**ABSTRACT** Heterotrimeric (αβγ) G proteins are crucial components of eukaryotic signal transduction pathways. G-protein-coupled receptors (GPCRs) act as guanine nucleotide exchange factors (GEFs) for Gα subunits. Recently, facilitated GDP/GTP exchange by non-GPCR GEFs, such as RIC8, has emerged as an important mechanism for Gα regulation in animals. RIC8 is present in animals and filamentous fungi, such as the model eukaryote *Neurospora crassa*, but is absent from the genomes of baker’s yeast and plants. In *Neurospora*, deletion of *ric8* leads to profound defects in growth and asexual and sexual development, similar to those observed for a mutant lacking the Gα genes *gna-1* and *gna-3*. In addition, constitutively activated alleles of *gna-1* and *gna-3* rescue many defects of Δ*ric8* mutants. Similar to reports in *Drosophila*, *Neurospora Δric8* strains have greatly reduced levels of G-protein subunits. Effects on cAMP signaling are suggested by low levels of adenyl cyclase protein in Δ*ric8* mutants and suppression of Δ*ric8* by a mutation in the protein kinase A regulatory subunit. RIC8 acts as a GEF for GNA-1 and GNA-3 *in vitro*, with the strongest effect on GNA-3. Our results support a role for RIC8 in regulating GNA-1 and GNA-3 in *Neurospora*.

**UKARYOTIC** cells sense many hormones, growth factors, neurotransmitters, and the presence of light via G-protein-signaling pathways. The G-protein heterotrimer consists of a Gα subunit, which binds and hydrolyzes GTP, and of tightly associated Gβ and Gγ subunits. G proteins interact with seven-transmembrane helix G-protein-coupled receptors (GPCRs) to regulate downstream signaling pathways (reviewed in Neves et al. 2002 and Wilkie and Kinch 2005). GDP-bound Gα subunits are associated with the Gβγ dimer and the GPCR (Wilkie and Kinch 2005). Ligand binding to the receptor leads to exchange of GDP for GTP on the Gα, leading to dissociation of the heterotrimer into Gα-GTP and Gβγ units, which can both interact with effector proteins to generate changes in cellular physiology (Neves et al. 2002). GPCRs thus act as guanine nucleotide-exchange factors (GEFs) for heterotrimeric Gα proteins. The activation cycle is terminated by hydrolysis of GTP to GDP by the Gα subunit and reassociation with Gβγ and the GPCR.

Recently a non-GPCR protein, RIC8, has been implicated as a positive regulator of Gα proteins in several animal species, including *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammalian cells (reviewed in Wilkie and Kinch 2005). Invertebrates such as *C. elegans* and *Drosophila* contain one RIC8 protein, while vertebrates contain two homologs, Ric-8A and Ric-8B (Tall et al. 2003). RIC8 is required for asymmetric cell division in zygotes and priming of synaptic vesicles in *C. elegans* (Miller and Rand 2000; Wilkie and Kinch 2005). In *Drosophila*, RIC8 is essential for responses to extracellular ligands and for maintenance of polarity during asymmetric cell division in embryogenesis (Hampoelz et al. 2005). Furthermore, RIC8 is also required for stability of a Gα and Gβ protein in *Drosophila* (Hampoelz et al. 2005). Of the two mammalian RIC8 proteins, only Ric-8A has demonstrated GEF activity toward the Gα proteins GαIP, Gαi1, Gαo, and Gα13 (Tall et al. 2003). Ric-8A binds the
GDP-bound Gα in the absence of Gβγ, and GDP is then released, forming a stable Ric-8A:Gα complex (Tall et al. 2003). Subsequently, GTP binds the Gα protein and Ric-8A is released. Similar to Drosophila RIC8, Ric-8B regulates the stability of a Gα subunit in mammalian cells (Nagai et al. 2010).

Neurospora crassa possesses three Gα subunits (GNA-1, GNA-2, and GNA-3), one Gβ (GNB-1), one Gγ (GNG-1), and >25 putative GPCRs (reviewed in Li et al. 2007). We have previously demonstrated that loss of gna-1 and gna-3 or of all three Gα genes leads to a severe decrease in extension of both basal and aerial hyphae. The relatively subtle phenotypes of GPCR mutants in comparison to strains lacking one or more Gα genes (Kim and Borkovich 2004; Krystofova and Borkovich 2006; Li and Borkovich 2006; Bieszke et al. 2007) prompted us to explore the possibility of additional non-GPCR regulators of G proteins in Neurospora. It was recently reported that a single protein homologous to RIC8 is present in Neurospora and other filamentous fungi, but is absent from the genomes of Saccharomyces cerevisiae and plants (Wilkie and Kinch 2005). It has been hypothesized that RIC8 was acquired by animals and certain fungi after the evolution of G proteins and GPCRs and is therefore a fairly recent addition to G-protein regulatory pathways (Wilkie and Kinch 2005). The only other study of a RIC8 homolog in filamentous fungi was reported very recently in the fungal rice pathogen Magnaporthe oryzae (Li et al. 2010). MoRic8 was shown to bind to the Gα subunit MagB in the yeast two-hybrid assay and is highly expressed in the appressorium, a specialized structure that invades plant tissue during pathogenesis. MoRic8 was also found to act upstream of the cAMP pathway, which regulates appressorium formation. However, this study did not investigate possible GEF activity for MoRic8.

Here we provide evidence that RIC8 is a Gα GEF that regulates growth and development in Neurospora. Loss of ric8 leads to several phenotypes also displayed by strains lacking the Gα genes gna-1 and gna-3, including extremely impaired growth, short aerial hyphae, inappropriate conidiation in submerged culture, and loss of female fertility. We also demonstrate that all three Neurospora Gα proteins bind GTP and that RIC8 interacts with and has GEF activity toward GNA-1 and GNA-3 in N. crassa.

