Subcellular Localization Directs Signaling Specificity of the *Cryptococcus neoformans* Ras1 Protein

Connie B. Nichols, Jessica Ferreyra, Elizabeth R. Ballou, and J. Andrew Alspaugh*

*Departments of Medicine and Molecular Genetics and Molecular Microbiology, Duke University Medical Center, Durham, North Carolina 27710*

Received 20 October 2008/Accepted 8 December 2008

In the human fungal pathogen *Cryptococcus neoformans*, Ras signaling mediates sexual differentiation, morphogenesis, and pathogenesis. By studying Ras prenylation and palmitoylation in this organism, we have found that the subcellular localization of this protein dictates its downstream signaling specificity. Inhibiting *C. neoformans* Ras1 prenylation results in the defective general membrane targeting of this protein and the loss of all Ras function. In contrast, palmitoylation mediates localization of Ras1 to the plasma membrane and is required for normal morphogenesis and survival at high temperatures. However, palmitoylation and plasma membrane localization are not required for Ras-dependent sexual differentiation. Likely as a result of its effect on thermotolerance, Ras1 palmitoylation is also required for the pathogenesis of *C. neoformans*. These data support an emerging paradigm of compartmentalized Ras signaling. However, our studies also demonstrate fundamental differences between the Ras pathways in different organisms that emphasize the functional flexibility of conserved signaling cascades.

Ras proteins are important determinants of cell morphology, differentiation, and stress response in diverse organisms. The activity of Ras is controlled primarily by its ability to bind and hydrolyze GTP. While the GDP-bound form of Ras is inactive, the exchange of GDP for GTP results in protein activation and interaction with downstream effectors. The proteins that precisely regulate the GTP cycling of Ras have been extensively studied (21). However, models of Ras function as a simple molecular switch must be able to accommodate the observation that Ras proteins often control multiple, distinct signaling pathways, not all of which are activated at the same time or in the same cellular location.

In mammalian cells, multiple Ras isoforms have been characterized, which include H-Ras, N-Ras, and K-RasB. These Ras isoforms share almost identical amino acid sequences but are functionally distinct (21). Recent studies have found that functional specificity of these isoforms is dictated at least in part by differential membrane localization, which in turn is due to posttranslational modifications within a C-terminal hydrophobic domain (HVR) that culminates with the CAAX motif (18, 21). Initial membrane association occurs by the prenylation of a specific cysteine residue in the CAAX motif, targeting Ras to the endoplasmic reticulum (ER). Inhibition of Ras prenylation results in defective membrane association and impaired protein function. Consistent with the observation that prenylation is required for Ras activity, this process is constitutive and irreversible (35).

Within the HVR is a second targeting motif that varies in the different Ras isoforms. For example, the HVR of K-RasB contains a polybasic stretch that mediates membrane association. In contrast, the HVRs of H-Ras and N-Ras contain a cysteine residue (two cysteines in the case of H-Ras) that is palmitoylated. In contrast to protein prenylation, Ras palmitoylation is a regulated and reversible process, occurring on the ER. Once palmitoylated, Ras proteins traffic through the Golgi to the plasma membrane (PM) (2). This process is necessary for Ras proteins with palmitoylation motifs.

Recent studies of Ras orthologs in other organisms also illustrate signaling specificity based on protein localization. The fission yeast *Schizosaccharomyces pombe* has a single Ras ortholog, Ras1 (10). Ras1 mediates two specific signaling pathways, morphogenesis and mating, via two effector proteins, Scd1 and Byr2, respectively (5, 11, 32). Using mutant Ras proteins with altered localization patterns, Onken et al. (22) demonstrated that the palmitoylation-defective forms of Ras1 were restricted to the endomembrane and were able to direct morphogenesis but not mating. In contrast, PM-restricted forms of Ras1 were mating competent but displayed altered morphogenesis. Therefore, the cellular localization of *S. pombe* Ras1 directs its downstream function.

