Upstream and Downstream Regulation of Asexual Development in *Aspergillus fumigatus*†

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The opportunistic human pathogen *Aspergillus fumigatus* produces a large quantity of asexual spores (conidia), which are the primary agent causing invasive aspergillosis in immunocompromised patients. We investigated the mechanisms controlling asexual sporulation (conidiation) in *A. fumigatus* via examining functions of four key regulators, GpaA (Gα), AfFlbA (RGS), AfFluG, and AfBrL, previously studied in *Aspergillus nidulans*. Expression analyses of gpaA, AfFlbA, AfFluG, AfBrL, and AfwetA throughout the lifecycle of *A. fumigatus* revealed that, while transcripts of AfFlbA and AfFluG accumulate constantly, the latter two downstream developmental regulators are specifically expressed during conidiation. Both loss-of-function AfFlbA and dominant activating GpaA203R mutations resulted in reduced conidiation with increased hyphal proliferation, indicating that GpaA signaling activates vegetative growth while inhibiting conidiation. As GpaA is the primary target of AfFlbA, the dominant interfering GpaA203R mutation suppressed reduced conidiation caused by loss of AfFlbA function. These results corroborate the hypothesis that functions of G proteins and RGSs are conserved in aspergilli. We then examined functions of the two major developmental activators AfFluG and AfBrL. While deletion of AfBrL eliminated conidiation completely, null mutation of AfFluG did not cause severe alterations in *A. fumigatus* sporulation in air-exposed culture, implying that, whereas the two aspergilli may have a common key downstream developmental activator, upstream mechanisms activating brlA may be distinct. Finally, both AfFluG and AfFlbA mutants showed reduced conidiation and delayed expression of AfBrL in synchronized developmental induction, indicating that these upstream regulators contribute to the proper progression of conidiation.

The genus *Aspergillus* represents the most widespread fungi in the environment and includes industrially, agriculturally, and medically important species. All aspergilli reproduce in asexual mode, which involves the formation of multicellular organs termed conidiophores bearing thousands of mitotically derived asexual spores (conidia). The study of asexual development (conidiation) in the model fungus *Aspergillus nidulans* has provided important information on the mechanisms controlling growth and development (reviewed in references 3 and 4). Conidiation in *A. nidulans* is a continual sequence from vegetative growth to asexual development. It is a precisely timed and genetically programmed event responding to internal and external cues. Previous studies demonstrated that vegetative growth signaling is primarily mediated by a heterotrimeric G protein system composed of FadA, SfaD, and GpgA (Gα, Gβ, and Gγ subunits, respectively); PhnA (a Gβγ activator); and the cyclic AMP (cAMP)-dependent protein kinases PkaA and PkaB (23, 25, 28, 29, 32, 39; reviewed in reference 43). Activation of this G protein signaling stimulates hyphal proliferation, which in turn represses conidiation and production of the mycotoxin sterigmatocystin (ST; Fig. 1A) (10, 25, 39). Constitutive activation of FadA signaling causes uncontrolled accumulation of hyphal mass and the absence of sporulation, resulting in the fluffy autolytic phenotype (39). Initiation of conidiation requires both inhibition of this G protein signaling and activation of development-specific functions. FíbB is an RGS (regulator of G protein signaling) domain protein, which plays a crucial role in antagonizing vegetative growth signaling, likely by facilitating the intrinsic GTPase activity of FadA (15, 39). FluG is a key upstream activator of conidiation and is associated with the production of a small diffusible molecule (16). Loss-of-function fluG mutants form colonies exhibiting the nonsporulating fluffy phenotype (16). Our recent study showed that this FluG-dependent commencement of development in *A. nidulans* occurs via removal of the negative regulation imposed by the novel Zn(II)2Cys6 domain protein SfgA (30; reviewed in reference 44). Derepression of conidiation caused by FluG activity leads to the activation of the key downstream developmental activator brlA encoding a C2H2 zinc finger transcription factor, which activates expression of other genes required for asexual development (Fig. 1A) (1, 8; reviewed in references 4 and 44). Further genetic and biochemical studies identified two additional regulators of conidiation, abaA and wetA, that function downstream of brlA. The abaA gene encodes another developmental regulator that is activated by brlA during the middle stages of conidiophore development (5). The wetA gene functions in the late phase of conidiation for the synthesis of crucial cell wall components (19, 31). These three genes act in concert with other genes to control conidiation-specific gene expression and determine the order of gene expression and development.

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* Supplemental material for this article may be found at http://ec.asm.org/.

