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The Putative Guanine Nucleotide Exchange Factor RicA Mediates Upstream Signaling for Growth and Development in *Aspergillus*

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Heterotrimeric G proteins (G proteins) govern growth, development, and secondary metabolism in various fungi. Here, we characterized *ricA*, which encodes a putative GDP/GTP exchange factor for G proteins in the model fungus *Aspergillus nidulans* and the opportunistic human pathogen *Aspergillus fumigatus*. In both species, *ricA* mRNA accumulates during vegetative growth and early developmental phases, but it is not present in spores. The deletion of *ricA* results in severely impaired colony growth and the total (for *A. nidulans*) or near (for *A. fumigatus*) absence of asexual sporulation (conidiation). The overexpression (OE) of the *A. fumigatus ricA* gene (Af*ricA*) restores growth and conidiation in the ΔAn*ricA* mutant to some extent, indicating partial conservation of RicA function in *Aspergillus*. A series of double mutant analyses revealed that the removal of RgsA (an RGS protein of the GanB Gα subunit), but not *sfgA*, *flbA*, *rgsB*, or *rgsC*, restored vegetative growth and conidiation in ΔAn*ricA*. Furthermore, we found that RicA can physically interact with GanB in yeast and *in vitro*. Moreover, the presence of two copies or OE of *pkaA* suppresses the profound defects caused by ΔAn*ricA*, indicating that RicA-mediated growth and developmental signaling is primarily through GanB and PkaA in *A. nidulans*. Despite the lack of conidiation, *brlA* and *vosA* mRNAs accumulated to normal levels in the Δ*ricA* mutant. In addition, mutants overexpressing *fluG* or *brlA* (OE*fluG* or OE*brlA*) failed to restore development in the ΔAn*ricA* mutant. These findings suggest that the commencement of asexual development requires unknown RicA-mediated signaling input in *A. nidulans*.

Heterotrimeric G proteins (G proteins) are conserved in all eukaryotes and are involved in almost all biological processes (22, 43, 58). Basic units of the heterotrimeric G protein signaling system include a G protein-coupled receptor (GPCR), a G protein, composed of α, β, and γ subunits, and a variety of effectors, which relay the signal into cells to elicit appropriate physiological and biochemical responses (6).

G proteins are regarded as biological switches that oscillate between on and off states (7). Under nonstimulated conditions, the inactive Gα-GDP::Gβγ trimeric complex prevails in the cell membrane, and the signaling pathway remains off. Typically, G proteins are turned on by the guanine nucleotide exchange caused by ligand-bound (sensitized) GPCRs, causing the dissociation of the GTP-bound Gα subunit and the Gβγ heterodimer, which then transduce signals by interacting with various effectors, including adenylyl cyclase-protein kinase A (PKA), phospholipase C, ionic channels, and mitogen-activated protein kinases (18, 45). The signal is turned off when Gα-GTP is hydrolyzed back to Gα-GDP by the intrinsic GTPase activity of the Gα subunit, forming the inactive trimeric complex. One key element facilitating inactivation is the regulator of G protein signaling (RGS), which accelerates GTP hydrolysis catalyzed by the Gα subunit (15). While GPCR-mediated signaling accounts for the majority of G protein-regulated cellular control mechanisms, the evolutionarily conserved RIC-8 (resistance to inhibitors of cholinesterase 8) protein is a proven critical guanine nucleotide exchange factor (GEF) that activates a subset of Gα subunits (24). Ric-8 interacts with monomeric Gα-GDP, stimulates the release of GDP, forms a stable nucleotide-free transition-state complex with the Gα subunit, and catalyzes the exchange of GDP for GTP (Fig. 1A) (5, 24).

In fungi, G protein signaling governs cell growth, morphogenesis, sexual/asexual development, mating, pathogenicity, secondary metabolism, and many more processes (33, 34, 69, 73). The model filamentous fungus *Aspergillus nidulans* contains three Gα subunits (FadA, GanB, and GanA) (10, 69, 74), one Gβ subunit (SfaD) (48), and one Gγ subunit (GpgA) (53). Genetic studies have revealed that both FadA (Gα) and SfaD::GpgA (Gβγ) mediate signaling that promotes vegetative growth while inhibiting development and biosynthesis of the carcinogenic mycotoxin sterigmatocystin (ST) (23, 48, 73, 74). Further studies have shown that FadA signaling is in part transduced via the cyclic AMP (cAMP)-dependent protein kinase PkaA (56). This FadA→PkaA-mediated signaling in turn inhibits asexual development (conidiation), which is activated by the FluG→BrlA pathway and completed by VosA (2, 44, 69, 70). FlbA is the cognate RGS protein, whose primary role is to negatively control FadA-mediated vegetative growth signaling (31, 74). Both Δ*flbA* and constitutively active FadA mutations (G42R, R178C, and Q204L, resulting in defective intrinsic GTPase) produce the fluffy autolytic phenotype (64, 74). Importantly, this FadA-mediated signaling for vegetative growth, development, and toxigenesis is conserved in the aflatoxin-producing fungi *Aspergillus parasiticus* and *Aspergillus flavus* (23, 49)

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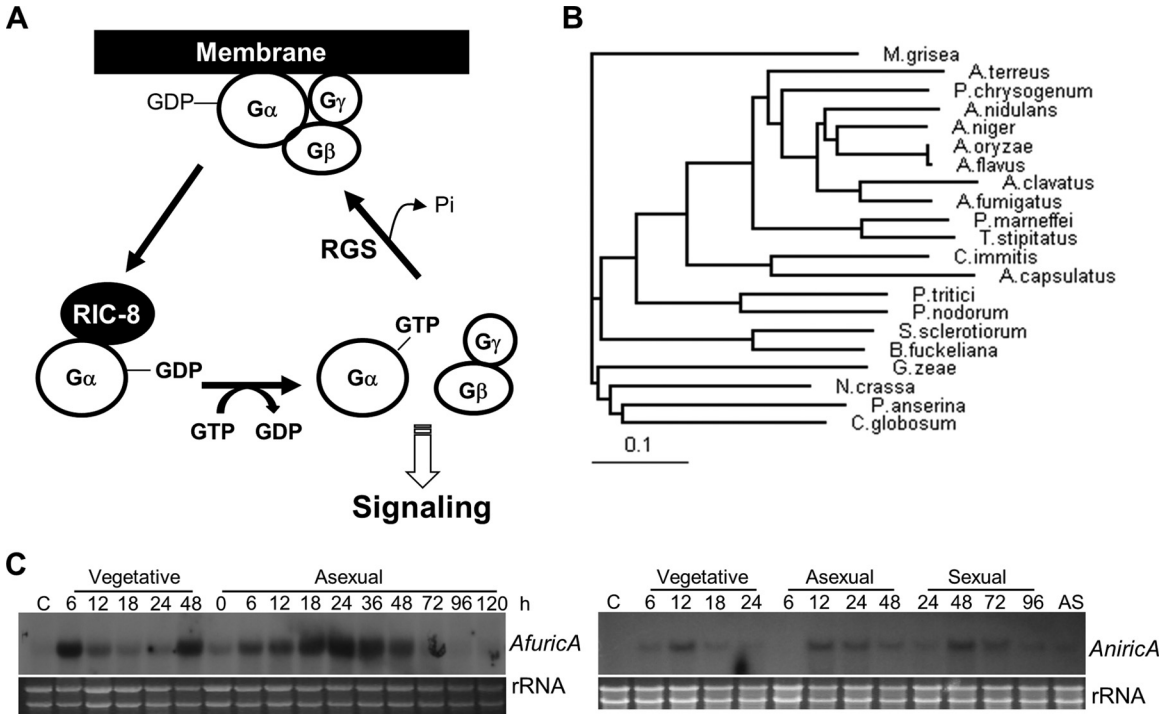

FIG 1 Summary of the *ricA* genes. (A) Proposed Ric-8-mediated G protein signaling. (B) Phylogenetic tree of the putative RicA proteins identified in various fungal species (from top to bottom: *Magnaporthe grisea*, *A. terreus*, *P. chrysogenum*, *A. nidulans*, *A. niger*, *A. oryzae*, *A. flavus*, *A. clavatus*, *A. fumigatus*, *Penicillium marneffei*, *Talaromyces stipitatus*, *Coccidioides immitis*, *Ajellomyces capsulatus*, *P. tritici*, *Phaeosphaeria nodorum*, *Sclerotinia sclerotiorum*, *G. zeae*, *Botryotinia fuckeliana*, *Podospora anserine*, and *Chaetomium globosum*). (C) mRNA levels of *ricA* during the life cycles of *A. fumigatus* and *A. nidulans*. C, conidia (asexual spores); AS, ascospores (sexual spores in *A. nidulans*). Numbers indicate the time (in hours) of incubation in liquid MMG or MMG plus 0.1% YE (vegetative) or after transfer onto solid MMG glucose under conditions favoring asexual or sexual development. Equal loading of total RNA was evaluated by ethidium bromide staining of rRNA.

and the opportunistic human pathogen *Aspergillus fumigatus* (38, 69).

The GanB Gα subunit negatively regulates conidiation and plays a positive role in the germination of conidia, whereas GanA's role is not yet understood (10). Additional studies have revealed that GanB and SfaD::GpgA constitute a functional heterotrimer controlling cAMP-PKA signaling and conidial germination in response to glucose, where GanB is the primary signaling element and SfaD::GpgA functions for proper activation of GanB (30). Among the three additional RGS proteins, RgsA, RgsB, and RgsC (69), RgsA acts as the negative regulator of GanB signaling in *A. nidulans* (21). The lack of RgsA results in phenotypes similar to those caused by constitutive activation of GanB (Q208L), i.e., germination of conidia in the absence of an external C source and an enhanced stress response (21). Furthermore, the overexpression of *rgsA* causes elaboration of asexual developmental structures (conidiophores) in liquid submerged cultures, as observed in Δ*ganB* or $GanB^{G207R}$ mutants (10, 21). In *A. fumigatus*, signaling mediated by GpaB (GanB homolog) is associated with the activation of the predominant PKA catalytic subunit PkaC1, which governs hyphal growth and development (36, 37).

