Role of the Npr1 Kinase in Ammonium Transport and Signaling by the Ammonium Permease Mep2 in *Candida albicans*

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The ammonium permease Mep2 induces a switch from unicellular yeast to filamentous growth in response to nitrogen limitation in *Saccharomyces cerevisiae* and *Candida albicans*. In *S. cerevisiae*, the function of Mep2 and other ammonium permeases depends on the protein kinase Npr1. Mutants lacking *NPR1* cannot grow on low concentrations of ammonium and do not form filamentous growth under limiting nitrogen conditions. A G349C mutation in Mep2 renders the protein independent of Npr1 and results in increased ammonium transport and hyperfilamentous growth, suggesting that the signaling activity of Mep2 directly correlates with its ammonium transport activity. In this study, we investigated the role of Npr1 in ammonium transport and Mep2-mediated filamentation in *C. albicans*. We found that the two ammonium permeases Mep1 and Mep2 of *C. albicans* differ in their dependency on Npr1. While Mep1 could function well in the absence of the Npr1 kinase, ammonium transport by Mep2 was virtually abolished in *npr1Δ* mutants. However, the dependence of Mep2 activity on Npr1 was relieved at higher temperatures (37°C), and Mep2 could efficiently induce filamentous growth under limiting nitrogen conditions in *npr1Δ* mutants. Like in *S. cerevisiae*, mutation of the conserved glycine at position 343 in Mep2 of *C. albicans* to cysteine resulted in Npr1-independent ammonium uptake. In striking contrast, however, the mutation abolished the ability of Mep2 to induce filamentous growth both in the wild type and in *npr1Δ* mutants. Therefore, a mutation that improves ammonium transport by Mep2 under nonpermissive conditions eliminates its signaling activity in *C. albicans*.

Microorganisms sense the availability of nutrients in their environment and express appropriate transporters and enzymes that are required for uptake and metabolism of these nutrients (11, 16). A preferred nitrogen source for many microorganisms is ammonium, which is transported into the cell by ammonium permeases of the Mep/Amt family (31). The yeast *Saccharomyces cerevisiae* possesses three ammonium permeases encoded by the *MEP1* to *MEP3* genes. Each of these transporters can support growth of *S. cerevisiae* on media containing low concentrations of ammonium as the only nitrogen source, but mutants lacking all three *MEP* genes are unable to grow on ammonium at concentrations below 5 mM (21). Expression of the *MEP* genes is induced under limiting nitrogen conditions and repressed at high ammonium concentrations (21). Under the latter conditions, sufficient ammonium may freely diffuse into the cell in the form of ammonia or be taken up by unspecific transporters to support growth.

In addition to its transport function, Mep2 is required for the transition from yeast to filamentous, pseudohyphal growth, which occurs under limiting nitrogen conditions on solid media (13, 19). It is believed that Mep2 is an ammonium sensor that induces pseudohyphal growth in response to the presence of extracellular ammonium. Current evidence suggests that the signaling activity of Mep2 is linked to ammonium transport, because amino acid substitutions that inhibit the transport activity of Mep2 also prevent pseudohyphal growth (20, 26, 30).

Ammonium transport by Mep1 to Mep3 requires the serine/threonine kinase Npr1 (nitrogen permease reactivator 1), which is important for the activity of several permeases mediating uptake of nitrogenous compounds (9, 14, 15, 29). *S. cerevisiae* npr1 mutants exhibit a growth defect on low-ammonium medium similar to that of mutants lacking the three ammonium permeases (10). Substitution of cysteine for the highly conserved glycine at position 349 in Mep2 results in a hyperactive transporter that is independent of Npr1 (4). The hyperactive Mep2 with the G349C mutation also induces filamentation more efficiently than does wild-type Mep2, further supporting a correlation between the ammonium transport and signaling activities of Mep2 (4).

The fungal pathogen *Candida albicans* also switches from budding yeast morphology to filamentous growth in response to different environmental signals, including nitrogen starvation (3). *C. albicans* has two ammonium permeases, Mep1 and Mep2, either of which is sufficient to enable growth in low ammonium concentrations (2). Similarly to the situation in *S. cerevisiae*, Mep2, but not Mep1, is required for filamentous growth of *C. albicans* in response to nitrogen limitation. The transport and signaling functions of Mep2 can be separated, as deletion of the C-terminal cytoplasmic tail of Mep2 abolishes filamentous growth without affecting ammonium uptake (2). Certain amino acid substitutions in Mep2 also disturb ammonium transport and/or filamentous growth (7). However, there is no direct correlation between the ammonium transport activity of mutated Mep2 proteins and their ability to stimulate filamentous growth. For example, mutation of the conserved residue W167 abolished filamentation without having a strong impact on ammonium transport. Vice versa, mutation of the conserved Y122, which is assumed to participate together with

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W167 in ammonium recruitment at the extracryostatic side of the cell membrane, reduced ammonium uptake more strongly than a W167A mutation but still allowed efficient filamentation (7). It is therefore possible that signaling by Mep2 is regulated in different ways in C. albicans and S. cerevisiae, and it has been proposed that a high transport activity of C. albicans Mep2 (CaMep2) in the presence of abundant extracellular ammonium may actually block its signaling activity and repress filamentous growth (2).

