Localization of RHO-4 Indicates Differential Regulation of Conidial versus Vegetative Septation in the Filamentous Fungus *Neurospora crassa*\(^\dagger\)

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**rho-4** mutants of the filamentous fungus *Neurospora crassa* lack septa and asexual spores (conidia) and grow slowly. In this report, localization of green fluorescent protein-tagged RHO-4 is used to elucidate the differences in factors controlling RHO-4 localization during vegetative growth versus asexual development. RHO-4 forms a ring at incipient vegetative septation sites that constricts with the formation of the septum toward the septal pore; RHO-4 persists around the septal pore after septum completion. During the formation of conidia, RHO-4 localizes to the primary septum but subsequently is relocalized to the cytoplasm after the placement of the secondary septum. Cytoplasmic localization and inactivation of RHO-4 are mediated by a direct physical interaction with RDI-1, a RHO guanosine nucleotide dissociation inhibitor. Inappropriate activation of the cyclic AMP-dependent protein kinase A pathway during vegetative growth causes mislocalization of RHO-4 away from septa to the cytoplasm, a process which was dependent upon RDI-1. An adenylate cyclase *cr-l* mutant partially suppresses the aconidial defect of *rho-4* mutants but only rarely suppresses the vegetative septation defect, indicating that conidial septation is negatively regulated by CR-1. These data highlight the differences in the regulation of septation during conidiation versus vegetative septation in filamentous fungi.

Cytokinesis couples the completion of mitosis with the compartmentalization of two individual cells. In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, well-characterized signal transduction pathways coordinate cytokinesis with the end of nuclear division. These pathways are referred to as the mitotic-exit network (3) and septation initiation network (20), respectively. In hyphae of the euascomycete species *Aspergillus nidulans*, mitosis and septation are linked, as indicated by mitotic defects in several septation mutants (5, 15). Although there is a coordination between mitosis and septation in *A. nidulans*, mutants have been isolated which bypass the need for this coordination (19). Because viable asciate mutants can be isolated from *A. nidulans* and other filamentous fungi, such as *Ashbya gossypii* and *Neurospora crassa* (2, 19, 31, 49), these organisms provide excellent systems for studying the process of cytokinesis.

Septa found in multinucleate vegetative hyphae of filamentous ascomycete species, such as *A. nidulans* and *N. crassa*, have important characteristic differences between those in fission and those in budding yeasts. Most notably, septa have a prominent septal pore, allowing the flow of cytoplasm and organelles, including nuclei, between hyphal compartments (14, 16). Thus, unlike most eukaryotic organisms, vegetative septa of filamentous ascomycete species do not separate cytoplasm and nuclei into distinct daughter compartments upon completion of nuclear division. The main purposes of septa are (i) to increase the structural integrity of hyphae; (ii) to provide a scaffold for the Woronin body, a structure which plugs septa upon injury to prevent excessive cytoplasmic leaking (17, 44); and (iii) to divide the mycelium into distinct compartments for developmental processes such as sexual structure formation and asexual spore production (14).

The formation of asexual spores, termed “conidiation,” is a developmental program occurring during the vegetative phase of growth in filamentous ascomycete fungi. *N. crassa* produces three types of asexual spores, macroconidia, microconidia, and arthroconidia (8). Macroconidiation (hereafter referred to as conidiation) begins with the development of aerial hyphae, followed by the formation of minor and major constrictions and the construction of single septa. A second septum is subsequently formed between conidia (referred to as the “double-doublet” stage). The conidia are held together by connective material which is eventually digested to release free conidia (43). In *N. crassa*, several mutants, such as the *fluffy* mutant, which encodes a zinc-finger transcription factor (32), or *acon-2* (23), are blocked before the formation of the major constrictions. Other mutants have late-stage defects in the conidiation pathway such as the conidial separation *csp-1* and *csp-2* mutants. The *csp* mutants, because they have no defects in vegetative septation or sexual development, are conidiation-specific mutants (39).

Recently, a small monomeric Rho-type GTPase has been shown to play a role during septation with both *S. pombe* and *N. crassa* (27, 31, 37). In *S. pombe*, a *rho4Δ* mutant makes multiple septa at elevated temperatures (27, 37) due to a cell separation defect caused by the inability to target glucanases to the septum (38). In contrast, *N. crassa rho-4* mutants do not form the F-actin ring required for septum formation and therefore lack septa (31). Rho-type GTPases are characterized by binding and hydrolysis of GTP and plasma membrane local-
ization via a conserved prenylation domain (11). Rhotype GTPases can be negatively regulated by guanosine nucleotide disassociation inhibitors (GDIs). GDI proteins have an immunoglobulin-like beta sandwich domain that envelopes the lipid-modified C terminus of Rhotype GTPases and forms an inactive cytoplasmic GDI-Rho complex. The GDI-Rho complex is inactive because membrane localization is required for Rhotype GTPase activity (9, 10).

