Mutational Analysis of the *Candida albicans* Ammonium Permease Mep2p Reveals Residues Required for Ammonium Transport and Signaling

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Received 14 July 2008/Accepted 24 November 2008

The ammonium permease Mep2p mediates ammonium uptake and also induces filamentous growth in the human-pathogenic yeast *Candida albicans* in response to nitrogen limitation. The C-terminal cytoplasmic tail of Mep2p contains a signaling domain that is not required for ammonium transport but is essential for Mep2p-dependent morphogenesis. Progressive C-terminal truncations showed Y433 to be the last amino acid that is essential for the induction of filamentous growth, thereby delimiting the Mep2p signaling domain. To understand in more detail how the signaling activity of Mep2p is regulated by ammonium availability and transport, we mutated conserved amino acid residues that have been implicated in ammonium binding or uptake. Mutation of D180, which has been proposed to mediate initial contact with extracellular ammonium, or the pore-lining residues H188 and H242 abolished Mep2p expression, indicating that these residues are important for protein stability. Mutation of F239, which together with F126 is thought to form an extracytosolic gate to the conductance channel, abolished both ammonium uptake and Mep2p-dependent filament formation, despite proper localization of the protein. On the other hand, mutation of W167, which is assumed to participate with Y122, F126, and S243 in the recruitment and coordination of the ammonium ion at the extracytosolic side of the cell membrane, also abolished filament formation without having a strong impact on ammonium transport, demonstrating that extracellular alterations in Mep2p can affect intracellular signaling. Mutation of Y122 reduced ammonium uptake much more strongly than mutation of W167 but still allowed efficient filament formation, indicating that the signaling activity of Mep2p is not directly correlated with its transport activity. These results provide important insights into ammonium transport and control of morphogenesis by Mep2p in *C. albicans*.

Microorganisms sense the availability of nutrients in the environment to induce the expression of genes whose encoded products are required for uptake and utilization of these nutrients (8, 11). Nutrient availability also governs developmental processes, like the induction of pseudohyphal growth of the budding yeast *Saccharomyces cerevisiae* in response to nitrogen limitation (9). In many cases, membrane proteins that are related to transporters mediating uptake of the nutrient into the cell but which have lost their transport function serve as extracellular nutrient sensors that activate signaling pathways to induce a cellular response (4, 12, 20, 31). However, in some cases, the transport proteins themselves also have a sensing and signaling function. Examples are the general amino acid permease Gap1p, the phosphate permeases Pho84p and Pho87p, and the ammonium permease Mep2p in *S. cerevisiae* (5, 10, 25).

*S. cerevisiae* possesses three genes, *MEP1* to *MEP3*, encoding ammonium permeases that mediate the uptake of this preferred nitrogen source into the cell (27). Any of the three ammonium permeases is sufficient to allow growth of the fungus when a low concentration of ammonium is the only available nitrogen source. However, only Mep2p induces pseudohyphal growth in response to limiting ammonium concentrations, and cells lacking *MEP2* do not form pseudohyphae under these conditions (3, 25). Interestingly, some other fungal ammonium permeases are able to rescue the pseudohyphal growth defect of *S. cerevisiae* *mep2Δ* mutants, indicating that the sensing and signaling function of Mep2p is preserved in specific members of the family across the fungal kingdom (1, 13, 17, 34, 35). How ammonium sensing by Mep2p and functionally homologous proteins is connected to the known signal transduction pathways that mediate pseudohyphal growth in *S. cerevisiae* has not yet been elucidated.

The human fungal pathogen *Candida albicans* also undergoes a transition from the budding yeast form to filamentous growth in response to nitrogen limitation. *C. albicans* possesses two genes, *MEP1* and *MEP2*, which allow growth on low concentrations of ammonium as the sole nitrogen source. *MEP2* can complement the pseudohyphal growth defect of *S. cerevisiae* *mep2Δ* mutants and is also required for filamentous growth of *C. albicans* in response to nitrogen limitation but not other signals that induce morphogenesis in this fungus (1). Therefore, the function of Mep2p in *C. albicans* is similar to that of its counterpart in *S. cerevisiae*. Mep2p is expressed at much higher levels than Mep1p, and this is a requirement for its ability to induce morphogenesis. Like other ammonium permeases, Mep2p contains 11 transmembrane domains, with the N terminus of the protein localized on the outer side of the cell membrane and the C terminus localized on the cytoplasmic side. Previous work has shown that the C-terminal cyto-
plasmic tail is not required for ammonium transport but is essential for the induction of filamentous growth in *C. albicans*, indicating that it contains a specific signaling domain (1).

The idea that Mep2p of *S. cerevisiae* is an ammonium sensor that induces pseudohyphal growth in response to extracellular ammonium was initially proposed because *mep2Δ* mutants were found to be defective for filamentous growth on solid media containing low concentrations of ammonium as the sole nitrogen source, whereas no filamentous growth defect was observed with other limiting nitrogen sources (25). In contrast, for *C. albicans*, *MEP2* was shown to be required for nitrogen starvation-induced filamentous growth, irrespective of the nature of the nitrogen source (1). Since high ammonium concentrations inhibit signaling by Mep2p, the latter findings suggested an alternative model for how the signaling activity of Mep2p might be regulated. In this model, Mep2p would induce filamentous growth as long as ammonium is absent or present at low concentrations, i.e., when most Mep2p proteins in the cell membrane are not engaged in ammonium transport. At higher ammonium concentrations, the transport activity of Mep2p would be increased, which in turn would inhibit its signaling activity and explain why *C. albicans* continues to grow in the budding yeast form instead of switching to filamentous growth under these conditions. Therefore, ammonium would not induce but would inhibit the signaling activity of the ammonium sensor Mep2p. Such a model would be supported if mutated Mep2p proteins that are unable to mediate ammonium transport but still induce filamentous growth could be identified. Recent experiments with *S. cerevisiae*, however, provided evidence that the signaling activity of Mep2p depends on its ammonium transport activity. Amino acid substitutions that blocked the transport activity of Mep2p prevented pseudohyphal growth, and a mutation that resulted in increased trans-

**Materials and Methods**

**Strains and growth conditions.** *C. albicans* strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at −80°C and subcultured on SD agar plates (6.7 g yeast nitrate base without amino acids [BIO 101, Vista, CA], 20 g glucose, 15 g agar per liter) at 30°C. For routine growth of the strains, YPD liquid medium (20 g peptone, 10 g yeast extract, 20 g glucose per liter) was used. To support growth of *ura3* strains, 100 μg/ml uridine was added to the medium. To observe MEP2 expression, strains were grown overnight in liquid SD-Pro medium, which contains 0.1% proline instead of ammonium, diluted 50-fold, and grown for 6 hours at 30°C in SD medium containing 100 μM ammonium (SLAD) or 100 μM proline (SLPD). To study filament formation, the strains were grown as single colonies for 6 days at 37°C on SD-2% agar plates containing 100 μM ammonium, proline, or urea as described in the text.

