The putative GEF RicA mediates upstream signaling for growth and
development in *Aspergillus*

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ABSTRACT

Heterotrimeric G proteins (G proteins) govern growth, development and secondary metabolism in various fungi. Here, we characterize ricA encoding a putative GDP/GTP exchange factor for G proteins in the model fungus Aspergillus nidulans (Ani) and the opportunistic human pathogen Aspergillus fumigatus (Afu). In both species, ricA mRNA accumulates during vegetative growth and early developmental phases, but is not present in spores. The deletion (Δ) of ricA results in severely impaired colony growth and the total (Ani) or near (Afu) absence of asexual sporulation (conidiation). The overexpression (OE) of AfuricA restores growth and conidiation in the ΔAniricA mutant to some extent, indicating partial conservation of RicA function in Aspergillus. A series of double mutant analyses reveal that the removal of RgsA (an RGS protein of the GanB Gα subunit), but not sfgA, flbA, rgsB, or rgsC, restores vegetative growth and conidiation in ΔAniricA. We further demonstrate 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 ΔAniricA, 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 accumulate at normal levels in the ΔricA mutant. In addition, OEfluG or OEbrlA fail to restore development in the ΔAniricA mutant. These suggest that the commencement of asexual development requires unknown RicA-mediated signaling input in A. nidulans.
INTRODUCTION

Heterotrimeric G proteins (G proteins) are conserved in all eukaryotes and 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 variety of effectors, relaying the signal into the cells to elicit appropriate physiological and biochemical responses (6).

G proteins are regarded as biological switches that oscillate between ON and OFF states (7). In non-stimulated 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-PKA, phospholipase C, ionic channels, and MAP kinases (18, 45). The signal is turned OFF when Gα-GTP is hydrolyzed back to Gα-GDP by the intrinsic GTPase activity of Gα subunit, forming the inactive trimeric complex.

One key element facilitating the inactivation is the regulator of G-protein signaling (RGS) that 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 evolutionary conserved RIC-8 (Resistance to Inhibitors of Cholinesterase-8) protein is a proven critical guanine nucleotide exchange factor (GEF) activating a subset of Gα subunits (24). Ric-8 interacts with a monomeric Gα-GDP, stimulates the release of GDP, forms a stable nucleotide free transition state complex with 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 showed that FadA signaling is in part transduced via the 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 *flbA*- and constitutively active FadA mutations (G42R, R178C and Q204L; defective intrinsic GTPase) result in 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) 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 in 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 external C-source and enhanced stress response (21). Furthermore, the overexpression of rgsA causes elaboration of asexual developmental structures (conidiophores) in liquid submerged culture as observed in the ΔganB or GanB_{G207R} mutants (10, 21). In A. fumigatus, GpaB (GanB homologue)-mediated signaling 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 have 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 have identified and characterized the Ric-8 ortholog RicA in A. nidulans and A. fumigatus. Functional studies of the ricA gene reveal that it plays a crucial (or essential) role in vegetative growth and development in both species, with partially conserved function. Genetic and biochemical studies further indicate 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 (Ani) and A. fumigatus (Afu) strains used in this study are listed in Table 1. Glucose minimal medium (MMG) and MMG with 0.5% (w/v) 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 + 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 the *alcA* (40, 63) promoter in A. nidulans and A. fumigatus, wild type (WT) and overexpression strains were inoculated on the MMG, MMT + 0.5% YE solid media, and incubated at 37°C for 5 days. Effects of overexpression of the target genes under the *niiA* (4) promoter in A. nidulans were examined by growing the strains in both MM with 0.2% (w/v) ammonium tartrate (MM + AT, non-inducing) and MMG (inducing, containing 0.6% (w/v) sodium nitrate). For Northern blot to confirm overexpression by the *alcA* promoter, strains were cultured in liquid MMG at 37°C, 220 rpm for 12 hours, 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 done by culturing the strains in liquid MMG 16 h at 37°C, 220 rpm. The *Saccharomyces cerevisiae* L40 strain (Clontech) was used to check the protein-protein interaction between RicA fused DNA binding domain and Gα subunits, FadA, GanA and GanB with the activation domain in Y2H. L40 strain was grown in the synthetic dropout minimal medium (SD) with necessary supplements (10 g/l leucine, 2 g/l tryptophan, and 2 g/l histidine) (55), and incubated at 30°C for 2-3 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. Oligonucleotides used in this study are listed in Table S1.

