The EMBO Journal vol.15 no.19 pp.5184–5190, 1996

The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development

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flbA **encodes an *Aspergillus nidulans* RGS (regulator of G protein signaling) domain protein that is required for control of mycelial proliferation and activation of asexual sporulation. We identified a dominant mutation in a second gene, *fadA*, that resulted in a very similar phenotype to *flbA* loss-of-function mutants. Analysis of *fadA* showed that it encodes the α-subunit of a heterotrimeric G protein, and the dominant phenotype resulted from conversion of glycine 42 to arginine (*fadA*^{G42R}). This mutation is predicted to result in a loss of intrinsic GTPase activity leading to constitutive signaling, indicating that activation of this pathway leads to proliferation and blocks sporulation. By contrast, a *fadA* deletion and a *fadA* dominant-interfering mutation (*fadA*^{G203R}) resulted in reduced growth without impairing sporulation. In fact, the *fadA*^{G203R} mutant was a hyperactive asexual sporulator and produced elaborate sporulation structures, called conidiophores, under environmental conditions that blocked wild-type sporulation. Both the *fadA*^{G203R} and the *fadA* deletion mutations suppressed the *flbA* mutant phenotype as predicted if the primary role of FlbA in sporulation is in blocking activation of FadA signaling. Because overexpression of *flbA* could not suppress the *fadA*^{G42R} mutant phenotype, we propose that FlbA's role in modulating the FadA proliferation signal is dependent upon the intrinsic GTPase activity of wild-type FadA.**

Keywords: *Aspergillus nidulans*/conidiophore/ heterotrimeric G protein/RGS domain/signal transduction

Introduction

The *Aspergillus nidulans flbA* gene (Lee and Adams, 1994b) encodes a member of an emerging protein family that includes proteins from a wide range of organisms including yeast and man (Dietzel and Kurjan, 1987; De Vries *et al.*, 1995; Druey *et al.*, 1996; Koelle and Horvitz, 1996). All of the proteins in this family share a conserved C-terminal domain of ~120 amino acids that has been implicated in negatively affecting heterotrimeric G protein signaling events and has therefore been called the RGS domain (for regulator of G protein signaling). The best characterized member of this protein family is *Saccharomyces cerevisiae* Sst2 which is required for adaptation to mating pheromone (Dietzel and Kurjan, 1987; Weiner *et al.*, 1993; Dohlman *et al.*, 1995). Genetic studies of Sst2 function have all been consistent with a model in which Sst2 acts by negatively regulating the signal response to pheromone either prior to or at the level of G protein activation (Dietzel and Kurjan, 1987; Hasson *et al.*, 1994; Dohlman *et al.*, 1995). Similarly, genetic analysis of *Caenorhabditis elegans* egg-laying behavior predicted that the RGS domain protein EGL-10 functions in antagonizing signaling by the Gα protein GOA-1 (Koelle and Horvitz, 1996). While the specific roles for mammalian RGS domain proteins in controlling signal response pathways are not as well defined genetically, the best biochemical evidence for direct interactions between the RGS domain and a Gα protein come from studies of human $G_i\alpha_3$ and GAIP (G alpha-interacting protein; De Vries *et al.*, 1995). GAIP is an RGS domain protein that was identified based on its ability to interact with $G_i\alpha_3$ in a yeast two-hybrid screen. Moreover, *in vitro* interaction experiments showed that the core RGS domain was required for this interaction (De Vries *et al.*, 1995). Amongst the other 15 mammalian RGS domain proteins identified to date, some function in yeast, partially complementing *SST2*-defective mutants, while others have *in vivo* activities affecting specific G protein signaling pathways (Druey *et al.*, 1996). All of these studies point to the RGS domain having a specific function in interacting with G proteins to negatively control intracellular signaling.

The *A.nidulans flbA* gene was identified by a mutation that blocks the asexual reproductive pathway prior to formation of specialized spore-bearing structures called conidiophores (Lee and Adams, 1994b; Wieser *et al.*, 1994). *flbA* mutants have a phenotype described as 'fluffy autolysis' because they fail to make the transition from vegetative growth to conidiophore formation that normally occurs early in colony development. This defect leads to proliferation of undifferentiated aerial hyphae that autolyse as colonies mature (Lee and Adams, 1994b; compare Figure 2A and C). By contrast, overexpression of *flbA* in vegetative cells has the opposite phenotype, i.e. inhibited hyphal growth coupled with conidiophore development even under growth conditions that normally prevent sporulation (Lee and Adams, 1994b, 1996). This ability of *flbA* overexpression to modulate growth and activate sporulation is dependent upon the activities of other genes, including *fluG*, which is apparently needed for production of a small diffusible molecule that signals initiation of conidiophore development (Lee and Adams, 1994a, 1996). Because *fluG*-driven activation of development has also been shown to be dependent on *flbA*, we have proposed that *flbA* is needed to regulate a response to the FluG signal (Lee and Adams, 1996).