**Plasmid construction.** Plasmids pMEP2K1 and pMEP2ΔC2, containing the full-length *MEP2* and *MEP2Δc4-100* alleles, respectively, have been described previously (1). Additionally, C-terminally truncated Mep2p alleles were generated by amplifying the *MEP2* gene from pMEP2K1 with primer MEP3 and one of the primers MEP81 to MEP87 or MEP59, which introduce a stop codon at the desired position (all primers used in this study are listed in Table 2). The truncated Mep2p alleles were cloned for full-length *MEP2* in pMEP2K1, resulting in plasmids pMEP2ΔC6 to pMEP2ΔC13, which contain the *MEP2Δc4-439* to *MEP2Δc4-532* alleles, respectively.

Plasmid pMEP21H2 contains a hybrid *MEP2Δc1-418, MEP1Δc17-534* allele (1). Truncated derivatives in which a stop codon was inserted behind codon 438 or 433 of *MEP2* were generated by amplification of part of the hybrid gene with the primer pair MEP39p-MEP23 or MEP39p-MEP91 and its substitution for the integrating region in pMEP21H2, resulting in plasmids pMEP21H4 or pMEP21H6, respectively.

*MEP2* alleles with single amino acid substitutions were generated in the following way. For pMEP2D180L, the N-terminal part of *MEP2* plus upstream sequences was amplified from pMEP2K1 with the primers MEP3 and MEP38p and the remaining part of the *MEP2* coding region was amplified with primers MEP39 and MEP32. The two fragments were digested with KpnI and BamHI, respectively, and ligated into the vector pBluescript. Plasmids pMEP2D180N, pMEP2Y122A, pMEP2F126A, pMEP2W165A, pMEP2W167A, pMEP2H188A, pMEP2S243A, and pMEP2H342A were generated in an analogous fashion, using the primer pairs MEP3-MEP67p and MEP39p-MEP32, MEP3-MEP88p and MEP39p-MEP32, MEP3-MEP99p and MEP39p-MEP32, and pMEP71p and pMEP77p-MEP32 to pMEP76p-MEP32, respectively. A *SacI* fragment from plasmid pMEP2K1 containing the C-terminal part of *MEP2*, the *ACT1* transcription termination sequence, the *URA3* marker, and *MEP2* downstream sequences was then ligated between the same sites of the plasmids listed above to generate plasmids pMEP2K4 to pMEP2K12, containing the *MEP2Δc1-418, MEP1Δc17-534, MEP2Δc4-100, MEP1Δc17-534, MEP2Δc4-100, MEP2Δc4-100, MEP2Δc4-100, MEP2Δc4-100, and MEP2Δc4-100* alleles, respectively. Plasmids pMEP2K6 containing the F239A mutation was obtained by ligating the *SacI* fragment from plasmid pMEP2K1 into pBluescript-digested pMEP2G16 (see below). Plasmids containing green fluorescent protein (GFP)-tagged versions of the mutated *MEP2* alleles were created by substituting the KpnI-BamHI fragments from plasmids pMEP2D180L, pMEP2D180N, and pMEP2Y122A for the corresponding region in pMEP21H2, resulting in plasmid pMEP2G2 (1), respectively.

To elucidate how the signaling activity of Mep2p is controlled in *C. albicans*, we undertook a mutational analysis of *C. albicans* Mep2p (CaMep2p) to further delimit the previously identified C-terminal cytoplasmic signaling domain and to investigate how the ammonium transport activity of the permease influences its ability to induce filamentous growth under limiting nitrogen conditions.
TABLE 1. *C. albicans* strains used in this study

| Strain | Parent | Relevant genotype| Reference |
|--------|--------|------------------|-----------|
| CAI4   | SC5314  | 
| mep2Δ single and mep1Δ mep2Δ double mutants | 
| MEP2M4A and -B | CAI4 | mep2-1Δ::FRT/mep2-2Δ::FRT | 1 |
| MEP2M5A | MEP2M4A | mep2-1Δ::FRT/mep2-2Δ::URA3 | 1 |
| MEP2M6B | MEP2M4B | mep2-1Δ::URA3/mep2-2Δ::FRT | 1 |
| MEP2M4A | MEP2M4A | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT | 1 |
| MEP2M4B | MEP2M4B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT | 1 |
| MEP2M4B | MEP2M4B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT | 1 |
| MEP12M6A | MEP12M4A | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::URA3 | 1 |
| MEP12M6B | MEP12M4B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::URA3/mep2-2Δ::FRT | 1 |

mep2Δ mutants expressing wild-type or mutated *MEP2* alleles

| Strain | Parent | Relevant genotype| Reference |
|--------|--------|------------------|-----------|
| MEP2M1A | MEP2M4A | mep2-1Δ::FRT/mep2-2Δ::MEP2-URA3 | 1 |
| MEP2M1B | MEP2M4B | mep2-1Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3 | 1 |
| MEP2M6B | MEP2M6B | mep1-1Δ::FRT/mep1-2Δ::MEP2::URA3 | This study |
| MEP2M7B | MEP2M7B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3 | This study |
| MEP2M9A | MEP2M9A | mep1-1Δ::FRT/mep1-2Δ::MEP2::URA3 | This study |
| MEP2M9B | MEP2M9B | mep1-1Δ::FRT/mep1-2Δ::MEP2::URA3 | This study |
| MEP2M17A and -B | MEP2M4B | mep1-1Δ::FRT/mep2-2Δ::MEP2::FRT | This study |

