Plasma Membrane Localization Is Required for RasA-Mediated Polarized Morphogenesis and Virulence of Aspergillus fumigatus

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Ras is a highly conserved GTPase protein that is essential for proper polarized morphogenesis of filamentous fungi. Localization of Ras proteins to the plasma membrane and endomembranes through posttranslational addition of farnesyl and palmitoyl residues is an important mechanism through which cells provide specificity to Ras signal output. Although the Aspergillus fumigatus RasA protein is known to be a major regulator of growth and development, the membrane distribution of RasA during polarized morphogenesis and the role of properly localized Ras signaling in virulence of a pathogenic mold remain unknown. Here we demonstrate that Aspergillus fumigatus RasA localizes primarily to the plasma membrane of actively growing hyphae. We show that treatment with the palmitoylation inhibitor 2-bromopalmitate disrupts normal RasA plasma membrane association and decreases hyphal growth. Targeted mutations of the highly conserved RasA palmitoylation motif also mislocalized RasA from the plasma membrane and led to severe hyphal abnormalities, cell wall structural changes, and reduced virulence in murine invasive aspergillosis. Finally, we provide evidence that proper RasA localization is independent of the Ras palmitoyltransferase homolog, encoded by erfB, but requires the palmitoyltransferase complex subunit, encoded by erfD. Our results demonstrate that plasma membrane-associated RasA is critical for polarized morphogenesis, cell wall stability, and virulence in A. fumigatus.

The importance of subcellular localization of Ras proteins for function in specific morphological processes has been explored in several fungi. Early evidence for this is found in the model yeast Schizosaccharomyces pombe. When the Ras1 palmitoylation motif of S. pombe is ablated, Ras1 localization is restricted to endomembranes, where it interacts with the guanine nucleotide exchange factor (GEF) Efc25p and signals through a Cdc42-mediated pathway to mediate cell morphology (34). In contrast, S. pombe Ras1p restricted to the plasma membrane is activated by a different GEF, Ste6p, and signals through a mitogen-activated protein (MAP) kinase pathway to mediate the mating response (34). Ras membrane distribution in the fungal pathogen Cryptococcus neoformans is also important for differential activation of morphology and mating pathways. In C. neoformans, plasma membrane-localized Ras1 is required for morphology, high-temperature growth, and virulence.
and virulence (32). Mutation of the highly conserved "dual-palmitoylation" motif of C. neoformans Ras1 results in a mislocalized Ras protein that cannot support morphology and high-temperature growth yet is still able to support the mating response (32). Most recently, the importance of Ras membrane localization has also been explored in the yeast pathogen Candida albicans. Cells expressing a palmitoylation-deficient C. albicans Ras1 are able to produce hyphae and grow at wild type-levels in rich media, although the mutant hyphae are more variable in length (36). However, polarized morphogenesis of the palmitoylation-deficient mutant is drastically reduced when cells are embedded in a matrix, a condition requiring Ras signaling for hyphal induction (36). In addition, expression of a constitutively activated Ras1 overcomes the embedded filamentation defect, arguing that mis-localization of C. albicans Ras1 may negatively affect activity (36). Together, these studies support the hypothesis that fungal Ras signal transduction from different cellular locations can lead to unique biological outputs, a general paradigm of Ras protein regulation that is apparent in many organisms.

The conservation of this paradigm among the yeast organisms described above leads to questions about the role of this form of Ras signaling in pathogenic molds, in which polarized morphogenesis is required for normal growth and virulence. In the human pathogen Aspergillus fumigatus, rasA is not essential, but it plays a critical role in hyphal morphogenesis and cell wall integrity (10, 12). From what membrane(s) does Ras mediate hyphal morphogenesis, and therefore likely virulence, in filamentous fungal organisms? Despite the emerging data describing its role in growth and development, the subcellular localization of Ras proteins in a mold has not been explored. Here, we show for the first time that RasA localizes to the A. fumigatus plasma membrane. Mutation of the highly conserved palmitoylation motif resulted in mislocalization of RasA from the plasma membrane to endomembrane compartments, leading to defects in hyphal morphogenesis, cell wall formation, and virulence. In addition, the putative RasA palmitoyltransferase subunit genes were identified and deleted: erfB, the homolog of S. cerevisiae ERF2, and erfD, the homolog of S. cerevisiae ERF4. Our data establish the importance of erfD for RasA plasma membrane localization in A. fumigatus, whereas erfB is not required. Our findings provide novel insights into the regulation of Ras signal transduction in A. fumigatus and may potentially be extrapolated for studies with other filamentous fungal pathogens.

**MATERIALS AND METHODS**

Strains, culture conditions, and growth rate analyses. Aspergillus fumigatus wild-type strain H237, the ΔrasA strain (10), and each of the green fluorescent protein (GFP)–RasA fusion strains were maintained on glucose minimal medium (GMM) (10). For quantification of growth rates, a 10-μl drop containing 10,000 conidia was inoculated onto GMM agar. The colony diameter of each strain was measured daily over the first 72 h of growth, and images were taken at the end of day 3.

