Genetic Involvement of a cAMP-Dependent Protein Kinase in a G Protein Signaling Pathway Regulating Morphological and Chemical Transitions in *Aspergillus nidulans*

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ABSTRACT

In the filamentous fungus *Aspergillus nidulans*, a heterotrimeric G protein α-subunit and an RGS domain protein, encoded by *fadA* and *flbA*, respectively, regulate production of the carcinogenic metabolite sterigmatocystin (ST) and asexual spores (*i.e.*, conidia). We investigated the genetic involvement of the cAMP-dependent protein kinase catalytic subunit (PkaA), a potential downstream target of FadA activity, in ST production and conidiation. Relative to wild type, sporulation was decreased in the *pkaA* overexpression strain but was not totally absent, as occurs in Δ*flbA* or *fadA*^{G42R} (*fadA*-dominant active) strains. Deletion of *pkaA* resulted in a hyper-conidiating strain with limited radial growth. This phenotype was epistatic to mutation in *flbA* or *fadA*; the double mutants Δ*pkaA;* Δ*flbA* and Δ*pkaA; fadA*^{G42R} recovered sporulation and their radial growth was severely restricted. PkaA overexpression also negatively regulated AflR, the ST biosynthesis-specific transcription factor, both transcriptionally and post-transcriptionally. Deletion of *pkaA* restored ST production in the Δ*flbA* background but not in the *fadA*^{G42R} background. These data provide genetic evidence that the FlbA/FadA signaling pathway regulating ST production and morphological development is partially mediated through PkaA.

THE polyketide sterigmatocystin (ST) is a carcinogenic secondary metabolite produced by *Aspergillus nidulans*. The biosynthetic genes and the gene encoding the pathway-specific transcription factor of this metabolic pathway have been cloned and functionally analyzed (Brown *et al.* 1996; Fernandes *et al.* 1998). Interestingly, ST biosynthesis is genetically linked with asexual development (*i.e.*, conidiation) in this fungus (Hicks *et al.* 1997). Two of the genes required for ST production and conidiation, *flbA* and *fadA*, are involved in a G protein signaling pathway. FlbA contains an RGS (*r*egulator of *G* protein *s*ignaling) domain in its C-terminal region, a conserved domain that is implicated in negatively regulating G protein-mediated signaling pathways through enhancing the endogenous GTPase activity in α-subunits of G proteins (de Vries and Gist Farquhar 1999). FadA, which is similar to the inhibitory α-subunit of heterotrimeric G proteins ($G_{(i)}\alpha$) of other organisms, appears to be a target of FlbA (Yu *et al.* 1996b). *aflR*, a gene encoding a ST biosynthesis pathway-specific transcription factor, and *brlA*, encoding a conidiation-specific transcription factor, require FlbA and FadA for their normal expression. When *flbA* is eliminated, or FadA is activated, *aflR* and *brlA* expression and subsequent ST production and conidiation are inhibited (Yu *et al.* 1996b; Hicks *et al.* 1997). On the other hand, overexpression of *flbA* or the presence of a dominant negative allele of *fadA* (*fadA*$^{d-}$) allows unscheduled and early expression of these transcription factors and subsequent ST and conidia production (Hicks *et al.* 1997). However, the genetic pathway connecting these four genes (or their products) has not been elucidated.

A candidate pathway genetically linking FadA activity to regulation of BrlA and AflR would be the adenylyl cyclase/cAMP/cAMP-dependent protein kinase (PKA) cascade. Activity of adenylyl cyclase, which synthesizes the intracellular messenger cAMP, has been shown to be regulated by α-subunits of heterotrimeric G proteins (Gα), which either stimulate ($G_{(s)}\alpha$) or inhibit ($G_{(i)}\alpha$) adenylyl cyclase activity in a number of diverse organisms (Dohlman *et al.* 1991; Quan *et al.* 1991; Isshiki *et al.* 1992; Chen *et al.* 1996; Korswagen *et al.* 1998; Ivey *et al.* 1999). When adenylyl cyclase is active, the intracellular concentration of the second messenger cAMP is elevated. cAMP activates PKA, which is composed of two catalytic subunits (PKAC) and two regulatory subunits, by binding to the regulatory subunits, which results in release of the active catalytic subunits. A connection between Gα-proteins, adenylyl cyclase, and PKA activation has been genetically characterized in *Saccharomyces cerevisiae* (Thevelein and de Winde 1999) and to some extent in the corn smut fungus *Ustilago maydis* (Kron-

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stad *et al.* 1998) and the rice blast fungus *Magnaporthe grisea* (Adachi and Hamer 1998).

Multiple PKAC genes have been characterized for two fungi, *U. maydis* and *S. cerevisiae*. *U. maydis* contains two PKAC genes, *adr1* and *uka1*. Inactivation of *adr1* resulted in constitutive filamentous growth and avirulence whereas deletion of *uka1* did not affect fungal morphology or pathogenicity (Dürrenberger *et al.* 1998). *S. cerevisiae* contains three PKAC proteins, encoded by *TPK1*, *TPK2*, and *TPK3*, with overlapping roles for viability (Toda *et al.* 1987), but recent studies showed that each catalytic subunit has a distinctive function for pseudohyphal growth, iron uptake, and respiration (Robertson and Fink 1998; Robertson *et al.* 2000). In both of these examples, environmental signals leading to developmental processes were transduced through PKA via upstream components including receptors, G proteins, and/or adenylyl cyclase.