Here, we describe the characterization of a dominant mutation in a gene called *fadA* that results in a fluffy autolysis phenotype like that described for *flbA* loss-of-function mutants (Figure 2). We found that the *fadA* gene

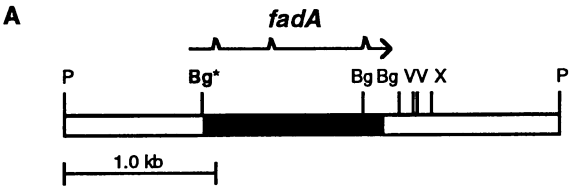
encodes a heterotrimeric G protein α-subunit and that the dominant phenotype apparently resulted from FadA gain-of-function because the mutation was in the FadA GTPase domain (FadA^{G42R}; Simon *et al.*, 1991; Kurjan, 1992; Neer, 1995). Thus, activation of FadA signaling leads to the same phenotype as loss of FlbA function, as expected if the normal role of the FlbA RGS domain protein is to interfere with FadA activity. This hypothesis is supported by the finding that a dominant-interfering mutation in FadA (FadA^{G203R}; Simon *et al.*, 1991; Kurjan, 1992) suppressed the developmental defect of *flbA* deletion mutants and stimulated sporulation in wild-type strains. The mechanism by which FlbA functions in blocking FadA signaling is not known, but it requires the GTPase activity because overexpression of *flbA* could not activate sporulation in the *fadA*^{G42R} mutant strain. However, overexpression of *flbA* in a *fadA* deletion mutant did activate sporulation, indicating that *flbA* must have additional functions besides inhibition of FadA, perhaps controlling the activity of other subunits of the heterotrimeric G protein. Finally, because development of the *fadA*^{G203R} mutant strain remains dependent on *fluG* function, we propose a model in which developmental activation by the FluG signal requires both modulation of growth through FlbA-dependent inhibition of FadA signaling and stimulation of development-specific events.

Results

fadA encodes the α-subunit of a heterotrimeric G protein

Our analysis of *flbA* function in controlling sporulation led us to characterize additional mutants with fluffy autolytic phenotypes similar to *flbA* loss-of-function strains. All of the recessive fluffy autolytic mutants identified in our original screen (Wieser *et al.*, 1994) could be complemented by the cloned *flbA* gene and therefore were considered likely to represent additional *flbA* alleles. In a separate screen to identify mutants blocked in asexual sporulation, we mutagenized a diploid strain with the goal of identifying dominant-acting mutations in genes with specific functions in development (J.Wieser and T.H. Adams, unpublished results). Our expectation was that such dominant mutations could define either novel inactivating alleles of genes identified in the original screen or a different set of genes that caused a fluffy phenotype through gain-of-function mutations. This approach led to the identification of several dominant fluffy mutants, 15 of which had an autolytic phenotype (called FAD for fluffy autolytic dominant) somewhat like *flbA* loss-of-function mutants. One such diploid mutant strain dFAD4 was particularly similar to *flbA* null mutants, and the characterization of this mutant is described here. Treatment of dFAD4 with the microtubule-destabilizing agent benomyl allowed isolation of both mutant and wild-type haploid strains, indicating that the original mutation was indeed dominant. One haploid fluffy strain (h1FAD4; Figure 2E) was selected for further studies.

To recover the gene responsible for the FAD phenotype, a library of h1FAD4 genomic DNA was constructed and used for transformation of a wild-type strain (see Materials and methods). Because *Aspergillus* transformation typically involves integration into the genome, recovery of the transforming plasmids from the two FAD transformants isolated required restriction digestion of genomic DNA followed by ligation and *Escherichia coli* transformation. The gene containing the dominant mutation was localized within an ~3.2 kb *Pst*I fragment and designated *fadA* (Figure 1A). This cloned region was used to recover the wild-type *fadA* gene by probing a partially ordered cosmid library, and four cosmids (pW04H05, pW30E01, pL09F12 and pL30F09) that are known to be derived from chromosome VIII (Brody *et al.*, 1991) were recovered. These same cosmids were also identified based on hybridization to the *benA* gene (May *et al.*, 1987), encoding β tubulin, indicating that *fadA* is physically linked to *benA*.