Live-cell microscopy was employed for analysis of morphogenesis during germination, as previously described (11). Briefly, conidia from each strain were preincubated in GMM for 4 h at 37°C in a glass-bottom tissue culture plate. Plates were subsequently used for live-cell analysis on a Zeiss Axio Observer inverted microscope equipped with a 37°C environmental chamber. For analysis of hyphal morphogenesis during branch formation, 100 conidia from each strain were inoculated onto a thin layer of GMM agar solidified on a glass-bottom petri dish. Cultures were incubated at 37°C for the indicated amount of time, and images were captured on an inverted Nikon Diaphot microscope with a mounted Nikon DS digital camera.

To analyze the effects of 2-bromopalmitate (2-BP) (Sigma) treatment, 10^6 conidia of the GFP-RasA strain were inoculated into GMM broth supplemented with the indicated concentrations of 2-BP and incubated for 24 h at 37°C. Mycelia from each treatment were collected by centrifugation, and excess water was expressed onto filter paper using a sterile spatula. Hyphal mats were then allowed to dry for 6 h at room temperature before weights were recorded. To assess the effect of 2-BP on RasA localization, 100-μl aliquots were removed from each culture prior to collection of mycelia and mounted for fluorescence microscopy.

**Construction of GFP-RasA fusion strains.** For construction of a vector expressing a green fluorescent protein (GFP)–RasA fusion, the rasA coding sequence was PCR amplified from pooled A. fumigatus cDNA using primers incorporating NotI restriction sites to both 5’ and 3’ ends. The amplified rasA cDNA was restriction digested with NotI and cloned into expression vector pAGRP. Vector pAGRP was generated by cloning enhanced GFP (eGFP), amplified as an N-terminal fusion from pUCGH (22), into vector pARP (11) as a BamHI and NotI restriction fragment. The GFP-RasA expression vector was then introduced into the ΔrasA mutant via protoplast transformation, as previously described (10). Eight transformants positive for fluorescence were found to have identical localization of GFP signal to the hyphal periphery. To introduce mutations in the dual-palmitoylation motif, the rasA cDNA was PCR amplified using reverse primers that incorporate the C206S, C207S, or C206S C207S mutations. The rasA palmitoylation mutated cDNAs were then cloned into pAGRP and verified by sequence analysis. Each GFP-RasA palmitoylation mutant vector was transformed into the ΔrasA background, and multiple transformants were analyzed for RasA localization patterns. To generate the GFP-DARasAΔC206/C207S strain, the previously constructed DARasA cDNA (12) was amplified using the same primers employed for the C206S C207S mutation described above. The DARasA and DARasAΔC206/C207S cDNAs were then cloned, sequenced, and transformed into the ΔrasA mutant. All strains were analyzed by Southern blot analysis to verify that a single, ectopic integration event occurred.

**Western blot analysis.** Western blot analysis for the GFP-RasA and GFP-RasAΔC206/C207S mutant was performed as previously described (22). In short, each strain was cultured in liquid GMM for 24 h at 37°C with shaking at 250 rpm. Total protein extracts were prepared by homogenizing mycelia in liquid nitrogen, followed by addition of extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM KCl, 0.01% Triton X-100, 1 μM phenylmethylsulfonyl fluoride [PMSF], and 1:100 protease cocktail inhibitor). Lysates were centrifuged at 4,000 rpm for 8 min to remove debris. Supernatants were removed, and protein content was quantitated by Bradford assay. Fifty micrograms of total protein from each strain was resolved on a 12% SDS-polyacrylamide gel using a Mini-protein tetracell (Bio-Rad). Proteins were then electroblotted to a polyvinylidene difluoride membrane (Bio-Rad) and probed with a polyclonal rabbit anti-GFP antibody. A horseradish peroxidase-conjugated anti-rabbit IgG was used as a secondary antibody, and detection was performed using SuperSignal West Pico chemiluminescent substrate (ThermoScientific).

**Identification and deletion of A. fumigatus erfB and erfD.** The A. fumigatus ERF2 homolog, erfB (Afu3G06470), was previously identified (14). The A. fumigatus ERF4 homolog, erfD (Afu5G7920), was identified using BLAST search analysis of the A. fumigatus genome database with the S. cerevisiae Erf4p sequence as a query. To delete erfB and erfD, 1.5 kb of upstream and downstream flanking genomic sequence was PCR amplified for each gene. Flanking sequences were cloned into vector pHygR, carrying a hygromycin resistance cassette, generating a deletion construct for each gene. The erfB and erfD deletion constructs were PCR amplified, and 2 μg of each purified product was used for transformation into A. fumigatus protoplasts, as previously described (10). Hygromycin-resistant transformants were screened for integration by PCR and homologous
recombination confirmed by Southern blot analysis. To generate strains expressing GFP-RasA in either the ΔerfB or ΔerfD background, the construct generated for GFP-RasA expression (vector pAGRP) was transformed into the ΔerfB and ΔerfD mutants as described above. Multiple phleomycin-resistant transformants were isolated for both strains, and RasA localization patterns were analyzed in each. Six ΔerfB+GFP-RasA isolates were identified and found to have unaltered RasA plasma membrane localization patterns. Five ΔerfB+GFP-RasA strains were isolated, and each displayed similar mislocalization of RasA to endomembrane structures.

