The Cyclic AMP-Dependent Protein Kinase A Network Regulates Development and Virulence in *Aspergillus fumigatus*

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*Aspergillus fumigatus* is an important pathogen of immunocompromised hosts, causing pneumonia and invasive disseminated disease with high mortality. To determine the importance of the cyclic AMP (cAMP) signaling pathway for virulence, the *pkaC1* gene encoding a protein kinase A (PKA) catalytic subunit was cloned and characterized. Deletion of *pkaC1* led to reduced conidiation and growth. PKA activity was not detectable in Δ*pkaC1*, Δ*gpaB*, and Δ*acyA* mutant strains. *gpaB* and *acyA* encode a G protein α subunit involved in cAMP signal transduction and adenylate cyclase, respectively. Addition of cAMP led to PKA activity in crude extracts of both the Δ*gpaB* and Δ*acyA* strains but not in crude extracts of the Δ*pkaC1* strain. These findings provide evidence that PKA represents the predominant form of PKA under the conditions tested, and GPA and ACYA are members of the cAMP signaling cascade. Analysis of a *pksP-lacZ* gene fusion indicated that the expression of the pathogenicity determinant-encoding *pksP* gene was reduced in Δ*pkaC1* mutant strains compared with the expression of the gene fusion in the parental strain. In a low-dose murine inhalation model, conidia of both the Δ*pkaC1* and Δ*gpaB* mutant strains were almost avirulent. Taken together, these findings indicate that the cAMP-PKA signal transduction pathway is required for *A. fumigatus* pathogenicity.

*Aspergillus fumigatus* has become the most important airborne fungal pathogen of humans. Alveolar macrophages form the first line of defense against fungal conidia, the infectious agents, entering the respiratory tract. Neutrophilic granulocytes, the second line of defense, primarily attack hyphae but also attack conidia. An important killing mechanism for both types of immune effector cells consists of the production of reactive oxygen species (ROS) (22, 31, 42). Improvement in transplant medicine and the therapy of hematological malignancies is often complicated by the threat of invasive aspergillosis. *A. fumigatus* accounts for approximately 90% of invasive aspergillosis cases. Specific diagnostics are still limited, as are the possibilities of therapeutic intervention, which leads to a high mortality rate (30 to 98%) for invasive aspergillosis (reviewed in references 9 and 31).

One of the important questions concerning *A. fumigatus* is the identification of pathogenicity determinants and their regulation. Recently, the group of J. Kwon-Chung and our group identified a gene that encodes a pathogenicity determinant. This gene was designated *pksP* (or alternatively *alb1*) for polyketide synthase involved in pigment biosynthesis (23, 24, 25, 28, 54). Conidia of a *pksP* mutant strain are white. Based on genetic and biochemical data, the conidial pigment consists of dihydroxynaphthalene (DHN)-melanin (7, 30, 52, 53). *pksP* mutant strains exhibited reduced virulence in a mouse infection model (23, 54). *pksP* mutant conidia were 20-fold more sensitive to ROS than wild-type conidia, suggesting that the pigment is able to scavenge ROS, thereby presumably detoxifying ROS (23, 24). Moreover, a key element of antimicrobial activity in macrophages is the formation of functional phagolysosomes that contain a large variety of degrading enzymes in an acidic environment. Recently, we showed that a functional *pksP* gene is associated with a reduction in phagolysosome fusion in human monocyte-derived macrophages (MDMs). Consistently, the intracellular killing of *pksP* mutant conidia by MDMs was significantly greater than the intracellular killing of wild-type conidia (25). By using the enhanced green fluorescent protein, it was shown that the *pksP* gene was expressed during sporulation. Interestingly, this gene was also expressed in outgrowing hyphae isolated from the lungs of infected immunocompromised mice (29). This finding suggested that certain stress conditions have an effect on *pksP* expression. Therefore, it is essential to understand the communication between host and pathogen, i.e., to elucidate the signaling pathways that enable pathogenic fungi to adapt and survive the drastically altered environmental conditions that they encounter upon infection of the host.

Recently, some elements of the cyclic AMP (cAMP) signaling pathway of *A. fumigatus* were identified (33, 37). These elements include the adenylate cyclase gene *acyA* and the G protein α subunit-encoding gene *gpaB* that was found to be an upstream stimulator [G(0)α] of adenylate cyclase. Deletion mutants with mutations in both genes showed reduced conidiation, and the Δ*acyA* mutant produced very few conidia. Whereas the growth rate of the Δ*acyA* mutant was reduced, a reduced growth rate was not observed in case of the Δ*gpaB* mutant. Addition of 10 mM dibutyryl-cAMP to the agar plates completely restored the wild-type phenotype of both mutant strains. Interestingly, the expression of the pathogenicity determinant-encoding gene *pksP* measured as a *pksP-lacZ* gene fusion was reduced in the Δ*gpaB* mutant. Moreover, the killing rates of conidia of both deletion strains (the Δ*acyA* and Δ*gpaB* strains) by human MDMs were significantly greater than the
killing rate of wild-type conidia. Taken together, these findings suggested that cAMP triggers the defense system of *A. fumigatus* to protect the organism against attack by host immune effector cells.

cAMP signaling was found to control virulence and development in several human-pathogenic fungi, such as *Candida albicans* and *Cryptococcus neoformans*, and in plant-pathogenic fungi, such as *Ustilago maydis* and *Magnaporthe grisea* (reviewed in references 5, 15, 27, and 32). However, for *A. fumigatus* final proof was lacking since the mutants generated previously (33) were not tested in a low-dose animal infection model. Therefore, to provide final evidence that the cAMP signaling pathway is required for virulence of *A. fumigatus*, in this study a gene encoding a putative catalytic subunit of protein kinase A (PKA), designated *pkaC1*, was cloned and characterized. ∆*pkaC1* and ∆*gpaB* mutants were almost avirulent in a low-dose inhalation mouse infection model.