The strongest evidence to support a genetic connection between PKA and other members of signaling pathways in fungi has been through mutagenesis and pathway studies detailed in *S. cerevisiae* and *U. maydis* (reviewed in Kronstad *et al.* 1998; Thevelein and de Winde 1999). However, biochemical experiments have also supported the existence of a G protein/adenylyl cyclase/cAMP/PKA signaling pathway in pathogenic fungi. For example, inactivation of the adenylyl cyclase gene, which decreases the intracellular concentration of cAMP and presumably concomitant PKA activity, has been associated with aberrations in sporulation, virulence, and mating (Gold *et al.* 1994; Choi and Dean 1997). The defects in adenylyl cyclase mutants resembled those of PKA catalytic subunit mutants (Mitchell and Dean 1995; Xu *et al.* 1997; Dürrenberger *et al.* 1998). Furthermore, exogenous cAMP partially restored wild-type phenotypes in *M. grisea* and *U. maydis* Gα or adenylyl cyclase mutants, thus suggesting that PKA is the downstream target of G protein and/or adenylyl cyclase (Gold *et al.* 1994; Choi and Dean 1997; Liu and Dean 1997; Regenfelder *et al.* 1997). In the human pathogen *Cryptococcus neoformens*, production of the pathogenicity factor melanin was affected by alterations in cAMP levels (Alspaugh *et al.* 1997). This report implies but does not genetically analyze a role for PKA in secondary metabolism.

Considering this accruing evidence that PKA is a member of the G protein signaling pathway in fungi, and furthermore, that this protein plays vital roles in regulating such diverse processes as spore production and, possibly, secondary metabolism in pathogenic fungi, we were interested in examining whether PKA is involved in linking FlbA and FadA activity to conidiation and ST production in the mycotoxigenic fungus *A. nidulans*. In this study, we demonstrate that a cAMP-dependent protein kinase catalytic subunit, PkaA, mediates FlbA and FadA effects on both asexual development and secondary metabolism in *A. nidulans*.

MATERIALS AND METHODS

Fungal strains and media: Fungal strains used in this study are listed in Table 1. All strains were maintained on Aspergillus minimal agar medium (MMG: 6.0 g $NaNO_3$, 0.52 g KCl, 0.52 g $MgSO_4 \cdot 7H_2O$, 1.52 g KH_2PO_4, 1 ml trace elements [2.2 g $ZnSO_4 \cdot 7H_2O$, 1.1 g H_3BO_3, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.16 g $CoCl_2 \cdot 5H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 5.0 g Na_4 EDTA in 100 ml distilled H_2O], 10 g glucose, 12.5 g agar, pH 6.5, in 1 liter distilled H_2O) with appropriate supplements (Käfer 1977). Glucose was replaced with 100 mm threonine (MMT) as indicated. Agar was not added for liquid medium. For genomic DNA extraction and protoplast preparation, strains were grown on appropriately supplemented liquid minimal medium, and 0.5% yeast extract was added when required. For RNA extraction from double mutant strains, complete medium (CM) was used as described previously (Hicks *et al.* 1997).

Cloning and sequencing of *pkaA*: The expressed sequence tag clone n8d03a1, whose nucleotide sequence was highly homologous to those of fungal genes encoding cAMP-dependent protein kinase catalytic subunit, was used to screen an *A. nidulans* cosmid library. Two overlapping cosmid clones, pWE08C4 and pWE06A11, were selected. A 5.4-kb *Hin*dIII fragment of pWE08C4 was cloned into pK19 to produce pKIS1. Restriction mapping and sequencing of pKIS1 revealed that the catalytic core of PkaA lay in the middle of the insert within a 1.5-kb *Bam*HI internal fragment. The nucleotide sequence of the *pkaA* gene was determined on both strands by the dideoxy chain termination method (Sanger *et al.* 1977).

Creation of *pkaA* deletion and *pkaA* overexpression strains: The 5.4-kb *Hin*dIII fragment from pKIS1 was placed into a modified pBluescript vector (pKIS16) in which the *Bam*HI site had been removed by ligating the blunt-ended *Bam*HI/*Sac*II sites together. The resulting plasmid, pKIS17, was then digested with *Bam*HI to release the 1.5-kb fragment, which was replaced with a 1.8-kb *Bam*HI fragment from pJYargB2 containing the *A. nidulans argB* gene. The resulting plasmid pKIS18, which eliminated most of the deduced PkaA, was used to transform *A. nidulans* strain FGSC851 to arginine prototrophy. The deletion in the transformant TKIS18.11 was confirmed by genomic Southern analysis (data not shown). The 2.3-kb *Sph*I fragment containing the entire coding region of the *pkaA* gene was ligated into the *Sph*I site of pBN55 (Lee and Adams 1994), which contains a 1.75-kb fragment containing the 5′ portion of the *trpC* gene, which can complement *trpC801* mutation, and *alcA(p)*, a promoter of the alcohol dehydrogenase gene. The resulting plasmid, pKIS20, has the *pkaA* fragment fused to the *alcA(p)*, which can be activated by supplementing media with specific carbon sources such as threonine or ethanol. pKIS20 was introduced into FGSC237, and a tryptophan prototroph containing the *alcA(p)::pkaA* construct was isolated and named TKIS20.1.

PKA activity assay: Two hundred and fifty milliliters of liquid minimal media was inoculated with 1×10^6 conidia/ml and incubated for 14 hr at 37° and 300 rpm. The mycelium was collected, washed, divided into equal parts, and transferred to flasks with 50 ml minimal media with 100 mm L-threonine as the sole carbon source or 50 ml minimal media with 1% glucose as the sole carbon source. Samples were incubated for an additional 12 hr at 300 rpm and 37° before harvesting, washing with phosphate buffered saline (0.2 g/liter KCl, 8.0 g/liter NaCl, 0.2 g/liter KH_2PO_4, 1.15 g/liter Na_2HPO_4), freezing in liquid nitrogen, and lyophilizing. The lyophilized mycelia were ground and mixed with phosphate buffer [50 mm Na_2HPO_4 (pH 7.0), 10 mm EDTA, 10 mm β-mercaptoethanol, 0.1% sarcosyl, 0.1% Triton X-100, 0.1 mm phenylmethyl-sulfonyl fluoride]. After centrifugation at 14,000 × *g* for 20 min,