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activation during conidiophore development and spore maturation (Fig. 1A) (22, reviewed in reference 4). The opportunistic human pathogen Aspergillus fumigatus is the most prevalent airborne fungal pathogen, and it causes severe and usually fatal invasive aspergillosis in immunocompromised patients (reviewed in reference 14). Moreover, the airborne A. fumigatus conidia are the primary agent for allergenic bronchopulmonary aspergillosis, a severe pulmonary complication resulting from hypersensitivity to A. fumigatus, the facts that these two fungi are distantly related (9) and are different in the reproductive processes, i.e., A. fumigatus lacks a sexual cycle and produces a structurally different conidiophore (lacking metulae), led us to the hypothesis that the two aspergilli may have both conserved and distinct mechanisms controlling the conidiation process. Via comparative genome analyses, we have identified the homologues of FadA, FiaB, FiaG, BrA, and WetA in A. fumigatus, which are designated GpaA (17), AfFiaB, AfFiaG, AfBrA, and AfWetA, respectively. Deletion and additional genetic analyses in conjunction with expression and phenotypic studies revealed that AfFiaB and GpaA constitute the crucial G protein signaling components that coordinate vegetative growth and asexual development, implying that functions of these signaling elements are conserved in both species. Moreover, as found in A. nidulans, AfBrA is essential for conidiophore formation, and AfFiaB and AfFiaG are necessary for proper conidiation and AfBrA expression in A. fumigatus. However, somewhat distinct from A. nidulans, AfFiaB or AfFiaG is not absolutely required for conidiation or activation of AfBrA in A. fumigatus. Taken together, we propose that, whereas a G protein (GpaA) signaling pathway and its regulation (AfFiaB) as well as downstream activation of conidiation are conserved in these distantly related model and pathogenic aspergilli, the imperfect fungus A. fumigatus has distinct and persistent mechanisms activating conidiation through AfBrA.

FIG. 1. Model for growth and developmental control in A. nidulans and downstream regulatory mechanisms of conidiation in A. fumigatus. The facts that these two fungi are distantly related (9) and are different in the reproductive processes, i.e., A. fumigatus lacks a sexual cycle and produces a structurally different conidiophore (lacking metulae), led us to the hypothesis that the two aspergilli may have both conserved and distinct mechanisms controlling the conidiation process. Via comparative genome analyses, we have identified the homologues of FadA, FiaB, FiaG, BrA, and WetA in A. fumigatus, which are designated GpaA (17), AfFiaB, AfFiaG, AfBrA, and AfWetA, respectively. Deletion and additional genetic analyses in conjunction with expression and phenotypic studies revealed that AfFiaB and GpaA constitute the crucial G protein signaling components that coordinate vegetative growth and asexual development, implying that functions of these signaling elements are conserved in both species. Moreover, as found in A. nidulans, AfBrA is essential for conidiophore formation, and AfFiaB and AfFiaG are necessary for proper conidiation and AfBrA expression in A. fumigatus. However, somewhat distinct from A. nidulans, AfFiaB or AfFiaG is not absolutely required for conidiation or activation of AfBrA in A. fumigatus. Taken together, we propose that, whereas a G protein (GpaA) signaling pathway and its regulation (AfFiaB) as well as downstream activation of conidiation are conserved in these distantly related model and pathogenic aspergilli, the imperfect fungus A. fumigatus has distinct and persistent mechanisms activating conidiation through AfBrA.

MATERIALS AND METHODS

Aspergillus strains, growth conditions, and transformation. A. fumigatus strains used in this study are listed in Table 1. Both A. fumigatus AF293 (wild type [WT] [57]) and AF293.1 (AfpyrG1 [36]) strains were used as WT. Standard culture and genetic techniques for A. nidulans were used (12, 24). The composition of minimal medium was as follows (per liter): 10 g glucose, 6 g NaNO₃, 0.52 g KCl, 1.52 g KH₂PO₄, and 1 ml of the 1,000× trace element solution [22 g/liter ZnSO₄·7H₂O, 7.0 g/liter KH₂PO₄, 1.52 g/liter KH₂PO₄, and 1 ml of the 1,000× trace element solution [22 g/liter ZnSO₄·7H₂O, 7.0 g/liter KH₂PO₄, 1.52 g/liter KH₂PO₄, and 1 ml of the 1,000× trace element solution [22 g/liter ZnSO₄·7H₂O, 7.0 g/liter KH₂PO₄, 1.52 g/liter KH₂PO₄, and 1 ml of the 1,000× trace element solution [22 g/liter ZnSO₄·7H₂O, 7.0 g/liter KH₂PO₄, 1.52 g/liter KH₂PO₄, and 1 ml of the 1,000× trace element solution [22 g/liter ZnSO₄·7H₂O, 7.0 g/liter KH₂PO₄].

TABLE 1. Aspergillus strains used in this study

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* mGF24⁺, -49, -80, -88, -104, -112, -114, -115, -129, -130, -131, and -132 are likely isogenic except for the AfFiaB mutant allele (Fig. 2C).