**Materials and Methods**

**Strains and growth conditions**

A list of Neurospora strains is provided in Table 1. Strains were maintained on Vogel’s minimal medium (VM) (Vogel 1964). Synthetic crossing medium was used to induce formation of female reproductive structures (protophertia) (Westergaard and Mitchell 1947). All submerged liquid and VM plate cultures were inoculated with conidia and grown as described previously (Krystofova and Borkovich 2006; Li and Borkovich 2006). Where indicated, ampicillin, histidine, and inositol were used at 100 μg/ml, hygromycin at 200 μg/ml, and phosphinothricin at 400 μg/ml.

**Identification and characterization of the ric8 gene**

The existence of a ric8 homologue in fungi was suggested by Wilkie and Kinch (2005). Homology searches (BLAST) (Cookson et al. 1997) of the N. crassa genome database at the Broad Institute (http://www.broad.mit.edu/annotation/fungi/neurospora_crasa_7/index.html) (Galagan et al. 2003) using the Xenopus laevis Ric8 protein sequence (accession no. NP_989159) revealed that the Neurospora RIC8 homolog corresponds to NCU02788. A ric8 subclone, pSM1, was generated by digesting cosmid pMOcosX G19 C10 with EcoRI, releasing a 12.6-kb fragment (corresponding to contiguous 7.7, nucleotides 24,473–37,042) that was subcloned into pGEM4 (Promega, Madison, WI). The clone was sequenced at the Institute for Integrative Genome Biology, Core Sequencing Facility, University of California, Riverside using primers T7 and SP6.

**Strain construction**

A transformant containing the ric8 deletion mutation was obtained from the Neurospora genome project (Colot et al. 2006). This Δric8 strain was crossed as a male to wild-type strain 74A (female), and progeny were plated on VM plates containing hygromycin to select for Δric8::hph+ strains. The Δric8 his-3 (R8H3) strain was isolated in a similar manner by crossing a his-3 strain [Fungal Genetics Stock Center (FGSC) 6103] as a male to the Δric8 heterokaryon (female) with selection on VM–hygromycin plates containing histidine. The presence of the Δric8 mutation in progeny was verified by growth on hygromycin and Southern analysis, and his-3 auxotrophs were identified by spot testing on VM medium lacking histidine.

To complement the Δric8 mutation in trans, the ric8 open reading frame (ORF) was cloned into vector pMF272 (Folco et al. 2003; Freitag et al. 2004). pMF272 contains the ccg-1 promoter and allows expression of an ORF with a 3′ GFP fusion. The ric8 ORF (including introns) was amplified from pSM1 using primers designed to add XbaI (5′ end, R8 GFP fw) and BamHI (3′ end, R8 GFP rv) sites to the ric8 gene (supporting information, Table S1). The fragment was ligated into pMF272 digested with BamHI and EcoRI, yielding plasmid pSM2. This vector was used to target the ric8-gfp fusion to the his-3 locus of the R8H3 strain (R8GFP, Table 1) (Aramayo et al. 1996). Transformants were plated on VM–hygromycin medium, and isolates were analyzed for integration of ric8-gfp at the his-3 locus using Southern analysis. Transformants with the ric8-gfp Southern pattern were purified by three rounds of colony isolation from VM–hygromycin plates.

Predicted GTpase-deficient, constitutively activated alleles of gna-1 (Q204L) (Yang and Borkovich 1999), gna-2 (Q205L) (Baasiri et al. 1997), and gna-3 (Q208L) were introduced into the Δric8 background to determine epistatic relationships between ric8 and the three Gα genes. These
vectors will be described elsewhere (S. Krystofova, S. Won, and K. Borkovich, unpublished results). Briefly, the ORFs corresponding to the three Ga alleles were subcloned into vector pMF272, with eviction of the GFP gene, producing plasmids pSVK51 (gna-1::T204L), pSVK52 (gna-2::T205L), and pSVK53 (gna-3::Q208L). These plasmids were targeted to the his-3 locus in R8H3 as described above, generating strains R81*, R82*, and R83*. Correct integration at the his-3 locus was verified by Southern analysis.

To construct a strain containing both gna-1::T204L and gna-3::Q208L alleles in the Δric8 background, the insert from plasmid pSVK51 (carrying gna-1::T204L) was introduced into a vector that would allow ectopic integration and confer resistance to phosphinothricin (Pall 1993). A 2.2-kb fragment from pSVK51 containing the ccr-1 promoter and gna-1::T204L allele was released using NotI and EcoRI. This fragment was cloned into pBARGEM7-2, which contains the bar+ gene and confers phosphinothricin resistance. The resulting vector, pSM3, was transformed into the R83* strain using electroporation. Transformants were plated on sorbose medium containing hygromycin and phosphinothricin, and colonies were analyzed for the ectopic integration of gna-1::T204L using Southern analysis.

The Δric8 mcb double mutant was generated from a cross with R8 a+1 (Δric8::hph + a+1 heterokaryon, Table S1) as a female and mcb inl as a male. Strain a+1 is a mating-type mutant that allows crossing of female-sterile strains, but does not passage its nuclei in a cross (Griffiths 1982). Since mcb is tightly linked to inl, inositol auxotrophy can be used to score the presence of the mcb mutation. Progeny were plated on VM medium containing hygromycin and inositol to isolate Δric8 mcb inl mutants (R8 mcb, Table 1).