TABLE 1

Fungal strains used in this study

Strain	Genotype	Source
FGSC237	*pabaA1, yA2; trpC801, veA1*	FGSC[a]
FGSC851	*pabaA1, yA2;* Δ*argB::trpC; trpC801, veA1*	FGSC
PW1	*biA1; argB2; methG1; veA1*	P. Weglenski
RKIS1	*pabaA1, yA2; veA1*	This study
TKIS18.11	*pabaA1, yA2,* Δ*pkaA::argB;* Δ*argB::trpC; trpC801, veA1*	This study
RKIS3.6	*pabaA1, yA2,* Δ*pkaA::argB,* Δ*flbA::argB; veA1*	This study
RKIS3.11	*pabaA1, yA2,* Δ*flbA::argB; veA1*	This study
RKIS3.15	*pabaA1, yA2,* Δ*pkaA::argB; pyroA4; veA1*	This study
TKIS15.11	*pabaA1, yA2,* Δ*pkaA::argB; fadA*^{G42R}*::pyroA; veA1*	This study
TKIS20.1	*pabaA1, yA2; alcA(p)::pkaA::trpC, veA1*	This study
RJH047	*biA1,* Δ*flbA::argB;* Δ*stcE::argB; pyroA4; veA1*	J. K. Hicks
RJH079	*biA1; argB2; alcA(p)::aflR::trpC, veA1*	J. K. Hicks
h1FAD4	*biA1; fadA*^{G42R}	Yu *et al.* (1996b)
DKIS2	$\frac{pabaA1,\ yA2;\ +;\ +;\ alcA(p)::pkaA::trpC,\ veA1}{+,\ +,\ biA1;\ argB2;\ alcA(p)::aflR::trpC,\ veA1}$	This study
DKIS3	$\frac{pabaA1,\ yA2,\ +;\ +;\ +,\ veA1}{+,\ +,\ biA2;\ argB2;\ alcA(p)::aflR::trpC,\ veA1}$	This study

[a] Fungal Genetics Stock Center.

the supernatants were collected and used to determine protein concentration (Bradford 1976) and were adjusted to 1.5 μg protein/μl. The assay for PKA activity was performed with SignaTECT cAMP-dependent protein kinase assay system (Promega, Madison, WI) according to the manufacturer's directions.

Creation of double mutant strains: RKIS3.6 (Δ*pkaA;* Δ*flbA*) was obtained from a sexual cross between TKIS18.11 and RJH047 (Table 1). The Δ*pkaA* and Δ*flbA* alleles were confirmed by genomic Southern hybridization. pKIS15 was constructed by introducing a 3.2-kb *Pst*I fragment of pJY8P2 containing the *fadA* dominant activating allele, *fadA*^{G42R}, into pSM3, which contains *pyroA* as a selectable marker. *A. nidulans* strain RKIS3.15, a progeny of the sexual cross between TKIS18.11 and RJH047, was transformed with pKIS15 to introduce *fadA*^{G42R} into the Δ*pkaA* background. Diploid strains DKIS2 and DKIS3 were constructed with haploid strains TKIS20.1 and RJH079 or RKIS1 and RJH079.

RESULTS

The *A. nidulans pkaA* gene encodes the catalytic subunit of a PKA protein: Sequence analysis of the putative cAMP-dependent protein kinase catalytic subunit gene (*pkaA*) revealed a 1416-bp open reading frame encoding a 472-amino-acid protein with a predicted molecular mass of 53.8 kD interrupted by three introns, 58 bp, 52 bp, and 51 bp in length (GenBank accession no. AF262987). Southern analysis showed that *A. nidulans* has only one copy of *pkaA* in the genome (data not shown). The catalytic core of protein kinase, as defined by Hanks and Hunter (1995), appears to be highly conserved in this gene. From amino acids 168 to 173 there is a consensus sequence for an ATP-binding site, GXGXXG, followed eight residues later by K (lysine), a motif found in protein kinases of other organisms (Hunter and Cooper 1985). A GenBank search showed that PkaA is similar (~32% identity) to numerous eukaryotic genes, including those of yeast, which encode PKACs. This was particularly true in comparing *A. nidulans* PkaA to that of other filamentous fungi (52–70% identity). Alignment of the amino acid sequence showed that all the fungal species examined have a highly conserved catalytic core, but the N termini of PKACs is quite variable except for the first 15 amino acids (data not shown).

PKA activity is increased in a *pkaA* overexpression strain and is decreased but not eliminated in a *pkaA* deletion strain: To assess the function of *pkaA* and its protein product, strains were created in which the gene was deleted and overexpressed. *pkaA* patterns are shown in Figure 1A. In wild-type strains, *pkaA* appears to be expressed at basal levels over the time points tested in both glucose and threonine media. The overexpression strain TKIS20.1, which contains both a wild-type allele and a copy of *pkaA* fused to the inducible promoter *alcA(p)*, showed much higher accumulation of *pkaA* in *alcA*-inducing medium (MMT). In addition, even in glucose media, slightly higher *pkaA* transcripts were detected in the overexpression strain as compared to the wild-type strain. This result indicates that *alcA(p)* is activated to a low level in 1% glucose. This was confirmed by probing the same RNA blot with the *alcA* gene (data not shown). As expected, no transcript was observed in either medium in the *pkaA* deletion strain TKIS18.11. Next we determined PKA activity in these three strains (Figure 2). TKIS20.1 showed much higher PKA activity in threonine medium compared to wild type. In TKIS18.11, PKA activity in both glucose and threonine