mep1Δ mep2Δ double mutants expressing wild-type or mutated *MEP2* alleles

| Strain | Parent | Relevant genotype| Reference |
|--------|--------|------------------|-----------|
| MEP12M2A | MEP12M4A | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3 | 1 |
| MEP12M2B | MEP12M4B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::URA3/mep2-2Δ::FRT | 1 |
| MEP12M6A | MEP12M6A | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::URA3/mep2-2Δ::URA3 | This study |
| MEP12M6B | MEP12M6B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::URA3/mep2-2Δ::URA3 | This study |

mep1Δ mep2Δ double mutants expressing GFP-tagged *MEP2* alleles

| Strain | Parent | Relevant genotype| Reference |
|--------|--------|------------------|-----------|
| MEP12M2A | MEP12M4A | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3/mep2-2Δ::FRT | 1 |
| MEP12M2B | MEP12M4B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3/mep2-2Δ::URA3 | 1 |
| MEP12M4A | MEP12M4A | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3 | This study |
| MEP12M4B | MEP12M4B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3 | This study |
| MEP12M4B | MEP12M4B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3 | This study |
| MEP12M4B | MEP12M4B | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3 | This study |
| MEP12M4A | MEP12M4A | mep1-1Δ::FRT/mep1-2Δ::FRT/mep2-1Δ::FRT/mep2-2Δ::FRT/mep2-1Δ::URA3 | This study |

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with a FACSCalibur flow cytometry system equipped with an argon laser emitting at 488 nm (Becton Dickinson, Heidelberg, Germany). Fluorescence was measured on the FL1 fluorescence channel equipped with a 530-nm-band-pass filter. Twenty thousand cells were analyzed per sample and were counted at a low flow rate. Fluorescence and forward scatter data were collected by using logarithmic amplifiers. The mean fluorescence values were determined with CellQuest Pro (Becton Dickinson) software.

**Ammonium removal assays.** Ammonium removal from the medium was assayed as described previously (25, 27). Briefly, strains were grown to late log phase in SD-Pro medium, and the cultures were diluted to an optical density at 600 nm of 1.0 in the same medium plus 250 μM ammonium sulfate. At the indicated times, a portion of the culture was taken, the cells were removed by centrifugation, and the ammonium concentration in the culture supernatant was determined using a glutamate dehydrogenase-linked assay in which NH₄⁺ plus NADH plus H⁺ plus α-ketoglutarate is converted to glutamate plus NAD⁺ plus H₂O. An increase in the NADH concentration (measured by determining the absorbance at 340 nm 1 min after the addition of NADH) indicates ammonium removal by the cells.

**RESULTS**

**Identification of the minimal region in the C-terminal cytoplasmic tail of Mep2p that is required for the induction of filamentous growth.** Previous experiments demonstrated that...
TABLE 2. Primers used in this study

| Primer   | Sequence (5′–3′)* |
|----------|-------------------|
| MEP3     | TAAATACGCTACCGAATCCTATTCGTCAGATGTCTG |
| MEP9     | CCAATGAGTTTGATGATCTCGATATCAGCTATACATCAGTAATCA |
| MEP23    | AAAGAATCATACGCATCAGTACACTACAGTAATCA |
| MEP32    | GCACCCACCGTTGGCGGAGCTATGCACTAAGC |
| MEP85    | CCATGAGTTGAGATCTCAGTATCATTGTG |
| MEP100   | ATTTAGAT |
| MEP98    | ATTTAGAT |
| MEP72p   | TGGCGGTCCAGTTTCACGAAAACTCTGG |
| MEP71p   | CCGCGAAATCCAATGACCTAAGC |
| MEP70p   | CACGTAAACACAGTCAACAGG |
| MEP74p   | CCTATTGCCTATTGGACA |
| MEP83    | TTGGTTTCAGGATAGAT |
| MEP38p   | CCGCGAA |
| MEP23    | AGAAATCAGAT |
| MEP3     | TAAATACGGTACCCAAACGATTGGCTTGA |
| MEP80p   | CCGGTCCAGGTCGTCATCAGAT |

* Restriction sites introduced into the primers are underlined; stop codons (reverse sequence) are highlighted in bold, and mutated codons are shown in italics. Primers with the suffix “p” are 5′ phosphorylated.

deletion of the last 40 amino acids of Mep2p did not impair its ability to induce filamentous growth of C. albicans in response to nitrogen limitation. Cells expressing the MEP2<sup>440–444</sup> allele even had a hyperfilamentous phenotype, because mRNA levels of the truncated allele were strongly increased compared with those of the wild-type allele (1). In contrast, deletion of 17 additional amino acids abolished the capacity of Mep2p to induce filamentous growth, although the MEP2<sup>424–427</sup> allele was expressed at equally high levels and ammonium uptake by the truncated Mep2p protein was not affected. This result indicated that at least some residues in the region between amino acids 424 and 440 of Mep2p are part of a signaling domain that is specifically required for morphogenesis but not ammonium transport (Fig. 1A). To define the minimal region that is essential for the induction of filamentous growth, we generated progressive truncations starting from amino acid 440 of Mep2p. The truncated alleles were expressed in mep1Δ mep2Δ double mutants, and the ability of the strains to undergo filamentous growth on SLAD plates was evaluated. As expected, all tested truncated alleles rescued the growth defect of the double mutants under limiting ammonium conditions, and strains expressing the MEP2<sup>424–439</sup> to MEP2<sup>432–433</sup> alleles also exhibited the hyperfilamentous phenotype (Fig. 1B, panels a to c, and data not shown). In contrast, strains expressing the MEP2<sup>432–433</sup> allele were unable to undergo filamentous growth (Fig. 1B, panel d), indicating that the tyrosine at position 433 is the last residue that is essential for signaling by Mep2p.