**MATERIALS AND METHODS**

**Fungal and bacterial strains, media, and growth conditions.** Fungal strains used in this study are listed in Table 1. *A. fumigatus* ATCC 46645 and CEA17 were used to generate pckC1 knockout strains by using different selection marker genes. *A. fumigatus* CEA17 is a uracil-auxotrophic (pyrG1) mutant that was used to generate strains CEA17*pyrG2-lacZ*, CEA17ΔpkaC1*pyrG2-lacZ1*, and CEA17ΔpkaC1*pyrG2-lacZ2*. *A. fumigatus* strains were cultivated at 37°C in *Agaricus* minimal medium (AMM) as previously described (56). Malt extract medium (2% [wt/vol] malt extract, 0.2% [wt/vol] yeast extract, 1% [wt/vol] glucose, 5 mM ammonium chloride, 1 mM dipotassium hydrogen phosphate) and AMM containing 5% (wt/vol) agar were used as solid media. Uridine (5 mM) or uracil (5 mM) was added to the media when required. For transformation of *Escherichia coli*, strains DH5α (Bethesda Research Laboratories, Gaithersburg, Md.), XL1-Blue (Stratagene, La Jolla, Calif.), INVαF’ (Invitrogen, Groningen, The Netherlands), and TOP10F’ (Invitrogen) were used. *E. coli* strains were grown at 37°C in Luria-Bertani medium supplemented, when required, with 50 μg of ampicillin per ml or 50 μg of kanamycin per ml.

** Colony radial growth rate determination.** Diameters of *A. fumigatus* colonies were measured twice a day for at least 10 colonies of each strain for up to 72 h (malt extract medium) or up to 96 h (AMM). The colony agar plates were inoculated centrally with a 2.5-μl drop of a suspension containing 1 × 10⁶ spores per ml. The colony radial growth rate (51) was calculated from the slope of the line between 40 and 72 h on a plot of colony radius versus time starting from the time of inoculation. Data were processed by least-square regression analysis.

**Quantification of sporulation.** Fifty micro liters of a spore suspension containing 1 × 10⁶ conidia prepared from a freshly harvested and filtered spore suspension was inoculated onto an AMM agar plate. Because of the high number of colonies the plate was covered by mycelium. The mass of mycelia was about the same for all plates irrespective of which strain was inoculated. Four plates containing each strain were incubated for 3 days, and the conidia produced on each plate were harvested with 10 ml of a saline solution containing 2% (vol/vol) Tween 80 (Merck, Darmstadt, Germany). The spore suspensions were filtered, and the numbers of conidia were determined by using a Thoma chamber.

**Microscopic analysis of growth.** For microscopic analysis of mycelial growth, spores were grown for 24 h on slides coated with a thin layer of AMM agar. Microscopic photographs were taken with a confocal laser scanning microscope (Leica, Bensheim, Germany).

**Analysis of conidial germination.** Fifty milliliters of AMM was inoculated with 10⁷ conidia. Cultures were incubated at 180 rpm and 37°C. Over a period of 16 h, samples were taken and deposited on microscope slides. To determine germination, at least 100 conidia of each sample were examined.

**Standard DNA techniques.** Standard techniques for the manipulation of DNA were carried out as described by Sambrook et al. (41). Chromosomal DNA of *A. fumigatus* was prepared as described previously for *Aspergillus nidulans* (3). For Southern blot analysis, chromosomal DNA of *A. fumigatus* was cut with different restriction enzymes. DNA fragments were separated on an agarose gel and blotted onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Labeling of the DNA probe, hybridization, and detection of DNA-DNA hybrids were performed by using the DIG High Prime labeling and detection system (Amersham Pharmacia Biotech) according to the manufacturer’s recommendations. Total RNA for cDNA synthesis was isolated by using an RNase plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The 5’ end of the pckC1 transcript was determined by 5’ rapid amplification of cDNA ends (RACE) by using the 5’ RACE system (Invitrogen) according to the manufacturer’s recommendations.

**Sequence analysis.** Cosmid or plasmid DNA was sequenced on both strands by primer walking by using a Big Dye terminator cycle sequencing kit (Applied Biosystems, Warrington, United Kingdom). The sequencing reaction mixtures were separated with an Applied Biosystems ABI 310 sequencer. DNA sequence data were edited by using the programs Sequence Navigator and Auto Assembler (Applied Biosystems). The analysis of the sequences was carried out by using Gene Works 2.2 (IntelliGenetics Inc.). Amino acid sequence comparisons were performed by using the BLAST 2.0 server (www.ncbi.nlm.gov/BLAST). The programs PepStat and ClustalW (Husar 4.0; DKFZ, Heidelberg, Germany) were used for predicting protein molecular masses and amino acid sequence alignments, respectively. Shading of aligned amino acid sequences was performed with MacBoxshade 2.15 (www.ch.embnet.org/software/BOX_form.html). Features and amino acid motifs of proteins were predicted by using the ISREC server (http://hits.isb-sib.ch/cgi-bin/PfSCAN).

**Cloning of the *A. fumigatus* pckC1 gene.** A 353-bp DNA fragment encoding part of the pckC1 gene was obtained by PCR amplification by using degenerate oligonucleotides PPKAC1deg (5’-ACXTYXGXXGAXCGXXWXTTYYG-3’) and PPKAC2deg (5’-TARTCXXGTXTGXXCRAXARXGT-3’), which were derived from conserved regions of known fungal homologues. Chromosomal DNA of *A. fumigatus* wild-type strain ATCC 46645 was used as the template. The DNA fragment obtained by PCR was ligated into plasmid pCR2.1 (Invitrogen). The cloned PCR product was sequenced and used as the probe to screen both a *pkaC1* knockout strain by using different selection gene library and the genomic DNA of *A. fumigatus* cosmids library (28) and *A. fumigatus* Uni-ZAP cDNA library (Stratagene, Heidelberg, Germany). This resulted in isolation of cosmids cospC1-1 and the cDNA-containing plasmid pBKSpC1-1.