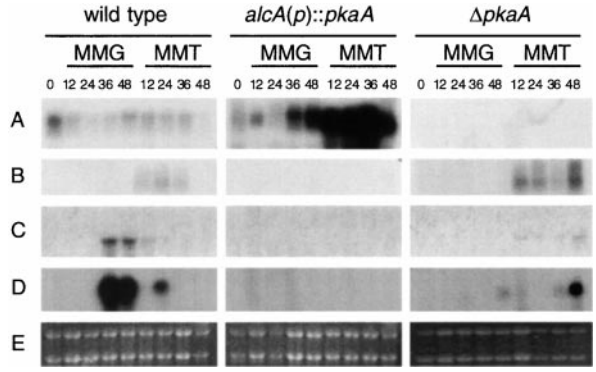

FIGURE 1.—*pkaA* mutants affect *pkaA*, *stcU*, and *brlA* expression. Total RNA was isolated from RKIS1 (wild type), TKIS20.1 (*alcA(p)::pkaA*), and TKIS18.11 (Δ*pkaA*) cultures 0, 12, 24, and 48 hr after transferring from MMG to MMG or from MMG to MMT (an *alcA*-inducing medium). A 1.5-kb *Bam*HI fragment of pKIS17 was used as a *pkaA*-specific probe (A). A 4.5-kb *Sal*I fragment of pTA111 (ADAMS *et al.* 1988) was used as a *brlA*-specific probe (B). A 1.3-kb *Eco*RV-*Xho*I fragment of pAHK25 (BROWN *et al.* 1996) was used as a *aflR*-specific probe (C). A 0.75-kb *Sst*II-*Sma*I fragment of pRB7 (YU *et al.* 1996a) was used as a *stcU*-specific probe (D). Equal loading of total RNA was evaluated by ethidium bromide staining (E).

was much lower than in the wild-type strain, but was still significantly greater than the negative control of boiled protein (data not shown).

PkaA inactivation and overexpression alter morphological development in *A. nidulans*: To examine if PkaA had an effect on differentiation in *A. nidulans*, morphological development of strains containing Δ*pkaA* and *alcA(p)::pkaA* alleles were compared and contrasted to wild type. If, as hypothesized, FadA signaling is mediated through PkaA, we would expect that the phenotype of *pkaA* mutants would mimic certain *fadA* and *flbA* mutants. For example, if FadA is a $G_{(i)}\alpha$-subunit resulting in decreased PKA activity, then the Δ*pkaA* might resemble a FadA activating mutant such as represented by a loss-of-function *flbA* allele or a $fadA^{G42R}$ allele. On the other hand, if FadA is a $G_{(s)}\alpha$-subunit, then one would expect that the enhanced PkaA activity of the *pkaA* overexpression strain would mimic the phenotype associated with Δ*flbA* or $fadA^{G42R}$. Δ*flbA* or $fadA^{G42R}$ mutants, which result in near-constitutive Gα-signaling, exhibit extreme aerial hyphal growth with a fluffy appearance and autolyse as colonies mature. They also are unable to produce conidia on MMG.

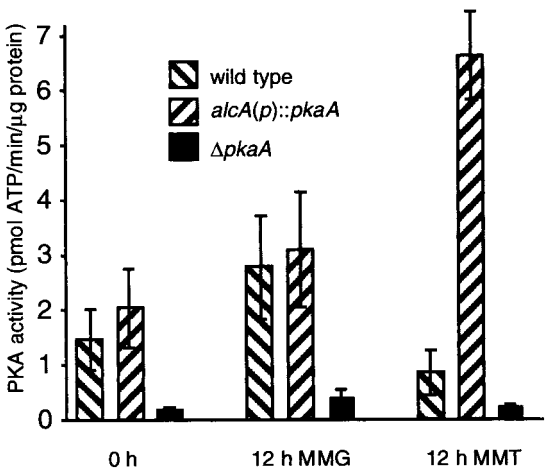

FIGURE 2.—PKA activity in *pkaA* mutant strains. Total protein was prepared from RKIS1 (wild type), TKIS20.1 (*alcA (p)::pkaA*), and TKIS18.11 (Δ*pkaA*) from cultures 0 and 12 hr after transferring from MMG to MMG or MMG to MMT (*alcA*-inducing condition). Standard error bars are shown for three replicates.

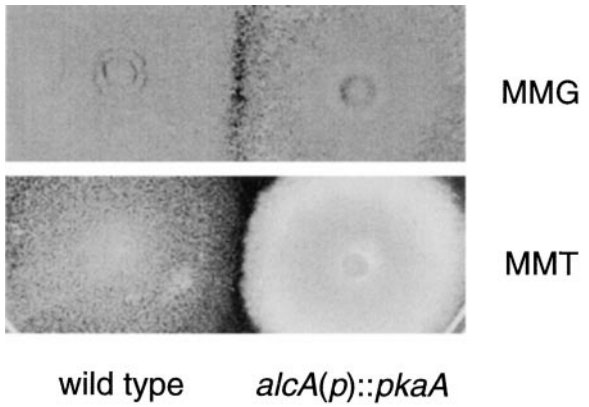

FIGURE 3.—Growth of mutant strains on solid media. Fungal strains, RKIS1 (wild type, left) and TKIS20.1 (*alcA(p)::pkaA*, right) were grown on either MMG or MMT solid media for 5 days at 37° after inoculation.

We examined the phenotype of the *pkaA* overexpression strain by evaluating growth on glucose agar medium, MMG (non-inducing), and threonine agar medium, MMT (inducing), and by comparing growth habits to that of an isogenic wild-type strain grown in the same conditions (Figure 3). There were considerably more aerial mycelia produced by TKIS20.1 when grown on MMT giving the colony a fluffy phenotype (Figure 3). In addition, TKIS20.1 also showed a significant reduction in the density of conidia per unit area when grown on MMT as compared to wild type (~84% reduction, Table 2). These growth and sporulation habits, while not as severe as those seen in the Δ*flbA* or $fadA^{G42R}$ phenotype, did nevertheless resemble this phenotype.

The growth habits of the Δ*pkaA* strain on MMG solid medium also differed from the growth habits of the wild type in two key aspects. First, radial growth rate was reduced by ~85% in TKIS18.11 (Δ*pkaA*) compared to the wild-type strain (Table 3). Second, conidial production per unit area of the Δ*pkaA* strain was significantly higher than wild type (~1.5-fold increase). The phenotype of TKIS18.11 showed some similarities to that of *A. nidulans* strains containing a $fadA^{G203R}$ allele, a dominant-interfering mutation that generates a Gα-protein constitutively bound to guanosine diphosphate (YU *et al.* 1996b).