⁸ FGSC, Fungal Genetic Stock Center.
gltier FeSO₄, 7H₂O, 1.6 g/liter CuCl₂·H₂O, 1.6 g/liter CuSO₄·5H₂O, 1.1 g/liter (NH₄)₂MoO₄·4H₂O, 50 g/liter Na₂EDTA. The mixture was then pH adjusted to 6.5 with 1.0 N NaOH. All strains were inoculated on solid (or liquid) minimal medium with appropriate supplements (5 mM uridine and 10 mM uracil; simplified as MM) and incubated at 37°C. If needed, yeast extract (YE) was added (0.1% or 0.5% final concentration). To observe development in liquid submerged culture, all strains were inoculated with 5 × 10⁶ conidia/ml in 100 ml liquid MM and incubated at 250 rpm at 37°C. The mycelial aggregates of each strain were observed microscopically every 3 h starting at 18 h of growth in liquid culture. Under these experimental conditions AF293 and AF293.1 elaborated conidiophores consistently. Standard \textit{A. nidulans} transformation techniques (21, 37) were used.

For synchronized asexual developmental induction, about 1 × 10⁶ conidia of WT and relevant mutant strains were inoculated in 100 ml liquid MM with 0.1% YE and incubated at 37°C and 250 rpm for 18 h (0 h for developmental induction). Then, mycelia were harvested by being filtered through Miracloth (Calbiochem, California), transferred to solid MM with 0.1% YE, and further incubated at 37°C. Samples for RNA isolation were collected (conidia, 12, 18, 24, and 36 h of liquid culture and 6, 12, 24, and 48 h post-axenic developmental induction; Fig. 1B), squeezed dried, stored at −80°C, and subjected to total RNA isolation.

The AfFlgG, AfFlbA, and AfGpaA null (deletion) mutants were generated by transforming AF293.1 with the individual PCR-generated deletion constructs (see below). The gpaA\textsuperscript{G203R} mutants were generated by transformation of AF293.1 with the PCR-generated gpaA\textsuperscript{G203R} construct along with the wild-type AfFlgG gene. The gpaA\textsuperscript{Q204L} mutants were introduced by introducing the gpaA\textsuperscript{Q204L} construct and AfFlgG* together into AF293.1 or gflgG8 (AfFlgG\textsuperscript{null}; Table 1). The strains were then screened for the presence of the mutant alleles by PCR followed by restriction enzyme digestion of the amplicons.

\textbf{Mutagenesis and isolation of AfFlbA loss-of-function mutants.} About 10⁵ conidia of AF293.1 were inoculated on solid MM with 0.5% YE and supplemented (5 mM uridine and 10 mM uracil) and incubated at 37°C for 4 days, and the conidia were collected for mutagenesis. Approximately 10⁶ conidia of AF293.1 were then treated with 1 µg/ml or 10 µg/ml (final concentration) of 4-nitroquinoline-1-oxide (6) for 0, 30, and 60 min, respectively, as previously described (26). The mean survival rate of a treatment with 1 µg/ml 4-nitroquinoline-1-oxide for 30 min was ~70%, and more than 110,000 survivors of this condition were screened for morphological abnormalities.

\textbf{Nucleic acid isolation and manipulation.} Genomic DNA isolation was carried out as previously described (41). Briefly, about 10⁶ conidia of individual strains were inoculated in 2 ml liquid MM with 0.5% YE in 10 ml test tubes and incubated at 37°C for 18 h (stationary culture), and the mycelial mats were harvested and squeezed dried. Samples (0.2 to 0.5 g) were transferred to microcentrifuge tubes containing 400 µl of 0.5-mm zirconia/silica beads (BioSpec Products, Oklahoma), 500 µl of breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 0.1 M NaCl, 10 mM Tris-Cl [pH 8.0], 1 mM EDTA), and 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1) and ground with a Mini Bead-Beater (BioSpec Products) for 2 min. The aqueous phase was separated by centrifugation, and genomic DNA was isolated as described previously (41).

Total RNA isolation and Northern blot analyses were carried out as described previously (26, 41). Total RNA was isolated from individual samples (about 0.2 g) by adding 400 µl of 0.5-mm zirconia/silica beads and 1 ml of Trizol reagent (Invitrogen, California) and grinding the mixture in a Mini Bead-Beater for 2-3 min. Subsequent RNA isolation was performed following the manufacturer’s instructions. Total RNA (6 µg/lane) was separated by electrophoresis using a 1.1% agarose gel containing 6% formaldehyde. The nucleic acids were transferred to the MagnaProbe nylon membrane (0.45 µm; Osmonics, Minnesota). Probes were prepared by amplifying the coding regions of the individual genes from WT (AF293) genomic DNA. Primers are listed in Table S1 in the supplemental material.

\textbf{RESULTS} Identification and expression of key developmental regulators in \textit{A. fumigatus}. To begin to understand the regulatory mechanisms of growth and development in \textit{A. fumigatus}, we first identified the \textit{A. fumigatus} homologs of the five key \textit{A. nidulans} regulators (Fig. 1A). Blastp analyses of the \textit{A. fumigatus} genome (TIGR [http://www.tigr.org/tdb/e2k1/afu1/]) using the \textit{A. nidulans} Fada, Flb, FlgG, BrlA, and EtaA proteins as queries have identified GpaA (97% identity, 98% similarity), GpaA\textsuperscript{Q204L}, GpaA\textsuperscript{G203R}, and AfFlbA (79% identity, 85% similarity), AfFlgG (69% identity, 83% similarity), AfBrlA (68% identity, 77% similarity), and AfEtaA (56% identity, 66% similarity). The locus numbers are presented in Fig. 1B.