In this paper, we show differential localization of RHO-4 during vegetative septation formation and during conidiation in N. crassa, indicating that different factors regulate septation during these distinct developmental programs. During vegetative septum formation, RHO-4 localization changes during the process of conidiation from primary septa to the cytoplasm during the double-doublet stage. Cytoplasmic localization of RHO-4 is dependent on its guanine nucleotide disassociation inhibitor, RDI-1; RHO-4 localization in rdi-1 mutants occurred primarily at the plasma membrane. Activation of the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway alters RHO-4 localization from septa to the cytoplasm, suggesting a role for the PKA pathway in negatively regulating septation in filamentous fungi.

### MATERIALS AND METHODS

**Strains, media, and growth conditions.** The strains used in this study are listed in Table 1. Strains were grown on Vogel's medium with the required supplements (47). Westergaard’s medium (50) was used for crossing. Linear growth of strains was determined by growth in race tubes at room temperature (35). Heterokaryons between mutant strains and Fungal Growth Stock Center (FGSC) 4564 a was used as female partners in most crosses in order to complement protoperithecial development defects or auxotrophic markers (28).

**DNA and RNA extraction.** DNA extraction was performed as described previously (21). Standard approaches for Southern blotting, cloning, and overlap PCR were used (36). FGSC 988 DNA was used as a template for PCR unless otherwise indicated. Sequencing was performed by the DNA Berkeley Sequencing Facility (http://mcb.berkeley.edu/barker/dnaseq). Primers used in this study are listed in Table S1 in the supplementary material. Total RNA was prepared using Trizol reagent (Invitrogen) from frozen mycelia. Reverse transcription (RT) was performed using RT Transcriptor (Roche). PCR amplification using primers RHO-4FOR646 and RHO-4REV1963. RT-PCR (RT) was performed using RT Transcriptor (Roche). PCR amplification using primers RHO-4FOR568 and BamH1RHO-4REV was followed by a second PCR amplification with primers RHO-4FOR646 and RHO-4REV1963. RT-PCR products were cloned into TOPO Blunt (Invitrogen), and their DNA sequences were determined.

**Strain construction.** A genetic method was used to introduce various constructs into a rho-4 mutant background, as described previously (31). Briefly, constructs were cloned into either pBM61 or PMF272 for targeting to the his-3 locus (12, 22) and introduced into FGSC 6103 (his-3) by electroporation (22). Transformed strains were crossed to rho-4 strain CR19-46 (Table 1), and progeny with the desired genotype were identified.

Overlap PCR was used to make an in-frame fusion between gfp (amplified from pMF272 with GFPCORPacI and GFPRevNoStop) and rhl-4 (amplified with primers GFPposRHO-4FOR and BamHI-RHO-4REV). This fragment was cloned into TOPO Blunt (Invitrogen). A PacI/EcoRI fragment containing the green fluorescent protein (GFP) fusion was cloned into pMF272. CR26-9 (Table 1) was used to cross gfp-rhl-4 into cwl-1, cwl-2, csp-1, csp-2, mcb, cr-1, and rdi-1 mutants. Strains containing cwl-1, cwl-2, csp-1, csp-2, cr-1, or mcb mutations were screened by morphology (4, 13, 30, 39, 45).

The knockout cassette for NCU06561 (rdi-1) was provided by Hildur Colot, and a ΔNCU06561 strain was generated using methods outlined in reference 7. One rdi-1 mutant from each mating type (CR59-2 A and CR59-4 a) can be obtained from the FGSC (FGSC 11144 and FGSC 11145, respectively).