As the Npr1 kinase is essential for the function of Mep2 and the other ammonium permeases in S. cerevisiae, we investigated the role of Npr1 in ammonium uptake by Mep1 and Mep2 and in Mep2-mediated filamentous growth of C. albicans.

MATERIALS AND METHODS

Strains and growth conditions. C. albicans strains used in this study are listed in Table 1. All strains were grown at 28°C in either YPD (10 g peptone, 10 g yeast extract, 20 g glucose, 20 g agar per liter) or YPM (10 g yeast extract, 20 g peptone, 20 g agar per liter) adjusted to pH 7.3 at 28°C. Strains were routinely grown in YPD liquid medium and subcultured on YPD agar plates (20 g peptone, 10 g yeast extract, 20 g glucose, 15 g agar per liter) plates (100 to 1000 colonies per plate) with appropriate chromogenic substrates until the KpnI-SacI fragment from pMEP3M3 was used to delete the corresponding marker from the genome of the wild-type S. cerevisiae. As the KpnI-SacI fragment was substituted for the corresponding fragment in the YPD-agar plates (20 g peptone, 10 g yeast extract, 20 g glucose, 15 g agar per liter) plates (100 to 1000 colonies per plate) with appropriate chromogenic substrates until the two independently generated series of homozygous mutant strains had become homozygous for chromosome R. However, in all assays described in this work, the two independently generated series of strains behaved identically, and the results obtained with these strains are therefore included.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from C. albicans strains was isolated as described previously (25). DNA was digested with appropriate restriction enzymes, separated by agarose gel electrophoresis, transferred to a positively charged nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence-labeled probes was performed with an Amersham ECL direct nucleic acid labeling and detection system (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

Fluorescence microscopy. C. albicans strains expressing green fluorescent protein (GFP)-tagged Mep2 were grown overnight in SD medium containing 100 μM proline. Two milliliters of the overnight cultures was washed three times in water, resuspended in 10 ml SD medium containing 100 μM ammonium chloride, and incubated for 6 h at 30°C or 37°C. Fluorescence of the cells was detected using a Zeiss Observer Z1 microscope with a Zeiss HXP120C illuminator. Images were taken successively with filter settings for GFP and transmission images. Cells were observed with a 100× immersion oil objective.

Ammonium uptake assay. To determine ammonium uptake rates of the strains, an ammonium removal assay was used. Cells were grown overnight in 50 ml SD medium containing 0.1% proline. Two milliliters of the overnight cultures was washed three times in water, resuspended in 10 ml SD medium containing 100 μM ammonium chloride, and incubated for 6 h at 30°C or 37°C. Fluorescence of the cells was detected using a Zeiss Observer Z1 microscope with a Zeiss HXP120C illuminator. Images were taken successively with filter settings for GFP and transmission images. Cells were observed with a 100× immersion oil objective.

RESULTS

Requirement of the Npr1 kinase for growth of C. albicans on ammonium. To investigate whether the Npr1 kinase is required for ammonium uptake in C. albicans, we deleted the Npr1 gene (orf19.6232) from the genome of the wild-type strain SC5314 by using the SAT1-flipping strategy (25). Two independent series of homozygous npr1Δ mutants and complemented strains (A and B series) were generated and tested for their ability to grow on agar plates containing...
### TABLE 1. *C. albicans* strains used in this study

