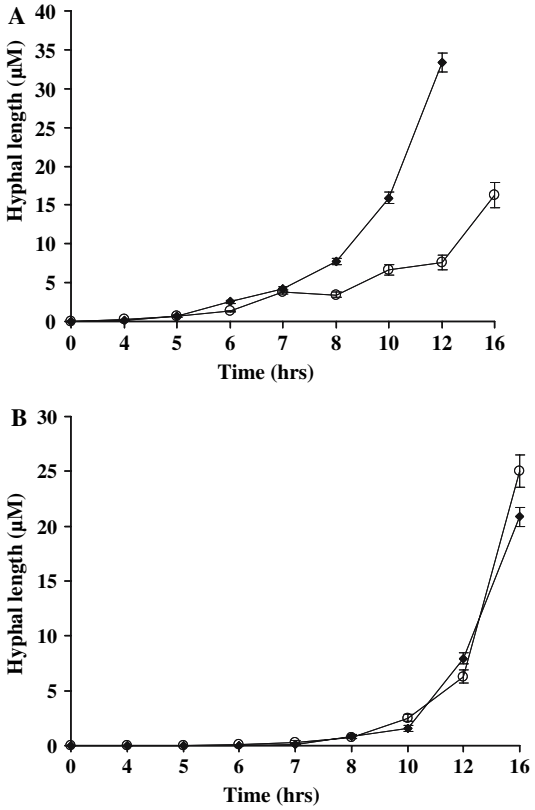

Fig. 3 Reduced growth rate of the *alcA-PKC* mutant in the presence of glucose. Hyphal growth rate and germination studies were performed by plating 10^3–10^5 freshly harvested spores ml^{-1} in **a** liquid MM or **b** MMG. At various time points, hyphal growth of the control wild-type (*filled diamond*) and *alcA-PKC* mutant (*open circle*) was observed under a grid-mounted Olympus CK inverted microscope at a magnification of ×200. Each time point was calculated as the mean ± SE of 60 hyphae

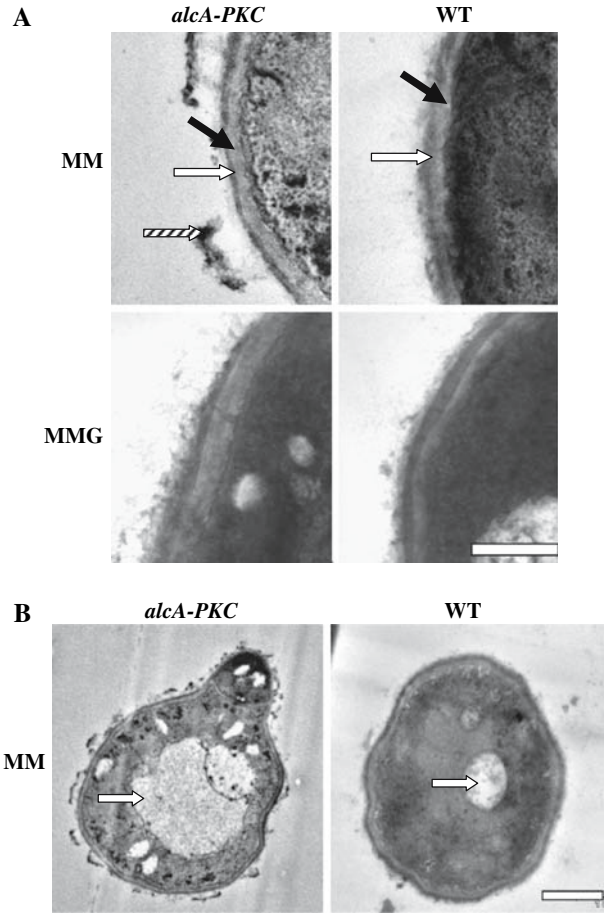

Fig. 4 Defects in the cell wall ultrastructure of the *alcA-PKC* mutant. Transmission electron micrograph (TEM) of the **a** *alcA-PKC* mutant (*left panel*) and wild-type (*right panel*) including spore body wall sections (**a**) and whole spore bodies (**b**) (*bar* 1 and 0.2 µm, respectively). The strains were grown for 16 h at 37°C on MM or MMG liquid medium prior to fixation. The inner layer of the cell wall is indicated by a *filled arrow*, the outer layer by an *open arrow*. Note the additional layers (*tiled arrow*) in the *alcA-PKC* mutant