B

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  1 MGCGMSTEDK EGKARNEEIE NQLKRDKMMQ RNEIKMLLLG AGESGKSTIL
 51 KQMKLIHEGG YSRDERESFK EIIYSNTVQS MRVILEAMES LELPLEDARN
101 EYHVQTVFMQ PAQIEGDSLP SEVGNAIAAL WQDAGVQECF KRSREYQLND
151 SAKYYFDSIE RIAQSDYLPT DQDVLRSRVK TTGITETTFI IGDLTYRMFD
201 VGGQRSERKK WIHCFENVTT ILFLVAISEY DQLLFEDETV NRMQEALTLF
251 DSICNSRWFV KTSIILFLNK IDRFKEKLPV SPMKNYFPDY EGGADYAAAC
301 DYILNRFVSL NQAEQKQIYT HFTCATDTTQ IRFVMAAVND IIIQENLRLC
351 GLI*
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Fig. 1. *fadA* gene structure. (**A**) The restriction map of a 3.2 kb *Pst*I fragment containing the *fadA* gene is shown. The direction of *fadA* transcription was deduced by analysis of a cDNA clone and is indicated by the arrow positioned above the map. Approximate intron positions are indicated by discontinuities in the arrow. The location of the *fadA* coding region is represented by the black box. The *Bgl*II site (Bg*) just upstream of the initation codon ATG was introduced by site-directed mutagenesis and used for constructing the *fadA* deletion plasmid, pJYFD3. Restriction sites are abbreviated: (Bg) *Bgl*II; (P) *Pst*I; (V) *Eco*RV; (X) *Xho*I. (**B**) The deduced amino acid sequence of FadA. A consensus myristoylation site and a putative pertussis toxin labeling site are underlined near the N- and C-terminal ends, respectively (see text). The dominant-activating (G42R) and a dominant-interfering (G203R) mutations described in the text are indicated by underlined bold text. The accession number for the *fadA* nucleotide and peptide sequences is U49917.

Sequence analysis of the wild-type and mutant *fadA* clones and of a nearly full-length *fadA* cDNA showed that *fadA* is predicted to encode a 353 amino acid polypeptide that is highly related to the α-subunits of heterotrimeric G proteins (Figure 1B). FadA shares >90% identity with the *Neurospora crassa* Gna2 (Turner and Borkovich, 1993) and the *Cryphonectria parasitica* CPG1 (Choi *et al.*, 1995) proteins and all three fungal proteins share ~55% identity with mammalian $G_i\alpha$ proteins. Moreover, FadA, Gna2 and CPG1 all share the diagnostic features of the $G_i\alpha$ family including a cysteine four amino acids from the C-terminal end which is part of the consensus pertussis toxin labeling site (West *et al.*, 1985) as well as a consensus myristoylation site (MGXXXS) at the N-terminal end (Buss *et al.*, 1987). Comparison of the

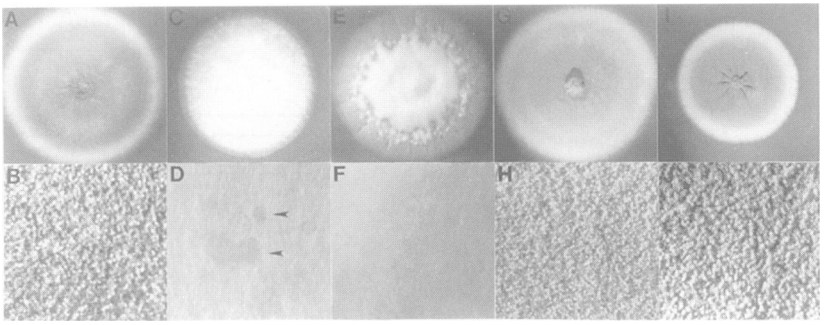

Fig. 2. Phenotypes of *flbA* and *fadA* mutant strains. The wild-type [FGSC26 (**A**) and (**B**)], *flbA*$^-$ [MJW100 (**C**) and (**D**)], *fadA*^{G42R} [h1FAD4 (**E**) and (**F**)], *flbA*$^-$; *fadA*^{G203R} [TJY12.5 (**G**) and (**H**)] and Δ*fadA* (RJY918.6 (**I**) and (**J**)] strains were point inoculated onto complete media (Käfer, 1977) and allowed to grow for 4 days at 37°C. (B), (D), (F), (H) and (J) represent a 30-fold magnification of samples in (A), (C), (E), (G) and (I), respectively. MJW100 (C) and h1FAD4 (E) both undergo autolysis after continued growth, and the arrowheads in (D) show the beginning of autolysis near the center of MJW100.

dominant *fadA* mutant allele with wild-type *fadA* revealed a G to A transition that resulted in conversion of Gly42 to arginine. This particular glycine is known to be required for GTPase activity so this mutation is predicted to result in Gα being constitutively GTP bound (Simon *et al.*, 1991; Kurjan, 1992). Because GTP binding is expected to activate signaling, we predict that the FadA^{G42R} results in constitutive activation of a signaling pathway that stimulates proliferation and blocks sporulation.