The human fungal pathogen *Cryptococcus neoformans* contains two Ras orthologs, Ras1 and Ras2 (33). Similar to that of *S. pombe* but distinct from that of *Saccharomyces cerevisiae*, *C. neoformans* Ras1 controls two distinct cellular processes, sexual differentiation and morphogenesis. Strains with loss-of-function *ras1* mutations are unable to mate, and this sterile phenotype involves a failure to induce expression of mating pheromones and other components of the pheromone response pathway (34). Additionally, Ras1 mediates cell morphology in a temperature-dependent fashion (1, 33). Strains with *ras1* mutations are impaired for growth at elevated temperatures, arresting at nonpermissive temperatures as unbudded cells with a depolarized actin cytoskeleton. This latter pathway is mediated by the Cdc24 nucleotide exchange factor, as well as by two Cdc42-like proteins (Cdc42 and Dcl2) (19).
To determine if palmitoylation plays a role in *C. neoformans* Ras1 signaling, we have identified and characterized specific cysteine residues within the C terminus of Ras1 and used mutational analysis to assess their roles in protein prenylation and palmitoylation. We also have assessed the phenotypic consequences of altered Ras1 protein localization, including its effects on microbial virulence. Our results support an emerging paradigm by which Ras localization directs its downstream function. However, we also explored the considerable functional flexibility of this otherwise highly conserved signaling pathway.

**MATERIALS AND METHODS**

**Strains and media.** *C. neoformans* strains used in this study are listed in Table 1. Strains were grown on yeast-peptone-dextrose (YPD) medium (27) or V8 mating medium (16). Mating assays were performed by coculturing strains of opposite mating types on V8 mating medium at 23°C.

The point mutations creating the various mutant *RAS1* alleles were also generated using PCR overlap extension, using the primers defined in Table 2. Each *RAS1* allele was cloned into the BamHI site of plasmid pCH233, placing the allele under the control of its native promoter and terminator and using the *nat* gene as a selectable marker, PCR overlap extension was used to replace the selectable marker, PCR overlap extension was used to replace the open reading frame with the neomycin resistance cassette (9). The linear overlap construct was used to create CBN45 (H99 mating-type *RAS1* nat::neo GFP-ras1) (25). The *ras1* allele was cloned into the BamHI site of plasmid pCN19 (25). The linear overlap construct was used to create CBN45 (H99 mating-type *RAS1* nat::neo GFP-ras1) (25). The *ras1* allele was cloned into the BamHI site of plasmid pCN19 (25). The linear overlap construct was used to create CBN45 (H99 mating-type *RAS1* nat::neo GFP-ras1) (25). The *ras1* allele was cloned into the BamHI site of plasmid pCN19 (25). The linear overlap construct was used to create CBN45 (H99 mating-type *RAS1* nat::neo GFP-ras1) (25). The *ras1* allele was cloned into the BamHI site of plasmid pCN19 (25). The linear overlap construct was used to create CBN45 (H99 mating-type *RAS1* nat::neo GFP-ras1) (25). The *ras1* allele was cloned into the BamHI site of plasmid pCN19 (25). The linear overlap construct was used to create CBN45 (H99 mating-type *RAS1* nat::neo GFP-ras1) (25). The *ras1* allele was cloned into the BamHI site of plasmid pCN19 (25). The linear overlap construct was used to create CBN45 (H99 mating-type *RAS1* nat::neo GFP-ras1) (25). The *ras1* allele was cloned into the BamHI site of plasmid pCN19 (25).

**Microscopy.** Differential interference microscopy and fluorescent images were captured with a Zeiss AxiosImager.A1 fluorescent microscope equipped with an AxioCam MRm digital camera. Confocal images were captured using a Zeiss LSM inverted confocal microscope with the Argon/2 488 laser.

**Pulmonary experiments.** Using the murine inhalation model of systemic cryptococcosis, we inoculated female A/Jcr mice intranasally with *5 × 10^4* *C. neoformans* cells as previously described (6). Briefly, groups of eight mice were inoculated with one of the following five strains: H99 (RAS1 wild type), CBN45 (ras1 mutant), CBN65A [ras1 plus ras1(C206S, C204S)], CBN71 [ras1 plus ras1(C206S, C204S)].
C. neoformans Ras1 contains a putative palmitoylation signaling motif conserved in filamentous fungi. To determine if palmitoylation plays a role in the function of C. neoformans Ras1, we first assessed the C-terminal sequence of Ras1 for possible palmitoylation motifs. In addition to the canonical cysteine of the CAAX motif, the C terminus contains two additional cysteines located adjacent to each other at positions 203 and 204. This motif is different than other characterized palmitoylation motifs; H-Ras has two palmitoylated cysteine residues spatially separated by two intervening amino acids, while other mammalian Ras proteins have only one palmitoylated cysteine (21).