### RT-PCR and Northern and Western analysis

There are two predicted introns in ric8 (see gene structure in Figure S1A). The presence of the 3’ predicted intron was confirmed by the sequence of a cDNA clone (NC006937; Broad Institute). The 5’ predicted intron and ric8 gene deletion were verified by semiquantitative RT-PCR (Kays and Borkovich 2004). Expression of ric8 was analyzed using 2 μg total RNA extracted from conidia (Puregene RNA isolation kit, Gentra Systems, Minneapolis) (Krystofova and Borkovich 2006). RT-PCR analysis was performed using the Access RT-PCR system (Promega) with gene specific primers (R81f and R81rv) designed to flank the 5’ intron (Table S1). Each reaction included 2 μg DNAase-treated RNA and both AMV reverse transcriptase and Taq polymerase (Promega). The genomic control contained the same primers with plasmid pSM2 (genomic fragment) as the template. The RT reaction was incubated at 48°C for 45 min and at 94°C for 2 min. The PCR thermocycling conditions were the following: 35 cycles at 94°C for 30 sec, at 50°C for 1 min and at 68°C for 90 sec with a final elongation step at 68°C for 7 min. The reactions were electrophoresed on an agarose gel, blotted onto a nylon membrane, and subjected to Southern analysis as described (Krystofova and Borkovich 2005), using the radiolabeled 410-bp fragment from the genomic control PCR as the probe. A control reaction for RNA quality was also run using 18S rRNA-specific primers as previously described (Bieszke et al. 2007).

For Northern analysis, total RNA was isolated and samples containing 20 μg were analyzed as described (Krystofova and Borkovich 2005). Probes for Northern detection of

### Table 1 Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Comments</th>
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</tr>
</thead>
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<td>FGSC</td>
</tr>
<tr>
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<td>Δric8 mutant</td>
<td>This study</td>
</tr>
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<td>This study</td>
</tr>
<tr>
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<td>Δric8::hph + his-3 matA</td>
<td>Δric8 his-3 strain</td>
<td>This study</td>
</tr>
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<td>R8H3 + a+1 heterokaryon</td>
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<td>Δgna-1 Δgna-2 Δgna-3 triple mutant</td>
<td>Kays and Borkovich (2004)</td>
</tr>
</tbody>
</table>

FGSC, Fungal Genetics Stock Center (Kansas City, MO).
* "X" denotes a genetic cross.
gna-1, gna-2, gna-3, and gnb-1 were generated as described previously (Krystofova and Borkovich 2005).

For Western analysis, the particulate fraction (GNA-1, GNA-2, GNA-3, and GNB-1) or whole-cell extracts (CR-1, R8GFP) were isolated as described previously (Ivey et al. 2006; Li and Borkovich 2006). Samples containing 100 μg of total protein were subjected to Western analysis as described (Yang et al. 2002; Krystofova and Borkovich 2005). The primary polyclonal antiserum for GNA-1, GNA-2, and GNB-1 were used at dilutions of 1:1000, while GNA-3 and CR-1 were diluted 1:750 and 1:5000, respectively. The primary polyclonal antibody against GFP (Abcam, Cambridge, MA) was used at a dilution of 1:2000. A horseradish peroxidase conjugate (Bio-Rad) was used as the secondary antibody at a dilution of 1:4000 (GNA-1 and GNA-2), 1:2000 (GNA-3), or 1:10,000 (CR-1), and chemiluminescent detection was performed as described previously (Krystofova and Borkovich 2005).

**Phenotypic analysis and microscopy**

Plate cultures were imaged by scanning. Intracellular localization of the RIC8 protein in conidia and vegetative hyphae was achieved by microscopic observation of GFP fluorescence from a Δric8 strain carrying a RIC8–GFP fusion protein at the his-3 locus (see **Strain construction**). Conidia were harvested as described (Krystofova and Borkovich 2006; Li and Borkovich 2006) and imaged directly or used to inoculate 30-ml liquid cultures at a concentration of 1 × 10⁶ cell/ml, followed by incubation for 16 hr at 30°C with centrifugation at 200 × g (for vegetative hyphae). Conidia and vegetative hyphae were viewed using differential interference contrast (DIC) and GFP fluorescence microscopy using an Olympus IX71 inverted microscope with a ×60 oil immersion objective (numerical aperture = 1.42). Images were captured using a QIClick digital CCD camera (Qimaging) and analyzed using Metamorph software (Molecular Devices).

**Yeast two-hybrid assay**

A ric8 ORF clone lacking introns (pSM6) was constructed using homologous recombination of PCR fragments in *S. cerevisiae* (Colot et al. 2006). All DNA fragments for cDNA were amplified by PCR using Pfu turbo DNA polymerase (Stratagene) according to the manufacturer’s protocols. To remove the first intron, the 5’ UTR up to the first intron was amplified (574-bp product; primers R8YR11fw and R8YR11rv; Table S1) as well as the first part of the second exon (756-bp product; primers R8YR12fw and R8YR12rv), using pSM1 as a template. The second intron was removed by amplifying the last part of the second exon (727-bp product; R8YR21fw and R8YR21rv) and the third exon and 3’ UTR (579 bp; R8YR22fw and R8YR22rv, Table S1).

The two sets of fragments were transformed separately into *S. cerevisiae* strain FY834 (Winston et al. 1995; Colot et al. 2006) along with vector pRS416 (Christianson et al. 1992; Gera et al. 2002) gapped with *XbaI* and *EcoRI*, with selection on SC medium lacking uracil. DNA was extracted from transformants, and plasmids were recovered by electroporation into *Escherichia coli* and plating on LB + Amp and sequenced.