To check whether these genes are expressed, we examined the mRNA levels of individual genes throughout the life cycle of an \textit{A. fumigatus} WT strain (AF293) by Northern blot analyses. As shown in Fig. 1B, the AfFlbA and AfFlgG genes were found to encode 3.3-kb and 3.4-kb transcripts, respectively, which were present at relatively constant levels throughout the life cycle. Hybridization with the \textit{gpaA} probe resulted in the detection of two (~3.0-kb and 2.4-kb) transcripts, whose levels are high in vegetative growth, low in conidiation, and absent in conidia. While it can be speculated that the \textit{gpaA} gene may encode two transcripts, a potential cross-hybridization cannot be excluded due to the high nucleotide level identity between \textit{gpaA} and \textit{gaba} (EAL90625; 63% identity) or \textit{afgA} (EAL92343; 61% identity).

The AfFlbA and AfEtaA genes were specifically expressed during conidiation. The AfFlbA transcript reached the highest level at 6 h
and then decreased, whereas the AfwetA mRNA began to accumulate at 12 h post-developmental induction and continued to accumulate. In accordance with the occurrence of conidiophore formation in WT, AfbrlA transcripts (reviewed in reference 4) were clearly visible at 18 and 24 h of liquid MM-submerged culture conditions (see below).

Deletion and 14 additional loss-of-function AfflbA mutations cause reduced conidiation. We first attempted to dissect

FIG. 2. Phenotypes of various AfflbA mutants. (A) WT (AF293 and FGSC26) and ΔflbA (ΔAfflbA and RJA5.9) strains of A. fumigatus and A. nidulans were point inoculated on solid MM with 0.5% YE and incubated for 3 days at 37°C. Note the differences between the A. nidulans and A. fumigatus flbA mutant phenotypes. (B) Fourteen AfflbA mutant strains and the ΔflbA mutant were point inoculated on solid MM with 0.5% YE and incubated for 2 days at 37°C. Numbers indicate mutant strains and alleles. (C) The AfflbA ORF composed of 2,375 nucleotides (nt) (including three introns, IntI, IntII, and IntIII) and the approximate position of each AfflbA mutation are schematically presented. In the table, the allele number, position, and nature of the nucleotide and resulting amino acid change(s) for each AfflbA mutant allele are presented. (D) Two graphs present the results of quantitative analyses of conidiation in WT (AF293 and AF293.1) and selected AfflbA mutant strains inoculated on solid MM with 0.5% YE. Error bars indicate standard deviations calculated from biological triplicates. Note that mutations in AfflbA result in a reduced number of conidia in both cases.
FIG. 3. GpaA is the cognate Gα for AfFlbA. WT (AF293.1 and AF293.1 with AfpyrG+), ΔAfFlbA, AfFlbA88, AfFlbA88 with AfpyrG+ alone, AfFlbA88 with AfFlbA+, AF293.1 with gpaA<sup>G203R</sup>, AF293.1 with gpaA<sup>G203R</sup>, and AfFlbA88 with gpaA<sup>G203R</sup> strains were point inoculated on solid MM with 0.5% YE (A, B, and C) and incubated for 3 days at 37°C. (A to C) Entire colonies (A) and close-up views of the center (B) and the edges (C) of individual colonies (bar, 200 μm). (D) Developmental status of tested strains in liquid-submerged culture (MM) was photographed at 24 h of incubation. Note that, while WT (AF293.1 and AF293.1 with AfpyrG+), AfFlbA88 with AfFlbA+, AF293.1 with gpaA<sup>G203R</sup>, and AfFlbA88 with gpaA<sup>G203R</sup> strains form conidiophores (marked by arrowheads), ΔAfFlbA, AfFlbA88, AfFlbA88 with AfpyrG+ alone, and AF293.1 with gpaA<sup>G203L</sup> strains do not sporulate (bar, 100 μm).
the role of AfFlbA in developmental regulation by generating the ΔAfFlbA mutant. Multiple ΔAfFlbA mutants were isolated and examined for phenotypic changes. Similar to the A. nidulans AfFlbA mutant, the ΔAfFlbA mutant exhibited the fluffy phenotype during the first 2 days of growth. In contrast, while the A. nidulans AfFlbA mutant continues to accumulate hyphal mass without development, resulting in autolysis of the colony (15), the ΔAfFlbA mutant started to produce conidiophores from the center of the colony and did not undergo hyphal disintegration (Fig. 2A). However, the levels of conidiation (and spore pigmentation) in the ΔAfFlbA mutant were dramatically reduced (~30% of WT), indicating that AfFlbA is necessary for the normal levels of conidiation in A. fumigatus, but the fungus can overcome the developmental defect caused by the lack of a key RGS protein.

To further confirm that reduced conidiation is due to loss of AfFlbA function, the AfFlbA coding regions from all 14 mutants were PCR amplified and the individual amplicons were directly sequenced. As presented in Fig. 2C, sequence analyses revealed that all 14 mutants had mutations within the AfFlbA ORF.