To determine if the two-cysteine palmitoylation motif in C. neoformans Ras1 was unique or conserved in other fungi, we examined the C termini of various fungal Ras orthologs. The Ras proteins of the fission and budding yeasts S. pombe and S. cerevisiae each contain a single cysteine residue in the hypervariable domains upstream of the CAAX motif. In contrast, we found that Ras orthologs from many filamentous fungi contained adjacent cysteines at positions −7 and −8 from the C terminus, a pattern identical to that of C. neoformans Ras1 (Table 2).

Palmitoylation plays a differential role in C. neoformans Ras1 function. We hypothesized that both cysteine residues C203 and C204 were capable of being palmitoylated and that each would be important for protein function. To test this hypothesis, we created cysteine–serine mutations for each residue singly as well as in combination. These ras1 mutant alleles were cloned under the control of the endogenous RAS1 promoter sequence and transformed into the ras1 mutant strain. In each case, multiple independent transformants for each allele were tested. Also, the sequence of each mutant ras1 allele was verified to ensure that there were no unintended mutations in the plasmids.

Each mutant ras1 allele was assessed for the ability to complement the phenotypes of the ras1 mutant strain. All three strains [ras1(C203S), ras1(C204S), and ras1(C203S, C204S)] grew well at 30°C, and the strains with the single mutations [ras1(C203S) and ras1(C204S)] also grew well at 37°C and 39°C. In contrast, the ras1(C203S, C204S) double mutant strain failed to complement the thermostolerance defect of the ras1 mutant (Fig. 1A). Microscopic examination of the mutant strains incubated at elevated temperatures revealed that both single mutant strains grew as small, ovoid, budding yeasts, with cell morphology identical to that of the wild type at all incubation temperatures. When incubated at 39°C overnight, the ras1(C203S, C204S) double mutant strain exhibited the large, unbudded cell morphology of the ras1 mutant strain (Fig. 1B).

In the fission yeast S. pombe, inhibition of Ras palmitoylation results in inhibition of mating but does not have any functional consequence on morphology (22). In C. neoformans, mutation of the palmitoylated cysteine residues in the Ras1 protein inhibits proper morphogenesis. However, all of the palmitoylation-defective ras1 mutants are mating competent. When coincubated on V8 mating medium with a MATa mating partner, the ras1(C203S), ras1(C204S), and ras1(C203S, C204S) strains all display normal mating structures after 1 week of incubation in a manner indistinguishable from that of the wild type (Fig. 2). This result is in contrast to unilateral crosses in which ras1 mutations present in either mating partner result in sterility.

To further determine whether the palmitoylation-defective RAS1 alleles would fully support mating, we crossed a MATa ras1(C203S, C204S) strain and a MATa ras1(C203S, C204S) strain so that there would be no wild-type Ras1 protein in this mating reaction (Fig. 2). Initial mating filaments were visualized within 48 h of incubation, similar to that for the wild type. The extent of mating filamentation in this bilateral mutant cross was slightly delayed at 1 and 2 weeks of incubation compared to that of either unilateral cross or compared to wild-type mating reactions. However, microscopic examination of the mating hyphae indicated that all of these crosses were indistinguishable to the morphological features of normal mating reactions, including closed clamp connections, basidia, and meiotic basidiospores. Therefore, Ras1 palmitoylation is required for normal morphogenesis and growth at elevated temperatures; however, this process is dispensable for C. neoformans mating.

In addition to assessing the phenotype of the ras1 palmitoylation-defective alleles, we also generated a ras1 prenylation mutant by generating a cysteine→alanine substitution in the CAAX motif. In contrast to the palmitoylation-defective strains, the ras1(C2074) mutant allele failed to complement either the ras1 morphogenesis or mating defects (Fig. 1 and 2).