PCR was used to amplify the ric8 cDNA and introduce restriction sites for yeast two-hybrid cloning. The 5’ fragment was amplified with primers R8YR2Hfw and R8YR12rv (744 bp), introducing the EcoRI restriction site 5’ to the initiator ATG and extending to include the restriction site *XhoI*. The 3’ fragment was amplified with primers R8YR21fw and R8YR2Hrv (836 bp), which include the Xhol site, and a BamHI site was inserted in place of the TGA stop codon. These fragments were digested and ligated into EcoRi- and BamHI-digested pGEM4 to create pSM6. pSM6 was digested using EcoRI and BamHI, and the ric8 insert was subsequently ligated into pGBK7 and pGAD424 (Clontech) to yield plasmids pSM7 and pSM8, respectively.

The yeast two-hybrid assay was performed as described previously (Li and Borkovich 2006). Yeast strains containing RIC8 gene plasmids (pSM7 or pSM8) were mated to the appropriate *Gα*-vector-containing yeast to isolate diploids. Diploids were then tested on SC medium lacking leucine, tryptophan, and histidine ± adenine to select for the expression of the HIS3 or HIS3 and ADE2 reporter genes, respectively. A colony lift assay for β-galactosidase activity was performed according to the manufacturer’s recommendations to screen for the lacZ reporter (Clontech).

**Expression and purification of RIC8 and Gα proteins from *E. coli***

For amplification of the *Gα* genes, primer pairs (NA13G1fw/NΔ13G1rv, NA13G2fw/NΔ13G2rv, and NA12G3fw/NΔ12G3rv; Table S1) were used to introduce a 36 (gna-3) or 39 (gna-1 and gna-2) nucleotide deletions at the 5’ end of each gene, along with restriction sites for cloning into pET16b, which expresses a 10-histidine N-terminal fusion protein (Novagen, Gibbstown, NJ). The ric8 primers (R8 NdEl fw/R8 BamHI rv; Table S1) were designed to clone full-length ric8 into pET16b.

*E. coli* strain BL21 (DE3) plysS was used to express HIS-tagged proteins. LB medium supplemented with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol was inoculated with 1/50 volume of an overnight *E. coli* culture and grown at 37°C for 2–3 hr with centrifugation at 225 × g. Heterologous protein expression was induced by the addition of IPTG to a final concentration of 100 μM, and incubation continued for an additional 2–3 hr at 30°C with centrifugation at 80 × g. The cells were centrifuged at 4,000 × g in an Avanti J-26 XP floor centrifuge using rotor JS-4.0 for 20 min at 4°C, and pellets were stored at −20°C.

For RIC8, 40 ml of His-Pur Lysis buffer [50 mM NaH₂PO₄ (pH 7.4), 300 mM NaCl, 10 mM imidazole (Sigma), 10% glycerol, 0.1 mM PMSF] containing 0.2 mg/ml lysozyme was added to cell pellets from a 1-liter culture, followed by incubation on ice for 20 min. The cell suspension was
tein concentration was near zero as determined using the Ni-NTA resin, and all buffers contained 50 mM imidazole. The column was washed with 10 ml wash buffer (lysis buffer containing 20 mM imidazole). The column was washed with 10–20 ml wash buffer (until protein concentration was near zero as determined using the Bradford protein assay). Protein was eluted in 1-ml fractions with lysis buffer containing increasing amounts of imidazole: 50, 75, and 100 mM (3 ml of each concentration).

The Gα proteins were purified similarly to RIC8 with the few differences noted below. The Gα proteins were purified using Ni-NTA resin, and all buffers contained 50 mM NaH₂PO₄ (pH 7.4), 500 mM NaCl, 10% glycerol, 0.1 mM PMSF. In addition, the lysis and elution buffers contained 20 μM GDP and 1 mM MgSO₄. Lysis buffer contained 20 mM imidazole, wash buffer contained 40 mM imidazole, and protein was eluted in 4-ml fractions with lysis buffer containing increasing amounts of imidazole: 60, 80, 100, and 120 mM.

For both RIC8 and Gα proteins, fractions with the highest protein concentration (typically 50–75 mM imidazole for RIC8 and 80–120 mM for Gα proteins) were pooled and simultaneously desalted and concentrated. The protein was transferred to an Amicon Ultra 30 column (Millipore, Billerica, MA) and centrifuged for 20 min at 4000 x g in an Avanti J-26 XP floor centrifuge using rotor JS-5.3. Exchange buffer contained 200 mM HEPES (pH 7.5), 100 mM NaCl, 10% glycerol, 1 mM EDTA, and 1 mM DTT. Gα exchange buffer also contained 1 mM MgSO₄, and 20 μM GDP was added to the concentrated protein (500–1000 μL) in 3- to 4-ml portions and centrifuged each time for 4–10 min for a total 10–12 ml of buffer exchange. The protein was then divided into aliquots and stored at ~80°C. The concentration of RIC8 and Gα proteins was determined by comparing to BSA after electrophoresis on an SDS-PAGE gel, followed by Coomassie staining. Samples containing 1–20 μl of purified protein were subjected to Western analysis according to the manufacturer's suggestions. The hexahistidine monoclonal antibody raised in rabbit (Bethyl Laboratories, Montgomery, TX) was used at a 1:1000 dilution, and goat anti-rabbit secondary antibody (Bio-Rad) was used at 1:10,000 dilution.

GTP-binding assays were modeled after protocols described previously (Tall et al. 2003). Reactions were carried out in exchange buffer [200 mM HEPES (pH 7.5), 100 mM NaCl, 10% glycerol, 1 mM EDTA, and 1 mM DTT] with a total volume of 20 μL. Each reaction contained 200 nM Gα protein and 0 or 200 nM RIC8. Solutions containing purified Gα proteins (400 nM) and 20 mM MgSO₄ in a total volume of 10 μL were prepared and kept on ice until reaction start.