fadA is not required for growth or sporulation

If *fadA* functions in signaling cell proliferation, we predicted that the phenotype of a *fadA* knockout mutant would be approximately opposite to that of the *fadA* dominant-activating mutation. Because *fadA*^{G42R} caused proliferation and blocked conidiation, we expected that a *fadA* null mutant would have inhibited growth and might hypersporulate or sporulate under conditions that suppress conidiation in wild-type strains. Alternatively, because free Gβγ heterodimer in some cases activates downstream effectors (Clapham and Neer, 1993; Rens-Domiano and Hamm, 1995), deletion of *fadA* might yield the same phenotype as *fadA*^{G42R} mutations. To distinguish between these hypotheses, a developmentally wild-type *argB*$^-$ strain (PW1) was transformed with a plasmid containing a fragment that spanned the *fadA* gene region from which *fadA* had been deleted and replaced with the wild-type *argB* gene (pJYFD3). As shown in Figure 2I, the growth of the *fadA* deletion mutant was reduced when compared with wild-type (Figure 2A) but sporulation was not greatly affected (Figure 2I and J), indicating that Gα (not Gβγ) must be primarily responsible for signaling proliferation.

We further examined the possibility that free Gβγ is at least partially involved in signaling growth or preventing sporulation by constructing a dominant-interfering allele of *fadA* that is expected to result in a Gα subunit that would remain associated with Gβγ regardless of whether it is bound to GDP or GTP. This was accomplished by changing the glycine residue of the conserved DXXG (Gly203) domain to arginine (*fadA*^{G203R}). This change is expected to prevent the conformational switch that accompanies GTP binding and is necessary for Gβγ release, thus resulting in dominant inactivation of signaling (Kurjan, 1992; Rens-Domiano and Hamm, 1995). Several mutant strains were recovered following transformation of a wild-type strain and in most cases were found to have both *fadA*$^+$ and one or more copies of *fadA*^{G203R} alleles. Growth of these mutant strains was reduced much more severely than growth of the *fadA* deletion mutant (Table I), and sporulation occurred more quickly than in wild-type as well as under growth conditions that normally inhibit sporulation, including submerged culture (Figure 3B). In one strain, the wild-type *fadA* gene had been replaced by *fadA*^{G203R} and the phenotype was virtually identical to that of the merodiploid, indicating that the *fadA*^{G203R} mutation was strongly dominant. These results support the hypothesis that the Gβγ heterodimer, as well as Gα, is associated with negatively controlling sporulation and possibly stimulating growth.

Table I. Genetic interactions between *flbA* and *fadA*

Genotype	Growth[a]	Sporulation	
		Aerial	Submerged
WT	0.55	++	–
fadA^{G42R}	0.55	–	–
fadA^{G203R}	0.32	+++[b]	++
fadA^{G203R}; *flbA*$^-$	0.46	+++[b]	++
Δ*fadA*	0.38	++	–
Δ*fadA*; Δ*fluG*	0.46	–	–
fadA^{G203R}; Δ*fluG*	0.33	–	–
flbA$^-$[c]	0.54	–	–
fadA^{G42R}; *alcA(p)::flbA*[d]	0.56	–	–
Δ*fadA*; *alcA(p)::flbA*	ND[e]	ND	++
alcA(p)::flbA	ND	ND	++

[a]Radial extension of colonies grown on complete medium (Käfer, 1977) at 37°C was determined as mm/h. All measurements come from three different colonies and deviation was <5%.
[b] Colonies formed beginning from single spores of strains with the *fadA*^{G203R} mutant allele produced conidiophores within 22 h of inoculation, as compared with 28 h in the wild-type (FGSC26) and a Δ*fadA* mutant strain.
[c]All *flbA* mutant strains used in this study behaved similarly in these experiments.
[d] *alcA*(p)::*flbA* induction was accomplished by transferring glucose-grown cultures to *alcA*-inducing medium as described previously (Lee and Adams, 1994b, 1996).
[e]Not determined.

Suppression of a flbA null mutation by fadA^{G203R} *and ΔfadA*

The observation that a gain-of-function *fadA* mutation (*fadA*^{G42R}) resulted in very similar phenotypes to loss-of-function mutations in the RGS domain protein FlbA led

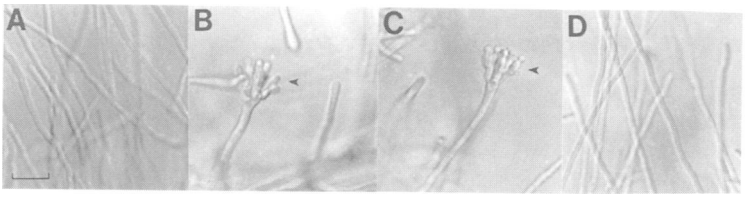

Fig. 3. *fadA*^{G203R} overcomes the need for air in activating sporulation. Conidia (5×10^5 conidia/ml) from (**A**) FGSC26 (wild-type), (**B**) RJY115.4 (*fadA*^{G203R}), (**C**) TJY12.5 (*flbA*$^-$; *fadA*^{G203R}) and (**D**) RJY918.6 (Δ*fadA*) were inoculated into 100 ml of liquid minimal media (Käfer, 1977) with supplements in 250 ml flasks and incubated at 37°C shaking at 300 r.p.m.. Mycelia from each culture were observed microscopically at 12, 14, 16, 18, 20, 24 and 36 h to assess development. Micrographs were taken at 20 h when RJY115.4 (B) and TJY12.5 (C) had each produced complete conidiophores (arrows). FGSC26 (A) and RJY918.6 (D) never produced conidiophores under these conditions. The scale bar shown in (A) is 10 μm.