Among 14 mutants, mGF104, mGF114, mGF49, mGF80, mGF88, and mGF1 are derived from nonsense mutations and exhibit phenotypes similar to those of the AfFlbA deletion mutant. Three mutants that have missense mutations prior to the RGS domain show various degrees of sporulation. mGF129 has a G-to-A transition, causing a mutation of the 461st amino acid Glu (acidic) to Lys (basic), which likely abolishes AfFlbA function. mGF112 has both missense (Arg373Gly) and silent (Leu380Leu) mutations, where the R373G substitution is likely responsible for the mutant phenotype. Sporulation of mGF132 that had the G337C substitution was affected less severely than that of other mutants. mGF24, mGF50, and mGF115 are all derived from deletion followed by frameshift and early termination within or before the RGS domain. The mGF131 mutant has a C insertion followed by frameshift and early termination. mGF130 is derived from a GT-to-CT transversion, which likely blocks the splicing of the second introns, resulting in frameshift and early termination.
In order to confirm whether the mutations in AfFlbA are solely responsible for the phenotype, mGF01, mGF88, and mGF104 were transformed with the wild-type AfFlbA gene and ApyrG\(^+\), and 40%, 54%, and 22% of the transformants in each case restored the WT phenotype (Fig. 3 shows results for AfFlbA88 [mGF88] and AfFlbA88 with AfFlbA\(^+\)). Interestingly, introduction of AfFlbA\(^+\) into mGF01, which produces white conidia, restored conidiation, but not spore pigmentation, to the WT level, indicating that mGF01 may have two mutations (AfFlbA1 and white1; Table 1).

Due to the evident reduction in conidiation levels of various AfFlbA\(^+\) mutants, quantitative analyses of spore formation were carried out by measuring the number of conidia produced by WT, ΔAf-flbA, and five selected AfFlbA\(^-\) strains. This was accomplished in two ways: (i) counting conidia from the entire point-inoculated colony grown for 2 to 5 days and (ii) counting conidia from the center (old region), middle, and edge (actively growing region) of the 5-day-old colony. In both cases, all AfFlbA\(^-\) mutants tested exhibited reduced (10 to 70% of WT) levels of conidiation (Fig. 2D). Moreover, unlike WT, AfFlbA\(^-\) mutants did not produce conidiophores in liquid-submerged culture (Fig. 3D). These results indicate that, while it is not absolutely required for conidiation, AfFlbA is needed for proper asexual sporulation in *A. fumigatus*.

**GpaA is the primary target of AfFlbA.** High (97%) identity between GpaA (17) and FadA led us to hypothesize that GpaA is the primary target of AfFlbA and that uncontrolled activation of GpaA-mediated signaling causes reduced conidiation. We tested this hypothesis by generating the constitutively active (Q204L) and dominant interfering (G203R) mutant GpaA alleles (references 39 and 40 and references therein) and examining the phenotypic changes caused by these mutations. If AfFlbA regulates GpaA negatively, GpaA\(^{G203R}\) should cause the phenotypic alterations similar to those resulting from loss of AfFlbA function and GpaA\(^{G203R}\) should suppress the altered conidiation phenotype caused by AfFlbA mutations in a dominant manner. Keeping this in mind, we first generated gpaA\(^{G204L}\), and gpaA\(^{G203R}\) mutant strains that are heterozygous for gpaA by cointroducing each construct with ApyrG\(^+\) into a WT strain (AF293.1, pyrG1). To generate the AfFlbA\(^+\) gpaA\(^{G203R}\) double mutant, mGF88 (AfFlbA88; pyrG1) was transformed with the gpaA\(^{G203R}\) construct and ApyrG\(^+\) or with ApyrG alone. As shown in Fig. 3, introduction of the gpaA\(^{G203R}\) allele into AF293.1 yielded the colonies exhibiting reduced conidiation and the absence of conidiophore formation in liquid-submerged culture as observed in AfFlbA\(^-\) mutants. Moreover, somewhat similarly to those found in *A. nidulans* (39), the gpaA\(^{G203R}\) mutants showed reduced radial growth with normal conidiation levels. Importantly, the introduction of the gpaA\(^{G203R}\) mutant allele into the AfFlbA88 mutant restored conidiation in both air-exposed and liquid-submerged culture conditions (Fig. 3, bottommost panel). Collectively, these results corroborate the hypothesis that GpaA is the cognate G for AfFlbA and that GpaA signaling stimulates hyphal growth while inhibiting asexual sporulation in *A. fumigatus*.