### TABLE 1. Strains used in this study

| Strain no. | Genotype                                      | Reference/origin |
|-----------|-----------------------------------------------|------------------|
| FGSC 988  | ORS8-1 a                                      | FGSC             |
| FGSC 2489 | OR74 A                                        | FGSC             |
| FGSC 2522 | csp-2 a                                       | FGSC             |
| FGSC 2555 | csp-1 a                                       | FGSC             |
| FGSC 4564 | ad-3B cyh-1 a^m^                             | FGSC             |
| FGSC 5068 | cr-1 a                                        | FGSC             |
| FGSC 5951 | (cwl-1 A + ad-3B cyh-1 a^m^)                 | FGSC             |
| FGSC 6103 | his-3 A                                       | FGSC             |
| FGSC 6875 | (cwl-2 A + ad-3B cyh-1 a^m^)                 | FGSC             |
| FGSC 7453 | mcb a                                         | FGSC             |
| FGSC 9719 | mus-52::bar a                                 | FGSC             |
| CR6561-1  | rdi-1::hph; mus-52::bar a                     | This study       |
| CR5-10    | ad-3A his-3; rho-4 A                          | 31               |
| CR19-46   | Sad-1::hph; rho-4 A                           | 31               |
| CR21-12   | his-3::Pgpd-HA-rho-4; rho-4 A                 | 31               |
| CR26-9    | Sad-1::hph his-3::Pcclg-sgfp-rho-4; rho-4 A   | CR26-9 × FGSC 5951 |
| CR34-2    | Sad-1::hph his-3::Pcclg-sgfp-rho-4; cwl-1 A   | CR26-9 × FGSC 7453 |
| CR48-1    | Sad-1::hph his-3::Pcclg-sgfp-rho-4; mcb A     | CR26-9 × FGSC 2555 |
| CR54-1    | Sad-1::hph his-3::Pcclg-sgfp-rho-4; csp-2 A   | CR26-9 × FGSC 2555 |
| CR55-1    | his-3::Pcclg-sgfp-rho-4; csp-1 A              | CR5-10 × FGSC 2489 |
| CR56-1    | rho-4 a                                       | CR26-9 × 6561-1  |
| CR58-4    | Sad-1::hph his-3::Pcclg-1-sgfp-rho-4; rdi-1::hph A | CR26-9 × 6561-1  |
| CR59-2    | rdi-1::hph A                                  | This study       |
| CR59-4    | rdi-1::hph a                                  | This study       |
| CR69-1    | cr-1; rho-4 a                                 | FGSC 5068 × CR26-9 |
| CR69-61   | Sad-1::hph his-3::Pcclg-sgfp-rho-4 cr-1 A     | FGSC 5068 × CR26-9 |
| GF-RHO-4-11| his-3::Pcclg-sgfp-rho-4 A                    | 31               |
| P21-12-6  | his-3::Pcpg-HA-rho-4; rho-4; Pd1-1-rdi-1-sgfp A | CR21-12          |
| P6103-5   | his-3::Pd1-1-rdi-1-sgfp A                     | This study       |

* ×, genetic crosses.

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FGSC 11144 and FGSC 11145, respectively.

One rdi-1 mutant from each mating type (CR59-2 A and CR59-4 a) can be obtained from the FGSC.
using primers SpeIRdi1-295FOR and PacIRdi-1REV that were cloned into TOPO Blunt (Invitrogen) and then subcloned into SpeI/PacI-digested pMP272 to create Pccg–Prdi1–rdi-1–sgfp. The cg–1 promoter sequence was removed by digestion with XbaI/NcoI, and blunt ended by treatment with Klenow polymerase (New England Biolabs). Prdi1–rdi-1–sgfp was cloned into pMP272 (12) and used for transformation to create Pr6103-5 (Table 1). Prdi1–rdi-1–sgfp was cloned into pCB1004 (6) and transformed into CR21-12, and the hygromycin-resistant transformants P21-12-6 was selected (Table 1).

**Coimmunoprecipitation.** Coimmunoprecipitation was performed using the protocol provided by Yi Liu (http://www.fgs.net/Neurospora/NeurosporaProtocollGuides.htm) with the following modifications. Complete mini-EDTA-free protease tablets (Roche) were used. Two milligrams of protein extracts was incubated with the primary antibody (dilution of 1:200 mouse anti-hemagglutinin [HA] clone 12CA5 or mouse anti-GFP clones 7.1 and 13.1 [Roche]) overnight at 4°C. Protein extracts (100 μg input) were loaded, and proteins were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Immunoblotting was performed as described previously (36).

**Microscopy.** Conidia or mycelia were inoculated onto Vogel's MM plates (47) and grown for 1 or 2 days. Squares of agar were cut out, placed on a glass slide, and covered with a coverslip. When it was used, calcofluor (100 μg/ml) was added to samples before coverslip placement. Photographs of hyphae and germinating conidia were taken using a Hamamatsu digital charge-coupled-device camera (Hamamatsu, Japan) and a Zeiss Axioskop II microscope. The files were further processed with Adobe Photoshop 7.0. Alternatively, samples were inverted onto a coverslip and examined using an API Delta Vision DV4 system (Applied Precision Instruments). Samples were optically sectioned using 0.2-μm Z-steps with the filter sets appropriate for either GFP (fluorescein isothiocyanate) or calcofluor. As shown in Fig. 2G to J, the images were deconvolved using Resolve3D function from SoftWoRx version 3.3.1 software, using a conservative ratio, point spread function, and fifteen iterations. For the image shown in Fig. 2H, the Volume-Viewer function of SoftWoRx was used to rotate the stacks around the axis of the septum.

At least six different conidiophores from FGSC 5068 (cr-1) and CR69-1 (cr-1; rho-4) were assessed for conidia and septa counts by using Openlab (Coventry, United Kingdom). Fisher's exact test was used for statistical analyses (http://www.matsforsk.no/ola/fisher.htm). Analysis of hyphal compartment lengths in wild-type (WT) and rdi-1 mutant (CR59-4) cells was performed as described previously (31).

Comparative fluorescence intensities for Pr6103-5 (Prdi1–rdi-1–sgfp [Table 1]) were measured using Openlab (Improvision). Exposure time was set to 200 ms. The interior hyphal pixel intensities (with the background subtracted) were arbitrarily set to 1 for comparison with tip hyphae or conidia intensities. The fluorescence intensity was measured using an average of more than eight samples.

