Heptahelical Receptors GprC and GprD of *Aspergillus fumigatus* Are Essential Regulators of Colony Growth, Hyphal Morphogenesis, and Virulence^{∇†}

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The filamentous fungus *Aspergillus fumigatus* normally grows on compost or hay but is also able to colonize environments such as the human lung. In order to survive, this organism needs to react to a multitude of external stimuli. Although extensive work has been carried out to investigate intracellular signal transduction in *A. fumigatus*, little is known about the specific stimuli and the corresponding receptors activating these signaling cascades. Here, two putative G-protein-coupled receptors, GprC and GprD, were characterized with respect to their cellular functions. Deletion of the corresponding genes resulted in drastic growth defects as hyphal extension was reduced, germination was retarded, and hyphae showed elevated levels of branching. The growth defect was found to be temperature dependent. The higher the temperature the more pronounced was the growth defect. Furthermore, compared with the wild type, the sensitivity of the mutant strains toward environmental stress caused by reactive oxygen intermediates was increased and the mutants displayed an attenuation of virulence in a murine infection model. Both mutants, especially the $\Delta gprC$ strain, exhibited increased tolerance toward cyclosporine, an inhibitor of the calcineurin signal transduction pathway. Transcriptome analyses indicated that in both the *gprC* and *gprD* deletion mutants, transcripts of primary metabolism genes were less abundant, whereas transcription of several secondary metabolism gene clusters was upregulated. Taken together, our data suggest the receptors are involved in integrating and processing stress signals via modulation of the calcineurin pathway.

Aspergillus fumigatus is a ubiquitous mold that can be isolated from different habitats all over the world. As a saprotroph, this filamentous fungus produces a large number of enzymes enabling its growth on different substrates that are available in the environment; e.g., compost heaps, hay, or soil (41). Moreover, *A. fumigatus* is also the most important airborne fungal pathogen of humans. In immunocompromised individuals, inhalation of conidia can cause invasive aspergillosis, a life-threatening disease (for an overview, see references 2 and 41).

A. fumigatus has to react specifically to many different kinds of environmental stimuli. To adapt to the availability of different nutrients, or to cope with stress, signal recognition and signal transduction are of major importance for the fungus. Signaling processes are also involved in host-pathogen interaction (7, 25, 38). Intracellular signaling is mediated by several conserved pathways: e.g., calcium signaling, cyclic AMP (cAMP) signal transduction, and mitogen-activated protein kinase (MAPK) cascades (26, 32). Central elements of these pathways are the Ca²⁺ signaling molecules calcineurin and

calmodulin, the cAMP-dependent protein kinase A (PKA), and different MAPKs (32). The cAMP-PKA pathway is well studied in eukaryotes. After perception of an extracellular signal by a G-protein-coupled receptor (GPCR), the intracellular second messenger molecule cAMP is produced and activates PKA. Activated PKA regulates the activity of specific target proteins by phosphorylation. In *A. fumigatus*, the cAMP-PKA network is responsible for regulation of essential physiological processes of the fungus (15, 26, 48). Impairment of PKA activity, as a result of deletion or overexpression of components of this pathway, drastically affects growth, sporulation, production of dihydroxynaphthalene (DHN)-melanin, and virulence (15, 26, 48).

Although for many eukaryotes the role of central components of this cascade was studied in detail, there is only limited information on upstream-acting GPCRs. In humans, more than 800 genes encode GPCRs sensing different stimuli: e.g., photons or light, hormones, lipids, or nucleotides (12). A common feature of GPCRs is the presence of seven membrane-spanning helices, localizing the receptor's N terminus to the outside of the cell and its C terminus into the cytoplasm. GPCRs are important therapeutic targets due to the fact that their function can be blocked or stimulated by specific ligands. Consequently, the majority of all modern therapeutics target the function of GPCRs (18).

Despite the importance of heptahelical receptors for drug development, in fungi only very few GPCRs were functionally characterized: e.g., pheromone and glucose receptors in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (3, 16, 19, 40). The availability of genome sequences of an increasing

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number of fungi enabled the *in silico* prediction of genes encoding GPCRs. By this means, several putative GPCRs in *Neurospora crassa* were identified (13) and in *Aspergillus nidulans* nine putative GPCRs, designated GprA to GprI, were annotated (17). Computational analysis of the genome of the plant-pathogenic fungus *Magnaporthe grisea* revealed a multiplicity of GPCRs (22). The majority of the 76 putative GPCRs were classified as PTH11-like receptors. PTH11 is essential for the formation of appressoria, the infectious structure of this plant pathogen (9). *In silico* analysis of putative heptahelical receptors and of components of cAMP-mediated signaling in aspergilli revealed the presence of 15 GPCRs in *A. fumigatus* (23). None of these receptors has been studied yet. Two of these receptors, GprC and GprD, showed sequence similarity to the glucose receptor Gpr1p from *S. cerevisiae* that activates cAMP-dependent protein kinase A. Interestingly, characterization of the homologous *gprD* mutant in *A. nidulans*, obtained by targeted gene deletion, implied a role for GprD in sexual development (17). Here, we set out to characterize in detail these GPCRs of *A. fumigatus* and investigated their potential physiological role by functional genomics.

MATERIALS AND METHODS

Fungal and bacterial strains, media, and growth conditions. The *A. fumigatus* strains used in this study were CEA10 (wild type) and CEA17 Δ akuB^{KU80} (PyrG⁻). *A. fumigatus* was cultivated at 37°C in *Aspergillus* minimal medium (AMM) as previously described (46). As solid medium, malt extract agar (1.8% [wt/vol] malt extract, 0.2% [wt/vol] yeast extract, 1% [wt/vol] glucose, 5 mM NH₄Cl, 1 mM K₂HPO₄) or AMM containing 1.5% (wt/vol) agar was used. Hygromycin B (80 µg/ml; Roche Applied Science, Germany), pyrithiamine (0.1 µg/ml; Sigma-Aldrich, Germany), or 10 mM uridine was added to the media when required. For propagation and amplification of plasmids, *Escherichia coli* strain Alpha-Select (Bioline, Germany) was used. *Escherichia coli* strains were grown at 37°C in LB medium supplemented with 100 µg/ml ampicillin.