Fluorescence microscopy for localization analysis and cell wall staining. Ten thousand conidia of each strain were inoculated in 3 ml of GMM broth and incubated in a petri dish on sterile coverslips. After 16 h of growth at 37°C, the strains were observed for fluorescence using an Axioskop 2 Plus microscope (Zeiss) equipped with a digital camera. Images were captured using AxioVision 4.6 imaging software.

Calcofluor white and aniline blue staining was performed as previously described (10, 25). Calcofluor white staining of the wild-type and RasAC206/207S strains was performed by culturing conidia on coverslips immersed in 5 ml GMM broth and grown for 16 h at 37°C. The coverslips were briefly rinsed in sterile water and inverted onto a 500-μl drop of 0.4-μg/ml fluorescent brightener 28 (Sigma) for 5 min at room temperature. Mycelia were then rinsed twice for 5 min each in sterile water and observed by fluorescence microscopy. β-Glucan staining with aniline blue was performed by rinsing coverslips in sterile water and inverting them onto a 500-μl drop of aniline blue (0.1 mg/ml) for 5 min at room temperature. Stained mycelia were directly observed by fluorescence microscopy.

Electron microscopy. For transmission electron microscopy (TEM) of the wild type and the RasC206/207S mutant, 10,000 conidia of each strain were inoculated into 10 ml GMM broth and grown for 48 h at 37°C. Mycelia were collected by centrifugation and washed three times in distilled water. Washed mycelia were then fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer. The mycelia were embedded in low-viscosity Spurr’s resin, ultrathin sectioned, and then stained with uranyl acetate and lead citrate. Images were obtained on Zeiss EM900 electron microscope, and negatives were scanned for image analysis.

For scanning electron microscopy (SEM), coverslip cultures of 10,000 conidia/strain were grown in 10 ml of GMM broth and incubated in a petri dish on sterile coverslips. After 16 h of growth at 37°C, the strains were observed for fluorescence using an Axioskop 2 Plus microscope (Zeiss) equipped with a digital camera. Images were captured using AxioVision 4.6 imaging software.

RESULTS
RasA localizes to the plasma membranes of germlings and actively growing hyphae. To investigate the distribution of RasA during A. fumigatus growth and development, a GFP-RasA fusion was generated and expressed in the ΔrasA background. To limit artificial cellular distribution, the endogenous rasA promoter was used to drive GFP-RasA expression (11). Expression of GFP-RasA in the ΔrasA mutant fully complemented the compact colony morphology and conidiation defects associated with loss of rasA (10), suggesting that the GFP-RasA fusion protein is functional (Fig. 1A and B). During early growth, GFP-RasA was visible at the cell periphery of swollen conidia (Fig. 1C, inset at upper left) and distributed around the periphery of nascent germlings (Fig. 1C). During fully polarized growth, GFP-RasA was visible at the outer edge of hyphae, with strong localization to septa, and no GFP signal was associated with internal compartments (Fig. 1D). These findings are consistent with RasA localization to the A. fumigatus plasma membrane, with the strong septal localization likely stemming from plasma membrane apposition along each side of the septum.

Palmitoylation of the Ras protein on conserved residues is required for plasma membrane localization and full function of Ras signaling in various organisms (4, 7, 32, 36, 49). However, nothing is known about the importance of Ras membrane distribution during invasive growth and virulence of a filamentous pathogen, such as A. fumigatus. To investigate, we cultured the GFP-RasA strain in the presence of increasing concentrations of 2-bromo-palmitate (2-BP) (1 to 50 μM), a nonreversible inhibitor of palmitoylation (20), and assayed hyphal mass. 2-BP inhibited hyphal growth in a dose-dependent manner (Fig. 1E), whereas dimethyl sulfoxide (DMSO) vehicle controls produced no effect (data not shown). To examine the effects of increasing concentrations of 2-BP on RasA localization, aliquots were removed from each culture and hyphae were observed using fluorescence microscopy. Although 1 μM 2-BP treatment reduced growth, no detectable disruption of RasA localization was noted (Fig. 1F). However, higher concentrations of 2-BP (5, 10, and 50 μM) caused mislocalization of GFP-RasA to internal patches (Fig. 1F). These results suggested that, similar to the case in C. albicans and C. neoformans, palmitoylation may be important for proper RasA localization during polarized growth and that mislocalization of RasA may contribute to decreased hyphal growth.