**Generation of pckC1 knockout plasmids encoding different selection markers.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and/or phenotype</th>
<th>Source or reference</th>
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<tr>
<td>ATCC 46645</td>
<td>Wild type</td>
<td>ATCC</td>
</tr>
<tr>
<td>CEA17</td>
<td>Derived from CBS144-89; pyrG1</td>
<td>This study</td>
</tr>
<tr>
<td>pkaC1</td>
<td>pkaC1::hph ΔpkaC1, Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgpaB</td>
<td>gpaB::hph ΔgpaB, Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>Δacy4</td>
<td>acy4::hph Δacy4, Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CEA17ΔgpaB</td>
<td>pyrG1 gpaB::(neo-A. niger pyrG-neo) ΔgpaB, PyrG&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CEA17ΔpkaC1</td>
<td>pyrG1 pkaC1::(neo-A. niger pyrG-neo) ΔpkaCl, PyrG&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CEA17ΔpkaC1pyrG2</td>
<td>pyrG1 pkaC1::(neo-A. niger pyrG-neo) ΔpkaC1, PyrG&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CEA17ΔpkaC1pyrG2 lacZ1</td>
<td>pyrG1 pkaC1::(neo-A. niger pyrG-neo) ΔpkaC1, PyrG&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>CEA17ΔpkaC1pyrG2-lacZ2</td>
<td>pyrG1 pkaC1::(neo-A. niger pyrG-neo) ΔpkaC1, PyrG&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<sup>a</sup> hph, *E. coli* hygromycin B phosphotransferase gene; neo, Tn5 neomycin phosphotransferase gene.

<sup>b</sup> ATCC, American Type Culture Collection.
To construct the pkaC1 deletion plasmids pUCpkac1pH1p and pUCpkac1Ura1, a 3.24-kbp PCR product spanning the pkaC1 gene and including a 0.64-kbp upstream sequence and a 0.92-kbp downstream sequence was generated by using oligonucleotides PPKAC12 (5′-TGAGAGCTGATAGGGTTGCA-3′) and PPKAC11 (5′-GGTTGAATTTAGAA-3′) for the transformation of A. fumigatus. After two different pkaC1 knock-out plasmids were generated. One plasmid contained the hygromycin B resistance gene hph from E. coli as a selection marker. The complete hph gene was amplified by PCR using oligonucleotides PHYG1 (5′-GGTTGAATTTAGAA CTTGGC-3′) and PHYG2 (5′-CGCTGTGAGACAAAGCCTG-3′) and plasmid pUCH1-pksPI (29) as the template. The 2.7-kbp PCR product generated was ligated into plasmid pCR2.1 (TA cloning kit; Invitrogen), yielding plasmid pTApkaC1.1. After transformation of E. coli HB101, plasmid pUCpkac1pH1p was used as the positive control. The incubation period for the phosphorylation reaction was 30 min at room temperature. This was the maximal period during which the control PKA activity increased at a constant rate. Protein concentrations were determined by the method of Bradford (6).

Animal infection model. The murine low-dose model for invasive aspergillosis (45) as optimized by Liebmann et al. (34) for BALB/c mice was used. Briefly, 18- to 20-g female BALB/c mice were immunosuppressed with 100 mg of cyclophosphamide (Sigma, Taufkirchen, Germany) per ml on days −4, −1, 2, 5, 8, and 11 prior to and after infection on day 0. A single dose of cortisone acetate (200 mg/kg of body weight; Sigma) was injected subcutaneously on day −1. A. fumigatus conidium suspensions were harvested with phosphate-buffered saline containing 0.1% (vol/vol) Tween 80 (Merck) and filtered twice through Miracloth (Calbiochem, Bad Soden, Germany). Mice were anesthetized by intraperitoneal injection of 200 μl of 1% (vol/vol) ketamine (WDTeG, Garbsen, Germany)-0.02% (vol/vol) rompun (Bayer, Leverkusen, Germany) and intranasally infected with a 25-μl drop of a fresh suspension containing 5 × 107 or 108 conidia. Survival was monitored daily, and moribund animals were sacrificed by intraperitoneal injection of 200 μl of 3.2% (vol/vol) narcuret (Rhone Merieux, Laupheim, Germany). The drinking water was supplemented with 0.5 mg of tetracycline (Sigma) per ml to prevent bacterial infections. Lungs were recovered and homogenized in Lysing MatrixD tubes (Q-Biogene, Heidelberg, Germany) containing 1 ml of phosphate-buffered saline (Nunc, Wiesbaden, Germany). To determine the number of CFU, 50-μl portions of homogenates were plated in duplicate on malt agar plates.

Results

Identification of two genes encoding A. fumigatus PKA catalytic subunits. To analyze the importance of the cAMP-PKA signaling pathway for A. fumigatus virulence, the pkaC1 gene encoding a homologue of PKA catalytic subunits was identified and isolated (accession no. AJ297841 and Q8J129). This gene was cloned by PCR by using degenerate oligonucleotides whose sequences matched conserved sequences of known fungal homologues (see Materials and Methods). The DNA fragment generated by PCR encoded part of the PKA catalytic subunit, and it was used as a probe to screen both a genomic cosmid library and a cDNA library of A. fumigatus. Starting from the known sequences present on both the isolated cosmide and the cDNA clone, the DNA sequence of the complete gene was determined by primer walking. The pkaC1 gene product is encoded by 1,687 bp, interrupted by three introns that are 65, 53, and 60 bp long. The intron positions were confirmed by sequencing the corresponding regions of the cDNA clone. The isolated cDNA clone contained a truncation at the 5′ end of the cDNA fragment lacking at least 420 bases up to the predicted start codon. Therefore, the 5′ end of the pkaC1 transcript was determined by 5′ RACE, and it was found to be located 136 bp upstream of the putative start codon. The deduced PKAC1 protein consists of 502 amino acid residues which result in a protein with a predicted molecular mass of 57 kDa. The amino acid sequence of PKAC1 revealed significant similarities to other fungal catalytic subunits of PKA (level of amino acid identity to A. nidulans PKAA, 76%; level of amino acid identity to A. niger PKAC, 76%; level of amino acid identity to M. grisea CPKA, 64%). A protein kinase domain characteristic of eukaryotic protein kinases is located between amino acids 191 and 446. Important motifs characteristic of eukaryotic protein kinases is located between amino acids 191 and 446.