Generation of *A. fumigatus* mutant strains. To obtain the deletion plasmids p Δ gprC_{pyrG} and p Δ gprD_{pyrG}, the *gprC* and *gprD* encoding regions, including 1.0 kb of up- and downstream sequences, were amplified by PCR using primer pairs GprC_F and GprC_R and GprD_F and GprD_R, respectively (see Table S1 in the supplemental material). The generated DNA fragments were cloned into plasmid pCR2.1 (Invitrogen, Germany). The obtained plasmids were subjected to an inverse PCR, employing primer pairs GprC_Not_F and GprC_Not_R and GprD_Not_F and GprD_Not_R, all encoding a half-NotI restriction site. After ligation, the resulting plasmids were cut with EcoRI (pCR Δ gprC) or XbaI (pUC Δ gprD) and subcloned into identically cut pUC18 vector. The newly formed NotI site between the up- and downstream regions was used to insert the *A. nidulans* *pyrG* gene, resulting in the final deletion plasmids. Finally, primer pairs GprC_F and GprC_R and GprD_F and GprD_R were used to amplify the deletion fragment by PCR. Transformation of *A. fumigatus* was carried out using protoplasts as previously described (45). To complement the deletion of the Δ gprC and Δ gprD mutant strains, the corresponding genes including up- and downstream regulatory sequences of around 1 kb were amplified by PCR using *A. fumigatus* wild-type genomic DNA as template by means of a proofreading polymerase (Phusion polymerase; Finnzymes, Finland) and the aforementioned primers. The DNA fragments obtained were used in a cotransformation approach together with plasmid pSK275 (a kind gift from S. Krappmann) which confers pyrithiamine resistance (21). Mutants were selected for pyrithiamine resistance and colony morphology. Single integration of the constructs at the *gprC* or *gprD* locus was verified by Southern blot analysis (data not shown). The resulting complemented strains were designated as *gprCc* and *gprDc*. To generate the enhanced green fluorescent protein (EGFP) fusion of *gprC* under the control of a constitutive promoter, genomic DNA was used as the template for amplification of the *gprC* gene with primers GprC_Ba_F and GprC_Ba_R. Thereby, BamHI restriction sites were introduced at the ends of the DNA fragment. The PCR product was cloned into plasmid pJET1.2 (Fermentas, Germany). The *gprC* gene was then inserted into the BamHI site of plasmid pUCGH (24) to give plasmid pGprC-GH. This plasmid, carrying the *gprC* gene under the control of the constitutive *otef* promoter (36) and the hygromycin

resistance gene *hph*, was used to transform *A. fumigatus* wild-type strain ATCC 46645. Using hygromycin resistance as selection marker, several mutants were obtained and analyzed by fluorescence microscopy and Southern blot analysis (data not shown). One of them, expressing the GprC-EGFP fusion, was used for further studies. A similar strategy was applied to generate a *gprC-egfp* construct under the control of the native *gprC* promoter. In brief, amplification of *gprC* including a 1-kb *gprC* promoter sequence was performed with primer pair GprC_Acc65I_F and GprC_Ba_R. The DNA fragment was inserted into pUCGH via Acc65I/BamHI simultaneously removing the *otef* promoter. Functionality of the GprC-EGFP fusion protein was tested by using plasmid pGprC-GH to transform the Δ gprC mutant and reconstitution of the wild-type phenotype.

Quantification of sporulation. Fifty microliters of a spore suspension containing 1×10^5 conidia prepared from a freshly harvested and filtered spore suspension was evenly spread onto AMM agar plates. Incubation at 37°C resulted in a mycelial mat covering the whole petri dish. Five plates for each strain were incubated for 4 days, and the conidia produced on each plate were harvested with 10 ml of a saline solution containing 2% (vol/vol) Tween 80 (Merck, Germany). The spore suspensions were filtered through 40-µm-pore cell strainer (BD Biosciences, Germany), and the number of conidia was determined using a CASY cell counter (model TT; Innovatis AG, Germany).

Microscopic analysis. For microscopic analysis, strains were grown in the media and for the time indicated. Microscopic photographs were taken on a Carl Zeiss Axiovert 200 microscope equipped with a LSM 5 live scan head (Carl Zeiss MicroImaging GmbH, Germany), which was also used for confocal imaging.

Analysis of conidial germination. Twenty milliliters of Sabouraud medium was mixed with 2×10^7 conidia and incubated in a petri dish with coverslips. At indicated time points, coverslips were removed and placed on microscope slides. To determine germination, at least 100 conidia of each sample were examined microscopically.

PKA activity assay. *A. fumigatus* strains were grown in AMM for 18 h at 37°C. After harvesting, mycelia were frozen in liquid nitrogen and mechanically disrupted using a homogenizer (FastPrep 120; MP Biomedicals) in extraction buffer (25 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 5 mM EDTA) and incubated on ice for 15 min. Samples were centrifuged at 4°C and $30,000 \times g$ for 10 min. Ten microliters of the supernatant adjusted to a protein concentration of 3 mg/ml was used in a nonradioactive assay for PKA activity by using fluorescent dye-coupled Kemptide peptide (Promega, Germany) as the phosphoacceptor. Activities of protein extracts obtained from three *A. fumigatus* cultures grown in parallel were measured. Where indicated, 1 µM cAMP was added to the assay. Purified cAMP-dependent PKA catalytic subunit from bovine heart (Promega, Germany) 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 (data not shown).