Mutation of the conserved RasA palmitoylation motif inhibits its hyphal growth and mislocalizes RasA. Nichols et al. recently characterized the importance of Ras palmitoylation to C. neoformans growth and virulence, describing a “dual-palmitoylation motif” that is conserved among the filamentous fungal Ras homologs (32). In C. neoformans, mutation of both cysteine residues to serine completely blocked palmitoylation of the Ras1 protein and caused mislocalization to internal membranes (32). Further, mutation of the single conserved palmitoylation residue of C. albicans Ras1 causes mislocalization from the plasma membrane to endomembrane structures (36). To investigate the role of the palmitoylation motif in RasA localization and function during A. fumigatus polarized morphogenesis, mutations were introduced to replace each conserved cysteine residue of the dual-palmitoylation motif with serine, either singly (RasAΔ206/207S) or in tandem (RasAΔ206/207S/H9262). Using the same vector constructed for GFP-RasA, each of the new RasA palmitoylation motif mutants
was expressed in the ΔrasA background. Expression of either GFP-RasAC206S or GFP-RasAC207S was able to fully complement the hyphal growth defects associated with ΔrasA, indicating that only one of the conserved cysteines is sufficient for RasA function in hyphal growth (Fig. 2A and B). However, expression of GFP-RasAC206/207S, representing complete blockade of RasA palmitoylation, only partially restored hyphal growth of the ΔrasA mutant (Fig. 2A and B). Because mutation of the palmitoylation domain in C. albicans Ras1 caused reduced protein abundance (36), we compared steady-state levels of the RasA protein in the GFP-RasA and GFP-RasAC206/207S strains. Western blot analysis of whole-cell protein lysates revealed the expected band size for a GFP-RasA fusion at ~51 kDa in both strains (Fig. 2C). In contrast to the C. albicans data, RasA protein levels in the GFP-RasA strain (Fig. 2C), suggesting that the RasAC206/207S mutation does not negatively affect protein abundance in A. fumigatus. These data suggest that the RasAC206/207S mutant phenotypes are not due to decreased protein abundance and are more directly attributable to mislocalization of RasA.

Under fluorescence microscopy, the GFP-RasAC206S mutant displayed no alteration in plasma membrane association in the subapical sections of hyphae, whereas the apical hyphal sections showed internal patches of GFP fluorescence (Fig. 2D, arrows). In contrast, the GFP-RasAC207S mutant displayed mislocalized GFP signal in both the subapical and apical regions (Fig. 2D). Although GFP-RasAC206S was associated with internal patches only in apical compartments whereas GFP-RasAC207S was found on internal patches in both the apical and subapical sections, both were still associated with the plasma membrane, as evidenced by clear lo-
calization of GFP signal to septa (Fig. 2D, arrowheads). In contrast to either single mutation, simultaneous mutation of both residues within the dual-palmitoylation motif (GFP-RasAC206/207S) resulted in a dramatic reduction of GFP signal at the plasma membrane. The GFP-RasAC206/207S mutant was mislocalized from the hyphal periphery and septa to the cytosol and internal patches, presumably to compartments of the endomembrane system (Fig. 2D). These data suggest that whereas a single palmitoylation site is sufficient for RasA function in supporting polarized morphogenesis, complete blockade of RasA palmitoylation through ablation of the entire dual-palmitoylation motif disrupts plasma membrane association and causes decreased hyphal growth. However, the radial growth difference between the RasA<sup>C206/207S</sup> and ΔrasA mutants suggests that the palmitoylation-deficient mutant is partially functional.

It is possible that the aberrant growth phenotypes noted in the GFP-RasA<sup>C206/207S</sup> strain were due to accumulation of RasA on the endomembranes and not to loss of RasA at the plasma membrane. To test this, we generated a strain expressing the rasA<sup>C206/207S</sup> allele in the wild-type A. fumigatus background. As with the GFP-RasA<sup>C206/207S</sup> mutant, this new strain (GFP-RasA<sup>C206/207S</sup>ΔrasA<sup>C206/207S</sup>) exhibited an enhanced GFP-RasA signal in the cytosol and on the endomembranes (data not shown). However, no alteration in growth or morphology was observed (data not shown). Taken together, these data argue that aberrant growth in the GFP-RasA<sup>C206/207S</sup>ΔrasA<sup>C206/207S</sup> mutant was due to loss of RasA localization at the plasma membrane.
Plasma membrane-localized RasA signaling controls early polarized growth and branch emergence. Normal vegetative growth of Aspergillus species progresses from resting conidia through germination to fully polarized hyphae. Deletion of rasA in A. fumigatus causes delayed germination and subsequent formation of wide, blunted hyphae that continually switch polarized growth axes (10). To examine the contribution of plasma membrane-localized Ras activity to these important morphogenetic processes, we compared polarization phenotypes during germination and early hyphal growth of the GFP-RasAC206/207S mutant to those of the wild-type and ΔrasA strains. Live-cell microscopy analysis revealed nearly identical growth defects in the ΔrasA and GFP-RasAC206/207S mutants during germination. Both mutants displayed low germination rates and formed stunted, highly branched germlings over the initial 15 h of culture (Fig. 3), suggesting a nonfunctional Ras protein. The only notable difference between the mutant strains throughout the initial growth stages was the exaggerated swelling of the conidium during germ tube extension in the GFP-RasAC206/207S strain (Fig. 3, arrowheads). In contrast, the germination rates and morphologies of the GFP-RasAC206S and GFP-RasAC207S mutants were similar to those of the GFP-RasA control strain (data not shown).