**Cytochalasin-A treatment.** CR26-9 conidia were grown in 20 ml of Vogel's MM for 12 to 17 h at 30°C and 250 rpm. The sample was split into two equal aliquots. One sample was treated with 14 μg/ml (29.32 μM) cytochalasin-A in dimethyl sulfoxide (DMSO), while the other was given DMSO only (46). Micrographs were taken at 15-min intervals over 2 h for cytochalasin-A-treated and control samples. This experiment was repeated three times with similar results.

**RESULTS**

* N. crassa* RHO-4 has an 86-amino-acid N-terminal extension. The protein structure of RHO-type GTPases is highly conserved and consists of GTP binding domains, a polybasic domain (41), and a prenylation domain at the C terminus (a CAAX box) for membrane localization (42). Inspection of the *N. crassa* predicted RHO-4 protein (NCU03407) showed conservation of these features (Fig. 1) but also a possible 86-aa N-terminal extension consisting of primarily serine (S), threonine (T), and alanine (A) residues. We first used nested RT-PCR (see Materials and Methods) to determine the correct start site of the rho-4 ORF; amplification products suggested that translation of rho-4 began after the predicted start site (http://mips.gsf.de/genre/proj/nccrassa/). To verify this result, stop codons were introduced into the predicted ORF by site-directed mutagenesis at Q16* and E79* (Fig. 1). The introduction of constructs containing the Q16*–rho-4 allele or the E79*–rho-4 allele into a rho-4 mutant (CR-56-1 or CR19-46; see Materials and Methods) failed to fully complement the rho-4 mutant phenotype, unlike the introduction of the WT rho-4 allele. Together, these data indicate that translation of rho-4 begins at the first methionine (Fig. 1); rho-4 encodes a 337-aa protein.

GFP–RHO-4 shows dynamic localization during septation in vegetative hyphae. HA epitope-tagged RHO-4 (HA–RHO-4) was undetectable by immunofluorescence when the rho-4 promoter was used (data not shown). However, HA–rho-4 driven by a gpd promoter (12) fully complemented a rho-4 mutant and was shown by immunofluorescence to localize to septa and the plasma membrane (31). To examine the dynamics of RHO-4 localization in vivo, a Pccg1 gfp–rho-4 allele was introduced into a rho-4 strain (CR26-9; Table 1). As with the HA–rho-4 allele, both the septation and growth defects of the rho-4 mutant were fully complemented by the introduction of gfp–rho-4. GFP–RHO-4 localized at the plasma membrane prior to visible septum formation (Fig. 2) and constricted toward the center of a hypha during septum formation (Fig. 2C to F). In fully developed septa, GFP–RHO-4 formed a persistent and prominent ring at the septal pore (Fig. 2H and I). Similar to observations of RHO-4 localization by immunofluorescence microscopy (31), GFP–RHO-4 fluorescence was observed at all completed septa. In addition to localization at septa, cytoplasmic and plasma membrane-localized GFP–RHO-4 fluorescence was also observed (Fig. 2J).

To determine whether overexpression of gfp–rho-4 may cause mislocalization of GFP–RHO–4 in vegetative hyphae, we assessed localization of GFP–RHO–4 under conditions where the ccg–1 promoter activity is moderate (using solid medium and 2% sucrose) versus conditions where ccg–1 promoter activity is repressed (using liquid medium and 2% sucrose). The ccg–1 promoter is repressed by a number of environmental signals including glucose (25) and growth in liquid medium (12). Although the GFP–RHO–4 signal in liquid medium was very faint in CR26-9 (data not shown), it showed a GFP–RHO–4 localization pattern identical to that of CR26-9 grown on an agar plate (as described above).

An intact actin cytoskeleton is required for the localization of GFP–RHO–4 to septal initiation sites but is not required for its maintenance at completed septa. Initiation of septation in fungi is characterized by the formation of an actin ring, which then constricts upon the deposition of septal wall material (20, 48). Previously, we showed that rho-4 mutations abolish the localization of actin rings at sites of septation in *N. crassa* (31). Treatment with cytochalasin-A, a drug which disrupts the polymerization of F-actin, has been used to show that F-actin is required for septum formation in fungi (15, 26) as well as for the localization of the septation machinery to sites of septation (37, 40). We therefore assessed whether *N. crassa* rho-4 localization to completed septa (maintenance) or to sites of septation initiation was dependent on an intact F-actin cytoskeleton. We define “sites of imminent septation” by the characteristically wide GFP–RHO–4 ring at the plasma membrane that precedes septum deposition. The location of a new septum in *N. crassa* hyphae prior to GFP–RHO–4 localization cannot be predicted with the tools currently available. Perturbation of the F-actin cytoskeleton in CR26-9 (gfp–rho-4) hy-
phe phycytchalin-A resulted in swollen tips and “starburst”-patterned hyphae as previously reported (1, 34). In DMSO-only treated samples, we observed a wide ring of GFP–RHO-4 localization at sites of imminent septation in 7% of 76 samples (Fig. 3A and B, arrowhead), in addition to GFP–RHO-4 fluorescence at all completed septa. By contrast, GFP–RHO-4 rings were never observed in cytochalasin-A-treated samples in the absence of observable septa (0/80; P = 0.03). However, GFP–RHO-4 localization at completed septa was indistinguishable between cytochalasin-A-treated samples and that of DMSO-only controls (data not shown). These data indicate that F-actin is not required for the maintenance of GFP–RHO-4 at completed septa but is required for the localization of GFP–RHO-4 to sites of imminent septation.