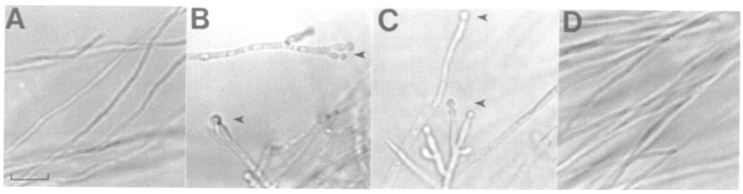

Fig. 4. Overexpression of *flbA* caused development without *fadA*. Cultures of (**A**) FGSC26 (wild-type), (**B**) RBN138 [*alcA(p)::flbA*], (**C**) RJYA21 [*alcA(p)::flbA*, Δ*fadA*] and (**D**) RJY918.6 (Δ*fadA*) were grown in *alcA(p)*-repressing medium (glucose) for 14 h and then shifted to *alcA(p)*-inducing medium (threonine). Micrographs were taken 12 h after *alcA(p)* induction. Overexpression of *flbA* with (B) or without *fadA* (C) caused conidiophore development (arrows), indicating that FlbA has additional role(s) in addition to negative control of FadA signaling (see text). The scale bar in (A) is 10 μm.

us to propose that the primary role of FlbA in controlling growth and activating sporulation is in negatively affecting FadA signaling. To test this hypothesis, we transformed a *flbA*$^-$ strain (RJW98) with the *fadA*^{G203R} dominant-interfering mutant allele (pJYFN9) to get TJY12.5. We also constructed the Δ*flbA; fadA*^{G203R} double mutant, that lacked the *fadA*$^+$ gene (RJYF01), by parasexual genetics (see Materials and methods), and it behaved in the same way as TJY12.5. As shown in Figure 2G and H, the *fadA*^{G203R} mutant allele overcame the need for *flbA* in sporulation. The *flbA*$^-$*; fadA*^{G203R} double mutant strain also sporulated in submerged culture (Figure 3C) like the *fadA*^{G203R} single mutant strain, indicating that *flbA* is not required for this process, consistent with the hypothesis that FlbA functions prior to FadA. Finally, we constructed a Δ*fadA; ΔflbA* double mutant strain (RJYE61) and observed that the Δ*fadA* mutation also overcame the need for *flbA* in sporulation, further supporting the hypothesis that the main function of FlbA is to inactivate FadA.

To address this question of how FlbA functions in another way, we tested to see if *flbA* overproduction could suppress the dominant-activating *fadA* mutation by constructing a *fadA*^{G42R} mutant strain that contained an *alcA(p)::flbA* fusion construct that allows induced overexpression of *flbA*. We showed previously that *flbA* overexpression, like *fadA*^{G203R}, could cause sporulation in submerged culture (Lee and Adams, 1994b, 1996). However, no sporulation was observed following *flbA* overexpression in the *fadA*^{G42R} mutant (Table I), indicating that *flbA* activation of development requires FadA GTPase activity.

fadA is not required for flbA overexpression-induced sporulation

If overproduction of FlbA activates development by interfering with FadA signaling, we would expect that *flbA* induction in a *fadA* deletion mutant would have no effect on growth or development. However, as shown in Figure 4, growth of a Δ*fadA*;*alcA(p)::flbA* mutant strain in *alcA(p)*-inducing medium caused sporulation just like that observed following *alcA(p)::flbA* induction in a wild-type strain. No development was observed in either wild-type (Figure 4A) or Δ*fadA* (Figure 4D) mutant strains lacking the *alcA(p)::flbA* fusion grown in *alcA(p)*-inducing medium.

fluG is required for sporulation in fadA^{G203R} *and ΔfadA mutants*

We proposed previously that the *fluG* gene product is required for the synthesis of a small diffusible factor that is required for endogenously regulated induction of asexual sporulation. Because *fluG* is needed for *flbA*-induced sporulation, we hypothesized that FlbA activity is dependent on the presence of FluG factor. We predicted that if the only function of FluG factor in developmental induction was activation of FlbA, the *fadA*^{G203R} allele should suppress the developmental defect in a *fluG* deletion mutant. To test this possibility, we constructed Δ*fluG*; *fadA*^{G203R} (and Δ*fluG*; Δ*fadA*) double mutant strains and examined their growth and sporulation phenotypes (Table I). We found that *fluG* was always required for sporulation but the double mutants did have the restricted growth phenotypes expected for *fadA*^{G203R} (and Δ*fadA*) mutant strains.