β-Galactosidase activity assays. A. fumigatus strains were grown in AMM for 24 h at 37°C. β-Galactosidase activities were measured in protein extracts obtained from three A. fumigatus cultures grown in parallel. Specific activities were calculated as previously described for A. nidulans (8).

PKA activity assay and determination of protein concentrations. A. fumigatus strains were grown in AMM for 24 h at 37°C. Harvesting, mycelia were frozen in liquid nitrogen and ground with a mortar and pestle. Mycelia were suspended in extraction buffer (25 mM Tris-HCl [pH 7.4], 1 mM dithiothreitol, 1 mM EDTA) and incubated on ice for 15 min. Samples were centrifuged at 4°C and 10,000 × g for 10 min. Ten microliters of the protein-containing supernatant was used as the assay for PKA activity by using fluorescent dye-coupled kemptide peptide (Promega, Mannheim, Germany) as the phosphoacceptor. Activities of protein extracts obtained from three A. fumigatus cultures grown in parallel were measured. Where indicated, 1 μM cAMP or 0.5 μg of a PKA inhibitor peptide (Promega) was added to the assay mixture. Purified cAMP-dependent PKA catalytic subunit from bovine heart (PTC1103) was used as the positive control. The incubation period for the phosphorylation reaction was 30 min at room temperature. This was the maximal period during which the control PKA activity increased at a constant rate. Protein concentrations were determined by the method of Bradford (6).
PKA catalytic subunits were found in the amino acid sequence of PKAC1. These motifs include the RDLKPEN sequence of the catalytic loop (amino acids 314 to 320) and the DFGFAKz sequence involved in Mg\(^{2+}\) binding (amino acids 333 to 338).

Additional conserved sequences include the nucleotide binding loop GTGSFG (amino acids 198 to 203) and three amino acid residues shown to be required for association of catalytic and regulatory subunits (H235, W344, and L346). An interesting additional feature of PKAC1 is a glutamine-rich stretch (amino acids 122 to 143) consisting of 21 glutamine residues interrupted by a proline residue. Glutamine stretches can be important for protein-protein interactions and are present in some other protein kinases in fungi and slime molds

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**PKC1 represents the predominant form controlling conidiation and vegetative growth.** To determine its biological function, the *pkaC1* gene was deleted by transformation and homologous recombination. To do this, plasmids pUCpkaC1hph1 and pUCpkaC1Ura1 (Fig. 1A and B) were generated (see Materials and Methods). These plasmids contained different selection markers, the hygromycin B resistance gene used as the selection marker gene in *A. fumigatus*. (B) Schematic map of the pkaC1 knockout plasmid pUCpkaC1Ura1. Abbreviations: amprar, ampicillin resistance gene; hygR, hygromycin B phosphotransferase gene hph used as the selection marker gene; neo, neomycin phosphotransferase genes.

(C) Southern blot analysis of the pkaC1 deletion strains. Chromosomal DNA of the parental strains ATCC 46645 (lane 1) and CEA17pksP-lacZ (lane 3), as well as mutant strains ΔpkaC1 (lane 2), CEA17ΔpkaC1 (lane 4), and CEA17ΔpkaC1pksP-lacZ (lane 5), was cut by BamHI. An 830-bp pkaC1-derived PCR fragment was used as the probe. In the ΔpkaC1, CEA17ΔpkaC1, and CEA17ΔpkaC1pksP-lacZ mutant strains, the band characteristic of the wild type (lanes 1 and 3) had disappeared. Instead, the bands characteristic of gene replacement at the pkaC1 locus were detected. The difference in the sizes of the bands in lane 2 (ΔpkaC1) and lane 4 (CEA17ΔpkaC1) is due to the different cassettes, hph and Ura-blaster, used to delete the pkaC1 gene. Strain CEA17ΔpkaC1pksP-lacZ (lane 5) resulted from a forced recombination in which the Ura-blaster was lost during selection on medium containing 5-FOA. Consequently, a copy of the neo gene remained at the pkaC1 gene locus, which led to a pkaC1 knockout band that migrated more quickly than the band of strain CEA17ΔpkaC1 (lane 4). (D) Schematic representation of the chromosomal pkaC1 locus of the wild type and the ΔpkaC1 deletion mutants. Restriction endonuclease cleavage sites and the position to which the probe hybridizes are indicated. The pkaC1 genes in mutants ΔpkaC1 and CEA17ΔpkaC1 lack the pkaC1 regions encoding amino acids 252 to 302 and 336 to 302, respectively, as well as 58 bp of the 3′ untranslated region.