| Strain     | Parent | Relevant characteristic or genotype | Reference or source |
|------------|--------|-------------------------------------|---------------------|
| SC5314     |        | Wild-type parental strain           | 12                  |
| *npr1Δ* mutants and complemented strains | | | |
| NPR1M1A    | SC5314 | *npr1-1::SAT1-FLIP/NPR1-2*                 | This study |
| NPR1M1B    | SC5314 | *NPR1-1*npr1-2::SAT1-FLIP               | This study |
| NPR1M2A    | NPR1M1A| *npr1-1::FRT/NPR1-2*                   | This study |
| NPR1M2B    | NPR1M1B| *NPR1-1*npr1-2::FRT                    | This study |
| NPR1M3A    | NPR1M2A| *npr1-1::FRT/npr1-2::SAT1-FLIP*         | This study |
| NPR1M3B    | NPR1M3A| *npr1-1::FRT/npr1-2::FRT*              | This study |
| NPR1M4A    | NPR1M3B| *npr1-1::FRT/npr1-2::FRT*              | This study |
| NPR1M4B    | NPR1M4A| *NPR1-SAT1-FLIP/npr1-2::FRT*           | This study |
| NPR1M5A    | NPR1M4B| *npr1-1::FRT/NPR1-SAT1-FLIP*           | This study |
| NPR1M5B    | NPR1M5A| *npr1-1::FRT/NPR1-2::FRT*              | This study |
| mep2Δ mutants | | | |
| SCMEP2M4A  | SC5314 | *mep2-1::FRT/mep2-2::FRT*               | 6                   |
| SCMEP2M4B  | SC5314 | *mep2-1::FRT/mep2-2::FRT*               | 6                   |
| mep1Δ* mep2Δ double mutants | | | |
| SCMEP12M2A | SCMEP2M4A| *MEP1-1*mep1-2::SAT1-FLIP*            | This study |
| SCMEP12M2B | SCMEP2M4B| *mep1-1::FRT/mep1-2::FRT*            | This study |
| SCMEP12M2C | SCMEP12M2A| *MEP1-1*mep1-2::FRT*                | This study |
| SCMEP12M2D | SCMEP12M2B| *mep1-1::FRT/mep1-2::FRT*            | This study |
| mep1Δ* mep2Δ* mep3Δ triple mutants | | | |
| SCMEP12M3A | SCMEP12M4A| *mep1-1::FRT/mep1-2::FRT*            | This study |
| SCMEP12M3B | SCMEP12M3A| *MEP1-1*mep1-2::SAT1-FLIP*           | This study |
| SCMEP12M3C | SCMEP12M3B| *mep1-1::FRT/mep1-2::FRT*            | This study |
| SCMEP12M3D | SCMEP12M3A| *MEP1-1*mep1-2::FRT*                | This study |
| mep1Δ* mep2Δ* npr1Δ triple mutants | | | |
| Δmep12NPR1M1A | SCMEP12M4A| *mep1-1::FRT/mep1-2::FRT*            | This study |
| Δmep12NPR1M1B | SCMEP12M4B| *mep1-1::FRT/mep1-2::FRT*            | This study |
| Δmep12NPR1M2A | SCMEP12M4A| *mep1-1::FRT/mep1-2::FRT*            | This study |
| Δmep12NPR1M2B | SCMEP12M4B| *mep1-1::FRT/mep1-2::FRT*            | This study |

*Continued on following page*
different concentrations of ammonium as the only nitrogen source. As can be seen in Fig. 1A, the npr1Δ mutants had a slight growth defect on these media. However, the growth defect was not as severe as that of mutants lacking the ammonium permeases Mep1 and Mep2, indicating that ammonium can still be taken up into the cells in the absence of Npr1, albeit not as efficiently as in the wild-type strain. A reduced growth rate of the npr1Δ mutants was also observed in liquid medium containing 1 mM ammonium (Table 3). Ammonium uptake assays demonstrated that ammonium uptake by the npr1Δ mutants was reduced to ca. 30% of wild-type uptake rates (Fig. 1B).

### TABLE 1—Continued

| Strain | Parent | Relevant characteristic or genotype* | Reference or source |
|--------|--------|--------------------------------------|---------------------|
| Δmep12NPR1M2B | Δmep12NPR1M1B | mep1-1::FRT/mep1-2::FRT | This study |
| Δmep12NPR1M3A | Δmep12NPR1M2A | mep1-1::FRT/mep1-2::FRT | This study |
| Δmep12NPR1M3B | Δmep12NPR1M2B | mep1-1::FRT/mep1-2::FRT | This study |
| Δmep12NPR1M4A | Δmep12NPR1M3A | mep1-1::FRT/mep1-2::FRT | This study |
| Δmep12NPR1M4B | Δmep12NPR1M3B | mep1-1::FRT/mep1-2::FRT | This study |

Strains expressing MEP1, MEP2, or MEP2\(^{G343C}\) in mep1Δ mep2Δ or mep1Δ mep2Δ npr1Δ backgrounds

| Strain | Parent | Relevant characteristic or genotype* | Reference or source |
|--------|--------|--------------------------------------|---------------------|
| SCmep12MEP1K1A | SCMEP12M4A | mep1-1::FRT/MEP1-caSAT1 | This study |
| SCΔmep12MEP1K1B | SCMEP12M4A | mep1-1::FRT/MEP1-caSAT1 | This study |
| SCΔmep12MEP2K1A | SCMEP12M4A | mep1-1::FRT/MEP2-caSAT1 | This study |
| SCΔmep12MEP2K1B | SCMEP12M4B | mep1-1::FRT/MEP2-caSAT1 | This study |
| SCΔmep12MEP2K2A | SCMEP12M4A | mep1-1::FRT/MEP2-caSAT1 | This study |
| SCΔmep12MEP2K2B | SCMEP12M4B | mep1-1::FRT/MEP2-caSAT1 | This study |