Despite such a pivotal role of G proteins in many aspects of *Aspergillus* biology, upstream mechanisms of signal activation remain to be understood. While at least 16 putative GPCRs have been identified in the genome of *A. nidulans* (69), none has been proven to specifically activate FadA- or GanB-mediated signaling. In an effort to understand the upstream activation of G protein signaling in *Aspergillus*, we identified and characterized the Ric-8 ortholog RicA in *A. nidulans* and *A. fumigatus*. Functional studies of the *ricA* gene revealed that it plays a crucial (or essential) role in vegetative growth and development in both species, with a partially conserved function. Genetic and biochemical studies further indicated that RicA primarily activates the GanB→PkaA signaling cascade in *A. nidulans*. Finally, as normal or elevated expression of key developmental activators fails to trigger conidiation in the absence of RicA, it is proposed that an unknown RicA-mediated signal input, independent of the FluG→BrlA→VosA pathway, is required for asexual development in *A. nidulans*.

MATERIALS AND METHODS

Strains and culture conditions. *A. nidulans* and *A. fumigatus* strains used in this study are listed in Table 1. Glucose minimal medium (MMG) and MMG with 0.5% (wt/vol) yeast extract (YE) with appropriate supplements were used for general culture of *A. nidulans* strains (26, 47). For *A. fumigatus* pyrimidine and arginine auxotrophic mutant strains (AF293.1 and AF293.6 [67]), MMG plus 0.1% YE was supplemented with 5 mM uridine, 10 mM uracil (for *pyrG1*), and 0.1% arginine (for *argB1*). Minimal medium with 100 mM threonine as a sole carbon source (MMT) with 0.5% YE was used for *alcA*p-mediated overexpression. To check the phenotype of the overexpression strains under control of the *alcA* promoter (40, 63) in *A. nidulans* and *A. fumigatus*, wild-type (WT) and overexpression strains were inoculated on MMG and MMT plus 0.5% YE solid media and incubated at 37°C for 5 days. Effects of overexpression of the target genes under the *niiA* promoter (4) in *A. nidulans* were examined by growing the strains in both MM with 0.2% (wt/vol) ammonium tartrate

TABLE 1 *Aspergillus* strains used in this study

Species and strain	Genotype	Source
A. nidulans strains		
FGSC4	*veA*$^{+}$ (wild type)	FGSC[a]
RJMP1.59	*pyrG89 pyroA4 veA*$^{+}$	54
TNJ21	*pyrG89 pyroA4* Δ*ricA*::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
TNJ36	*pyrG89* Af*pyrG*$^{+}$ *pyroA4 veA*$^{+}$	28
TNJ42	*pyrG89* Δ*flbA*::An*pyroA*$^{+}$ *pyroA4 veA*$^{+}$	This study
TNJ49	*pyrG89* Δ*flbA*::An*pyroA*$^{+}$ *pyroA4* Δ*ricA*::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
TNJ57	*pyrG89* Δ*sfgA*::Af*pyrG*$^{+}$ *pyroA4 veA*$^{+}$	This study
TNJ58	*pyrG89* Δ*sfgA*::Af*pyrG*$^{+}$ *pyroA4* Δ*ricA*::An*pyroA*$^{+}$ *veA*$^{+}$	This study
TNJ59[b]	*pyrG89 pyroA4 niiA*(p)::*fluG*::Flag::3/4*pyroA*$^{+}$ *veA*$^{+}$	This study
TNJ60[b]	*pyrG89 pyroA4 niiA*(p)::*fluG*::Flag::3/4*pyroA*$^{+}$ Δ*ricA*::An*pyroA*$^{+}$ *veA*$^{+}$	This study
TNJ61	*pyrG89 pyroA4* Δ*rgsA*::An*pyroA*$^{+}$ *veA*$^{+}$	This study
TNJ62	*pyrG89 pyroA4* Δ*rgsA*::An*pyroA*$^{+}$ Δ*ricA*::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
TNJ63	*pyrG89* Δ*rgsB*::Af*pyrG*$^{+}$ *pyroA4 veA*$^{+}$	This study
TNJ64	*pyrG89* Δ*rgsB*::Af*pyrG*$^{+}$ *pyroA4* Δ*ricA*::An*pyroA*$^{+}$ *veA*$^{+}$	This study
TNJ65	*pyrG89 pyroA4* Δ*rgsC*::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
TNJ66	*pyrG89 pyroA4* Δ*ricA*::An*pyroA*$^{+}$ Δ*rgsC*::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
TNJ68	*pyrG89 pyroA4 ganB*^{Q208L}::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
TNJ69	*pyrG89 pyroA4* Δ*ricA*::An*pyroA*$^{+}$ *ganB*^{Q208L}::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
TNJ85[b]	*pyrG89 pyroA4 niiA*(p)::*brlA*::Flag::3/4*pyroA*$^{+}$ *veA*$^{+}$	This study
TNJ86[b]	*pyrG89 pyroA4 niiA*(p)::*brlA*::Flag::3/4*pyroA*$^{+}$ Δ*ricA*::An*pyroA*$^{+}$ *veA*$^{+}$	This study
TNJ87[b]	*pyrG89 pyroA4 ricA*(p)::*ricA*::*ricA*(t)::3/4*pyroA*$^{+}$ Δ*ricA*::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
TNJ89[b]	*pyrG89 pyroA4 niiA*(p)::*pkaA*::$^{3/4}$*pyroA*$^{+}$ *veA*$^{+}$	This study
TNJ90[b]	*pyrG89 pyroA4 niiA*(p)::*pkaA*::$^{3/4}$*pyroA*$^{+}$ Δ*ricA*::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
TNJ94[b]	*pyrG89 pyroA4 alcA*(p)::Af*ricA*::$^{3/4}$*pyroA*$^{+}$Af*veA*$^{+}$	This study
TNJ95[b]	*pyrG89 pyroA4 alcA*(p)::Af*ricA*::$^{3/4}$*pyroA*$^{+}$ Δ*ricA*::Af*pyrG*$^{+}$ *veA*$^{+}$	This study
A. fumigatus strains		
AF293	Wild type	9
AF293.1	*pyrG1*	67
AF293.6	*pyrG1 argB1*	67
FNJ12	*pyrG1* Δ*ricA*::An*pyrG*$^{+}$	This study
FNJ13[c]	*pyrG1 ricA*(p)::*ricA*::*ricA*(t)::$^{3/4}$*pyrG*$^{+}$ Δ*ricA*::An*argB*$^{+}$ *argB1*	This study

[a] FGSC, Fungal Genetics Stock Center.
[b] The 3/4 *pyroA* marker in pHS causes targeted integration at the *pyroA* locus.
[c] The 3/4 Af*pyrG* marker in pNJ25 causes targeted integration at the *pyrG* locus.

(MM plus AT; noninducing) and also MMG (containing 0.6% [wt/vol] sodium nitrate; inducing). For Northern blot assays to confirm overexpression by the *alcA* promoter, strains were cultured in liquid MMG at 37°C, 220 rpm, for 12 h, and the mycelial aggregates were collected, rinsed with liquid MMT, transferred into liquid MMT, and further induced at 37°C, 220 rpm, for 6 h. Overexpression under *niiA*(p) was performed by culturing the strains in liquid MMG for 16 h at 37°C, 220 rpm. The *Saccharomyces cerevisiae* L40 strain (Clontech) was used to check the protein-protein interactions between the RicA-fused DNA binding domain and Gα subunits FadA, GanA, and GanB with the activation domain in a yeast two-hybrid assay. The L40 strain was grown in synthetic dropout minimal medium (SD) with the necessary supplements (10 g/liter leucine, 2 g/liter tryptophan, and 2 g/liter histidine) (55) and incubated at 30°C for 2 to 3 days. *Escherichia coli* DH5α and DH10B were grown in the Luria-Bertani (LB) medium with ampicillin (50 μg/ml; Sigma) or zeocin (20 μg/ml; Invitrogen) for plasmid amplification and construction. The oligonucleotides used in this study are listed in Table S1 of the supplemental material.

Database analyses, nucleic acid isolation, and manipulation. The putative RicA proteins were retrieved from an NCBI BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search based on *A. nidulans ricA*. A phylogenetic tree of the 21 putative RicA proteins was created by using information in EMBL-EBI (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The construction and analysis of the phylogenetic tree were carried out within EMBL-EBI (http://www.ebi.ac.uk/Tools/clustalw2/help.html#tree). The An*ricA* and Af*ricA* genes were PCR amplified from *A. nidulans* (FGSC4) and *A. fumigatus* (AF293) genomic DNA. cDNA of An*ricA* was isolated from an *A. nidulans* cDNA library (provided by K. Y. Jahng, Chonbuk National University, Jeonju, Korea) with the primer pairs oNK-39 and oNK-394. Af*ricA* cDNA was isolated from reverse transcriptase-treated total RNA and primers oNK-391 and oNK-392. Isolation of genomic DNA and total RNA for Northern blot analysis was carried out as described previously (38, 72). About 10 μg of total RNA isolated from each sample was separated by electrophoresis, using a 1.1% (wt/vol) agarose gel containing 3% (wt/vol) formaldehyde and ethidium bromide, then transferred onto a Hybond-N^{+} membrane (Amersham). The probes for *brlA* (1) and *vosA* (44) in *A. nidulans* and *brlA*, *abaA*, and *wetA* in *A. fumigatus* for Northern blot analyses were prepared by PCR amplification with primer pairs oNK-556/oNK-557 (An*brlA*), oNK-14/oNK-15 (An*vosA*), oNK-594/oNK-595 (Af*brlA*), oHS-382/oHS-383 (Af*abaA*), and oTL-7/oTL-8 (Af*wetA*), respectively (see Table S1 of the supplemental material).