**FIG. 1.** Deletion of *A. fumigatus* PKA catalytic subunit-encoding gene *pkaC1*. (A) Schematic map of the pkaC1 knockout plasmid pUCpkaC1hph1. Abbreviations: amprar, ampicillin resistance gene; hygR, hygromycin B phosphotransferase gene hph used as the selection marker gene in *A. fumigatus*. (B) Schematic map of the pkaC1 knockout plasmid pUCpkaC1Ura1. Abbreviations: amprar, ampicillin resistance gene; pyrG, orotidine 5′-monophosphate decarboxylase gene of *A. niger* used as the selection marker gene; neo, neomycin phosphotransferase genes. (C) Southern blot analysis of the pkaC1 deletion strains. Chromosomal DNA of the parental strains ATCC 46645 (lane 1) and CEA17pksP-lacZ (lane 3), as well as mutant strains ΔpkaC1 (lane 2), CEA17ΔpkaC1 (lane 4), and CEA17ΔpkaC1pksP-lacZ (lane 5), was cut by BamHI. An 830-bp pkaC1-derived PCR fragment was used as the probe. In the ΔpkaC1, CEA17ΔpkaC1, and CEA17ΔpkaC1pksP-lacZ mutant strains, the band characteristic of the wild type (lanes 1 and 3) had disappeared. Instead, the bands characteristic of gene replacement at the pkaC1 locus were detected. The difference in the sizes of the bands in lane 2 (ΔpkaC1) and lane 4 (CEA17ΔpkaC1) is due to the different cassettes, hph and Ura-blaster, used to delete the pkaC1 gene. Strain CEA17ΔpkaC1pksP-lacZ (lane 5) resulted from a forced recombination in which the Ura-blaster was lost during selection on medium containing 5-FOA. Consequently, a copy of the neo gene remained at the pkaC1 gene locus, which led to a pkaC1 knockout band that migrated more quickly than the band of strain CEA17ΔpkaC1 (lane 4). (D) Schematic representation of the chromosomal pkaC1 locus of the wild type and the ΔpkaC1 deletion mutants. Restriction endonuclease cleavage sites and the position to which the probe hybridizes are indicated. The pkaC1 genes in mutants ΔpkaC1 and CEA17ΔpkaC1 lack the pkaC1 regions encoding amino acids 252 to 302 and 336 to 302, respectively, as well as 58 bp of the 3′ untranslated region.
strain ATCC 46645 were isolated. Ten of these transformants were tested by Southern blot analysis, which resulted in identification of four strains which exhibited the expected gene replacement. Three of these four transformants carried an additional ectopic integration of the knockout construct (data not shown). The remaining transformant exhibited only the hybridization pattern characteristic of a gene replacement event (Fig. 1C and D). This strain was designated ΔpkaC1 and was used for further studies. All of the uracil-prototrophic transformants of strain CEA17 were tested by Southern blot analysis, and five of the strains tested showed a gene replacement event. However, two of these transformants carried an additional ectopic integration of the DNA fragment used for transformation (data not shown). One of the transformants that showed a hybridization pattern characteristic of a gene replacement at the pkaC1 locus was designated CEA17ΔpkaC1 and was used for further studies (Fig. 1C and D).

Growth of strains ΔpkaC1 and CEA17ΔpkaC1 on agar plates revealed that the pkaC1 deletion affected growth, sporulation, and germination of A. fumigatus (Fig. 2). pkaC1 dele-
tion strains formed small colonies with few conidiophores (Fig. 2A). The growth rate was quantified on both AMM and malt extract agar plates. AMM was used as a minimal medium to characterize the phenotypes of mutants. Malt extract medium was used as a complete medium in order to analyze whether some phenotypes could be complemented just by the presence of complex nutrients. Quantification of the growth rates on AMM and malt extract agar plates revealed that the specific growth rate of the \textit{pkaC1} mutant was strongly reduced (90 and 81\%, respectively) compared with the specific growth rate of the wild type (Fig. 2B and C). Accordingly, on AMM and malt extract agar plates, strain CEA17/H9004\textit{pkaC1} showed reductions in growth of 88 and 80\%, respectively, compared with the corresponding parental strains (Fig. 2B and C). It turned out that on both AMM and malt extract agar plates the difference in colony radius after 24 h was only moderate (Fig. 2B and C). Therefore, microscopic inspection was used as a more sensitive method to detect subtle differences in germination and growth. Microscopic inspection revealed that after 24 h of growth on AMM agar, both \textit{pkaC1} mutant strains had produced only a few hyphae compared with wild-type strains ATCC 46645 and CEA17\textit{pksP-lacZ} (Fig. 2D). In order to evaluate the role of \textit{pkaC1} during conidial germination, germ tube outgrowth for conidia germinated in AMM was measured. The results (Fig. 2E) demonstrate that the \textit{pkaC1} deletion affected the kinetics of germ tube outgrowth. After 10 h of incubation, 94 and 90\% of the conidia of parental strains ATCC 46645 and CEA17\textit{pksP-lacZ}, respectively, had germinated (Fig. 2E). By contrast, only 49 and 60\% of the conidia of mutant strains \textit{ΔpkaC1} and CEA17\textit{ΔpkaC1}, respectively, had formed germ tubes after 10 h (Fig. 2E). Prolongation of the incubation time up to a maximum of 16 h led to germination of 90\% of the \textit{pkaC1} mutant conidia (Fig. 2E). Taken together, these results suggest that \textit{pkaC1} deletion resulted in delayed germination kinetics of \\textit{A. fumigatus}. Moreover, compared to the wild-type and parental strains, which produced significant numbers of conidia after incubation on AMM agar plates for 2 to 3 days, macroscopic inspection revealed that the \textit{pkaC1} deletion strains produced far fewer conidia (Fig. 2A). Compared with

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<th>TABLE 2. Conidiation of \textit{A. fumigatus} parental and deletion strains</th>
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<td>Strain</td>
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<td>ATCC 46645</td>
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<td>\textit{ΔpkaC1}</td>
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FIG. 3. PKA activities of mutant strains \textit{ΔpkaC1}, CEA17\textit{ΔpkaC1}, \textit{ΔacyA}, and CEA17\textit{ΔgpaB} and parental strains ATCC 46645 and CEA17\textit{pksP-lacZ}. (A) Enzyme activity as monitored by gel electrophoresis. A phosphorylated substrate migrated toward the anode. (B) Spots shown in panel A quantified by spectrophotometry. Abbreviations: PKI, PKA inhibitor peptide; WT, wild type.
the numbers of conidia produced by wild-type strains, on AMM agar plates the numbers of conidia produced by strains \( \Delta pkaC1 \) and CEA17\( pkaC1 \) were reduced by 89 and 94%, respectively (Table 2). The reduction did not change even when the incubation was extended for 3 days. These data indicate that PKAC1 has a direct effect on formation of conidia. As expected, suppression of the \( \Delta pkaC1 \) deletion phenotype was not observed upon addition of 10 mM dibutylryl-cAMP to the agar plates (data not shown), suggesting that PKAC1 acts downstream of adenylate cyclase.