Susceptibility to cyclosporine and ROI. The sensitivity of the mutant strains against cyclosporine and reactive oxygen intermediate (ROI)-generating agents was measured by agar plate diffusion assays. A total of 1.5×10^7 conidia of the strains tested were mixed with 15 ml YAG agar (0.5% [wt/vol] yeast extract, 2% [wt/vol] agar, 2% [wt/vol] glucose, and trace elements) and poured in a petri dish. A hole 1 cm in diameter was punched in the middle of the agar plate. The well was filled with 150 µl of 0.1 mg/ml cyclosporine (Sigma-Aldrich, Germany), 150 µl of 3% (vol/vol) H₂O₂ (Fluka, Germany), 150 µl of 0.1 M diamide (N,N,N',N'-tetramethylazodicarboxamide; Sigma-Aldrich, Germany), or 150 µl of 5 mM menadione (2-methyl-1,4-naphthoquinone; Sigma, Germany). After incubation at 37°C for 24 h, the diameter of the inhibition zone was determined.

Standard DNA techniques. Standard techniques in the manipulation of DNA were carried out as described previously (33). Genomic DNA was isolated using the MasterPure yeast DNA purification kit (Epicenter Biotechnologies). For Southern blot analysis, DNA was restricted with HindIII. After separation on an agarose gel, nucleic acids were blotted onto a Hybond N⁺ membrane (GE Healthcare Bio-Sciences, Germany). Probe labeling, hybridization, and detection were performed using the digoxigenin (DIG) labeling mix, DIG Easy Hyb, and the CDP-Star ready-to-use kit according to the instructions of the manufacturer (Roche Applied Science, Germany).

Northern blot analysis. For the determination of mRNA steady-state levels, *A. fumigatus* strains were cultivated in AMM and on solid AMM agar plates. Mycelia were harvested at different time points. To obtain sufficient mycelial mass for RNA isolation from samples grown on solid AMM agar plates, *A. fumigatus* was precultivated for 16 h in AMM. Then, the mycelium was separated by filtration and transferred to AMM agar plates. For Northern blot analysis, RNA isolation was performed with the TriSure reagent according to the manufacturer's instructions (Bioline, Germany). Ten micrograms of RNA was separated on a denaturing agarose gel and transferred onto a Hybond N⁺ mem-

brane (GE Healthcare Bio-Sciences, Germany). Probe labeling, hybridization, and detection were performed as described above.

Sequence analysis. Sequence information was obtained from the Central *Aspergillus* Data REpository CADRE (www.cadre.man.ac.uk) (29). DNA was sequenced at Eurofins (Germany). All bioinformatic analyses were done using the Vector NTI Advanced software suite (Invitrogen, Germany). Topology and PFAM motifs of proteins were predicted by using the publicly available databases at www.ncbi.nih.gov and www.expasy.org.

Animal infection model. A murine low-dose model for invasive aspergillosis was applied with modifications as previously described (26, 43). In brief, female BALB/c mice were immunosuppressed with 120 mg cyclophosphamide (Sigma-Aldrich)/kg 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; Sigma-Aldrich) was injected subcutaneously on day -1. *A. fumigatus* conidial suspensions were harvested with phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween 80 (Merck, Germany) and filtered through a 40- μ m cell strainer (BD Biosciences, Germany). Mice were anesthetized and infected intranasally with 25 μ l of a fresh suspension containing 3×10^4 conidia. The health status was monitored twice daily, and moribund animals were sacrificed. A control group remained uninfected (inhalation of PBS) to monitor the influence of the immunosuppressive regime. Infections were performed with two groups of 5 mice for each tested strain, and the whole experiment was carried out in duplicate. Mice were cared for in accordance with the principles outlined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series, no. 123; <http://conventions.coe.int/Treaty/en/Treaties/Html/123>). All animal experiments were in compliance with the German Animal Protection Law and were approved by the responsible Federal State authority and ethics committee.

Transcriptome analysis. Full genome transcriptomic analyses were performed at Febit (Germany). Details on the Febit experimental procedures for gene expression profiling services are available on the company's homepage (<http://www.febit.de>). In brief, each array on the biochip comprises 15,000 probes. These probes are 30-mers covering all postulated gene transcripts that were designed based on the available genome sequence. Probes were selected according to a preliminary experiment that determined probes with highest specificity. The best probe was calculated for each gene fragment of interest. The second-best probes were chosen for gene fragments longer than 1,200 bp. The intensities of blank probes which consist only of one single T nucleotide are used for background corrections. Blank, labeling control and hybridization control probes are not included in the data analysis.

For microarray analyses, whole RNA was isolated from cultures grown for 18 h in AMM at 37°C as described above. Additionally, the samples were treated with DNase (TurboDNA-free kit, Ambion, Germany). Labeling was performed with the MessageAmp II enhanced biotin kit for mRNA labeling (Ambion, Germany). Raw data were analyzed according to Febit's protocols.

Microarray data accession numbers. The results of the microarray analyses have been deposited in the OmniFung Data Warehouse [<http://www.omnifung.hki-jena.de>; see *A. fumigatus* collection "Receptor deletion (GprC/GprD)"].

RESULTS

Generation and phenotypical characterization of mutant strains of the heptahelical receptors GprC and GprD. To assess the biological role of the putative carbon source-sensing receptors GprC and GprD in *A. fumigatus*, corresponding gene deletion mutants were created by transformation of *A. fumigatus* with DNA fragments obtained from plasmid p Δ *gprC*_pyrG or p Δ *gprD*_pyrG. Southern blot analyses of the transformant strains resulted in the identification of several deletion mutants for each gene (see Fig. S1 in the supplemental material). The growth of the Δ *gprC* and Δ *gprD* deletion mutants and the wild type was examined on minimal and complex medium agar plates. Under all conditions tested, the sizes of the colonies of the mutant strains were drastically reduced (Fig. 1A). The mutants displayed compact colonies compared to the wild type and the complemented strains. On complex medium, the growth of the mutant strains was as severely impaired as on minimal medium agar plates. The phenotype of the deletion

