Published in final edited form as:
Mol Microbiol. 2011 February ; 79(4): 1045–1062. doi:10.1111/j.1365-2958.2010.07509.x.

Divergent Protein Kinase A isoforms co-ordinately regulate conidial germination, carbohydrate metabolism and virulence in *Aspergillus fumigatus*

Kevin K. Fuller[1,†], **Daryl L. Richie**[1,‡], **Xizhi Feng**[1], **Karthik Krishnan**[1], **Timothy J. Stephens**[1], **Kathryn A. Wikenheiser-Brokamp**[1,2], **David S. Askew**[1], and **Judith C. Rhodes**[1,*]

[1]Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0529, USA.

[2]Divisions of Pathology and Laboratory Medicine and Pulmonary Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA.

Summary

The genome of *Aspergillus fumigatus* encodes two isoforms of the catalytic subunit of the cAMP-dependent Protein Kinase (PKA). Although deletion of the class I isoform, *pkaC1*, leads to an attenuation of virulence, the function of the class II subunit, PkaC2, was previously uninvestigated. In this report, we demonstrate that both isoforms act in concert to support various physiologic processes that promote the virulence of this pathogen. Whereas *pkaC1* and *pkaC2* single-deletion mutants display wild-type conidial germination, a double-deletion mutant is delayed in germination in response to environmental nutrients. Furthermore, PkaC1 and PkaC2 interact to positively regulate flux through the carbohydrate catabolic pathway and, consequently, the Δ*pkaC1*Δ*pkaC2* mutant is unable to grow on low glucose concentrations. Importantly, the reduced germinative capacity and inability to utilize glucose observed for the Δ*pkaC1*Δ*pkaC2* strain correlated with an inability of the mutant to establish infection in a murine model. Conversely, overexpression of *pkaC2* both promotes the *in vitro* growth on glucose, and restores the fungal burden and mortality associated with the Δ*pkaC1* to that of the wild-type organism. Taken together, these data demonstrate the functional capacity of *pkaC2* and emphasize the importance of PKA-mediated metabolic control in the pathogenic potential of *A. fumigatus*.

Introduction

Aspergillus fumigatus has emerged as the predominant mould pathogen among severely immunocompromised patients (Latge, 1999; Upton *et al*., 2007). Current evidence suggests that the clinical prevalence of *A. fumigatus* is related to attributes that have evolved in the fungus that promote its survival in its *ex vivo* niche of the compost. This environment confronts the fungus with a variety of stresses: elevated temperatures, changes in pH,

*For correspondence. judith.rhodes@uc.edu; Tel. (+1) 513 558 0130; Fax (+1) 513 558 8687.
†Present addresses: Dartmouth Medical School, 1 Rope Ferry Road, Hanover, NH 03755, USA
‡Novartis Institutes for Biomedical Research, 250 Massachusetts Avenue, Cambridge, MA 02139, USA.

Supporting information

Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

nutritional limitations and fluctuations in oxygen and carbon dioxide levels, as examples (Beffa *et al*., 1998; van Heerden *et al*., 2002; Askew, 2008). As these stresses are also present within the host, the adaptability of *A. fumigatus* translates into its success as an opportunistic pathogen. Accordingly, understanding the signalling pathways that detect these environmental conditions and mediate appropriate physiological responses will provide significant insights into the virulence of this organism.

The cAMP-dependent Protein Kinase A (PKA) pathway is a key regulator of fungal biology, and in many cases, virulence (Adachi and Hamer, 1998; Dürrenberger *et al*., 1998; Yang and Dickman, 1999; D'Souza and Heitman, 2001; D'Souza *et al*., 2001; Chen *et al*., 2007). The central core of the pathway is well conserved among all eukaryotes, with the holoenzyme consisting of two catalytic subunits that are bound and inactivated by a dimer of regulatory subunits. Activation occurs after the second messenger, cAMP, binds to the regulatory subunits, thereby inducing a conformational change that releases the active kinases. In the model yeast *Saccharomyces cerevisiae*, nutrients, particularly fermentable sugars, are important stimulators of the pathway (Thevelein *et al*., 2005). Downstream targets of PKA include proteins that fall within a variety of functional groups, including enzymes, transcriptional factors, and other signalling effectors that co-ordinately regulate fungal metabolism, morphogenesis and stress response. In this way, PKA is strategically positioned to drastically alter cellular physiology in response to diverse extracellular cues.

In an early effort to identify *A. fumigatus* genes involved in virulence, we analysed patterns of fungal gene expression following co-culture with human endothelial and epithelial cells (Rhodes *et al*., 2001; Oliver *et al*., 2002a). These studies revealed an upregulation of core components of the PKA pathway, including the catalytic subunit gene, *pkaC1*. Deletion of *pkaC1* resulted in attenuation of virulence in a murine model of invasive aspergillosis (IA), demonstrating an essential role for PKA signalling in pathogenesis (Liebmann *et al*., 2004).

Despite the advances, the contribution of PKA to the virulence of *A. fumigatus* remains incompletely understood. The Δ*pkaC1* mutant is growth impaired *in vitro* (Liebmann *et al*., 2004), which may partially explain the attenuated virulence of the strain. But other studies in *A. fumigatus* suggest that a decreased *in vitro* growth rate cannot fully explain the virulence of other mutants in the PKA pathway. For example, GpaB is the Gα subunit of a heterotrimeric G–protein complex that stimulates adenylate cyclase (AcyA) to produce cAMP. As expected, deletion of either *gpaB* or *acyA* leads to a loss of detectable PKA activity in *A. fumigatus* (Liebmann *et al*., 2003). However, although both mutants were severely attenuated for virulence, only the Δ*acyA* strain showed a reduction in growth rate *in vitro* (Liebmann *et al*., 2003; Liebmann *et al*., 2004). This implies that PKA signalling governs specific physiologic requirements for growth within the host environment.

Analysis of PKA signalling in fungi is complicated by the fact that most organisms contain two or more genes encoding a PKA catalytic subunit. For instance, *S. cerevisiae* encodes three PKA catalytic isoforms, Tpk1p, 2p and 3p. Signalling through any one of the isoforms is sufficient to support normal growth of the organism, whereas deletion of all three is synthetically lethal (Toda *et al*., 1987). In addition to their functional redundancy, phenotypic and transcriptional analyses have revealed non-overlapping roles for each of these genes. Tpk2p positively regulates pseudohyphal filamentation, whereas Tpk1p and Tpk3p are repressive in this respect (Robertson and Fink, 1998; Pan and Heitman, 1999). Moreover, Tpk2p uniquely downregulates a variety of genes involved in high-affinity iron uptake, and Tpk1p is the sole isoform involved in the biosynthesis of branched chain amino acids that function in mitochondrial homeostasis. In this regard, Tpk1p and Tpk2p play opposite roles in regulating processes involved in the maintenance of respiratory growth (Robertson *et al*., 2000).

Despite the independent functionalities associated with the PKA catalytic isoforms of yeast, the three proteins share a high degree of sequence identity (Robertson and Fink, 1998). This is in contrast with the filamentous fungi, in which the PKA isoforms are more evolutionarily divergent from one another. For example, *Aspergillus nidulans* encodes two catalytic subunits, PkaA and PkaB (Ni *et al*., 2005). PkaA demonstrates high homology to the TPK proteins of *S. cerevisiae*, and together, they form a phylogenetic group called the class I PKAs. However, *pkaB* and its homologues form a separate phylogenetic group of PKA-related kinases, the class II PKAs, which are uniquely found in filamentous fungi (Ni *et al*., 2005). The lack of appreciable phenotypes associated with class II deletion mutants, as exemplified by Δ*pkaB* in *A. nidulans*, is in strong contrast to the significant growth and developmental defects imparted by the deletion of class I isoforms (Dürrenberger *et al*., 1998; Ni *et al*., 2005). However, deletion of both *pkaA* and *pkaB* in *A. nidulans* is synthetically lethal, confirming that the class II gene retains some important function. Moreover, differential roles between the two catalytic isoforms with regard to germination, asexual development and oxidative stress response have been described (Ni *et al*., 2005). These findings underscore the importance of studying both PKA isoforms in order to obtain a comprehensive understanding of PKA signalling within filamentous fungi.

In this study, we report the first functional characterization of the class II PKA, PkaC2, in *A. fumigatus*. Our data demonstrate that, although PkaC2 activity alone is largely redundant to that of PkaC1, both isoforms act in concert to regulate germination, cell wall homeostasis and growth on fermentable carbon substrates. Importantly, these PkaC2-controlled processes contribute to the pathogenic potential of *A. fumigatus*, as deletion of *pkaC2* eliminates any residual virulence associated with Δ*pkaC1*, and overexpression of *pkaC2* fully restores virulence to the Δ*pkaC1* mutant. Taken together, these data underscore the importance of PKA signalling to the expression of virulence-related attributes of *A. fumigatus* that facilitate its adaptation to, and growth within the host.

Results

A. fumigatus genome encodes two PKA catalytic subunits that are not essential for viability

Aspergillus fumigatus, like other filamentous fungi, contains two PKA catalytic genes within its genome, *pkaC1* and *pkaC2*. PkaC1 is the canonical class I PKA, the homologues of which have been studied in multiple species and shown to play a role in *A. fumigatus* growth and virulence (Liebmann *et al*., 2004). In comparison, *pkaC2* encodes a member of the class II PKA subunits found uniquely in filamentous fungi, the function of which is not yet known in this fungal pathogen.

Sequence analysis of the *pkaC2* cDNA identified three exons spanning 1188 base pairs, confirming the genomic organization predicted in the GenBank database (data not shown, Accession No. AJ575357.1 and Q70KQ0). The PkaC2 protein contains a typical serine/threonine kinase domain as well as a C-terminal AGC domain, which is representative of a large family of kinases that includes PKA (Pearce *et al*., 2010) (Fig. 1). The PkaC1 and PkaC2 proteins share approximately 32% sequence identity overall, with the highest degree of divergence existing at the N-terminal region. PkaC1 and its class I homologues in filamentous fungi contain N-terminal extensions that are absent in the class II PKAs. As would be expected, higher degrees of conservation between the two isoforms exist across the two kinase domains, with approximately 48% identity and 65% similarity.

To determine the relative contributions of PkaC1 and PkaC2 to the biology and virulence of *A. fumigatus*, deletion mutants of each gene were constructed by homologous replacement with the hygromycin and phleomycin resistance genes respectively (Fig. S1). In addition, a

strain deficient in both *pkaC1* and *pkaC2* was isolated (Fig. S1), demonstrating that PKA signalling is not essential for the viability of *A. fumigatus*. This is in striking contrast to *A. nidulans*, where loss of the both PKA isoforms has been reported to be lethal (Ni *et al*., 2005).

