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Aspergillus fumigatus rasA and *rasB* regulate the timing and morphology of asexual development

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Abstract

Expression of *rasA* plays an important role in conidial germination in *Aspergillus nidulans*. Conidial germination is required to initiate both infection and asexual development in the opportunistic pathogen *Aspergillus fumigatus*. Therefore, we sought to determine the requirements for Ras proteins in conidial germination and asexual development of *A. fumigatus*. A second homolog, *rasB*, has been identified that characterizes a new subclass of Ras genes. Dominant active (DA) and dominant negative (DN) mutations of each gene were introduced into protoplasts as transgenes. DA*rasA* expression led to reduced conidiation, malformed conidiophores, and altered mitotic progression, whereas expression of DN*rasA* caused a significant reduction in the rate of conidial germination. In contrast, expression of DN*rasB* slightly delayed the initiation of germination and caused the development of conidiophores in submerged culture. DA*rasB* expression led to reduced conidiation. RasA and RasB appear to play different, but overlapping, roles in the vegetative growth and asexual development of *A. fumigatus*.

Keywords: *Aspergillus*; Ras; Asexual development; Conidial germination; Fungi

1. Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen that is the cause of a broad spectrum of illnesses, ranging from allergic reactions to invasive disease (Latge, 1999). The population at risk for the most severe and life-threatening form of disease, invasive aspergillosis, continues to expand for reasons including increases in bone marrow and solid organ transplantation and the aggressive use of chemotherapeutic regimens for solid tumors and hematologic malignancies (Lin et al., 2001). Treatment of invasive aspergillosis with anti-fungal therapies is still largely inadequate, resulting in high morbidity and up to 100% mortality (Denning et al., 2002).

Recently, much attention has been given to signal transduction cascades that regulate fungal morphogenetic networks, as aberrations in these pathways may produce less virulent phenotypes (Lengeler et al., 2000). Among these networks are multiple cascades controlled by the highly conserved family of Ras proteins. Ras genes encode small, membrane-associated GTPases that exist in two states: active (GTP bound) and inactive (GDP bound). Cycling between these two states is aided by interaction with GTPase activating proteins and guanine exchange factors (Reuther and Der, 2000). Thus, the main route for regulation of Ras activity is through protein–protein interactions. Studies involving mutations in the Ras signaling pathways of several fungi have revealed a role for these conserved proteins in many developmental processes. Mutations in the *rasA* gene of *Aspergillus nidulans*, a close relative of *A. fumigatus*, cause aberrations in germination and asexual development, the severity of which depends on the levels of mutant transgene expression (Som and Kolaparthi, 1994). Overexpression of a dominant negative (DN) form of *rasA* causes delayed conidial germination, whereas overexpression of dominant active (DA) *rasA* causes initiation of germination in the absence of a

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germinant (Osherov and May, 2000). In the model fungal organism *Neurospora crassa*, deletion of a Ras homolog, *NC-ras2*, produces defects in apical growth of hyphae, cell wall biosynthesis, and conidia formation (Kana-uchi et al., 1997). Among the pathogenic fungi, Ras activity has been shown to be essential for virulence in both *Cryptococcus neoformans* and *Candida albicans* (Alspaugh et al., 2000; Leberer et al., 2001; Waugh et al., 2002). Although one Ras homolog, *rasA*, has previously been identified in *A. fumigatus*, the role that Ras proteins play in the asexual developmental program of this important human pathogen is not known. Here we report the identification of a second Ras homolog in *A. fumigatus*, *rasB*, which is a member of a distinct class of Ras proteins, and provide evidence for a role for both *rasA* and *rasB* in the conidial germination and asexual development of *A. fumigatus*.

Table 2
Initiation of germination in *A. fumigatus*

Supplement	% Germination ± SE[a]
Fructose[b]	97
Acetate[b]	80
Glycerol[b]	96
Galactose[b]	97
Sucrose[b]	99
Raffinose[b]	99
Maltose[b]	100
Glucose[b]	98.7 ± 0.7
Citrate[b]	6 ± 3.2
2-Deoxyglucose[c]	92.3 ± 5.2
6-Deoxyglucose[c]	68.7 ± 15.4
Cyclic-AMP[c]	52 ± 18.1
No addition	10 ± 3.2

[a] Conidia (10^6/ml) were incubated at 37 °C for 14 h with shaking at 250 rpm.
[b] 1% (w/v).
[c] 10 mM.

2. Materials and methods

2.1. Culture conditions

All strains used and created in this study are listed in Table 1. Conidia were harvested from strains grown on *Aspergillus* minimal medium, modified to contain 1 mM ammonium tartrate as the nitrogen source (Cove, 1966). For studies involving the quantitation of germination, conidia were incubated in *Aspergillus* minimal medium at 37 °C with shaking at 250 rpm, using one of the carbon sources listed in Table 2. For each germination study, 100 conidia were counted in each of three independent assays. Germination was defined as the production of a germ tube.

For developmental analysis, cultures enriched for a specific stage of development were obtained based on a previously described method (Law and Timberlake, 1980). For each strain, four replicate flasks containing 50 ml of YG liquid medium were inoculated with 1×10^7 conidia and incubated at 37 °C with shaking at 250 rpm. At 6 and 12 h, cultures representing primarily germlings and early vegetative hyphae, respectively, were harvested by centrifugation and examined by differential interference contrast (DIC) microscopy. At 12 h, the remaining cultures were vacuum filtered onto paper discs and floated on 5 ml of YG medium in a petri dish, using glass beads as a support. This step produced the air/water interface needed for asexual differentiation allowing examination by DIC microscopy for up to 48 h. To monitor the development of conidiophore structures in submerged culture, the same protocol was applied, omitting the vacuum filtration step. These cultures were then harvested by centrifugation and examined.