Database analyses, nucleic acid isolation and manipulation: The putative RicA proteins were retrieved from NCBI BlastX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using A. nidulans ricA. Phylogenetic tree of the putative 21 RicA proteins was created by EMBL-EBI, http://www.ebi.ac.uk/Tools/clustalw2/index.html. The construction and analysis of phylogenetic tree was carried out from EMBL-EBI, http://www.ebi.ac.uk/Tools/clustalw2/help.html#tree. The AniricA and AfuricA genes were PCR-amplified from A. nidulans (FGSC4) and A. fumigatus (AF293) genomic DNA. cDNA of AniricA was isolated from A. nidulans cDNA library (provided by K.Y. Jahng, Chonbuk National University, Korea) with the primer pairs oNK-39 and 394. AfuricA cDNA was isolated from reverse-transcriptase treated total RNA using oNK-391 and 392. Genomic DNA and total RNA isolation for Northern blot analysis was carried out as described (38, 72). About 10 g of total RNA isolated from each sample was separated by electrophoresis using the 1.1% (w/v) agarose gel containing 3% (w/v) formaldehyde and ethidium bromide, then transferred onto Hybond-N+ membrane (Amersham). The probes of 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, 557 (AnibrlA), oNK-14, 15 (AnivosA), oNK-594, 595 (AfubrlA), oHS-382, 383 (AfuabaA) and oTL-7, 8 (AfuwetA), respectively (Table S1).

Construction of deletion, complementation and overexpression strains: The ricA deletion (∆ricA) mutants in A. nidulans and A. fumigatus were generated by double-joint PCR (DJ-PCR) as described (72). The flanking regions of each ricA gene were amplified by PCR with primer pair, oNK-352, 353 (Ani5’ with AfupyrG tail), oNK-354, 355 (Ani3’ with AfupyrG tail), oNK-
352, 474 (Ani5’ with AnipyroA tail), oNK-475, 355 (Ani3’ with AfupyroA tail), oNK-358, 359 (Afu5’ with AnipyroA tail), oNK-360, 361 (Afu3’ with AfupyroA tail), oNK-358, 933 (Afu5’ with AnipyrG tail), oNK-358, 361 (Afu3’ with AniargB tail), oNK-934, 361 (Afu5’ with AniargB tail) and oNK-934, 361 (Afu3’ with AniargB tail) from the both genomic DNAs, respectively. The AnipyroA, AnipyroA, AniargB and AfupyroG 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) and 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 293.6 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, 413 (5’ flbA with AnipyroA tail), oNK-414, 415 (3’ flbA with AnipyroA tail), oNK-397, 398 (5’ sfgA with AfupyroA tail), oNK-399, 400 (3’ sfgA with AfupyroA tail), oNK-540, 541 (5’ rgsA with AfupyroA tail), oNK-542, 543 (3’ rgsA with AfupyroA tail), oNK-562, 563 (5’ rgsB with AfupyroA tail), oNK-564, 567 (3’ rgsB with AfupyroA tail), oNK-568, 569 (5’ rgsC with AfupyroA tail) and oNK-603, 604 (3’ rgsC with AfupyroA tail), respectively. The final deletion constructs were amplified with oNK-416, 417 (AniflbA), oNK-401, 402 (AnisfgA), oNK-544, 545 (AnirgsA), oNK-605, 606 (AnirgsB) and oNK-607, 608 (AnirgsC). These deletion mutants were used to generate double deletion mutants with ΔAnirica by subsequent transformation.