PkaC2 has a secondary role to PkaC1 in maintaining colonial growth

Whereas deletion of *pkaC1* resulted in the formation of small compact colonies, the radial growth of the Δ*pkaC2* mutant was indistinguishable from wild type (WT). However, the Δ*pkaC1*Δ*pkaC2* mutant was notably growth impaired relative to the Δ*pkaC1* strain, indicating that both catalytic subunits contribute to colonial growth of *A. fumigatus* (Fig. 2A). The normal growth of the Δ*pkaC2* strain suggested that PkaC2 functionality could best be elucidated in the context of the loss of PkaC1 activity. Accordingly, in addition to the double-deletion mutant, we overexpressed *pkaC2* in the Δ*pkaC1* background by placing it under the control of the strong, constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene (Fig. S1). The Δ*pkaC1::PgpdA-C2* strain demonstrated a modest increase in radial growth relative to the Δ*pkaC1* strain, which further supports a role for PkaC2 in hyphal proliferation. However, *pkaC2* overexpression did not fully complement the *in vitro* growth defects seen in the Δ*pkaC1* mutant, which is consistent with previous reports that PkaC1 is the dominant isoform controlling colonial growth in *A. fumigatus* (Liebmann *et al*., 2004) (Fig. 2A).

PkaC2 shares a primary role with PkaC1 in regulating conidial germination

Protein Kinase A is known to regulate fungal morphogenesis in response to extracellular cues in a variety of fungal species (Gold *et al*., 1994; Adachi and Hamer, 1998; Pan and Heitman, 1999; Bockmühl *et al*., 2001). As the transition from a conidium to an invasive hypha is critical for the establishment of infection, we analysed the relative contributions of PkaC1 and PkaC2 to germination. Conidia of both the *pkaC1* and *pkaC2* single gene deletion mutants displayed germination kinetics identical to that of WT, with all three populations reaching 100% germ-tube formation by 8 h incubation in a complete growth medium (Fig. 2B and data not shown). When the Δ*pkaC1*Δ*pkaC2* mutant was tested, only 34% of those conidia had germinated at 8 h, and 100% germ-tube formation was not achieved until about 11 h. In minimal medium, this defect was even more pronounced, with only 2% of the Δ*pkaC1*Δ*pkaC2* conidia producing germ tubes by 11 h (Fig. 2B). By 24 h of incubation, substantial hyphal growth could be appreciated in the Δ*pkaC1*Δ*pkaC2* population, indicating the mutant maintained its capacity to germinate, albeit with a markedly delayed time-course (data not shown).

During germination, polarized growth occurs after the quiescent conidium becomes metabolically activated (d'Enfert, 1997). Therefore, the delay in germ-tube emergence observed for the Δ*pkaC1*Δ*pkaC2* mutant may represent a defect in polarized growth and/or a delay in conidial activation. To resolve this issue, we analysed mitochondrial respiration as a marker for metabolic activation by staining conidia with the dye Mitotracker Orange CMTMRos, as previously described (Fuller *et al*., 2009) (Fig. 2C). Whereas positively staining conidia of WT and Δ*pkaC1* were detected after 3.5 h of incubation in YG, no positive cells within the Δ*pkaC1*Δ*pkaC2* population were evident at that time. Although all Δ*pkaC1*Δ*pkaC2* mutant conidia stained positively at 5.5 h, initiation of germ-tube emergence was further delayed. Because trehalose breakdown provides glucose for energy required for germination, we hypothesized that trehalose levels in resting conidia might vary among the mutants. However, the trehalose content was equivalent among mutant and WT strains (Fig. S2). Nevertheless, loss of phosphorylation of neutral trehalases in the PKA mutants following nutrient stimulation could still have adversely affected the germination rates among the PKAmutants (d'Enfert *et al*., 1999). These data therefore support a model in

which PkaC1 and PkaC2 act in concert to regulate metabolic activation and initiation of polarized growth in *A. fumigatus* conidia.

PkaC2 has limited influence on asexual development

Aspergillus fumigatus produces numerous pigmented asexual conidia, which give the mycelium a blue-green colour when grown on solid medium. The colonial appearance of the Δ*pkaC2* mutant was indistinguishable from WT, indicating that PkaC2 is dispensable for asexual development (Fig. 2A). In contrast, all strains harbouring the *pkaC1* deletion demonstrated reduced conidiation, as previously demonstrated (Liebmann *et al*., 2004), which is consistent with a dominant role for PkaC1 in this process. Quantification of conidia revealed that the Δ*pkaC1*Δ*pkaC2* mutant produced even fewer conidia than did Δ*pkaC1*, whereas the Δ*pkaC1::PgpdA-C2* strain produced more, indicating that PkaC2 can positively influence this developmental pathway (Fig. 3A). Furthermore, many conidiophores of the Δ*pkaC1* and Δ*pkaC1*Δ*pkaC2* mutants were underdeveloped or displayed aberrant morphologies (Fig. 3B), demonstrating that PKA signalling is required for normal conidiophore formation. Interestingly, osmotic stabilization of the medium largely rescued the conidiation deficiency of each of the *pkaC1* mutants, but addition of sorbitol was not able to correct the growth rate (Fig. 2A and data not shown). Sorbitol is an osmostabilizer that has been shown to protect a variety of fungal cell wall mutants from osmotic stress-induced lysis (Fortwendel *et al*., 2008;Richie *et al*., 2009). Therefore, these finding suggest that PKA mutants have underlying defects, which influence cell wall integrity, leading to impaired conidiophore development.

PkaC2 acts in concert with PkaC1 to support cell wall homeostasis

The ability of osmotic stabilization to rescue the conidiation defect of the PKA mutants suggested that PkaC1 and PkaC2 contribute to cell wall homeostasis. To test this, we analysed growth of the various mutants in the presence of the cell wall-damaging agent Congo red. The susceptibility of the Δ*pkaC2* mutant was indistinguishable from that of WT, whereas the strains harbouring the *pkaC1* deletion were unable to grow at concentrations of Congo red that had limited effect on either the WT or the Δ*pkaC2* strains (Fig. 4A). Furthermore, the Δ*pkaC1*Δ*pkaC2* mutant was more sensitive than the *pkaC1* single-deletion mutant, suggesting that both isoforms act synergistically to regulate cell wall physiology. Transient exposure to Congo red induced swelling of the hyphal tips in the mutants, which suggests that areas of active cell wall remodelling are more susceptible to the defect imparted by the absence of PKA (data not shown). Similar susceptibility profiles were observed in the presence of SDS, another agent to which fungal cell wall mutants are typically hypersusceptible (Fig. S3). Taken together, these data indicate that both PkaC1 and PkaC2 play active roles in maintaining cell wall homeostasis in *A. fumigatus*.

PkaC2 influences carbohydrate metabolism of the Δ*pkaC1* mutant

In *S. cerevisiae*, PKA mediates carbon catabolite repression by activating glycolytic and fermentative pathways, while concurrently downregulating respiration and alternative carbon utilization pathways in the presence of glucose (Thevelein *et al*., 2005). Three lines of evidence support a similar role for PKA in *A. fumigatus*. First, PKA activity is higher in cultures grown in glucose than those grown in glycerol, demonstrating that the pathway is influenced by the available carbon source (Oliver *et al*., 2002b). Second, the addition of exogenous cAMP, which would be expected to activate PKA, reduces growth of *A. fumigatus* on glycerol (Oliver *et al*., 2002b). Finally, overexpression of *pkaC1* prevents the organism from growing on acetate as the sole carbon source (Grosse *et al*., 2008). These findings indicate that PKA signalling in *A. fumigatus* plays a role in regulating carbon metabolism, although the relative contributions from PkaC1 and PkaC2 in this process are unknown.

To determine the requirement for PkaC2 in the utilization of carbon sources, the growth rates of the PKA mutants were compared on different nutritional substrates. The Δ*pkaC2* mutant was indistinguishable from WT on all carbon sources tested (data not shown), suggesting that PkaC2 is dispensable for regulating carbon metabolism in *A. fumigatus*. Since functional redundancy between PkaC1 and PkaC2 could account for the apparent lack of activity of PkaC2, we examined whether the deletion or overexpression of *pkaC2* in the background of the Δ*pkaC1* strain would have discernible influence upon growth on different carbon sources (Fig. 5A). Neither loss (Δ*pkaC1*Δ*pkaC2*) nor overexpression (Δ*pkaC1::PgpdA-pkaC2*) of PkaC2 altered the ability of the Δ*pkaC1* mutant to grow on acetate or on glycerol, consistent with the idea that *pkaC2* is not involved in regulating growth on non-fermentable carbon sources. However, overexpression of *pkaC2* increased the ability of the Δ*pkaC1* mutant to grow on both glucose and fructose, whereas the double-deletion mutant was further growth impaired, relative to the *pkaC1* mutant, on those fermentable carbon sources.

The above data suggested that PkaC2 plays a specific role in catabolic carbohydrate pathway(s). To test this hypothesis, we analysed the relative transcript levels of the pyruvate dehydrogenase beta subunit gene, *pdbA*. In filamentous fungi, such as *Aspergillus oryzae* (Maeda *et al*., 2004) and *Neurospora crassa* (Xie *et al*., 2004), glucose increases the expression of this gene, allowing it to serve as a marker for activation of carbohydrate catabolism. We found that the steady-state levels of *pdbA* were significantly lower in the Δ*pkaC1*Δ*pkaC2* mutant, relative to the WT, Δ*pkaC1* or Δ*pkaC1::PgpdA-C2* strains (Fig. 5B). This suggested that there is reduced flux through the carbon catabolic pathway in the double mutant, which may account for the reduced growth on glucose seen in that strain. A defect in glucose utilization would be expected to reduce growth under glucose-limiting conditions. Consistent with that, the double mutant could not grow when the glucose concentration was reduced to 0.1%. In contrast, Δ*pkaC1*Δ*pkaC2* was able to utilize 0.1% and 0.01% acetate as a carbon source for growth (Fig. 5C and data not shown). These data suggest that PkaC1 and PkaC2 act co-ordinately to regulate flux through the glycolytic/pyruvate metabolic pathways and therefore positively regulate carbohydrate utilization in *A. fumigatus*.

The composition of the fungal cell wall is greater than 90% carbohydrate, consisting of interconnecting chains of modified glucose (glucan) or amino-glucose (chitin) polymers (Latgé, 2007). Therefore, the proper synthesis and maintenance of the cell wall is dependent upon a continual flow of glucose monomers to the site of cell wall assembly. Accordingly, we hypothesized that the cell wall defect observed for the various mutants could be attributed, in part, to the involvement of PKA in controlling glucose assimilation. In support of this possibility, we found that the increased susceptibility of the Δ*pkaC1* mutant to Congo red could be recovered by elevating the glucose concentrations of the medium from 1% to 10% (Fig. 4B). Although partial osmotic stabilization by increased solute cannot be ruled out, this result is consistent with a model in which elevated glucose overcomes the reduction of sugar influx into the cell caused by *pkaC1* deletion. Interestingly, neither the increase in glucose concentration nor addition of the amino sugar *N*-acetylglucosamine rescued the hypersensitivity of the Δ*pkaC1*Δ*pkaC2* mutant to Congo red (Fig. 4B and data not shown). It is possible that the defect in the double mutant is simply too severe to be overcome by amending the medium, or that there is insufficient uptake to reach efficacious levels.