Table 1
Strains employed in this study

Strain	Background + transgene	Source
H237	*wt*	Human isolate
DN*rasA*	*wt* + $rasA^{S22N}$	This study
DA*rasA*	*wt* + $rasA^{G17V}$	This study
DN*rasB*	*wt* + $rasB^{T21N}$	This study
DA*rasB*	*wt* + $rasB^{G16V}$	This study

2.2. Molecular cloning of A. fumigatus rasB

The *rasB* genomic sequence was identified in a search of the *A. fumigatus* genome (http://tigrblast.tigr.org/ufmg), using the *Saccharomyces cerevisiae* Ras1p as a query sequence. Based on the predicted coding region, the entire *rasB* cDNA was PCR amplified from reverse transcribed cDNA using the forward primer 5′-CACC ATGGATCCTATGTCGGGGAAAATGACG-3′ and the reverse primer 5′-ATTATCGCGGCCGCATGGA TGTTCGTGGACGC-3′.

2.3. Construction of mutant Ras strains

The full-length cDNAs for the *rasA* and *rasB* genes were amplified from reverse-transcribed cDNA using primers that contained 5′ *Nde*I and 3′*Not*I sites and the products cloned into pCR 2.1-TOPO (Invitrogen) by TA-cloning. Primers used for *rasA* amplification are: (*Nde*I) 5′-TGCGCCATATGGCTTCAAAGGTACGC CG-3′ and (*Not*I) 5′-GCGACGCGGCCGCCTTTCCT ATTGACCTGCG-3′ and for *rasB* amplification are: (*Nde*I) 5′-ATCGTTCATATGTCGGGGAAAATGAC GTTG-3′ and (*Not*I) 5′-ATTATCGCGGCCGCATG GATGTTCGTGGACGC-3′. A DA or DN mutation was introduced into each of the *rasA* and *rasB* cDNA clones by site-directed mutagenesis (Stratagene). Oligo-

nucleotides used to introduce the specific mutations were as follows: DA*rasA* 5′-CCTTAGAGAGTACAA ***A***CTAGTTGTTGTCGGTGGTGTTGGTGTCGG-3′ (G17V/*Spe*I); DN*rasA* 5′-GGTGTCGGAAAGAACT GCTTAACAATCC***A***GCTGATTCAGAGTCACTTC G-3′ (S22N/*Pvu*II); DA*rasB* 5′-GGTGGTGTTGGGA GA***C***GTCGGTGTTGGGAAGACCGC-3′ (G16V/*Aat*II); and DN*rasB* 5′-GGTGGTGTTGGGAAGAAC GCGCTCACGATTCAGCT***G***TGTTTGAATCACTTC G-3′ (T21N/*Pvu*II). In each case, the reverse complement of the above oligonucleotides was used as the reverse primer. Underlined bases indicate those that were changed to encode the mutation conferring DA and DN properties, and bases changed to introduce novel restriction sites for screening purposes are shown in bold italics. The amino acids changed and restriction sites created by each mutation are listed in parentheses at the end of each oligonucleotide. Nucleotides changed to construct novel restriction sites did not affect the encoded amino acid. Before further cloning, all introduced mutations in cDNAs were confirmed by sequencing. Promoter segments (1 kb upstream of the coding region) for both *rasA* and *rasB* were PCR amplified from genomic DNA using primers that added a 5′ *Bam*HI site and a 3′ *Nde*I site to each; products were cloned into pCR 2.1-TOPO. The cloned DA and DN *rasA* and *rasB* cDNAs were then digested with *Nde*I and *Not*I and subcloned downstream of their respective endogenous promoters. The entire promoter-cDNA cassette was then moved by *Bam*HI–*Not*I restriction and ligated into vector pTHP. Vector pTHP was constructed by moving the hygromycin resistance marker from pAN7-1 (Punt et al., 1987) as a *Sal*I–*Hin*dIII fragment into pSL1180 (Pharmacia) digested with *Xho*I–*Hin*dIII. The vector contains the hygromycin resistance gene driven by the *Aspergillus nidulans gpdA* promoter and ending with the *A. nidulans trpC* terminator. The *trpC* terminator is also cloned upstream of the *gpdA* promoter to function as a terminator sequence for genes inserted into this vector. This allows all cDNAs to be cloned in the appropriate orientation as a *Bam*HI–*Not*I fragments, creating an expression construct for each mutant allele, which would be driven by the native promoter and allow for hygromycin selection. Each expression construct was linearized at a unique restriction site in the pTHP backbone and introduced into strain H237 by protoplast transformation as previously described (Yelton et al., 1984). Single copy integration at an ectopic site was confirmed by Southern blot analysis using the *rasA* or *rasB* cDNA as a probe. Expression of the mutant transgenes was confirmed by reverse transcription-PCR (RT-PCR) using specific primers for the *rasA* transgenes (Forward: 5′-ATGGCTTCAAAGTTCCTTAGAGA G-3′, Reverse: 5′-AGCTGTATCTGGAAGAGG-3′) and for the *rasB* transgenes (Forward: 5′-ATGTCGGGGAAAATGA CG-3′, Reverse: 5′-AGCTGTATCTGGAAGAGG-3′). Initial phenotypic analysis was performed on at least three transformants per strain and the data presented are from a representative transformant of each strain.

2.4. RNA isolation and RT-PCR analysis

At the 6 h developmental timepoint, total RNA was isolated from each strain using the Qiagen RNeasy Mini Kit following the Yeast III protocol. For all other timepoints, RNA was extracted using the Qiagen RNeasy Plant Mini Kit following the Filamentous Fungi protocol. For RT-PCR analysis, 1 μg of total RNA from each sample was digested with RNase-free DNase (Promega) following the manufacturer's protocol. According to manufacturer's specifications, the SuperScript II system (Invitrogen) was then used to generate first strand cDNA from these samples. The first strand cDNA was PCR amplified using gene specific primers for 500 bp fragments of the *A. fumigatus brlA* and *gpdA* (loading control) genes. Primers for the *A. fumigatus brlA* gene were designed from a search of the *A. fumigatus* genome database using the *A. nidulans brlA* nucleotide sequence and are: (forward) 5′-GGATA TGAGATCCCAGGG-3′ and (reverse) 5′-TGGTGG CAGGCGCTCGGG-3′. Sequencing of the cloned PCR product confirmed its identity as *brlA*. PCR specifications for all reactions in this study were as follows: denaturation at 94 °C for 90 s, annealing at 52 °C for 60 s, and extension at 72 °C for 60 s. Amplification was repeated for 20 cycles.

