

The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*

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In response to nitrogen starvation, diploid cells of the budding yeast *Saccharomyces cerevisiae* differentiate into a filamentous, pseudohyphal growth form. This dimorphic transition is regulated by the G α protein GPA2, by RAS2, and by elements of the pheromone-responsive MAP kinase cascade, yet the mechanisms by which nitrogen starvation is sensed remain unclear. We have found that MEP2, a high affinity ammonium permease, is required for pseudohyphal differentiation in response to ammonium limitation. In contrast, MEP1 and MEP3, which are lower affinity ammonium permeases, are not required for filamentous growth. $\Delta mep2$ mutant strains had no defects in growth rates or ammonium uptake, even at limiting ammonium concentrations. The pseudohyphal defect of $\Delta mep2/\Delta mep2$ strains was suppressed by dominant active GPA2 or RAS2 mutations and by addition of exogenous cAMP, but was not suppressed by activated alleles of the MAP kinase pathway. Analysis of MEP1/MEP2 hybrid proteins identified a small intracellular loop of MEP2 involved in the pseudohyphal regulatory function. In addition, mutations in *GLN3*, *URE2* and *NPR1*, which abrogate MEP2 expression or stability, also conferred pseudohyphal growth defects. We propose that MEP2 is an ammonium sensor, generating a signal to regulate filamentous growth in response to ammonium starvation.

Keywords: ammonium permease/MEP2/pseudohyphal differentiation/yeast

Introduction

Many fungi interconvert between multiple growth patterns, including yeast and filamentous forms, depending on environmental conditions. Filamentous growth may enable these non-motile organisms to seek preferable environments. For example, conjugation of compatible cell types in the maize pathogen *Ustilago maydis* results in the formation of a filamentous heterokaryon, the cell type responsible for host infection (Banuett, 1991; Hartmann *et al.*, 1996). Polymorphism between yeast, hyphal and pseudohyphal forms in the opportunistic human pathogen *Candida albicans* has been proposed to be a key means of tissue invasion and dissemination during infection, and non-filamentous *C.albicans* mutants are avirulent in a

mouse model (Lo *et al.*, 1997). Similarly, in the human pathogen *Cryptococcus neoformans*, a filamentous growth form accompanied by spore formation (haploid fruiting) has only been observed in cells of the α mating type, which are more virulent than the **a** mating type (Kwon-Chung *et al.*, 1992; Wickes *et al.*, 1996).

In the budding yeast *Saccharomyces cerevisiae*, severe nitrogen starvation induces diploid cells to differentiate into a filamentous, pseudohyphal growth form (Gimeno *et al.*, 1992). This developmental pathway has been proposed to be a scavenging mechanism under nutrient limiting conditions (Gimeno *et al.*, 1992). Similar to the filamentous states of other fungi, pseudohyphal cells are elongated and have an altered budding pattern and cell cycle compared with yeast form cells (Gimeno *et al.*, 1992; Kron *et al.*, 1994). In addition, pseudohyphal cells invade the growth substrate.

Pseudohyphal differentiation is regulated by GPA2, the α subunit of a guanine nucleotide binding protein (Kübler *et al.*, 1997; Lorenz and Heitman, 1997). Diploid $\Delta gpa2/\Delta gpa2$ strains have a severe defect in filamentation, whereas a dominant active GPA2 allele stimulates pseudohyphal differentiation, even under conditions of nitrogen excess. GPA2 has been proposed to be a component of the nitrogen sensor that regulates this dimorphic switch (Lorenz and Heitman, 1997). G proteins play similar roles in other fungi, including *U.maydis* in which the G α protein Gpa3 is required for mating, a necessary precursor to filamentous growth (Regenfelder *et al.*, 1996), and in *C.neoformans*, in which the G α protein Gpa1 regulates both mating and virulence (Tolkacheva *et al.*, 1994; Alspaugh *et al.*, 1997).

Several observations suggest that GPA2 and the small G protein RAS2 coordinately regulate vegetative and pseudohyphal growth. Mutational activation of either GPA2 or RAS2 stimulates filamentous growth (Gimeno *et al.*, 1992; Lorenz and Heitman, 1997), both RAS2 and GPA2 modulate cAMP levels (Toda *et al.*, 1985; Field *et al.*, 1988; Nakafuku *et al.*, 1988), and $\Delta ras2$ and $\Delta gpa2$ mutations exhibit a synthetic growth defect (Kübler *et al.*, 1997; Lorenz and Heitman, 1997; Y.Xue and J.Hirsch, personal communication). cAMP promotes filamentous growth, even under nutrient rich conditions, and increased cAMP levels suppress both the $\Delta gpa2$ pseudohyphal defect (Lorenz and Heitman, 1997) and the $\Delta gpa2 \Delta ras2$ vegetative growth defect (Kübler *et al.*, 1997). cAMP also regulates mating and dimorphism in other fungi, including *U.maydis*, *C.albicans*, *Neurospora crassa*, and *Schizosaccharomyces pombe* (Niimi *et al.*, 1980; Maeda *et al.*, 1990; Sabie and Gadd, 1992; Yarden *et al.*, 1992; Gold *et al.*, 1994; Kronstad, 1997).

Activation of a signaling pathway that is independent of the G protein/cAMP pathway and includes elements of the haploid pheromone response MAP kinase cascade is

also required for pseudohyphal differentiation. Mutations in the STE20, STE11 and STE7 protein kinases and the STE12 transcription factor block filamentation in diploid cells (Liu *et al.*, 1993). In response to nitrogen starvation the MAP kinase cascade stimulates the transcription of a reporter gene under the control of regulatory sequences from the transposon Ty1 (Laloux *et al.*, 1994; Möscher *et al.*, 1996). These sequences include a binding site for a STE12/TEC1 heterodimer (Madhani and Fink, 1997). In addition to the role of RAS2 in regulating cAMP synthesis, it has been suggested that the dominant active *RAS2*^{Val19} allele may also activate the MAP kinase cascade (Möscher *et al.*, 1996).

Although pseudohyphal differentiation is induced by nitrogen starvation, the mechanisms by which this environmental signal is sensed are not known. GPA2 is involved, but potential GPA2-linked receptors have not yet been reported. Nutrient-based regulation has been extensively studied with respect to transcriptional and cell cycle control (reviewed in Grenson, 1992; Magasanik, 1992), yet little is known about the direct sensing of nutrient availability. This signaling is likely to be complex as yeast, like most microorganisms, can utilize a wide variety of compounds to satisfy nutritional requirements. Several recent studies have reported that transmembrane permeases have a role as receptors in such signaling pathways. In yeast, the glucose transporter homologs RGT2 and SNF3 are required for transcriptional induction of other glucose transporters based on glucose availability (Liang and Gaber, 1996; Ozcan *et al.*, 1996), and dominant mutations in both RGT2 and SNF3 have been identified which signal in the complete absence of glucose (Ozcan *et al.*, 1996). Despite the homology of RGT2 and SNF3 to glucose permeases, neither transports glucose (Liang and Gaber, 1996). A related glucose transporter homolog, Rco-3, regulates conidiation in *N.crassa* in response to changes in sugar availability (Madi *et al.*, 1997).

Transport through plasma membrane permeases is an early step in the metabolism of any nutrient and thus nutrient permeases are in a unique position to both sense and import their substrates. Yeast has specific, high-affinity plasma membrane permeases for numerous nutrients (reviewed in Andre, 1995). Standard media for induction of pseudohyphal differentiation contains low concentrations (50 μ M) of ammonium as the sole nitrogen source (Gimeno *et al.*, 1992). We have therefore examined the role of the ammonium permeases, MEP1, MEP2 and MEP3 in the regulation of dimorphism in *S.cerevisiae*. We find that the high affinity ammonium permease MEP2 is required for pseudohyphal differentiation under standard conditions, whereas the homologous permeases MEP1 and MEP3 are not. Strains lacking MEP2 have no apparent defects in ammonium uptake, metabolism, or growth, even under the low ammonium concentrations that promote filamentation. The pseudohyphal defect of Δ *mep2*/ Δ *mep2* mutant strains is not suppressed by activation of the MAP kinase cascade, but is suppressed by activated alleles of *GPA2* or *RAS2*, or by exogenous cAMP. We propose that MEP2 serves as an ammonium sensor to regulate pseudohyphal growth and functions in a signaling pathway upstream of GPA2, RAS2 and cAMP.