To further explore requirements for RHO-4 localization to sites of imminent septation, we evaluated RHO-4 localization in two aseptate mutants, cwl-1 and cwl-2 (13, 30). GFP–RHO-4 formed ring-like structures at presumptive sites of septation in the cwl-1 strain CR34-2, although the ring morphology was often irregular (Fig. 4B, arrowheads). These observations indicate that CWL-1 is not required for GFP–RHO-4 localization to septation sites but may be involved in the stabilization of RHO-4 at these sites. Unlike cwl-1 mutants, cwl-2 mutants containing the gfp–rho-4 cassette completely lacked GFP–RHO-4 rings (data not shown). These data indicated that the cwl-2 gene product is required for RHO-4 localization to sites of imminent septation.

GFP RHO-4 localization in conidiophores and conidial separation csp-1 and csp-2 mutants.

In addition to the lack of vegetative septa, rho-4 mutants do not produce conidia, conidiophores, or aerial hypha (31). However, conidiation requires septum formation in N. crassa, suggesting that RHO-4 may also play a role in this process. Conidial septa are morphologically distinct from vegetative septa; conidia are separated by two complete septa which are not perforated by a septal pore (43). Additionally, release of conidia requires the digestion of the connective material between these two septa. In the WT (CR26-9), GFP–RHO-4 fluorescence was observed at primary conidial septa but was mostly localized to the cyto-
plasm in maturing conidia after the formation of the double septum ("double doublet") (Fig. 5A to C). Thus, in contrast to the persistent localization of GFP–RHO-4 at vegetative septa, RHO-4 was lost from conidial septa sometime during the septation process.

We sought to determine the developmental step at which GFP–RHO-4 became cytoplasmically localized during conidiation by using the csp-1 and csp-2 mutants (39). The csp-1 mutant is slow to complete conidial development and makes single or double septa less often than the wild type. The csp-2 mutant, because it is unable to digest connective material between conidia, is blocked at the double-septum stage (43). Both mutants rarely form free conidia. GFP–RHO-4 localization in the csp-1 mutant, CR55-1 (csp-1; gfp–rho-4) was observed at primary conidial septa (Fig. 5D to F) but was cytoplasmically localized in conidia abutting rare double doublets (data not shown). In the csp-2 mutant, CR54-1 (csp-2; gfp–rho-4), GFP–RHO-4 was mostly localized to the cytoplasm at the double-doublet stage (Fig. 5G to I). These data indicate that RHO-4 localizes to conidiation-specific single septa and becomes cytoplasmically localized at some point during or after the formation of the septum.

FIG. 2. GFP–RHO-4 localization in vegetative hyphae. (A) Differential interference contrast image of a hypha with a completed septum (arrow) and an incipient septum (arrowhead). (B) Fluorescence image of the GFP–RHO-4 localization in hypha in panel A. Note that GFP–RHO-4 localization appears as a wide ring at the incipient septum site and is constricted in the completed septum. (C to F) Time course of GFP–RHO-4 localization by fluorescence microscopy during septum formation. GFP–RHO-4 localized to an incipient septum defined as time zero (C), 2 min (D), 4 min (E), and 9 min (F). (G to J) Deconvolved fluorescence images of GFP–RHO-4 localization in hyphae. (G) An optical slice of a hypha early in septation shows GFP–RHO-4 localization at the plasma membrane. (H) Calcofluor-stained (blue) hypha reconstructed from optical sections into a three-dimensional view and rotated such that the septal pore is obvious. The completed septum shows bright GFP–RHO-4 localization (green) at the septal pore. (I) GFP–RHO-4 localization around the septal pore (arrow). (J) GFP–RHO-4 fluorescence was also observed at the plasma membrane and in the cytoplasm. Bar, 10 μm (except for panel H, where the bar is 5 μm.

FIG. 3. Localization of GFP–RHO-4 after treatment with cytochalasin-A. (A) Differential interference contrast (DIC) and (B) corresponding fluorescence images of GFP–RHO-4 at an imminent septation site (A and B, arrowheads) in a sample without cytochalasin-A treatment. (C) DIC micrograph shows a completed septum and (D) corresponding fluorescence image showing GFP–RHO-4 localization at completed septa in cytochalasin-A-treated sample. Bar, 10 μm.
second septum but before the degradation of the connective material (cell separation).