Construction of deletion, complementation, and overexpression strains. The *ricA* deletion (Δ*ricA*) mutants for *A. nidulans* and *A. fumigatus* were generated by double-joint PCR (DJ-PCR) as described previously (72). The flanking regions of each *ricA* gene were amplified by PCR with primer pairs oNK-352/oNK-353 (An5′ with Af*pyrG* tail), oNK-354/oNK-355 (An3′ with Af*pyrG* tail), oNK-352/oNK-474 (An5′ with An*pyroA* tail), oNK-475/oNK-355 (An3′ with Af*pyroA* tail), oNK-358/oNK-359 (Af5′ with An*pyrG* tail), oNK-360/oNK-361 (Af3′ with An*pyrG* tail), oNK-358/oNK-933 (Af5′ with An*argB* tail), and oNK-934/oNK-361

(Af3′ with An*argB* tail) from both genomic DNAs, respectively. The An*pyrG*, An*pyroA*, An*argB*, and Af*pyrG* markers were amplified with the primer pairs oBS-08/oBS-09, oNK-395/oNK-396, oNK-104/oNK-105, and oJH-83/oJH-86, respectively. The final deletion constructs were amplified with the nested primer set oNK-356/oNK-357 (*A. nidulans*) or oNK-362/oNK-363 (*A. fumigatus*), respectively. The final PCR products were introduced into RJMP1.59 (N. P. Keller; *veA*$^{+}$) and RNIW3 (M. Ni; *veA1*) for *A. nidulans* AF293.1 or AF293.6 for *A. fumigatus* (67) using the Vinoflow FCE lysing enzyme (Novo Nordisk) (59). For the deletion mutants of *flbA*, *sfgA*, *rgsA*, *rgsB*, and *rgsC* in *A. nidulans*, each flanking region was PCR amplified using primer pairs oNK-412/oNK-413 (5′ *flbA* with An*pyroA* tail), oNK-414/oNK-415 (3′ *flbA* with An*pyroA* tail), oNK-397/oNK-398 (5′ *sfgA* with Af*pyroA* tail), oNK-399/oNK-400 (3′ *sfgA* with Af*pyroA* tail), oNK-540/oNK-541 (5′ *rgsA* with Af*pyroA* tail), oNK-542/oNK-543 (3′ *rgsA* with Af*pyroA* tail), oNK-562/oNK-563 (5′ *rgsB* with Af*pyroA* tail), oNK-564/oNK-567 (3′ *rgsB* with Af*pyroA* tail), oNK-568/oNK-569 (5′ *rgsC* with Af*pyroA* tail), and oNK-603/oNK-604 (3′ *rgsC* with Af*pyroA* tail), respectively. The final deletion constructs were amplified with oNK-416/oNK-417 (An*flbA*), oNK-401/oNK-402 (An*sfgA*), oNK-544/oNK-545 (An*rgsA*), oNK-605/oNK-606 (An*rgsB*), and oNK-607/oNK-608 (An*rgsC*). These deletion mutants were used to generate double-deletion mutants with ΔAn*ricA* by subsequent transformation.

To generate the complemented strains, genomic DNA fragments of An*ricA* and Af*ricA* were PCR amplified using the primer pairs oNK-870/oNK-871 and oNK-868/oNK-869 from each genomic DNA, then digested with BamHI (followed by treatment with Klenow fragment) and NotI, and cloned between PvuII and NotI of pHS3 (28) containing the ¾An*pyroA* (46) marker and pNJ25 (29) containing the ¾Af*pyrG* (14) marker with the *alcA* promoter (19), FLAG tag (DYKDDDDK), and the *trpC* terminator (68), respectively. Each construct was introduced into the recipient ΔAn*ricA and* ΔAf*ricA* strains, where preferentially a single copy is inserted into the An*pyroA* or Af*pyrG* locus, respectively. The complemented strains were confirmed by PCR amplification using the primers of each vector from the genomic DNA of transformants.

To generate the *ricA* overexpression mutant, the *ricA* genes were amplified by primer pairs, oNK-393/oNK-394 (An*ricA*) and oNK-391/oNK-392 (Af*ricA*) from each genomic DNA. The amplified genes were digested with restriction enzymes EcoRI and NotI and ligated between the *alcA* promoter and the *trpC* terminator in pHS3 and pNJ25, respectively. The final plasmids were used to transform TNJ36 and AF293.1, and single integration at *pyroA* in *A. nidulans* and *pyrG* locus in *A. fumigatus* was confirmed.

Autolysis and cell death assays. The alamarBlue (AB) assay to assess the cell viability based on the percent reduction of alamarBlue was used as described previously (57). A total of 10^6 conidia of the WT and ΔAf*ricA* strains were cultured in MMG plus 0.1% YE liquid medium at 37°C for 7 days. Aliquots (0.5 ml) of cultures according to time (days), including the mycelial aggregates and liquid medium, were transferred into 24-well plates (Nunc), 1 ml of fresh liquid medium containing 150 μl of alamarBlue (AbD Serotec) was added to each sample, and then the cultures were incubated for a further 6 h at 37°C. The solution samples were transferred into 96-well plates excluding mycelial aggregates, and absorbance was read at 570 and 600 nm. The percent alamarBlue reduction was detected with a Synergy HT apparatus (Bio-Tek) and the KC4 v3.1 software and was calculated using the following formula: $[(117{,}216 \times A_{570}\text{ of sample}) - (80{,}586 \times A_{600}\text{ of the sample})]/[(155{,}677 \times A_{600}\text{ of the medium}) - (14{,}652 \times A_{570}\text{ of the medium})] \times 100$ (39), as described by Shin et al. (57)

Yeast two-hybrid assay. Open reading frames (ORFs) of RicA (oNK-521/oNK-394), FadA (oNK-507/oNK-508), GanA (oNK-509/oNK-510), and GanB (oNK-511/oNK-512) were PCR amplified from the cDNA library of *A. nidulans*. The *ricA* ORF was cloned into the pTLexA vector (provided by S. K. Chae, Paichai University, Daejeon, Republic of Korea) (11) carrying the LexA DNA binding domain (DBD) (8), which was generated by modifying pHybLex/Zeo (Invitrogen) via insertion of the *TRP1* marker from pGBT9 (Clontech). The cDNA-derived ORFs of *fadA*, *ganA*, and *ganB* were each fused under the activation domain of pGAD424 (Clontech). Plasmids were sequence verified and cointroduced into *S. cerevisiae* L40 by lithium acetate-polyethylene glycol-mediated yeast transformation (25). The yeast transformants were selected on SD medium in the absence of uracil, tryptophan, and leucine (-UWL). To test the reporters β-galactosidase and histidine (H) by the interaction of RicA with Gα subunits in yeast, the transformants were inoculated on the -UWL medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml; Sigma) and the -UHWL medium, and the growth and color of the colonies were examined. To confirm and quantify the *trans*-activation activity, five transformants per each test set were tested for β-galactosidase activity (32) with a yeast β-galactosidase assay kit (Pierce).

GST pulldown assay. The An*ricA* ORF was cloned between EcoRI and NotI sites into pGEX-5X-1 (GE Healthcare) and then introduced into *E. coli* BL21(DE3) to express the glutathione *S*-transferase (GST)–AnRiA fusion protein. The *E. coli* strain was grown to an optical density at 600 nm of 0.5 to 0.6 at 37°C, 220 rpm, and 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside; Sigma) was added to induce fusion protein expression. The culture was then further incubated at 30°C, 220 rpm, for 3 h. Subsequently, cells were lysed by sonication in ice-cold *E. coli* lysis buffer (0.1% Triton X-100, 0.1 mM EDTA, 500 mM NaCl, with 1 protease inhibitor cocktail tablet [Roche] per 50 ml added before use). Then, the cell lysates were cleared of cellular debris by centrifugation, and the supernatant was collected and incubated with glutathione-Sepharose 4B beads (GE Healthcare) on a mixer at 4°C overnight. The beads were washed with the lysis buffer three times and resuspended in 500 μl lysis buffer. In addition, ORFs for *A. nidulans fadA*, *ganA*, *ganB*, and *ricA* were each cloned into pcDNA3 (Invitrogen) to translate *in vitro* by using the TNT T7 quick-coupled transcription/translation system (Promega). One-microgram aliquots of individual plasmids were incubated with 20 μCi of [^{35}S]methionine (PerkinElmer) in TNT mixture for 90 min at 30°C. Equal amounts of *in vitro*-translated proteins were added to glutathione bead-GST-RicA or glutathione bead-GST (control) suspensions. The mixtures were incubated on a mixer at 4°C overnight. After washing with *E. coli* lysis buffer five times, the samples were mixed with Laemmli sample buffer (Bio-Rad) and loaded onto SDS-PAGE gels. After electrophoresis, the gels were dried under a vacuum onto three layers of Whatman 3MM filter paper. Autoradiography was performed at −80°C with Fuji SuperRX film.

Microscopy. The colony photographs were taken using a Sony DSC-T30 digital camera. Photomicrographs were taken using a Zeiss M2Bio microscope equipped with AxioCam and AxioVision digital imaging software.

Nucleotide sequence accession numbers. Our newly determined AnRicA and AfRicA sequences and annotations have been deposited in GenBank under the accession numbers JN410838 and JN582330, respectively.