2.5. Fluorescent staining and microscopy

Staining of nuclei, septa, and cell walls were performed by a modification of previously described methods (Momany and Taylor, 2000; Ovechkina et al., 1999). In short, freshly harvested conidia were incubated for the specified timepoints on coverslips immersed in YG media. At the specified times, coverslips were removed, washed twice in 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.7, and fixed in 3 ml of fixative solution [8% formaldehyde in 50 mM Pipes, pH 6.7; 25 mM EGTA, pH 7.0; 5 mM $MgSO_4$; and 5% DMSO] for 30 min. After fixation, coverslips were washed twice for 10 min in 50 mM Pipes, pH 6.7, RNAse (0.1 mg/ml) was added and samples incubated at 37 °C for 1 h. The coverslips were then washed twice for 10 min in 50 mM Pipes, pH 6.7, and inverted onto 0.5 ml of freshly made staining solution [propidium iodide (12.5 μg/ml; Sigma) and Fluorescent Brightener 28 (0.4 μg/ml; Sigma) in 50 mM Pipes, pH 6.7] for 5 min at room temperature. Finally, the coverslips were washed twice for 10 min in 50 mM Pipes, pH 6.7, and mounted in 50% glycerol. Fluorescence microscopy was performed on an Olympus AX80 microscope using a digital camera equipped with a DAPI/FITC/TRITC triple cube

filter. Digital images were analyzed using Magnafire 2.1 and Photoshop 6.0 software. Brightfield and DIC analysis were performed on an Olympus BH-2 microscope.

2.6. GenBank accession numbers

The *rasA* cDNA, *rasB* genomic and cDNA sequences, and the *A. fumigatus brlA* partial coding sequence were submitted to the GenBank database. Their accession numbers are: AY327892 (*rasA* cDNA), AY266674(*rasB* cDNA), AY327893 (*rasB* genomic) and AY327894 (partial *brlA* sequence). The *A. nidulans* and *Magnaporthe grisea rasB* sequences were retrieved from the Whitehead Institute Center for Genome Research. The retrieval information is *A. nidulans* 1.100 (scaffold 7) for *A. nidulans rasB* and *M. grisea* contig 2.1817 for *M. grisea rasB*. The *Histoplasma capsulatum rasB* (F HCG217B.contig_pc_5.3) sequence was retrieved from the Genome Sequencing Center of Washington University, St. Louis. *Schizosaccharomyces pombe* and *S. cerevisiae* protein sequences were retrieved from the National Center for Biotechnology Information protein database.

3. Results

3.1. Identification of Aspergillus fumigatus rasB

A blast search of the *A. fumigatus* genome database, using *S. cerevisiae* Ras1p as a query sequence, identified a Ras homolog defined by the presence of the four domains important for GTP/GDP interaction, an effector domain, and a CAAX motif (Valencia et al., 1991). The full-length cDNA for this uncharacterized Ras homolog, termed *rasB*, was cloned by RT-PCR and a comparison of the genomic and cDNA sequence revealed a genomic organization consisting of two exons separated by a single intron of 79 bp. The predicted protein encoded by *rasB* contains 243 amino acids with a molecular weight of 26.9 kDa. Expression of the *rasB* gene in vegetative hyphae was very low, as *rasB* mRNA was evident by RT-PCR but barely detectable on a Northern blot of total RNA. This was in contrast to the *rasA* gene, which was expressed at high levels in vegetative hyphae (data not shown). When aligned with Ras protein sequences from humans and other fungi, phylogenetic comparisons (Fig. 1) revealed that the RasB proteins are more closely related to the *S. cerevisiae* Rsr1p, a Ras-GTPase involved in bud site-selection in yeast (Chant and Herskowitz, 1991), than to Ras1p or Ras2p. An

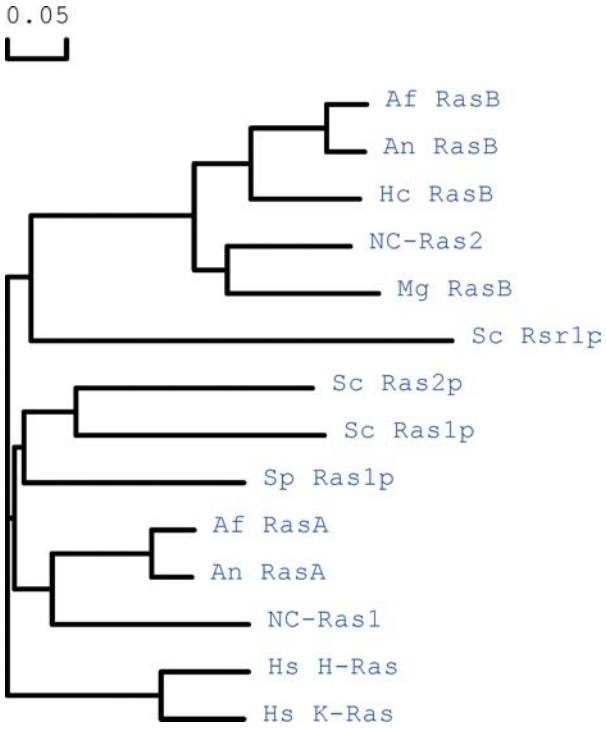

Fig. 1. Phylogenetic tree showing evolutionary relationships of fungal and human Ras proteins. Phylogenies were inferred using a PHYLIP based program (DNAMAN version 5.2.9) to create an unrooted phylogenetic tree. Organisms that were used for analysis were: *Aspergillus fumigatus* (Af), *Aspergillus nidulans* (An), *Histoplasma capsulatum* (Hc), *Neurospora crassa* (Nc), *Magnaporthe grisea* (Mg), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), and *Homo sapien* (Hs).