TABLE 2

Comparison of conidiation and growth rate of overexpression *pkaA* to wild-type *A. nidulans* on solid media

Relevant genotype	No. of conidia/mm^2		Growth rate (mm/day)	
	MMG[a] ($\times 10^6$)	MMT[b] ($\times 10^4$)	MMG	MMT
alcA(p)::pkaA	1.31 a[c]	1.69 a	6.70 a	5.98 a
Wild type	1.52 a	10.9 b	6.63 a	5.98 a

Conidia (1×10^6) of each strain were spread on media plates and incubated at 37° for 5 days, and then a plug of agar (1 cm^2) was removed for spore calculation. For determination of growth rate, each strain was inoculated on the center of media plates and grown at 37° for 5 days. Results shown above are average of three replicates. The *P* value for each comparison of *alcA(p)::pkaA vs.* wild type for each growth condition was calculated by two-sample *t*-test.

[a] MMG, minimal media with 1% glucose as a sole carbon source.

[b] MMT, minimal media with 100 mM threonine as a sole carbon source.

[c] The same letter per column indicates no significant difference ($P < 0.01$).

Morphological observations were also made on growth habits in submerged culture. Wild-type strains of *A. nidulans* normally do not sporulate in submerged glucose culture, and, as shown in Figure 4, Δ*pkaA* and *alcA(p)::pkaA* strains also did not sporulate under these conditions at the time points tested. In this instance, the Δ*pkaA* strain did not resemble the *fadA*^{G203R} strain as the latter does sporulate in submerged culture (Hicks *et al.* 1997). Microscopic examination revealed, however, that the Δ*pkaA* strain had a thinner hyphal diameter than the other two strains (Figure 4C). When grown in threonine media, TKIS20.1 did not develop conidia (Figure 4E) whereas both the wild type and TKIS18.11 produced conidia from conidiophores (Figure 4, D and F). Both the wild type and the deletion strain sporulated within 12 hr after shifting from glucose to threonine medium. Notably, the Δ*pkaA* strain sporulated with little mycelial growth in contrast to wild type, which produced copious amounts of mycelia. Expression of *brlA*, which is required for conidiophore development, was not detected in the *pkaA* overexpression strain, but was detected both in the wild type and to a higher level in the *pkaA* deletion strain grown in threonine medium (Figure 1B). This was consistent with the results of observations shown in Figure 4, D–F. In addition to producing conidiophores, the Δ*pkaA* strain also formed many large spherical cells in threonine medium (Figure 4F). The fact that the overexpression strain did not sporulate in submerged threonine-inducing media, unlike wild-type and Δ*pkaA* strains, and that it exhibited reduced conidiophore formation on MMT agar medium (Table 2 and Figure 3) demonstrates that PKA activity inhibits asexual development, a phenotype associated with constitutive FadA signaling.

TABLE 3

Conidiation and growth rates of *pkaA* mutants of *A. nidulans*

Relevant genotype	No. of conidia/ mm^2 ($\times 10^5$)	Growth rate (mm/day)
Δ*pkaA*	27.8 a[a]	0.97 c
Δ*pkaA;* Δ*flbA*	2.85 d	0.93 c
Δ*pkaA; fadA*^{G42R}	7.45 c	0.88 c
Δ*flbA*	0.00 e	4.80 b
fadA^{G42R}	0.00 e	6.59 a
Wild type	18.3 b	6.73 a
LSD ($P = 0.05$)	2.58	0.20

Conidia (1×10^6) of each strain were spread on media plates and incubated at 37° for 5 days, and then a plug of agar (1 cm^2) was removed for spore calculation. For determination of growth rate, each strain was inoculated on the center of media plates and grown at 37° for 5 days. Results shown above are average of three replicates. ANOVA was used to test for effects of phenotype on the numbers of conidia and growth rate. Multiple comparison was performed with Fisher's least significant difference (LSD) value.

[a] The different letters per column indicate significant differences ($P < 0.05$).

PkaA mediates FadA and FlbA growth signaling: To determine genetically if PkaA was involved in mediating

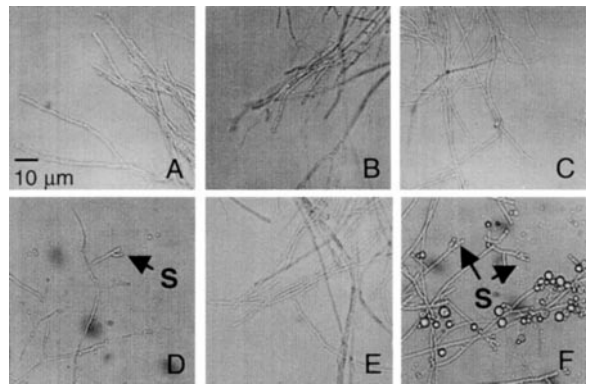

Figure 4.—Growth of different *pkaA* mutant strains in submerged cultures. RKIS1 (wild type, A and D), TKIS20.1 (*alcA (p)::pkaA*, B and E), and TKIS18.11 (Δ*pkaA*, C and F) were grown in MMG liquid shake cultures at 37° for 24 hr and transferred to MMG (A–C) or MMT (D–F) for an additional 12 hr at 37°. S, asexual spore.

the FadA signal in asexual development, we introduced *flbA* and *fadA* mutant alleles into the Δ*pkaA* background to examine epistatic relationships among these genes. Morphological characteristics of these strains are summarized in Table 3. A striking phenotype of strains carrying either loss-of-function *flbA* (Δ*flbA*) or dominant positive *fadA* alleles (*fadA*^{G42R}) is the inability to produce conidiophores, abundant aerial mycelia, and autolysing of the mature colony (Yu *et al.* 1996b). As described in the previous section, the distinct phenotype of Δ*pkaA* strains is the severe restriction of the radial growth rate and an increase in the number of conidia/area. Both double mutant strains, Δ*pkaA; fadA*^{G42R} and Δ*pkaA;* Δ*flbA*, exhibited the restricted radial growth phenotype of a Δ*pkaA* strain and an intermediate sporulation phenotype compared to the single mutant strains (Table 3). Thus in *A. nidulans*, colony expansion seems to be largely contributed by PkaA activity, but normal asexual sporulation may require not only inactivation of PkaA but also active FlbA and subsequent inactivation of FadA. Interestingly, the Δ*pkaA;* Δ*flbA* strain but not the Δ*pkaA; fadA*^{G42R} strain developed aerial hyphae. Additionally, unlike the Δ*flbA* or *fadA*^{G42R} single mutant strains, neither the Δ*pkaA;* Δ*flbA* nor the Δ*pkaA; fadA*^{G42R} strain autolysed. Mycelial autolysis may require both activated FadA and PkaA in *A. nidulans*.