However, after 24 h of growth, the highly branched, stunted hyphae of the GFP-RasAC206/207S mutant developed into morphologically normal hyphae after the initial 24 h of culture, subsequent branching resulted in reversion to the early growth phenotype (Fig. 4C). The altered morphology of germination and branch formation in the GFP-RasAC206/207S mutant mimicked the phenotype of the ΔrasA strain (10). Taken together, these results further suggest partial Ras functionality of the GFP-RasA C206/207S mutant during invasive growth. Although exogenous 2-BP inhibited hyphal growth (Fig. 1E and F), 2-BP treatment did not phenocopy the RasAC206/207S phenotypes noted in Fig. 4. These findings are likely due to the inability of 2-BP to completely inhibit RasA palmitoylation and therefore to perfectly mimic the ΔrasA mutation.

Plasma membrane localization is required for full Ras function in cell wall integrity, β-glucan deposition, and virulence. Deletion of RasA causes decreased cell wall integrity as evidenced by increased susceptibility to cell wall-destabilizing agents (10). To examine the contribution of plasma membrane-localized RasA activity to cell wall integrity, we first compared the susceptibilities of the wild type and the GFP-RasAC206/207S mutant to cell wall-damaging agents. The GFP-RasAC206/207S mutant displayed greater sensitivity to the chitin-perturbing compounds nikkomycin Z and calcofluor white than the wild-type strain. Nikkomycin Z (0.25 g/ml) treatment yielded 10% versus 52% inhibition (P < 0.01) of radial growth rates for the wild-type and GFP-RasAC206/207S strains, respectively. Furthermore, growth in the presence of calcofluor white (3 μg/ml) yielded 3% inhibition of the wild-type strain versus 32% inhibition of the GFP-RasAC206/207S strain (P < 0.01). The GFP-RasAC206/207S and
GFP-RasAC207S mutants displayed no increase in sensitivity to either cell wall-damaging agent compared to the wild type (data not shown). Because increased sensitivity to cell wall-disruptive agents was apparent in the GFP-RasAC206/207S mutant, cell wall organizational differences were examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM analysis revealed the presence of an extracellular matrix associated with cultures of the GFP-RasAC206/207S mutant that was not evident in the wild

![FIG 4](image1.png)

**FIG 4** Plasma membrane-localized RasA regulates morphology of branch formation. Conidia of the GFP-RasAC206/207S strain were inoculated onto a thin layer of GMM agar solidified on a glass-bottom petri dish and incubated at 37°C. Bright-field microscopy images were taken during germination (A) and at 24 h (B) and 48 h (C) of hyphal growth. Arrows indicate the appearance of morphologically normal hyphae after 24 h of growth. Arrowheads denote formation of branches after 48 h of growth that revert to the early growth phenotype.

![FIG 5](image2.png)

**FIG 5** RasA contributes to cell wall structure and integrity from the plasma membrane. (A) Twenty-four-hour coverslip cultures of the wild-type (WT) and GFP-RasAC206/207S strains were used for scanning electron microscopy analysis. The lower panels are magnified images of the areas indicated in the upper panels by dashed boxes. Arrowheads indicate extracellular matrix produced by the GFP-RasAC206/207S mutant. (B) Twenty-four-hour coverslip cultures of each strain were utilized for transmission electron microscopy analysis. Note the disorganized outer layer and translucent middle layer of the GFP-RasAC206/207S cell wall. (C) Conidia of the wild-type (WT), ΔrasA, and GFP-RasAC206/207S strains were grown on glass coverslips for 16 h at 37°C. Following incubation, coverslips were rinsed briefly and hyphae stained in 0.1% aniline blue. Arrowheads indicate low staining of hyphal tips in the ΔrasA and GFP-RasAC206/207S mutants.
type (Fig. 5A, arrowheads). Subsequent TEM analysis revealed a GFP-RasA C206/207S cell wall that was disorganized compared to that of the wild type, with fibrillar material protruding from the outer layers of hyphae (Fig. 5B).