mutant strains was independent from the glucose concentration used in the medium. Neither low (5 mM) nor high (0.5 M) glucose concentrations suppressed the growth defect of the deletion strains (data not shown). Addition of exogenous dibutyryl cAMP to the mutant strains did not restore wild-type growth, and also PKA activities did not differ between wild-type and receptor mutant strains (data not shown). Interestingly, the effect of deletion of the receptors on radial growth was found to be temperature dependent. At an elevated incubation temperature (48°C), the growth defect of the mutant strains on agar plates was even more pronounced, whereas incubation at 28°C nearly restored wild-type growth (Fig. 1A, lower panel). One of the first mutants of the ascomycota for which a similar phenotype was described is the temperature-sensitive *cot-1* mutant of *N. crassa* (14). In *N. crassa*, the effects of the *cot-1* mutation could be suppressed by external stress like high salt, high osmolarity, or addition of ethanol to the medium. In contrast, similar experiments did not lead to reversion of the phenotypic defects caused by the deletion of *gprC* or *gprD* in *A. fumigatus* (data not shown). Growth of the mutant strains was also monitored in liquid medium. Conidia of the wild type and the two mutant strains were incubated in AMM at 48°C for up to 45 h. Under these conditions, the Δ *gprC* and Δ *gprD* mutants displayed retarded germination and restricted hyphal elongation, in contrast to the wild type (Fig. 1B). After 13 h of incubation, spores of the Δ *gprC* and Δ *gprD* strains were swollen and exhibited only stunted germ tubes. After 45 h of incubation, the wild type revealed an increase in the number of vacuoles resulting from temperature stress. In contrast, the mutants displayed disturbed growth and heavily swollen, vacuolar hyphae which tended to lyse. Comparison of the strains in a shift experiment from 30 to 48°C was not possible as the deletion mutants started to lyse 1 h after the shift (data not shown). Furthermore, both mutants displayed a severe polarity defect which resulted in abnormal branching of the hyphae in a temperature-dependent manner (Fig. 1B). When incubated at 37°C, the growth defect of the mutant strains was less pronounced: i.e., swelling and lysis of hyphae did not occur. However, retarded germination and restricted hyphal elongation were still observed in the mutant strains (Fig. 1B). Incubation at room temperature nearly restored wild-type growth of the mutants (data not shown).

As development seemed to be retarded in the mutant strains, influence of the deletion of both *gprC* and *gprD* on germination was studied. The results indicate that the deletion of either *gprC* or *gprD* clearly affected germination in the respective mutant strains (Fig. 1C). After 9 h, 90% of the wild-type conidia had germinated, whereas only 65% of the *gprC* deletion strain and 60% of the *gprD* mutant showed germ tubes. Prolonged incubation times led to a germination rate of more than 95% for the mutants after 13 h, indicating that the spores were viable but the germination was delayed. Monitoring of nuclear division in DAPI (4',6-diamidino-2-phenylindole)-stained germinating spores did not reveal differences compared to the wild type (data not shown). This corroborates the hypothesis that deletion of the receptors affects polarization and does not result in delayed breakage of dormancy.

The effect of cAMP-PKA signaling on sporulation was shown by Liebmann et al. (26): i.e., reduced spore production was demonstrated for the *pkaC1* deletion mutant. Therefore, the effect of

FIG. 1. Phenotypical characterization of $\Delta gprC$ and $\Delta gprD$ mutant strains. (A) Strains were grown on AMM and malt agar plates at the indicated temperatures for 72 h. (B) Differential interference contrast (DIC) microscopic pictures of *A. fumigatus* grown at 48°C or 37°C for the indicated time in liquid AMM (scale bar, 10 μ m). (C) Determination of germ tube formation for *A. fumigatus* conidia incubated in liquid Sabouraud medium at 37°C. The number of conidia with an emerging germ tube was determined at the indicated time points. The experiment was repeated three times, and for each time point, at least 100 conidia were counted. The percentage of germinated conidia based on the total number of conidia is shown. (D) Determination of sporulation capacity. Colonies were grown for 4 days on AMM agar plates at 37°C. Conidia were harvested, filtered, and counted. Data result from five independent experiments. (E) Resistance against cyclosporine. The wild-type (WT) and mutant strains were tested for their resistance against cyclosporine, an inhibitor of the calcineurin/calmodulin signal pathway. The cyclosporine-resistant $\Delta calA$ mutant (7) was included as a control. The inhibition zone induced by cyclosporine was determined after incubation at 37°C for 36 h.

deletion of *gprC* and *gprD* on the sporulation capacity of the mutant strains was examined (Fig. 1D). The wild type and $\Delta gprC$ mutant produced equal numbers of conidia. Interestingly, the $\Delta gprD$ mutant produced nearly twice the number of asexual spores as the wild type and the $\Delta gprC$ strain, indicating that only the deletion of *gprD* and not that of *gprC* positively affected sporulation.

Sensitivity of $\Delta gprC$ and $\Delta gprD$ mutant strains to reactive oxygen intermediates and cyclosporine. To further analyze the stress response of the *gprC* and *gprD* deletion strains, their sensitivity against reactive oxygen intermediates (ROI) was tested in an inhibition zone assay using H₂O₂, diamide, and menadione. The deletion mutants were more susceptible to H₂O₂, with the $\Delta gprC$ mutant displaying a 10% larger inhibition zone and the $\Delta gprD$ mutant displaying a 17% larger inhibition zone (Table 1). Furthermore, the $\Delta gprD$ mutant showed an increased sensitivity toward diamide and menadione,

whereas no difference between the $\Delta gprC$ mutant and the wild type was detected after incubation with these two substances.