**A potential role of AffluG in sporulation.** As the upstream developmental activator FluG is required for the commencement of conidiation in *A. nidulans*, the ΔfluG mutant exhibits the nonconidial fluffy (but not autolytic) phenotype (Fig. 4A) (16). To test whether the *A. fumigatus* FluG homologue is needed for conidiation in *A. fumigatus*, the AffluG deletion mutant was generated. Somewhat unexpectedly, the AffluG deletion mutant could sporulate normally like WT but formed slightly increased levels of asexual hyphae in air-exposed culture (solid medium) conditions (Fig. 4A), indicating that activation of *A. fumigatus* conidiation in the presence of air does not

**FIG. 5. Requirement of AfbrlA in *A. fumigatus* conidiation.** (A) WT (AF293 and FGSC26) and brlA mutant (ΔAfbrlA7 and AJC11.32) strains of *A. fumigatus* and *A. nidulans* were point inoculated on solid MM with 0.5% YE and incubated at 37°C for 3 days. (B) Close-up views of the edges of the colonies (marked by squares; bar, 100 μm). Note that the AfbrlA deletion mutant fails to produce conidiophores.
mental induction is shown (bar, 100 μm). Numbers indicate time (hours) of post-asexual developmental induction. Note that AfFluG and AfFlbA mutant strains exhibit enhanced formation of aerial hyphae and reduced (and delayed) sporulation. (B) Corresponding Northern blots of the samples shown in panel A (0 h = 18 h in culture submerged in liquid MM with 0.1% YE). Equal loading of total RNA was evaluated by ethidium bromide staining of rRNA. Note that mutational inactivation of AfFluG or AfFlbA causes delayed and reduced AflbA mRNA accumulation. The AfFlbA88 with AflbA* strain shows slightly elevated AflbA expression levels, which is likely due to the presence of two copies of AflbA* (data not shown).

require the activity of AfFluG. However, while *A. fumigatus* WT strains sporulate within 24 h in liquid-submerged culture, the AfFluG deletion mutant never produced conidiophores up to 32 h. Moreover, the AfFluG deletion mutant formed less compact mycelial aggregates (Fig. 4B). Taken together, it can be speculated that, while AfFluG may play a certain role in conidiation, the presence of air can bypass the need for AfFluG in conidiophore development in *A. fumigatus*. A potential role of AfFluG in conidiation was further tested by examining the expression of AflbA (see below).

**AflbA is required for conidiophore formation.** The result suggesting that *A. fumigatus* may have a distinct upstream regulatory mechanism(s) for the activation of sporulation led us to test whether downstream regulation of conidiation by BrlA is divergent in the two aspergilli. To test this, multiple AflbA deletion mutant strains were generated. As shown in Fig. 5, deletion of AflbA completely eliminated asexual development in *A. fumigatus*, resulting in the colonies displaying elongated aerial hyphae and increased hyphal mass. These characteristics of the ΔAflbA mutant are more similar to those of the *A. nidulans* fluffy mutants than the *A. nidulans* brlA mutants, which form flat colonies. In any case, it is clear that AflbA is essential for the formation of conidiophores in *A. fumigatus* and that the role of the key downstream developmental activator BrlA in asexual development is conserved in the two aspergilli.

**AfFluG and AflbA are required for proper expression of AflbA.** In *A. nidulans*, both fluG and flbA are required for the expression of brlA (15, 16). The facts that AflbA is necessary for conidiophore formation and that accumulation of its transcript(s) is specifically coupled with conidiation (Fig. 1B) led us to test whether AfFlbA and AfFluG affect AflbA expression. Synchronous developmental induction of WT (AF293), ΔAfFluG, AflbA88, and AflbA88 with AflbA* strains was carried out, and changes in the development and brlA expression patterns were examined. We found that the ΔAfFluG and AflbA88 mutants, but not WT or AflbA88 with AflbA*, exhibited delayed conidiation with increased aerial hyphae during the early phase (6 to 12 h) of post-asexual developmental induction (Fig. 6A). Furthermore, while WT and AflbA88 with AflbA* strains accumulated high levels of AflbA transcript at 6 h post-developmental induction, the ΔAfFluG and AflbA88 mutants showed the highest level of AflbA accumulation at 12 h (Fig. 6B). These results indicate that AfFluG and AfFlbA (at least partially) function in conidiation by influencing expression of AflbA. However, in contrast to *A. nidulans*, these two upstream regulators are not absolutely required for activation of AflbA expression or conidiation in *A. fumigatus*.

**DISCUSSION**

The ease of genetic analyses and the availability of various experimental tools have made *A. nidulans* an excellent model system for studying signal transduction, multicellular development, and secondary metabolism (20, 34, 42, 43). While the study of developmental regulation in *A. nidulans* has provided valuable information, the potential use of such knowledge in dissecting the mechanisms controlling growth and development in other aspergilli remained to be tested. In this study, we examined the roles of the four key *A. nidulans* regulators in...
controlling development of the pathogenic fungus _A. fumigatus_ and demonstrated that these two _Aspergillus_ species have conserved Gα-RGS signaling components and a core downstream activator of sporulation, but _A. fumigatus_ may have distinct upstream mechanisms activating _AfbrlA_.