We hypothesized that the extracellular fibrillar matrix was likely to be cell wall material that was inadequately incorporated at the growing hyphal tip. To visualize the distribution of chitin and β-glucan, major cell wall components of *A. fumigatus*, hyphae from each strain were stained with calcofluor white or aniline blue. Although calcofluor white staining of the ΔrasA and GFP-RasA C206/207S hyphae revealed slightly brighter fluorescence than for the wild type, all strains displayed similar patterns of chitin distribution (data not shown). This finding coincided with the TEM analysis, which revealed that the innermost portion of the cell wall, consisting mostly of chitin, was relatively unaltered in the mutant strain (Fig. 5B). In contrast, hyphal staining with aniline blue revealed an altered distribution of β-1,3-glucan in the ΔrasA and GFP-RasA C206/207S cell walls. After 16 h of culture, the wild-type strain stained intensely at the hyphal tip, whereas the subapical hyphal walls stained at relatively low levels (Fig. 5C). In contrast, both the ΔrasA and RasA C206/207S mutants displayed reduced aniline blue staining at the hyphal tips accompanied by intense staining along the subapical hyphal walls (Fig. 5C). Thus, normal β-glucan deposition appears to be dependent on plasma membrane-associated RasA.

In *C. neoformans*, the deletion of Ras1 and mutation of the Ras1 palmitoylation domain both result in avirulence, due to lack of growth at 37°C (32). To determine the contribution of plasma membrane localization of RasA to *A. fumigatus* virulence, the ΔrasA and GFP-RasA C206/207S mutants were compared to the wild-type strain in a murine model of invasive aspergillosis. This model utilizes a single dose of triamcinolone acetonide (40 mg kg⁻¹) on day −1 and inoculated intranasally with 2 × 10⁶ conidia from each strain. Survival was assessed over a 15-day period. The ΔrasA and GFP-RasA C206/207S mutants displayed statistically significantly reduced virulence compared to the wild type.

Plasma membrane localization is required for full induction of the activated RasA phenotypes of the ΔrasA1 mutant. We recently reported novel phenotypes caused by the expression of constitutively activated *A. fumigatus* RasA, including unchecked vacuolar expansion, hyphal swelling, and lysis of hyphal compartments (11). Recent studies with *C. albicans* revealed that constitutively active CαRas1p can overcome the effects of mislocalization, suggesting that CαRas1p localization at the plasma membrane may be more critical for full Ras protein activity than for engagement of the proper effector proteins (36). To test this hypothesis in *A. fumigatus*, we mutated the dual-palmitoylation motif of the previously generated DARasA1 cDNA to generate a palmitoylation-deficient ΔrasA1 mutant (ΔrasA1 C206/207S). The ADrasA1 and ΔrasA1 C206/207S alleles were then expressed as GFP fusion proteins in the ΔrasA background using the pAGRP vector. Expression of GFP-ΔrasA1 produced the same phenotypes as previously reported for the untagged DARasA1 strain (11), including development of large, vacuolated subapical hyphal segments intermingled with normal apical compartments (Fig. 7A). Similarly to the wild-type RasA protein, GFP-ΔrasA1 localized primarily to the plasma membrane, along the hyphal periphery, and at septa (Fig. 7A). In contrast, mutation of the dual-palmitoylation motif caused the accumulation of GFP-ΔrasA1 C206/207S in the cytosol and on punctate endomembrane structures but not at the plasma membrane (Fig. 7B). Phenotypic analysis revealed that the GFP-ΔrasA1 C206/207S mutant developed hyphae that were stunted and highly branched (Fig. 7B). These hyphal phenotypes were more similar to those of the GFP-RasA C206/207S mutant (Fig. 3 and Fig. 4A) than to those of the GFP-ΔrasA1 mutant (Fig. 7A) (11), suggesting that constitutively activated RasA does not overcome the defects caused by RasA mislocalization. Although the hyphal morphology was more similar to that of the GFP-RasA C206/207S strain, the radial growth and colony morphology of the GFP-ΔrasA1 C206/207S mutant resembled that of the ΔrasA1 strain. Both strains formed more compact, slowly growing colonies, compared to the more substantial radial outgrowth generated by the GFP-RasA C206/207S mutant (Fig. 7C). Together, these data show that constitutively active RasA mislocalized to the endomembranes only partially induces activated Ras phenotypes. Therefore, although plasma membrane localization of RasA may be required for full induction of RasA activity, our studies do not rule out the lack of proper effector engagement by RasA restricted to the endomembrane system as a plausible scenario in *A. fumigatus*.