The drastic reduction of the radial colony extension resembled that of the $\Delta calA$ mutant of *A. fumigatus* (7), which is

TABLE 1. Sensitivity against ROI

Strain	Inhibition zone diam (mm) with ^a :		
	H ₂ O ₂	Diamide	Menadione
Wild type	29.9 ± 0.6	28.3 ± 0.7	29.1 ± 0.8
$\Delta gprC$ mutant	33.0 ± 0.5*	28.4 ± 0.5	28.3 ± 0.7
$\Delta gprD$ mutant	35.1 ± 0.8*	32.4 ± 0.7*	33.9 ± 0.6*

^a Sensitivity of the mutant strains and the wild type against H₂O₂, diamide, and menadione was determined by measuring the inhibition zone in a plate diffusion assay. For each measurement, the mean value ± standard deviation is indicated. *, values obtained from the $\Delta gprC$ and/or $\Delta gprD$ mutant strain significantly differ from those from the wild-type strain ($P < 0.01$).

FIG. 2. Northern blot analysis of *gprC* and *gprD*. Mycelium of the wild type grown in liquid AMM or on solid AMM agar plates was harvested at the time points indicated. As a control, the $\Delta gprC$ and the $\Delta gprD$ mutant strains were cultivated in AMM and the mycelium was harvested after 16 h. Ethidium bromide (EtBr) staining of the gel is shown as a loading control.

defective in Ca^{2+} -mediated signal transduction due to deletion of the calcineurin catalytic subunit *calA*. $\Delta calA$ mutants were shown to be resistant against the calcineurin inhibitor cyclosporine (7). To analyze a possible connection of the receptors GprC and GprD with calcineurin signaling, sensitivity of the $\Delta gprC$ and $\Delta gprD$ mutants to cyclosporine was tested in a plate diffusion assay. The sensitivity of the $\Delta gprD$ mutant was only slightly reduced, whereas the $\Delta gprC$ mutant was nearly as resistant against cyclosporine as the $\Delta calA$ mutant (Fig. 1E). The reduction of the cyclosporine-induced inhibition zone indicates a role of the receptors in calcineurin signal transduction.

Determination of *gprC* and *gprD* transcript levels and receptor localization. The expression of the two genes *gprC* and *gprD* was monitored during vegetative growth in liquid and on solid media (according to reference 17). The steady-state level of *gprC* mRNA was rather constant throughout the experiment, with the highest level in air-exposed mycelia (Fig. 2). The *gprD* mRNA level reached a maximum in mycelia grown for 24 h in AMM.

To localize the receptor GprC and to elucidate its role during growth of the fungus, the *A. fumigatus* GprC-EGFP mutant strain was generated, expressing a GprC-EGFP fusion protein under the control of the constitutive *otef* promoter. By transformation of the $\Delta gprC$ mutant with the *gprC-egfp* construct, the wild-type phenotype could be reconstituted verifying the functionality of the receptor-GFP fusion. Fluorescence microscopy revealed a localization of the fusion protein along the hyphal membrane in a gradient with an apparent maximum at the apical site (Fig. 3). The GprC-EGFP fusion was also visualized in vesicles and vacuoles, as demonstrated by the green fluorescence inside FM4-64-stained vacuoles. In the older parts of the hyphae, the fusion protein was mainly detected in vacuoles. Remarkably, GprC-EGFP also localized to septae. The same GprC-EGFP localization pattern was also observed when the native *gprC* promoter was used; however, the fluorescence signal was much weaker (data not shown). Microscopic analysis of calcofluor white-stained hyphae revealed that a regular septation process occurred, indicating that the growth defect in the *gprC* mutant does not result from improper cytokinesis (data not shown).

Virulence of the $\Delta gprC$ and $\Delta gprD$ mutant strains. To test whether *gprC* and *gprD* are involved in virulence, we infected neutropenic mice with conidia of the wild type, the *gprC* and *gprD* deletion strains and the corresponding complemented strains. The animals' state of health was monitored over a

FIG. 3. Fluorescence microscopy for localization of the GprC-EGFP fusion protein. (A) EGFP-fluorescence; (B) staining with calcofluor for visualization of cell wall and septae; (C) staining with FM4-64 to detect vacuoles. Panel D shows an overlap of all fluorescence images (scale bar, 5 μ m). (E and F) EGFP fluorescence of multiple hyphae cultivated overnight in AMM on coverslips.

period of 2 weeks. Mortality of mice infected with the *gprCc* and *gprDc* complemented strains was comparable to that of mice infected with the wild type. The $\Delta gprD$ mutant was significantly attenuated ($P = 0.005$ compared to *gprDc* and $P = 0.023$ compared to wild type) (Fig. 4). In comparison to both the wild-type and *gprCc* strains, the onset of mortality after infection with the $\Delta gprC$ strain was delayed (Fig. 4). Although this was not statistically significant, we observed delayed and reduced mortality in two independent experiments, suggesting that *gprC* has a minor influence on virulence.

Transcriptome analysis. In order to gain a deeper insight into the cellular processes affected by the deletion of *gprC* and *gprD*, a transcriptomic approach was applied. The wild-type and mutant strains were grown for 18 h at 37°C in AMM. In total, 199 genes for the $\Delta gprC$ mutant (see Table S2 in the

FIG. 4. Virulence of the mutant strains in a mouse infection model. Survival of neutropenic mice infected intranasally with 3×10^4 conidia of different *A. fumigatus* strains was monitored over a period of 14 days.