PKA signalling through both isoforms is essential for WT virulence

Although loss of PkaC1 has previously been shown to reduce the virulence of *A. fumigatus* (Liebmann *et al*., 2004), the contribution to virulence by PkaC2 is unknown. To determine whether the class II isoform also plays a role in regulating virulence, the PkaC2 mutants were analysed in a murine model of IA. The virulence of the Δ*pkaC2* strain was

indistinguishable from that of WT, indicating that PkaC2 is dispensable for virulence in the presence of PkaC1. This was in contrast to the Δ*pkaC1* single mutant, which, consistent with the previous report, demonstrated attenuated virulence (Fig. 6A). However, deletion of both PKA catalytic subunit genes rendered the organism avirulent, and strikingly, overexpression of *pkaC2* in the Δ*pkaC1* background completely rescued the attenuated virulence of Δ*pkaC1*. This suggests that there are processes controlled by PkaC2 that are important for regulating virulence-related attributes that are not directly related to radial growth in *A. fumigatus* (Fig. 6B). We were unable to complement the double mutant due to its poor conidiation and the inability to obtain sufficient numbers of viable protoplasts. Therefore, two additional, independent isolates of the Δ*pkaC1*Δ*pkaC2* mutant were selected and tested for virulence in the murine model; these additional isolates were also avirulent (Fig. S4B).

Analysis of the histopathology of the lungs at 24 h post infection revealed no fungal growth in the animals infected with the Δ*pkaC1*Δ*pkaC2* strain, whereas focal fungal colonization was observed in lungs from WT, Δ*pkaC1* and Δ*pkaC1::PgpdA-pkaC2*-infected mice (Fig. 7). At 48 and 72 h post infection, fungal lesions in WT, Δ*pkaC1* and Δ*pkaC1::PgpdA-pkaC2*-infected mice were much more prominent and characterized by abundant fungal hyphae associated with a marked inflammatory infiltrate and focal necrosis. Rarely, fungal hyphae were detected in the Δ*pkaC1*Δ*pkaC2*-infected lungs, which verified inoculation; however, the lesions were smaller and not associated with necrosis nor a significant influx of inflammatory cells (Fig 8 and Fig S5). Consistent with the histological findings, morphometric analysis demonstrated that the extent of fungal growth mirrored what would be predicted from cumulative mortality of the strains.

Based on the outcome of the virulence studies, the established role of cell wall homeostasis in virulence, and the cell wall defect observed in the PKA mutants, the transcripts of selected cell wall genes were measured to evaluate their possible contributions to the PKA mutant virulence phenotypes. The message levels of *ecm33* and *ags3* were measured in the WT and in the Δ*pkaC1* mutant (Maubon *et al*., 2006; Romano *et al*., 2006; Ejzykowicz *et al*., 2009). Although there was not a significant change in *ecm33* transcript, the level of *ags3* message was significantly increased in the mutant lacking PkaC1 (Fig. S6). The attenuated virulence of the Δ*pkaC1* is compatible with reports of increased virulence-related phenotypes, such as germination rate, in *ags3* deletion mutants. Taken as a whole, these data demonstrate that interaction of PkaC1 and PkaC2 plays a critical role in controlling processes that contribute to the success of *A. fumigatus* as an opportunistic pathogen.

Discussion

In order to successfully establish an invasive infection, *A. fumigatus* must adjust to and grow within the milieu of the mammalian lung. Although previous studies have implicated the cAMP–PKA pathway in this adaptation (Rhodes *et al*., 2001; Oliver *et al*., 2002a; Liebmann *et al*., 2003; 2004), the relevant processes controlled by PKA within the host remain incompletely understood. A major challenge in understanding PKA signalling in fungi is that the pathway cannot be easily defined through a single catalytic subunit. Rather, most organisms, including all filamentous fungi examined to date, contain multiple isoforms that may display both overlapping and unique functions. *A. fumigatus* contains a divergent PKA isoform, PkaC2, but its contribution to growth and pathogenesis is unknown. In this report, we demonstrate that PkaC2 acts in concert with PkaC1 to support germination, cell wall homeostasis and carbohydrate catabolism. Furthermore, these functions likely account for the ability of PkaC2 to strongly influence the attenuated virulence of the Δ*pkaC1* mutants of *A. fumigatus*, improving our insight into the PKA-dependent processes required for pathogenesis.

Similar to the Δ*pkaB* mutant of *A. nidulans* (Ni *et al*., 2005), the Δ*pkaC2* strain of *A. fumigatus* displayed no discernable phenotype, indicating that the class II isoform is dispensable for WT growth *in vitro*. Surprisingly, we were able to delete both *pkaC1* and *pkaC2*, demonstrating that a complete loss of PKA activity is also dispensable in *A. fumigatus*. Since PKA represents an essential pathway among ascomycetous fungi that have been tested, including *A. nidulans*, it is interesting to speculate that *A. fumigatus* has developed novel signalling redundancies that allow other pathways to suffice in the absence of PKA (Toda *et al*., 1987; Bockmühl *et al*., 2001; Ni *et al*., 2005).

Although *pkaC2* alone was dispensable for growth, its deletion or overexpression modulated various phenotypes of the Δ*pkaC1* mutant, thereby demonstrating the functional capacity of the PkaC2 isoform. Interestingly, however, we did not detect phosphorylation of the synthetic PKA substrate kemptide in any of the strains in which *pkaC1* was deleted (Fig. S7). Loss of kemptide phosphorylation in a Δ*pkaC1* mutant of *A. fumigatus* led previous authors to conclude that PkaC2 is nonfunctional, perhaps due to neutral mutations in the nucleotide-binding loop (Liebmann *et al*., 2004;Grosse *et al*., 2008). However, an *S. cerevisiae TPK1*/*TPK2* deletion strain (*tpk1tpk2TPK3*) similarly failed to display kemptide phosphorylation, despite the capability of Tpk3p activity alone to support normal vegetative growth (Mazon *et al*., 1993). This result, along with our data, suggests that the level of kinase activity required to detect phosphorylation of the kemptide substrate *in vitro* does not predict physiologic relevance *in vivo*.

If the results of the kemptide assay are indicative of reduced intrinsic kinase activity of the PkaC2 isoform, what then can account for this finding? One possibility is that PkaC2, and the fungal class II kinases in general, are slowly losing function over evolutionary time. In *Cryptococcus neoformans* (serotype A), the loss of function of the Pka2 isoform illustrates this point (Hicks *et al*., 2004). After duplication of the ancestral gene, it has been proposed that one allele is under selective pressure to maintain its function, whereas the other is free to accumulate mutations and lose activity, ultimately becoming a pseudogene (Lynch and Conery, 2000; Hicks and Heitman, 2007; Hahn, 2009). Comparative sequence analysis among various class II isoforms reveals conserved point mutations in the nucleotide-binding loop (S201 to T86 and G203 to A88), mentioned above, and in the catalytic site (I311 to V201 and L320 to I211), both of which result in neutral amino acid changes (Fig. 1). Although the impact of these conservative mutations has not been substantiated experimentally, they may explain, at least in part, the reduced activity of these class II kinases. Another intriguing possibility is that PkaC2 acts upon a unique phosphorylation site and therefore maintains a separate phosphoproteome from that of PkaC1. Although perhaps less likely, based on overlapping functions described herein, experiments are currently underway to explore this exciting possibility.

The proper maintenance of the cell wall is a crucial aspect of fungal morphogenesis and stress resistance (Latgé, 2007). Our data demonstrate that both PKA isoforms contribute to the regulation of cell wall homeostasis in *A. fumigatus*. To our knowledge, this is the first report describing the involvement of PKA in cell wall homeostasis of a filamentous fungus. In both *S. cerevisiae* and *Candida albicans*, constitutive cAMP–PKA signalling, resulting from deletion of the high-affinity phosphodiesterase *PDE2*, leads to altered expression of genes involved in cell wall biogenesis and architecture. Consequently, these *pde2*Δ mutants demonstrate hypersensitivity to several cell wall modulating agents (Jones *et al*., 2003; Jung *et al*., 2005). Although we did not detect a cell wall phenotype in the *A. fumigatus* Δ*pkaR* mutant, which also displays constitutive PKA activity (data not shown), we do show that reduced PKA signalling leads to hypersensitivity to cell wall agents (Fig. 4). This suggests that PKA is a positive regulator of cell wall homeostasis in *A. fumigatus*, and that the

relationship between PKA and the cell wall is fundamentally different between this organism and *S. cerevisiae*.

The basis for the cell wall defect among the PKA-deficient strains is incompletely understood. However, we demonstrate that the central role for PKA in carbohydrate metabolism may be an important contributing factor. Recently, an *A. fumigatus* mutant deficient in the glucose phosphorylating enzymes, glucokinase and hexokinase, was shown to be hypersensitive to cell wall perturbation, thereby underscoring the relationship between glucose utilization and the cell wall (Fleck and Brock, 2010). In the case of the PKA mutants, however, there was no detectable difference in hexokinase activity between the WT and the mutants lacking PkaC1 or PkaC1 and PkaC2 (data not shown). Furthermore, efforts to relieve the cell wall stress by providing exogenous *N*-acetyl glucosamine were not successful, which suggests that limiting quantities of amino sugars were not responsible for the cell wall defect. Indeed, other than osmotic stabilization, only high levels of glucose were able to ameliorate cell wall hypersensitivity in the PKA mutants (Fig. 4). In addition, it is interesting to speculate that the cAMP–PKA pathway co-ordinately regulates the response to cell wall stress along with the classical cell wall integrity (PKC–MAP kinase) pathway (Valiante *et al*., 2008), since both pathways may be activated by similar upstream signals, e.g. nutrients, and can regulate similar downstream processes, such as morphogenesis (Pandey *et al*., 2004; Xue *et al*., 2004; Reyes *et al*., 2006).