**RDI-1** is a guanosine nucleotide disassociation inhibitor (GDI) of RHO-4. Cytoplasmic localization of RHO-4 in conidia suggested that a negative regulator removed RHO-4 from the plasma membrane, thus inactivating it. A potential negative regulator for RHO-4 was RDI-1, a GDI similar to the S. pombe Rdi1 (27). We hypothesized that mutations in the *N. crassa* ortholog of *rdi-1* might alter RHO-4 localization in *N. crassa*. The best bidirectional hit from the *S. pombe* Rdi1 in the *N. crassa* genome was the predicted protein encoded by NCU06561. NCU06561 is the only protein predicted to be similar to Rdi1 in the *N. crassa* genome, suggesting that it may function as a GDI for all of the small monomeric GTPases, including RHO-4.

The NCU06561 ORF was disrupted with a hygromycin cassette (CR59-4; see Materials and Methods). Consistent with its predicted role as a negative regulator of septation, the *rdi-1* mutant (CR59-4) had dramatically decreased hyphal compartment lengths (Fig. 6B) and a slow growth rate of mutant (CR59-4) had dramatically decreased hyphal compartment lengths (Fig. 6B) and a slow growth rate of the wild type (compare Fig. 2B with Fig. 7B). In immature conidia and conidiophores of CR58-4, GFP–RHO-4 localized to single septa and to the plasma membrane. At the double-doublet stage of conidiation, GFP–RHO-4 was observed in the plasma membrane of the *rdi-1* mutant (Fig. 5J to L), in contrast to the wild type, which exhibited cytoplasmic GFP–RHO-4 localization (Fig. 5A-C). Additionally, an apparent endomembrane localization of GFP–RHO-4 was revealed in the *rdi-1* mutant background (Fig. 5K, arrow).

Based on the plasma membrane localization of GFP–RHO-4 in the *rdi-1* mutant, we predicted that RDI-1 physically interacts with RHO-4 to remove it from the plasma membrane. A strain bearing an HA epitope-tagged *rho-4* allele, CR21-12 (31), was transformed with *rdi-1–gfp* to create strain P21-12-6 (see Materials and Methods). Protein extracts were isolated from strain P21-12-6 and subjected to immunoprecipitation with anti-HA antibodies. When an immunoblot of the anti-HA-immunoprecipitated fraction was probed with anti-GFP antibodies, a protein of ~50 kDa, the predicted size of GFP–RDI-1, was detected (Fig. 7C, lane 2). In a reciprocal immunoprecipitation with anti-GFP antibodies, HA–RHO-4 was detected by immunoblot with anti-HA antibodies in the anti-GFP immunoprecipitated fraction (Fig. 7D, lane 2). Together with the altered GFP–RHO-4 localization observed for *rdi-1* mutants, these data indicate that RDI-1 is a direct negative regulator of RHO-4 in vivo.

**GFP–RHO-4 localization at septa is altered in a mutant with activated cAMP-responsive protein kinase A**. In other systems, it has been shown that phosphorylation of Rho-type GTPases by cAMP-dependent PKA can increase the binding affinity between Rho and its Rho GDI, thereby inactivating the Rho-type GTPase via sequestration (9, 10). In *N. crassa*, the *mcb* mutant has a temperature-sensitive mutation in the negative regulatory subunit of PKA (4). After a shift to the nonpermissive temperature, the *mcb* mutant produces aberrant septa and loses polar growth, causing the hyphae to swell isotropically and eventually lyse, effects which are attributed to inappropriate activation of PKA (4). Similar to previous reports, we observed septa in the *mcb* mutant that appeared to bisect hyphal compartments that had already lost polar growth (Fig. 8A, arrow). These septa were often incomplete, suggesting that PKA activation may inhibit septum completion (Fig. 8A, inset). We then evaluated whether inappropriate activation of the PKA pathway in the *mcb* mutant would alter GFP–RHO-4 localization. At a permissive temperature, GFP–RHO-4 showed normal septal localization in the *mcb* mutant (CR48-1) (data not shown). After 3 to 4 h at the nonpermissive temperature, CR48-1 showed apolar growth and septa with aberrant positions (i.e., not perpendicular to hyphae or within 10 μm of another septum). GFP–RHO-4 was present at these aberrant septa (Fig. 8A and B). However, after 6 h at a nonpermissive temperature (37°C), GFP–RHO-4 localization to all septa was lost; only cytoplasmic fluorescence was observed (Fig. 8C and D).

To determine if GFP–RHO-4 localization could be reestablished at completed septa, we shifted CR48-1 to a permissive temperature (~22°C) for 2 h, following the 6-h incubation at a nonpermissive temperature. After this shift to the permissive temperature, GFP–RHO-4 relocalized to all “incomplete” and complete septa, forming bright rings (Fig. 8E and F). These
data indicate that activation of the PKA pathway initially resulted in aberrant septum formation with associated GFP–RHO-4 localization, followed by GFP–RHO-4 disassociation from septa. Relocalization of GFP–RHO-4 to the septa (both complete and incomplete) upon restoration of PKA regulation suggests that the factor(s) that controls RHO-4 localization to the septa was not disrupted by PKA activation.