Interestingly, Mep2p is highly similar in this region to Mep1p, which does not normally induce filamentous growth in C. albicans (Fig. 1A). It was previously shown that substitution of the Mep1p C-terminal tail for the C terminus of Mep2p abolished filamentous growth (1). To exclude the possibility that the C terminus of Mep1p, which is longer than that of Mep2p, contains an inhibitory domain, we created new hybrid alleles in which the last 22 and 17 codons of the hyperactive MEP2<sup>424–440</sup> and MEP2<sup>424–435</sup> alleles, respectively, were replaced by the corresponding sequence of MEP1. Strains expressing hybrid alleles did not undergo filamentous growth (Fig. 1B, panels e and f), demonstrating that residues in the region between amino acids 419 and 433 of Mep2p, in which it differs from Mep1p, must be essential for signaling. To investigate which of these eight amino acids are required for the signaling function of Mep2p, each of them was exchanged individually with the corresponding Mep1p amino acid in the MEP2<sup>424–435</sup> allele. The amino acid substitutions D419N, E420G, L423A, T425V, T426C, L427E, V428I, and A428D mutations did not undergo filamentous growth (Fig. 1B, panels g, h, i, l, m, and p). In contrast, strains expressing the MEP2<sup>424–435</sup> alleles with the M422E, L427E, and A428D mutations did not undergo filamentous growth or very weakly formed filaments on SLAD plates (Fig. 1B, panels k, n, and o). Therefore, the presence of a negatively charged amino acid (Glu or Asp) instead of the alanine or valine at position 427 influences the signaling activity of Mep2p, explaining the signaling defect of the Mep2p-Mep1p hybrid proteins.

Expression of mutated Mep2p proteins. It is thought that ammonium binding or transport by the Mep2p proteins of S. cerevisiae and C. albicans influences the signaling activity of the
proteins (1, 25). Although there is experimental evidence that ammonium transport is required for signaling by Mep2p in *S. cerevisiae* (3, 26, 32, 39), an alternative possibility that was suggested for *C. albicans* (1) is that Mep2p can induce filamentous growth only when it is not engaged in ammonium transport (i.e., when ammonium is absent or present only at low concentrations) and that transport activity inhibits signaling. To test the latter hypothesis, we attempted to generate transport-deficient Mep2p derivatives. The conserved aspartate at position 160 of *Escherichia coli* AmtB (in the mature protein after removal of the signal sequence) has been suggested to be an initial ammonium binding site (36). We therefore mutated D180 of Mep2p, which corresponds to D160 of AmtB, to either leucine or asparagine (also see Discussion). The resolution of the AmtB crystal structure revealed additional highly conserved residues that might stabilize ammonium in an outer vestibule of the protein (F103, F107, W148, and S219) as well as two pore-lining histidines (H168 and H318) that are believed to make contacts with ammonium during transport (19, 21). Since mutation of these residues might abolish ammonium transport, we changed the corresponding amino acids in Mep2p (Y122, F126, W167, H188, S243, and H342) as well as W165, which is located near W167, to alanine. The mutated alleles were introduced into *mep1Δ mep2Δ* double mutants, and their capacity to restore growth of the mutants on plates containing limiting ammonium concentrations was assessed. As shown in Fig. 2, strains expressing the *MEP2*<sub>W165A</sub>, *MEP2*<sub>W167A</sub>, and *MEP2*S243A alleles grew as well as control strains containing a wild-type *MEP2* allele, indicating that the mutated proteins were still able to transport ammonium. Growth was also restored by the *MEP2*<sub>Y122A</sub> and *MEP2*<sub>F126A</sub> alleles, albeit at reduced levels compared with the wild-type *MEP2* allele. In contrast, the D180L, D180N, H188A, and H342A mutations abolished growth of strains expressing the corresponding *MEP2* alleles, indicating that these mutations rendered the ammonium permease nonfunctional. To test if the mutated Mep2p proteins were correctly expressed in the cell membrane, we introduced C-terminally GFP-tagged versions of all alleles into *mep1Δ mep2Δ* double mutants and observed expression of the fusion proteins by fluorescence microscopy. Mep2p-GFP fusion proteins containing the Y122A, F126A, W165A, W167A, and S243A mutations were all correctly localized at the cell periphery (Fig. 3A). However, while cells expressing GFP-tagged *MEP2*<sub>W165A</sub> and *MEP2*S243A alleles exhibited similar fluorescence to that of cells expressing a GFP-tagged wild-type *MEP2* gene, the Y122A, F126A, and W167A mutations resulted in reduced fluorescence of the corresponding transformants. In contrast,
GFP-tagged Mep2p proteins containing the D180L, D180N, H188A, and H342A mutations could not be detected in the cells by fluorescence microscopy (data not shown). Therefore, the latter mutations, which abolished growth at low ammonium concentrations, did not specifically affect ammonium transport but rather impaired Mep2p expression or protein stability. To compare the expression levels of the mutated Mep2p proteins, the strains carrying the GFP-tagged alleles were analyzed by flow cytometry. As shown in Fig. 3B, cells containing the MEP2\textsuperscript{D180L}, MEP2\textsuperscript{D180N}, and MEP2\textsuperscript{H342A} alleles exhibited only background fluorescence, confirming that these proteins were not expressed at detectable levels. The fluorescence of cells containing the MEP2\textsuperscript{H188A} allele was marginally higher (3.5-fold) than that of control cells without a GFP-tagged MEP2 gene. The W165A and S243A mutations resulted in only slightly reduced fluorescence compared with that of the wild-type control (53% and 62% of the wild-type level, respectively). In contrast, and in line with the microscopic observations, the fluorescence of the cells expressing the MEP2\textsuperscript{Y122A}, MEP2\textsuperscript{F126A}, and MEP2\textsuperscript{W167A} alleles was more strongly reduced (20%, 23%, and 21% of the wild-type level, respectively). Similar results were obtained when MEP2 expression was induced in medium containing proline instead of ammonium as the limiting nitrogen source, in which a functional Mep2p protein is not required for growth (Fig. 3C).

Ammonium transport and signaling activity of mutated Mep2p proteins. To assess the effects of the various amino acid substitutions on the signaling activity of Mep2p, the ability of strains expressing the corresponding MEP2 alleles to undergo filamentous growth in response to nitrogen limitation was tested. As expected, MEP2 alleles containing the D180L, D180N, H188A, and H342A mutations, whose encoded proteins were not detectably expressed in the cell membrane, did not restore filamentous growth of mep1Δ mep2Δ double mutants on plates containing limiting concentrations of different nitrogen sources (data not shown). In contrast, strains expressing the MEP2\textsuperscript{Y122A} and MEP2\textsuperscript{S243A} alleles exhibited wild-type filament formation on SLAD plates (Fig. 4). Filamentous growth was slightly reduced in strains expressing the MEP2\textsuperscript{W167A} allele compared with that of the wild-type control, which could be explained by the fivefold reduced expression levels of the mutated Mep2p proteins (see above). However, filament formation on SLAD plates was completely abolished in strains containing the MEP2\textsuperscript{F126A} and MEP2\textsuperscript{W167A} alleles (Fig. 4), although the corresponding Mep2p proteins were expressed at similar levels to that of Mep2p containing the Y122A substitution (Fig. 3), indicating that the F126A and W167A mutations impaired the signaling activity of Mep2p.