***aflR* and *stc* gene expression and ST production is repressed in *pkaA* overexpression strains:** Production of the mycotoxin ST has been genetically linked to conidiation through FadA signaling (Hicks *et al.* 1997). It was therefore of interest to determine whether the *pkaA* mutant strains also were affected in ST production. Because ST production is heralded by the appearance of ST gene cluster transcripts (Butchko *et al.* 1999), we examined expression of *aflR*, the ST pathway-specific transcription factor, and *stcU*, one of the biosynthetic genes involved in ST biosynthesis (Brown *et al.* 1996; Fernandes *et al.* 1998), in *pkaA* mutant strains. In the wild-type strain, both *aflR* and *stcU* transcripts were detected at 36 and 48 hr after shifting from MMG to MMG and at 24 hr when shifted from MMG to MMT (Figure 1, C and D). In the Δ*pkaA* strain, expression of both genes was delayed in both media and decreased in intensity in MMG medium. Neither *aflR* nor *stcU* transcript was detected in the *pkaA* overexpression strain, whether grown in MMG or MMT media. This suggested that even the small increase of PkaA activity observed in 1% glucose medium was sufficient to inhibit ST production.

PkaA activity regulates *aflR* not only transcriptionally but also post-transcriptionally: Because the expression of *aflR* was shown to be negatively regulated by PkaA activity, we were interested to see if we could remediate this regulation by forced expression of *aflR* in the *pkaA* overexpression background. Thus, we constructed diploid strains carrying *alcA(p)::aflR* and *alcA(p)::pkaA* alleles or *alcA(p)::aflR* alone and grew these strains in *alcA*-inducing and non-inducing conditions. As expected,

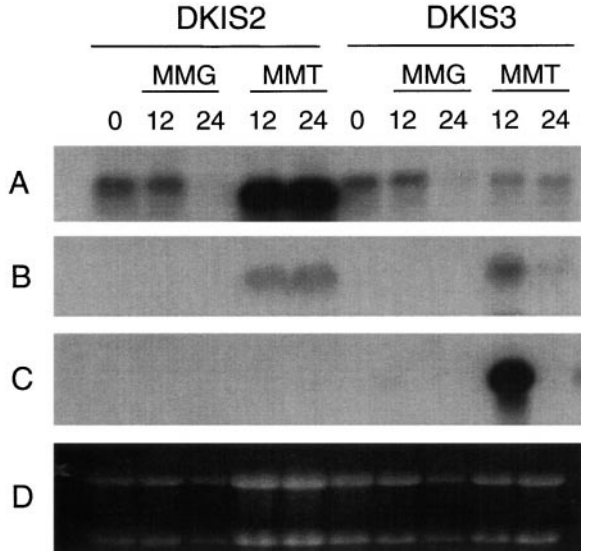

Figure 5.—*stc* gene expression in the *pkaA/aflR* overexpression strains. Fungal diploid strains, DKIS2 (*alcA(p)::aflR/alcA(p)::pkaA*) and DKIS3 (*alcA(p)::aflR*), were grown in liquid shaking MMG for 14 hr at 37°, transferred to MMG or MMT, and incubated for additional 12 or 24 hr at 37°. (A) *pkaA* transcript, (B) *aflR* transcript, (C) *stcU* transcript; (D) staining of total RNA with ethidium bromide.

significant expression of *aflR* was not observed in MMG medium but was observed in both diploid strains in MMT (Figure 5). However, despite high *aflR* expression, *stcU* expression in MMT was detected in only the wild-type *pkaA* diploid and not in the *pkaA* overexpression diploid, suggesting that *aflR* activity is post-transcriptionally regulated by PkaA. Taken together with the results shown in the previous section, we conclude that *aflR* is negatively regulated both transcriptionally and post-transcriptionally by PkaA activity. At present, the mechanism by which PkaA regulates *aflR* is not known, but it is interesting to note that the *A. nidulans* AflR protein contains three putative PKA-specific phosphorylation motifs (data not shown).

Deletion of *pkaA* remediates the inhibitory effect of a loss-of-function *flbA* allele on ST production: Because of poor growth of the double mutant strains in liquid minimal medium, fungal cultures were grown in CM to assay *stc* expression. In the wild-type strain, both *aflR* and *stcU* transcripts were observed at 48 hr and lower expression was seen at 72 hr after inoculation (Figure 6, lanes 1 and 2). In the Δ*pkaA* strain expression of both genes was slightly less than that of the wild-type strain at both time points (Figure 6, lanes 3 and 4). Both the Δ*flbA* and *fadA*^{G42R} strains showed no expression of either *aflR* or *stcU* (Figure 6, lanes 5–8). The Δ*pkaA;* Δ*flbA* double mutant strain showed both *aflR* and *stcU* transcripts (Figure 6, lanes 9 and 10). In contrast, however, the Δ*pkaA; fadA*^{G42R} strain did not show either *aflR* or *stcU* transcript in this growth condition (Figure 6, lanes 11 and 12). Analysis of extracts from these strains re-

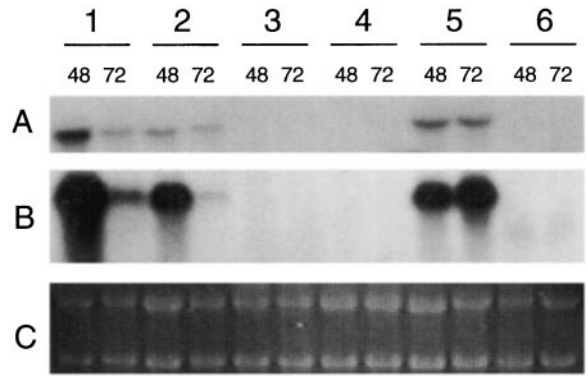

FIGURE 6.—*aflR* and *stc* gene expression in *pkaA* deletion strains. Fungal strains were grown in liquid stationary culture (CM) and harvested at 48 and 72 hr post-inoculation: lane 1, wild type; lane 2, Δ*pkaA*; lane 3, Δ*flbA*; lane 4, $fadA^{G42R}$; lane 5, Δ*pkaA;* Δ*flbA*; lane 6, Δ*pkaA; fadA*^{G42R}. (A) *aflR* transcript, (B) *stcU* transcript; (C) staining of total RNA with ethidium bromide.

vealed a corresponding pattern of ST production (data not shown).