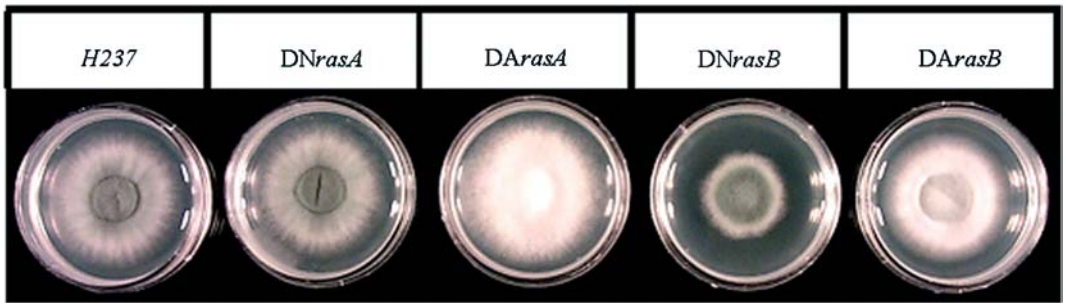

Fig. 4. Expression of *rasA* and *rasB* mutants alters colony morphology. Conidia (10^4) of the wild type strain, DN*rasA* and DA*rasA* strains, and DN*rasB* and DA*rasB* strains were spotted on *Aspergillus* minimal media and incubated for 48 h at 37 °C. Radial outgrowth was measured at 24 and 48 h and expressed as mm/h.

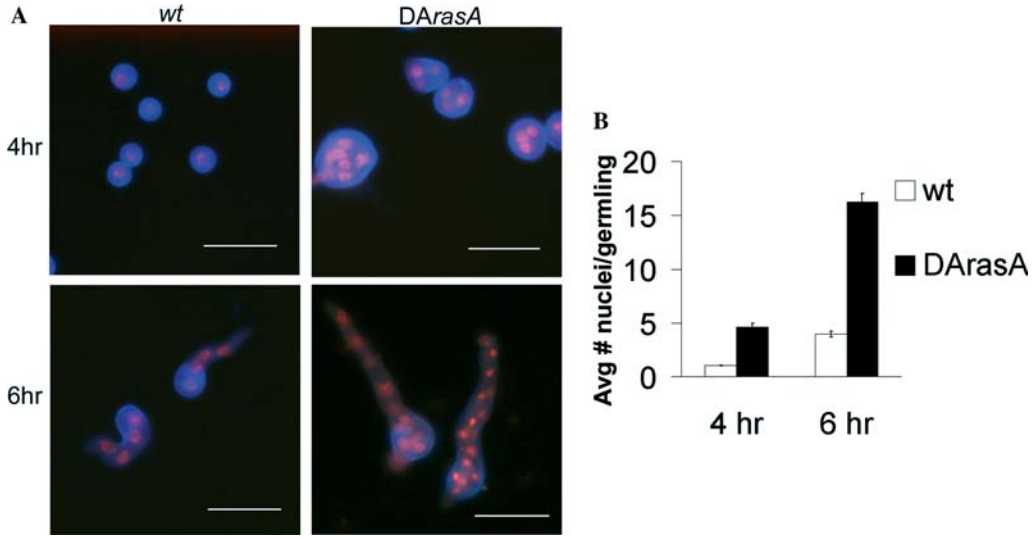

Fig. 8. Expression of DA*rasA* causes alteration in mitotic events. (A) Propidium iodide staining of nuclei in wild type (*wt*) vs. DA*rasA* strains at 4 and 6 h after inoculation. (B) Quantitation of nuclei (average number/germling) of wild type vs. DA*rasA* strains. For each strain, the nuclei of 50 conidia and/or germlings from three independent samples were counted and data were analyzed with Excel software. Error bars represent standard deviation. Scale bars = 10 μm.

homology-based alignment between an internal segment of the predicted amino acid sequences of RasA and RasB, and other Ras homlogs, is shown in Fig. 2. The most striking difference between the two proteins is a 20 amino acid insertion that is located just upstream of the third GDP/GTP binding domain (Fig. 2). This region is present in all of the RasB homologs including NC-ras2 of *N. crassa*, the only previously identified gene in this group (Kana-uchi et al., 1997). The insertion is not present in members of the RasA group.

3.2. Generation of strains expressing ras mutations

In order to investigate the Ras signaling pathways in *A. fumigatus*, mutant alleles of both *rasA* and *rasB* were constructed by site-directed mutagenesis. These mutations are predicted to produce proteins with either dominant active ($rasA^{G17V}$, $rasB^{G16V}$) or dominant negative signaling properties ($rasA^{S22N}$, $rasB^{T21N}$), based on known functions of Ras proteins (Valencia et al., 1991). The mutated cDNAs, each under the control of its endogenous promoter, were transformed into *A. fumigatus* as transgenes, and strains carrying single copy integrations were identified by Southern blot analyses. Expression of the mutant alleles was confirmed by RT-PCR (data not shown).

3.3. Initiators of germination for A. fumigatus

Conidial germination in *A. nidulans* has been reported to be both Ras- and carbon source-dependent (Osherov and May, 2000; Som and Kolaparthi, 1994). Therefore, we compared the carbon source requirements for conidial germination in *A. fumigatus* with what has been reported for *A. nidulans* (Table 2). Although most of the results were comparable with what was reported for *A. nidulans* (Osherov and May, 2000), *A. fumigatus* differed from *A. nidulans* in its ability to initiate germination in the presence of the non-metabolizable glucose analogs 2- and 6-deoxyglucose. Also, in contrast to *A. nidulans* data, exogenous cAMP, in the absence of a carbon source, induced germination in *A. fumigatus* to levels significantly higher than in the control (Table 2). Although the data presented here were obtained from shake culture at 37 °C, similar results were seen if the incubations were carried out in static cultures (data not shown).

To define a possible role for Ras activity in the germination of *A. fumigatus*, the germination rates for each mutant strain were compared in medium containing glucose as the sole carbon source. The DN*rasA* strain showed a reduced germination rate when compared to wild type (Fig. 3A), whereas the DN*rasB* strain showed a delay in initiation of germination, but then progressed at a wild type rate once germination began (Fig. 3B). Although the DA*rasA* and DA*rasB* mutants germinated at a rate that was indistinguishable from wild type (Figs. 3A and B), the DA*rasA* mutant was able to germinate in water alone (data not shown). In *A. nidulans*, the expression of DA*rasA* also causes initiation of germination in the absence of a carbon source (Osherov and May, 2000).