Mixtures containing RIC8 (0 or 400 nM), 4 μM GTPγS, and 1 μCi GTPγ35S (Perkin Elmer, Waltham MS) were also prepared and kept on ice until reaction start. Reactions were initiated by mixing 10 μl of the RIC8 solution with 10 μl of each Gα solution and then incubated at 30°C for up to 60 min. Reactions were quenched using 100 μl of cold stop solution [20 mM Tris–Cl (pH 8.0), 100 mM NaCl, 2 mM MgSO₄ and 1 mM GTP]. Proteins were separated from unbound GTPγS by vacuum filtration using 0.45 μM of HA mixed cellulose membranes (Millipore), and filters were washed three times with 5 ml cold wash solution [50 mM Tris–Cl (pH 8.0), 100 mM NaCl, and 2 mM MgSO₄] using vacuum filtration. Filters were dried at room temperature and placed in vials, and scintillation fluid (Scintiverse BD cocktail, Fisher Scientific, 4–5 ml) was added prior to counting using a Beckman LS 6500 liquid scintillation counter.

**Results**

**ric8 isolation, gene structure analysis, and protein alignment**

RIC8 belongs to a unique protein family that does not exhibit any conserved domains when compared to known proteins (Wilkie and Kinch 2005; Figueroa et al. 2009). However, analysis of the primary sequence of *Xenopus laevis* xRic-8 reveals an armadillo-type folding pattern, which is a group of three α-helices folded into a triangle structure (Coates 2003; Figueroa et al. 2009). This armadillo fold is repeated several times in the xRic-8 protein to form a compact armadillo repeat structure. This is of interest because armadillo proteins are known to interact with several protein partners and to be involved in multiple cellular pathways (Coates 2003). These *in silico* results were corroborated by circular dichroism studies that revealed a high number of α-helices in the xRic-8 protein (Figueroa et al. 2009).

RIC8 is found only in fungi and animals (Wilkie and Kinch 2005). In *N. crassa*, the closest homolog to animal RIC8 is a predicted 53-kDa protein designated NCU02788 (http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/index.html). The predicted gene structure in the Broad Institute database was confirmed by sequence analysis and verification of the predicted introns. The presence of the 3' intron was confirmed by cDNA sequence NCW06G3T7 (cDNA clone W06G3 3'; Broad Institute), and the 5' predicted intron was verified by RT-PCR (Figure S1).

Of the animal RIC8 homologs, *Neurospora* RIC8 is most similar to *Xenopus tropicalis* Ric-8A and also exhibits significant identity to human RIC-8A (Figure 1). Although RIC8 proteins are highly conserved among filamentous fungi (Figure S2), there are no significant (*E < 1e-4*) homologs in the yeast *S. cerevisiae*, and RIC8 is also absent from the genomes of plants.

**Epistatic relationships between RIC8 and Gα subunits**

Vegetative growth in *Neurospora* involves apical polar growth of basal hyphae away from the point of inoculation...
and parallel to the solid medium surface, with branching occurring back from the tips of the hyphae (reviewed in Springer 1993). Under nutrient limiting conditions or in the presence of an air/water interface, specialized aerial hyphae branch perpendicularly from basal hyphae and produce asexual spore-forming structures called conidiophores, which in turn elaborate strings of asexual spores called macroconidia or conidia (Springer 1993). Once mature, conidia can be dispersed by wind currents or animals in nature. In nutrient-rich submerged cultures, wild-type Neurospora does not form conidiophores.

Since RIC8 has been shown to be a GEF for Ga proteins in other systems, we investigated a possible role for Neurospora RIC8 as an upstream regulator of the Ga subunits GNA-1, GNA-2, and GNA-3. A gene deletion mutant was created by replacement of the ric8 gene with the hph gene and analyzed for growth and/or developmental phenotypes (Figure 2A). Our laboratory has previously demonstrated that simultaneous loss of the Ga genes gna-1 and gna-3 causes severe defects in vegetative growth, absence of female sexual structures (protoperithecia), and inappropriate formation of conidia in submerged cultures (Kays and Borkovich 2004). Mutation of ric8 leads to phenotypes similar to those observed for Δgna-1 Δgna-3 double mutants. Δric8 mutants have thin basal hyphae that grow extremely slowly and produce short aerial hyphae, accumulate less mass, and appropriately form conidia in submerged cultures (Figure 2A). During the sexual cycle, Δric8 strains do not differentiate protoperithecia and are thus female-sterile (data not shown).

The shared defects of Δric8 and Ga mutants prompted us to measure Ga protein levels in the Δric8 mutant. Loss of ric8 causes a decrease in GNA-1, GNA-2, GNA-3, and GNB-1 protein levels (Figure 3A). In contrast, transcript amounts for the respective genes are similar in wild type and the Δric8 strain (Figure 3B), suggesting a post-transcriptional control mechanism. Interestingly, a similar requirement for ric8 in maintaining normal levels of a Ga i protein and an associated Gb subunit has been observed in Drosophila (Hampoelz et al. 2005). In mammals, Ric-8B inhibits ubiquitination and proteasomal turnover of Ga i (Nagai et al. 2010). We have previously shown that loss of the Neurospora Gb gene gnb-1 leads to decreased levels of GNA-1 and GNA-2, while GNA-3 protein levels are unaffected in submerged cultures (Yang et al. 2002). Although a decreased level of GNB-1 in the Δric8 mutant could account for low amounts of GNA-1 and GNA-2, the reduction in GNA-3 levels in Δric8 mutants cannot be explained by the loss of GNB-1. This suggests that GNA-3 stability is regulated by RIC8 independently of GNB-1.
During the G-protein cycle, GTP-bound Gα is released from Gβγ and the GPCR and is then free to interact with downstream effectors. Hydrolysis of GTP to GDP leads to reassociation of the Gα protein with Gβγ and termination of signaling. Because of these attributes, a GTPase-deficient Gα allele is constitutively active and independent of Gβγ. We therefore tested whether introduction of GTPase-deficient alleles of the three Gα genes (under control of the cog-1 promoter) into the Δric8 background would rescue Δric8 phenotypes.