Results

The MEP2 ammonium transporter is required for pseudohyphal differentiation

Previous studies had identified two ammonium-specific permeases, MEP1 and MEP2 (Dubois and Grenson, 1979; Marini *et al.*, 1994). A third permease, MEP3, was identified through BLAST searches during the course of this work and independently by Marini *et al.* (1997). These proteins share significant sequence identity: MEP1 and MEP3 share 80% identity; MEP2 is less similar, 41% identical with MEP1 and 39% identical with MEP3. MEP2 is the highest affinity permease, with a K_m for ammonium of 1–2 μ M whereas MEP1 (K_m ~5–10 μ M) and MEP3 (K_m ~1.4–2.1 mM) are lower affinity ammonium transporters (Marini *et al.*, 1997). Deletion of all three MEP permeases renders a cell inviable on media containing <5 mM ammonium as the sole nitrogen source (Marini *et al.*, 1997; see Figure 1A and B), indicating that these proteins are the only specific ammonium permeases in yeast.

To test whether any of these permeases affect pseudohyphal differentiation, we constructed a series of homozygous diploid strains that each lack a single permease. Our initial hypothesis was that mutations in these permeases might impair ammonium uptake, thus increasing nitrogen starvation and possibly enhancing pseudohyphal differentiation. In contrast, we observed that strains lacking MEP2 have a severe defect in filamentous growth under limiting ammonium conditions (Figure 1A). Deletions of *MEP1* or *MEP3*, however, had no effect on pseudohyphal growth (Figure 1A). As previously reported (Marini *et al.*, 1997), strains lacking MEP2 had no apparent growth defect, even on low ammonium media. Δ *mep2*/ Δ *mep2* mutant strains do form filaments on media containing limiting concentrations of glutamine, proline, asparagine or arginine as the sole nitrogen source (data not shown), indicating that the pseudohyphal defect conferred by the Δ *mep2* mutation is only observed in the presence of its substrate, ammonium.

Next we constructed strains lacking multiple MEP permeases. Both the Δ *mep1* Δ *mep2* and Δ *mep2* Δ *mep3* homozygous diploid strains exhibited the pseudohyphal defect conferred by the Δ *mep2* mutation (Figure 1A). The Δ *mep1* Δ *mep2* strain also had a growth defect when grown under limiting ammonium conditions, consistent with previous studies (Figure 1A; Dubois and Grenson, 1979; Marini *et al.*, 1994). Δ *mep1*/ Δ *mep1* Δ *mep2*/ Δ *mep2* cells apparently experience nitrogen starvation severe enough to result in a growth defect, yet still do not undergo filamentous growth. These findings suggest that MEP2 might have a role in regulating pseudohyphal differentiation in addition to its role in ammonium uptake.

When incubated on synthetic media containing 5 μ M to 5 mM ammonium as the sole nitrogen source, the triple Δ *mep1* Δ *mep2* Δ *mep3* mutant strain completes a few cell divisions before arresting (Figure 1A), possibly when vacuolar stores of amino acids are exhausted. On YNB medium (38 mM ammonium), the triple mutant does grow, albeit with a significant growth defect (data not shown), suggesting that an additional non-specific ammonium transport system exists. As shown in Figure 1B, expression of either *MEP1*, *MEP2* or *MEP3* from a plasmid is

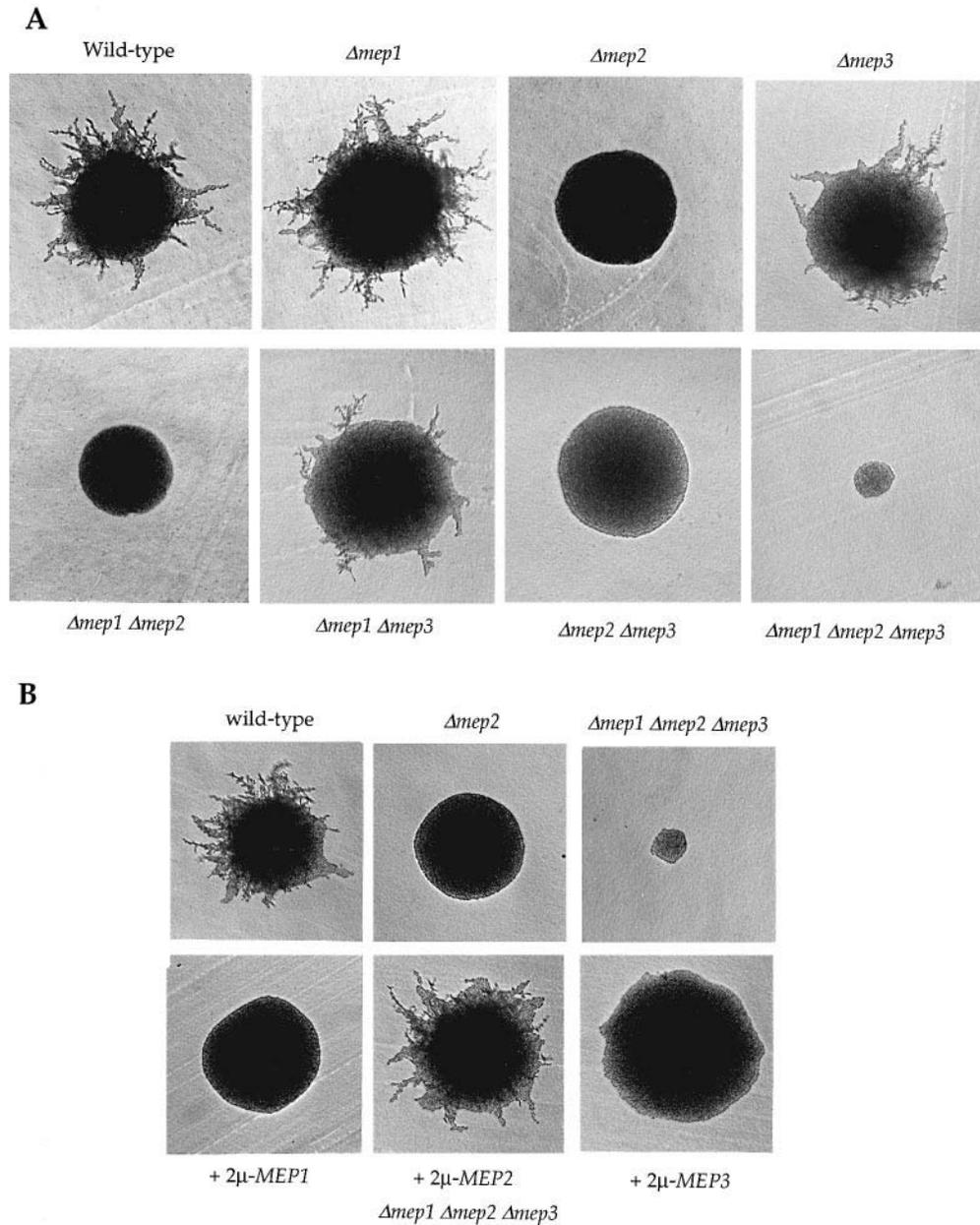


Fig. 1. MEP2 is required for pseudohyphal differentiation. **(A)** Homozygous diploid strains of the indicated genotypes (see Materials and methods; Table II) were incubated on low-ammonium SLAD medium for 4 days at 30°C. **(B)** The diploid $\Delta mep1/\Delta mep1 \Delta mep2/\Delta mep2 \Delta mep3/\Delta mep3$ strain (MLY131a/ α) with plasmids expressing no MEP gene, MEP1 (pML100), MEP2 (pML151) or MEP3 (pML113) were incubated on SLAD medium for 4 days at 30°C. Isogenic wild-type (MLY61) and $\Delta mep2/\Delta mep2$ strains (MLY108a/ α) are included as controls.

sufficient to complement the growth defect of the homozygous diploid $\Delta mep1 \Delta mep2 \Delta mep3$ mutant strain, demonstrating that all three proteins are functional ammonium transporters. In contrast, only expression of MEP2 also restored pseudohyphal differentiation in the diploid $\Delta mep1 \Delta mep2 \Delta mep3$ strain, indicating that MEP2 has a specific role in the regulation of filamentous growth that is not shared with the homologous permeases MEP1 and MEP3.