3.4. Ras signaling modulates colony morphology

The radial growth rate of the DA*rasA* and DN*rasA* mutants were indistinguishable from the wild type rate (data not shown). However, the DA*rasA* strain produced much more aerial hyphae, and conidiation was reduced

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Af rasA   QILRVKDKD.......................YFPIIVVGNK    122
An rasA   QILRVKDKD.......................YFPIIVVGNK    122
NC-ras1   QILRVKDRD.......................SFPMIIVGNK    122
Sc ras1   QIQRVKDSD.......................YIPVVVVGNK    124
Sc ras2   QILRVKDTD.......................YVPIVVVGNK    124
Sp ras1   QILRVKDKD.......................TFPVVLVANK    122
Af rasB   QIKMVKESAHSGSPSGASYLGS.PMNAPSGPPLPVPVMLVGNK   144
An rasB   QIKMVKESANSSSPSGASYLGS.PMSSPSGPPLPVPVMLVGNK   144
Hc rasB   QIQLVKESASSGSPTGASYLGS.PITAPS.PGMKVPVMLVGNK   143
NC-ras2   QIQRVKESTSSPS....AYPGSSPLAATN.PSAPVPIMLVGNK   141
Mg rasB   QIQRVKESCASSP....SYPGS.PIATVT.TQAPVPIMLVGNK   139
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Fig. 2. RasB homologs contain an internal amino acid insertion. Multiple sequence alignment showing conservation of the unique domain found in the RasB proteins. Sequence analysis was performed with DNAMAN software. The organisms used for comparison are *Aspergillus fumigatus* (Af), *Aspergillus nidulans* (An), *Neurospora crassa* (NC), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Histoplasma capsulatum* (Hc), and *Magnaporthe grisea* (Mg).

(Fig. 4). The DN*rasB* strain displayed abnormalities in colony morphology, having a decreased peripheral growth zone (Fig. 4). When compared with the rate of growth of the wild type organism (0.648 ± 0.0064 mm/h), the DN*rasB* and DA*rasB* strains had decreased (0.567 ± 0.0096 mm/h) and increased (0.693 ± 0.0097 mm/h) growth rates, respectively.

3.5. Activity of RasA influences the temporal and spatial organization of A. fumigatus development

The differences in colony morphology among the Ras mutants suggested an effect on the asexual developmental of *A. fumigatus*. To determine how Ras activity influences the timing of appearance and the spatial organization of asexual structures, we compared the developmental progression in cultures that were enriched for various stages of development. After 6 h of incubation, the germinating conidia of the DA*rasA* strain were more swollen and had longer germ tubes, relative to wild type (Fig. 5A). Between 18 and 24 h, the wild type initiates and completes vesicle and conidiophore development, whereas conidiophores were not present in the DA*rasA* strain until 48 h. Even after completion of development, marked morphological abnormalities were seen in conidiophore structure. The conidiophores in the DA*rasA* mutant had minimally swollen vesicles with irregular phialides that sometimes projected from the

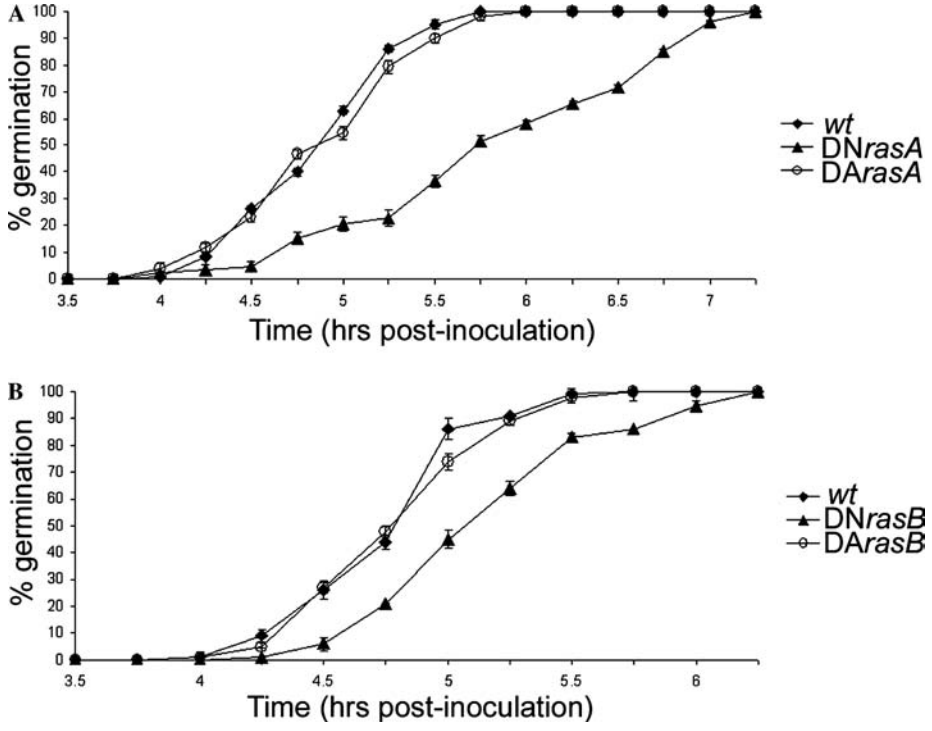

Fig. 3. Expression of DN*rasA* and DN*rasB* causes defects in germination. (A) Germination rates of *rasA* mutant strains *vs.* wild type. (B) Germination rates of *rasB* mutant strains *vs.* wild type. Data shown (means ± SE) are from three independent experiments for each strain. Germinations were carried out in media containing dextrose as the carbon source and incubations were at 37 °C with shaking at 250 rpm.