We previously showed that the _A. nidulans_ RGS protein FlbA (fluffy low brlA locus A) has a major role in determining the balance between vegetative growth and development through its ability to down-regulate FadA (39). When FadA-dependent signaling is activated in response to some unknown factor, it stimulates growth and blocks both asexual and sexual development. Attenuation of FadA-mediated vegetative growth signaling by FlbA allows development to occur. Inactivation of FlbA or constitutive activation of FadA, e.g., by the G42R, R178L, G183S, R178C, or Q204L mutation predicted to cause reduced (or lack of) intrinsic GTPase activity of FadA, results in uncontrolled FadA signaling and leads to proliferation of undifferentiated aerial hyphae that autolyze as colonies mature (35, 39, 40). In contrast, overexpression of _flbA_ or a dominant interfering mutation in FadA (G203R) results in inhibited hyphal growth coupled with hyperactive conidiation (15, 39). The _flbA_ loss-of-function or dominant activating _fadA_ mutations result in the fluffy-autolytic phenotype regardless of the _veA1_ or _veA+_ alleles (our unpublished data). _VeA_ is a novel multifunctional protein balancing sexual and asexual development in _A. nidulans_ and influencing production of pigments and secondary metabolite in other aspergilli (see reference 42 and references therein).

Due to such a critical function of FlbA in _A. nidulans_, we first investigated the role of the FlbA homologue AfiFlbA in _A. fumigatus_. Deletion and 14 other loss-of-function AfiFlbA mutations resulted in reduced levels of conidiation and conidial pigmentation. Furthermore, it also caused increased hyphal proliferation during the early period of growth (up to 2 days), and the mutant colonies exhibited an expanded growing edge with delayed conidiation, while WT colonies showed vigorous production of conidiophores (Fig. 3A and C). In addition, the Δ_AfiFlbA or AfiFlbA88 mutants did not produce conidiophores in liquid-submerged culture conditions, whereas WT and AfiFlbA complemented (AfiFlbA88 with AfiFlbA+) strains elaborated conidiophores abundantly. Collectively, these findings suggest that AfiFlbA functions in down-regulation of hyphal proliferation and (indirect) activation of development in _A. fumigatus_, too. However, there is a noticeable difference between the phenotypes of the _A. nidulans_ Δ_flbA and Δ_AfiFlbA mutants, where the latter never undergoes autolysis. Such a difference can be explained by a speculation that _A. fumigatus_ has multiple mechanisms activating development, which bypass the requirement of AfiFlbA in sporulation and allow the AfiFlbA mutants to escape hyphal disintegration. It is important to note that the _A. nidulans_ Δ_flbA mutant cannot proceed to development. This speculation is further studied by examining the role of AfiFluG (see below).

As the FadA homologue GpaA is the primary target of AfiFlbA, the constitutively active GpaA_G204R, mutation caused increased hyphal proliferation and reduced sporulation in a dominant manner. Moreover, the dominant interfering GpaA_G203R mutation restored conidiation in the AfiFlbA88 mutant to the WT level in both air-exposed and liquid-submerged culture conditions (Fig. 3). The G203R mutation is predicted to block the conformational change in the switch II region of GpaA and thereby prevent dissociation of GTP-Gα from Gβγ (39, 40). These results indicate that inactivation of GpaA signaling circumvents the need for AfiFlbA in proper progression of conidiation and corroborate the idea that GpaA and AfiFlbA constitute a Gα-RGS pair, which functions as a major coordinator of growth and development in _A. fumigatus_. Interestingly, the levels of _gpaA_ mRNA(s) appeared to be low during asexual development and absent in conidia in comparison to those observed during vegetative growth. If this is a part of the means by which the level of GpaA signaling is controlled, it indicates that the two aspergilli may have different regulatory mechanisms, because both the mRNA and the protein levels of _fadA_ were relatively constant throughout the life cycle of _A. nidulans_ (39).

In _A. nidulans_, both the Fada and GanB (another Gα signaling pathways are involved in activation of CAM-dependent protein kinase A (PKA [13, 32]). Two PKA catalytic subunits, PkaA and PkaB, have been shown to stimulate vegetative growth, where PkaA plays a primary role (23, 32). Deletion of _pkaA_ resulted in hyperactive conidiation with restricted vegetative growth and suppressed developmental defects caused by _Δ_flbA_ as well as the dominant activating _fadA_G42R_ mutation. Furthermore, overexpression of _pkaA_ led to reduced sporulation with elevated hyphal proliferation (32). Later, PkaA was also shown to function downstream of GanB for conidial germination (13). Similarly, in _A. fumigatus_, GpaB (GanB homologue)-mediated signaling is associated with activation of the predominant PKA catalytic subunit Pkac1 (18). However, one critical difference between two fungi is that, while deletion of _pkaC1_ also resulted in restricted hyphal growth, it caused drastically reduced sporulation (18). These findings indicate that, whereas the Gα-RGS level regulatory mechanism is conserved, asexual development is regulated differently at the level of PKA in the two aspergilli. The potential involvement of Pkac1 in the GpaA signaling pathway remains to be investigated.