RESULTS

Identification of RicA in *A. nidulans* and *A. fumigatus*. A genome search with the *Caenorhabditis elegans* RIC-8 protein (GenBank accession number AF288812.1) resulted in the identification one putative ortholog in each *Aspergillus* species: Afu4g08820 (AfRicA; score of 134 and e-value of 2.5e−05) and AN1661 (AnRicA; score of 131 and e-value of 6.5e−05). To verify the corresponding ORFs, we isolated and analyzed the *ricA* cDNA from the *A. fumigatus* and *A. nidulans* cells. Briefly, the AnRicA protein is composed of 466 amino acids (aa; ORF of 1,401 bp with 6 introns), and AfRicA is composed of 461 aa (ORF of 1,386 bp with 6 introns), with predicted masses of 51.7 kDa and 51.2 kDa, respectively. Employing these protein sequences, we further identified putative RicA orthologs in other fungi and carried out alignments (see Fig. S1 in the supplemental materal) and phylogenetic analyses (Fig. 1B). The RIC8 ortholog is absent in the genomes of *S. cerevisiae*

and plants (65). As presented, the *A. nidulans* RicA is close to that of *A. niger*, *A. oryzae*, and *A. flavus*, while the *A. fumigatus* RicA is close to the *A. clavatus* RicA. To characterize the *ricA* gene, levels of *ricA* mRNA at different time points in the life cycle of the two species were examined. As shown in Fig. 1C, in both species the *ricA* transcript (each ~2.5 kb) was detectable in a somewhat undulant manner during growth and developmental phases, but not in conidia. It appears that An*ricA* mRNA is present at low levels in sexual spores (ascospores [Fig. 1C]).

Characterization of *ricA* in *A. fumigatus*. To understand the function of RicA in *A. fumigatus*, we generated the Af*ricA* deletion mutant (ΔAf*ricA*) by replacing its coding region with An*pyrG*+ (for AF293.1) or An*argB*+ (for AF293.6; for further complementation information, see Table 1). Subsequently, by using a ΔAf*ricA*:: An*argB*+ *pyrG1* strain as a transformation host, we further generated Af*ricA*-complemented strains by introducing the Af*ricA* WT allele into the *pyrG* locus, via pNJ25 (57). We then examined the phenotypes of ΔAf*ricA* (FNJ12), WT (AF293), and complemented (FNJ13) strains (Table 1). Most markedly, the ΔAf*ricA* mutant exhibited highly restricted colony growth (about 20% of WT) and produced very few and abnormal conidia at the center of colonies (Fig. 2A). Conidiation at this point was less than 1% that of the WT at 5 days after point inoculation on solid MMG with YE. To test whether the ΔAf*ricA* abnormal conidia were viable, we tested the rates of conidial germination on solid MMG plus 0.1% YE. As shown in Fig. 2B, WT conidia showed germination rates of 90% ± 1.78% (mean ± standard deviation) at 6 h and 100% at 12 h postinoculation. On the contrary, the ΔAf*ricA* conidia displayed extremely delayed and defective germination: 0%, 2.9% ± 0.18%, and 34.5% ± 2.8% of conidia germinated at 6 h, 12 h, and 18 h, respectively (Fig. 2B), whereas only ~35% of conidia germinated eventually (data not shown). These results indicate that AfRicA is necessary for the functionality (germination) of conidia.

Aspergillus conidiophore formation requires sequential activities of the central regulatory components *brlA*, *abaA*, and *wetA* (2, 61, 70). As shown in Fig. 2A (middle and bottom panels), the ΔAf*ricA* mutant produced abnormal conidiophores (black arrowheads) showing improper septation in stalks and incomplete formation of phialides and conidia. As shown by Northern blot analysis, the ΔAf*ricA* mutant exhibited severely delayed and reduced levels of Af*brlA*, Af*abaA*, and Af*wetA* mRNA during the progression of conidiation (Fig. 2C). In the WT, Af*brlA* mRNA levels increased at 6 h, peaked at 12 h, began to decrease at 24 h, and became almost undetectable at 72 h post-developmental induction. Accumulation of the Af*abaA* and Af*wetA* transcripts followed the Af*brlA* mRNA accumulation pattern. However, in the ΔAf*ricA* mutant, Af*brlA* mRNA accumulated at low levels at 0 h (vegetative growth at 16 h) and gradually increased until 72 h, i.e., there was reduced, delayed, and uncontrolled accumulation of Af*brlA*. Moreover, transcripts of Af*abaA* and Af*wetA* accumulated at low levels even at 48 and 72 h post-developmental induction. These results indicate that AfRicA is necessary for proper expression and regulation of key developmental regulators that coordinate the formation, integrity, and vitality of spores.

Finally, we tested whether the deletion of Af*ricA* affects cell death and autolysis in *A. fumigatus* by using AB reduction (57) and dry weight assays, respectively. As shown in Fig. 2D and E, the absence of AfRicA resulted in delayed cell death and autolysis. Whereas both WT and complemented strains exhibited reduced AB reduction rates at day 4 and levels of only 20% at day 7, the ΔAf*ricA* mutant exhibited 100% AB reduction at day 5, a decreased rate from day 6 (to about 82%), and retained ~58% AB reduction even at day 7 (Fig. 2D). Similarly, while WT and complemented strains exhibited maximum dry weight at day 2 and reduced dry weights from day 3, the ΔAf*ricA* mutant showed a peak dry weight at day 5 and gradually reduced weight at days 6 and 7 (Fig. 2E). These findings suggest that AfRicA is necessary for proper vegetative proliferation and normal progression of cell death and autolysis in *A. fumigatus*.

Characterization of An*ricA* and cross-species complementation. The deletion of An*ricA* resulted in severely impaired growth and a complete lack of asexual and sexual development in *A. nidulans*. As clearly noticeable from the colonies of WT (TNJ36), ΔAn*ricA* (TNJ21), and complemented (TNJ87) strains grown on solid MMG at 37°C for 5 days, the ΔAn*ricA* mutant formed a very small colony (~30% of the WT colony diameter) composed of hyphae without any conidiophores, conidia, cleistothecia (sexual fruiting bodies), or Hülle cells (Fig. 3A). These growth and developmental defects could not be alleviated by changing the growth conditions, e.g., high salt (0.6 M KCl or 0.8 M NaCl), the lack of carbon source, sexual induction, or rich nutrient (0.5% YE) (data not shown). Thus, all further experiments employing the ΔAn*ricA* mutant were done by collecting and inoculating hyphal fragments from a high number of air-exposed mutant colonies. In submerged shake cultures following inoculation of the ΔAn*ricA* aerial hyphae, the ΔAn*ricA* mutant exhibited extremely slow hyphal proliferation compared to the WT, and the size of ΔAn*ricA* mycelial aggregates was only 15% of that of WT and complemented strains at 3 days (data not shown). We then asked whether introduction and/or overexpression of the Af*ricA* WT allele could restore growth and development in the ΔAn*ricA* mutant. We found that the presence of a genomic fragment of Af*ricA*, including its own promoter, coding region, and terminator, was not sufficient to restore growth and development in the ΔAn*ricA* mutant (data not shown). However, as shown in Fig. 3B, the overexpression of Af*ricA* under the control of the An*alcA* promoter partially enhanced growth, and there was fully restored conidiation in the ΔAn*ricA* mutant under the inducing condition (MMT plus 0.5% YE). Supplementation with 0.6 M KCl enhanced the developmental restoration by Af*ricA* in the ΔAn*ricA* mutant grown on MMT plus 0.5% YE, and even on MMG (noninducing) (Fig. 3B, bottom, +KCl). These results indicate that AnRicA plays a crucial and essential role for growth and development in *A. nidulans*, and AfRicA can partially replace AnRicA.

Suppression of Δ*ricA* by Δ*rgsA* in *A. nidulans*. If RicA is an ortholog of Ric-8 in animals, it likely functions in activation of heterotrimeric G protein signaling (24). As RicA is clearly needed for growth and development, we carried out a series of double-mutant analyses to examine the genetic interactions of RicA with other key regulators in *A. nidulans*. The *flbA* gene encodes an RGS protein required for the attenuation of vegetative proliferation signaling mediated by FadA and activation of conidiophore development in *Aspergillus* (23, 31, 38, 74). SfgA (suppressor of *fluG*) is a negative regulator of conidiation, functioning downstream of FluG but upstream of other key developmental activators, including FlbD, FlbC, FlbB, and BrlA (52). As shown in Fig. 4A, the Δ*ricA* Δ*flbA* and Δ*ricA* Δ*sfgA* double mutants exhibited phenotypes identical to the Δ*ricA* single mutant. These findings indicate that the removal of the key negative regulators of growth (FlbA) or conidiation (SfgA) could not alleviate the growth and developmental defects caused by Δ*ricA* and that RicA functions either