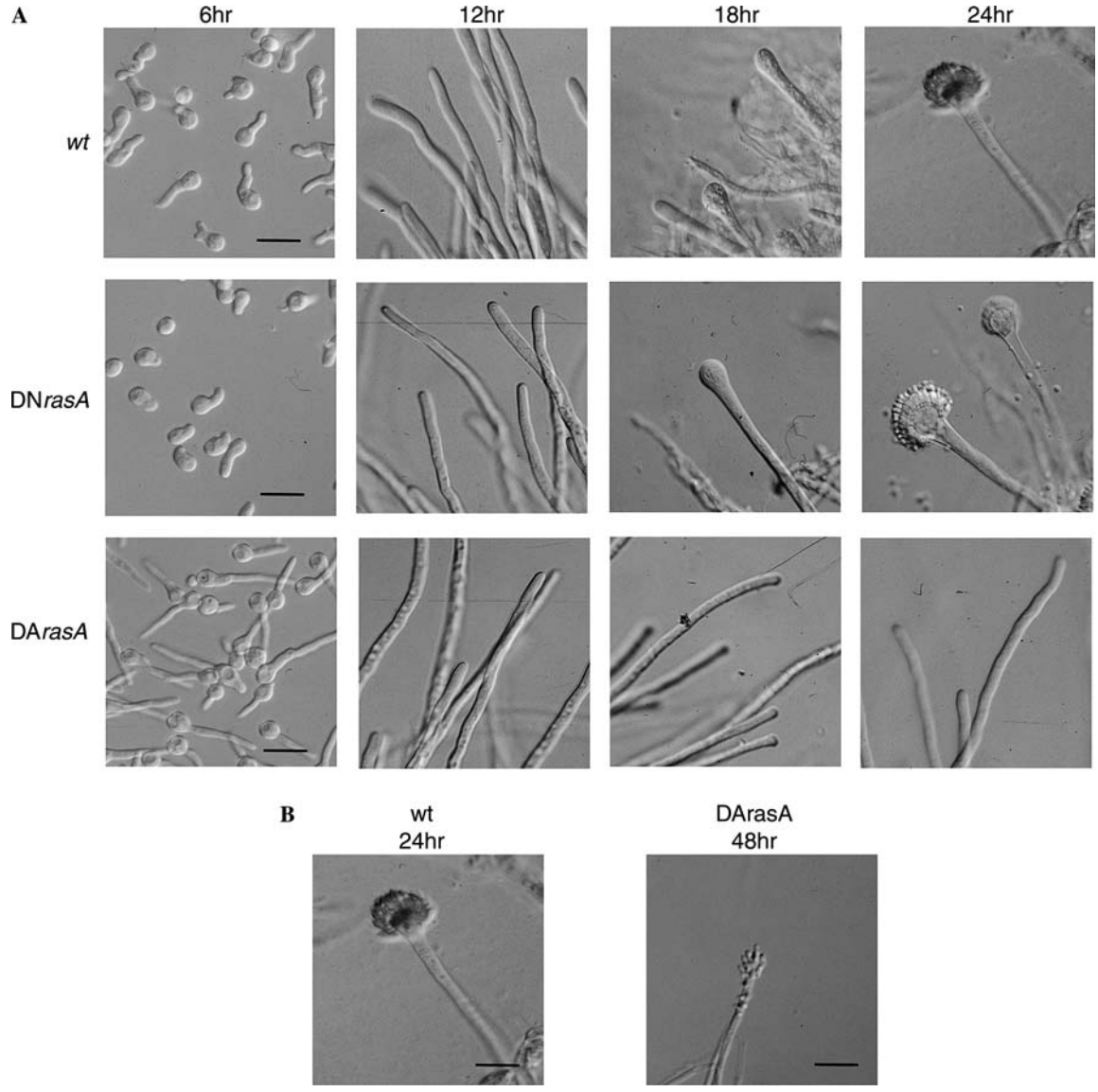

Fig. 5. Alteration of RasA activity affects the timing and morphology of development. (A) Developmental progression of wild type vs. DN*rasA* and DA*rasA* mutants. (B) Comparison of conidiophores of wild type and DA*rasA* strains at 24 and 48 h, respectively. Scale bar = 10 μm. All pictures were taken at the same magnification.

conidiophore stalk (Fig. 5B). Asexual development of the DN*rasA* mutant was identical to that of wild type (Fig. 5A). These data support a role for properly regulated RasA in asexual development.

3.6. RasB activity influences both hyphal morphology and conidiophore development

The initial steps in development in the DA*rasB* strain appeared normal through 12 h. Subsequently, the conidiophores that formed were smaller and the phialides, although properly positioned, were longer than wild type and fewer in number than in the wild type (Fig. 6). After 6 h of germination, the germlings of *rasB* mutants were morphologically indistinguishable from wild type. But by 12 h, the DN*rasB* strain exhibited a defect in hyphal morphology characterized by irregular apical branching (Fig. 6). In this mutant, conidiophore development was comparable to wild type. These data suggest that normal RasB activity is required for proper hyphal morphology and asexual reproduction.

3.7. DNrasB promotes asexual differentiation in submerged culture

Since expression of some of the mutant Ras genes caused obvious aberrancies in conidiophore morphology in developmental cultures, each mutant was tested for inappropriate initiation of asexual reproduction in submerged culture. In contrast to wild type, the DN*rasB*