To investigate if the signaling activities of mutated Mep2p proteins correlated with their ability to transport ammonium, we compared ammonium uptake by strains expressing wild-type, MEP2\textsuperscript{Y122A}, MEP2\textsuperscript{F126A}, MEP2\textsuperscript{W167A}, and MEP2\textsuperscript{S243A} alleles in an ammonium removal assay (see Materials and Methods). As shown in Fig. 5A, the S243A mutation, which did not impair growth and filament formation on SLAD plates, had no detectable effect on ammonium uptake. In contrast, ammonium uptake was reduced to different degrees in cells expressing the MEP2\textsuperscript{Y122A} and MEP2\textsuperscript{W167A} alleles (Fig. 4), which still allowed filamentous growth. Therefore, the signaling activity of the mutated Mep2p proteins was not directly correlated with their ammonium transport activity. The ammonium uptake capacity of cells expressing the mutated MEP2 alleles corresponded well with their ability to grow in liquid medium (Fig. 5B) as well as on solid medium (Fig. 4) containing limiting ammonium concentrations. The F126A mutation, which had the severest effect on ammonium uptake, also reduced growth of the cells most strongly. The Y122A mutation, which had an intermediate effect on ammonium transport, also resulted in a significant growth reduction. In contrast, the minor effect of the W167A mutation on ammonium uptake did not translate into a detectable growth defect of the cells.

In order to test the filament formation capacity of cells expressing mutated MEP2 alleles independently of their ability to grow under nitrogen-limiting conditions, we assessed filamentous growth of the strains on plates containing limiting concentrations of other nitrogen sources, i.e., urea.
or the amino acid proline. Under these conditions, ammonium permeases are not required for growth, but Mep2p is still necessary for the induction of filament formation (1). The effects of the various mutations on filamentous growth on plates containing 100 μM urea corresponded to those seen on SLAD plates, i.e., the Y122A substitution resulted in a slight reduction of filament formation, whereas the F126A and W167A mutations abolished filamentous growth (Fig. 6). Interestingly, on plates containing 100 μM proline, strains expressing the \( \text{MEP2}^{F126A} \) allele produced filamentous colonies, whereas the W167A mutation also abolished filament formation under these conditions. Proline is known to induce filament formation in \( \text{C. albicans} \) (23), although at the limiting concentrations used in our assays Mep2p is nevertheless required for filamentous growth. It therefore seems that Mep2p containing the F126A mutation can still

![Image of fluorescence microscopy results](image-url)

FIG. 3. Expression of GFP-tagged Mep2p proteins. (A) \( \text{mep1Δ mep2Δ} \) double mutants carrying wild-type or mutated \( \text{MEP2} \) alleles were grown for 6 h at 30°C in liquid SLAD medium and observed by fluorescence microscopy to confirm proper localization of GFP-tagged Mep2p. Similar results were obtained when the cells were grown in SLPD medium containing proline instead of ammonium (data not shown). (B) Fluorescence of strains grown in SLAD medium was quantified by flow cytometry. The bars represent the means and standard deviations from five (control, wild type, and Y122A, F126A, W165A, W167A, and S243A mutants) or two (D180L, D180N, H188A, and H342A mutants) experiments performed with each of two independently constructed series of strains. The \( \text{mep1Δ mep2Δ} \) double mutants MEP12M6A and -B served as controls. (C) Fluorescence of strains grown in SLPD medium was quantified by flow cytometry. The bars represent the means and standard deviations from two independent experiments.
support filament formation when additional signaling pathways are activated.

In a complementary approach to study the effects of MEP2 mutations on signaling separately from their effects on growth of the cells, we introduced the MEP2<sup>Y122A</sup>, MEP2<sup>F126A</sup>, and MEP2<sup>W167A</sup> alleles into mep2Δ single mutants, which can grow normally on SLAD plates because they express Mep1p. As shown in Fig. 6 (right panels), the

![Fig. 4](image)

FIG. 4. Filament formation of strains expressing the indicated MEP2 alleles in a mep1Δ mep2Δ background. Individual colonies were photographed after 6 days of growth at 37°C on SLAD plates. The two independently constructed series of strains behaved identically, and only one of them is shown. Strains MEP12M6A and -B (mep1Δ mep2Δ) were used as controls. All pictures are shown at the same magnification.

![Fig. 5](image)

FIG. 5. (A) Ammonium uptake by mep1Δ mep2Δ double mutants expressing wild-type or mutated MEP2 alleles. Ammonium uptake was measured as described in Materials and Methods. Two independently constructed strains were used in each case. Strains MEP12M6A and -B (mep1Δ mep2Δ mutants) served as controls. (B) Growth of the same strains in liquid medium under limiting ammonium conditions. Precultures of the strains grown in SD-Pro medium were diluted 10<sup>-2</sup> in SD medium containing 1 mM ammonium as the sole nitrogen source, and growth was monitored by measuring the optical densities (OD<sub>600</sub>) of the cultures over time.
Filament formation behaviors of these strains corresponded to those of the \textit{mep1\Delta mep2\Delta} double mutants expressing the same alleles, i.e., the Y122A mutation resulted in reduced filamentous growth and the F126A and W167A mutations abolished filament formation.