Cysteines203 and cysteine204 are palmitoylated in C. neoformans. Our functional data suggests that palmitoylation of either C203 or C204 is sufficient to support Ras1 function. Although cysteine residues immediately upstream of the CAAX motif are often palmitoylated in other species, we sought to confirm that this specific posttranslational modification actually occurred at these sites. To assess protein palmitoylation, we generated GFP-tagged versions of the wild-type and mutant Ras1 proteins. Each GFP-tagged variant behaved in a manner similar to the corresponding untagged wild-type or mutant allele in the ras1 deletion mutant. The GFP-RAS1, GFP-ras1(C203S), GFP-ras1(C204S), and GFP-ras1(C203S, C204S) strains were incubated to mid-logarithmic phase in YPD medium, and total protein lysates were obtained from these cell suspensions.

To access palmitoylation, we used a modified version of the acyl-biotinyl switch assay (14, 31). In this assay, NEM was added to the protein lysates to block all free sulphydryl groups. Thioester groups, such as those present at palmitoylated cysteines, were then cleaved with hydroxylamine, and the resulting sulphydryl groups were labeled with biotin. The biotinylated proteins were then precipitated using NeutrAvidin beads and immunoblotted with anti-GFP antibodies. Each protein lysate was split into thirds and processed as an experimental palmitoylation sample, a negative control without hydroxylamine to confirm that the labeling was specific, or a positive control for loading and immunoblotting.
We found that Ras1 palmitoylation occurred in the GFP-
RAS1 sample as well as in the GFP-ras1(C203S) and
GFP-ras1(C204S) samples (Fig. 3). However, no Ras1 protein pal-
mitoylation was detected in the GFP-ras1(C203S, C204S)
sample. These results confirm that both cysteines C203 and
C204 are true sites for palmitoylation. They also confirm that
palmitoylation at either residue results in sufficient Ras1 func-
tion to allow proper morphogenesis and growth at high tem-
peratures.

Ras1 palmitoylation is required for PM localization. In
other palmitoylated Ras proteins, palmitoylation plays an im-
portant role in specifying membrane localization (18, 21). We
assessed the localization of Ras1p using the GFP-tagged
strains. Each strain was incubated to mid-logarithmic growth
phase in rich medium at 30°C and assessed by epifluorescent
and confocal microscopy. The strain expressing the fusion pro-
note of GFP and wild-type Ras1p (Gfp-Ras1p) demonstrated a
strong fluorescent signal at the PM. Additionally, Gfp-Ras1p
was detected in punctate patches within the cells in a pattern
similar to proteins sorting through the Golgi (21) (Fig. 4). We
confirmed that the internal structures were indeed vesicular by
staining these cells with FM4-64, a vesicle vital dye, and found
overlap staining between Ras1p-GFP and FM4-64 (data not
shown).

The strains expressing RAS1 alleles with the single-palmi-
toylation mutations (GFP-Ras1p<sup>C203S</sup> and GFP-Ras1p<sup>C204S</sup>)
demonstrated a normal Ras1 localization signal. In contrast,
the double mutation did not allow Ras1p localization to the
PM; instead, the GFP-Ras1p<sup>C203S, C204S</sup> protein was restricted
to endomembrane structures, such as the ER and Golgi. We
also localized GFP-Ras1p<sup>C207A</sup>, defective in prenylation, and
found that it did not localize to either the PM or endomem-
branes. Instead, the fluorescent signal was diffusely present
throughout the cytoplasm (Fig. 4). Together these data suggest
that Ras1 farnesylation is required for initial association of the
protein with endomembranes and that palmitoylation pro-
motes trafficking of Ras1 to the PM. Furthermore, our results
indicate that palmitoylation of either C203 or C204 is sufficient
to direct localization of Ras1p to the PM and to mediate
morphogenesis at high temperatures.