To determine if the cytoplasmic localization of RHO-4 in the mcb mutant at a nonpermissive temperature was regulated by RDI-1, the localization of GFP–RHO-4 was assessed in the mcb rdi-1 double mutant (CR64-16). At a permissive temperature, CR64-16 showed GFP–RHO-4 localization at all septa and at the plasma membrane (data not shown). However, unlike the mcb mutant, GFP–RHO-4 was still observed at all septa, the plasma membrane and endomembrane structures in the mcb rdi-1 double mutant, after a 6-h incubation at a non-permissive temperature (Fig. 8H). These data indicate that RHO-4 cytoplasmic localization in a mutant with activated PKA is mediated through RDI-1.

The cr-1 mutation partially restores conidiation to rho-4 mutants. The cr-1 mutant, which has a mutation in the adenylate cyclase gene, suppresses the phenotype of the mcb mu-

**FIG. 5.** GFP–RHO-4 localization during conidiation in a wild-type strain (CR26-9) (panels A to C), in the csp-1 mutant (CR55-1) (panels D to F), in the csp-2 mutant (CR54-1) (panels G to I), and in the rdi-1 mutant (CR58-4) (panels J to L). (A, D, G, and J) Calcofluor-stained conidiophores. (B, E, H, and K) Fluorescence micrographs showing GFP–RHO-4 localization. (C, F, I, and L) Merged images of calcofluor (magenta) and GFP–RHO-4 (green). When the calcofluor and GFP–RHO-4 signals overlap, the resulting signal is white. (A to C) In CR26-9 (WT), the GFP–RHO-4 signal becomes cytoplasmic after formation of the second septum (arrow). (D to F) In the csp-1 mutant (CR55-1), GFP–RHO-4 localization at primary conidial septa is apparent (arrow). (G to I) In the csp-2 mutant (CR54-1), GFP–RHO-4 localization is mostly cytoplasmic, although a faint plasma membrane accumulation can be seen at sites of septation (arrow). (J to L) In the rdi-1 mutant (CR58-4), GFP–RHO-4 is localized predominantly at the plasma membrane after the formation of the double doublet. Arrowhead in panel K indicates endomembrane localization of GFP–RHO-4 in the rdi-1 mutant. Bar, 10 μm.
tant; the mcb cr-1 double mutants show polar growth at the nonpermissive temperature (4). In addition, cr-1 mutants are colonial and show constitutive conidiation (45) (Fig. 9A, inset). We hypothesized that GFP–RHO-4 would incorrectly localize to the plasma membrane in the cr-1 mutant; the cytoplasmic localization observed for CR48-1 [cr-1 rho-4] had fewer conidial septa than the cr-1 strain (FGSC 5068; Table 2) and a higher frequency of primary septa compared with the cr-1 strain, suggesting that the rate-limiting step in the cr-1 rho-4 mutant was primary septum formation. An extremely low rate of vegetative septa was observed for CR69-1 (three septa in five calcofluor-stained colonies). Thus, the cr-1 mutation suppressed the aseptate phenotype of vegetative hyphae. Together, these data indicate that in the absence of rho-4, another factor(s), negatively regulated by CR-1, initiates conidial septum formation.

**DISCUSSION**

In N. crassa, rho-4 mutants lack septa, grow slowly, and are aconidial (31). In this study, we show that RHO-4 localizes to sites of imminent septation and constricts during the process of septum formation. After the completion of septum formation, RHO-4 localization persists at the septal pore. This localization contrasts with the transient localization of other components required for septation in filamentous fungi, such as the formin SEPA (40) and actin (26, 31) as well as other Rho-type GTPases (29). Our speculation is that the persistent localization of RHO-4 at septa may serve as a marker that defines the ends of hyphal compartments. Treatment with cytochalasin-A showed that RHO-4 localization to sites of imminent septation...
requires an intact actin cytoskeleton, but its localization at completed septa is F-actin independent. These data suggest that a component of the early acting septation machinery may recruit RHO-4 to sites of imminent septation in an F-actin-dependent manner. Two candidates for recruiting RHO-4 to sites of septation may be the guanine nucleotide exchange factor or the GTPase activating protein, similar to that reported for mammalian cells (18, 51, 52). Because RHO-4 localization was not observed for the cwl-2 mutant, CWL-2 may be a good candidate for an early acting septation protein, whereas CWL-1 may be important for the stabilization of RHO-4 at sites of septation.