The $\Delta mep2$ mutation does not affect ammonium utilization

Although the $\Delta mep2$ mutation did not appear to have phenotypes indicative of defects in nitrogen metabolism, we wished to exclude the possibility that alterations in

ammonium metabolism in $\Delta mep2$ mutant strains might contribute to the defect in pseudohyphal growth. Isogenic MEP2/MEP2 and $\Delta mep2/\Delta mep2$ strains grew at similar rates, even under low ammonium conditions, as determined from growth curves in liquid media (data not shown). We next directly assayed the ability of diploid Δmep strains to extract ammonium from the extracellular medium, a measure of the ammonium uptake capacity of these strains (see Materials and methods). As shown in Figure 2, only the $\Delta mep1 \Delta mep2$ and $\Delta mep1 \Delta mep2 \Delta mep3$ homozygous diploid strains exhibited reduced ammonium uptake capacity. These findings are consistent with the reduced growth rates of these strains on ammonium-limiting media (Figure 1A and data not shown) and with a previous report (Marini *et al.*, 1997). We also assayed the activity of the nitrogen

metabolic enzymes glutamine synthase (GLN1) and glutamate dehydrogenase (GDH1 and GDH2) after growth at a range of ammonium concentrations from 50 μ M to 50 mM. Activities of these enzymes were similar in wild-type and Δ *mep2*/ Δ *mep2* strains (data not shown), indicating that neither ammonium uptake nor metabolism are significantly altered in cells lacking the MEP2 ammonium permease.

MEP2 regulation of pseudohyphal growth is independent of the MAP kinase cascade

The findings that the Δ *mep2* mutation does not alter ammonium uptake or metabolism yet blocks pseudohyphal

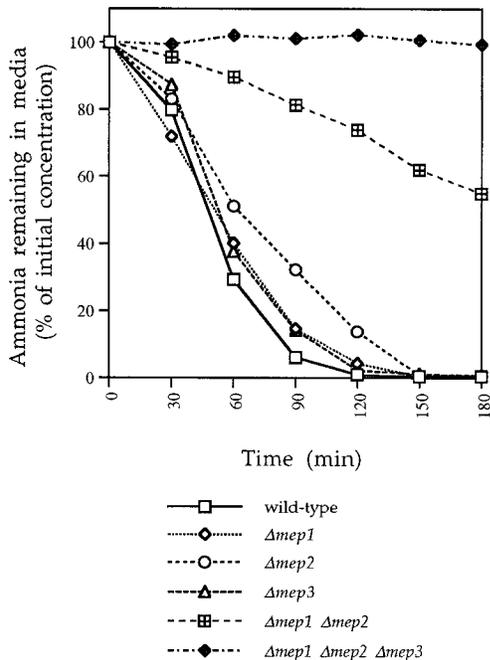


Fig. 2. Ammonium import in ammonium permease mutants. Ammonium uptake capacity was assayed as described in Materials and methods in homozygous diploid strains: wild-type (MLY61), Δ *mep1* (MLY104a/ α), Δ *mep2* (MLY108a/ α), Δ *mep3* (MLY128a/ α), Δ *mep1* Δ *mep2* (MLY115a/ α) and Δ *mep1* Δ *mep2* Δ *mep3* (MLY131a/ α). The ammonium remaining in the media is expressed as a percentage of the starting concentration (~500 μ M).

differentiation led to the hypothesis that MEP2 might have a signaling function in the regulation of pseudohyphal differentiation. One previously characterized element of the signaling machinery is the pheromone responsive MAP kinase cascade. Expression of the dominant active *STE11-4* allele suppresses mutations in upstream components (e.g. Δ *ste20*) to restore either mating response or pseudohyphal growth, while overexpression of the *STE12* transcription factor greatly enhances filamentation (Liu *et al.*, 1993). These alleles allowed us to test the point of action of MEP2 with respect to the MAP kinase cascade through genetic epistasis. The pseudohyphal defect of the Δ *mep2*/ Δ *mep2* strain was not suppressed by the dominant active *STE11-4* allele or by overproduction of the *STE12* transcription factor (Figure 3), suggesting that MEP2 does not function upstream of these proteins. Likewise, the Δ *mep2* mutation was not suppressed by overexpression of *PHD1*, a DNA binding protein that enhances pseudohyphal differentiation in both wild-type strains (see Figure 3; Gimeno and Fink, 1994) and Δ *ste* mutant strains. The pseudohyphal deficiency conferred by the Δ *mep2* mutation was modestly suppressed by high copy expression of *TEC1* (Figure 3), which encodes a transcription factor that dimerizes with *STE12* to modulate expression of a nitrogen-regulated reporter gene [FG(TyA)::*lacZ*; Laloux *et al.*, 1994; Madhani and Fink, 1997]. This disparity between the *STE12* and *TEC1* epistasis results may result from lower expression of *STE12*, as high level overproduction of *STE12* is lethal (Liu *et al.*, 1993). To moderate *STE12* expression we used a low concentration of glucose to reduce the activity of the galactose-inducible promoter (Liu *et al.*, 1993; see Materials and methods). Alternatively, *TEC1* may have a *STE12*-independent function also important in regulation of filamentation, or *TEC1* may be regulated by a signaling pathway other than the MAP kinase cascade that activates *STE12*.

To examine the relationship between MEP2 and the MAP kinase pathway further, we employed the FG(TyA)::*lacZ* reporter known to be induced 2- to 10-fold by nitrogen starvation (Mösch *et al.*, 1996; Lorenz and Heitman, 1997; Madhani and Fink, 1997). Nitrogen starvation induced reporter gene expression to a similar extent in wild-type, Δ *mep1*, Δ *mep2* or Δ *mep1* Δ *mep2* homozygous

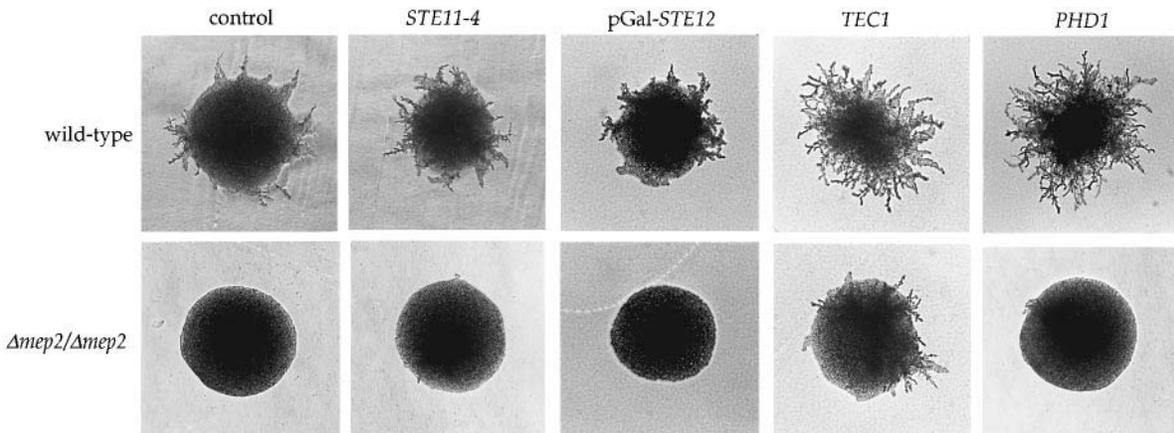


Fig. 3. MEP2 does not interact with the MAP kinase cascade. Epistasis tests with elements of the MAP kinase cascade were performed in homozygous wild-type (MLY61) and Δ *mep2*/ Δ *mep2* (MLY108a/ α) diploid strains. Each strain expresses a control vector, the dominant *STE11-4* allele (pSL1509), or high copy plasmids with *TEC1* (p2.5-2) or *PHD1* (pCG38) grown on SLAD medium for 4 days at 30°C. Expression of the pGal-*STE12* allele (pNC252) on inducing SLADG media was assayed after 6 days at 30°C.

Table I. Δmep mutations do not alter FG(TyA)::lacZ induction by nitrogen starvation

Strain	Genotype	Relative β -galactosidase activity	
		50 μ M NH ₄ ⁺	5 mM NH ₄ ⁺
MLY97	wild-type	1.8	1.0
MLY230a/ α	$\Delta mep1/\Delta mep1$	2.1	1.0
MLY225a/ α	$\Delta mep2/\Delta mep2$	2.4	0.8
MLY231a/ α	$\Delta mep1/\Delta mep1$ $\Delta mep2/\Delta mep2$	2.4	0.7

Values are the average of two independent transformants, each tested in duplicate and are reported as relative to the wild-type strain on nitrogen rich (5 mM ammonium sulfate) medium. Assays were performed as described in Materials and methods.