To generate the complemented strains, genomic DNA fragments of Anirica and Afurica were PCR-amplified using the primer pairs oNK-870, 871 and oNK-868, 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 ¾ AnipyroA (46) marker and pNJ25 (29)
containing the AfupyrG (13) marker with the alcA promoter (19), FLAG (DYKDDDDK) tag and the trpC terminator (68), respectively. Each construct was introduced into the recipient ΔAniricA and ΔAfuricA strains, where preferentially a single copy is inserted into the AnipyroA or AfupyrG 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, 394 (AniricA) and oNK-391, 392 (AfuricA) 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 the 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 by the percent reduction of AlamarBlue was used as described previously (57). 10^6 conidia of the WT and ΔAfuricA strains were cultured in MMG + 0.1% YE liquid at 37°C for 7 days. Aliquots (0.5 ml) of cultures according to time (day) including the mycelial aggregates and liquid medium were transferred into 24-well plates (Nunc), 1 ml fresh liquid medium containing 150 µl of AlamarBlue® (AbD Serotec) was added to each sample, and then further incubated for 6 h at 37°C. The solution was transferred into 96 well plates excluding mycelial aggregates, and absorbance was read at A_570 and A_600. A percent AlamarBlue reduction was detected by Synergy HT (BIO-TEK) using a KC4™ v3.1 software, and was calculated by a formula, (117216 x A_570 of sample – 80586 x A_600 of sample) / (155677 x A_600 of media – 14652 x A_570 of media) x 100 (39) as described in Shin et al., (57)
Yeast two hybrid assay: ORFs of RicA (oNK521, 394), FadA (oNK-507, 508), GanA (oNK-509, 510) and GanB (oNK-511, 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, Korea) (11) carrying the LexA DNA binding domain (DBD) (8) generated by modifying pHybLex/Zeo (Invitrogen): insertion of the *TRP1* marker from pGBT9 (Clontech). The cDNA derived ORF of *fadA*, *ganA* and *ganB* was each fused under the activation domain of pGAD424 (Clontech), respectively. Plasmids were sequence verified and co-introduced into *S. cerevisiae L40* using the 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) using a yeast β-galactosidase assay kit (Pierce).

GST pull down assay: The *AnriA* ORF was cloned between *EcoRI* and *NolI* into pGEX-5X-1 (GE Healthcare), then introduced into *E. coli* BL21 (DE3) to express the GST-AniRiA fusion protein. *E. coli* strain was grown up to OD$_{600}$ = 0.5–0.6 at 37°C, 220 rpm, and 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside, Sigma) was added to induce the 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 by the lysis buffer three times and resuspended in 500 μl lysis buffer. In addition, ORF of *A. nidulans fadA*, *ganA*, *ganB* and *ricA* was each cloned into pcDNA3 (Invitrogen) to translate *in vitro* by TNT T7 quick coupled transcription/translation system (Promega). One μg of individual plasmids was incubated with 20 μCi of [35S]-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 mixture was 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 (BioRad) and loaded to SDS-PAGE gel. After electrophoresis, the gel was dried under a vacuum to three layers of Whatman 3MM filter paper. Autoradiography was performed at -80°C with the Fuji super RX 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.

**RESULTS**

**Identification of RicA in *A. nidulans* and *A. fumigatus**

Genome search with the *Caenorhabditis elegans* RIC-8 protein (GenBank# AF288812.1) resulted in the identification one putative ortholog in each *Aspergillus* species; Afu4g08820 (*AfRicA*; score = 134, e-value = 2.5e-05) and AN1661 (*AnRicA*; score = 131, e-value = 6.5e-05). To verify its ORF, we isolated and analyzed the *ricA* cDNA from the *A. fumigatus* and *A. nidulans*
cells, and our new *AniRicA* and *AfuRicA* sequences and annotations have been deposited in Genbank with the accession numbers JN410838 and JN582330, respectively. Briefly, the *AniRicA* protein is composed of 466 aa (ORF = 1401 bp with 6 introns) and *AfuRicA* is composed of 461 aa (ORF=1386 bp with 6 introns) with a predicted mass 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 alignment (Fig. S1) and phylogenetic analyses (Fig. 1B).

The RIC8 ortholog is absent in the genomes of *S. cerevisiae* and plants (65). As presented, *A. nidulans* RicA is close to that of *A. niger*, *A. oryzae*, and *A. flavus*, while *A. fumigatus* RicA is close to *A. clavatus* RicA. To characterize the *ricA* gene, levels of *ricA* mRNA at different time points in the lifecycle of the two species was examined. As shown in Fig. 1C, in both species, the *ricA* transcript (each ~2.5 kb) is detectable in a somewhat undulant manner during growth and developmental phases, but not in conidia. It appears that *AniricA* mRNA is present at low levels in sexual spores (ascospores; AS in Fig. 1C).