Strains expressing GFP-tagged MEP2 in mep1Δ mep2Δ or mep1Δ mep2Δ npr1Δ backgrounds

| Strain | Parent | Relevant characteristic or genotype* | Reference or source |
|--------|--------|--------------------------------------|---------------------|
| SCΔmep12MEP2G7A | SCMEP12M4A | mep1-1::FRT/MEP2-GFP-caSAT1 | This study |
| SCΔmep12MEP2G7B | SCMEP12M4B | mep1-1::FRT/MEP2-GFP-caSAT1 | This study |
| SCΔmep12npr1MEP2G7A | Δmep12NPR1M4A | mep1-1::FRT/MEP2-GFP-caSAT1 | This study |
| SCΔmep12npr1MEP2G7B | Δmep12NPR1M4B | mep1-1::FRT/MEP2-GFP-caSAT1 | This study |

* SAT1-FLIP denotes the SAT1 flipper cassette. The NPR1, MEP1, MEP2, and MEP3 alleles in strain SC5314 can be distinguished by HaeII, ClaI, EcoRI, and BamHI restriction site polymorphisms, respectively. The NPR1 allele containing the polymorphic HaeII site at position +1002 was arbitrarily designated NPR1-2, the MEP1 allele with the polymorphic upstream ClaI site MEP1-1, the MEP2 allele with the polymorphic upstream EcoRI site MEP2-1, and the MEP3 allele with the polymorphic downstream BamHI site MEP3-2.
Differential requirement of Npr1 for ammonium transport by Mep1 and Mep2. The phenotype of the npr1Δ mutants suggested that, in contrast to the situation in S. cerevisiae, one or both of the C. albicans ammonium permeases Mep1 and Mep2 can function in a partially Npr1-independent fashion. To address the specific requirement of Npr1 for Mep1 and/or Mep2 activity, we deleted NPR1 in a mep1Δ mep2Δ double mutant background and then reintroduced a functional copy of MEP1 or MEP2 into the double and triple mutants. Reinsertion of MEP1 into the mep1Δ mep2Δ double mutants restored growth and ammonium uptake to wild-type levels (Fig. 2 and Table 3). MEP2 also complemented the growth defect of the

TABLE 2. Primers used in this study

| Primer name | Primer sequencea |
|-------------|------------------|
| ACT38       | 5'-ATATGGGCCCTTGAGACATTTATGGAATGGAATTGGG-3' |
| NPR1-1      | 5'-ATATGGGCTTTCAAGGAGCAAGAC-3' |
| NPR1-2      | 5'-ATATCTGGGAGTGTACATTAGAAGTTG-3' |
| NPR1-3      | 5'-ATATGGCCGGTGGAGATCTATTGAAGATC-3' |
| NPR1-4      | 5'-ATATGGCTTTCCGAGTGGAGATCTATTGAC-3' |
| NPR1-5      | 5'-ATATAGATTTTCTTATTCAAAAGC-3' |
| MEP3        | 5'-TACACCTGGTACCCCGATTGGCTTGAATGTC-3' |
| MEP46       | 5'-TACCACTGGTACCCGATTAGCTTTCTC-3' |
| MEP47       | 5'-TACCACTGGTACCCGATTAGCTTTCTC-3' |
| MEP48       | 5'-TACCACTGGTACCCGATTAGCTTTCTC-3' |
| MEP49       | 5'-TACCACTGGTACCCGATTAGCTTTCTC-3' |
| MEP79       | 5'-TACCACTGGTACCCGATTAGCTTTCTC-3' |
| MEP107      | 5'-TACCACTGGTACCCGATTAGCTTTCTC-3' |
| MEP108      | 5'-CCACACACACATGGGAATTGGG-3' |

a Restriction sites introduced into the primers are underlined; stop codons (reverse sequence) are highlighted in bold and mutated codons in italics.

FIG. 1. (A) Growth of the wild-type strain SC5314, mep1Δ mep2Δ double mutants, and npr1Δ mutants and complemented strains on different concentrations of ammonium as the sole nitrogen source. Tenfold dilution series of the strains were spotted on a YPD control plate or on SD plates containing the indicated ammonium concentrations and incubated for 1 day (YPD) or 3 days (SD) at 30°C. The following strains were used: SC5314 (wild type), SCMEP12M4A and -B (mep1Δ mep2Δ), NPR1M4A and -B (npr1Δ), and NPR1MK2A and -B (npr1Δ + NPR1). (B) Ammonium uptake by the same strains. Uptake rates were determined in the presence of 1 mM ammonium as described in Materials and Methods. Ammonium uptake by the wild-type strain SC5314 was set to 100%, and uptake rates by the mutants are given as percentages of the wild-type uptake rate. Shown are the means and standard deviations from three independent experiments (one with the A series and two with the B series of mutants).
mep1Δ mep2Δ double mutants on agar plates containing limiting ammonium concentrations (Fig. 2A), but ammonium uptake was still reduced (Fig. 2B), in agreement with a slightly slower growth of the strains in liquid medium (Table 3).