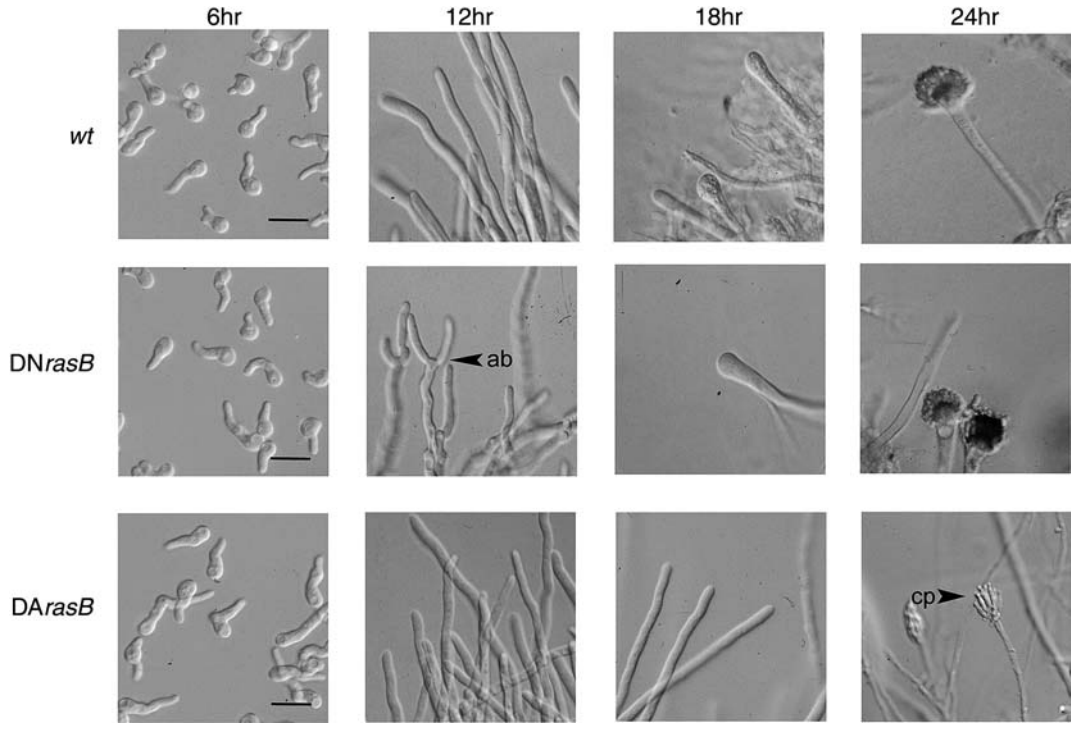

Fig. 6. RasB activity influences hyphal morphology and conidiophore development. Developmental progression of wild type and mutant *rasB* strains (as described in Fig. 5). Arrowheads denote apically branched hyphae (ab) and conidiophore (cp). Scale bar = 10 μm. All pictures were taken at the same magnification.

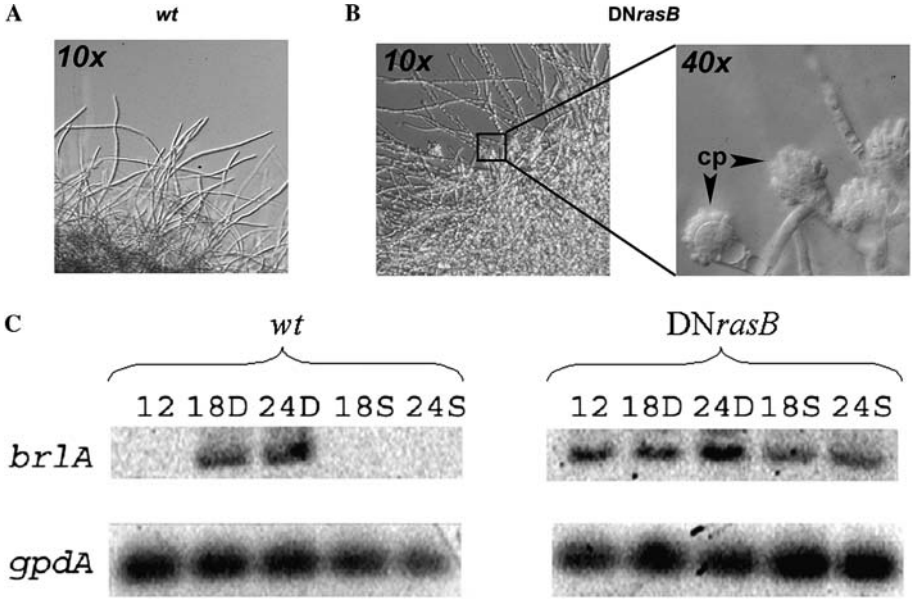

Fig. 7. DN*rasB* mutants produce conidiophores in submerged culture. (A) Wild type *A. fumigatus* (10×) after 24 h of growth in submerged culture. (B) DN*rasB* (10× and 40×) after 24 h of submerged growth, showing fully developed conidiophores. Arrows denote conidiophores (cp) developed in submerged culture. (C) Reverse transcription-PCR analysis of *brlA* expression in wild type (wt) and DN*rasB* strains following 12 h submerged growth and under both developmental (D) and submerged (S) culture conditions at 18 and 24 h, repectively.

strain formed fully differentiated conidiophores with conidia in submerged culture within 24 h (Fig. 7B). Asexual development is a complex process in *Aspergillus* species, requiring the timed expression of many genes. Activation of the primary regulator of this process in *A. nidulans*, the transcription factor encoded by *brlA*, is

both necessary and sufficient to induce conidiophore development (Adams et al., 1988, 1998). As a molecular marker for this event, RT-PCR analysis revealed that expression of the *A. fumigatus brlA* transcript in the DN*rasB* strain was detectable in submerged culture at the 12-, 18-, and 24-h timepoints (Fig. 7C), whereas *brlA* expression in the wild type was only observed under developmental culture conditions at 18 and 24 h (Fig. 7C), as would be expected.

3.8. DArasA displays alterations of mitosis

Because introduction of an activated form of *rasA* has been shown to cause initiation of mitosis in *A. nidulans* (Som and Kolaparthi, 1994), nuclear division and distribution during the early stages of growth (prior to septation) were examined for each strain. After 4 h of culture in YG medium, wild type conidia contained one to two nuclei, whereas conidia from the DA*rasA* strain contained up to 8 nuclei (Fig. 8A). After two additional hours of culture, the wild type strain had completed an average of two rounds of mitosis (~4 nuclei/germling). At the same time, the DA*rasA* mutant contained many more nuclei, with an average of 16 nuclei/germling (Fig. 8B). In contrast to the DA*rasA* strain, the DA*rasB* mutant showed no abnormalities in the initial stages of mitosis when compared to the wild type (data not shown).

4. Discussion

Ras proteins are highly conserved molecular switches that regulate a wide variety of cellular processes and are found in all eukaryotes. Homologs of *rasA* have been found throughout the fungal kingdom, whereas *rasB* homologs appear to be unique to fungi that grow as filamentous organisms during, at least, part of their life cycle. The filamentous fungi share several growth and differentiation processes, including growth by apical extension, development of branching hyphae, and formation of asexual developmental structures, suggesting a role for RasB in one or more of these activities. The RasB proteins contain a conserved 20 amino acid insertion that is absent in RasA. Phylogenetic analysis revealed that the RasB proteins are more closely related to Rsr1p of *S. cerevisiae* than to other fungal Ras subfamily proteins. This finding is interesting in that Rsr1p, like RasB, contains an internal amino acid extension not found in Ras1p or Ras2p. The similarities in sequence and conserved location among the different fungi suggest that this domain contributes to the unique functions of the RasB proteins.