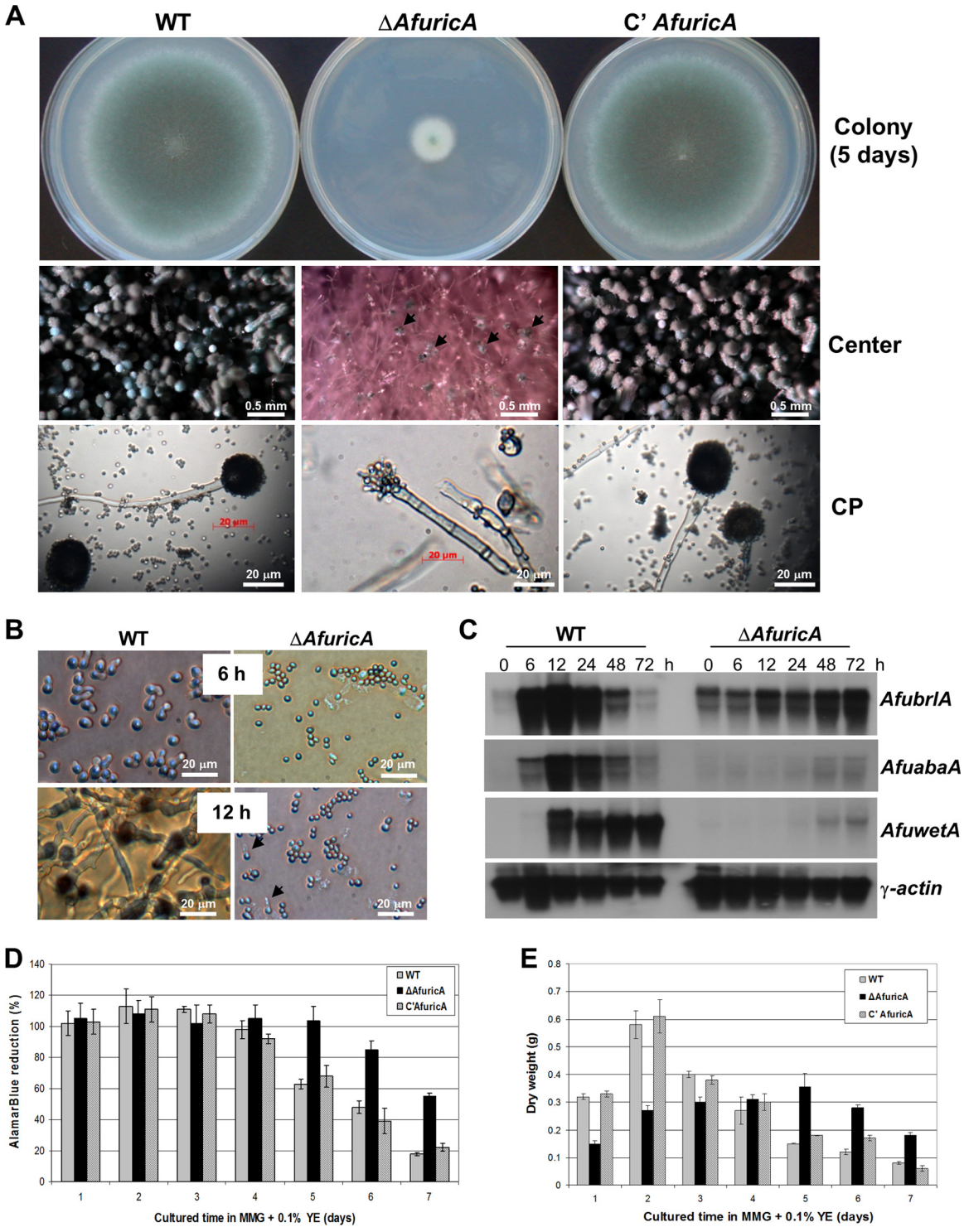

FIG 2 Phenotypes caused by ΔAf*ricA*. (A) WT (AF293), ΔAf*ricA* (FNJ12), and complemented (C′ Af*ricA*; FNJ13) strains were point inoculated on solid MMG plus 0.1% YE and incubated at 37°C for 5 days. Photographs of colony sizes and close-up views of the centers of the colonies and conidiophores are shown. Black arrows indicate abnormal conidiophores at the center of the ΔAf*ricA* colony. (B) Germination of WT (AF293) and ΔAf*ricA* (FNJ12) conidia inoculated on MMG plus 0.1% YE plates and incubated for 6 and 12 h. Black arrows in the image for ΔAf*ricA* indicate germinated conidia at 12 h. (C) Accumulation of *brlA*, *abaA*, *wetA*, and γ-actin mRNA post-asexual developmental induction of WT (AF293) and ΔAf*ricA* (FNJ12) strains. Development at 0 h indicates vegetative growth in MMG plus 0.1% YE liquid for 18 h. The *A. fumigatus* γ-actin gene (16, 29) was used as a control. (D) alamarBlue reduction data, indicating relative cell death rates. The mycelial aggregates of WT, ΔAf*ricA*, and complemented strains were mixed with the AB reagent to check the cell viability for 7 days. (E) Dry weights of WT, ΔAf*ricA*, and the complemented strain in MMG plus 0.1% YE submerged cultures were quantified for 7 days at 37°C, 220 rpm.

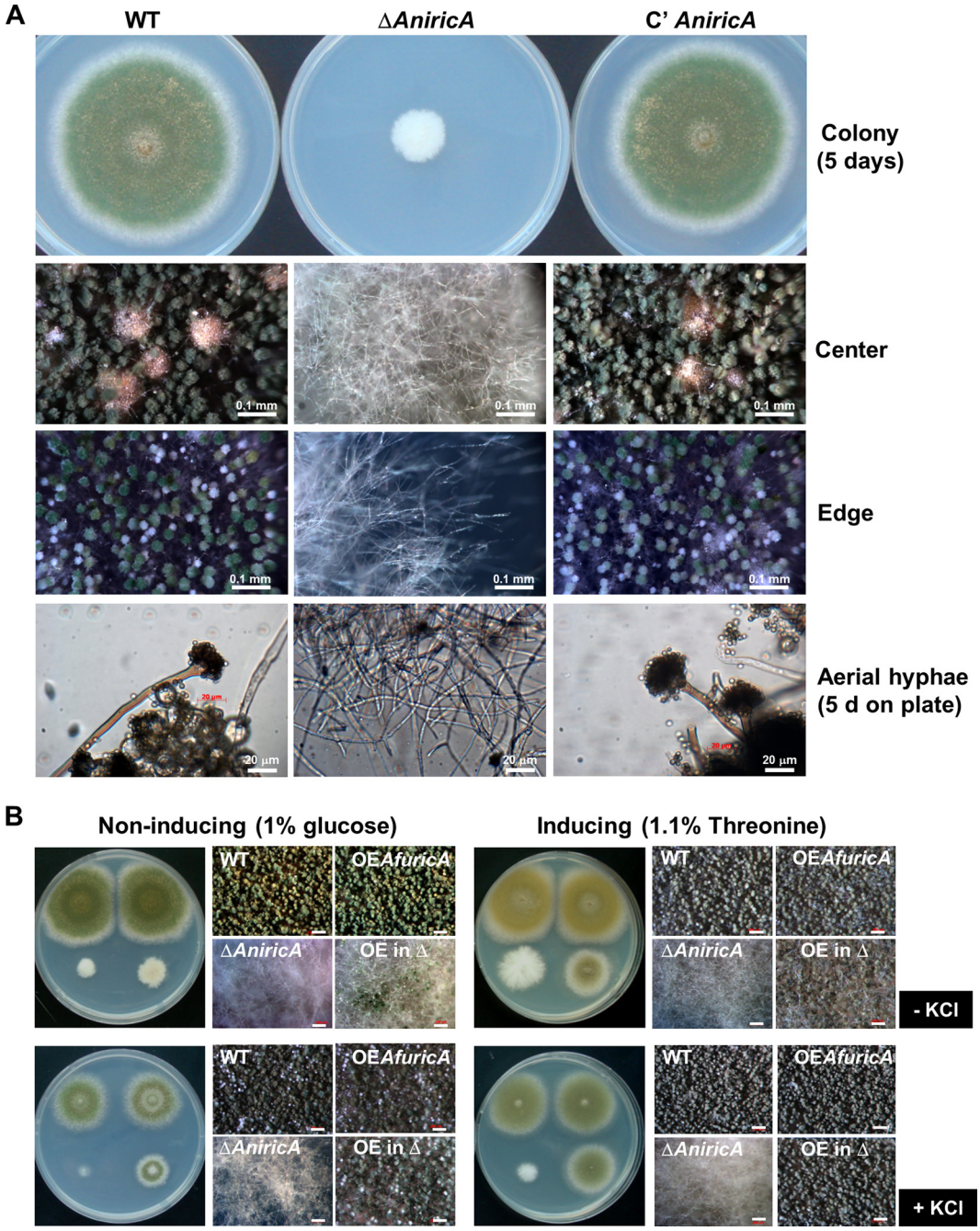

FIG 3 Requirement of RicA in *A. nidulans* growth and development. (A) *A. nidulans* WT (TNJ36), Δ*AnricA* (TNJ21), and complemented (C′ *AnricA*; TNJ87) strains were inoculated on MMG and cultured for 5 days at 37°C. Colonies and conidiophores of the 5-day-old culture on solid MMG were observed under a stereomicroscope. (B) Partial complementation by *AfricA* overexpression in Δ*AnricA*. WT (TNJ36), OEA*fricA* (TNJ94), Δ*AnricA* (TNJ21), and OEA*fricA* Δ*AnricA* (TNJ95; OE in Δ) were point inoculated on solid MMG (noninducing), MMT plus 0.5% YE (induction by the *alcA* promoter), MMG without KCl, or MMT plus 0.5% YE with 0.6 M KCl and incubated for 3 days 37°C. Bar, 200 μm.

upstream or independently of the FlbA/FadA growth and FluG/SfgA developmental control pathways. We then asked whether RicA is associated with activation of other G protein pathways. RgsA is an RGS protein that inhibits GanB-mediated signaling for germination/developmental control and stress response (10, 21). RgsB and RgsC are putative RGS proteins that remain to be characterized (21). As shown in Fig. 4B, while the deletion of *rgsB* or *rgsC* failed to suppress Δ*ricA*, the absence of *rgsA* restored growth and development to the Δ*rgsA* level in the Δ*ricA* mutant. Furthermore, as found in the Δ*rgsA* mutant (21), the Δ*ricA* Δ*rgsA* mutant conidia germinated proficiently in liquid medium lacking an external carbon source, whereas WT conidia did not show any sign of germination (Fig. 4C). These results indicate that RicA mediates signaling for growth and development, primarily through the RgsA/GanB signaling pathway. It might do so by activating GanB in the absence of the (unidentified) corresponding GPCR(s).