An F239A mutation abolishes ammonium uptake and induction of filamentous growth by Mep2p. Very recently, Javelle et al. demonstrated that mutation of the conserved phenylalanine at amino acid position 215 in AmtB of \textit{E. coli} abolished ammonium transport (14). F215, together with F107, is thought to form an extracytosolic gate to the conductance channel in AmtB, and they concluded that F215 is essential for the deprotonation of ammonium to allow transport of ammonium into the cell. In order to obtain a similarly transport-deficient Mep2p protein in \textit{C. albicans}, we replaced the corresponding residue, F239, with an alanine. Indeed, expression of the \textit{MEP2^F239A} allele did not restore growth of \textit{mep1\Delta mep2\Delta} double mutants on SLAD plates, indicating that the F239A mutation abolished ammonium uptake by Mep2p (Fig. 7A). Fluorescence microscopy of cells expressing a GFP-tagged Mep2p^F239A protein demonstrated that the mutated protein was properly localized (Fig. 7B), and quantification of the cellular fluorescence by flow cytometry showed that the expression levels of the mutated protein reached approximately 35% of those of wild-type Mep2p in cells grown in SLAD medium and approximately 50% of those of wild-type Mep2p in cells grown in SLPD (data not shown). We then investigated if the transport-deficient Mep2p protein was able to induce filamentation. As shown in Fig. 7C, \textit{mep1\Delta mep2\Delta} double mutants expressing the \textit{MEP2^F239A} allele were unable to undergo filamentous growth on agar plates containing limiting concentrations of different nitrogen sources (left three panels), and the mutated allele also did not restore filamentous growth of \textit{mep2\Delta} single mutants on SLAD plates (right panels). Therefore, F239 is essential for both ammonium transport and induction of filamentous growth by Mep2p in \textit{C. albicans}.

**FIG. 6.** Filament formation of \textit{mep1\Delta mep2\Delta} double mutants (left two panels) and \textit{mep2\Delta} single mutants (right panels) expressing wild-type or mutated \textit{MEP2} alleles on agar plates containing 100 \(\mu\text{M}\) of the indicated nitrogen sources. Individual colonies were photographed after 6 days of growth at 37°C. The \textit{mep1\Delta mep2\Delta} double mutants MEP12M6A and -B and the \textit{mep2\Delta} single mutants MEP2M5A and -B were used as controls. The two independently constructed series of strains behaved identically, and only one of them is shown.
DISCUSSION

The yeast ammonium permease Mep2p and functionally related proteins from other fungi are believed to be signaling proteins that sense ammonium in the environment and, as a response, activate signal transduction pathways to induce developmental processes in these organisms (1, 17, 25, 33–35). How exactly ammonium controls the signaling activity of the ammonium permeases and how these sensor permeases activate the signal transduction pathways have not yet been resolved. An N-terminal domain has been implicated in the signaling function of Mep2p in *S. cerevisiae* (25), and the C-terminal cytoplasmic tail of *C. albicans* Mep2p was shown to contain a specific signaling domain that is required for the induction of filament formation but not for ammonium uptake (1). A signaling function of the C-terminal cytoplasmic tail has also been shown for bacterial ammonium permeases. AmtB proteins of *E. coli* and other bacteria bind via the C-terminal tail to the PII protein GlnK, which controls ammonium uptake and the activities of enzymes involved in nitrogen metabolism, such as glutamine synthetase and nitrogenase (16, 38). In addition, the C-terminal tails of Amt1;1 and Amt1;2 in the plant *Arabidopsis thaliana* mediate cross talk between the monomers in the trimeric ammonium permeases to regulate ammonium transport. In this case, phosphorylation of the C-terminal tail seems to inhibit ammonium uptake at high ammonium concentrations (24, 30).

Here we have delimited the C-terminal signaling domain of CaMep2p by generating progressive truncations and assessing the ability of the mutated proteins to induce filamentous growth. This analysis demonstrated that the C-terminal 47 amino acids of CaMep2p are dispensable for nitrogen starvation-induced filament formation and that Y433 is the last

FIG. 7. (A) An F239A mutation abolishes ammonium uptake by Mep2p. *mep1Δ mep2Δ* double mutants expressing wild-type *MEP2* or the *MEP2*<sup>F239A</sup> allele were grown for 4 days at 30°C on plates containing 2 mM ammonium. (B) The F239A mutation does not affect localization of Mep2p. *mep1Δ mep2Δ* double mutants carrying *GFP*-tagged wild-type or *MEP2*<sup>F239A</sup> alleles were grown for 6 h at 30°C in liquid SLAD medium and observed by fluorescence microscopy. (C) The F239A mutation in Mep2p prevents the induction of filamentous growth in response to nitrogen limitation. *mep1Δ mep2Δ* double mutants (left three panels) and *mep2Δ* single mutants (right panels) expressing wild-type *MEP2* or the *MEP2*<sup>F239A</sup> allele were grown for 6 days at 37°C on agar plates containing 100 μM of the indicated nitrogen sources, and individual representative colonies were photographed. The two independently constructed series of strains behaved identically in all experiments, and only one of them is shown in panels A to C. The *mep1Δ mep2Δ* double mutants MEP12M6A and -B and the *mep2Δ* single mutants MEP2M5A and -B were used as controls.
amino acid that is required for signaling. A hybrid protein in which the region between amino acids 419 and 435 of Mep2p was replaced by the corresponding region from Mep1p was unable to induce filament formation, demonstrating that one or more of the eight amino acids in which the two proteins differ in this region are critical for the signaling function of Mep2p. Indeed, replacement of the hydrophobic amino acids M422, L427, and A428 by the negatively charged amino acid Glu or Asp, located at the corresponding positions in Mep1p, abolished the ability of the mutated Mep2p proteins to induce filamentous growth. However, it is likely that other residues in this region which are present in both Mep1p and Mep2p are also part of the signaling domain. Notably, Mep1p has been shown to induce a low level of filament formation when it is overexpressed from the MEP2 promoter, indicating that specific contacts of the C-terminal tail with the remainder of the protein also govern the signaling capacity of the ammonium permeases (1). Further support for this conclusion comes from the observations that ammonium permeases from other fungi can also restore pseudohyphal growth in S. cerevisiae mep2Δ mutants (13, 17, 34, 35) and that, like CaMep1p, some of these contain Glu and/or Asp at positions corresponding to M422 and L427 of CaMep2p. It is therefore likely that the signaling competence of an ammonium permease does not depend on universally conserved amino acid residues but on specific interactions between the C-terminal signaling domain and adjacent loops in its own and/or neighboring monomers, similar to the control of ammonium uptake by intra- and intersubunit interactions in ammonium transporters of A. thaliana (24, 30). Nevertheless, one must assume that all of these ammonium permeases act similarly on the same signal transduction pathway to induce pseudohyphal growth when expressed in S. cerevisiae. An understanding of how this is achieved requires the identification of the interaction partners of the proteins, which are currently not known for any of these fungi. Recently, a different model was proposed for how Mep2p regulates filamentous growth in S. cerevisiae (2). It was shown that ScMep1p and ScMep2p have different pH optima and proposed that the two proteins may differ in the mechanism of ammonium transport. Transport by Mep2p would involve a deprotonation step, whereas transport by Mep1p would not, with opposite consequences on internal pH variations, and it was suggested that Mep2p regulates filamentous growth indirectly, by influencing pH. At least for C. albicans, such a model of indirect control via pH regulation is difficult to reconcile with our finding that the C-terminal cytoplasmic tail of Mep2p contains a signaling domain that is dispensable for ammonium transport but essential for signaling, as it seems unlikely that removal of the cytoplasmic tail would affect the deprotonation step at the extracellular gate and alter the mechanism of ammonium transport through the channel.