Ras1 palmitoylation is required for pathogenesis. Inhibition
of Ras1 function results in the complete loss of virulence in
animal models of *C. neoformans* disease (1). This virulence defect is due to the growth and morphological defects that occur in the ras1 mutant at host physiological temperatures and not to its mating defects; other sterile strains have no defects in virulence. Since the ras1(C203S, C204S) palmitoylation defective strain shares the impaired thermotolerance of the ras1 mutant, we tested the palmitoylation mutants for virulence in a murine inhalational model of cryptococcosis (6). Female A/Jcr mice were inoculated by inhalation with 10^5 CFU of each of the following strains: wild-type RAS1, ras1(C203S), ras1(C204S), and ras1(C207A). The single-palmitoylation mutant strains displayed no virulence defects, resulting in a lethal infection identical to that of the wild type. In contrast, the strain with a mutation of both Ras1 palmitoylation sites was avirulent \( P < 0.01; \) wild type versus ras1(C203S, C204S), consistent with its ras1-like defects in morphogenesis and growth at elevated temperatures (Fig. 5).

**DISCUSSION**

In this series of experiments, we have demonstrated that posttranslational modifications direct the *C. neoformans* Ras1 protein to various membrane compartments. Prenylation of Ras proteins is required for early membrane association, and mutations that prevent prenylation are associated with altered Ras function. This process has been intensively studied as a potential therapeutic target in cancer since many human malignancies are associated with altered Ras function (12). Previously we demonstrated that pharmacologic inhibition of protein prenylation in *C. neoformans* resulted in reduced mating and altered morphogenesis, consistent with reduction in Ras protein activity (29, 30). In the current study, we definitively showed that a prenylation-defective form of Ras1p failed to localize to membranes and did not complement any phenotypes of the ras1 mutant strain.

In contrast to the constitutive prenylation process, protein palmitoylation is a regulated and reversible process. It is this variable modification that allows Ras proteins to target different membranes, thus affecting different downstream signaling pathways. As has been demonstrated in other systems, we confirmed that palmitoylation of *C. neoformans* Ras1 is involved in the targeting of this protein to the PM. However, the functional consequence of PM localization is variable in different species. For example, *S. pombe* Ras1p activates primarily the mating pathway when present on the PM; inhibition of
Ras1p palmitoylation results in a sterile mutant strain with otherwise normal morphogenesis (22). In contrast, mating is unaffected in C. neoformans mutants with defective Ras1p palmitoylation.

There are several potential mechanisms by which C. neoformans Ras might mediate mating from endomembranes rather than from the PM, even though many of the cellular events of mating are predicted to occur at the cell surface. It is possible that the mating response pathway proteins are actually present primarily on endomembranes. The identification and localization of mating-specific guanine nucleotide exchange factors and other effectors of Ras1 will help to distinguish between these two models. The C. neoformans Ras2 protein may also contribute to the mating response, but its failure to support mating in a ras1 mutant strain argues that its contribution to the mating response will be minor.

The S. pombe morphogenesis pathway does not require Ras1p palmitoylation or PM localization (22). In contrast, the morphogenic functions of C. neoformans Ras1p are specifically directed from the PM. We previously demonstrated that Ras1p is required for correct polarization of the actin cytoskeleton after exposure to nonlethal stresses such as incubation at 37°C to 39°C. In the absence of functional Ras1 protein, C. neoformans arrests at high temperatures as large, unbudded yeast cells with depolarized actin. Interestingly, the S. cerevisiae ras2 mutant displays a similar thermotolerance defect, as well as a failure to properly restore actin polarization in response to heat stress (15). Normal morphogenesis in the presence of temperature stress is important for all microorganisms as they encounter and respond to changes in their environment. However, one could argue that this process is especially important for microbial pathogens such as C. neoformans that require frequent adaptation to mammalian physiological temperatures.

As we demonstrated in these experiments, inhibition of protein palmitoylation mimics the thermotolerance/morphogenesis defects of the loss-of-function ras1 mutation without affecting mating. Moreover, our studies conclusively demonstrated that inhibition of Ras protein palmitoylation resulted in a major effect in pathogenesis. Neither of these results would have been predicted from the models developed in nonpathogenic microbial systems such as in S. pombe, in which Ras palmitoylation is required only for mating. Therefore, C. neoformans and S. pombe Ras proteins both mediate distinct mating and morphogenesis pathways. However, these conserved signaling elements have been “rewired” in these different fungal species to affect the same cellular processes from different subcellular locations.