**Characterization of ricA in A. fumigatus**

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

Aspergillus conidiophore formation requires sequential activities of the central regulatory components brlA → abaA → wetA (2, 61, 70). As shown in Fig. 2A (middle and bottom panels), the ΔAfuricA mutant produced abnormal conidiophores (marked by black arrow heads) showing improper septation in stalk and incomplete formation of phialides and conidia. As shown with Northern blot analysis, the ΔAfuricA mutant exhibited severely delayed and reduced levels of AfubrlA, AfuabaA and AfuwetA mRNA during the progression of conidiation (Fig. 2C). In WT, AfubrlA 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 AfuabaA and AfuwetA transcripts followed AfubrlA mRNA accumulation. However, in the ΔAfuricA mutant, AfubrlA mRNA accumulated at low levels at 0 h (= vegetative growth 16 h), gradually increased until 72 h, i.e., reduced, delayed and uncontrolled accumulation of AfubrlA. Moreover, transcripts of AfuabaA and AfuwetA accumulated at low levels even at 48 and 72 h post developmental induction. These results indicate that AfuRicA is necessary for proper expression and regulation of key developmental regulators that coordinate formation, integrity and vitality of spores.
Finally, we tested whether the deletion of *AfuricA* affects cell death and autolysis in *A. fumigatus* by AlamarBlue (AB) reduction (57) and dry weight assays, respectively. As shown in Fig. 2D-E, the absence of *AfuRicA* resulted in delayed cell death and autolysis. Whereas both WT and complemented strains exhibited reduced AB reduction rates at day 4 and only 20% at day 7, the ∆*AfuricA* mutant exhibited 100% AB reduction at day 5, a decreased rate from day 6 (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 ∆*AfuricA* mutant showed peak dry weight at day 5 and gradually reduced weight at day 6 and 7 (Fig. 2E). These suggest that *AfuRicA* is necessary for proper vegetative proliferation and normal progression of cell death and autolysis in *A. fumigatus*.

### Characterization of *AniricA* and cross-species complementation

The deletion of *AniricA* resulted in severely impaired growth and the complete lack of asexual and sexual development in *A. nidulans*. As clearly noticeable from the colonies of WT (TNJ36), ∆*AniricA* (TNJ21) and complemented (TNJ87) strains grown on solid MMG 37°C for 5 days, the ∆*AniricA* mutant formed a very small colony (~30% of 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 ∆*AniricA* mutant were done by collecting and inoculating hyphal fragments from a high number of air-exposed mutant colonies. In submerged shake culture following inoculation of the ∆*AniricA* aerial hyphae, the ∆*AniricA* mutant exhibited extremely slow hyphal proliferation compared to WT, and the size of ∆*AniricA* 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 AfuricA WT allele could restore growth and development in the ΔAniricA mutant. We found that the presence of a genomic fragment of AfuricA including its own promoter, coding region and terminator was not sufficient to restore growth and development in the ΔAniricA mutant (data not shown). However, as shown in Fig. 3B, the overexpression of AfuricA under the control of the AnialcA promoter partially enhanced growth, and fully restored conidiation in the ΔAniricA mutant under the inducing condition (MMT + 0.5% YE). Supplementation with 0.6 M KCl enhanced the developmental restoration by AfuricA in the ΔAniricA mutant grown on MMT + 0.5% YE and even on MMG (non-inducing; Fig. 3B bottom +KCl). These results indicate that AniRicA plays a crucial and essential role for growth and development in A. nidulans, and AfuRicA can partially replace AniRicA.