To determine the contribution of PKAC1 to total PKA activity, PKA activity in cell extracts of wild-type strains and both \( \Delta pkaC1 \) deletion strains was assayed. CAMP-dependent PKA activity was detectable in both wild-type strains (Fig. 3A, lanes 2 and 12, and Fig. 3B). Activation with 5 \( \mu M \) cAMP led to significantly increased PKA activity of protein extracts of the wild-type strains (Fig. 3A, lanes 3 and 13, and Fig. 3B). After addition of the PKA-specific inhibitor, no activity was measured, confirming the validity of the assay method (Fig. 3A, lanes 4 and 14, and Fig. 3B). In contrast to wild-type strains, no PKA activity was detected in the \( \Delta pkaC1 \) deletion strains \( \Delta pkaC1 \) and CEA17\( \Delta pkaC1 \) (Fig. 3A, lanes 5 to 7 and 15 to 17, and Fig. 3B), irrespective of whether cAMP was added to the assay mixture (Fig. 3A, lanes 6 and 16, and Fig. 3B). These findings provided evidence that \( pkaC1 \) encodes a PKA catalytic subunit that represents the predominant form of the enzyme under the conditions tested.

\( acyA \) and \( gpaB \) mutants lack PKA activity without activation by cAMP. PKA activity was not detected in either of the adenylate cyclase and G protein \( \alpha \) subunit mutant strains, \( acyA \) and CEA17\( \Delta gpaB \) (Fig. 3A, lanes 8 and 18, and Fig. 3B). However, addition of CAMP to the assay mixture led to significant PKA activity in protein extracts of both strains, although the levels did not reach wild-type levels (Fig. 3A, lanes 9 and 19, and Fig. 3B). This finding indicated that PKA is present but not activated in these mutant strains, most likely due to the lack of significant amounts of CAMP in either strain. This result further confirms the finding that GPAB is an element of the CAMP signaling pathway. Furthermore, it shows that cellular PKAC1 activity depends on CAMP.

CAMP-PKA-dependent expression of a \( pksPp-lacZ \) gene fusion. To analyze whether signaling via PKA is involved in the expression of \( pksP \), \( A. fumigatus pkaC1 \) deletion strains carrying a \( pksPp-lacZ \) gene fusion were generated. Thus, the Ura-blaster of strain CEA17\( \Delta pkaC1 \), which was initially introduced to delete the \( pkaC1 \) gene, was removed by selection on 5-FOA-containing agar plates, resulting in strain CEA17\( \Delta pkaC1 \)pyrG. Strain CEA17\( \Delta pkaC1 \)pyrG was transformed with circular DNA of plasmid pUCpyrG2pks-lacZ (33), which complemented the uracil auxotrophy of the recipient strain by integration of the plasmid at the chromosomal \( pyrG \) locus. The results of a Southern blot analysis of transformants are shown in Fig. 4A. The 8-kbp band characteristic of the wild-type \( pyrG \) gene (Fig. 4A, lane 1) had disappeared. Instead, parental strain CEA17\( pksPp-lacZ \) (Fig. 4A, lane 2) and two \( \Delta pkaC1 \) transformants (Fig. 4A, lanes 3 and 4) had an 18-kbp band due to the integration of the plasmid at the \( pyrG \) gene locus. The newly created transformants were designated CEA17\( \Delta pkaC1 \)pksP-lacZ1 and CEA17\( \Delta pkaC1 \)pksP-lacZ2. In addition, the \( \Delta pkaC1 \) genotype of transformant CEA17\( \Delta pkaC1 \)pksP-lacZ1 was checked by Southern blot analysis (Fig. 1C). The band characteristic of the wild-type control (Fig. 1C, lane 3) had disappeared in the \( \Delta pkaC1 \) mutant strain CEA17\( \Delta pkaC1 \)pksP-lacZ1. Instead, a 4.8-kbp DNA fragment caused by deletion of \( pkaC1 \) and the subsequent removal of the Ura-blaster by selection on 5-FOA was detected (Fig. 1C, lane 5).

The expression of the \( pksPp-lacZ \) gene fusion of the parental strain, the \( \Delta pkaC1 \) deletion strains, and the \( \Delta gpaB \) deletion strain was determined. The results are shown in Fig. 4B. There was a moderate but significant difference between the \( \Delta pkaC1 \) mutants and the wild-type strain. The \( \Delta pkaC1 \)-1 (CEA17\( \Delta pkaC1 \)pksP-lacZ1) and \( \Delta pkaC1 \)-2 (CEA17\( \Delta pkaC1 \)pksP-lacZ2) mutant strains showed 70 and 60%, respectively, of \( pksPp-lacZ \) expression measured for the wild-type strain (Fig. 4B). This level of expression is more than that of the \( \Delta gpaB \) strain, which showed 50% of the wild-type \( pksP \) expression (Fig. 4B). These results suggest that the CAMP-PKA signaling network is involved in the regulation of the \( pksP \) gene.

Mutants with mutations in elements of the CAMP-PKA signal transduction pathway were almost avirulent. To assess the roles of GPAB and PKAC1 in pathogenesis, the corresponding deletion mutants were tested in an animal infection model of invasive aspergillosis. Groups of 10 immunosuppressed mice were infected by inhalation with \( 5 \times 10^5 \) or \( 10^6 \) conidia of the different strains. The results are shown in Fig. 5. In the groups infected with wild-type conidia (strain ATCC 46645), irrespective of the number of conidia given, the mortality was 90% after 9 days (Fig. 5). By contrast, when mice were infected with conidia of the \( \Delta gpaB \) mutant strain, the mortality was drastically reduced to 30 and 20% in the animal groups infected with \( 5 \times 10^3 \) and \( 10^4 \) conidia, respectively (Fig. 5A). When mice
were infected with conidia of the ΔpkaC1 mutant, 90 and 100% of the animals survived in the groups infected with 5 × 10^{3} and 10^{4} conidia, respectively (Fig. 5B). The lungs of 15 of 18 animals which died after infection with wild-type conidia were found to be colonized with *A. fumigatus* at levels of 168 ± 101 CFU/g of tissue. *A. fumigatus* could not be reisolated from the lungs of either survivor infected with wild-type conidia. With one exception, the lungs of mice infected with ΔpkaC1 mutant conidia were not colonized by *A. fumigatus*. In the lungs of animals which died after infection with ΔgpaB mutant conidia, we found *A. fumigatus* at levels of 245 ± 215 CFU/g of tissue. Interestingly, the lungs of five survivors of ΔgpaB infection were also colonized with the fungus at levels of 503 ± 345 CFU/g of tissue. These results indicate that in some cases, the *A. fumigatus* gpaB mutant was able to germinate in the lungs of immunosuppressed mice. However, after germination the fungus seemed to persist in the lungs without invasive growth because the corresponding mice did not show symptoms of disease. Taken together, deletion of components of the cAMP signaling pathway of *A. fumigatus* resulted in significantly reduced virulence. The strains were almost avirulent. However, in contrast to the ΔgpaB mutant, ΔpkaC1 strains grew poorly, which needs to be taken into account when virulence is assessed (see Discussion).