In contrast to the persistent localization of RHO-4 at vegetative septa, RHO-4 is transiently localized to septa during the conidiation process. First, RHO-4 localizes to primary conidial septa. It subsequently becomes cytoplasmically localized in conidia during or after the formation of the second septum (the “double doublet”) but before the digestion of the connective material. In S. pombe, Rho4 is required for septum degradation and cell separation (27, 37); the localization of two glucanases, Eng1 and Agn1, to the septum requires Rho4 (38). Our original hypothesis was that RHO-4 would be required for cell separation during conidiation, similar to that in S. pombe. However, RHO-4 is cytoplasmically localized (and therefore inactive) in the csp-2 mutant, which is defective in cell separation, making it unlikely that RHO-4 regulates this process in N. crassa.

A knockout of rdi-1, the predicted GDI for RHO-4, was constructed to evaluate its role as a potential negative regulator of RHO-4. The rdi-1 mutant has increased vegetative septation and aberrant plasma membrane-biased RHO-4 localization, consistent with the predicted negative regulatory role of RDI-1. In addition, direct interaction between RDI-1 and RHO-4 was shown by coimmunoprecipitation experiments. These data strongly suggest that RDI-1 interacts directly with RHO-4 in vivo, thereby negatively regulating the RHO-4 function by sequestering it in the cytoplasm. In S. cerevisiae, Rd11 localizes preferentially to the bud tip or the cytokinetic ring and shows a temporally regulated localization pattern during

![FIG. 8. GFP–RHO-4 localization in the mcb mutant CR48-1 and mcb; rdi-1 strain CR64-16.](image)

![FIG. 9. Phenotype of the cr-1 (FGSC 5068) and cr-1; rho-4 (CR69-1) mutants.](image)

| TABLE 2. Comparison of conidial septation between the cr-1 and cr-1; rho-4 mutant strains |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Strain  | Genotype | No. of conidia | % of single septa | % of double septa | % Mark |
|---------|-----------|----------------|------------------|------------------|--------|
| FGSC 5068 | cr-1 | 902 | 9.3 | 33.6 | 2.1 |
| CR69-1 | cr-1; rho-4 | 971 | 2.1* | 11.7* | 7.62* |

* Fisher’s exact test, P < 0.001.
the cell cycle (33). In *N. crassa*, RDI-1 shows a spatially regulated localization pattern. Cytoplasmic RDI-1–GFP showed increased fluorescence at hyphal tips versus at the interior of a hypha, which correlates with previously observed increased hyphal compartment lengths (i.e., decreased septation) at the tip (16). RDI-1–GFP fluorescence was also higher in conidia, where RHO-4 is cytoplasmically localized due to RHO-4–RDI-1 interaction. Thus, increased RDI-1–GFP fluorescence was correlated with decreased septation and with cytoplasmically localized (and thus, inactive) RHO-4.

In a number of systems, it has been shown that Rho-type GTPases can be direct phosphorylation targets of PKA. In cytotoxic T lymphocytes, phosphorylation of RhoA by PKA is associated with increased affinity of RhoA for its Rho GDI and subsequent removal of RhoA from membranes (9, 10). We therefore evaluated whether the localization of RHO-4 was altered in a mutant with constitutively active PKA (the mcb mutant) or in the CAMP-deficient cr-1 mutant, in which the PKA pathway is not active. In the mcb mutant, the earliest phenotype associated with a shift to a nonpermissive temperature is the loss of polarity at the hyphal tip, which is associated with actin patch mislocalization. However, actin rings are still associated with aberrant septa (4). Consistent with these data, we observed RHO-4 (which is required for actin ring formation [31]) at the septa during early stages of PKA activation, followed by its delocalization to the cytoplasm. Through analysis of an mcb crdi-1 double mutant, we observed that the cytoplasmic localization of RHO-4 after PKA activation was dependent on RDI-1. These data are consistent with the hypothesis that PKA phosphorylates RHO-4, thus leading to an increased RDI-1–RHO-4 affinity and subsequent inactivation of RHO-4 via cytoplasmic localization. An alternate hypothesis is that PKA activation prevents RHO-4 localization to septa, eventually leading to the loss of RHO-4 septal localization due to degradation or removal without replacement.

Although we did not observe mislocalization of RHO-4 in the cr-1 mutant, the cr-1 mutant restored conidiation to the rho-4 mutant. It is possible that the cr-1 mutant, because it has abnormally early conidiophore development (45), simply bypasses the developmental step where the rho-4 mutant is blocked, resulting in the formation of conidia and conidio- phores. However, we believe RHO-4 is normally required for the formation of conidial septa, which is consistent with both the localization of RHO-4 to conidial-specific primary septa and the conidial septation defect observed for the cr-1; rho-4 mutant. Although the cr-1 mutation partially suppressed the conidial septation defect of the rho-4 mutant, vegetative septa were extremely rare. These observations suggest that vegetative versus conidial septation has important regulatory differences, a developmental issue that can only be addressed experimentally with filamentous fungi. Our data indicate that the cAMP/PKA pathway may play a negative regulatory role, particularly in the conidial-specific septation pathway. Future experiments will address both differences and commonalities in the initiation and the regulation of the septation process in the vegetative versus conidial septation pathways.

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