Suppression of ΔricA by ΔrgsA in A. nidulans

If RicA is a 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 ΔricA ΔflbA and ΔricA ΔsfgA double mutants exhibited the phenotypes identical to the ΔricA single mutant. These indicate that the removal of the key negative regulator of growth (FlbA) or conidiation (SfgA) could not alleviate the growth and developmental defects caused by ΔricA, and that RicA functions either upstream
or independently of the FlbA/FadA growth and FluG/SfgA developmental control pathways. We then asked whether RicA is associated with activating other G protein pathways. RgsA is a RGS protein that inhibits GanB-mediated signaling for germination/developmental control and stress response (10, 21). RgsB and RgsC are putative RGS proteins remain to be characterized (21). As shown in Fig. 4B, while the deletion of rgsB or rgsC failed to suppress ΔricA, the absence of rgsA restored growth and development to the ΔrgsA level in the ΔricA mutant. Furthermore, as found in the ΔrgsA mutant (21), the ΔricA ΔrgsA 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).

Physical interaction between RicA and GanB

To further test the hypothesis that the primary target of AniRicA is AniGanB, we first tested the physical interaction between AniRicA and individual Gα subunits employing yeast two hybrid (Y2H) assay. The AniricA ORF PCR fragment derived from cDNA was fused with the LexA DNA binding domain (LexA DBD) in the pTLexA vector (11) and each Gα ORF, AniFadA, AniGana or AniGanB 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 the β-galactosidase reporter. As shown in Fig. 5A, only the AniRicA-AniGanB pair exhibited blue color on the X-gal SD medium lacking uracil, tryptophan and leucine. Quantification of the β-galactosidase activity of each pair in yeast using o-nitrophenyl-galactosidase (ONPG) further demonstrated that only the AniRicA-AniGanB pair resulted in high levels of reporter expression in yeast (Fig. 5B), i.e., about 90% of that of the well-known
transcriptional activator *Ani*AflR (positive control) (44, 71). To map the critical interacting domains, we further tested physical interaction between the truncated *Ani*RicA (aa 1-398 or 51-466 of the full length 466 aa) and *Ani*GanB (aa 1-325 or 35-356 of the full length 356 aa), and found that the full length *Ani*RicA and *Ani*GanB are necessary for the interaction in yeast (data not shown). The physical interaction of *Ani*RicA and *Ani*GanB was further tested *in vitro* by employing the GST-pull down assay (Fig. 5C). The *AniricA* ORF was fused with GST in the pGEX 5X-1 vector (GE Healthcare), and the *Ani*RicA protein was expressed and purified in *Escherichia coli*. The ORF region of *AnifadA*, *AniganA* or *AniganB* was cloned under the T7 promoter of pCDNA3, and each Gα subunit was translated *in vitro* and labeled by S35. An equal amount of *in vitro* translated proteins was added to Glutathione bead-GST-*Ani*RicA or Glutathione bead-GST (control) suspensions and subjected to pull-down. As shown in Fig. 5C, S35 labeled *Ani*GanB, but not *Ani*FadA or *Ani*GanA, could be co-purified with GST-*Ani*RicA specifically, indicating that *Ani*RicA directly binds to *Ani*GanB *in vitro*. Collectively, these data suggest that GanB is a primary target of RicA-mediated signaling in *A. nidulans*, and the full length *Ani*RicA and *Ani*GanB are necessary for their physical interaction.

### 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 is proven to be a primary target of RicA, we asked whether the elevated expression of *pkaA* could restore growth and development in the *ΔricA* deletion mutant. While supplementation of exogenous (up to 10 mM) cAMP and dibutyryl cAMP failed to restore (data not shown), two (one native and an ectopic) copies or the overexpression of *pkaA* was sufficient to restore growth and development in the *ΔricA* mutant. As shown in Fig. 6, the *niiA(p)::pkaA*
single and ΔricA niiA(p)::pkaA double mutants exhibited the identical phenotypes on solid medium with 0.2% ammonium tartrate (non-inducing) and 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 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 ΔricA is due to the defective expression of the key regulators *brlA* (1) and *vosA* (44). The mycelial mats of WT, ΔAniricA, niiA(p):ricA (OEricA), ΔflbA, ΔflbA ΔricA, ΔsfgA, and ΔsfgA ΔricA 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 ΔricA 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 ΔricA 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 (OE) *fluG* and *brlA* in the absence of *ricA*. Neither OEfluG nor OEbrlA could restore conidiation in the ΔricA mutant on solid medium (Fig. 7B). Moreover, while OEbrlA ricA+ caused the formation of conidia at the hyphal tip in liquid submerged culture, the OEbrlA ΔricA 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 \textit{fluG} \rightarrow \textit{brlA} pathway, is necessary for conidiophore development in \textit{A. nidulans} (see Fig. 8 and Discussion).