The study of asexual development in _A. nidulans_ has identified a number of genes required for the activation of conidiation, where FluG functions most upstream (reviewed in reference 4; see also reference 44). The FluG-dependent sporulation requires the key downstream transcription factor BrlA, a C2H2 zinc finger DNA-binding protein, which activates development-specific gene expression beginning at the time of conidiophore vesicle formation (1, 2). Since FluG and BrlA represent key upstream and downstream activators of conidiation, we attempted to characterize the homologues of these regulators in _A. fumigatus_. Expression patterns of _AfiFluG_ and _AfiBrlA_ were almost identical to those found in _A. nidulans_ (reviewed in reference 4). However, mRNA of _AfiBrlA_ accumulates highly at 6 h post-developmental induction, whereas it takes about 12 h for the _A. nidulans_ _brlA_ mRNA to reach the same level (Fig. 1B) (30). Moreover, _AfiBrlA_ mRNA(s) started to accumulate as early as 18 h of vegetative growth in liquid-submerged culture conditions, at which time no _A. nidulans_ _brlA_ mRNA is clearly detectable. These results are in agreement with our observations that _A. fumigatus_ WT strains sporulate in liquid-submerged culture and produce conidiophores much faster than _A. nidulans_ does under synchronous developmental induction conditions (not shown).
Deletion of \textit{AfbrlA} completely eliminated conidiation in all conditions tested, indicating that the activation of \textit{AfbrlA} expression early in conidiophore development also represents a foremost and essential control point for initiating the conidiation pathway in \textit{A. fumigatus} and that the two aspergilli have a common core downstream activator for conidiophore development. However, somewhat unexpectedly, \textit{AfFluG} is found to be dispensable for conidiation in the presence of air, which is in contrast to the necessity for \textit{FluG} in \textit{A. nidulans} conidiation. On the other hand, we also found that \textit{AfFluG} (at least partially) contributes to the commencement of development under different culture conditions, i.e., liquid MM-submerged culture and synchronous developmental induction, through affecting expression of \textit{AfbrlA} (Fig. 4 and 6). We also demonstrated that \textit{AfFlbA} is necessary for the proper expression of \textit{AfbrlA} and thereby progression of conidiation. Collectively, our phenotypic, expression, and genetic studies all suggest that the pathogenic fungus \textit{A. fumigatus} may have more than one mechanism activating \textit{AfbrlA} and unique and powerful strategies for its asexual reproduction. In \textit{A. nidulans}, both inhibition of G protein-mediated growth signaling by FlbA and activation of developmental functions by FluG must occur in order for the development to proceed (reviewed in reference 4). Thus, together with the fact that both \textit{AfFluG} and \textit{AfFlbA} are required for proper expression of \textit{AfbrlA}, it will be interesting to test whether removal of both \textit{AfFlbA} and \textit{AfFluG} functions would cause additive detrimental effects on development of \textit{A. fumigatus}.

Regarding a possible \textit{FluG}-independent developmental activation branch, it is noteworthy that the newly identified \textit{A. nidulans} \textit{tmpA} gene regulates conidiation independently of the \textit{FluG} pathway (33). The TmpA protein belongs to a novel family of putative membrane flavoproteins that may be involved in the synthesis of a (different) developmental signal. The absence of \textit{tmpA} resulted in decreased \textit{brlA} expression and conidiation on solid medium, and overexpression of \textit{tmpA} tagged alleles caused conidiation in liquid-submerged culture. Three lines of evidence indicate that TmpA and FluG regulate conidiation through independent pathways: (i) conidiation of the \textit{\textDelta tmpA} mutant could be restored by juxtaposed growth with WT or the \textit{\textDelta fluG} mutant, (ii) overexpression of \textit{fluG} induced conidiation independently of \textit{tmpA}, and (iii) the \textit{\textDelta tmpA \textDelta fluG} double mutants exhibited an additive fluffy phenotype (33). If the homologue of TmpA plays a similar role in \textit{A. fumigatus} conidiation, it can be speculated that the presence of either \textit{AfFluG} or \textit{AfTmpA} function alone may be sufficient to confer the progression of conidiation in \textit{A. fumigatus}.

Finally, based on our findings, we present a genetic model for regulation of asexual development and vegetative growth in \textit{A. fumigatus} (Fig. 7). In this model, similar to the one proposed for \textit{A. nidulans}, \textit{AfFlbA} functions as the major negative regulator of GpaA-mediated signaling that stimulates vegetative growth, which in turn inhibits sporulation. The GpaB-PkaC signaling pathway has been proposed to induce both hyphal growth and conidiation (18). \textit{AfBrlA} is essential for the activation of conidiophore formation, and its expression is influenced in part by \textit{AfFluG} and \textit{AfFlbA}. The potential presence of an upstream mechanism(s) activating \textit{AfbrlA} that is independent of \textit{AfFluG} is indicated. The roles of other G protein components and FLB genes in conidiation as well as the involvement of negative regulators of conidiation including SfG on \textit{A. fumigatus} remain to be studied. Experiments testing the roles of these \textit{A. fumigatus} developmental regulators in gliotoxin production (reviewed in reference 14) and virulence establishment are currently being carried out.

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