The cell wall phenotypes associated with the PKA mutants of *A. fumigatus* may contribute to their attenuated virulence. A variety of *A. fumigatus* mutants harbouring mutations of specific cell wall biosynthetic genes, including a glucanosyltransferase and an α-mannosyltransferase, display cell wall abnormalities that ultimately manifest as changes in virulence (Mouyna *et al*., 2005; Wagener *et al*., 2008). Although these examples illustrate that cell wall aberrations may be detrimental for the fungus during infection, the precise relationship between the cell wall and *A. fumigatus* virulence is unclear. For instance, several glucan and chitin synthase mutants with morphological defects, as well as a MAPK mutant that is hypersensitive to cell wall stress, were all reported to have WT-like virulence (Aufauvre-Brown *et al*., 1997; Beauvais *et al*., 2005; Valiante *et al*., 2008). Indeed, cell wall mutant strains of *A. fumigatus* in which *ecm33* or *ags3* have been deleted or downregulated actually have increased virulence-related properties, when compared with WT (Maubon *et al*., 2006; Romano *et al*., 2006; Ejzykowicz *et al*., 2009). The increased level of *ags3* message seen in the Δ*pkaC1* mutant would be compatible with the decreased virulence seen in strains lacking PkaC1, suggesting that loss of cell wall homeostasis may contribute to the pathogenic defect in these strains.

The germination of inhaled conidia represents the first essential step in the development of IA. Our data revealed that PkaC2 signalling alone is sufficient to support germination in *A. fumigatus*, as a germination defect was only observed in the Δ*pkaC1*Δ*pkaC2* mutant. Therefore, a major impact of PkaC2 on virulence could be through its involvement in germination. The trehalose pathway has recently been linked to virulence in *A. fumigatus*, and since trehalose is the primary storage carbohydrate in the conidia, it is possible that PKA may influence germination (d'Enfert *et al*., 1999; Al-Bader *et al*., 2010; Puttikamonkul *et al*., 2010). Although trehalose levels in conidia were similar among the PKA mutants (Fig. S2), we are unable to rule out the role that regulation of the neutral trehalase by PKA phosphorylation may play in trehalose catabolism, which provides glucose to drive early steps in germination (d'Enfert *et al*., 1999). Indeed, PKA phosphorylation has been shown to regulate the rate of germination in *A. nidulans*. So, whereas fungal foci were visible in WT, Δ*pkaC1* and Δ*pkaC1::PgpdA-pkaC2*-infected groups within 24 h post infection, no growth was observed in the Δ*pkaC1*Δ*pkaC2*-infected mice until 48 h, suggesting that germination was delayed, but not blocked. However, since 48 h also correlated with the influx of

neutrophils at sites of fungal growth, it is likely that residual or recovering immune effector cells are better able to clear the germlings of Δ*pkaC1*Δ*pkaC2*, relative to the other strains that have established a more advanced infection.

Following germination, *A. fumigatus* must utilize nutrients available within the host environment. Indeed, transcriptional profiling of germlings recovered from murine bronchoalveolar lavage fluid demonstrated an upregulation of genes involved in carbohydrate transport and a downregulation of respiratory genes (McDonagh *et al*., 2008), which supports the critical role for metabolic reprogramming of carbohydrate utilization *in vivo*. We show here that the Δ*pkaC1*Δ*pkaC2* mutant has reduced message level of *pdbA*, a gene encoding the beta subunit of pyruvate dehydrogenase, which catalyses oxidative decarboxylation of pyruvate to acetyl-coA, thus linking glycolysis with the citric acid cycle. The effect on *pbdA*, then, suggests that both PKA isoforms function to regulate flow through the carbohydrate catabolic pathway. This finding is consistent with the inability of the Δ*pkaC1*Δ*pkaC2* mutant to grow in the presence of low glucose concentrations, such as what would be expected within the lung (de Prost and Saumon, 2007). Moreover, although the Δ*pkaC1::PgpdA-pkaC2* strain grew better than the Δ*pkaC1* mutant on glucose, all three mutants grew equally well on acetate. The large disparity in *in vivo* fungal burden among the mutants may reflect that the fungus must use glucose within the host. Since recent evidence suggests that *A. fumigatus* is under hypoxic stress *in vivo* (Willger *et al*., 2008), limiting oxygen levels may lead to downregulation of respiratory pathways that increases the reliance upon glycolysis for ATP generation. Thus, the infected lung likely provides an environment of reduced glucose and reduced oxygen, in which the disparity in growth rates between Δ*pkaC1*, Δ*pkaC1*Δ*pkaC2* and Δ*pkaC1::PgpdA-pkaC2* would be accentuated.

The capacity of *A. fumigatus* and other pathogenic fungi to grow rapidly at 37°C is considered to be an essential virulence trait. Indeed, *in vitro* growth rate has been shown to correlate with virulence in a number of studies (Latgé, 2001; Rhodes *et al*., 2001; Paisley *et al*., 2005). It is therefore striking that the mortality curve of the Δ*pkaC1::PgpdA-pkaC2* strain was indistinguishable from WT, whereas the Δ*pkaC1*Δ*pkaC2* was avirulent, even though both strains have significantly impaired growth phenotypes. This observation adds the Δ*pkaC1::PgpdA-pkaC2* mutant to an emerging list of *A. fumigatus* mutants in which *in vitro* growth rate does not predict the virulence phenotype. For example, the *A. fumigatus* Δ*gpaB* mutant displays WT-like growth *in vitro*, yet is markedly attenuated for virulence (Liebmann *et al*., 2003; 2004). In contrast, mutants of two genes involved in MAPK signalling of *A. fumigatus*, Δ*mpkA* and Δ*sho1*, display WT-like virulence, despite the strong inhibition of radial outgrowth on plates (Ma *et al*., 2008; Valiante *et al*., 2008). Although several scenarios could be constructed to explain these discrepant results, we favour one in which growth in the host environment is the critical determinant of virulence. This is consistent with our observation that the fungal burden of the Δ*pkaC1::PgpdA-pkaC2* mutant is indistinguishable from that of the WT. Because our *in vitro* systems are, at very least, inaccurate predictors of the *in vivo* environment, the *in vitro* growth phenotype alone does not necessarily predict virulence. Taken together, our results suggest that PkaC2, in cooperation with PkaC1, regulates additional processes that are critical to growth within the host.

In conclusion, we have characterized the relative contributions of the two PKA isoforms, PkaC1 and PkaC2, in the mould pathogen *A. fumigatus*. We show that whereas PkaC1 is the predominant isoform with respect to radial growth and asexual development, PkaC2 maintains important roles in regulating germination, cell wall homeostasis and carbohydrate metabolism. Furthermore, the processes controlled by PkaC1 and PkaC2 play a critical role in adaptation to the host niche, and have expanded upon our understanding of how PKA contributes to pathogenesis. Future studies analysing the downstream targets of the two

isoforms and how they may be differentially regulated will provide important insight into the complex and important pathway.

Experimental procedures

Strains and culture conditions

Aspergillus fumigatus strain H237, a clinical isolate, was used to generate the *pkaC1* and *pkaC2* deletion strains. Conidia were harvested from osmotically stabilized medium (OSM), which consisted of *Aspergillus* minimal medium (AMM) (Cove, 1966) supplemented with 1.2 M sorbitol. Colony images were taken after 72 h incubation at 37°C on either AMM or OSM media, as indicated.

Construction of the various mutant strains

All primers used for vector construction are listed in Table S1 and all products were amplified using PFU Turbo polymerase (Stratagene).

The *A. fumigatus pkaC2* gene was deleted using the splitmarker method (Catlett *et al*., 2002). Briefly, the first two-thirds of the phleomycin resistance cassette were amplified from pBCphleo using primers 398 and 408. This product was combined with the *pkaC2* left arm product by overlap PCR using primers 100 and 408 to yield the left deletion construct. The second two-thirds of the phleomycin cassette were amplified using primers 410 and 409. This product was combined with the *pkaC2* right arm product with primers 410 and 104 to generate the right deletion construct. Both the left and right deletion constructs were cloned separately into pCR-Blunt II-TOPO (Invitrogen). The inserts were liberated from their plasmids using an EcoRI and NotI/SpeI digestions respectively. Ten micrograms of each was used for transformation of H237 protoplasts, as previously described (Bhabhra *et al*., 2004). The probe used for the Southern blot was amplified from H237 genomic DNA with primers 109 and 110.

The *pkaC1* gene was similarly deleted in both the H237 and Δ*pkaC2* strains by a split-marker approach. The first two-thirds of the hygromycin resistance cassette were amplified form pan7-1 using primers 398 and 395. This product was combined with the *pkaC1* left arm product by overlap PCR using primers 105 and 395 to generate the left deletion construct. The second two-thirds of the hygromycin cassette were amplified using 396 and 399. This product was combined with the *pkaC1* right arm using primers 396 and 108 to generate the right deletion construct. Both the left and right deletion constructs were cloned into pCR-Blunt II-TOPO (Invitrogen). Following amplification from their respective plasmids, 10 μg of each insert was co-transformed into the H237 WT strain to generate the Δ*pkaC1* mutant or the Δ*pkaC2* strain to generate the Δ*pkaC1*Δ*pkaC2* mutant. The probe used for the Southern blots was isolated by digesting the *pkaC1* right deletion plasmid with BamHI and HindIII and purifying the 568 bp fragment.

The construct for the overexpression of *pkaC2* was generated by overlap PCR of three products. The first product is the *pkaC2* left arm, which was amplified with primers 201 and 202 from genomic DNA. The second product is the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*), which was amplified from pBC-phleo with primers 203 and 204. Primers 202 and 203 contain 20 base pairs of reverse complementarity to mediate fusion of the two pieces by PCR. The third product is the *pkaC2* ORF amplified with primers 205 and 206 from genomic DNA. Primers 205 and 204 contain a 20-base-pair region of reverse complementarity to mediate fusion of the two pieces by PCR. All three products were fused by a single overlap PCR reaction with primers 201 and 206. The overlap construct was cloned into pCR-Blunt II-TOPO. Ten micrograms of linearized plasmid (SmaI digest) along with 1 μg of linearized p402R (containing the phleomycin

resistance cassette) was co-transformed into protoplasts of the Δ*pkaC1* mutant. Phleomycin resistance colonies were screened for the presence of the construct with primers 203 and 206.

Analyses of conidial germination and mitochondrial activation

For the germination assay, 5.0×10^5 conidia ml^{-1} were inoculated onto coverslips in 3 ml of YG (2% glucose, 0.5% yeast extract) or AMM and then incubated at 37°C. At the indicated time points, coverslips were mounted onto a microscope slide and a total of 200 conidia were scored for the presence or absence of a germ tube. Experiments were repeated with similar results.

To analyse mitochondrial activation, conidia were stained with Mitotracker Orange CM-H2 TMRos (Invitrogen), as previously described (Fuller *et al*., 2009). Briefly, conidia were inoculated onto coverslips in YG medium and incubated for the indicated times at 37°C. Following incubation, the medium was aspirated and the samples were then stained (Fuller *et al*., 2009). Samples were observed by fluorescence microscopy (TRITC filter) and images were taken with Adobe ImageReady 7.0.1 and analysed with Adobe Photoshop 6.0 software.