\section*{DISCUSSION}

During last 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; (24). Ric-8 is a cytoplasmic protein initially identified by a genetic screening of the \textit{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 G\textalpha subunits (27, 60). Later, it was revealed that RIC-8 is involved in the asymmetric division of \textit{C. elegans} embryos (3, 12, 42) and \textit{Drosophila melanogaster} neuroblasts (14, 20, 62). The RIC-8 orthologs are present in genomes of animals and filamentous fungi, but not in the baker's yeast and plants. Whereas \textit{C. elegans} and \textit{Drosophila} genome contains 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 G\textalpha subunit associated with G\beta\gamma, i.e., the inactive heterotrimeric complex. Thus, Ric-8 proteins can only function on dissociated (free) monomeric G\textalpha-GDP (see 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 study, we present the experimental evidence that the Ric-8 ortholog (RicA) plays a crucial role in governing vegetative growth and development in two \textit{Aspergillus} species. The lack of
AniRicA function results in the profound defects in hyphal proliferation and asexual/sexual fruiting. Particularly, the AniricA null mutants were severely impaired in hyphal growth and unable to form conidia or ascospores in A. nidulans. Thus, the ΔAniricA cultures were derived from the mutant hyphal fragments and all double mutants were generated by deleting AniricA from individual single mutants through transformation. Likewise, the ΔAfuricA mutant produces defective conidia many of which are unable to germinate, making it extremely difficult to study the 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 AfuRicA only partially restored growth and development in A. nidulans. We show here that RicA interacts with G alpha 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. described that the deletion of ganB, but not fadA or ganA, suppressed the developmental and metabolic defects caused by ΔrgsA, and concluded that RgsA negatively regulates GanB-mediated signaling in A. nidulans (21). We show that RicA physically interacts with GanB, and only ΔrgsA suppresses the defective growth and development phenotypes caused by ΔricA, and the ΔrgsA ΔricA double mutant exhibits the phenotype identical to the ΔrgsA single mutant (Fig. 4B & C). All these suggest that RicA-mediated signaling is transduced primarily via GanB in A. nidulans (Fig. 8).

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 (Nc) ric8 deletion mutant shows severe defects in growth and development. The phenotypes caused by Nric8 are similar to those observed in the mutant lacking the Gα genes gna-1 and gna-3. Moreover, Δ Nric8 results in greatly reduced levels of all three Gα and one Gβ...
(GNB-1) subunits (66). Further studies have revealed that NcRIC8 positively regulates of GNA-1 and GNA-3, and physically interact with and acts as a GEF for GNA-1 (equivalent to AniFadA) and GNA-3 (AniGanB) in vitro, with the strongest effect on GNA-3. The rice blast fungus *M. oryzae* RIC8 (*MoRIC8*) is known to be a novel component of G protein signaling during the infection into plants (35). The deletion of *MoRIC8* results in non-pathogenicity and impairment in cellular differentiation associated with sporulation, sexual development, and plant infection. MoRic8 is highly expressed in the appressorium, a specialized plant tissue invading structure, and physically interacts with MagB (AniFadA) but not MagA (AniGanB) in the yeast two-hybrid system. Collectively, these indicate that RIC8/RicA plays a crucial role in upstream activation of various signaling pathways in filamentous fungi and that the primary targets of RicA can 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*, ΔNcric8 results in low levels of adenylyl cyclase protein. Moreover, ΔNcric8 can be suppressed by a mutation in the PKA regulatory subunit (66). *MoRic8* is thought to act upstream of the cyclic AMP response pathway that is necessary for appressorium morphogenesis. In accordance with these, we have shown that two copy and/or overexpression of the primary catalytic subunit of 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 *MoRIC8* causes the down-regulation of Gα subunits, *MAGA, MAGB* and *MAGC* as well as Gγ(*MGG1*) subunit but not Gβ subunit (*MGB1*) (35). Taken together, these suggest that maintenance of normal levels of G proteins and adenylyl cyclase (and perhaps other yet-unknown regulatory components) is an important conserved...
function of the RIC-8 protein (66) and that RicA-mediated signaling is transduced primarily through GanB→PkaA in Aspergillus (Fig. 8).