Discussion

The genetic data presented here support the model that FlbA (like Sst2 and EGL-10) functions upstream of or at the same level as a Gα protein (FadA) to negatively affect signaling. The final result of this activity is reduced growth coupled with sporulation, and this certainly involves the activities of other genes. Both protein and mRNA encoded by *fadA* and *flbA* (Lee and Adams, 1994b) can be detected at relatively constant levels throughout the *A.nidulans* lifecycle (data not shown), indicating that regulation of the FlbA–FadA interaction occurs at some level other than expression.

As described in the model presented in Figure 5, we

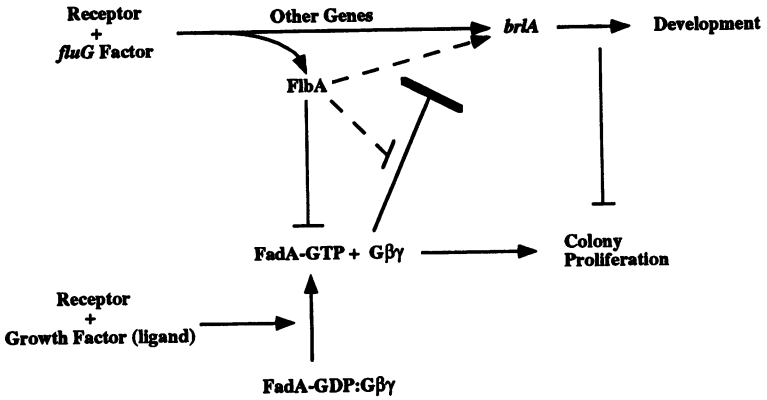

Fig. 5. Proposed model for *flbA* and *fadA* control of *A.nidulans* growth and development. We propose that FadA functions in responding to an unknown factor to stimulate growth and block sporulation. This FadA-dependent signaling pathway is modulated by *fluG* and *flbA* activities. *fluG* is responsible for production of a diffusible signal that controls initiation of conidiophore development (Lee and Adams, 1994a). This signal could function in part by activating FlbA, which then blocks FadA signaling, but must also have a role in activating sporulation-specific functions. The other genes required for sporulation in response to the FluG signal include *flbB*, *flbC*, *flbD* and *flbE* (Wieser *et al.*, 1994; Wieser and Adams, 1995). The role of FlbA in controlling development cannot be explained fully by its role in antagonizing FadA signaling because overexpression of *flbA* causes sporulation in a Δ*fadA* mutant. It is possible that FlbA interferes with Gβγ signaling or has a direct role in activating sporulation-specific genes like *brlA*. Finally, *brlA* activation has also been shown to result in growth inhibition (Adams *et al.*, 1988; Adams and Timberlake, 1990a).

propose that during early colony growth some unknown factor interacts with a receptor to activate the FadA proliferation signal. As hyphal cells mature, a different signaling pathway controlling sporulation and blocking proliferation is activated. The likely activator for this sporulation pathway is the small diffusible signal produced by FluG (Lee and Adams, 1994a, 1996). One possible result of FluG signaling is activation of FlbA, which then interferes with FadA signaling of proliferation. The growth reduction resulting from FlbA blocking FadA activity is apparently essential for sporulation to take place because FluG-induced development normally requires FlbA (Lee and Adams, 1996). However, interfering with the growth signal by either overproducing FlbA or by rendering FadA non-functional cannot cause sporulation without FluG factor, indicating that *fluG* must have additional functions in activating development. Thus, the interdependence previously described for *fluG* and *flbA* activities in developmental induction can be explained by these apparent co-requirements for growth control with other FluG-dependent signaling, to activate sporulation.

The demonstration that a *flbA* deletion mutation can be suppressed by a dominant-interfering mutation in *fadA* or a *fadA* deletion mutation supports the hypothesis that the primary function of FlbA in development is in negatively affecting FadA signaling. The mechanism by which FlbA-related RGS domain proteins interfere with G protein-mediated signaling is not clear. There is substantial evidence that Sst2 and GAIP function through direct interactions with specific Gα subunits and this has led to the hypothesis that RGS domain proteins could function as GAPs, activating the GTPase (Angelson and Wensel, 1993; Otto-Bruc *et al.*, 1994; De Vries *et al.*, 1995; Dohlman *et al.*, 1995; Druey *et al.*, 1996). In keeping with this model, we found that overexpression of *flbA* was not able to activate sporulation in the FadA^{G42R} GTPase mutant strain. However, FlbA must have functions in addition to inhibiting FadA activity because overexpression of *flbA* was able to activate sporulation in a *fadA* deletion mutant. This Gα-independent function of FlbA is in stark contrast to Sst2 which is completely dependent on the Gα protein encoded by *GPA1* for its function (Dohlman *et al.*, 1995). We consider it likely that the *fadA*-independent activity of *flbA* involves interactions with the Gβγ subunits. This hypothesis is based on the observation that the *fadA*^{G203R} dominant-interfering mutation causes sporulation in submerged culture but deletion of *fadA* does not. Because the major difference between these two mutations is that the *fadA*^{G203R} mutation is predicted to interfere with Gβγ as well as Gα signaling, and this is apparently sufficient to cause sporulation, the *flbA* overexpression result could be explained if FlbA inactivates signaling by all three G protein subunits.