Asexual developmental studies

Conidia were streaked onto AMM plates and incubated at 37°C for several days. Total conidia were harvested from the plates, diluted and counted with a haemocytometer. The experiment was performed in triplicate and mean values were analysed statistically with a Student's *t*-test.

To analyse conidiophore morphology, conidia were inoculated along the sides of AMM plugs and a coverslip was placed on top of the plug. The samples were then incubated at 37°C for 48 h. Following incubation, coverslips were photographed (40×) using differential interference contrast (DIC) microscopy. Images were analysed using Adobe Photoshop 6.0.

Susceptibility assays to cell wall modulating agents

Two microlitres of a suspension of 4.0×10^3 conidia was point-inoculated across wells that contained the indicated concentrations of Congo red or SDS inAMM solid medium. In some experiments, the concentration of glucose in the medium was increased to 10%. Plates were incubated for 3 days at 37°C.

Analysis of growth on different carbon sources

To determine radial growth rates on the various carbon sources, 1.0×10^4 conidia in a 5 ml suspension were point-inoculated onto AMM plates that contained 1% (w/v) dextrose, fructose or sodium acetate or 1% (v/v) ethanol. The plates were made with 1.5% noble agar (Difco Laboratories) to reduce potential utilization of the agar components as a nutrient source. Plates were incubated at 37°C and colony diameter was measured every 24 h. Experiments were performed in triplicate and changes in colony diameter between 24 h and 48 h were subject to statistical analysis (Student's *t*-test).

To analyse the ability of the strains to grow on reducing carbon concentrations, 1.0×10^4 conidia were spotted onto AMM plates that contained the indicated concentrations (w/v) of dextrose or sodium acetate. The media contained 1.5% agarose. Plates were incubated for 3 days at 37°C. The WT organism was included in the study and could grow on all carbon sources and concentrations tested. However, growth was diffuse and was difficult to visualize by photography.

Trehalose concentration in conidia

Conidia were harvested from AMM plates and counted in a haemocytometer. Equal quantities were heated at 100°C for 20 min to lyse the conidia. The concentration of trehalose, as glucose, was measured in the conidial lysate, as described by Puttikamonkul *et al*. (2010).

RNA isolation and quantitative RT-PCR analysis

Strains were grown overnight (13–16 h) in YG media at 37°C, shaking at 200 r.p.m. Total RNA was isolated (RNeasy minikit, Qiagen) from vacuum-filtered mycelia that were crushed with liquid nitrogen. Five micrograms of total RNA were then DNased (RQ1 Dnase, Qiagen) for 45 min at 37°C. Following the DNase incubation, 6 μl of sample was used for first-strand cDNA synthesis (Superscript III, Invitrogen) or for a no reverse transcriptase control. For quantitative PCR analysis of *pdbA* or *ags3* mRNA levels, 1 μl of the cDNA was used in a 25 μl Sybr Green reaction (SYBR GreenER, Invitrogen) (SmartCycler II, Cepheid). ΔCt values for each strain were determined based on the mean of three to four separate quantitative PCR runs. The relative expression levels are based on the $2^{-\Delta\Delta Ct}$ values comparing the each mutant strain with WT. Error bars are based on standard error for the ΔCt values of the mutant strain. Statistical analysis was performed comparing the ΔCt values by Student's *t*-test. Primers used are listed in Table S1.

Virulence studies

Conidia were harvested from OSM plates and viable conidia were enumerated by dilution plating and colony-forming unit (cfu) counting. Samples were then diluted in sterile saline prior to inoculation. Groups of 12–16 CF-1 outbred, female mice (20–28 g) were immunosuppressed with a dose of 40 mg kg^{-1} triamcinolone acetonide (Kenalog-10, Bristol-Myers Squibb) subcutaneously on day −1 and with a dose of 20 mg kg^{-1} on day +4. The mice were anaesthetized with 3.5% isofluorane and inoculated intranasally with 1.0×10^5 viable conidia in a 20 μl suspension. Mortality was monitored for 12 days and statistical analysis was assessed by the log rank test using Sigma Stat 3.5. For testing of additional strains of Δ*pkaC1*Δ*pkaC2*, groups of eight were used.

Histopathology and morphometric analysis of fungal burden

For the histopathology studies, additional groups of CF-1, female mice were infected as described above and sacrificed at the 0, 1, 2 or 3 days post infection ($n = 2$ mice per group per day). Lungs were removed and inflated with 10% phosphate-buffered formalin on the day of sacrifice. Lung tissues were then embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) or Grocott's methenamine silver (GMS). All pictures are at 10×.

Semi-quantitative analysis of fungal burden was performed by morphometric analysis of the histological sections described above. First, the GMS sections of all strains at the 72 h time point were randomly assigned a number by an outside person, such that the investigator was blinded to the strain associated with the section. Second, the sections were observed by microscopy and all regions that contained observable hyphae were photographed at 10× magnification. The photomicrographs were then analysed using the Volocity 4 software (Improvision). Briefly, a selection tool was used to outline the hyphae (stained black) and the area of the selections was calculated to determine the hyphal area associated with each photomicrograph. The hyphal areas associated with a particular section were then added together to give the total hyphal area of the section. The average area associated with WT was set to 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Jay Card for his excellent assistance with photography. We also thank the Pathology Research Core at Cincinnati Children's Hospital Medical Center for the help with histology. We acknowledge support from the National Institutes of Health (J.C.R. – AI061497, D.S.A. – AI072297, K.A.W.-B. – HL079193, CA102357), the American Cancer Society (K.A.W.-B. – RSG-10-194-01-TBG) and St. Baldrick's Foundation (K.A.W.-B. – 18033).

References

Adachi K, Hamer JE. Divergent cAMP signaling pathways regulate growth and pathogenesis in the rice blast fungus *Magnaporthe grisea*. Plant Cell. 1998; 10:1361–1374. [PubMed: 9707535]

Al-Bader N, Vanier G, Liu H, Gravelat FN, Urb M, Hoareau CM-Q, et al. Role of trehalose biosynthesis in *Aspergillus fumigatus* development, stress response, and virulence. Infect Immun. 2010; 78:3007–3018. [PubMed: 20439478]

Askew DS. *Aspergillus fumigatus*: virulence genes in a street-smart mold. Curr Opin Microbiol. 2008; 11:331–337. [PubMed: 18579432]

Aufauvre-Brown A, Mellado E, Gow NAR, Holden DW. *Aspergillus fumigatus chse*: a gene related to *chs3* of *Saccharomyces cerevisiae* and important for hyphal growth and conidiophore development but not pathogenicity. Fungal Genet Biol. 1997; 21:141–152. [PubMed: 9126623]

Beauvais A, Maubon D, Park S, Morelle W, Tanguy M, Huerre M, et al. Two alpha(1–3) glucan synthases with different functions in *Aspergillus fumigatus*. Appl Environ Microbiol. 2005; 71:1531–1538. [PubMed: 15746357]

Beffa T, Staib F, Lott Fischer J, Lyon P, Gumowski P, Marfenina O, et al. Myl and surveillance of biological waste and compost. Med Mycol. 1998; 36 Suppl. I:137–145. [PubMed: 9988502]

Bhabhra R, Miley MD, Mylonakis E, Boettner D, Fortwendel J, Panepinto JC, et al. Disruption of the *Aspergillus fumigatus* gene encoding nucleolar protein CgrA impairs thermotolerant growth and reduces virulence. Infect Immun. 2004; 72:4731–4740. [PubMed: 15271935]

Bockmühl DP, Krishnamurthy S, Gerads M, Sonneborn A, Ernst JF. Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of *Candida albicans*. Mol Microbiol. 2001; 42:1243–1257. [PubMed: 11886556]

Catlett NL, Lee BN, Yoder OC, Turgeon BG. Split-marker recombination for efficient targeted deletion of fungal genes. Fungal Genet Newsl. 2002; 50:9–11.

Chen D, Janganan TK, Chen G, Marques ER, Kress MR, Goldman GH, et al. The cAMP pathway is important for controlling the morphological switch to the pathogenic yeast form of *Paracoccidioides brasiliensis*. Mol Microbiol. 2007; 65:761–779. [PubMed: 17635191]

Cove D. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochim Biophys Acta. 1966; 113:51–56. [PubMed: 5940632]

D'Souza CA, Heitman J. Conserved cAMP signaling cascades regulate fungal development and virulence. FEMS Microbiol Rev. 2001; 25:349–364. [PubMed: 11348689]

D'Souza CA, Alspaugh JA, Yue C, Harashima T, Cox GM, Perfect JR, Heitman J. Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. Mol Cell Biol. 2001; 21:3179–3191. [PubMed: 11287622]

Dürrenberger F, Wong K, Kronstad JW. Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis*. Proc Natl Acad Sci USA. 1998; 95:5684–5689. [PubMed: 9576944]

Ejzykowicz DE, Cunha MM, Rozental S, Solis NV, Gravelat FN, Sheppard DC, Filler SG. The *Aspergillus fumigatus* transcription factor Ace2 governs pigment production, conidiation and virulence. Mol Microbiol. 2009; 72:155–169. [PubMed: 19220748]

d'Enfert C. Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. Fungal Genet Biol. 1997; 21:163–172.

d'Enfert C, Bonini BM, Zapella PDA, Fontaine T, da Silva AM, Terenzi HF. Neutral trehalases catalyse intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. Mol Microbiol. 1999; 32:471–483. [PubMed: 10320571]

Fleck CB, Brock M. *Aspergillus fumigatus* catalytic glucokinase and hexokinase: expression analysis and importance for germination, growth, and conidiation. Eukaryot Cell. 2010; 9:1120–1135. [PubMed: 20453072]

Fortwendel JR, Fuller KK, Stephens TJ, Bacon WC, Askew DS, Rhodes JC. *Aspergillus fumigatus* RasA regulates asexual development and cell wall integrity. Eukaryot Cell. 2008; 7:1530–1539. [PubMed: 18606827]

Fuller KK, Zhao W, Askew DS, Rhodes JC. Deletion of the protein kinase A regulatory subunit leads to deregulation of mitochondrial activation and nuclear duplication in *Aspergillus fumigatus*. Eukaryot Cell. 2009; 8:271–277. [PubMed: 19124579]

Gold S, Duncan G, Barrett K, Kronstad J. cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. Genes Dev. 1994; 8:2805–2816. [PubMed: 7995519]

Grosse C, Heinekamp T, Kniemeyer O, Gehrke A, Brakhage AA. Protein kinase A regulates growth, sporulation, and pigment formation in *Aspergillus fumigatus*. Appl Environ Microbiol. 2008; 74:4923–4933. [PubMed: 18539819]

Hahn M. Distinguishing among evolutionary models for the maintenance of gene duplicates. J Hered. 2009; 5:605–617. [PubMed: 19596713]

van Heerden I, Cronje C, Swart S, Kotze J. Microbial, chemical and physical aspects of citrus waste composting. Bioresour Technol. 2002; 81:71–76. [PubMed: 11708757]