DISCUSSION

In many fungi, a correlation between asexual development (*e.g.*, conidiation) and secondary metabolism has been recognized, but the cellular machinery linking these two processes is largely unclear. We have initiated an investigation of the pathways connecting sporulation and secondary metabolism in the genetic model system *A. nidulans*. At least two genes, *flbA* and *fadA*, have been identified as common factors in regulating conidiation and ST production by controlling expression of the specific transcription factor genes *brlA* (conidiation) and *aflR* (ST production; YU *et al.* 1996b; HICKS *et al.* 1997). FlbA and FadA are members of a G protein signal transduction pathway and the molecular mechanisms connecting these two proteins with *aflR* and *brlA* activity remain unknown. One logical candidate for transmitting a signal from FlbA and FadA to *aflR* and *brlA* would be cAMP-dependent PKA.

To determine if PKA could be a component in this transduction pathway, we cloned and characterized the gene encoding the cAMP-dependent protein kinase catalytic subunit, *pkaA*. Amino acid analysis of PkaA shows it to contain the highly conserved catalytic core common to numerous eukaryote PKAC proteins. The N-terminal domain shows little similarity to any of these proteins with the exception of the first dozen amino acids, which were similar to those of other filamentous fungal PKACs (data not shown). Little is known about the function of the N-terminal region; in *Dictyostelium discoideum*, deletion of this portion showed no phenotype (ETCHEBEHERE *et al.* 1997). Comparison of the three yeast PKAC proteins, however, suggests that the variation in their N-terminal domain contributes to the differences in their function (ROBERTSON and FINK 1998). We have seen an inhibitory effect on ST production and altered morphological development by adding an extra copy of the *A. nidulans* N-terminal region (K. SHIMIZU and N. P. KELLER, unpublished results).

Expression of *pkaA* was detected at nearly the same level in all the time points tested in this study in the wild-type strain, suggesting that *pkaA* is expressed constitutively. In other fungal species such as *C. trifolii*, *Ct-PKAC* expression also has been detected at all time points examined (YANG and DICKMAN 1999). Basal level expression of a gene sometimes indicates an absolute requirement for the activity of the gene product in the cell. However, we found that while deletion of *pkaA* did exhibit a severe phenotype, it was not lethal in *A. nidulans*. This may be because *A. nidulans* possesses another PKAC protein as indicated by the low but measurable level of PKA activity in the Δ*pkaA* strain. This would not be without precedent because both *U. maydis* and *S. cerevisiae* have more than one gene encoding PKAC (TODA *et al.* 1987; DÜRRENBERGER *et al.* 1998). In these two fungi the different PKAC proteins appear to play different but overlapping roles in the cell (DÜRRENBERGER *et al.* 1998; ROBERTSON and FINK 1998; ROBERTSON *et al.* 2000). In *S. cerevisiae* lethality is exhibited only when all three genes encoding PKAC are disabled (TODA *et al.* 1987).

Examination of *pkaA* deletion and overexpression strains indicated a role for PkaA in several aspects of Aspergillus development. Consistent observations included the complete inhibition of *stcU* expression and ST production and partial inhibition of asexual development in *pkaA* overexpression strains and the reduced radial growth and increased sporulation of Δ*pkaA* strains. Interestingly, the data shown here suggest that the ST biosynthesis-specific transcription factor AflR is transcriptionally and post-transcriptionally regulated by PkaA activity, a hypothesis we are presently examining in our laboratory. These results strongly support a major role of PkaA activity in regulating conidiation, vegetative growth, and secondary metabolism in this fungus. Notably, the phenotype of *pkaA* overexpression strains was reminiscent of loss-of-function FlbA and FadA constitutive activating mutants of *A. nidulans* and suggested that, as in other organisms, RGS/G protein signaling is mediated in part through the cAMP/PkaA cascade. This also implies that FadA may function as a $G_{(s)}\alpha$-subunit despite its sequence similarity to mammalian $G_{(i)}\alpha$-subunits. Interestingly, most fungal Gα-genes cloned to date show this same sequence similarity to $G_{(i)}\alpha$-subunits but have been found to more likely function as activators of adenylyl cyclase, while that of *Cryphonectria parasitica* seems to work as $G_{(i)}\alpha$ (ISSHIKI *et al.* 1992; CHEN *et al.* 1996; SAVINON-TEJEDA *et al.* 1996; ALSPAUGH *et al.* 1997; LIU and DEAN 1997; KRÜGER *et al.* 1998; IVEY *et al.* 1999).