Regardless of how FlbA and FadA interact to control proliferation, it is increasingly clear that proteins with RGS domains are able to negatively affect G protein signaling. The existence of at least 15 RGS homologs in mammals is proposed to provide cells with a mechanism for independently regulating various G protein targets (Druey *et al.*, 1996). Although the extent to which G protein control of intracellular signaling regulates growth and development in filamentous fungi has not been investigated fully, at least two distinct Gα proteins have been identified in *C.parasitica* (CPG-1 and CPG-2; Choi *et al.*, 1995) and *N.crassa* (Gna-1 and Gna-2; Turner and Borkovich, 1993). CPG-1, like FadA, is associated with growth control and, interestingly, is also necessary in *C.parasitica* for a virulent interaction with chestnut trees (Choi *et al.*, 1995). The general relevance of FlbA-like RGS domain proteins in controlling these and other fungal G protein-related activities requires further study.

Materials and methods

Aspergillus strains, growth conditions and genetics

The *A.nidulans* strains used in this study are described in Table II. Standard *A.nidulans* transformation and genetic techniques were used (Pontecorvo *et al.*, 1953; Yelton *et al.*, 1984). When appropriate, genotypes of strains generated for this study were confirmed by genomic DNA Southern blot analyses. The *fadA* deletion strain TJYFD3 was generated by transforming PW1 with pJYFD3, and RJY918.6 and RJY918.8 were then derived from a sexual cross between FGSC237 and TJYFD3. RJYA21 and RJYB27 were generated by sexual crosses of

Table II. *Aspergillus nidulans* strains

Strain	Genotype	Source
FGSC237	*pabaA1, yA2; trpC801, veA1*	FGSC[a]
FGSC26	*biA1; veA1*	FGSC
h1FAD4	*biA1; fadA*^{G42R}*; veA1*	J.Wieser and T.H.Adams
MJW100	*pabaA1, yA2, flbA100; veA1*	Wieser *et al.* (1994)
PW1	*biA1; argB2; methG1; veA1*	P.Weglenski
RBN138	*wA3; pyroA4; alcA(P)::flbA::trpC, veA1*	Lee and Adams (1996)
RJW98	*pabaA1, yA2, flbA98; argB2; veA1*	J.Wieser and T.H.Adams
RJY115.2	*pabaA1, yA2; argB2; fadA*^{G203R}*, veA1*	this study
RJY115.3	*biA1; argB2; methG1; fadA*^{G203R}*, veA1*	this study
RJY115.4	*yA2; fadA*^{G203R}*, veA1*	this study
RJY918.6	*argB2; methG1; ΔfadA::argB, veA1*	this study
RJY918.8	*pabaA1, yA2, biA1; ΔfadA::argB, trpC801, veA1*	this study
RJYA21	*biA1, wA3; ΔfadA::argB, alcA(p)::flbA::trpC, veA1*	this study
RJYB27	*pabaA1, yA2; ΔfluG::trpC; ΔfadA::argB, veA1*	this study
RJYC28	*pabaA1, yA2; ΔfluG::trpC; fadA*^{G203R}*, veA1*	this study
RJYE61	*pabaA1, biA1, ΔflbA::argB; ΔfadA::argB, trpC801, veA1*	this study
RJYF01	*biA1, ΔflbA::argB; fadA*^{G203R}*, veA1*	this study
TBN39.5	*biA1, ΔflbA::argB; methG1; veA1*	Lee and Adams (1994b)
TJY12.5[b]	*pabaA1, yA2, flbA98; argB2; argB::fadA*^{G203R}*, fadA*$^{+}$*,veA1*	this study
TJY13.1	*pabaA1, wA3; pyroA4; pyroA::fadA*^{G42R}*, alcA(p)::flbA::trpC, fadA*$^{+}$*,veA1*	this study
TTA11	*pabaA1, yA2; veA1*	Adams and Timberlake (1990b)
TTA127.4	*pabaA1, yA2; ΔfluG::trpC; trpC801, veA1*	Lee and Adams (1994a)
TJYFN9.1[b]	*biA1; argB2; methG1; argB::fadA*^{G203R}*, fadA*$^{+}$*, veA1*	this study
TJYFN9.2	*biA1; methG1; fadA*^{G203R}*, veA1*	this study

[a]Fungal Genetics Stock Center.
[b]These strains have approximately two copies of the *fadA*^{G203R} allele as well as wild-type *fadA*$^{+}$.