**DISCUSSION**

Here we analyzed the importance of the cAMP-PKA signal transduction pathway for *A. fumigatus* growth, development, and virulence. The pkaC1 gene encoding a PKA catalytic subunit was identified. Moreover, the gpaB gene previously shown to encode a putative member of the cAMP signaling network was shown here to be required for virulence. Previously, Oliver et al. (37) reported the isolation of a gene from *A. fumigatus* designated pkaC. This gene encodes a PKA catalytic subunit with 490 amino acids and a deduced molecular mass of 56 kDa. Until now, the gene and the encoded protein had not been characterized further. Sequence alignments revealed that the previously identified gene is identical to the pkaC1 gene described here. However, PKAC1 is composed of 502 amino acids and has a predicted molecular mass of 57 kDa. Sequence comparisons showed that the PKAC protein described by Oliver et al. (37) lacks 12 glutamine residues present in the N-terminal Q stretch of PKAC1, whose presence was confirmed by repeated sequencing of both pkaC1 genomic DNA and cDNA.

To determine its biological functions, the pkaC1 gene of *A. fumigatus* was deleted. Deletion was carried out by using two different selection markers. Both mutants with mutations in the *A. fumigatus* catalytic subunit (ΔpkaC1 and CEA17ΔpkaC1) displayed the same phenotype (i.e., delayed germ tube outgrowth and severely impaired sporulation and growth). The ΔpkaC1 phenotypes resembled those of the *A. fumigatus* adenylate cyclase mutant (ΔacyA), whose growth was also reduced; in contrast, the adenylate cyclase mutant showed almost no conidiation on agar plates (33). With respect to sporulation this phenotype was also observed for the *A. fumigatus* ΔgpaB mutant, which is defective in a stimulatory G protein subunit, which most likely leads to decreased activation of adenylate cyclase and therefore reduced cAMP levels. This mutant produced only one-half as many conidia as the wild-type strains. Wild-type phenotypes of both ΔacyA and ΔgpaB mutants were restored by addition of dibutyryl-cAMP to the medium (33). As expected, this was not the case for the ΔpkaC1 mutants, because PKA acts downstream of cAMP synthesis. Elevated cAMP levels did not produce an increase in an alternative cellular PKA activity, which could, at least in part, replace the PKAC1 function. This finding was confirmed by data which showed that there was no measurable PKA activity in crude extracts of the ΔpkaC1 deletion strains. Moreover, no PKA activity was detectable in either the ΔgpaB or ΔacyA mutants, most likely due to increased levels or lack of cAMP in these strains. PKA activity in the ΔacyA and ΔgpaB deletion mutants was partially restored by addition of cAMP to the assay mixtures. These results indicate that PKAC1 activity depends on the cAMP level and, in addition, that PKAC1 acts downstream of GPAB and ACYA in the same pathway. Taken together, our results indicate that pkaC1 encodes an active PKA catalytic subunit which is part of the cAMP-PKA signal transduction pathway regulating growth and sporulation of *A. fumigatus*.

Interestingly, the observed ΔpkaC1 phenotypes only partially resembled those of, e.g., *A. nidulans* ΔpkaA and *A. niger* ΔpkaC mutants, which had mutations in PKA catalytic subunit genes that showed the highest levels of similarity to *A. fumiga-
The latter two mutants also displayed reduced growth rates on agar plates and delayed germination (17, 43, 47). Interestingly, deletion of pkaA in A. nidulans led to hyperconidiation, while pkaA overexpression resulted in decreased sporulation (43). This is in contrast to the findings reported here for A. fumigatus. ∆pkaC1 strains showed drastically reduced sporulation, indicating that asexual development of conidia is regulated differently at the level of PKA in the two fungi. Disruption of the A. niger PKA regulatory subunit-encoding gene pkaR resulted in very small colonies on plates, the absence of sporulation, and a complete loss of growth polarity during submerged growth (47). These phenotypes were even more drastic than those described for the A. niger ∆pkaC strain, which formed sporulating colonies whose diameters were two- to threefold smaller than those of wild-type colonies (47). Taken together, PKA mutants of fungi closely related to A. fumigatus exhibited similar growth phenotypes but different sporulation phenotypes.

The remaining, weak sporulation of A. fumigatus ∆pkaC1 strains opens up the possibility of a minor role for other catalytic PKA subunits in regulation of sporulation. One of these subunits could be encoded by the pkaC2 gene which was identified in the genome of A. fumigatus. However, in ∆pkaC1 strains this subunit and putative additional existing catalytic subunits did not show any activity under the conditions tested. This result suggests that PKAC1 plays the predominant role in cAMP-PKA signaling. Alternatively, PKAC2 and putative additional existing catalytic subunits were not active under the conditions used or could not be measured with the assay used. It is also conceivable that a cAMP-PKA-independent signal cascade is involved in sporulation, which, under certain conditions, could overlap the PKA-cAMP pathway. For example, a novel transcription factor regulating sporulation of A. nidulans was identified. This transcription factor, designated DevR, was not required for sporulation when the fungus was grown on 0.6 M NaCl but was essential for sporulation when the fungus was grown under standard conditions. There is a homologous gene in A. fumigatus (55). The function of pkaC2, if there is any, will be analyzed in future experiments.