Hicks JK, Heitman J. Divergence of protein kinase A catalytic subunits in *Cryptococcus neoformans* and *Cryptococcus gattii* illustrates evolutionary reconfiguration of a signaling cascade. Eukaryot Cell. 2007; 6:413–420. [PubMed: 17189488]

Hicks JK, D'Souza CA, Cox GM, Heitman J. Cyclic AMP-dependent protein kinase catalytic subunits have divergent roles in virulence factor production in two varieties of the fungal pathogen *Cryptococcus neoformans*. Eukaryot Cell. 2004; 3:14–26. [PubMed: 14871933]

Jones DL, Petty J, Hoyle DC, Hayes A, Ragni E, Popolo L, et al. Transcriptome profiling of a *Saccharomyces cerevisiae* mutant with a constitutively activated ras/cAMP pathway. Physiol Genomics. 2003; 16:107–118. [PubMed: 14570984]

Jung WH, Warn P, Ragni E, Popolo L, Nunn CD, Turner MP, Stateva L. Deletion of *PDE2*, the gene encoding the high-affinity cAMP phosphodiesterase, results in changes of the cell wall and membrane in *Candida albicans*. Yeast. 2005; 22:285–294. [PubMed: 15789349]

Latge J-P. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev. 1999; 12:310–350. [PubMed: 10194462]

Latgé J-P. The pathobiology of *Aspergillus fumigatus*. Trends Microbiol. 2001; 9:382–389. [PubMed: 11514221]

Latgé J-P. The cell wall: a carbohydrate armour for the fungal cell. Mol Microbiol. 2007; 66:279–290. [PubMed: 17854405]

Liebmann B, Gattung S, Jahn B, Brakhage AA. cAMP signaling in *Aspergillus fumigatus* is involved in the regulation of the virulence gene *pksP* and in defense against killing by macrophages. Mol Genet Genomics. 2003; 269:420–435. [PubMed: 12734751]

Liebmann B, Muller M, Braun A, Brakhage AA. The cyclic AMP-dependent protein kinase a network regulates development and virulence in *Aspergillus fumigatus*. Infect Immun. 2004; 72:5193–5203. [PubMed: 15322014]

Lynch M, Conery J. The evolutionary fate and consequences of duplicate genes. Science. 2000; 290:1151–1155. [PubMed: 11073452]

Ma Y, Qiao J, Liu W, Wan Z, Wang X, Calderone R, Li R. The Sho1 sensor regulates growth, morphology, and oxidant adaptation in *Aspergillus fumigatus* but is not essential for development of invasive pulmonary aspergillosis. Infect Immun. 2008; 76:1695–1701. [PubMed: 18227163]

McDonagh A, Fedorova N, Crabtree J, Yu Y, Kim S, Chen D, et al. Sub-telomere directed gene expression during initiation of invasive aspergillosis. PLoS Pathog. 2008; 4:e1000154. [PubMed: 18787699]

Maeda H, Sano M, Maruyama Y, Tanno T, Akao T, Totsuka Y, et al. Transcriptional analysis of genes for energy catabolism and hydrolytic enzymes in the filamentous fungus *Aspergillus oryzae* using cDNA microarrays and expressed sequence tags. Appl Environ Microbiol. 2004; 65:74–83.

Maubon D, Park S, Tanguy M, Huerre M, Schmitt C, Prévost MC, et al. *ags3*, an a(1–3)glucan synthase gene family member of *Aspergillus fumigatus*, modulates mycelium growth in the lung of experimentally infected mice. Fungal Genet Biol. 2006; 43:366–375. [PubMed: 16531086]

Mazon M, Behrens M, Morgado E, Portillo F. Low activity of the yeast cAMP-dependent protein kinase catalytic subunit Tpk3 is due to the poor expression of the *TPK3* gene. Eur J Biochem. 1993; 213:501–506. [PubMed: 8386630]

Mouyna I, Morelle W, Vai M, Monod M, Léchenne B, Fontaine T, et al. Deletion of *gel2* encoding for a beta(1–3)glucanosyltransferase affects morphogenesis and virulence in *Aspergillus fumigatus*. Mol Microbiol. 2005; 56:1675–1688. [PubMed: 15916615]

Ni M, Rierson S, Seo J-A, Yu J-H. The *pkaB* gene encoding the secondary protein kinase A catalytic subunit has a synthetic lethal interaction with *pkaA* and plays overlapping and opposite roles in *Aspergillus nidulans*. Eukaryot Cell. 2005; 4:1465–1476. [PubMed: 16087751]

Oliver B, Panepinto J, Fortwendel J, Smith D, Askew D, Rhodes J. Cloning and expression of *pkaC* and *pkaR*, the genes encoding the cAMP-dependent protein kinase of *Aspergillus fumigatus*. Mycopathologia. 2002a; 154:85–91. [PubMed: 12086105]

Oliver BG, Panepinto JC, Askew DS, Rhodes JC. cAMP alteration of growth rate of *Aspergillus fumigatus* and *Aspergillus niger* is carbon-source dependent. Microbiology. 2002b; 148:2627–2633. [PubMed: 12177357]

Paisley D, Robson G, Denning D. Correlation between *in vitro* growth rate and *in vivo* virulence in *Aspergillus fumigatus*. Med Mycol. 2005; 43:397–401. [PubMed: 16178367]

Pan X, Heitman J. Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. Mol Cell Biol. 1999; 19:4874–4887. [PubMed: 10373537]

Pandey A, Roca MG, Read ND, Glass NL. Role of a mitogen-activated protein kinase pathway during conidial germination and hyphal fusion in *Neurospora crassa*. Eukaryot Cell. 2004; 3:348–358. [PubMed: 15075265]

Pearce L, Komander D, Alessi D. The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol. 2010; 11:9–22. [PubMed: 20027184]

Puttikamonkul S, Willger SD, Grahl N, Perfect JR, Movahed N, Bothner B, et al. Trehalose 6-phosphate phosphatase is required for cell wall integrity and fungal virulence but not trehalose biosynthesis in the human fungal pathogen *Aspergillus fumigatus*. Mol Microbiol. 2010; 77:891–911.

de Prost N, Saumon G. Glucose transport in the lung and its role in liquid movement. Respir Physiol Neurobiol. 2007; 159:331–337. [PubMed: 17369109]

Reyes G, Romans A, Nguyen CK, May GS. Novel mitogen-activated protein kinase MpkC of *Aspergillus fumigatus* is required for utilization of polyalcohol sugars. Eukaryot Cell. 2006; 5:1934–1940. [PubMed: 16998074]

Rhodes JC, Oliver BG, Askew DS, Amlung TW. Identification of genes of *Aspergillus fumigatus* up-regulated during growth on endothelial cells. Med Mycol. 2001; 39:253–260. [PubMed: 11446528]

Richie D, Hartl L, Aimanianda V, Winters M, Fuller K, Miley M, et al. A role for the unfolded protein response (upr) in virulence and antifungal susceptibility in *Aspergillus fumigatus*. PLoS Pathog. 2009; 5:e1000258. [PubMed: 19132084]

Robertson LS, Fink GR. The three yeast A kinases have specific signaling functions in pseudohyphal growth. Proc Natl Acad Sci USA. 1998; 95:13783–13787. [PubMed: 9811878]

Robertson LS, Causton HC, Young RA, Fink GR. The yeast A kinases differentially regulate iron uptake and respiratory function. Proc Natl Acad Sci USA. 2000; 97:5984–5988. [PubMed: 10811893]

Romano J, Nimrod G, Ben-Tal N, Shadkchan Y, Baruch K, Sharon H, Osherov N. Disruption of the *Aspergillus fumigatus* ECM33 homologue results in rapid conidial germination, antifungal resistance and hypervirulence. Microbiology. 2006; 152:1919–1928. [PubMed: 16804168]

Thevelein JM, Geladé R, Holsbeeks I, Lagatie O, Popova Y, Rolland F, et al. Nutrient sensing systems for rapid activation of the protein kinase A pathway in yeast. Biochem Soc Trans. 2005; 33:253–256. [PubMed: 15667319]

Toda T, Cameron S, Sass P, Zoller M, Wigler M. Three different genes in *S. cerevisiae* encode the catalytic subunit of the cAMP-dependent protein kinase. Cell. 1987; 50:277–287. [PubMed: 3036373]

Upton A, Kirby KA, Carpenter P, Boeckh M, Marr KA. Invasive aspergillosis following hematopoietic cell transplantation: outcomes and prognostic factors associated with mortality. Clin Infect Dis. 2007; 44:531–540. [PubMed: 17243056]

Valiante V, Heinekamp T, Jain R, Härtl A, Brakhage AA. The mitogen-activated protein kinase MpkA of *Aspergillus fumigatus* regulates cell wall signaling and oxidative stress response. Fungal Genet Biol. 2008; 45:618–627. [PubMed: 17981060]

Wagener J, Echtenacher B, Rohde M, Kotz A, Krappmann S, Heesemann J, Ebel F. The putative {alpha}-1,2-mannosyltransferase AfMnt1 of the opportunistic fungal pathogen *Aspergillus fumigatus* is required for cell wall stability and full virulence. Eukaryot Cell. 2008; 7:1661–1673. [PubMed: 18708564]

Willger S, Puttikamonkul S, Kim K, Burritt J, Grahl N, Metzler L, et al. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. PLoS Pathog. 2008; 4:e1000200. [PubMed: 18989462]

Xie X, Wilkinson HH, Correa A, Lewis ZA, Bell-Pedersen D, Ebbole DJ. Transcriptional response to glucose starvation and functional analysis of a glucose transporter of *Neurospora crassa*. Fungal Genet Biol. 2004; 41:1104–1119. [PubMed: 15531214]

Xue T, Nguyen CK, Romans A, May GS. A mitogen-activated protein kinase that senses nitrogen regulates conidial germination and growth in *Aspergillus fumigatus*. Eukaryot Cell. 2004; 3:557–560. [PubMed: 15075285]

Yang Z, Dickman MB. *Colletotrichum trifolii* mutants disrupted in the catalytic subunit of cAMP-dependent protein kinase are nonpathogenic. Mol Plant Microbe Interact. 1999; 12:430–439. [PubMed: 10226376]

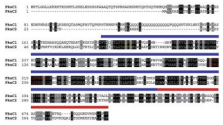

Fig. 1. Sequence analysis of PkaC1 versus PkaC2

The protein sequences of PkaC1 and PkaC2 were aligned using DNAMAN software (Lynnon Corp. Canada) using default parameters and shaded using BOXSHADE 3.21. The blue and red bars span the conserved serine/threonine kinase domain and the conserved AGC domain respectively. The catalytic active site is highlighted in red font and the ATP-binding loop is highlighted in yellow font.