RJY918.6 with RBN138 and TTA127.4, respectively. The *fadA*^{G203R} strains (RJY115.2, RJY115.3, RJY115.4) are sexual progeny resulting from a cross between FGSC237 and TJYFN9.2, a pJYFN9 transformant of PW1 in which the wild-type *fadA* locus had been replaced by the mutant *fadA*^{G203R} allele. RJYC28 was isolated as the progeny of a sexual cross between RJY115.3 with TTA127.4. RJYE61 and RJYF01 were generated by first forming diploids between RJY918.8 and TBN39.5 or RJY115.2 and TBN39.5, followed by benomyl treatment and identification of haploid sectors. TJY12.5 (*flbA*$^{-}$; *fadA*^{G203R}) was generated by transforming RJW98 (*flbA98*) with pJYFN9 and was shown to have two copies of the *fadA*^{G203R} allele. TJY13.1 [*fadA*^{G42R}, *alcA(p)::flbA*] was generated by transforming RBN138 with a plasmid carrying the *fadA*^{G42R} allele (pJYSM3).

All strains were grown in appropriately supplemented minimal or complete medium (Käfer, 1977). Colony growth rates and developmental timing were determined as described (Lee and Adams, 1994b; Wieser *et al.*, 1994). Cultures for the *alcA(p)::flbA* induction experiments were inoculated at a density of 5×10^5 spores/ml and grown as described previously (Lee and Adams, 1994b, 1996).

Nucleic acid manipulations

To isolate the mutant *fadA* allele, we constructed a library of h1FAD4 genomic DNA with an average insert size of ~7 kb using a plasmid vector that contained the *A.nidulans argB*$^{+}$ gene (pPK1; Wieser and Adams, 1995). This library was used to transform a developmentally wild-type *argB*$^{-}$ strain selecting for arginine prototrophy and screening visually to identify transformants with the FAD phenotype. Two FAD mutants were identified among 2000 *argB*$^{+}$ transformants, and each was used to recover the transforming DNA. Genomic DNA from FAD transformants was digested with either *Xba*I or *Xho*I followed by phenol–chloroform extraction and self-ligation. The self-ligated genomic DNA was used to transform *E.coli* HB101 to ampicillin resistance, and plasmids recovered from selected transformants were used to screen the FAD mutant genomic DNA library. A 3.2 kb *Pst*I fragment containing the *fadA*^{G42R} allele was used to isolate the wild-type *fadA* gene from pLORIST and pWE cosmid libraries (Brody *et al.*, 1991). The *fadA* cDNA was isolated by screening the λUNI-ZAP library made from vegetatively grown wild-type tissue (O'Donnel *et al.*, 1991). Total RNA and proteins were purified from a wild-type strain (TTA11) following developmental induction and were analyzed as previously described (Lee and Adams, 1994b, 1996). The CPG-1 antiserum used for the FadA Western blot was supplied by D.L.Nuss (Choi *et al.*, 1995).

The *fadA* disruption vector was constructed by first introducing a *Bgl*II site 25 bases 5′ of the ATG codon using site-directed mutagenesis with the synthetic oligonucleotide CACTCAgATcTATCCGACTTT-CTAAAATCC (lower case letters represent mismatches) as described (Kunkel, 1985). The entire *fadA* coding region was then deleted by *Bgl*II digestion to remove a 1.3 kb fragment that extended 110 bases beyond the *fadA* stop codon (see Figure 1). This fragment was replaced by a 1.8 kb *Bam*HI fragment containing the *argB* gene to yield pJYFD3 which was used to transform strain PW1. The *fadA*^{G203R} dominant-interfering mutation was generated by site-directed mutagenesis with the synthetic oligonucleotide CGTTGGTcGaCAGCGTTCTGAGCG which introduced a *Sal*I site for screening convenience. The 3.2 kb *Pst*I fragment with the *fadA*^{G203R} allele was moved into pPK1 to give pJYFN9 which was used for transformation of wild-type and *flbA*$^{-}$ strains. pJYSM3 was constructed by moving the 3.2 kb *Pst*I fragment with the *fadA*^{G42R} allele into a plasmid vector containing the *A.nidulans pyroA* gene (pSM3, kindly provided by Dr L.Yager, Temple University) and was used to transform RBN138.

Microscopy

Photomicrographs of hyphal development were taken using an Olympus BH2 compound microscope and differential interference contrast optics. All other microscopy was carried out using an Olympus SZ-11 stereo microscope and transmitted light.

Acknowledgements

We thank Debby Siegele and Jim Golden for critically evaluating the manuscript, Donald Nuss for providing CPG-1 antiserum, and our colleagues in the lab for many helpful discussions. This work was supported by NIH grant GM45252 and a gift from Myco Pharmaceuticals Inc. to T.H.A.

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Received on May 17, 1996; revised on June 12, 1996