While the absence of RicA function essentially abolished conidiation in both species, mRNA of the key developmental activator brlA accumulates to some extent in both A. nidulans and A. fumigatus. Moreover, while abaA and wetA mRNA levels were very low in A. fumigatus ricA null mutant, AnivosA mRNA accumulates 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 (see Fig. 8). As a possible explanation, we propose that RicA mediated activation of GanB and PkaA singling is necessary for the acquisition of developmental competence (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 (2 and references therein), supporting the hypothesis that the early aspect of Aspergillus conidiophore development occurs as an integral part of the lifecycle 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 GanB→PkaA (Fig 8). In a previous model, it was proposed that GanB and PkaA signaling inhibit expression of brlA (10, 56). We further speculate that GanB and PkaA play a role in down-
regulating brlA expression in order to prevent precocious conidiation and to confer vegetative
growth for a certain period of time during the lifecycle 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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^aFungal Genetics Stock Center.
The pyroA marker in pHS causes the targeted integration at the pyroA locus.
The AfupyrG marker in pNJ25 causes the targeted integration at the pyrG locus.
FIGURE LEGENDS

FIG. 1. Summary of the ricA genes

(A) Proposed Ric-8 mediated G protein signaling. (B) A 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. triticum, Phaeosphaeria nodorum, Sclerotinia sclerotiorum, G. zeae, Botryotinia fuckeliana, Podospora anserine and Chaetomium globosum). (C) mRNA levels of ricA during the lifecycles of A. fumigatus and A. nidulans. C and AS indicate conidia (asexual spores) and ascospores (sexual spores in A. nidulans), respectively. Numbers indicate the time (hours) of incubation in liquid MMG or MMG + 0.1% YE (Vegetative) or after transfer onto solid MMG glucose under the conditions favoring asexual development (Asexual) or sexual development (Sexual). Equal loading of total RNA was evaluated by the ethidium bromide (EtBr) staining of rRNA.

FIG. 2. Phenotypes caused by ΔAfuricA

(A) Wild type (WT; AF293), ΔAfuricA (FNJ12) and complemented (C’ AfuricA; FNJ13) strains were point inoculated on solid MMG + 0.1% YE, and incubated at 37°C for 5 d. Photographs of colony size and close-up views of the center of the colony and conidiophores are shown. Black arrows indicate abnormal conidiophores at the center of the ΔAfuricA colony. (B) Germination of WT (AF293) and ΔAfuricA (FNJ12) conidia inoculated on MMG + 0.1% YE plates and incubated for 6 and 12 h. Black arrows in ΔAfuricA indicate germinated conidia at 12 h. (C) Accumulation of brlA, abaA, wetA and γ-actin mRNA post asexual developmental induction of WT (AF293) and ΔAfuricA (FNJ12) strains. Development 0 h indicates vegetative growth in...
MMG + 0.1% YE liquid for 18 h. The predicted \textit{A. fumigatus} \(\gamma\)-actin gene (Afu6g04740) (29) was identified by \textit{A. nidulans} \(\gamma\)-actin (16). (D) AlamarBlue reduction data indicating relative cell death rates. The mycelial aggregates of WT, \(\Delta AfuricA\) and complemented strains were mixed with the AlamarBlue (AB) reagent to check the cell viability for 7 days. (E) Dry weights of WT, \(\Delta AfuricA\) and complemented strain in MMG + 0.1% YE submerged culture were quantified for 7 days at 37°C, 220 rpm.

**FIG. 3. Requirement of RicA in \textit{A. nidulans} growth and development**

(A) \textit{A. nidulans} WT (TNJ36), \(\Delta AniricA\) (TNJ21) and complemented (C' \textit{AniricA}; TNJ87) strains were inoculated on MMG and cultured for 5 days at 37°C. Colonies and conidiophores of the 5 day old culture solid MMG were observed under a stereo-microscope. (B) Partial complementation by \textit{AfuricA} overexpression in \(\Delta AniricA\). WT (TNJ36), OE\textit{AfuricA} (TNJ94), \(\Delta AniricA\) (TNJ21) and OE\textit{AfuricA} \(\Delta AniricA\) (TNJ95; OE in \(\Delta\)) were point inoculated on solid MMG (non-inducing), MMT + 0.5% YE (inducing by the \textit{alcA} promoter), MMG without KCl and MMT + 0.5% YE with 0.6 M KCl, and incubated for 3 days 37°C. Bar = 200 \(\mu\)m.