A more striking difference between MEP1 and MEP2 was seen in strains lacking the Npr1 kinase. Reintegration of MEP1 into the mep1Δ mep2Δ npr1Δ triple mutants strongly improved growth both on solid and in liquid medium, although ammonium uptake was only partially restored. In contrast, expression of MEP2 in the same strains only slightly ameliorated ammonium uptake and growth (Fig. 2 and Table 3), indicating that Mep2 does not function efficiently in the absence of Npr1.

To evaluate whether expression or localization of Mep2 was impaired in the absence of Npr1, we expressed a GFP-tagged

### TABLE 3. Doubling times of *C. albicans* strains in liquid medium with 1 mM NH₄⁺

| Strain Description or genotype | Doubling time (min)* |
|-------------------------------|----------------------|
| SC5314 Wild type              | 111                  |
| NPR1ΔM4 mep1Δ + NPR1          | 229 ± 14             |
| SCMEP12M4 mep1Δ mep2Δ         | 1.215 ± 0.70         |
| SCMEP12MEP1K1 mep1Δ mep2Δ + MEP1 | 113 ± 5           |
| SCMEP12MEP2K2 mep1Δ mep2Δ + MEP2 | 145 ± 27           |
| Δmep1Δmep2ΔNPR1Δ mep1Δ mep2Δ npr1Δ | 1.385 ± 0.15      |
| Δmep1Δmep2Δnpr1ΔMEP1K1 mep1Δ mep2Δ npr1Δ + MEP1 | 266 ± 21           |
| Δmep1Δmep2Δnpr1ΔMEP2K1 mep1Δ mep2Δ npr1Δ + MEP2 | 1.056 ± 0.64       |
| Δmep1Δmep2Δnpr1ΔMEP2K2 mep1Δ mep2Δ npr1Δ + MEP2 | 193 ± 4            |

a Means ± standard deviations (SD) from four biological replicates (two each for the A and B series). The doubling time of strain SC5314 is the means from two biological replicates (106 min and 116 min).

FIG. 2. (A) Growth of the wild-type strain SC5314, mep1Δ mep2Δ double mutants, mep1Δ mep2Δ npr1Δ triple mutants, and strains in which a functional copy of MEP1 or MEP2 was reinserted on different concentrations of ammonium as the sole nitrogen source. Tenfold dilution series of the strains were spotted on a YPD control plate or on SD plates containing the indicated ammonium concentrations and incubated for 1 day (YPD) or 3 days (SD) at 30°C. The following strains were used: SC5314 (wild type), SCMEP12M4A and -B (mep1Δ mep2Δ), SCΔmep1ΔMEP1K1A and -B (mep1Δ mep2Δ + MEP1), SCΔmep1ΔMEP2K1A and -B (mep1Δ mep2Δ + MEP2), Δmep1Δnpr1ΔMEP1K1A and -B (mep1Δ mep2Δ + MEP1), and Δmep1Δnpr1ΔMEP2K1A and -B (mep1Δ mep2Δ + MEP2). (B) Ammonium uptake by the same strains. Uptake rates were determined in the presence of 1 mM ammonium as described in Materials and Methods. Shown are the means and standard deviations from three independent experiments (two with the A series and one with the B series of mutants).
Mep2 in the same strains. As can be seen in Fig. 3, Mep2 was localized at the cell periphery and expressed at similar levels in the presence or absence of Npr1, demonstrating that Npr1 is not required for expression and correct localization of Mep2. Altogether, these results argue that ammonium transport by Mep2 strongly depends on Npr1, while Mep1 can efficiently support growth in the absence of the kinase.

**Npr1 is required for ammonium uptake by unspecific transporters.** Of note, deletion of *NPR1* in a *mep1Δ mep2Δ* double mutant background resulted in a further growth impairment (Fig. 2A and Table 3), although no difference between *mep1Δ mep2Δ* double mutants and *mep1Δ mep2Δ npr1Δ* triple mutants could be detected in ammonium uptake assays (Fig. 2B). These results indicated that Npr1 is required for the activity of other proteins that contribute to ammonium uptake/utilization. The most obvious candidate for an Npr1-dependent ammonium transporter was orf19.4446, which encodes a putative protein with 34% amino acid identity to Mep1 and Mep2. To test whether this protein, which we designated Mep3 for the purpose of the present study, is responsible for the residual ammonium uptake in the absence of Mep1 and Mep2, we deleted the corresponding gene in a *mep1Δ mep2Δ* double mutant background. However, no growth differences between *mep1Δ mep2Δ* double mutants and *mep1Δ mep2Δ mep3Δ* triple mutants were observed at a range of ammonium concentrations (Fig. 4), indicating that orf19.4446 does not encode a functional ammonium permease. It is likely that Npr1 is required for the activity of unspecific cation transporters that allow some ammonium uptake into the cells in the absence of the specific ammonium transporters Mep1 and Mep2, especially at increased ammonium concentrations.