The conclusion that FadA activates PkaA is also supported by examining the phenotypes of double mutants

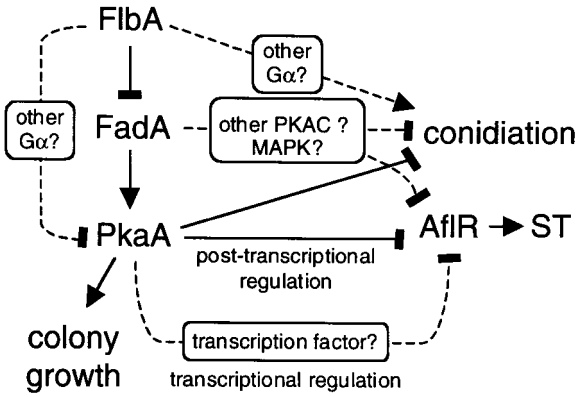

FIGURE 7.—Proposed model for a signal transduction pathway regulating both ST production and asexual development. The model is described in the text. Solid lines indicate genetically determined steps and dashed lines hypothesized steps. PkaA may phosphorylate an unidentified transcription factor, which triggers transcription of *aflR*, and the transcribed *aflR* is also regulated by PkaA activity. Regulation of morphological development by FlbA and FadA is only partially mediated through PkaA.

with regard to several key differentiation processes. One of the most dramatic effects of constitutive activation of G protein signaling in *A. nidulans* is the inhibition of asexual sporulation. We found that introduction of the Δ*pkaA* allele into Δ*flbA* and *fadA*^{G42R} backgrounds restored conidiation; this provides strong evidence that FadA inhibition of asexual development is mediated through PkaA. However, remediation was not to wild-type levels (Δ*pkaA;* Δ*flbA* showing ~15% and Δ*pkaA; fadA*^{G42R} showing ~40% sporulation levels of wild type). Previous studies (YU *et al.* 1996b; HICKS *et al.* 1997) suggested that FlbA has some additional function(s) in regulating sporulation other than through modification of FadA activity. Therefore, one explanation as to why sporulation was not fully restored in the Δ*pkaA;* Δ*flbA* strain is that Δ*pkaA* cannot complement a FadA-independent role for FlbA. Sporulation in the Δ*pkaA; fadA*^{G42R} strain was statistically greater than in the Δ*pkaA;* Δ*flbA* strain but still less than that of wild type. This indicates a PkaA-independent role for FadA in asexual sporulation. As mentioned earlier, other fungi possess more than one PKAC protein and as our data provide evidence for more than one PKAC in *A. nidulans* (Figure 2), we speculate that FadA activity may also negatively impact sporulation through another PKAC or, although untested in this work, perhaps mitogen-activated protein kinase (MAPK) cascade. A model for this complex regulation of asexual development is presented in Figure 7.

The genetic linkage of asexual sporulation and ST production was evident from examination of the various *pkaA* mutants. The inhibitory effect of overexpression of *pkaA* on *aflR* expression and ST biosynthesis was identical to the inhibitory effects of Δ*flbA* and *fadA*^{G42R} alleles on *aflR* expression. This suggested that FlbA and FadA regulation of ST production is mediated through PkaA, leading therefore to the prediction that introducing Δ*pkaA* into Δ*flbA* and *fadA*^{G42R} backgrounds would restore ST production. Interestingly, this was the case for the Δ*pkaA;* Δ*flbA* strain but not the Δ*pkaA; fadA*^{G42R} strain under the conditions tested. The inability to detect ST or *stc* expression in the Δ*pkaA; fadA*^{G42R} strain suggests that FadA also has a PkaA-independent role in inhibiting ST production (Figure 7).

PkaA activity also affected several aspects of Aspergillus vegetative growth including radial expansion rate, aerial mycelium formation, and mycelial autolysis. Strains containing Δ*flbA* and *fadA*^{G42R} alleles produce extensive aerial mycelium and have a habit of autolysing as the colony matures. *pkaA* overexpression strains exhibited the former characteristic but not the latter. The radial expansion rate appears tightly linked to PkaA activity because all strains carrying the Δ*pkaA* allele (Δ*pkaA,* Δ*pkaA;* Δ*flbA*, and Δ*pkaA; fadA*^{G42R}) showed a similarly restricted expansion rate. Aerial mycelium formation, although enhanced by *pkaA* overexpression (Figure 3), was not as prolific as seen in Δ*flbA* or *fadA*^{G42R} strains and, furthermore, was not fully dependent on PkaA activity as judged by the fact that aerial mycelium formation was not eliminated in the Δ*pkaA;* Δ*flbA* strain. Mycelial autolysis appears to require PkaA activity because deletion of *pkaA* eliminated the autolytic habit of the Δ*flbA* and *fadA*^{G42R} alleles as observed in Δ*pkaA;* Δ*flbA* and Δ*pkaA; fadA*^{G42R} strains. The fact that overexpression of *pkaA* did not result in mycelial autolysis suggests that autolysis requires both activation of FadA and subsequent increased PkaA activity.

Consideration of all data presented in this article leads us to conclude that FlbA/FadA signaling of asexual sporulation, ST production, and vegetative growth is mediated only in part through a cAMP/PkaA cascade (Figure 7). Obviously there must be other cellular mechanisms impacting all of these differentiation processes. In other fungi, transduction pathways are complex and can involve interactions between β- and γ-subunits of heterotrimeric G proteins, RAS proteins, MAPK cascades, and protein kinase C pathways (BANUETT 1998; RUPP *et al.* 1999; THEVELEIN and DE WINDE 1999). Two other pertinent *A. nidulans* genes that have been shown to be involved in sporulation and colony formation are *rasA*, encoding a RAS protein (SOM and KOLAPARTHI 1994) and *sfaD*, encoding a β-subunit of a heterotrimeric G protein (Gβ; ROSÉN *et al.* 1999). In submerged culture conditions, deletion of *sfaD* exhibits conidiation, a similar phenotype to a *fadA*^{G203R} strain (ROSÉN *et al.* 1999), but unlike a *fadA*^{G203R} strain, spore production is decreased on agar growth medium (K. SHIMIZU and N. P. KELLER, unpublished results). Dominant-acting mutations in *rasA* (RAS) result in developmental defects similar to overexpression of *pkaA* (SOM and KOLAPARTHI 1994; K. SHIMIZU and N. P. KELLER, unpublished re-

sults). In addition to these proteins, *A. nidulans* likely contains at least two additional Gα-subunits (J. Yu, personal communication) as well as a possible additional PkaA as suggested by the data in Figure 2. One current goal in our laboratory is to determine if these different members of signal transduction pathways converge to impact morphogenesis and ST production in *A. nidulans*.

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