Conidial germination of *A. nidulans* can be initiated by a variety of carbon sources, but does not occur in the presence of the non-metabolizable glucose analogs 2- or 6-deoxyglucose or with the addition of exogenous cAMP in the absence of a carbon source (Osherov and May, 2000). In contrast, we found that both 2- and 6-deoxyglucose initiated germ tube formation in *A. fumigatus* at efficiencies similar to those seen with metabolizable carbon sources (Table 2) and that exogenous cAMP could support the early stages of *A. fumigatus* germination. Growth in the presence of either of the glucose analogs or of cAMP was not maintained for longer than 30 h (data not shown). These finding suggest that the non-metabolizable glucose analogs and exogenous cAMP act as signals to activate molecular pathways important for initiation of germination but are insufficient to sustain growth. In support of this interpretation, Fillinger et al., showed that the addition of 6-deoxyglucose to conidia of *A. nidulans* activates trehalose breakdown, a pre-germination event in fungi, but does not lead to germ tube formation (Fillinger et al., 2002), as shown with *A. fumigatus*. From these data, it appears that these two closely related fungi differ in signaling downstream of carbon source recognition and that these differences precede the step leading to germ tube emergence.

To delineate further the role of Ras activity in the asexual developmental program of *A. fumigatus*, site directed mutagenesis was employed to produce strains expressing constitutively active and inactive transgenes of *rasA* and *rasB* in a wild type background. Although strains expressing inactivated forms of either Ras protein had defects in germination, they were not the same defects. Strains expressing DN*rasA* displayed an overall slower rate of germination, whereas DN*rasB* strains seemed only to exhibit an initial lag in germination, progressing at the same rate once the process had begun. These findings are in keeping with studies from *A. nidulans* that show that low-level expression of an inactivated form of *rasA* leads to slower germination rates (Som and Kolaparthi, 1994). Deletion of components of the Ras2 signaling pathway of *S. cerevisiae* also impairs ascospore germination (Herman and Rine, 1997), supporting a conserved role for Ras proteins in the germination of fungal spores and conidia.

Introduction of the mutant transgenes, with the exception of DN*rasA*, produced remarkable macroscopic and microscopic phenotypes. Expression of DA*rasA* induced overproduction of aerial hyphae and greatly reduced conidiation. Microscopic analysis revealed swollen conidia that emitted narrow germ tubes, aberrant conidiophore morphology, and a delay in the completion of conidiophore development. It has been reported that expression of varying levels of DA*rasA* in *A. nidulans* induces aerial hyphae formation, reduces conidiation, and causes initiation of mitosis while inhibiting germ tube formation (Osherov and May, 2000; Som and Kolaparthi, 1994). One difference reported here is that expression of DA*rasA* in *A. fumigatus* did

not lead to inhibition of germ tube formation. This difference between the fungi may be explained in part by the use of the endogenous promoter for the DA*rasA* transgene in *A. fumigatus* compared with expression from the *alcA* promoter used for *A. nidulans*. Endogenous expression levels would be expected to be lower and appropriately regulated when compared with those achieved by a*lcA* induction (Osherov and May, 2000; Som and Kolaparthi, 1994).

The events of mitosis, germ tube formation, nuclear migration and septum formation occur in an ordered fashion during the early stages of growth in *A. fumigatus* and *A. nidulans* (Momany and Taylor, 2000). Expression of DA*rasA* led to an uncoupling of the timing of mitosis and germ tube formation in *A. fumigatus*, which resulted in accumulation of many nuclei in the cells. However, the production of septa and migration of nuclei along hyphae was not affected in our studies (data not shown). This finding implies that, in *A. fumigatus*, initiation of cell cycle lies downstream of Ras signaling (Pichova et al., 1997), whereas migration of nuclei and formation of septa may reside in a parallel pathway.

Expression of *rasB* mutant transgenes also produced notable phenotypic abnormalities. Strains expressing DA*rasB* exhibited abnormal conidiophore morphology and decreased conidiation, although the phenotypes were milder than those seen with DN*rasA* expression. Because *rasB* expression is very low, this difference could be explained by activation of different pathways or by different levels of activation of the same pathways. Expression of DN*rasB* did not impair conidiation but caused a decreased growth rate and a hyperbranching phenotype in submerged growth. This phenotype is similar to that displayed by the *N. crassa* mutant lacking functional RasB homolog, *NC-ras2*. This mutant had defects in hyphal morphology and cell wall biosynthesis that led to a decrease in growth rate when compared with wild type *N. crassa* (Kana-uchi et al., 1997). However, this mutant also displays defects in conidium formation (Kana-uchi et al., 1997), a process that seemed unaffected in the *A. fumigatus* DN*rasB* strains. Further analysis of DN*rasB* strain growth revealed inappropriate development of conidiophore structures. In nutrient rich culture, conidiophore generation in*Aspergillus* species requires a specified growth time to reach competency and exposure to an air–liquid interface (Adams et al., 1988, 1998). After 18 h of growth in submerged culture, the DN*rasB* strain developed fully differentiated conidiophores that produced conidia. This morphological observation was correlated with the expression of the transcription factor, *brlA*, known to be necessary for proper completion of the asexual developmental process (Adams et al., 1998). The RasB molecular switch appears to be involved in the pathway regulating conidiophore production.

In summary, these studies have led to the identification of a novel class of Ras genes. Similar to findings with *A. nidulans* and *N. crassa*, Ras proteins in *A. fumigatus* seem to play a role in germination, hyphal formation and asexual differentiation. However, in contrast to data from *A. nidulans*, *A. fumigatus* conidia germinate in non-metabolizable carbon sources, revealing differences between the model organism, *A. nidulans*, and the human pathogen, *A. fumigatus*, in the control of germ tube emergence. In addition, the finding that expression of constitutively active RasA leads to uncoupling of mitosis and hyphal growth in germlings, suggest that these conserved signaling proteins play an important role in coordinating the landmarks of vegetative growth and asexual development.

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