**Npr1 is required for growth of *C. albicans* on other nitrogen sources.** In *S. cerevisiae*, Npr1 is required for growth on other nitrogen sources in addition to ammonium, in part because the ammonium permeases contribute to growth under these conditions by retrieval of excreted ammonium (4). We therefore tested growth of the *C. albicans npr1Δ* mutants on a panel of these alternative nitrogen sources (Fig. 5). Similarly to *S. cerevisiae* *npr1Δ* mutants, *C. albicans* mutants lacking *NPR1* exhibited a growth defect on isoleucine, tyrosine, and tryptophan. However, there were also notable differences in the phenotypes of *npr1Δ* mutants of the two species. While Npr1 has been shown to be required for growth of *S. cerevisiae* on arginine, ornithine, and urea, little or no growth defect of the *C. albicans* *npr1Δ* mutants was observed on these nitrogen sources. Vice versa, inactivation of *NPR1* in *C. albicans* severely affected the ability of the mutants to utilize threonine and phenylalanine, whereas Npr1 is not required for growth of *S. cerevisiae* on these amino acids. In *C. albicans*, the ammonium permeases Mep1 and Mep2 contributed only marginally to growth on most of the tested alternative nitrogen sources, except for ornithine and isoleucine, where a relatively strong growth defect of the *mep1Δ mep2Δ* mutants was observed. These results suggest that the activity of certain amino acid transporters or enzymes involved in the metabolism of the corresponding amino acids depends on Npr1. However, the exacerbated growth defect of the *mep1Δ mep2Δ npr1Δ* triple mutants compared to that of the *npr1Δ* single mutants demonstrates that ammonium retrieval by Mep1 is important for the residual growth of *npr1Δ* mutants on various amino acids.

Mep2-mediated ammonium transport and filamentous growth become independent of Npr1 at elevated temperatures. In both *S. cerevisiae* and *C. albicans*, Mep2 also acts as a signaling protein and stimulates filamentous growth in response to nitrogen limitation. As explained in the introduction, the ability of *S. cerevisiae* Mep2 (ScMep2) to induce filamentation correlates with its ammonium transport activity. Consequently, *S. cerevisiae* *npr1Δ* mutants have a filamentation defect, because Npr1 is required for Mep2 activity (4, 19). As our previous experiments demonstrated that Npr1 is important for ammonium transport by Mep2 also in *C. albicans*, we investigated whether the *npr1Δ* mutants had a filamentation defect under limiting nitrogen conditions. Filamentous growth is usu-
ally stimulated at elevated temperatures in *C. albicans*. Surprisingly, we observed that Npr1 is much less important for Mep2-dependent growth at 37°C than at 30°C, as growth of *mep1Δ mep2Δ npr1Δ* triple mutants expressing a single copy of *MEP2* was largely restored at the elevated temperature (Fig. 6A). Analysis of strains expressing a GFP-tagged Mep2 showed that Mep2 was expressed at comparable levels at 30°C and 37°C, both in the absence and in the presence of Npr1 (data not shown). In line with the Npr1-independent activity of Mep2 at 37°C, the *C. albicans npr1Δ* mutants exhibited fila-

![FIG. 5](image_url)  
**FIG. 5.** Growth of the wild-type strain SC5314 and different mutants on SD agar plates containing the indicated amino acids (10 mM) as the sole nitrogen source. Tenfold dilution series of the strains were spotted on the plates and incubated for 3 days at 30°C. The following strains were used: SC5314 (wild type), SCMEP12M4A and -B (*mep1Δ mep2Δ*), Δ*mep2*NPR1M4A and -B (*mep1Δ mep2Δ npr1Δ*), NPR1M4A and -B (*npr1Δ*), and NPR1MK2A and -B (*npr1Δ + NPR1*). The two independently generated series of mutants behaved identically, and only one of them is shown in each case.

![FIG. 6](image_url)  
**FIG. 6.** (A) Mep2-dependent growth becomes independent of the Npr1 kinase at elevated temperatures. Tenfold dilution series of the strains were spotted on SD plates containing the indicated ammonium concentrations and incubated for 3 days at 30°C or 37°C. The following strains were used: SC5314 (wild type), SCMEP2M4A and -B (*mep2Δ*), NPR1M4A and -B (*npr1Δ*), and NPR1MK2A and -B (*npr1Δ + MEP2*). (B) Filamentous growth of the wild-type strain SC5314, *mep2Δ* mutants, and *npr1Δ* mutants and complemented strains on SD agar plates containing 100 μM ammonium as the sole nitrogen source. The plates were incubated for 6 days at 37°C. The following strains were used: SC5314 (wild type), SCMEP2M4A and -B (*mep2Δ*), NPR1M4A and -B (*npr1Δ*), and NPR1MK2A and -B (*npr1Δ + NPR1*). The two independently generated series of mutants behaved identically, and only one of them is shown in each case.
mentous growth on media containing limiting ammonium concentrations, although filamentation was slightly reduced in the absence of Npr1 (Fig. 6B).