The *A. fumigatus* Erf4p homolog, ErfD, is important for proper RasA localization, whereas the Erf2p homolog, ErfB, is not required. In *S. cerevisiae*, the Ras palmitoyltransferase complex is known to consist of two proteins, Erf2p and Erf4p (27, 50). Erf2p is a DHHC palmitoyltransferase enzyme responsible for transferring the palmitoyl moiety onto the Ras protein (1, 30, 31). The second subunit, Erf4p, was originally copurified with Erf2p, and later found to be a peripheral ER membrane protein (1, 21). Although Erf4p does not contain the DHHC domain required for palmitoyl transfer, deletion of either subunit causes mislocalization of Ras2p from the plasma membrane (30). The *A. fumigatus* ERF2 homolog, erfB, was previously identified in a study of fungal DHHC palmitoyltransferases (14). To explore the importance of the palmitoyltransferase complex to RasA localization, we first identified the putative Erf4p homolog in *A. fumigatus*. The *S. cerevisiae* Erf4p sequence was used in a BLAST search of the A.
In the present study, mutations of the conserved Ras palmitoylation motif were introduced into A. fumigatus to delineate the importance of this domain to RasA localization and to understand the role of properly localized RasA in polarized morphogenesis. We found that RasA localized primarily to the plasma membrane, including at septa. Mislocalization of RasA from the plasma membrane caused defects in germination and hyphal morphogenesis, aberrant cell wall formation, and decreased virulence. The majority of these phenotypes were shared by the ΔrasA mutant (10), suggesting a mostly nonfunctional RasA protein. However, hyphal growth of the RasAΔC206/207S mutant was significantly greater than that seen in the complete absence of RasA, suggesting that mislocalized RasA remains partially competent to mediate some aspects of fungal growth and/or morphogenesis. This partial functionality was not attributable to reduced protein abundance, as was seen with a similar mutation in C. albicans Ras1 (36), because steady-state RasA protein levels were not negatively affected by the palmitoylation mutation in our model. Although temporal regulation of RasA protein activity is also important for RasA-mediated growth (12, 35, 44), our findings support the hypothesis that spatial regulation of RasA through proper localization to the plasma membrane is required for engagement of RasA signaling pathways controlling polarized morphogenesis. At present, three possible models for spatial regulation of Ras signal transduction in fungi are as follows: (i) fungal Ras proteins employ the plasma membrane and endomembranes as separate signaling platforms, engaging distinct effectors at each membrane; (ii) fungal Ras proteins can potentially interact with all effectors equally from either membrane, but plasma membrane localization is required for activation and, therefore, function; or (iii) Ras activity is not affected by membrane distribution, and the partial functionality of the RasAΔC206/207S mutant could be explained by the presence of a pool of Ras that is still active at or near the plasma membrane. Each of these models is at least partially supported by the current literature, and some combination of the three is likely to provide a more complete picture of spatiotemporal control of Ras signaling in fungi.

Differential localization of human Ras proteins to the plasma membrane and endomembranes propagates distinct signal transduction events through the utilization of distinct effector proteins in human and yeast (3, 4). Our data, along with recent reports for the yeasts, provide some support for a model of spatially segregated Ras-effector interactions in fungal organisms as well. For example, the utilization of specific membrane platforms for Ras signaling to morphogenetic programs appears to be conserved for C. neoformans, C. albicans, S. pombe, and, as our data show, A. fumigatus. At the plasma membrane, C. neoformans Ras1 controls...
cell shape and high-temperature growth, while \textit{C. albicans} Ras1 regulates the yeast-to-hypha transition (32). Our data similarly suggest that \textit{A. fumigatus} RasA may utilize the plasma membrane as a platform to interact with distinct effectors mediating polarized growth and hyphal morphogenesis. Further, \textit{C. neoformans} Ras1 restricted to endomembranes is functional for the support of the mating response (32). In contrast, \textit{S. pombe} utilizes plasma membrane-localized Ras1 for the mating response, whereas endomembrane-localized Ras1 mediates proper morphology (4, 32). Although the specific membrane platforms utilized for Ras signal output differ, the current data argue that both \textit{C. neoformans} and \textit{S. pombe} may be able to spatially segregate the activation of Ras-mediated morphology and mating pathways through activation of distinct effectors (4, 32). As a sexual cycle for \textit{A. fumigatus} has recently been described, it will be of interest to analyze the ability of a RasAC206/207S mutant to mate (33). Such studies would reveal whether activation of the mating pathway in a mold can be accomplished when Ras localization or activity is not concentrated at the plasma membrane. In addition to morphogenesis, \(\beta\)-glucan display properties and cell wall integrity were altered in both the \(\Delta\text{rasA}\) and RasAC206/207S mutants, again suggesting that plasma membrane localization is important for these roles. Host recognition of \(\beta\)-glucan of the \textit{A. fumigatus} cell wall is an important mediator of host-pathogen interactions (25, 28). Future work in this area will focus on quantitative measurement of \(\beta\)-glucan exposure and the subsequent immune response generated by RasA mutant strains.