(CFW) (4-fold and 16-fold reduced MIC compared to wild type, respectively), the antifungal drug caspofungin (CAS) which inhibits 1,3-β-glucan biosynthesis (256-fold reduced MIC compared to wild type), and the PKC inhibitor staurosporine (SSP) (8-fold reduced MIC compared to wild type) (Table 3). Importantly, the *alcA-PKC* strain did not exhibit hypersensitivity to the antifungal drugs amphotericin B (AMB), which binds ergosterol and disrupts the fungal plasma membrane, or voriconazole (VRZ), an inhibitor of ergosterol biosynthesis (Table 3), suggesting that it may be specifically sensitive to cell wall damage.

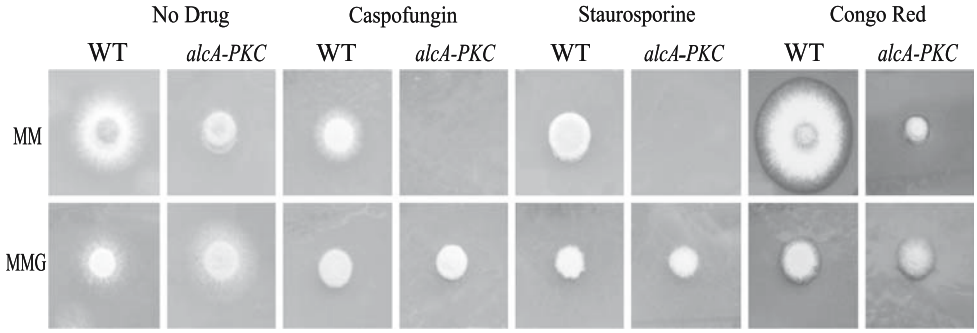

Fig. 5 Hypersensitivity of the *alcA-PKC* mutant towards cell wall disrupting agents. Conidia from the mutant and wild-type strain were point inoculated on MM (*top panel*) or MMG (*lower panel*) agar plates containing either no drug (*control*) or 0.25 μg ml^{-1} caspofungin, 1.25 μg ml^{-1} staurosporine or 15 μg ml^{-1} congo red. The strains were grown for 48 h at 37°C and photographed

Table 3 MIC results for the wild-type control strain R153 (WT) and the *alcA-PKC* mutant on minimal glucose (MM) and glycerol (MMG) liquid media

	AMB	VRZ	CAS	CR	CFW	SSP
MM						
alcA-PKC	1.25[a]	0.039	0.125	5	3.125	1.25
WT	1.25	0.039	32	20	50	10
MMG						
alcA-PKC	10	0.078	32	20	25	5
WT	10	0.078	32	20	25	5

AMB amphotericin B, *VRZ* voriconazole, *CAS* caspofungin, *CR* congo red, *CFW* calcofluor white, *SSP* staurosporine

[a] *MIC* minimal inhibitory concentration-the lowest drug concentration resulting in complete inhibition of hyphal growth

Discussion

In this work, we describe the generation and phenotypic analysis of a conditional *pkcA* mutant in *A. nidulans*. We chose to study this gene because the role of PKC family proteins in the filamentous fungi is poorly understood. Their important role in yeast in the maintenance of cell wall integrity suggests that they could serve as important targets for the development of novel antifungal drugs (Sussman et al. 2004).

Previous work has demonstrated that knockdown of *pkcA* expression by antisense RNA leads to reduced growth, conidiation and penicillin production (Herrmann et al. 2006).

Our main findings revealed that *pkc*A is involved in additional processes, including the maintenance of cell wall integrity. Repression of PKCA production in *A. nidulans* resulted in conidiospore swelling, defects in the morphology of the cell wall and sensitivity to cell wall destabilizing agents. Similar results have been demonstrated in the yeasts *S. cerevisiae* (Paravicini et al. 1992), *Schizosaccharomyces pombe* (Kobori et al. 1994) and *C. albicans* (Paravicini et al. 1996), but never in filamentous fungi. Furthermore, we show that under *pkcA*-repressing conditions, conidial germination is partially blocked (on solid medium) or delayed (in liquid medium) and that the rate of hyphal elongation is strongly reduced. This suggests that the PKCA protein is involved in cell wall maintenance and construction during both isotropic and polarized growth.