Similar observations were reported for both the human-pathogenic basidiomycete C. neoformans and the rice pathogen M. grisea. ∆pka1 and ∆cpkA mutants, respectively, showed almost no detectable PKA activity (1, 14), but a second PKA isoform for each fungus was identified in the corresponding genome databases (14; K. Adachi and J. E. Hamer, EMBL database accession no. AA021201). Until now, however, these PKA isoforms have not been characterized. The existence of multiple isoforms of catalytic PKA subunits has been described for several other fungi, including Saccharomyces cerevisiae, in which PKA is essential for viability (48, 49). The three subunits identified (TPK1 to TPK3) have redundant functions, including regulation of intermediary metabolism, especially carbohydrate metabolism (reviewed in reference 49), response to stress conditions (44), cell cycle control (39), and pseudohyphal morphogenesis (38, 40). In the human pathogen C. albicans two catalytic PKA isoforms, Tpk1 and Tpk2, were found. Both of these isoforms play distinct and redundant roles in morphogenesis, growth, and virulence (4, 46). The corn smut fungus U. maydis also contains two PKA catalytic subunits, Adr1 and Uka1. Only Adr1 is responsible for the main cellular PKA activity and is important for mating and virulence (16).

The key question concerns the importance of cAMP-PKA signaling for virulence. In order to answer this question, two different aspects were considered: (i) a possible influence of PKAC1 on the expression of the pathogenicity determinant-encoding gene pksP; and (ii) the importance of elements of the cAMP signaling cascade for the pathogenicity of the fungus in a low-dose animal model. As shown previously, the pksP gene is expressed mainly during sporulation, but it was also expressed in outgrowing hyphae of A. fumigatus when conidia germinated in the lungs of immunocompromised mice (28, 29, 53, 54). In both ∆pkaC1 mutant strains, reduced pksP-lacZ expression was measured in hyphae under standard growth conditions. This result agrees well with the previous finding that pksP expression is decreased in an A. fumigatus ∆gpaB mutant (33) (Fig. 4). The ∆gpaB effect is most likely due to the lowered cAMP level and therefore the lack of stimulation of adenylate cyclase activity by the Gsα subunit GPAB. Lower cAMP levels then lead to decreased activation of PKA. However, the significantly higher pksP-lacZ expression in the ∆pkaC1 mutants compared with the expression in the ∆gpaB strain suggests that in addition to the GPAB-cAMP-PKA network, there is a GPAB-dependent PKA-independent signaling cascade that influences pksP expression.

Deletion of gpaB or pkaC1 led to drastically attenuated virulence of A. fumigatus. In fact, the mutants were almost avirulent. This is in agreement with the previous finding that conidia of A. fumigatus cAMP signaling mutants (∆gpaB and ∆acy1A) were killed more efficiently by human MDMs than conidia of the wild-type strains were killed (33). However, the decreased virulence of the ∆pkaC1 mutant needs to be seen in the context of its poor growth. We cannot exclude the possibility that the survival of mice infected with the mutant was due to delayed germination or the reduced growth rate of the organism. Similar observations were made for PKA mutants of C. neoformans and U. maydis (14, 16). However, in contrast to the ∆pkaC1 deletion mutants, the ∆gpaB mutant did not show any growth retardation (33). Nevertheless, as shown here, the virulence of the ∆gpaB strain was strongly attenuated, confirming the importance of cAMP signaling for virulence in A. fumigatus. Moreover, the A. fumigatus virulence determinant-encoding gene pksP is also regulated by cAMP-PKA signaling, and decreased expression of pksP, as shown here for both the ∆gpaB and ∆pkaC1 mutants, could contribute to better killing of the mutants in the animal model.

The polypeptide synthase PKSP catalyzes the initial step of DHN-melanin biosynthesis in A. fumigatus. In both M. grisea and C. neoformans, melanins play an important role in virulence. This led to the question of whether in melanin-producing fungi the expression of melanin biosynthesis genes is a general mechanism required for fungal pathogenicity (reviewed in reference 30). At least one of the signal transduction pathways triggering the expression of melanin biosynthesis genes in A. fumigatus appears to be similar to the pathways in C. neoformans and M. grisea. In the latter two fungi, both melanin biosynthesis and virulence were affected by deletion of genes encoding Gs protein subunits, adenylate cyclases, and catalytic subunits of PKA (2, 11, 12, 14, 19, 20, 21, 35, 36, 50). However, the situation in A. fumigatus appears to be rather
complex. Analysis of pigmentless pksP mutant conidia which showed reduced virulence in a murine infection model and were more sensitive to ROS indicated that the DHN-melanin-containing pigment is able to scavenge ROS (23, 28, 53, 54). Therefore, both pigments might act as protective agents against oxidant-based host defense mechanisms and thus contribute to the relative resistance of conidia to both alveolar macrophage and neutrophil attack. However, these findings do not explain why A. fumigatus conidia can be pathogenic, whereas this is rarely the case for A. nidulans conidia. An attractive hypothesis is that the pksP gene product of A. fumigatus is involved in the production of a compound which is immunosuppressive. This could be the DHN-melanin pigment, an intermediate or by-product of DHN-melanin, or even another compound in whose biosynthesis PKSP is involved (7, 9). This hypothesis is supported by the notion that the presence of a functional pksP gene in A. fumigatus conidia is associated with inhibition of the fusion of phagosomes and lysosomes in human MDMs (25). As shown here, both ΔpkaC1 and ΔgpaB mutant conidia are grey-green. Therefore, pigment was produced, although the possibility that the amount was lower than that in the wild type cannot be excluded yet. It therefore seems unlikely that ΔpkaC1 and ΔgpaB mutant conidia exhibit a strong increase in ROS sensitivity. This finding suggests that the pigment plays only a minor role in virulence. Consequently, the attenuated virulence of the ΔgpaB mutant in particular supports the alternative model of formation of an immunosuppressive compound by PKSP and, furthermore, strongly suggests that there are genes other than pksP that are involved in pathogenicity and are also regulated by the cAMP-PKA cascade.

In conclusion, the involvement of cAMP signaling in virulence appears to be a common theme in plant- and human-pathogenic fungi. For A. fumigatus it will be interesting to discover which other genes are regulated by the cAMP-PKA network and whether other pathogenicity determinants are found among them.

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