supplemental material) and 182 genes for the $\Delta gprD$ mutant (see Table S3 in the supplemental material) were differentially transcribed in the respective mutant strain in comparison to the wild type. Table 2 presents a selection of differentially transcribed genes, and Fig. 5 summarizes the functional assignment of these transcripts to different cellular processes. In particular, transcripts belonging to the primary metabolism of *A. fumigatus* and genes putatively involved in signaling processes were affected in the $\Delta gprC$ strain. A more than 10-fold downregulation was detected for a flavin-binding monooxygenase-encoding transcript (AFUA_3G15050). Similarly regulated was a polyamine oxidase (PAO; AFUA_6G03510). PAOs were shown to generate hydrogen peroxide in plants as a signal molecule (47). Northern blot analyses affirmed the microarray result showing reduced mRNA steady-state levels for AFUA_3G15050 and AFUA_6G03510 in the deletion strains (see Fig. S2 in the supplemental material). Furthermore, a pyruvate decarboxylase, a phosphoketolase, and a fructose-6-phosphate kinase, which act as central regulators of glycolysis, were downregulated in the *gprC* deletion mutant. Genes that belong to known secondary metabolism gene clusters (e.g., the toxin fumitremorgin or pseurotin A) were positively affected in the receptor mutants. Additionally, transcripts of gene clusters with so-far-unknown synthesis products were similarly deregulated in the receptor deletion strains. A putative glycogen phosphorylase (Gph1p)-encoding gene was expressed at a higher level in the *gprC* mutant. Gph1p has been shown to be involved in the stress adaptation of *S. cerevisiae* via the HOG map kinase pathway (39). Similar to the *gprC* deletion mutant, the majority of transcripts that are deregulated in the $\Delta gprD$ strain can be grouped into the categories of carbon metabolism and secondary metabolite biosynthesis. Among the gene clusters that were differentially regulated in the mutant strain there were two orphan nonribosomal peptide synthetase (NRPS)-containing gene clusters. Additionally, the transcription factor BrlA was upregulated in the *gprD* mutant. BrlA is essential for the biogenesis of conidiophores and sporulation in *A. fumigatus* (30).

The differentially expressed genes in the two receptor mutants had a high degree of overlap compared to the wild type.

More than 80 transcripts of the same genes were in the same way up- or downregulated in the deletion strains: e.g., the toxin fumitremorgin, pseurotin A, and three additional gene clusters without known synthesis products. Accordingly, transcripts of carbon metabolism genes, like fructose-6-phosphate kinase, a phosphoketolase, two pyruvate decarboxylases, and a maltase were similarly regulated, pointing at signaling pathways which substantially overlap but which have a distinct output.

DISCUSSION

To shed light on the repertoire of receptors that the filamentous fungus *A. fumigatus* uses to cope with diverse ecological niches such as compost or the mammalian lung, we aimed at investigating the role of putative carbon source-sensing receptors. Therefore, we focused on the receptors GprC and GprD, which are similar to Gpr1p, the glucose receptor of *S. cerevisiae* that activates cAMP-dependent protein kinase A (20). In *A. fumigatus*, the cAMP-PKA pathway is a major regulator of growth, development, morphogenesis, and virulence and was also shown to regulate carbon source sensing (15, 31). However, analysis of $\Delta gprC$ and $\Delta gprD$ mutant strains revealed that GprC and GprD most likely are not involved in glucose sensing and cAMP signal transduction. There are several lines of evidence supporting this view: deletion of the receptor-encoding genes resulted in a severe growth defect, independent of the growth media tested for the fungus. If the receptors' function were sensing glucose or other carbon sources, the phenotype of the mutants should be influenced by the carbon sources. Furthermore, localization studies also contradicted a possible role of GprC in glucose sensing. In general, receptors located in the cellular membrane need to be desensitized after activation upon ligand binding to avoid the generation of a constitutive signal. Therefore, receptors are internalized which apparently represents a conserved modus throughout eukaryotic organisms. After internalization, the receptors can be either degraded or recycled (see reference 28 for an overview). The analysis of the distribution of the GprC-EGFP fusion protein revealed that the fusion protein was never exclusively detected in the cell membrane, which would be expected in case no ligand was present. Additionally, usage of acetate, ethanol, or a medium without any C source did not lead to increased vesicle transport or cytoplasmic membrane localization of the fusion protein (data not shown). This piece of data indicates that the receptors were not specifically activated under the conditions tested. Instead, the fluorescence was distributed in a gradient along the hyphae with a maximum at the apical tip, whereas in older parts of the hyphae the fluorescence protein mainly localized to the vacuoles. A similar subcellular distribution was described for Bgs1p from *S. pombe*, a putative catalytic subunit of the $\beta(1,3)$ -D-glucan synthase. A GFP fusion of this protein was observed at sites of active cell growth and polarized growth (4). Moreover, Bgs1p was shown to be responsible for proper septation and generation of linear $\beta(1,3)$ -D-glucan polysaccharides (5). These findings supported the proposed role of Bgs1p being primarily responsible for generation of $\beta(1,3)$ -D-glucan. In analogy, the *A. fumigatus* receptor, GprC, and probably GprD as well, analyzed here, might act at sites of active cell wall/cell membrane synthesis.