Another important question that we tried to address in this study is how the signaling activity of Mep2p is controlled by ammonium availability. Mutational analyses of Mep2p of S. cerevisiae have shown that Mep2p-dependent pseudohyphal growth usually correlates with the ammonium transport capacity of mutated proteins (3, 26, 32, 39). Current models therefore propose that ammonium transport by Mep2p is required for its sensory role in the induction of pseudohyphal growth. Since filamentous growth of C. albicans is repressed at higher ammonium concentrations even when Mep2p is still expressed, it was previously suggested that ammonium transport activity may actually repress signaling by Mep2p and that Mep2p would induce filamentous growth only at low extracellular ammonium concentrations, i.e., when Mep2p proteins in the cell membrane are not engaged in ammonium transport, to allow a fine-tuning of the growth behavior of C. albicans in response to nitrogen availability in the environment (1). Such a model would be supported by the identification of transport-deficient Mep2p proteins that are still able to induce filamentous growth. We therefore mutated several conserved amino acid residues in CaMep2p, which from structural and functional analyses of ammonium permeases of other organisms were supposed to be required for ammonium transport, and tested the ability of the mutated proteins to mediate ammonium uptake and filamentous growth in C. albicans. The phenotypes caused by amino acid exchanges in Mep2p are summarized in Table 3.

Methylation of several amino acid residues (D180, H188, and H342) abolished the expression of Mep2p, indicating that these amino acids are important for the stability of the protein. We first changed D180 to leucine, as the negatively charged aspartate at the analogous position of AmtB of E. coli (D160) had been proposed to function as an initial binding site for the

### Table 3. Phenotypes caused by amino acid exchanges in Mep2p

| Mutation | Mep2p level<sup>a</sup> | Growth phenotype<sup>b</sup> | Ammonium transport phenotype<sup>g</sup> | Filament formation phenotype<sup>c</sup> |
|----------|-----------------------|-----------------|--------------------------------|--------------------------------|
| Y122A    | Reduced               | Slightly reduced | Reduced                       | Slightly reduced               |
| Y126A    | Reduced               | Strongly reduced | Strongly reduced               | Normal                         |
| W165A    | Slightly reduced      | Normal          | Slightly reduced Normal<sup>f</sup> | Normal                         |
| W167A    | Reduced               | Normal          | Slightly reduced Normal<sup>f</sup> | Abolished                      |
| D180L    | Absent                | No growth       | Normal                        | NT Abolished                  |
| D180N    | Absent                | No growth       | Normal                        | Abolished                      |
| H188A    | Absent                | No growth       | Normal                        | Abolished                      |
| S243A    | Slightly reduced      | Normal          | Normal                        | Normal                         |
| F239A    | Reduced               | No growth       | NT Abolished                  | Abolished                      |
| H342A    | Absent                | No growth       | NT Abolished                  | Abolished                      |
| D419N    | NT                    | Normal          | Normal                        | NT Abolished                  |
| E420G    | NT                    | Normal          | Normal<sup>b</sup>            | Normal<sup>b</sup>             |
| M422E    | NT                    | Normal          | Normal<sup>b</sup>            | Normal<sup>b</sup>             |
| L423A    | NT                    | Normal          | Normal<sup>b</sup>            | Normal<sup>b</sup>             |
| T425V    | NT                    | Normal          | Normal<sup>b</sup>            | Normal<sup>b</sup>             |
| L427E    | NT                    | Normal          | Normal<sup>b</sup>            | Abolished                      |
| A428D    | NT                    | Normal          | Normal<sup>b</sup>            | Strongly reduced Normal<sup>b</sup> |
| Y433F    | NT                    | Normal          | Normal<sup>b</sup>            | Normal<sup>b</sup>             |

<sup>a</sup> Determined by fluorescence-activated cell sorter analysis of strains expressing GFP-tagged Mep2p (Fig. 3).

<sup>b</sup> Growth in liquid SLAD medium and/or on agar plates containing limiting ammonium concentrations (Fig. 1, 2, 4, 5, and 7).

<sup>c</sup> Determined by ammonium removal assay (Fig. 5A).

<sup>d</sup> Filament formation on agar plates containing 100 μM ammonium, proline, or urea (Fig. 1, 4, 5, and 7).

<sup>e</sup> Normal filament formation on plates containing 100 μM proline, but no filament formation on plates containing other limiting nitrogen sources (Fig. 4 and 6).

<sup>f</sup> NT, not tested.

<sup>g</sup> Data not shown.

<sup>h</sup> Hyperfilamentation because of the ΔC435 truncation.