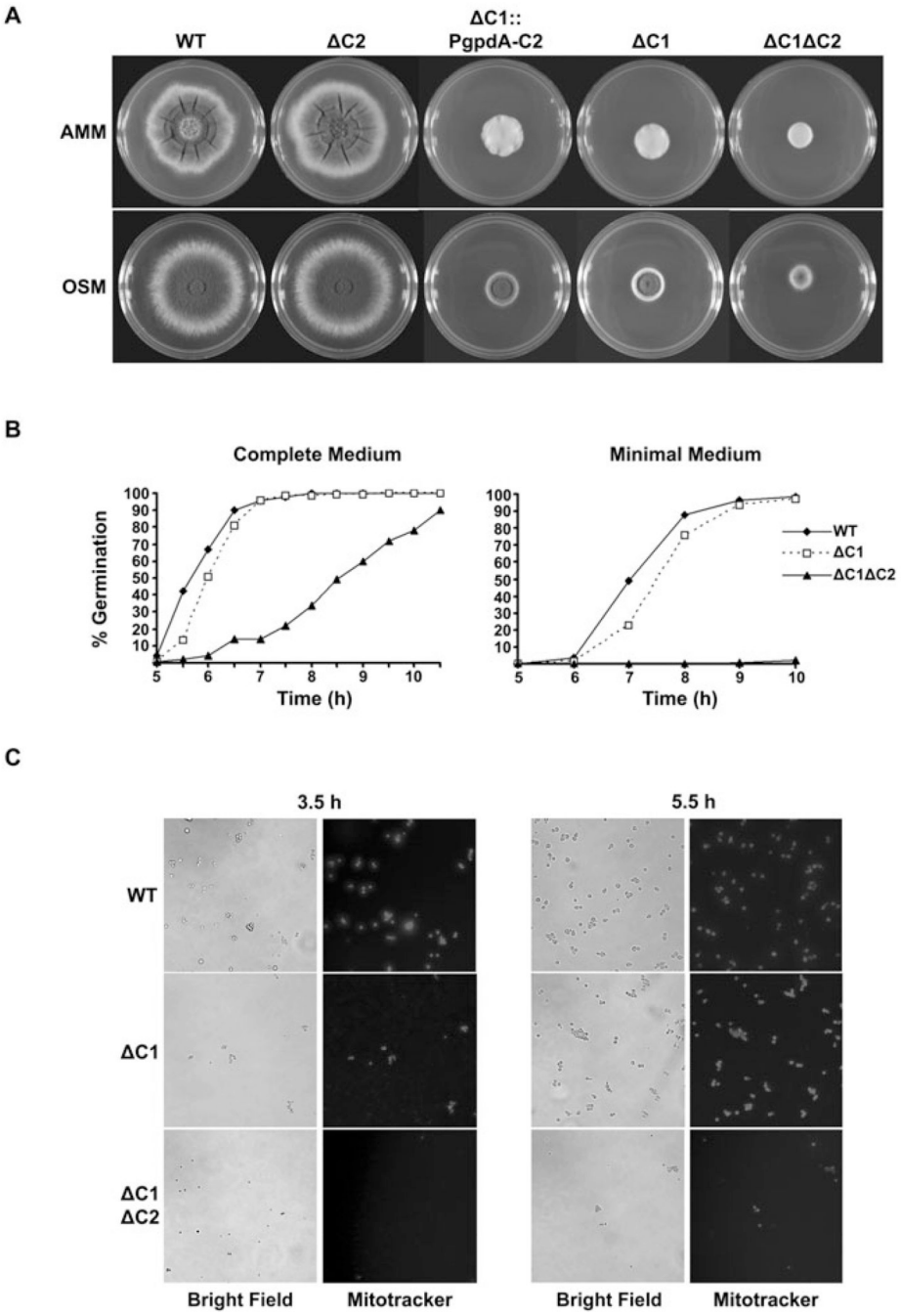

Fig. 2. Growth and germination of the PKA mutants

A. Colony appearance of the PKA mutants grown on AMM or OSM plates for 3 days at 37°C.

B. PkaC1 and PkaC2 cooperatively regulate germination. Conidia were germinated on coverslips in YG (left) or AMM (right) at 37°C. Germ-tube emergence was scored at the indicated times (n = 200).

C. Conidia of the Δ*pkaC1*Δ*pkaC2* mutant are delayed in metabolic activation. Conidia were incubated in YG at 37°C and stained with Mitotracker Orange. Only conidia harbouring active mitochondria fluoresce.

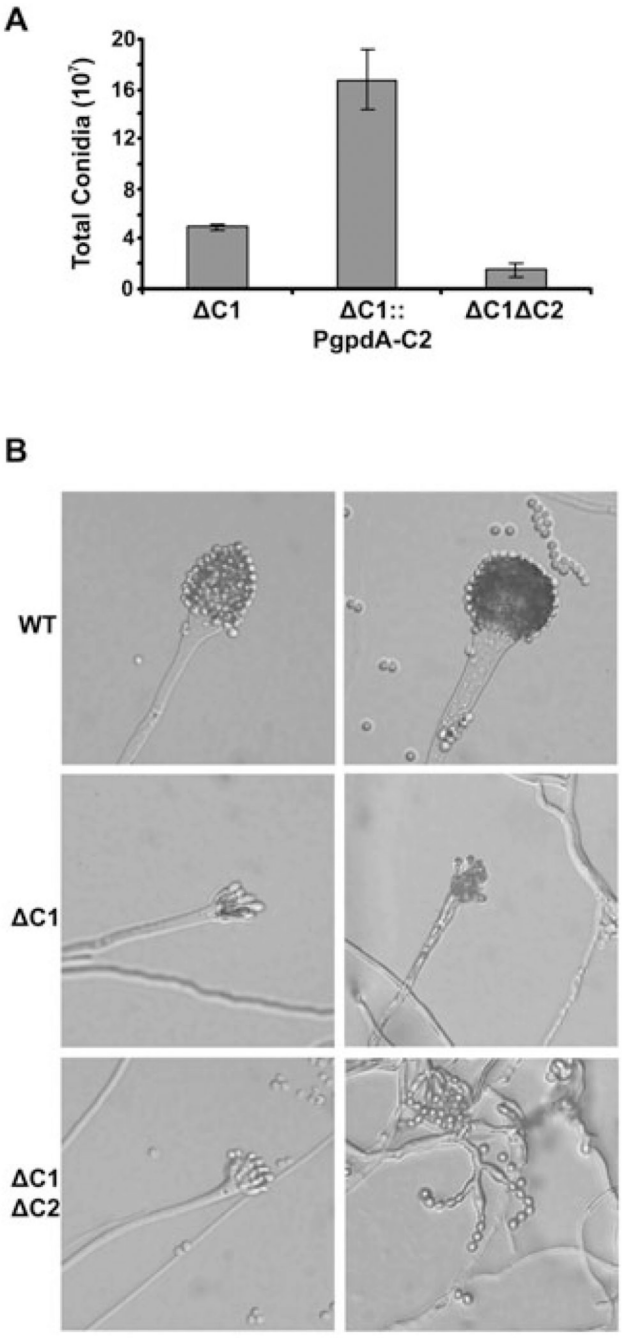

Fig. 3. PkaC1 and PkaC2 positively regulate asexual development

A. *pkaC2* positively affects conidiation of Δ*pkaC1*. Total conidia were harvested from AMM plates incubated at 37°C and were counted with a haemocytometer (mean ± SD from an experiment performed in triplicate).

B. PKA affects conidiophore morphogenesis. DIC images (40×) of conidiophores from 48 h cultures on AMM agar at 37°C.

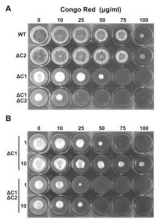

Fig. 4. PKA contributes to cell wall homeostasis

A. Loss of PKA leads to a hypersensitivity to cell wall damage. Conidia were point-inoculated into wells that contained increasing concentrations of Congo red in AMM and were incubated at 37°C for 3 days.

B. Conidia were incubated as above, in the presence of either 1% or 10% glucose.

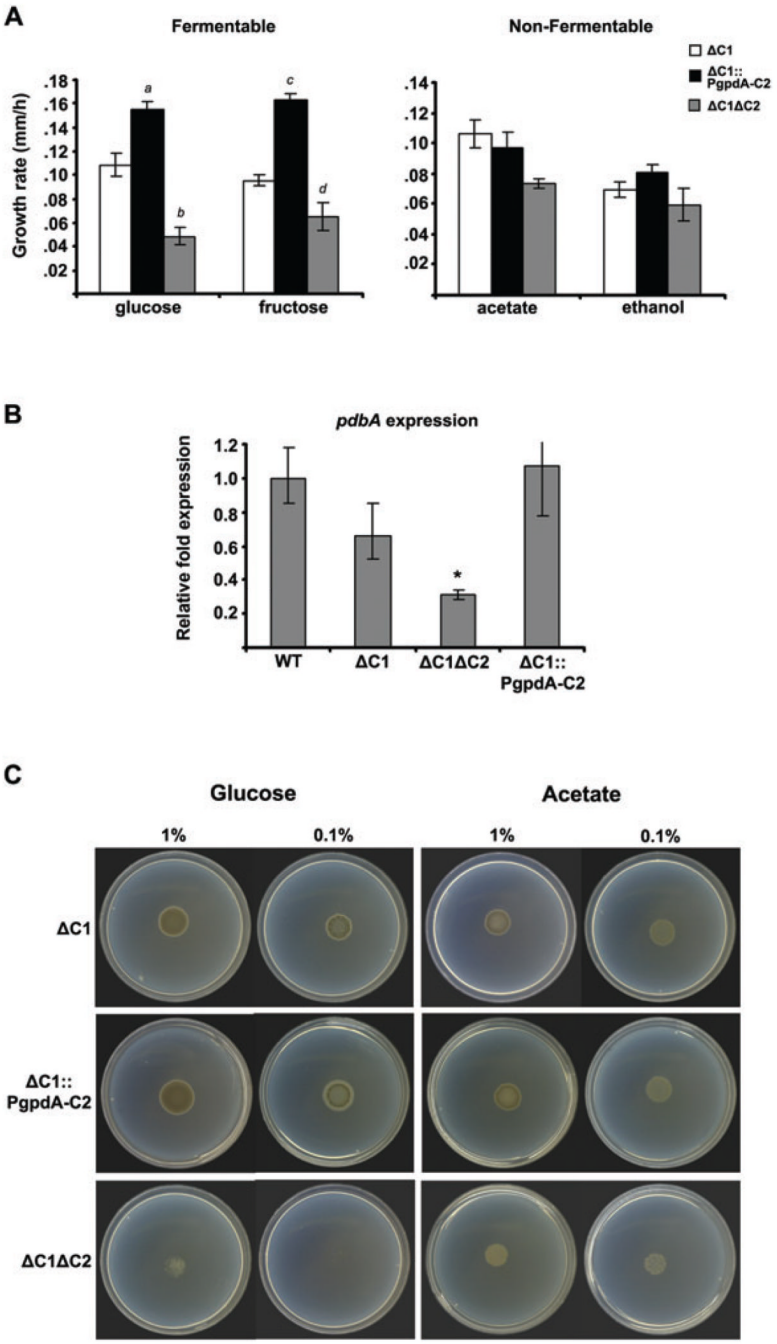

Fig. 5. PkaC1 and PkaC2 regulated carbohydrate catabolism

A. Overexpression of *pkaC2* enhances growth of Δ*pkaC1* on fermentable substrates (1%), whereas deletion of *pkaC2* exacerbates the defect. The data are pooled data from three separate experiments and represent changes in colony diameter between 24 h and 48 h of incubation at 37°C. Error bars represent ± SEM (*a*, Δ*pkaC1* versus Δ*pkaC1::PgpdA-pkaC2* *P*= 0.007; *b*, Δ*pkaC1* versus Δ*pkaC1*Δ*pkaC2* *P* = 0.005; *c*, Δ*pkaC1* versus Δ*pkaC1::PgpdA-pkaC2* *P* < 0.001; *d*, Δ*pkaC1 versus* Δ*pkaC1*Δ*pkaC2* *P* = 0.016).