TABLE 2. Selection of differentially expressed genes^a

Fold change in gene	Gene product description	Locus tag no.
Downregulated in <i>ΔgprC</i> mutant		
0.09	Flavin-binding monooxygenase, putative	AFUA_3G15050
0.1	Flavin containing polyamine oxidase	AFUA_6G03510
0.14	PTH11-like integral membrane protein (GPCR)	AFUA_5G11245
0.15	Pyruvate decarboxylase	AFUA_5G14810
0.17	Ankyrin repeat protein	AFUA_3G02830
0.18	UDP-glucose dehydrogenase Ugd1, putative	AFUA_8G00920
0.18	NADH oxidase, partial mRNA	AFUA_1G12210
0.2	Dienelactone hydrolase	AFUA_2G05810
0.2	Extracellular dipeptidyl-peptidase Dpp4	AFUA_4G09320
0.24	Phosphoketolase	AFUA_3G10760
0.25	DEAD/DEAH box helicase	AFUA_3G06922
0.26	α-1,3-Glucanase/mutanase, putative	AFUA_1G03352
0.26	Extracellular lipase	AFUA_8G02530
0.28	Cytochrome P450 phenylacetate 2-hydroxylase	AFUA_5G01710
0.29	Mitochondrial integral membrane protein	AFUA_1G03090
0.3	Sulfate transporter	AFUA_1G05020
0.3	Sodium P-type ATPase	AFUA_6G03690
0.31	Potassium uptake transporter, putative	AFUA_4G13540
0.31	NADH-dependent flavin oxidoreductase	AFUA_5G01450
0.32	cAMP-mediated signaling protein Sok1	AFUA_4G07280
0.33	6-Phosphofructo-2-kinase 1	AFUA_1G07220
Upregulated in <i>ΔgprC</i> mutant		
2.44	Polyketide synthase	AFUA_8G00370
2.47	Glycogen phosphorylase GlpV/Gph1, putative	AFUA_1G12920
2.48	Iron-sulfur cluster-binding protein	AFUA_6G03920
2.5	α-Glucosidase	AFUA_1G16250
2.52	Adenylate-forming enzyme	AFUA_6G13920
2.53	2-Oxoisovalerate dehydrogenase complex alpha subunit	AFUA_6G08830
2.54	AAA family ATPase	AFUA_7G05752
2.55	Sad1/UNC domain protein	AFUA_5G06480
2.7	Rieske [2Fe-2S] domain protein	AFUA_7G06700
3.16	Class V chitinase	AFUA_6G09310
3.2	Cytochrome P450 monooxygenase, putative	AFUA_6G13945
3.21	Acyl coenzyme A dehydrogenase	AFUA_7G06510
3.25	Squalene-hopene-cyclase	AFUA_4G14770
3.26	Dimethylallyl tryptophan synthase, putative	AFUA_8G00620
3.32	Cytochrome P450 oxidoreductase, putative	AFUA_8G00560
3.42	Ankyrin repeat protein	AFUA_4G01580
3.57	C6 transcription factor	AFUA_2G05360
3.67	Maltase partial mRNA	AFUA_7G06380
3.67	Cytochrome P450 monooxygenase, putative	AFUA_3G03930
3.89	Methionine aminopeptidase, type II, putative	AFUA_8G00410
4.17	Squalene-hopene-cyclase	AFUA_5G00110
4.37	α/β-Hydrolase	AFUA_8G00530
4.42	Cytochrome P450 oxidoreductase/alkane hydroxylase	AFUA_5G00120
6.46	C6 finger transcription factor, putative	AFUA_8G00420
7.29	MFS transporter ^b	AFUA_2G05350
7.7	O-Methyltransferase	AFUA_7G05130
Downregulated in <i>ΔgprD</i> mutant		
0.18	Sulfate transporter	AFUA_1G05020
0.2	Flavin-containing polyamine oxidase	AFUA_6G03510
0.2	Dienelactone hydrolase	AFUA_2G05810
0.22	Ankyrin repeat protein	AFUA_3G02830
0.23	Flavin-binding monooxygenase, putative	AFUA_3G15050
0.23	Cytochrome P450 monooxygenase, putative	AFUA_6G13945
0.24	NACHT domain protein	AFUA_2G00960
0.24	Amino acid permease	AFUA_6G11100
0.27	Hydroxymethylglutaryl-coenzyme A synthase, putative	AFUA_8G07210
0.28	PE repeat family protein	AFUA_4G13630
0.29	α-1,3-Glucanase/mutanase, putative	AFUA_1G03352
0.3	ABC multidrug transporter SitT	AFUA_3G03430
0.3	6-Phosphofructo-2-kinase 1	AFUA_1G07220
0.31	Glutamate decarboxylase	AFUA_6G13490
0.32	Potassium uptake transporter, putative	AFUA_4G13540
0.33	Sodium P-type ATPase	AFUA_6G03690

Continued on following page

TABLE 2—Continued

Fold change in gene	Gene product description	Locus tag no.
0.34	Extracellular dipeptidyl-peptidase Dpp4	AFUA_4G09320
0.34	Proline permease	AFUA_8G02200
0.35	Phosphoketolase	AFUA_3G00370
0.35	MFS multidrug transporter	AFUA_3G14560
0.35	GYF domain protein	AFUA_2G13290
0.35	GMC oxidoreductase	AFUA_3G01580
0.35	PTH11-like integral membrane protein (GPCR)	AFUA_5G11245
0.36	MFS multidrug transporter	AFUA_1G10370
0.36	Pyruvate decarboxylase	AFUA_5G14810
0.37	ABC multidrug transporter Mdr1	AFUA_5G06070
0.38	Phosphatidate cytidyltransferase, putative	AFUA_4G09060
0.38	Carboxylesterase	AFUA_5G13710
0.39	Pyruvate decarboxylase PdcA, putative	AFUA_3G11070
Upregulated in <i>ΔgprD</i> mutant		
3.65	β-Glucosidase	AFUA_1G05770
3.76	C6 transcription factor	AFUA_2G05360
3.79	Methionine aminopeptidase type II, putative	AFUA_8G00410
3.8	Dimethylallyl tryptophan synthase, putative	AFUA_8G00620
3.89	MAK1-like monooxygenase	AFUA_6G12060
3.96	Flavin adenine dinucleotide binding domain protein	AFUA_6G12070
3.97	Adenylate-forming enzyme	AFUA_6G13920
4.18	Ankyrin repeat protein	AFUA_4G01580
4.19	MFS multidrug transporter	AFUA_8G06410
4.42	C6 finger domain protein	AFUA_6G03430
4.82	Maltase, partial mRNA	AFUA_7G06380
4.97	Spore-specific catalase CatA	AFUA_6G03890
5.42	α/β-Hydrolase	AFUA_8G00530
5.51	Adenylate-forming enzyme AfeA	AFUA_5G12510
5.99	C ₂ H ₂ -type conidiation transcription factor BrlA	AFUA_1G16590
6.47	Cytochrome P450 monooxygenase, putative	AFUA_3G03930
7.43	MFS transporter	AFUA_2G05350
8.5	C6 finger transcription factor, putative	AFUA_8G00420
8.73	O-Methyltransferase	AFUA_7G05130
12.11	Cytochrome P450 monooxygenase, putative	AFUA_4G14810
18.13	Squalene-hopene-cyclase	AFUA_4G14770

^a Transcripts that were up- or downregulated in the *ΔgprC* or *ΔgprD* mutant in comparison to the wild type.