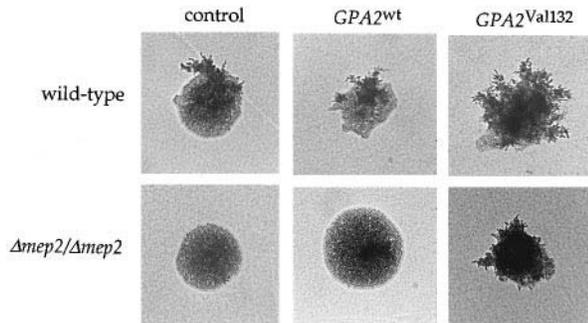


Fig. 4. Activated GPA2 suppresses the $\Delta mep2/\Delta mep2$ pseudohyphal defect. Wild-type (MLY61) and $\Delta mep2/\Delta mep2$ (MLY108a/ α) strains expressing a control vector (pSEYC68), wild-type *GPA2* (pML180), or the dominant, constitutively active *GPA2-2* allele (pML160) under the control of a galactose-inducible promoter were incubated on SLARG medium for 4 days at 30°C.

diploid strains (Table I), thus MEP1 and MEP2 are not required for induction of the FG(TyA)::lacZ reporter. While this finding is in contrast to mutations in the MAP kinase cascade, which reduce induction of this reporter (Mösch *et al.*, 1996; Madhani and Fink, 1997), expression of the FG(TyA)::lacZ gene is also unaffected by either loss of function or activated alleles of *GPA2* (Lorenz and Heitman, 1997).

The $\Delta mep2$ pseudohyphal defect is suppressed by activation of GPA2 or RAS2, or by exogenous cAMP

The findings presented above indicate that MEP2 regulates filamentous growth independent of the MAP kinase cascade. We recently identified a signaling pathway including GPA2, RAS2 and cAMP that also regulates pseudohyphal differentiation independent of this MAP kinase pathway (Lorenz and Heitman, 1997). We tested the relationship between MEP2 and GPA2 using a dominant active allele, *GPA2-2* (Gly132Val), which stimulates pseudohyphal differentiation, even in nutrient rich conditions, and suppresses the filamentation defect of Δste mutant strains (Lorenz and Heitman, 1997). Expression of *GPA2-2* restored filamentation in $\Delta mep2/\Delta mep2$ mutant strains (Figure 4), suggesting that MEP2 functions upstream of GPA2 in signaling pseudohyphal growth.

If MEP2 acts in the same pathway as GPA2, conditions that restore filamentation in a $\Delta gpa2/\Delta gpa2$ strain should also suppress the pseudohyphal defect of $\Delta mep2/\Delta mep2$

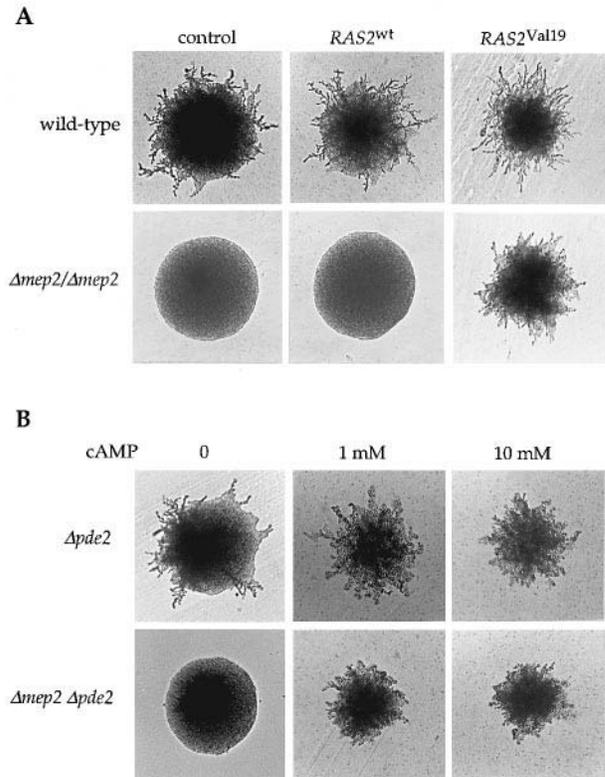


Fig. 5. The $\Delta mep2$ pseudohyphal defect is suppressed by *RAS2*^{Val19} and by cAMP. (A) Wild-type (MLY61) and $\Delta mep2/\Delta mep2$ (MLY108a/ α) strains with a control plasmid, or expressing *RAS2*^{wt} (pMW1) or *RAS2*^{Val19} (pMW2) were grown on SLAD medium for 4 days at 30°C. (B) $\Delta pde2/\Delta pde2$ (MLY162a/ α) and $\Delta mep2/\Delta mep2 \Delta pde2/\Delta pde2$ (MLY229a/ α) strains were grown on low ammonium SLAD media in the absence of cAMP or in the presence of 1 mM or 10 mM cAMP for 4 days at 30°C.

strains. Indeed, one suppressor of $\Delta gpa2$, the dominant *RAS2*^{Gly19Val} allele, also suppressed the $\Delta mep2$ mutation (Figure 5A). In strains lacking the high-affinity cAMP phosphodiesterase PDE2, cAMP enhances filamentation and suppresses the $\Delta gpa2$ mutation (Lorenz and Heitman, 1997). Similarly, cAMP restored pseudohyphal differentiation in a $\Delta mep2/\Delta mep2 \Delta pde2/\Delta pde2$ strain (Figure 5B). These findings support a model in which GPA2, RAS2 and cAMP function downstream of MEP2 in a signaling pathway regulating filamentous growth.

An intracellular loop is important for MEP function in pseudohyphal growth

To address the unique structural features of MEP2 required for pseudohyphal differentiation, we took advantage of the significant similarities between MEP1 and MEP2 in sequence (66% similarity), topology (10 predicted transmembrane domains), and function (as ammonium permeases), though MEP2 regulates filamentous growth while MEP1 does not. Deletion of the C-terminus of MEP2, 50 amino acids in length and predicted to be cytoplasmic, had no effect on either the pseudohyphal growth or ammonium transport functions of MEP2 (Figure 6; pML153). We constructed a series of MEP1-MEP2 hybrids, with junctions in the predicted extracellular loops and expressed from the *MEP2* promoter (Figure 6). Each of these hybrid permeases complemented the growth defect of the $\Delta mep1 \Delta mep2 \Delta mep3$ homozygous diploid

Plasmid	Structure	Construct	Pseudohyphal Growth	Growth on low ammonium
pML100		all MEP1	-	+
pML151		all MEP2	+	+
pML153		MEP2ΔC-term	+	+
pML155		N-term hybrid	-	+
pML156		1st loop hybrid	+	+
pML157		2nd loop hybrid	+	+
pML158		3rd loop hybrid	+	+
pML161		1st loop swap	+	+
pML162		1st loop swap	+	+

Fig. 6. MEP1/MEP2 hybrid analysis. The hybrids diagrammed above (MEP1 in grey, MEP2 in black) expressed from the high copy YEplac195 plasmid, each under control of the *MEP2* promoter, were assayed for growth and filamentation in the $\Delta mep1/\Delta mep1 \Delta mep2/\Delta mep2 \Delta mep3/\Delta mep3$ strain (MLY131a/ α). '+' indicates complementation of either the pseudohyphal growth or ammonium uptake defects. Hybrids were constructed as described in the Materials and methods.

strain on low ammonium media (50 μ M), consistent with restoration of ammonium transport. A hybrid in which only the sequence at the N-terminus is derived from MEP2 (through the first transmembrane domain; pML155) did not complement the pseudohyphal defect of $\Delta mep2/\Delta mep2$ mutant strains (Figure 6). This hybrid, composed almost entirely of *MEP1* coding sequences, uses the *MEP2* promoter; thus differences in expression are unlikely to account for the functional differences between MEP1 and MEP2. Expression of a hybrid protein including the three N-terminal transmembrane domains of MEP2 fused to C-terminal sequences of MEP1 (pML156) restored pseudohyphal differentiation in $\Delta mep2/\Delta mep2$ strains (Figure 6). These observations suggest that the region of MEP2 between the first and third transmembrane domains (e.g. the first intracellular loop) participates in the pseudohyphal regulatory function.

Next we constructed a fusion protein in which only the first intracellular loop plus the adjacent membrane spanning segments (87 amino acids) was derived from MEP2 (pML162). This hybrid protein complemented to restore both the pseudohyphal and ammonium transport functions of MEP2. Surprisingly, the reciprocal swap (pML161), in which the majority of the protein is MEP2 and only the first intracellular loop is derived from MEP1, also complemented both MEP2 functions. Thus the first intracellular loop is sufficient, but not strictly necessary, for the signaling function of MEP2; hence other regions of MEP2 are likely to also participate in signaling.