A mutation that restores Mep2-mediated ammonium transport in the absence of Npr1 abolishes the signaling activity of Mep2. As a G349C mutation in ScMep2 results in a hyperactive, Npr1-independent ammonium transporter, we investigated the effect of a corresponding G343C mutation in CaMep2. Similarly to the situation in S. cerevisiae, the MEP2G343C allele restored growth of C. albicans mep1Δ mep2Δ npr1Δ triple mutants on media containing ammonium as the sole nitrogen source (Fig. 7A and Table 3), and ammonium uptake rates by the mutated Mep2 were similar in the presence and absence of Npr1 (Fig. 7B). Therefore, the G343C mutation resulted in Npr1-independent ammonium transport activity of Mep2 also in C. albicans. When expressed in a mep1Δ mep2Δ mutant, MEP2G343C restored ammonium uptake and growth slightly less efficiently than did wild-type MEP2.

We then tested the ability of the wild-type MEP2 and MEP2G343C alleles to complement the filamentation defect of mep1Δ mep2Δ mutants in the presence and absence of Npr1. Unexpectedly, the MEP2G343C allele was unable to induce filamentous growth under limiting nitrogen conditions, in contrast to wild-type MEP2, which induced morphogenesis regard-

FIG. 7. (A) Growth of the wild-type strain SC5314, mep1Δ mep2Δ double mutants, mep1Δ mep2Δ npr1Δ triple mutants, and strains in which a wild-type copy of MEP2 or the MEP2G343C allele was reinserted on different concentrations of ammonium as the sole nitrogen source. Tenfold dilution series of the strains were spotted on a YPD control plate or on SD plates containing the indicated ammonium concentrations and incubated for 1 day (YPD) or 3 days (SD) at 30°C. The following strains were used: SC5314 (wild type), SCMEP12M4A and -B (mep1Δ mep2Δ), SCΔmep12MEP2K1A and -B (mep1Δ mep2Δ + MEP2), SCΔmep12MEP2K2A and -B (mep1Δ mep2Δ + MEP2G343C), Δmep12NPR1M4A and -B (mep1Δ mep2Δ npr1Δ), Δmep1ΔΔmep2ΔΔnpr1ΔMEP2K1A and -B (mep1Δ mep2Δ npr1Δ + MEP2), and Δmep1ΔΔmep2ΔΔnpr1ΔMEP2K2A and -B (mep1Δ mep2Δ npr1Δ + MEP2G343C). (B) Ammonium uptake by the same strains. Uptake rates were determined in the presence of 1 mM ammonium as described in Materials and Methods. Shown are the means and standard deviations from three independent experiments (two with the A series and one with the B series of mutants). Data are from the same experiments with results shown in Fig. 2, and the values of the control strains are included for comparison.
less of the presence of Npr1 (Fig. 8). Therefore, a mutation in Mep2 that restores the ammonium transport capacity in the absence of the Npr1 kinase abolishes the ability to induce filamentous growth.

**DISCUSSION**

The results presented in this work demonstrate that the importance of the Npr1 kinase for ammonium permease function in *S. cerevisiae* is different from that in *C. albicans*. The transport activity of all three ammonium permeases of *S. cerevisiae* strongly depends on Npr1, and none of the Mep proteins can support growth at low ammonium concentrations in the absence of this kinase (4, 10). In contrast, the two ammonium permeases of *C. albicans* differ in their dependency on Npr1. While Mep1 was sufficiently active in cells lacking Npr1 to support growth under low-ammonium conditions, ammonium transport by Mep2 was largely abolished in *npr1Δ* mutants at the standard growth temperature of 30°C. Interestingly, however, at 37°C Mep2 functioned well in the absence of Npr1 and enabled growth on ammonium as the sole nitrogen source.