The results show that Δric8 strains carrying a GTPase-deficient activated Gα allele restored the encoded protein to wild-type levels (Figure 3A). This suggests that overexpression and/or a constitutively GTP-bound Gα can override the reduced protein levels observed in Δric8 mutants. Transformation of the gna-2ΔQ205L activated allele into the Δric8 background had no effect. However, the presence of the gna-1ΔQ204L- or gna-3ΔQ208L-activated allele partially rescued the slow growth and reduced mass and thin basal hyphal phenotypes of Δric8 (Figure 2B), with gna-3ΔQ208L having the greatest effect on growth. When both gna-1ΔQ204L and gna-3ΔQ208L are introduced into the Δric8 background, the phenotype resembles that obtained after transformation with gna-3ΔQ208L alone (Figure 2B). Taken together, these results suggest that RIC8 acts upstream of and is a positive regulator of GNA-1 and GNA-3, but not of GNA-2 in Neurospora.

**RIC8 is cytoplasmic in conidia and vegetative hyphae**

To determine the localization of RIC8 in Neurospora, we produced a vector containing a C-terminal fusion of GFP to RIC8. This construct complemented Δric8 phenotypes and restored Function of the Gα protein with Gβγ and termination of signaling. Because of these attributes, a GTPase-deficient Gα allele is constitutively active and independent of Gβγ. We therefore tested whether introduction of GTPase-deficient alleles of the three Gα genes (under control of the cog-1 promoter) into the Δric8 background would rescue Δric8 phenotypes.

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expression of the ric8 transcript (Figure S1, B and C). Production of a full-length RIC8-GFP fusion protein was observed by Western analysis using whole-cell extracts and GFP antiserum (Figure S1D). Analysis of strains carrying the GFP fusion showed that RIC8 is cytosolic in mature conidia and vegetative hyphae; this was confirmed by comparison to a control strain expressing a cytosolic GFP protein (Figure 4). GFP fluorescence images for a wild-type strain without a GFP construct were completely black using the same settings, indicating negligible background fluorescence (data not shown).

This observation of a cytosolic location for RIC8 is consistent with observations in animals (Hampoelz et al. 2005) and in M. oryzae (Li et al. 2010). In higher eukaryotes, RIC8 has been shown to have cytoplasmic localization, which moves to the mitotic machinery at specific times during embryonic mitosis. In Drosophila embryos, RIC8 is located in the cytoplasm, with concentrated amounts at the mitotic spindle (Hampoelz et al. 2005). In C. elegans embryos, RIC8 is localized at the cortex, mitotic spindle, nuclear envelope, and around chromatin (Couwenbergs et al. 2004). The absence of nuclear localization for RIC8 in the two fungi analyzed could result from a number of factors, including growth conditions, time in the life cycle, or absence of an environmental trigger.

**Loss of ric8 affects the cAMP-signaling pathway, which is regulated by Gα proteins in Neurospora**

Adenylyl cyclase has been extensively characterized in eukaryotes and has been shown to be activated and repressed by G-protein signaling in various systems (reviewed in Neves et al. 2002). Activated adenylyl cyclase produces cAMP, which binds to the regulatory subunits of protein kinase A (PKA), releasing the catalytic subunits of PKA to phosphorylate a wide variety of target proteins (Neves et al. 2002). In the C. elegans synaptic signaling pathway, RIC8 has been linked to cAMP metabolism through the identification of mutations that suppress defects of ric8 loss-of-function mutants (Schade et al. 2005; Charlie et al. 2006). These mutations include dominant activated alleles of gsa-1 (Gαs) and acy-1 (adenylyl cyclase) and reduction-of-function alleles of kin-2 (regulatory subunit of PKA) and pde-4 (cAMP phosphodiesterase). These results are consistent with RIC8 as an upstream activator of the cAMP pathway during synaptic signal transmission in C. elegans. In Neurospora, previous work from our group suggests that adenylyl cyclase (CR-1) is activated by GTP-bound GNA-1, while GNA-3 is required to maintain normal levels of CR-1 protein (Ivey et al. 1999; Kays et al. 2000). Furthermore, a mutant allele of the PKA regulatory subunit (mcb) that is predicted to lead to hyperactivation of the catalytic subunit is epistatic to gna-3 and suppresses the Δgna-1 mutation (Bruno et al. 1996; Kays and Borkovich 2004).

On the basis of the known relationship between RIC8 and cAMP metabolism in C. elegans as well as the genetic relationship between RIC8, GNA-1 and GNA-3 in Neurospora, we investigated whether cAMP signaling is affected in Neurospora Δric8 mutants. We first assessed levels of the CR-1 adenylyl cyclase using western analysis with a CR-1 antiserum (Figure 5A). The amount of CR-1 protein was reduced in the Δric8 background (Figure 5A), similar to results observed for Δgna-3 mutants and consistent with low cAMP levels (Kays et al. 2000). However, expression of the activated forms of GNA-1, GNA-2, or GNA-3 did not return the CR-1 protein levels to normal (Figure 5A). These results suggest a Gα-independent role for RIC8 in modulation of CR-1 protein levels.