Regulation of MEP2 protein expression: nitrogen regulatory genes are required for filamentous growth

Next we addressed whether regulation of MEP2 expression could underlie a signaling function of this ammonium permease. Northern analysis demonstrated that *MEP2* is preferentially expressed under nitrogen limiting conditions

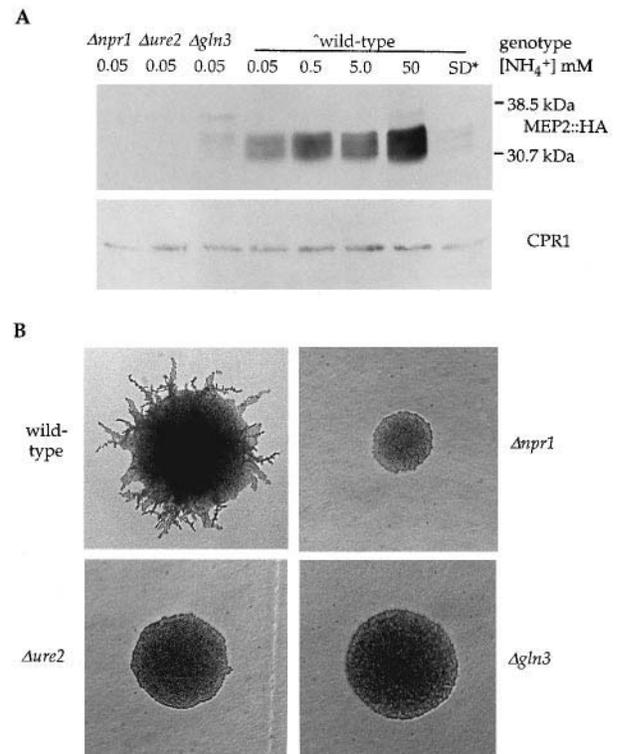


Fig. 7. MEP2 protein expression requires GLN3, URE2, and NPR1. (A) Western blot analysis of the epitope tagged MEP2-HA protein. Otherwise wild-type *MEP2::HA/MEP2::HA* strain (MLY220), or $\Delta gln3/\Delta gln3$ (MLY194a/ α), $\Delta ure2/\Delta ure2$ (MLY198a/ α), and $\Delta npr1/\Delta npr1$ (MLY221) mutant strains were grown in minimal media containing the indicated concentration of ammonium sulfate for 4 h at 30°C. Proteins were detected using an α -HA monoclonal antibody or α -cyclophilin A (α -CPR1) as a control for protein loading. (B) Homozygous diploid strains wild-type (MLY61), $\Delta gln3/\Delta gln3$ (MLY139a/ α), $\Delta ure2/\Delta ure2$ (MLY140a/ α), and $\Delta npr1/\Delta npr1$ (MLY54a/ α) were incubated on low ammonium media (SLAD) for 4 days at 30°C.

(Marini *et al.*, 1997). Because many nutrient permeases are regulated both post-translationally and transcriptionally (reviewed in Grenson, 1992), we analyzed MEP2 protein levels. To detect the MEP2 protein directly we used an integrative technique to tag the genomic *MEP2* locus such that three repeats of the hemagglutinin epitope (HA) are fused to the C-terminus (Schneider *et al.*, 1995). This approach utilizes the native *MEP2* promoter and terminator sequences. The epitope-tagged protein complemented both the pseudohyphal and ammonium transport functions of the native MEP2 (data not shown).

The MEP2::HA protein was detected in cell lysates through Western blot analysis as shown in Figure 7A. MEP2 is present at very low levels in cells grown in rich medium (SD-Ura). In media in which ammonium is the sole nitrogen source, MEP2 was expressed at high levels, and expression did not increase at lower ammonium concentrations (Figure 7A), in contrast with *MEP2* mRNA which does accumulate at lower ammonium concentrations (Marini *et al.*, 1997). Since MEP2 expression is not significantly different between high and low ammonium conditions, regulation of MEP2 protein levels does not explain its unique role in pseudohyphal differentiation.

Marini *et al.* (1997) found that the nitrogen regulatory genes *GLN3* and *NIL1* are required for the transcription

of *MEP2*. We found that *MEP2* protein was markedly reduced in strains that lack *GLN3* (Figure 7A). *GLN3*, a zinc-finger DNA binding protein, is required for the transcriptional induction of a number of nitrogen catabolic genes, probably including *MEP1* and *MEP2* (Minehart and Magasanik, 1991; Stanbrough *et al.*, 1995). *URE2*, a prion analog with homology to glutathione *S*-transferases and which antagonizes *GLN3* function in some cases (Coschigano and Magasanik, 1991; Wickner, 1994; Xu *et al.*, 1995), was also required for *MEP2* accumulation (Figure 7A). Finally, the *MEP2* protein was also undetectable in $\Delta npr1/\Delta npr1$ mutant strains (Figure 7A). *NPR1* is a protein kinase that maintains the activity of a variety of plasma membrane permeases, including *MEP1* and *MEP2*, under conditions of nitrogen starvation (Grenson and Acheroy, 1982; Grenson and Dubois, 1982; Grenson, 1983b; Vandenbol *et al.*, 1990).

One prediction from these findings is that the *GLN3*, *URE2* and *NPR1* regulatory proteins might also regulate pseudohyphal differentiation. This is indeed the case, as $\Delta gln3/\Delta gln3$, $\Delta ure2/\Delta ure2$ and $\Delta npr1/\Delta npr1$ mutant strains were all defective in pseudohyphal differentiation (Figure 7B). The filamentation defect of $\Delta gln3/\Delta gln3$, $\Delta ure2/\Delta ure2$ and $\Delta npr1/\Delta npr1$ strains was also observed when glutamine or proline were present as the sole nitrogen source (data not shown), in contrast to $\Delta mep2/\Delta mep2$ strains in which the pseudohyphal deficiency is limited to ammonium-grown cells. When under the control of a heterologous promoter (the inducible *Gall1,10* promoter), *MEP2* complements the low ammonium growth defect of the *mep* triple mutant, indicating that *MEP2* is expressed, but did not restore filamentation in $\Delta gln3/\Delta gln3$ or $\Delta ure2/\Delta ure2$ mutant strains. Thus these regulatory proteins are likely to have targets in addition to *MEP2* that are critical for the regulation of dimorphism.

Other permeases may also regulate pseudohyphal differentiation

Next we addressed whether our findings with *MEP2* could be extended to other nitrogen permeases. Since *MEP2* does not alter pseudohyphal differentiation when nitrogen sources other than ammonium are present, we hypothesized that other permeases may play a similar role to *MEP2* when in the presence of their substrates. Unfortunately, most other nitrogen sources are imported by a single specific uptake system, in addition to non-specific systems such as the general amino acid permease. Analysis of other permeases, then, is more difficult than for the *MEPs* as the presence of multiple ammonium permeases made this system ideal for this type of analysis. A genetic screen was designed to address this issue. The *NPR1* protein kinase, required for pseudohyphal growth and stable *MEP2* expression, post-translationally regulates many permeases, including those for glutamine (*GNP1*), proline (*PUT4*), general amino acids (*GAP1*) and several others (Grenson and Dubois, 1982; Grenson, 1983b; Vandenbol *et al.*, 1987). These permeases are significantly less active in $\Delta npr1$ mutant strains than in wild-type strains; *GAP1* activity, for example, is undetectable in the absence of *NPR1* (Grenson, 1983b). Genetic evidence suggests that *NPR1* antagonizes the activity of the essential *NPI1/RSP5* protein-ubiquitin ligase (Grenson, 1983b; Hein *et al.*, 1995; Huibregste *et al.*, 1995). We capitalized on this

phenotype to examine the role of other permeases in the regulation of dimorphism.

We isolated suppressors of the $\Delta npr1$ growth defect, reasoning that some mutations might also suppress the $\Delta npr1$ filamentation defect. UV-induced mutants with improved growth on either ammonium-limiting SLAD or tryptophan-citrulline (TC) media were isolated. Of 36 mutants identified (in seven allele groups), one mutant exhibited constitutively derepressed *GAP1* activity, as measured by sensitivity to toxic levels of D-histidine and L-lysine under repressing (high ammonium) growth conditions, the behavior expected of an *npi1/rsp5* mutant (Grenson, 1983b). This phenotype was complemented by expression of the wild-type *NPI1* from a low copy plasmid; moreover, the mutation is allelic with *NPI1* based on tight linkage of the mutant phenotype to an *NPI1-URA3* tagged allele integrated at the mutant *npi1* locus (see Materials and methods). The $\Delta npr1$ growth and pseudohyphal defects were strongly suppressed by the *npi1* mutation on glutamine and weakly on proline, but were not suppressed on media containing ammonium as the sole nitrogen source (Figure 8). Genetic studies have indicated that *NPI1* targets the glutamine (*GNP1*) and proline (*PUT4*) permeases for degradation, but does not affect the *MEP* ammonium permeases (Dubois and Grenson, 1979; Grenson and Dubois, 1982; Grenson, 1983a,b). The only known targets for both *NPR1* and *NPI1* action are plasma membrane permeases; thus, this finding suggests that other permeases may play a role analogous to *MEP2*.