B. *pdbA* expression is reduced in the Δ*pkaC1*Δ*pkaC2* mutant. Quantitative PCR data are displayed as a fold expression (±SE of ΔCt values calculated across triplicate runs) relative

to WT in hyphae following overnight incubation in YG at 37°C. Data are normalized to the *gpdA* reference control. (**P* = 0.002).

C. The Δ*pkaC1*Δ*pkaC2* mutant is unable to grow on reduced glucose concentrations. Conidia were point-inoculated onto agarose plates containing glucose or acetate and incubated for 3 days at 37°C.

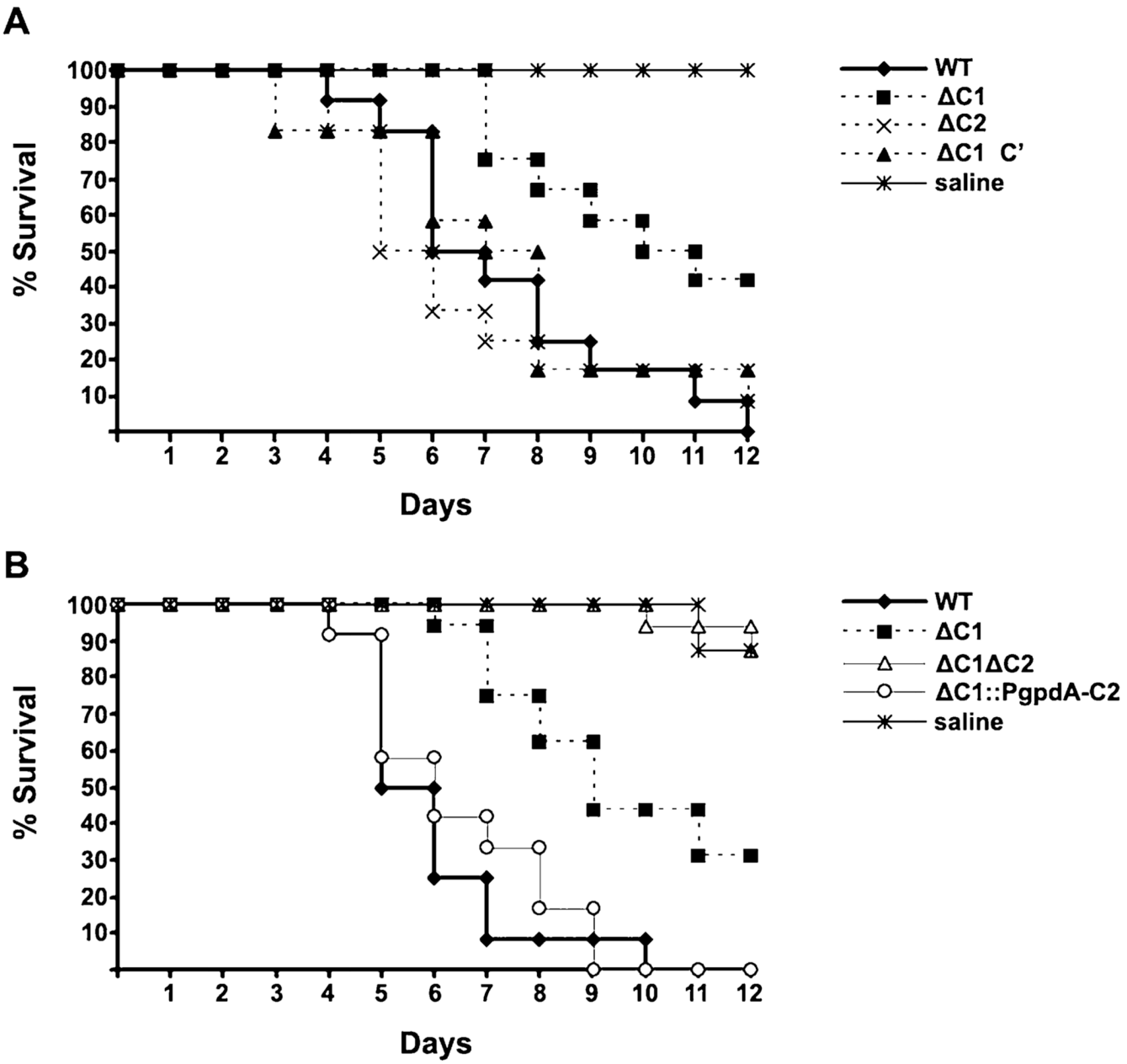

Fig. 6. PkaC2 modulates the virulence of the Δ*pkaC1* mutant

CF-1 outbred mice were immunosuppressed with triamcinolone acetonide and inoculated intranasally with 10^5 conidia.

A. Δ*pkaC1* is attenuated in virulence relative to WT ($P = 0.003$), whereas Δ*pkaC2* and the Δ*pkaC1*-complemented strain (Δ*pkaC1* C′) are indistinguishable from WT.

B. The five strains can be divided into three statistically distinct groups: (i) WT and Δ*pkaC1::PgpdA-pkaC2*, (ii) Δ*pkaC1* and (iii) Δ*pkaC1*Δ*pkaC2* and saline ($n = 12–16$ per group). Pairwise log rank values; WT versus Δ*pkaC1* ($P < 0.001$), WT versus Δ*pkaC1::PgpdA-pkaC2* ($P = 0.6$), Δ*pkaC1* versus Δ*pkaC1*Δ*pkaC2* ($P < 0.001$), Δ*pkaC1*Δ*pkaC2* versus saline ($P = 0.99$).

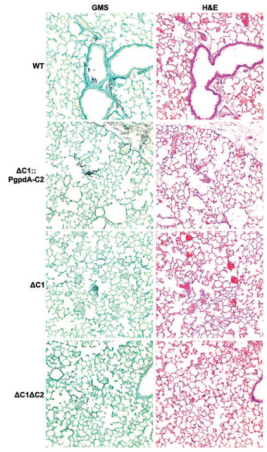

Fig. 7. Histopathology of infected mice at 24 h post infection

CF-1 mice were immunosuppressed with triamcinolone acetonide and inoculated with 2.0×10^5 conidia. Following staining with Gomori's methenamine silver (GMS) and haematoxylin and eosin (H&E), small fungal foci were seen in animals from each of the groups, except those infected with the Δ*pkaC1*Δ*pkaC2* mutant. All images are at 10x.

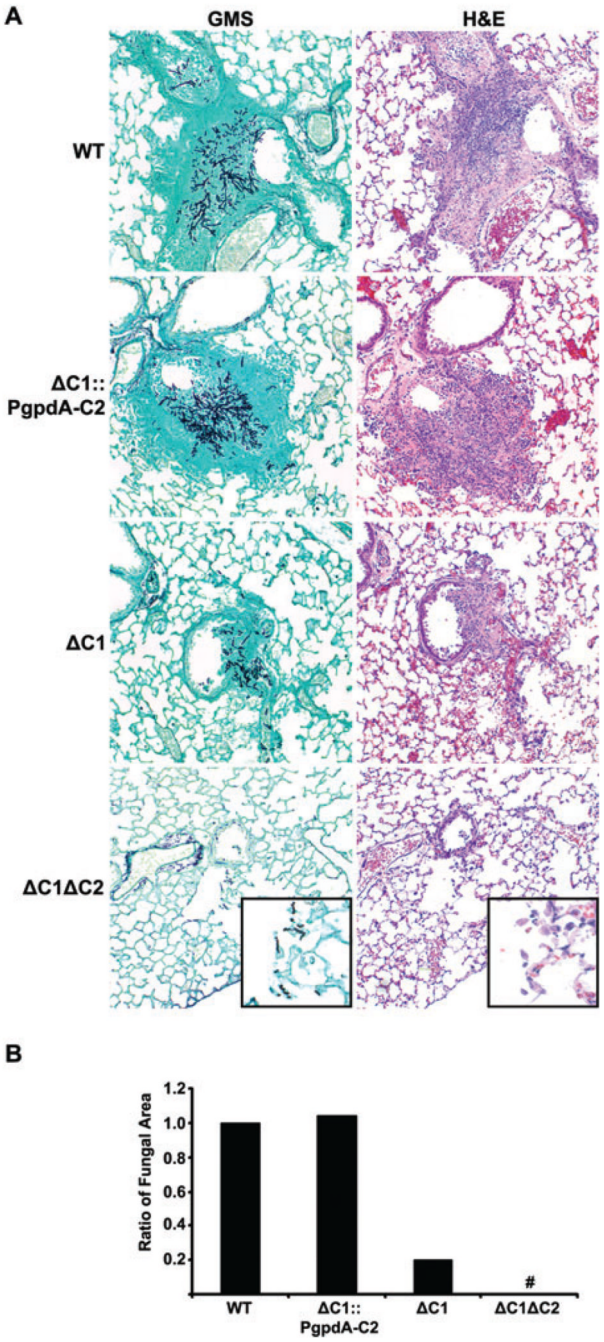

Fig. 8. Histopathology of infected mice at 72 h post infection

A. Numerous fungal lesions with associated inflammation were seen in mice from each of the infection groups, with the exception of the Δ*pkaC1*Δ*pkaC2*-infected mice. The inset shows the only hyphae seen in any of the animals in the Δ*pkaC1*Δ*pkaC2* group.

B. Morphometric analysis of the observable fungus in time-matched histological sections correlated with the survival curves associated with the strains.