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positively charged ammonium ion (36) and its mutation to alanine resulted in a complete loss of transport activity, despite wild-type expression levels (18). However, we could not detect expression of the mutated Mep2p protein in C. albicans. Recently, a similar mutation in Mep2p of S. cerevisiae (D186A) was also reported to result in stacking of the protein in the endoplasmic reticulum, but it was found that a D186N substitution allowed normal expression of the protein and abolished ammonium transport and Mep2p-dependent pseudohyphal growth (26). We therefore also changed D180 to asparagine in Mep2p of C. albicans to investigate if this would similarly affect ammonium transport and signaling. Again, no expression of the mutated protein could be detected, which prevented us from assigning a specific function to D180, apart from its importance in protein stability. Mutated Mep2p proteins in which the highly conserved histidines H188 and H342 were changed to alanine were also not detected in the cell membrane. In contrast, mutation of the analogous residues inAmtB of E. coli (H168 and H318) or Rhodobacter capsulatus (H193 and H342) allowed normal expression of the protein but abolished ammonium transport (15, 37). Similarly, mutation of H194 and H348 to alanine in Mep2p of S. cerevisiae also did not affect expression and localization of the proteins, but Mep2p with the H194A mutation did not restore growth of mep1Δ mep2Δ mep3Δ mutants at low ammonium concentrations (32). Therefore, Mep2p of C. albicans seems to be more sensitive to mutations in these highly conserved amino acid residues than ammonium permeases from other organisms, which are still normally expressed.

Mutation of four other residues (Y122, F126, W167, and S243) predicted to participate in the recruitment and coordination of the ammonium ion (19, 21) allowed functional expression of Mep2p. An S243A substitution did not affect ammonium transport and signaling, indicating that S243 is not important for the function of Mep2p. A similar result was recently reported forAmtB of E. coli, where an analogous S219A mutation even increased transport activity (14). Substitution of alanine for Y122, F126, and W167 also allowed functional expression of the mutated ammonium permeases in the cytoplasmic membrane, although at strongly reduced levels, and it affected ammonium transport to different degrees. These three residues have also been mutated in other organisms. A Y133I mutation in Amt1;1 from Lycopersicon esculentum (corresponding to Y122 in CaMep2p) resulted in decreased methylammonium transport despite normal expression levels and cellular localization (28). An F131A mutation inAmtB of R. capsulatus completely abolished ammonium transport, although the protein was normally expressed and localized to the membrane (37). In contrast, we found that an analogous F126A substitution still allowed some ammonium transport by CaMep2p. A W178L substitution in LeAmt1;1 (corresponding to W167 of CaMep2p) also decreased ammonium transport, despite normal expression levels and localization of the protein (28). In contrast, an analogous W148L mutation inAmtB of E. coli even increased ammonium flux (6). We found a minor effect of the W167A mutation on ammonium transport by CaMep2p, which may be explained by the reduced expression level of the mutated protein. These results indicate that the importance of different amino acid residues for the function of ammonium permeases may vary between different organisms. As shown in the present study, several residues that can be mutated in other ammonium permeases without affecting their expression and localization seem to be indispensable for the stability of Mep2p in C. albicans.

It was previously demonstrated that the transport and signaling functions of C. albicans Mep2p can be separated (1); however, so far this conclusion was based on mutated proteins lacking the C-terminal cytoplasmic tail, which contains (part of) the signaling domain. In contrast, W167 is located in an extracytoplasmic loop of Mep2p, and its mutation abolished its ability to induce filamentous growth, although it had only a minor effect on ammonium transport. This finding demonstrates that alterations in the extracellular portion of Mep2p can affect intracellular signaling. For S. cerevisiae, several lines of evidence suggest that the signaling activity depends on its ammonium transport activity. D186N and H194A mutations inScMep2p abolished both ammonium transport and pseudohyphal growth, despite normal localization of the proteins (26, 32). Conversely, a G349C mutation inScMep2p resulted in increased ammonium transport and concomitantly enhanced pseudohyphal growth (3). On the other hand, an H194E mutation inScMep2p abolished pseudohyphal growth despite the fact that ammonium transport by the mutated protein was increased (2). In our present work, we were able to generate one mutated CaMep2p protein that was defective for ammonium transport despite normal localization. Mutation of F239, whose counterpart, F215, in E. coli AmtB was recently shown to be essential for ammonium transport (14), abolished both ammonium uptake and induction of filamentous growth by Mep2p in C. albicans, supporting the model that signaling by Mep2p depends on ammonium transport. However, our results indicate that the effects of mutations on signaling may also be caused by an alteration of protein structure and not necessarily by altered ammonium transport, as exemplified by the W167A mutation, which abolished signaling without having a strong effect on ammonium transport. In contrast, the Y122A mutation had a stronger impact on ammonium transport than the W167A mutation but nevertheless hardly influenced Mep2p-dependent filamentous growth. Therefore, a definite conclusion about how ammonium transport affects signaling by Mep2p cannot be drawn from mutational analyses alone.

Apart from the effect of ammonium on the signaling activity of Mep2p, there are differences in the regulation of filamentous growth by ammonium availability in C. albicans and S. cerevisiae. Dominant-active RAS1 or GPA2 alleles, which activate the cyclic AMP (cAMP)-protein kinase A signaling pathway, or the addition of exogenous cAMP can bypass the requirement of MEP2 for filamentous growth in response to limiting nitrogen concentrations in both C. albicans and S. cerevisiae. However, increased ammonium concentrations nevertheless suppress morphogenesis even in C. albicans strains expressing dominant-active RAS1 or GPA2 alleles or in the presence of exogenous cAMP, indicating that ammonium or its metabolic products also inhibit filament formation downstream of these regulators (1). In contrast, dominant-active RAS1 or GPA2 alleles or exogenous cAMP stimulates pseudohyphal growth even at high ammonium concentrations in S. cerevisiae (25). In addition, forced overexpression of MEP2 from a galactose-inducible promoter also induced

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pseudohyphal growth under nitrogen-replete conditions, demonstrating that ammonium limitation per se is not required for the induction of the ammonium-responsive dimorphic switch in S. cerevisiae (32). In contrast, filamentous growth was suppressed in C. albicans cells containing the hyperactive MEP2ΔC440 allele at an increased ammonium concentration (10 mM) at which MEP2 is still expressed (1), and the same was found when the wild-type or hyperactive MEP2ΔC440 allele was expressed from the constitutively active ADH1 promoter (our unpublished results). Therefore, the control of morphogenesis by nitrogen availability differs between C. albicans and S. cerevisiae, and these species-specific differences may well extend to the control of Mep2p signaling activity itself.

ACKNOWLEDGMENT

This study was supported by the Deutsche Forschungsgemeinschaft (DFG grant MO 846/4).

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