**FIG. 4. Double mutant analyses in \textit{A. nidulans}**

(A) WT (TNJ36), \(\Delta AniricA\) (TNJ21), \(\Delta AnisfgA\) (TNJ57), \(\Delta AnisfgA \Delta AniricA\) (TNJ58) \(\Delta AniflbA\) (TNJ42) and \(\Delta AniflbA \Delta AniricA\) (TNJ49) strains grown on solid MMG for 5 d at 37°C. (B) WT (TNJ36), \(\Delta ricA\) (TNJ21), \(\Delta rgsA\) (TNJ61), \(\Delta ricA \Delta rgsA\) (TNJ62), \(\Delta rgsB\) (TNJ63), \(\Delta ricA \Delta rgsB\) (TNJ64), \(\Delta rgsC\) (TNJ65), \(\Delta ricA \Delta rgsC\) (TNJ66) were inoculated on MMG, and incubated at 37°C for 5 days. (C) Germination of WT (TNJ36), \(\Delta rgsA\) (TNJ61) and \(\Delta ricA \Delta rgsA\) (TNJ62)
conidia in the absence of an external carbon source. Photographs were taken after inoculating 1 X $10^6$ conidia into liquid MM without an external C source, and cultured for 16 h at 37°C and 220 rpm. Bar = 25 µm.

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 AniRicA protein and the Gal4 activation domain fused Gα proteins, AniFadA, AniGanA and AniGanB on the X-Gal medium without uracil, tryptophan and leucine (-UWL/X-Gal). (B) Quantitative analyses of β-galactosidase activities using ONPG in yeast strains including the positive (AflR) (71) and negative (pTLex vector; LexA DNA binding domain alone) controls shown left. (C) GST pull down assay for GST-AniRicA and in vitro translated $^{35}$S-Gα proteins. The right lane of each panel shows the in vitro translated FadA, GanA and GanB protein, respectively (10 µl from 50 µl translation reaction volume). The in vitro translated proteins were divided into two parts (each 20 µl) and mixed with the GST-AniRicA protein (left lane) or GST alone (middle lane). The expected protein size of FadA, GanA and GanB is about 39, 40 and 39 kDa, respectively.

FIG. 6. Suppression of ΔAniricA by an ectopic copy or overexpression of pkaA

Colony (top) photographs and close-up views (bottom) of A. nidulans WT (TNJ36), ΔAniricA (TNJ21), OEpkaA (TNJ89) and OEpkaA ΔAniricA (TNJ90) strains are shown. These strains were point inoculated on non-inducing (NI; MMG containing 0.2% ammonium tartrate as a nitrogen source) and inducing (I; MMG containing 0.6% sodium nitrate as a nitrogen source) media, and incubated for 5 days at 37°C.
FIG. 7. Requirement of AniricA in conidiation independent of fluG and brlA

(A) mRNA levels of brlA and vosA in WT (TNJ36), ΔricA (TNJ21), OEricA (TNJ88), ΔflbA; ΔricA (TNJ49) and ΔsfgA ΔricA (TNJ58) cells collected from MMG liquid surface culture. Note the strains including ΔricA were cultured for 3 days on the surface of liquid MMG to obtain equal amount of the hyphal aggregates. (B) WT, ΔricA (TNJ21), OEfluG (TNJ59), OEfluG ΔricA (TNJ60), OEbrlA (TNJ85) and OEbrlA ΔricA (TNJ86) strains were point inoculated on non-inducing (NI; MMG with 0.2% ammonium tartrate) and inducing (I; MMG with 0.6% sodium nitrate) solid media, and incubated at 37°C for 5 days.

FIG. 8. Model for RicA governing A. nidulans growth and development (see 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/BrI, is indicated. It is further speculated that brlA is down-regulated by GanB and PkaA during early phases of vegetative growth in A. nidulans.