In earlier work studying Ras protein palmitoylation in S. cerevisiae, mutation of the C-terminal palmitoylated cysteine resulted in defective Ras2 protein function, similar to a ras2 mutation. Interestingly, overexpression of the palmitoylation-defective ras2 allele was able to partially suppress all ras2 mutant phenotypes (8). In our system, we did not observe full functional complementation of Ras activity when the RAS1 (C237S, C204S) mutant allele was expressed behind either the HIS3 or native RAS1 promoter. Therefore, at near-physiological levels of expression, complete Ras function in C. neoformans is dependent upon intact protein prenylation.

The presence of two adjacent palmitoylated cysteines in the C. neoformans Ras1 C terminus is a different motif than that found in many other well-characterized Ras proteins, such as mammalian H-Ras and N-Ras, and the Ras proteins of budding and fission yeasts. However, the dual and adjacent palmitoylated cysteines present in C. neoformans Ras1p also exist in the C-termini of Ras proteins in many filamentous fungi. In fact, this appears to be a highly conserved feature of many fungal Ras proteins that is absent in S. pombe and S. cerevisiae. Rap2, a member of the mammalian Ras subgroup that also
FIG. 4. Ras1 palmitoylation is required for membrane localization. The localization patterns of the GFP-RAS1, GFP-ras1(C203S), GFP-ras1(C204S), GFP-ras1(C203S, C204S), and GFP-ras1(C207A) transformants were determined using confocal microscopy. Cells were imaged (×63 objective). Bar, 10 μm.
includes H-Ras and N-Ras also contains a similar dual cysteine motif (13). Similar to C. neoformans Ras1, the two Rap2 cysteines are located at positions −7 and −8. Rap2, which has two isoforms, Rap2A and Rap2B, localizes primarily to the Golgi (4, 24). Although palmitoylation is required for Rap2 function, the role of its two cysteines in palmitoylation has not been determined.

Is there any advantage to having two palmitoylated cysteines? Studies in mammalian H-Ras would argue that the presence of dual-palmitoylated cysteines may have important functional consequences. The H-Ras protein contains two palmitoylated cysteines at amino acid positions 181 and 184. Mutational studies in H-Ras reveal that monopalmitoylation of C181 is sufficient to traffic H-Ras from the Golgi to the PM. In contrast, the majority of monopalmitoylated C184 H-Ras is trapped at the Golgi (26). Differential palmitoylation of H-Ras also mediates localization within PM microdomains. Activated H-Ras signals in the PM from cholesterol-independent microdomains, while N-Ras signals from cholesterol-dependent microdomains. Similar to wild-type H-Ras, monopalmitoylated C184 H-Ras signals from cholesterol-independent microdomains. However, monopalmitoylated C181 H-Ras signals from cholesterol-dependent microdomains in a pattern similar to that of N-Ras, which is palmitoylated only at C181 (26).

In contrast to H-Ras, we did not observe any differential function or localization of either monopalmitoylated Ras1 protein in C. neoformans (Ras1pC203S or Ras1pC204S). In S. cerevisiae, the palmitoylated cysteine residue is directly adjacent to the prenylated cysteine. It has been postulated that the side-by-side location of these residues strengthens PM binding. It is possible that a similar scenario may exist for the adjacent palmitoylated cysteines in C. neoformans Ras1; i.e., that the double palmitoylation may strengthen membrane localization. However, our results do not preclude the possibility that only one site is palmitoylated at any given time.

A subset of fungal species, including Ustilago maydis, Neurospora crassa, and Aspergillus species, also has a second Ras ortholog. Similar to C. neoformans Ras2p, these second Ras proteins do not possess classic palmitoylation motifs. Instead, these proteins contain lysine-rich, poly-basic regions upstream of the CAAX motif. In mammalian K-RasB, the presence of multiple lysine residues directs localization to the PM independent of palmitoylation and Golgi trafficking. Similarly, fungal Ras proteins may direct protein localization by various mechanisms.

ACKNOWLEDGMENTS

This work was supported by PHS grants AI0163242 and AI050128. We thank Sam Johnson and the Duke Light Microscopy Core Facility for instruction and help with confocal imaging. We also thank the Fungal Genomes Initiative at the Broad Institute and the Washington University Medical Center Genome Sequencing Center for sequence data.

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