^b MFS, major facilitator superfamily.

Polarity defects in *A. fumigatus* result from deletion of a multitude of various genes: e.g., those coding for GTPases of the Ras family (10, 11), components of the calcineurin-calmodulin signaling cascade (6–8, 37), or the MpkA-MAPK signaling pathway (43, 44), although this does not imply mandatory interconnection of these phenotypical changes. When comparing the phenotypes of the receptor mutants with mutants of the calcineurin-calmodulin pathway (7, 37) striking similarities were observed. Both the *ΔcalA* strain and the receptor mutants showed increased resistance against the calcineurin inhibitor cyclosporine. Admittedly, the sensitivity of the *ΔgprD* mutant was only slightly lower, whereas the *ΔgprC* mutant was nearly as resistant as the *ΔcalA* mutant. This indicates a connection of the receptors, with emphasis on GprC, to the calcineurin-calmodulin signaling pathway. Moreover, the *calA* calcineurin mutant is characterized by a severely stunted growth. The *ΔcalA* conidia grow isotropically for longer time periods, and the germlings display heavily branched hyphae which show characteristic dichotomous bifurcation. Also, the hyphae grew more densely (7) and the virulence of the mutant in a low-dose murine infection model was reduced, which can be attributed to the growth defect and the missing rodlet structure. The latter might contribute to tissue adhesion (8) and was recently

shown to hide conidia from recognition by the immune system (1). Furthermore, in response to infection with *A. fumigatus* conidia the animal's body temperature transiently increases by 1 to 2°C. Due to the temperature-dependent growth phenotype of the mutant strains, an influence of this slight increase in body temperature cannot be excluded. However, a growth defect does not need to be the cause for a reduction in virulence. For example, the *mpkA* deletion mutant of *A. fumigatus* showed a reduced growth rate and compact colonies but was still as virulent in a mouse infection model as the wild type (43). Therefore, the cause of the reduced virulence of the *ΔgprC* and *ΔgprD* receptor mutants remains to be elucidated.

To investigate the molecular function of the receptors, we analyzed the transcriptomes of the mutant strains and compared them with the transcriptome of the wild type. The most striking result was the remarkable overlap in the subsets of differentially regulated transcripts in the two mutants. Altogether 80 genes displayed a similar regulation in both mutants compared with the wild type. As some genes of the primary metabolism were downregulated, it is tempting to speculate that the receptors affect the regulation of C-source metabolism, although not being involved in their sensing. In a proteomic study (data not shown), the glucose-6-phosphate dehy-

FIG. 5. Classification of differentially transcribed genes according to KEGG (Kyoto Encyclopedia of Genes and Genomes). All differentially transcribed genes of the transcriptome analysis of the wild-type strain compared to the $\Delta gprC$ and $\Delta gprD$ mutants were grouped according to their function. Genes with unknown function were not included.

drogenase was found to be upregulated in the $\Delta gprC$ mutant. This enzyme represents the bottleneck for the pentose phosphate cycle which, in turn, is needed to supply reduction equivalents such as NADPH to the cell to maintain a proper balanced thioredoxin-glutathione system. This has already been shown for *A. nidulans* (42). Furthermore, a direct correlation between glutathione levels and the glucose-6-phosphate dehydrogenase activity was found for the model yeast *S. cerevisiae* (27). Recently, Semighini and Harris (35) characterized the effects of redox imbalance on the polar growth of *A. nidulans*. The authors suggested that apical dominance depends on a gradient of reactive oxygen intermediates (ROI). Apical dominance apparently suppresses lateral hyphal branching at the hyphal tip, where a high concentration of ROI was detected. In the absence of an NADPH oxidase or similarly functional flavoproteins, the ROI gradient was not detected and the hyphal morphology was altered: i.e., hyphae showed extensive branching (35). The $\Delta gprC$ and $\Delta gprD$ receptor mutants exhibited a transcriptomic pattern which reflects an imbalance in the cellular redox state or at least in the cellular signaling via ROI because in both mutants, transcripts of genes encoding flavin-containing polyamine oxidases, monooxygenases, and NADH oxidases were deregulated. This observation led us to conclude that the phenotypic defects are, at least in part, due to a missing or imperfectly established ROI gradient. Another factor determining polar growth in filamentous fungi seems to be the calcium concentration. This was first reported to be essential in *N. crassa* (34). Upon addition of a calcium ionophore, hyphae displayed a higher degree of branching and the branches were formed closer to the hyphal tip. An important role for calcium is the activation of the protein phosphatase calcineurin via calmodulin. *A. fumigatus* mutants with defects in the calcineurin-calmodulin pathway exhibited severe growth defects that resulted from improper polar growth and temperature sensitivity (7, 37). Interestingly, in an *A. fumigatus* calcineurin null mutant, five secondary metabolites (e.g., spirotryprostatin A) were produced which were absent in the wild-type strain (M. T. Pupo et al., presented at the 47th

Annual Meeting of the American Society of Pharmacognosy, Arlington, VA, 5 to 9 August 2006). Both mutants characterized here (i.e., the $\Delta gprC$ and $\Delta gprD$ strains) revealed a growth defect comparable to that of the $\Delta calA$ mutant. Moreover, several putative gene clusters for the biosynthesis of secondary metabolites were found to be upregulated in the transcriptomic profile of the receptor mutants.

Taken together, our results indicate that the heptahelical proteins GprC and GprD encode G protein-coupled receptors that are essential for the defined regulation of fungal metabolism and important cellular processes, such as germination, hyphal elongation, and branching. Furthermore, these receptors regulate resistance toward environmental stress caused by ROI and elevated temperatures, and they play a role during the infection process, as the mutant strains were attenuated in virulence. We propose a connection of the receptors with calcineurin-mediated signal transduction.

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