Although differential activation of distinct effectors from each membrane is an attractive model, we cannot yet rule out the possibility that mislocalization of RasA results in greatly decreased activation due to the inability of Ras to interact with its cognate GEF. Data from studies with \textit{C. albicans} seem to support this alternative, as expression of a constitutively activated palmitoylation-deficient Ras1 can overcome the embedded filamentation defect of the Ras1 palmitoylation mutant (36). However, in \textit{S. cerevisiae}, where expression of activated Ras2p results in hypersensitivity to heat shock and nutrient stress, loss of Ras2p palmitoylation reverses activated Ras phenotypes (6). This supports a model in which Ras must be localized to the plasma membrane to engage proper effectors. Our data suggest that regulation of \textit{A. fumigatus} RasA may involve a combination of these two scenarios. Expression of DARasA1C026/207S caused loss of some hyphal phenotypes associated with DARasA1 expression, suggesting that the constitutively activated RasA was unable to interact with needed effectors. However, mislocalization of DARasA1 did not cause complete reversion to the RasAC206/207S phenotype, as colony morphology was compact, similar to that of the DARasA1 mutant. A specific, biochemical analysis of Ras activity will be required to decipher this question. Similarly, we cannot rule out the potential for a pool of active RasA near the plasma membrane that may be able to propagate the signals needed to produce the limited hyphal growth observed in the RasAC206/207S mutant. This caveat was also noted in recent studies with the Ras1 protein of \textit{C. neoformans} (32). Current models of human Ras protein trafficking suggest a
complicated system involving Golgi-mediated exocytosis through recycling endosome (RE) and non-RE pathways accompanied by palmitoylation-mediated, reversible association with the plasma membrane (29). It is possible that RasA could signal, albeit less efficiently, from recycling endosomes near the plasma membrane to support limited hyphal growth. More-in-depth studies of Ras trafficking in fungal organisms will elucidate the complex relationships between Ras localization and effector engagement.

Another finding of the current study is the ability of the palmitoylation inhibitor 2-BP to disrupt RasA localization and to inhibit A. fumigatus hyphal growth in a dose-dependent manner. Although 2-BP has been used to inhibit palmitoylation of yeast proteins in vitro (20) and to inhibit Trypanosoma brucei growth in culture (9), this is the first study providing data on the sensitivity of hyphal growth, increasing concentrations of 2-BP did not phenocopy the RasA(C206/207S) mutant. This discrepancy could be explained by an inability of 2-BP to completely inhibit RasA palmitoylation, therefore not mimicking phenotypes rendered by complete mutation of the dual palmitoylation motif. In this situation, it may be more likely that decreased growth caused by 2-BP treatment is due to inhibition of palmitoylation of the myriad other proteins involved in hyphal growth. Regardless, these findings highlight the importance of palmitoylation as a posttranslational modification controlling cellular processes required for invasive growth.

The importance of palmitoyltransferases to various cellular activities of microbes has now been studied in depth for both yeast (41) and T. brucei (9). The A. fumigatus genome contains six predicted DHHC palmitoyltransferases, including one with significant homology to S. cerevisiae Erf2p, the yeast Ras-palmitoyltransferase (14). Although Erf2p is considered the main palmitoyltransferase for Ras2p, palmitoylation of S. cerevisiae Ras2p occurs in the absence of Erf2p (41). These findings suggest that palmitoyltransferase activity may be promiscuous, such that nonspecific palmitoylation by other palmitoyltransferase enzymes may compensate for Erf2p loss. In fact, recent data suggest that palmitoylation of many peripheral membrane proteins (e.g., Ras) may actually be a promiscuous process, whereas integral membrane protein palmitoylation occurs via specific palmitoyltransferase enzymes (13, 39). Because deletion of erfB did not affect RasA localization, our data suggest that RasA may be a peripheral membrane protein whose palmitoylation can be accomplished through the activity of multiple palmitoyltransferases. In contrast, deletion of the ERF4 homolog, erfD, caused partial disruption of RasA localization. However, mislocalization of RasA due to erfD deletion did not cause morphological abnormalities mimicking those of the RasA(C206/207S) mutant, likely due to incomplete inhibition of RasA plasma membrane association. It is tempting to speculate that the ErfD protein may be an important subunit of palmitoyltransferase complex formation in A. fumigatus, regardless of the DHHC enzyme that is present. Further studies on the roles of the various palmitoyltransferases and the protein makeup of the complexes in which they function will help to elucidate their importance to Ras localization and fungal growth in general.

In summary, the present study reports the importance of RasA plasma membrane localization for full function in the hyphal morphogenesis, cell wall integrity, and virulence of A. fumigatus. Regardless of the mechanistic reason for decreased protein function of the RasA(C206/207S) mutant (i.e., decreased RasA activity versus inability to interact with distinct effectors), plasma membrane-directed RasA signaling is required for the majority of roles played by RasA in A. fumigatus growth and virulence. What are those signaling pathways that are engaged by RasA at the plasma membrane to control the phenotypes mentioned above? Effectors likely to be immediately downstream of RasA signaling are the conserved GTPase proteins ModA (Aspergillus Cdc42 homolog) and RacA, for activation of mitogen-activated protein kinase or p21-activated kinase pathways (18, 24, 47). Other interesting candidates that may physically or genetically interact with RasA at the plasma membrane or endomembranes are the septins and Rho proteins, both of which play roles in germination and branch formation of A. nidulans (16, 26, 48). Future work in this area will focus on the identification of RasA effectors to construct improved molecular models of fungal growth and morphogenesis.

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