The presence of multiple nuclei in the enlarged spore bodies of the *alcA-PKC* mutant suggests that the cell cycle can progress in the absence of PKCA. A multinucleate phenotype has been previously observed following deletion of the *A. nidulans* MAPK homolog *mpkA* (Bussink and Osmani 1999), and the mutation of several cytoskeletal-associated dynactin-complex proteins (Xiang et al. 1999), and is suggestive of a defect in nuclear migration.

The *alcA-PKC* mutant contains greatly enlarged vacuoles. Fungal vacuoles are complex organelles involved in multiple functions including osmoregulation, metabolite storage, proteolysis, protein transport and sorting (Klionsky et al. 1990). Vacuole enlargement can result from impairment of vesicle trafficking leading to and from the vacuole (Geissenhoner et al. 2001) or from water uptake as a result of defects in the cell wall and membrane leakage (Klionsky et al. 1990).

Interestingly, unlike in yeast, the *alcA-PKC* mutant does not require osmotic stabilization to grow. In fact, the addition of osmotic stabilizers does not remediate the increased spore-body swelling and reduced growth rate of this mutant. This suggests that in *A. nidulans*, *pkcA* probably performs other functions, which are not remediated by osmotic stabilization.

The *alcA-PKC* mutant we have generated provides an opportunity to identify the components of the cell wall integrity pathway in a filamentous fungus. In *S. cerevisiae*, the small G-protein Rho1p activates Pkc1p and the MAPK cascade in response to cell wall damaging agents. Interest-

ingly, constitutive activation of *rhoA* expression in *A. nidulans* results in osmotic instability and hypersensitivity to calcofluor and caspofungin (Guest et al. 2004). Assuming that like in *S. cerevisiae*, *A. nidulans* RHOA activates PKCA, these results suggest that deregulated hyperactivity of this kinase can also result in cell wall defects. In *S. cerevisiae*, the MAPK Slt2p functions in the cell wall integrity pathway downstream of Pkc1p (Schmitz and Heinisch, 2003). Deletion of *mpkA*, encoding the *A. nidulans* MAPK most similar to Slt2p, results in the formation of enlarged multinucleate conidia and swollen hyphal tips, which are only partially remediated in the presence of osmotic stabilization (Bussink and Osmani 1999). This suggests that *A. nidulans* PKCA and MPKA function in the same pathway. Availability of the *alcA-PKC* strain described in this work, the putative PKC-pathway mutants described above and the completed genomic sequence of *A. nidulans* enables the reconstruction of the cell wall integrity pathway in a filamentous fungus based on homology analysis with known yeast genes and its experimental verification. Future work will identify the epistatic relationships among these genes and attempt to identify the MAPKK and MAPKKK genes downstream of *pkcA*.

It is worth noting that the *alcA-PKC* mutant is hypersensitive to cell wall damaging agents and not to drugs that damage the fungal plasma membrane. This finding suggests that this mutant may prove useful in the identification of antifungal compounds that specifically target the cell wall. Such an approach has been used successfully to identify microtubule-disrupting antifungal agents in an *A. nidulans* temperature-sensitive microtubule mutant (Kiso et al. 2004) and to determine the mode of action of antifungal compounds in the yeast *S. cerevisiae* (Buurman et al. 2005). We have initiated a high-throughput screen of a combinatorial library of compounds using the *alcA-PKC* strain grown under both *pkcA*-repressing and activating conditions to identify novel cell wall destabilizing agents in *A. nidulans*.

Aspergillus nidulans is closely related to *A. fumigatus*, the primary mold pathogen in immunocompromised patients, and there is therefore a high likelihood that the identified compounds will be applicable to research in the latter organism.

In summary, our findings suggest that the *A. nidulans pkcA* gene is involved in key aspects of cell wall architecture. The increased conidial swelling, decreased hyphal growth rates and susceptibility to cell wall-destabilizing agents due to the conditional expression of *alcA-PKC* suggest that significant changes in the cell wall have occurred. Further elucidation of the mechanisms responsible for these changes may shed new light on the maintenance and biogenesis of the cell wall in *A. nidulans* at the molecular level.

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