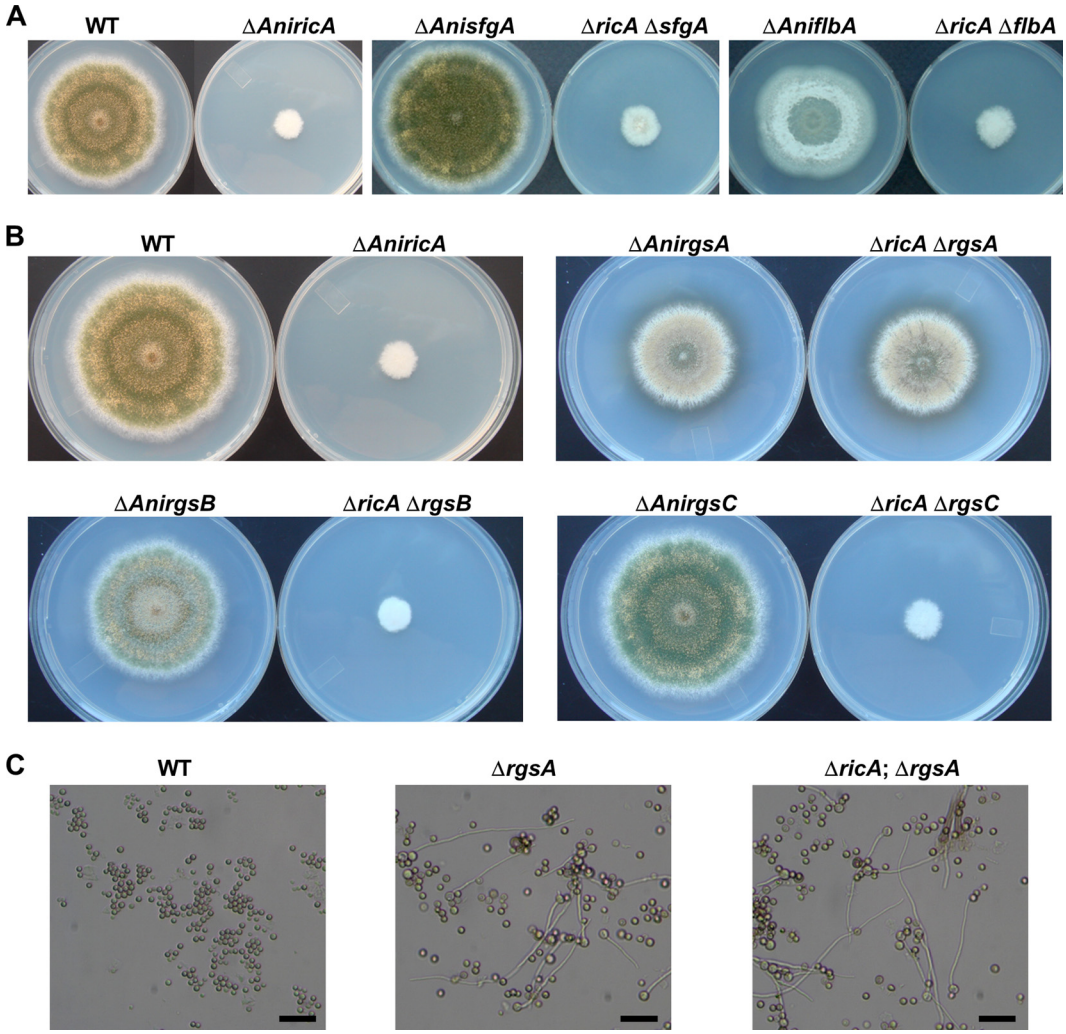

FIG 4 Double mutant analyses in *A. nidulans*. (A) WT (TNJ36), ΔAn*ricA* (TNJ21), ΔAn*sfgA* (TNJ57), ΔAn*sfgA* ΔAn*ricA* (TNJ58) ΔAn*flbA* (TNJ42), and ΔAn*flbA* ΔAn*ricA* (TNJ49) strains grown on solid MMG for 5 days at 37°C. (B) WT (TNJ36), Δ*ricA* (TNJ21), Δ*rgsA* (TNJ61), Δ*ricA* Δ*rgsA* (TNJ62), Δ*rgsB* (TNJ63), Δ*ricA* Δ*rgsB* (TNJ64), Δ*rgsC* (TNJ65), and Δ*ricA* Δ*rgsC* (TNJ66) were inoculated on MMG and incubated at 37°C for 5 days. (C) Germination of WT (TNJ36), Δ*rgsA* (TNJ61), and Δ*ricA* Δ*rgsA* (TNJ62) conidia in the absence of an external carbon source. Photographs were taken after inoculating 1×10^6 conidia into liquid MM without an external C source and cultured for 16 h at 37°C and 220 rpm. Bar, 25 μm.

Physical interaction between RicA and GanB. To further test the hypothesis that the primary target of AnRicA is AnGanB, we first tested the physical interaction between AnRicA and individual Gα subunits by employing a yeast two-hybrid assay. The An*ricA* ORF PCR fragment derived from cDNA was fused with the LexA DBD in the pTLexA vector (11), and each Gα ORF, AnFadA, AnGanA, and AnGanB, was fused with the Gal4 activation domain (Gal4 AD) in the pGAD424 vector (Clontech). Individual pairs of plasmids were introduced into the yeast and examined for levels of β-galactosidase reporter activity. As shown in Fig. 5A, only the AnRicA-AnGanB pair exhibited a blue color on the X-Gal SD medium lacking uracil, tryptophan, and leucine. Quantification of the β-galactosidase activity of each pair in yeast by using *o*-nitrophenyl-galactosidase (ONPG) further demonstrated that only the AnRicA-AnGanB pair resulted in high levels of reporter expression in yeast (Fig. 5B), i.e., about 90% of that of the well-known transcriptional activator AnAflR (positive control) (44, 71). To map the critical interacting domains, we further tested the physical interaction between the truncated AnRicA (aa 1 to 398 or 51 to 466, of the full-length 466 aa) and AnGanB (aa 1 to 325 or 35 to 356 of the full-length 356 aa), and we found that the full-length AnRicA and AnGanB are necessary for the interaction in yeast (data not shown). The physical interaction of AnRicA and AnGanB was further tested *in vitro* in a GST-pulldown assay (Fig. 5C). The An*ricA* ORF was fused with GST in the pGEX 5X-1 vector (GE Healthcare), and the AnRicA protein was expressed and purified in *Escherichia coli*. The ORF regions of An*fadA*, An*ganA*, and An*ganB* were cloned under the T7 promoter of pCDNA3, and each Gα subunit was translated *in vitro* and labeled with ^{35}S. An equal amount of *in vitro*-translated proteins was added to glutathione bead-GST-AnRicA or glutathione bead-GST (control) suspensions and subjected to pulldown. As shown in Fig. 5C, ^{35}S-labeled AnGanB, but not AnFadA or AnGanA, could be copurified with GST-AnRicA specifically, indicating that AnRicA directly binds to AnGanB *in vitro*. Collectively, these data suggest that GanB is a primary target of RicA-mediated signaling in *A. nidulans*, and the full-length AnRicA and AnGanB are necessary for their physical interaction.

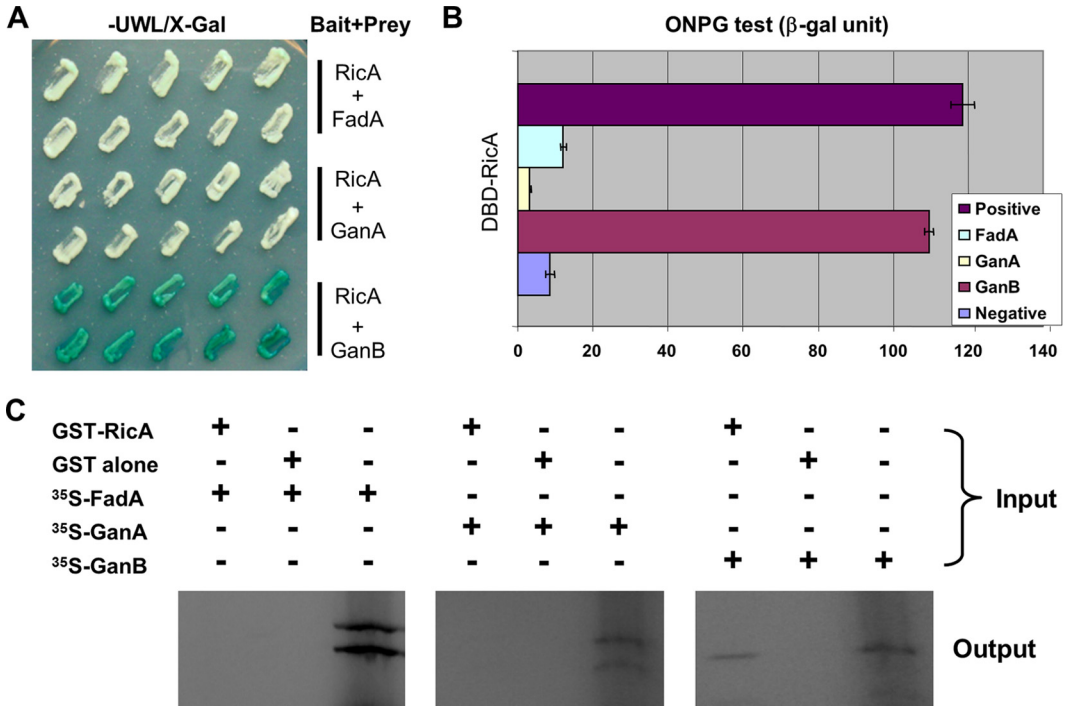

FIG 5 RicA and GanB physically interact in yeast and *in vitro*. (A) Colony photographs of yeast strains expressing both the LexA DNA binding domain fused the AnRicA protein and the Gal4 activation domain fused to Gα proteins, AnFadA, AnGanA, and AnGanB on X-Gal medium without uracil, tryptophan, or leucine (-UWL/X-Gal). (B) Quantitative analyses of β-galactosidase activities, using ONPG in yeast strains, including the positive control (AflR) (71) and negative control (pTLex vector; LexA DNA binding domain alone), shown on the left. (C) GST pulldown assay for GST-AnRicA and *in vitro*-translated [^{35}S]Gα proteins. The right lane of each panel shows the *in vitro*-translated FadA, GanA, and GanB proteins, respectively (10 μl from 50-μl translation reaction volumes). The *in vitro*-translated proteins were divided into two parts (each 20 μl) and mixed with the GST-AnRicA protein (left lane) or GST alone (middle lane). The expected protein sizes of FadA, GanA, and GanB were about 39, 40, and 39 kDa, respectively.