Discussion

The downstream events in nutrient based signaling have been well studied—regulatory events at the transcriptional, translational, and post-translational levels ensure the presence of the appropriate enzymatic systems to utilize the available nutrients (reviewed in Grenson, 1992; Magasanik, 1992). In contrast, the initial events that sense the quantity and quality of these nutrients are poorly understood. We have studied these processes during pseudohyphal differentiation, a nitrogen-regulated developmental transition in budding yeast.

We find that the ammonium permease *MEP2* is required for filamentous growth in yeast. These observations suggest *MEP2* may function both to transport and to sense ammonium levels. First, *MEP2* is one of three related ammonium permeases, any one of which are sufficient for growth on ammonium-limiting media, yet *MEP2* is the only permease required for pseudohyphal differentiation. The role of *MEP2* in pseudohyphal growth exhibits signal specificity; the filamentation defect is observed only when ammonium is present as the sole nitrogen source in limiting concentrations. Finally, ammonium import and nitrogen metabolic pathways (as measured by the activity of *GLN1*, *GDH1* and *GDH2* in ammonium limiting media) are normal in $\Delta mep2/\Delta mep2$ mutant cells.

Based on these observations, we propose that *MEP2* serves as both an ammonium transporter and as a component of a nitrogen sensor that signals when conditions are appropriate for pseudohyphal growth.

Our findings are consistent with a model (Figure 9) in which *MEP2* functions upstream of the $G\alpha$ protein *GPA2* in the regulation of pseudohyphal growth. Mutational

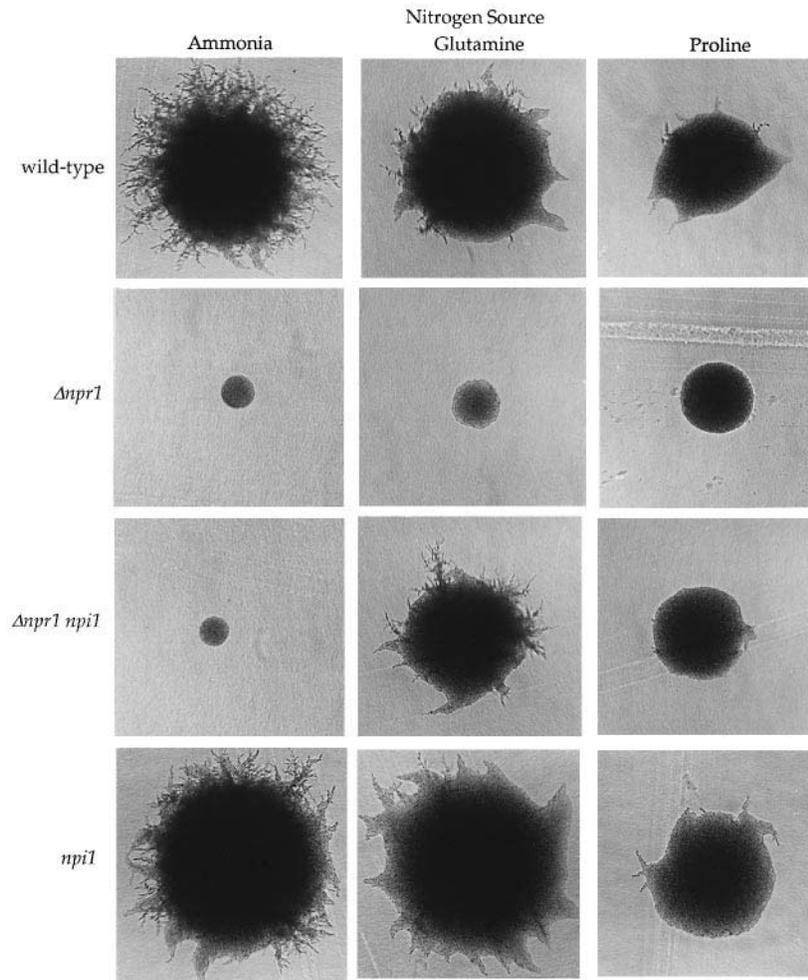


Fig. 8. Other nitrogen permeases may regulate pseudohyphal differentiation. Homozygous diploid wild-type (MLY61), $\Delta npr1/\Delta npr1$ (MLY54a/ α), $\Delta npr1/\Delta npr1 npi1/npi1$ (MLY92a/ α), and $npi1/npi1$ (MLY96) strains were incubated on media containing 100 μ M ammonium, glutamine or proline as the sole nitrogen sources, for 4 days at 30°C.

activation of GPA2 (the dominant *GPA2-2* allele) suppresses the $\Delta mep2/\Delta mep2$ pseudohyphal defect. Moreover, both *RAS2*^{Val19} and exogenous cAMP suppress the filamentation defect conferred by both the $\Delta gpa2$ and $\Delta mep2$ mutations. Importantly, this signaling pathway is independent of the pheromone responsive MAP kinase cascade, which also regulates pseudohyphal differentiation (see Figure 9).

While the model presented in Figure 9 is consistent with our data, there are other possible explanations as well. We have no evidence directly linking MEP2 and GPA2; thus MEP2 may function in a signaling pathway separate from either the GPA2/cAMP or MAP kinase pathways. A critical reagent to test this hypothesis would be a dominant *MEP2* allele; we have been unable to isolate such an allele to date. Alternative models are that MEP2 might be required to secrete ammonium or a related compound with an extracellular signaling function, or to sequester ammonium in an intracellular compartment. There is some very recent evidence that ammonia secretion may facilitate intercolony communication in yeast (Palkova *et al.*, 1997).

The suggestion that a permease such as MEP2 may function as a receptor is not unprecedented. Recent findings indicate a similar role for the glucose transporter homologs

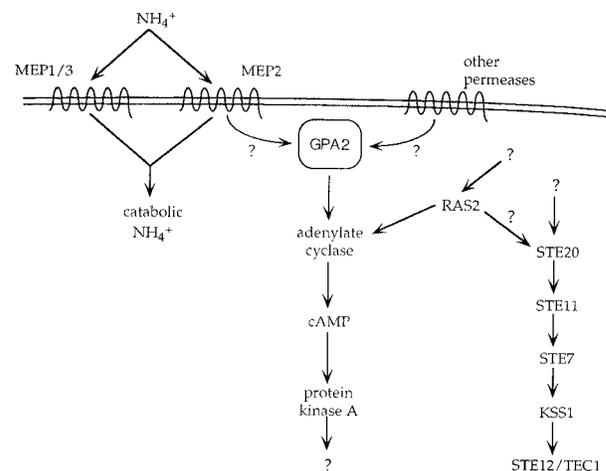


Fig. 9. A model for MEP2 regulation of pseudohyphal differentiation. Ammonium starvation is sensed via MEP2 to produce a signal that activates GPA2 and a signaling pathway that regulates filamentous growth independently of the MAP kinase cascade.

RGT2 and SNF3 in yeast. These proteins activate a signaling cascade to regulate transcription of additional hexose permeases and a similar role has recently been ascribed to the Rco-3 glucose transporter during

conidiation in *N.crassa* (Liang and Gaber, 1996; Ozcan *et al.*, 1996; Madi *et al.*, 1997). Dominant mutations in both *RGT2* and *SNF3* have been described that constitutively activate this signaling cascade, even in the complete absence of glucose (Liang and Gaber, 1996; Ozcan *et al.*, 1996).