We followed up on the above finding by testing the effect of the mcb PKA regulatory subunit mutation described above (Table 1). The mcb mutation partially suppresses the Δric8 growth and conidiation phenotypes (Figure 5B). These results support a role for RIC8 as a positive regulator of the cAMP-signaling pathway through the activation of Gα subunits and at least in part via a Gα-independent pathway that influences the stability of cAMP-signaling pathway components.
RIC8 interacts with and acts as a GEF toward Gα subunits

Results from the genetic epistasis experiments suggest that RIC8 is a positive regulator of GNA-1 and GNA-3. We next tested for a possible physical interaction between Gα proteins and RIC8 using the yeast two-hybrid assay. RIC8 was observed to interact with both GNA-1 and GNA-3, but not with GNA-2 (Figure 6). Interestingly, RIC8 interacts with GNA-1 only as a Gal4p DNA-binding domain fusion and with GNA-3 only as a Gal4p activation domain fusion (Figure 6). This may indicate a different physical relationship and/or binding site for RIC8 on these two Gα subunits.

Having established a physical association between RIC8 and a subset of Neurospora Gα subunits using the yeast two-hybrid assay, we next performed experiments to directly test whether RIC8 can accelerate exchange of GDP for GTP on Gα proteins. Since Gα proteins had not been previously purified or assayed for GTP binding in any filamentous fungal species, we had to devise methods to facilitate expression of soluble, active proteins and their subsequent purification. A previous study reported that efficient purification of mammalian Gα proteins from E. coli and subsequent protein crystallization were facilitated either by removal of the disordered N-terminal region or by replacement with the N terminus of another Gα subunit (Kimple et al. 2002). These proteins were shown to retain normal GTP binding (Kretz et al. 2006). We predicted that the first 12 (GNA-3) or 13 (GNA-1 and GNA-2) amino acids of Neurospora Gα proteins are disordered using the DisEMBL Intrinsic Protein Disorder Prediction program (http://dis.embl.de/). We therefore produced E. coli vectors expressing deca-histidine (10H) N-terminal tagged and N-terminal truncated versions of all three Gα subunits, termed 10H-N^{Δ13}-GNA-1, 10H-N^{Δ13}-GNA-2, and 10H-N^{Δ12}-GNA-3. For clarity, we refer to these proteins as GNA-1, GNA-2, and GNA-3. A vector encoding full-length 10H-tagged RIC8 was also produced for expression in E. coli. Proteins were overexpressed in E. coli and then purified using nickel or cobalt affinity chromatography (see Materials and Methods).

GTPγS-binding assays were conducted in the absence and presence of RIC8. All three Gα proteins bind GTP in the absence of RIC8, demonstrating that the fusions are active and confirming the identity of the three Neurospora proteins as G proteins (Figure 7). Addition of RIC8 had different effects on GTPγS binding to the three Gα proteins. RIC8 does not appreciably influence binding of GTPγS to GNA-2 (Figure 7B). However, binding of GTPγS by GNA-1 is increased twofold by the addition of RIC8 at early time points (5–30 min, Figure 7C). These results suggest that RIC8 acts as a GEF for GNA-1 and GNA-3, playing a major role in the activation of GNA-3. Importantly, the findings from the GEF assays are consistent with those obtained from the genetic epistasis analysis and yeast two-hybrid assays and support a model in which RIC8 activates GNA-1 and GNA-3, but not GNA-2, in Neurospora.

Discussion

The discovery and subsequent study of RIC8 in metazoan cells has shown that this protein is required for major cellular processes, including asymmetric cell division and synaptic signaling. Here we demonstrate that RIC8 is involved in critical functions, including vegetative growth and sexual and asexual development in Neurospora. Mutations that activate the GNA-1 and GNA-3 Gα proteins in the Δric8 background partially suppress Δric8 phenotypes, and RIC8 interacts with GNA-1 and GNA-3 in the yeast two-hybrid assay. Most importantly, we show that RIC8 acts as a GEF for GNA-1 and GNA-3 in vitro. The data suggest that RIC8...
Figure 7 GTP binding of \( \alpha \) proteins and GEF activity of RIC8. GNA-1 (A), GNA-2 (B), and GNA-3 (C) were incubated with \[^{35}S\]GTP\(^{\gamma}\S\) in the absence (○) or presence (●) of RIC8. All protein concentrations were 200 nM. Reactions were performed in triplicate, as indicated in Materials and Methods. A representative result from several independent assays is shown for each protein.

participates in pathways involving GNA-1 and GNA-3 to regulate growth and development.

Loss of ric8 leads to lower levels of G-protein subunits in Neurospora, Drosophila, C. elegans, and mammalian cells. In Drosophila, RIC8 is required for proper plasma membrane localization and normal protein stability of \( \alpha \) subunits, but only correct membrane localization of \( \alpha \) subunits (Hampeolz et al. 2005). In C. elegans, RIC8 is essential for membrane localization and normal protein stability of \( \alpha \) subunits, while \( \alpha \) subunits do not depend on RIC8 for protein stability or localization (Afshar et al. 2004, 2005). In Drosophila, it has been proposed that effects on \( \alpha \)-protein localization and stability in the absence of RIC8 may stem from defective assembly of the heterotrimer and inhibited transport to the plasma membrane (Hampeolz et al. 2005). In mammalian cells, it has been demonstrated that Ric-8B inhibits ubiquitination of \( \alpha \) subunits, thus preventing degradation of the protein (Nagai et al. 2010). The mechanism underlying how loss of RIC8 leads to lower levels of \( \alpha \) proteins in Neurospora is unknown, but may be a consequence of both mislocalization and accelerated protein turnover (A. Michkov, S. Won, and K. Borkovich, unpublished observations).