The RicA→GanB→PkaA signaling cascade. In *A. nidulans*, early events of conidial germination in response to sensing carbon sources are controlled by GanB (Gα) and SfaD::GpgA (Gβγ) and the cAMP-dependent protein kinase PkaA (17, 30, 56). As GanB has been proven to be a primary target of RicA, we asked whether the elevated expression of *pkaA* could restore growth and development in the *ricA* deletion mutant. While supplementation of exogenous (up to 10 mM) cAMP and dibutyryl cAMP failed to restore growth and development (data not shown), two copies (one native and an ectopic) or the overexpression of *pkaA* was sufficient to restore growth and development in the Δ*ricA* mutant. As shown in Fig. 6, the *niiA*(p)::*pkaA* single and Δ*ricA niiA*(p)::*pkaA* double mutants exhibited identical phenotypes on solid medium with 0.2% ammonium tartrate (noninducing) or 0.6% sodium nitrate (inducing) as a nitrogen source. The overexpression of *pkaA* regardless of the presence or absence of RicA resulted in enhanced production of aerial hyphae and a reduced density of conidia per unit area on inducing medium, as described by Shimizu and Keller (56). These results suggest that RicA likely mediates signaling through GanB→PkaA in *A. nidulans*.

Unknown role of RicA-mediated signaling in *A. nidulans* development. Finally, we checked whether the developmental defect caused by Δ*ricA* is due to the defective expression of the key regulators *brlA* (1) and *vosA* (44). The mycelial mats of WT, ΔAn*ricA*, *niiA*(p)::*ricA* (OE*ricA*), Δ*flbA*, Δ*flbA* Δ*ricA*, Δ*sfgA*, and Δ*sfgA* Δ*ricA* strains grown on the surface of liquid stationary MMG were collected and subjected to Northern blot analyses. It is important to note that the mutant strains containing Δ*ricA* were cultured for 3 days in order to generate enough hyphal mass on the surface of liquid MMG. Despite the complete lack of conidia, all mutants containing Δ*ricA* showed comparable levels of *brlA* and *vosA* mRNAs (Fig. 7A), suggesting that expression of *brlA* is not sufficient to activate conidiation in the absence of RicA and that RicA-mediated signaling may provide a critical input for conidiophore development. This was further tested by overexpressing *fluG* and *brlA* in the absence of *ricA*. Neither OE*fluG* nor OE*brlA* could restore conidiation in the Δ*ricA* mutant on solid medium (Fig. 7B). Moreover, while OE*brlA ricA*$^{+}$ caused the formation of conidia at the hyphal tip in liquid submerged culture, the OE*brlA* Δ*ricA* double mutant showed only reduced mycelial growth without forming conidia (data not shown). These results suggest that expression of *brlA* is not sufficient for conidiation, and unknown RicA-mediated developmental signaling, likely independent of the *fluG* →*brlA* pathway, is necessary for conidiophore development in *A. nidulans* (Fig. 8).

DISCUSSION

During the past decade, novel families of proteins that can modulate the on-off state of G proteins have been identified, raising the complexity of the regulation of signal transduction (50). Among the newly identified components that may function as positive modulators of G proteins is Ric-8 (also known as synembryn

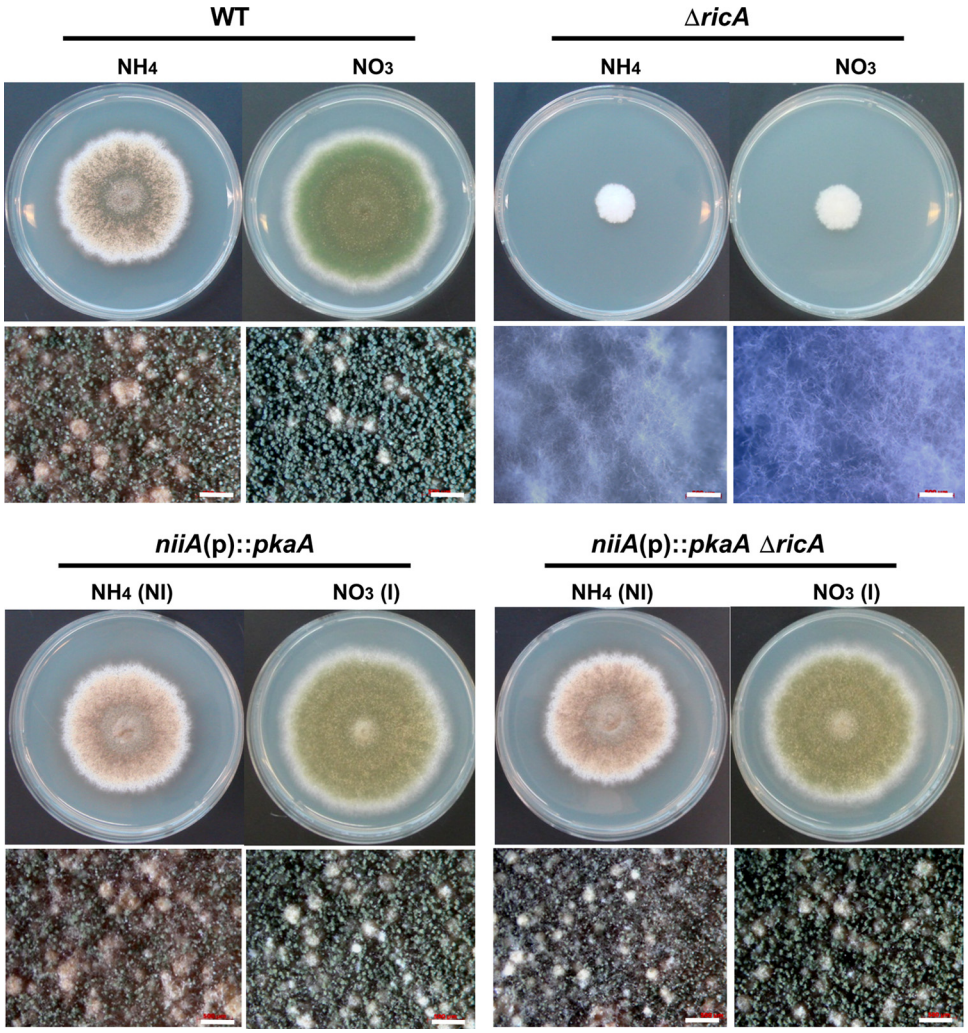

FIG 6 Suppression of ΔAn*ricA* by an ectopic copy or overexpression of *pkaA*. Colony photographs (top) and close-up views (bottom) of *A. nidulans* WT (TNJ36), ΔAn*ricA* (TNJ21), OE*pkaA* (TNJ89), and OE*pkaA* ΔAn*ricA* (TNJ90) strains are shown. These strains were point inoculated on noninducing medium (NI; MMG containing 0.2% ammonium tartrate as a nitrogen source) or inducing medium (I; MMG containing 0.6% sodium nitrate as a nitrogen source) and incubated for 5 days at 37°C.

[24]). Ric-8 is a cytoplasmic protein that was initially identified by a genetic screening of *C. elegans* mutants that are resistant to the cholinesterase inhibitor aldicarb (41) and by a yeast two-hybrid screen searching for interacting partners of mammalian Gα subunits (27, 60). Later, it was revealed that RIC-8 is involved in the asymmetric division of *C. elegans* embryos (3, 12, 42) and *Drosophila melanogaster* neuroblasts (13, 20, 62). The RIC-8 orthologs are present in genomes of animals and filamentous fungi, but not in baker's yeast or plants. Whereas the *C. elegans* and *Drosophila* genomes contain a single Ric-8 gene, mammals possess two Ric-8 orthologs, Ric-8A and Ric-8B (24). Unlike GPCRs, Ric-8 proteins cannot stimulate the guanine nucleotide exchange activity of the Gα subunit associated with Gβγ, i.e., the inactive heterotrimeric complex. Thus, Ric-8 proteins can only function on dissociated (free) monomeric Gα-GDP (Fig. 1A) (24, 60). It has been hypothesized that RIC-8 was acquired by animals and certain fungi after G proteins and GPCRs evolved and is therefore a fairly recent addition to G protein regulatory pathways (65).

In this report, we present experimental evidence that the Ric-8 ortholog (RicA) plays a crucial role in governing vegetative growth and development in two *Aspergillus* species. The lack of AnRicA function results in profound defects in hyphal proliferation and asexual/sexual fruiting. In particular, the An*ricA* null mutants were severely impaired in hyphal growth and unable to form conidia or ascospores in *A. nidulans*. Thus, the ΔAn*ricA* cultures were derived from the mutant hyphal fragments, and all double mutants were generated by deleting An*ricA* from individual single mutants through transformation. Likewise, the ΔAf*ricA* mutant produces defective conidia, many of which are unable to germinate, making it extremely difficult to study RicA function in both species. Whereas the primary structure of the two *Aspergillus* RicA proteins is highly conserved (75% identity, 89% similarity, and 0% gaps [http://blast.ncbi.nlm.nih.gov/Blast.cgi]), the overexpression of AfRicA only partially restored growth and development in *A. nidulans*. We found here that RicA interacts with the Gα subunit GanB, but not with FadA or GanA, in *A. nidulans*. Furthermore, RicA is incapable of forming a homodimer or a multimer (Fig. 5). Han et al. reported that the deletion of *ganB*,