How might MEP2 function as an ammonium sensor? One possibility is that MEP2 exists in different conformational states depending on its transport status (active, idling or off) and that associated effector proteins recognize these different conformations. Extracellular ammonium concentration, then, would be communicated to effectors via the structure of MEP2. MEP2 may directly interact with GPA2; such an interaction would be novel in G protein signaling systems, typically regulated by seven-transmembrane domain proteins such as the pheromone receptors, STE2 and STE3. Efforts using the two hybrid system have not provided evidence for a MEP2–GPA2 complex. Instead, other protein intermediates may couple MEP2 conformational changes to the activation of GPA2. As an alternative, MEP2 could be required to regulate the production of a signaling molecule, possibly a nitrogen metabolite, under nitrogen starvation conditions that would activate GPA2 through a more conventional signaling mechanism. The phenotypic differences between MEP2 and its homologs MEP1 and MEP3 could result from a direct interaction of MEP2 with ammonium assimilating enzymes or other enzymatic machinery that generates such a signaling molecule, or with other downstream effectors. The first intracellular loop of MEP2, which our evidence suggests plays a role in MEP2 signaling, may mediate such an interaction with downstream effectors.

The pseudohyphal defects of the $\Delta mep2/\Delta mep2$ mutant strain are only observed when ammonium, the substrate of MEP2, is the sole nitrogen source. In contrast, yeast cells initiate this dimorphic switch in response to general nitrogen starvation, regardless of the nitrogenous compounds present. Thus permeases for other compounds may have a regulatory role similar to MEP2 in the presence of their substrates. In accord with this hypothesis, we find that the NPR1 protein kinase is required for pseudohyphal differentiation on media containing ammonium (Figure 7B), proline (Figure 8) or glutamine (Figure 8). A mutation in the *NPII/RSP5* gene suppressed this $\Delta npr1$ phenotype, but only with proline or glutamine as the sole source of nitrogen. Genetic evidence indicates that NPI1, an essential protein-ubiquitin ligase (Hein *et al.*, 1995; Huibregste *et al.*, 1995), destabilizes the proline (PUT4) and glutamine permeases (GNP1) but not the MEP ammonium permeases (Dubois and Grenson, 1979; Grenson and Dubois, 1982; Grenson, 1983a,b). Our finding that NPR1 and NPI1 regulate pseudohyphal growth suggests that permeases other than MEP2 may regulate this dimorphic transition in the presence of their substrates. Like NPR1, the GPA2 G α protein is required for filamentous growth in response to starvation for any nitrogen source (Lorenz and Heitman, 1997), and hence signaling from each of these permeases could be mediated by GPA2. The multiplicity of ammonium permeases in yeast made this system ideal for dissecting the role of the individual permeases; in the case of many other nitrogen sources there is only a single high affinity transporter, making similar studies more difficult.

Finally, as nitrogen starvation plays a broad role in

regulating growth and differentiation pathways in diverse fungi, related transport proteins may have a conserved function in regulating these developmental events. As one example, mating in *S.pombe* and *C.neoformans* requires nitrogen starvation, which is signaled in both organisms via a conserved G α protein with marked identity to yeast GPA2: GPA2 in *S.pombe* and GPA1 in *C.neoformans* (Isshiki *et al.*, 1992; Tolkacheva *et al.*, 1994; Alspaugh *et al.*, 1997). The mechanisms by which these G proteins are activated by nitrogen starvation are not yet known but, based on our findings, may involve a conserved role for related nitrogen permeases. Experiments to test this hypothesis in *C.neoformans* are currently in progress.

Materials and methods

Yeast strains, media and microbiological techniques

Yeast strains are listed in Table II. Standard yeast media and genetic manipulations were as described (Sherman, 1991). Limiting nitrogen media (SLAD; Gimeno *et al.*, 1992) contains 0.17% Yeast Nitrogen Base without amino acids or ammonium sulfate, 50 μ M ammonium sulfate, 2% dextrose and 2% Bacto agar. SLARG, to induce *GPA2* alleles, contains 0.5% galactose and 2% raffinose (Lorenz and Heitman, 1997). SLADG, to induce the pGal-*STE12* construct, contains 2% galactose and 0.13% glucose (Liu *et al.*, 1993). YNB media contains 0.67% yeast nitrogen base (minus amino acids plus ammonium sulfate) and 2% glucose. Media which use alternative nitrogen sources contained proline, glutamine or ammonium sulfate at 100 μ M and 2% Noble agar (Difco). TC media (Grenson, 1983b) contains 250 μ g/ml tryptophan, 250 μ g/ml citrulline, 2% glucose and 2% Bacto agar.

Yeast transformations were performed as described (Schiestl *et al.*, 1993). Disruption alleles for *MEP1*, *MEP2* and *NPR1* were constructed by replacement of coding sequences by the *LEU2* selectable marker. The $\Delta mep1::G418$, $\Delta mep2::G418$, $\Delta mep3::G418$, $\Delta gln3::G418$ and $\Delta ure2::G418$ mutations were created through PCR mediated disruption (Wach *et al.*, 1994) in either Leu⁺ (MLY40, MLY41; see Table II) or $\Delta leu2::hisG$ (MLY42, MLY43) host strains.

Plasmids

Plasmids are listed in Table III. The plasmid-borne *MEP1* (pML100), *MEP2* (pML151), *MEP3* (pML113) and *NPII* (pML95) genes were derived from PCR amplification from genomic DNA of strain MLY54 α . Plasmids for the analysis of MAP kinase function have been described (Liu *et al.*, 1993; Gimeno and Fink, 1994; Möscher *et al.*, 1996), as have *GPA2* plasmids (Lorenz and Heitman, 1997).

Hybrid analysis

Hybrids between *MEP1* and *MEP2* were constructed by PCR overlap. The fusion proteins are diagrammed in Figure 6. Each hybrid uses the native MEP2 promoter sequences. Junction points are in the predicted extracellular loops. The hybrids were cloned into the multicopy *URA3* plasmid YEplac195 (Gietz and Sugino, 1988). The amino acid residues that comprise the junction points are shown in Table IV, numbered as in Marini *et al.* (1997).

Photomicroscopy

All single colony photographs were taken directly from Petri plates using a Nikon Labophot-2 microscope with a 10 \times primary objective (Zeiss) and a 2.5 \times trinocular camera adaptor (Nikon). Unless otherwise stated, colonies were incubated at 30 $^{\circ}$ C for 4 days.

Ammonium import assays

Ammonium uptake was assayed as in Marini *et al.* (1994). The Δmep mutant strains were grown to late log phase in minimal proline media (Mpro; 0.1% proline, 0.17% yeast nitrogen base, 2% glucose). Cultures were diluted to an OD₆₀₀ of 1.0 in Mpro and 10 mM ammonium sulfate was added to a final concentration of 500 μ M. At the indicated times, a portion of the culture was removed and the cells removed by centrifugation. The ammonium concentration in the culture supernatant was assayed using a glutamate dehydrogenase-linked assay as described (Tabor, 1971).

Growth rates of Δmep mutant strains were determined by OD₆₀₀ at various time points in either liquid SLAD or SD-Ura media. Assays for

Table II. *Saccharomyces cerevisiae* strainsAll strains are congenic to Σ 1278b (Grenson *et al.*, 1966)

Strain	Genotype	Reference
MLY40	<i>ura3-52 MATα</i>	Lorenz and Heitman (1997)
MLY41	<i>ura3-52 MATa</i>	Lorenz and Heitman (1997)
MLY42	<i>ura3-52 Δleu2::hisG MATα</i>	Lorenz and Heitman (1997)
MLY43	<i>ura3-52 Δleu2::hisG MATa</i>	Lorenz and Heitman (1997)
MLY54a	<i>Δnpr1::LEU2 Δleu2::hisG <i>ura3-52 MATa</i></i>	this study
MLY54 α	<i>Δnpr1::LEU2 Δleu2::hisG <i>ura3-52 MATα</i></i>	this study
MLY54a/ α	<i>Δnpr1::LEU2/Δnpr1::LEU2 <i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i></i>	this study
MLY61	<i>ura3-52/ura3-52 MATa/α</i>	Lorenz and Heitman (1997)
MLY92a/ α	<i>npi1/npi1 Δnpr1::LEU2/Δnpr1::LEU2 <i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i></i>	this study
MLY97	<i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i>	Lorenz and Heitman (1997)
MLY104a/ α	<i>Δmep1::LEU2/Δmep1::LEU2 <i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i></i>	this study
MLY108a/ α	<i>Δmep2::LEU2/Δmep2::LEU2 <i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i></i>	this study
MLY115a/ α	<i>Δmep1::LEU2/Δmep1::LEU2 Δmep2::LEU2/Δmep2::LEU2 <i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i></i>	this study
MLY128a/ α	<i>Δmep3::G418/Δmep3::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY129a/ α	<i>Δmep1::LEU2/Δmep1::LEU2 Δmep3::G418/Δmep3::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY130a/ α	<i>Δmep2::LEU2/Δmep2::LEU2 Δmep3::G418/Δmep3::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY131a/ α	<i>Δmep1::LEU2/Δmep1::LEU2 Δmep2::LEU2/Δmep2::LEU2 Δmep3::G418/Δmep3::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY132a/ α	<i>Δgpa2::G418/Δgpa2::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	Lorenz and Heitman (1997)
MLY135a/ α	<i>Δgpa2::G418/Δgpa2::G418 Δmep1::LEU2/Δmep1::LEU2 Δleu2::hisG/Δleu2::hisG <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY139a/ α	<i>Δgln3::G418/Δgln3::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY140a/ α	<i>Δure2::G418/Δure2::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY141a/ α	<i>npi1/npi1 <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY162a/ α	<i>Δpde2::G418/Δpde2::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	Lorenz and Heitman (1997)
MLY194a/ α	<i>MEP2::HA/MEP2::HA Δgln3::G418/Δgln3::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY198a/ α	<i>MEP2::HA/MEP2::HA Δure2::G418/Δure2::G418 <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY220	<i>MEP2::HA/MEP2::HA <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY221	<i>MEP2::HA/MEP2::HA Δnpr1::LEU2/Δnpr1::LEU2 <i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i></i>	this study
MLY225a/ α	<i>Δmep2::G418/Δmep2::G418 <i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i></i>	this study
MLY229a/ α	<i>Δpde2::G418/Δpde2::G418 Δmep2::LEU2/Δmep2::LEU2 Δleu2::hisG/Δleu2::hisG <i>ura3-52/ura3-52 MATa/α</i></i>	this study
MLY230a/ α	<i>Δmep1::G418/Δmep1::G418 <i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i></i>	this study
MLY231a/ α	<i>Δmep1::G418/Δmep1::G418 Δmep2::G418/Δmep2::G418 <i>ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG MATa/α</i></i>	this study