In *S. cerevisiae*, Npr1 is required to maintain the general amino acid permease Gap1, which is expressed when the cells are grown in a nitrogen-poor medium, at the cytoplasmic membrane by preventing ubiquitination-dependent endocytosis. In the absence of Npr1, Gap1 is endocytosed and targeted to the vacuole for degradation, and newly synthesized Gap1 is directly sorted to the vacuole and never reaches the plasma membrane (8, 26). There is evidence that Npr1 does not phosphorylate Gap1 directly but acts in an indirect fashion by phosphorylating the α-arrestin Aly2, thereby interfering with Gap1 sorting to the vacuole (23). On the other hand, the nitrate transporter Yrt1 of *Hansenula polymorpha* is protected from ubiquitinylation-mediated sorting to the vacuole by direct, Npr1-dependent phosphorylation in response to nitrogen deprivation, allowing its delivery to the plasma membrane (22). Npr1 itself is inhibited under nutrient-rich conditions by TOR-dependent dephosphorylation (17, 27). Inactivation of *NPR1* confers increased resistance to rapamycin in *S. cerevisiae* (27), and we observed the same phenotype for the *C. albicans npr1Δ* mutants (data not shown), indicating that Npr1 activity is controlled by TOR also in *C. albicans*.

We found that the role of Npr1 in maintaining the transport activity of Mep2 in *C. albicans* is different from its role in stabilizing Gap1 in *S. cerevisiae*, as Mep2 was properly expressed and localized in *C. albicans npr1Δ* mutants at the restrictive temperature of 30°C. Similar results have been reported for *S. cerevisiae*, where Mep2 was also correctly targeted to the plasma membrane in the absence of Npr1 (26). Therefore, instead of preventing endocytosis and degradation of Mep2, Npr1 seems to enable Mep2 to adopt a transport-competent conformation. Apparently, CaMep2 can attain its active conformation at higher temperatures also in an Npr1-independent fashion. Additional observations suggest that the role of Npr1 in maintaining ammonium permease function in *S. cerevisiae* is not protection from ubiquitination-dependent degradation. Gap1 degradation in the absence of Npr1 is prevented when ubiquitination is inhibited by an *rsp5* mutation (8). In contrast, nitrogen catabolite repression (NCR)-sensitive genes were still expressed at normally repressing ammonium concentrations in an *npr1Δ* mutant even when *RS5* was also inactivated, indicating that ammonium permease activity in the *npr1Δ* mutant was not restored by the *rsp5* mutation (10). These findings support the idea that the role of Npr1 in maintaining Mep function is different from its role in stabilizing Gap1 at the plasma membrane.

Our results indicate that ammonium permeases can attain a transport-competent state by different mechanisms in *C. albicans*. Mep2 becomes transport proficient in the presence of a functional Npr1 kinase, at elevated temperatures, or by a G343C mutation. Interestingly, Mep1 also contains the conserved glycine, mutation of which to cysteine in Mep2 of *S. cerevisiae* and *C. albicans* renders these transporters Npr1 independent. Therefore, the ability of CaMep1 to efficiently transport ammonium in the absence of Npr1 must be caused by some other feature of this permease in which it differs from Mep2.

In general, the signaling activity of mutated Mep2 proteins

![FIG. 8. Filamentous growth of mep1Δ mep2Δ double mutants and mep1Δ mep2Δ npr1Δ triple mutants expressing wild-type MEP2 or the MEP2G343C allele on SD agar plates containing 100 μM ammonium or urea as the sole nitrogen source. The plates were incubated for 6 days at 37°C. The following strains were used: SCΔmep1Δmep2Δ, SCΔmep1Δmep2ΔΔnpr1ΔMEP2Δ, SCΔmep1Δmep2ΔΔnpr1ΔMEP2ΔK1A and -B (mep1Δ mep2Δ npr1Δ + MEP2Δ), SCΔmep1Δmep2ΔΔnpr1ΔMEP2ΔK2A and -B (mep1Δ mep2Δ npr1Δ + MEP2ΔG343C). The two independently generated series of mutants behaved identically, and only one of them is shown in each case.](image-url)
in \textit{S. cerevisiae} correlates with their transport activity, supporting the model that ammonium transport is required for signaling by Mep2 (4, 20, 26, 30). On the other hand, an H194E mutation abolished pseudohyphal growth despite the fact that it increased ammonium transport by Mep2 (5). Therefore, the possibility that the signaling activity of Mep2 is in fact repressed when it is engaged in ammonium transport, especially in the presence of relatively high ammonium concentrations, cannot be ruled out. So far, no mutations in Mep2 that prevent ammonium transport and result in constitutive signaling, which would be in favor of such an alternative model for the regulation of Mep2 signaling activity, have been described. Nevertheless, our finding that one mechanism by which Mep2 achieves a transport-competent state, namely, the G343C mutation, abolishes its signaling activity is compatible with this hypothesis. However, the ammonium uptake rate of Mep2 was slightly reduced by the G343C mutation in a wild-type background, which may be the reason for the filamentation defect. In \textit{S. cerevisiae}, the analogous G349S mutation has a different effect in that it increases both the transport and signaling activities of Mep2 above those of the wild-type protein. It is therefore possible that the signaling activity of Mep2 is controlled in different ways by ammonium availability in the two yeast species.

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