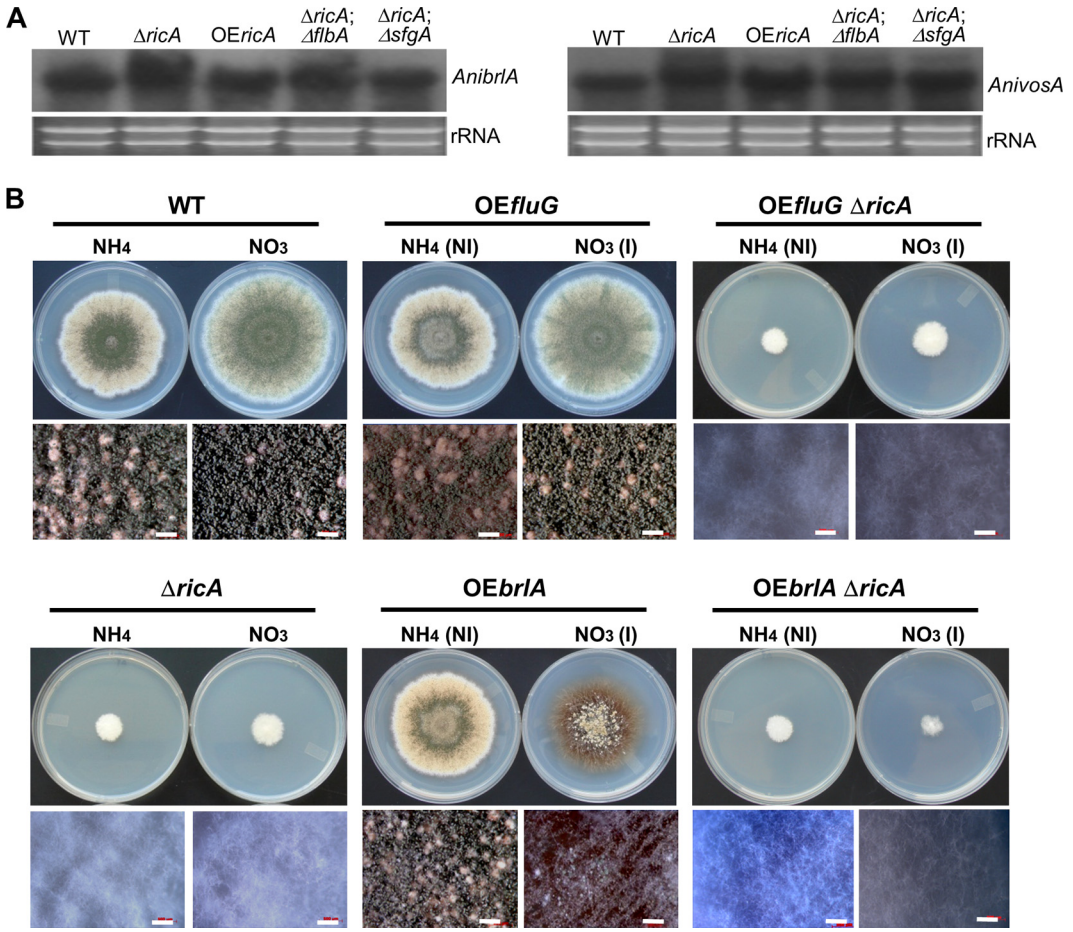

FIG 7 Requirement of An*ricA* in conidiation independent of *fluG* and *brlA*. (A) mRNA levels of *brlA* and *vosA* in WT (TNJ36), Δ*ricA* (TNJ21), OE*ricA* (TNJ88), Δ*flbA* Δ*ricA* (TNJ49), and Δ*sfgA* Δ*ricA* (TNJ58) cells collected from MMG liquid surface culture. Note the strains, including Δ*ricA*, were cultured for 3 days on the surface of liquid MMG to obtain equal amounts of the hyphal aggregates. (B) WT, Δ*ricA* (TNJ21), OE*fluG* (TNJ59), OE*fluG* Δ*ricA* (TNJ60), OE*brlA* (TNJ85), and OE*brlA* Δ*ricA* (TNJ86) strains were point inoculated on noninducing (NI; MMG with 0.2% ammonium tartrate) or inducing (I; MMG with 0.6% sodium nitrate) solid medium and incubated at 37°C for 5 days.

but not *fadA* or *ganA*, suppressed the developmental and metabolic defects caused by Δ*rgsA*, and they concluded that RgsA negatively regulates GanB-mediated signaling in *A. nidulans* (21). We found that RicA physically interacts with GanB, and only Δ*rgsA* suppresses the defective growth and development phenotypes caused by Δ*ricA*; the Δ*rgsA* Δ*ricA* double mutant exhibited a phenotype identical to the Δ*rgsA* single mutant (Fig. 4B and C). All these findings suggest that RicA-mediated signaling is transduced primarily via GanB in *A. nidulans* (Fig. 8).

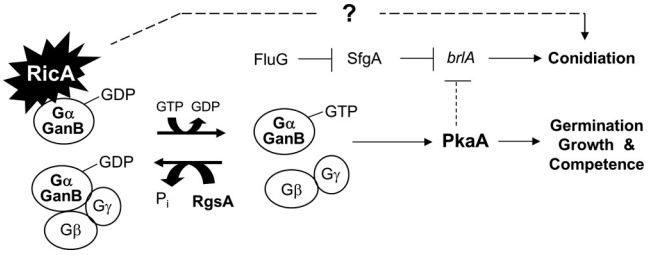

FIG 8 Model for how RicA governs *A. nidulans* growth and development (see the text). The putative GEF RicA governs upstream signaling for spore germination, vegetative growth, and development in *Aspergillus*, primarily through the GanB→PkaA signaling cascade. RicA is also (indirectly) required for conidiophore development, which may involve the acquisition of developmental competence via modulating GanB→PkaA signals. In addition, a potential direct role of RicA in activating conidiation, independent of FluG/BrlA, is indicated. It is further speculated that *brlA* is downregulated by GanB and PkaA during early phases of vegetative growth in *A. nidulans*.

Studies of the Ric-8 ortholog in *Neurospora crassa* (66) and *Magnaporthe oryzae* (35) have revealed that RIC-8 plays a highly conserved role in filamentous fungi. As found in *Aspergillus*, the *N. crassa ric-8* (Nc*ric-8*) deletion mutant shows severe defects in growth and development. The phenotypes caused by Nc*ric-8* are similar to those observed in the mutant lacking the Gα genes *gna-1* and *gna-3*. Moreover, ΔNc*ric-8* results in greatly reduced levels of all three Gα subunits and one Gβ subunit (GNB-1) (66). Further studies have revealed that NcRIC8 positively regulates GNA-1 and GNA-3 and physically interacts with and acts as a GEF for GNA-1 (equivalent to AnFadA) and GNA-3 (AnGanB) *in vitro*, with the strongest effect on GNA-3. The rice blast fungus *M. oryzae RIC-8* (Mo*RIC-8*) is known to be a novel component of G protein signaling during infection into plants (35). The deletion of Mo*RIC-8* results in nonpathogenicity and impairment in cellular differentiation associated with sporulation, sexual development, and plant in-

fection. MoRic-8 is highly expressed in the appressorium, a specialized plant tissue-invading structure, and physically interacts with MagB (AnFadA) but not MagA (AnGanB) in the yeast two-hybrid system. Collectively, these results indicate that Ric-8/RicA plays a crucial role in upstream activation of various signaling pathways in filamentous fungi and that the primary targets of RicA vary.

Studies in these three fungi (*Aspergillus*, *Neurospora*, and *Magnaporthe*) indicate that RIC-8-mediated signaling likely involves cAMP-dependent protein kinase signaling and might affect G protein levels. In *N. crassa*, ΔNc*ric-8* results in low levels of adenylyl cyclase protein. Moreover, ΔNc*ric-8* can be suppressed by a mutation in the PKA regulatory subunit (66). MoRic-8 is thought to act upstream of the cyclic AMP response pathway that is necessary for appressorium morphogenesis. In accordance with these concepts, we have shown that two copies and/or overexpression of the primary catalytic subunit of the cAMP-dependent protein kinase PkaA suppresses the profound defects caused by the lack of RicA function. Previous studies have also revealed that RIC-8 is also required for normal levels of various G proteins in *Neurospora*, *Drosophila*, *C. elegans*, and mammalian cells. Likewise, the deletion of Mo*RIC-8* causes the downregulation of Gα subunits, *MAGA*, *MAGB*, and *MAGC*, as well as the Gγ (*MGG1*) subunit, but not the Gβ subunit (*MGB1*) (35). Taken together, these results suggest that maintenance of normal levels of G proteins and adenylyl cyclase (and perhaps other yet-unknown regulatory components) are important in the conserved function of the RIC-8 protein (66) and that RicA-mediated signaling is transduced primarily through the GanB→PkaA pathway in *Aspergillus* (Fig. 8).

While the absence of RicA function essentially abolished conidiation in both species, mRNA of the key developmental activator *brlA* accumulated to some extent in both *A. nidulans* and *A. fumigatus*. Moreover, while *abaA* and *wetA* mRNA levels were very low in the *A. fumigatus ricA* null mutant, An*vosA* mRNA accumulated in hyphal cells. Furthermore, neither the removal of the key repressor of conidiation SfgA (51, 52) nor the overexpression of the key activators FluG/BrlA rescued the developmental defects caused by the lack of RicA. This suggests that, in addition to the activation of *brlA* expression, an unknown RicA-mediated signaling input is essential for conidiation in *A. nidulans*. As the deletion of *rgsA* and the overexpression of PkaA restored the production of conidia in the *ricA* null mutant, we speculate that the RicA→GanB→PkaA signaling input is somehow (indirectly) necessary for the activation of conidiophore development (Fig. 8). As a possible explanation, we propose that RicA-mediated activation of GanB and PkaA signaling is necessary for the acquisition of developmental competence (reference 2 and references therein). As previously reported, conidiation does not usually occur in *A. nidulans* until cells have gone through a defined period of vegetative growth (reference 2 and references therein), supporting the hypothesis that the early aspect of *Aspergillus* conidiophore development occurs as an integral part of the life cycle rather than as a response to unfavorable environmental conditions. We speculate that the absence of *ricA* function abolishes transduction of signals for carbon source sensing, spore germination, vegetative proliferation, and thereby the acquisition of developmental competence; all are thought to be primarily mediated by the GanB→PkaA pathway (Fig. 8). In a previous model, it was proposed that GanB and PkaA signaling inhibited expression of *brlA* (10, 56). We further speculate that GanB and PkaA play a role in downregulating *brlA* expression in order to prevent precocious conidiation and to confer vegetative growth for a certain period of time during the life cycle of *A. nidulans*. Further investigation of RicA-mediated signaling and the PkaA downstream components that are associated with the developmental regulation/competence needs to be carried out in *Aspergillus* species.

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