glutamine synthetase (GLN1) or glutamate dehydrogenase (GDH1 and GDH2) activity were performed on extracts from wild-type or Δ mep2/ Δ mep2 strains inoculated into liquid minimal media (0.17% yeast nitrogen base, 2% glucose) with increasing concentrations of ammonium (from 50 μ M to 50 mM) and SD-Ura and grown for 4 h at 30°C. Assays were performed as described (Doherty, 1970; Mitchell and Magasanik, 1983).

FG(TyA)::lacZ reporter gene assays

These assays were performed in strains MLY97 (wild-type), MLY225a/ α (Δ mep2/ Δ mep2), MLY230a/ α (Δ mep1/ Δ mep1) and MLY231a/ α (Δ mep1/ Δ mep1 Δ mep2/ Δ mep2) expressing the FG(TyA)::lacZ reporter gene from plasmid pIL30-LEU2. Assays were performed as described (Lorenz and Heitman, 1997).

Epitope tagging and Western blotting

MEP2 was epitope tagged with the hemagglutinin (HA) epitope using the integrative method of Schneider *et al.* (1995). Three direct repeats of the nine amino acid HA epitope were fused in-frame to the MEP2 C-terminus. This fusion gene (*MEP2::HA*) is under the control of the endogenous *MEP2* promoter.

Cultures of homozygous diploid *MEP2::HA* strains MLY220 (wild-type), MLY221 (Δ npr1/ Δ npr1), MLY194a/ α (Δ gln3/ Δ gln3) and MLY198a/ α (Δ ure2/ Δ ure2) were grown overnight in YNB, washed once with water and diluted into minimal media containing ammonium sulfate (50 μ M to 50 mM; Figure 7A) for 4 h at 30°C. Extracts were prepared by glass bead agitation in lysis buffer (50 mM Tris-Cl, pH 8, 1 mM DTT, 1 mM EDTA, 100 U/ml aprotinin, 0.5 mM PMSF, 1 μ g/ml TPCK, 1 μ g/ml pepstatin, 1 μ g/ml benzamide). Protein concentrations were determined through a modified Bradford assay (Bio-Rad). Equal amounts of protein were boiled in sample buffer (250 mM Tris-Cl, pH 6.8, 25%

Table III. Plasmid list

Plasmid	Construct	Reference
YEplac195	<i>URA3-2μ</i>	Gietz and Sugino (1988)
pRS316	<i>URA3-CEN</i>	Sikorski and Hieter (1989)
pSEY68	pGal1,10 <i>CEN URA3</i>	S.Elledge
pML95	<i>NPI1/RSP5</i> in pRS316	this study
pML100	<i>MEP1</i> in YEplac195	this study
pML104	<i>Δmep1::LEU2</i> in pUC18	this study
pML111	<i>Δmep2::LEU2</i> in pUC18	this study
pML113	<i>MEP3</i> in YEplac195	this study
pML151	<i>MEP2</i> in YEplac195	this study
pML154	<i>NPI1-URA3</i> in YIp211	this study
pML160	pGal- <i>GPA2-2</i> (Gly132Val)	Lorenz and Heitman (1997)
pML180	pGal- <i>GPA2</i>	Lorenz and Heitman (1997)
p2.5-2	<i>TEC1 URA3 2μ</i>	M.Lorenz, unpublished
pSL1509	<i>STE11-4 URA3 CEN</i>	Stevenson <i>et al.</i> (1992)
pNC252	pGal- <i>STE12 URA3 CEN</i>	Liu <i>et al.</i> (1993)
pMW1	<i>RAS2^{wt} URA3 CEN</i>	M.Ward, unpublished
pMW2	<i>RAS2^{Val19} URA3 CEN</i>	M.Ward, unpublished
pCG38	<i>PHD1 URA3 2μ</i>	Gimeno and Fink (1994)
pIL30-LEU2	FG(TyA)::lacZ <i>LEU2</i>	Laloux <i>et al.</i> (1994)

glycerol, 10% β -mercaptoethanol, 5% SDS, 0.01% Bromophenol Blue) and separated by SDS-PAGE.

The MEP2::HA protein was detected by Western blot using an α -HA monoclonal antibody (Berkeley), a horseradish peroxidase conjugated α -mouse secondary antibody and the ECL system (Amersham). Cyclo-

Table IV. Amino acid residues comprising junction points

Plasmid	Construct	Junction point amino acids	
		Last MEP2	First MEP1
pML100	full-length MEP1	NA	NA
pML151	full-length MEP2	NA	NA
pML152	C-terminus truncation	449	NA
pML155	N-terminal	58	46
pML156	1st loop	146	134
pML157	2nd loop	211	199
pML158	3rd loop	285	268
Plasmid	1st loop swap	MEP2 amino acids	MEP1 amino acids
pML161	mostly MEP1	59–146	1–46, 134–492
pML162	mostly MEP2	1–58, 146–499	46–133

philin A (CPR1) was used as a loading control and was detected with a polyclonal α -CPR1 antibody (Cardenas *et al.*, 1995) and an α -rabbit secondary antibody.

$\Delta npr1$ suppressor analysis

$\Delta npr1$ strains grow poorly on SLAD (likely due to inhibition of MEP activity) and on TC media (tryptophan–citrulline, due to inhibition of GAP1; Grenson and Acheroy, 1982). To identify suppressors of the growth defect, 10^5 cells of strains MLY54a or MLY54 α were plated to either SLAD + uracil or TC + uracil media and UV irradiated at 7500 $\mu\text{J}/\text{m}^2$ to 60% survival. Thirty six mutants in six recessive and one dominant allele groups were identified. The only mutant in this collection which did not suppress the $\Delta npr1$ mutant phenotype on all nitrogen sources tested also showed the behavior expected of an *npi1/rsp5* mutant; that is, derepression of GAP1 activity as assayed by the GAP1-mediated uptake of toxic levels of L-lysine and D-histidine on derepressing (ammonium) media (Grenson, 1983b). A low copy vector (pML95) containing the *NPII* gene generated by PCR complemented this phenotype. We also cloned *NPII* in the yeast integrating vector YIp211, and used a linearized form to transform the *npi1* mutant strain MLY141a. Transformants displayed *NPII*⁺ phenotype in 15/16 Ura⁺ strains. The *npi1* mutant phenotype did not reappear in 29 complete tetrads of a cross between the tagged *NPII-URA3* strain and a wild-type strain.

Acknowledgements

We thank G.Fink, M.Ward and S.Garrett for generously providing strains and plasmids, J.Nevins, R.Wharton and M.Cardenas for comments on the manuscript, and S.Garrett, D.Lew and the members of the Heitman lab for helpful discussions. J.H. is an assistant investigator of the Howard Hughes Medical Institute.

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Received October 6, 1997; revised December 22, 1997;
accepted December 29, 1997