Work in C. elegans has demonstrated the importance of cAMP metabolism to RIC8-related functions in the synaptic signaling pathway. In addition, stabilization of \( \alpha \) subunit by RIC-8B in mouse embryonic fibroblast cells is required for increased cAMP in response to isoproterenol (Nagai et al. 2010). The similarities between the genetically tractable C. elegans and Neurospora with regards to positive regulation of the cAMP pathway by RIC8 are particularly striking. In both systems, a mutation in the PKA regulatory subunit suppresses a ric8 mutation. Importantly, we also demonstrate that loss of RIC8 leads to a reduced level of adenylyl cyclase protein, a finding that has not been previously reported in any system. This observation may explain why a hyperactive allele of adenylyl cyclase was recovered in a ric8 suppressor screen in C. elegans (Schade et al. 2005). As mentioned above, RIC8 is also required for normal levels of various G proteins in Neurospora, Drosophila, C. elegans, and mammalian cells. Taken together, these results 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 RIC8 protein and one that directly impacts cAMP signaling.

A prediction based on our results is that RIC8 regulates \( \alpha \) activity and subsequent cAMP production in response to a specific developmental signal. However, the mechanism by which RIC8 is regulated at the pre- or post-translational level is currently unknown. Regarding transcriptional regulation, microarray analysis indicates that ric8 is expressed ubiquitously throughout the vegetative colony in Neurospora (Kasuga and Glass 2008). Results from additional microarray studies using Neurospora grown on medium that mimics either symbiotic or pathogenic plant relationships suggest that ric8 expression is not induced during fungal/plant interactions (http://bioinfo.townsend.yale.edu/browse.jsp?scope=4&tabid=1&query=ncu02788).

Heterotrimeric \( \alpha \) proteins are activated by the exchange of GDP for GTP. This exchange is facilitated by a GEF, such as a GPCR or RIC8. Our results provide the first evidence that filamentous fungal \( \alpha \) subunits do indeed bind GTP and that RIC8 functions as an apparent GEF by increasing GDP–GTP exchange on two of the three subunits. The main target of RIC8 appears to be GNA-3, as GNA-3 is activated most strongly by RIC8 in vitro, accelerating the GTP binding by fourfold. This activation is comparable to the more than sevenfold activation of Gα11 by RIC8 in mammals (Tall et al. 2003) and to the fourfold activation of Gpa1 by another GEF, Arr4, in S. cerevisiae (Lee and Dohlman 2008). The twofold activation of GNA-1 by RIC8 is similar to that observed...
for mammalian $\alpha_{Go}$, while mammalian $\alpha_{G}$ is not affected by RIC8 (Tall et al. 2003). The finding that RIC8 acts preferentially on GNA-3 is supported by the observations that the GTPase-deficient allele of GNA-3 suppresses the $\Delta ric8$ mutation to the greatest extent and that CR-1 protein levels are reduced in both $\Delta ric8$ and $\Delta gna-3$ mutants.

Our results reveal important parallels between RIC8 functions during cell division, cAMP signaling, and perhaps regulated protein turnover in *Neurospora* and metazoans. *Neurospora* is a haploid organism that has a relatively short doubling time, well-developed genetic tools, and a sequenced genome. There is also a comprehensive collection of knockout mutants for most genes, and it is relatively easy to isolate and clone suppressor mutations. Future investigations will capitalize on these tools to reveal roles for known proteins, as well as to screen for novel components that participate in RIC8-regulated processes, thus illuminating conserved functions for RIC8 during growth and development in *Neurospora* and animals.

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*Communicating editor: E. U. Selker*
RIC8 Is a Guanine-Nucleotide Exchange Factor for Gα Subunits That Regulates Growth and Development in *Neurospora crassa*

Sara J. Wright, Regina Inchausti, Carla J. Eaton, Svetlana Krystofova, and Katherine A. Borkovich
Figure S1  ric8 gene structure, mutant verification, and expression RIC8-GFP.  A) The ric8 genomic region from Neurospora crassa. Intron locations (black triangles) and restriction enzyme sites (black lines) are shown.  B) Complementation of Δric8 by expression of a ric8-gfp allele.  A VM plate culture is shown for wild-type (2489), Δric8 (R81a), and Δric8, ric8-gfp+::his-3′(R8GFP).  C) RT-PCR analysis of ric8 mRNA levels.  Samples containing 2 μg of total RNA isolated from conidia of strains used in (B), with a duplicate R81a reaction, were subjected to RT-PCR with primers designed to flank the first predicted intron of ric8 (R8I1fw and R8I1rv, TABLE S1).  The predicted intron size is 56 bp, making the size of the RT-PCR fragment about 354 bp.  For the control and probe DNA, a 410 bp fragment of genomic DNA was amplified by PCR from plasmid pSM2 using the same primers.  Expression of the 18S rRNA gene was assessed under identical RT-PCR conditions (See methods).  D) Western analysis.  Protein from a whole cell extract isolated from a 16 hr submerged culture of strain R8GFP was subjected to Western analysis using anti-GFP antiserum (See methods).  The approximately 93 KDa protein is indicated by the black arrow.
Figure S2  RIC8 homologs in other fungi. ClustalW (http://www.ch.embnet.org/software/ClustalW.html) was used to align RIC8 sequences from Neurospora crassa (Nc; NCU02788.3), Podospora anserina (Pa; Pa_1_3900), Magnaporthe grisea (Mg; MGG_14008), Fusarium graminearum (Fg; FGSG_01511), Sclerotinia sclerotiorum (Ss; SS1G_02787.1), Aspergillus nidulans (An; AN1661.3), Histoplasma capsulatum (Hc; HCAG_03335.1), Botrytis cinerea (Bc; BC1G_02317.1), Schizosaccharomyces japonicus (Sj; SIAG_02027), Yarrowia lipolytica (Yl; ACCESSION# XP_505953). Shading indicates identical (black) and similar (grey) amino acid residues (http://www.ch.embnet.org/software/BOX_form.html).

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