Regulators of Pseudohyphal Differentiation in *Saccharomyces cerevisiae* Identified Through Multicopy Suppressor Analysis in Ammonium Permease Mutant Strains

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ABSTRACT

Nitrogen-starved diploid cells of the yeast *Saccharomyces cerevisiae* differentiate into a filamentous, pseudohyphal growth form. Recognition of nitrogen starvation is mediated, at least in part, by the ammonium permease Mep2p and the Gα subunit Gpa2p. Genetic activation of the pheromone-responsive MAP kinase cascade, which is also required for filamentous growth, only weakly suppresses the filamentation defect of Δ*mep2*/Δ*mep2* and Δ*gpa2*/Δ*gpa2* strain. Surprisingly, deletion of Mep1p, an ammonium permease not previously thought to regulate differentiation, significantly enhances the potency of MAP kinase activation, such that the *STE11-4* allele induces filamentation to near wild-type levels in Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* and Δ*mep1*/Δ*mep1* Δ*gpa2*/Δ*gpa2* strains. To identify additional regulatory components, we isolated high-copy suppressors of the filamentation defect of the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* mutant. Multicopy expression of *TEC1*, *PHD1*, *PHD2* (*MSS10*/*MSN1*/*FUP4*), *MSN5*, *CDC6*, *MSS11*, *MGA1*, *SKN7*, *DOT6*, *HMS1*, *HMS2*, or *MEP2* each restored filamentation in a Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* strain. Overexpression of *SRK1* (*SSD1*), *URE2*, *DAL80*, *MEP1*, or *MEP3* suppressed only the growth defect of the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* mutant strain. Characterization of these genes through deletion analysis and epistasis underscores the complexity of this developmental pathway and suggests that stress conditions other than nitrogen deprivation may also promote filamentous growth.

WHEN diploid cells of the budding yeast *Saccharomyces cerevisiae* are deprived of nitrogen, they differentiate into a filamentous, pseudohyphal form. Pseudohyphal cells are characterized by an elongated morphology, altered budding pattern and cell cycle, and the ability to invade the growth substrate (Gimeno *et al.* 1992; Kron *et al.* 1994). Pseudohyphal differentiation shares both regulatory and morphological similarities with filamentous growth forms of other fungi, including the maize fungus *Ustilago maydis* and the animal pathogen *Candida albicans.* The filamentous forms of these organisms have been associated with tissue invasion and dissemination, and they are critical for virulence (Banuett 1991; Lo *et al.* 1997). Similarly, pseudohyphal differentiation may serve to disperse yeast cells under adverse conditions, particularly nutrient stresses.

The regulation of pseudohyphal differentiation involves at least two signaling pathways. One includes Gpa2p, the α subunit of a heterotrimeric guanine nucleotide-binding protein (Kübler *et al.* 1997; Lorenz and Heitman 1997). Genetic evidence indicates that Gpa2p regulates cAMP metabolism coordinately with Ras2p: the pseudohyphal defect of Δ*gpa2*/Δ*gpa2* strains and the synthetic growth defect of Δ*gpa2* Δ*ras2* strains are both suppressed by increasing cAMP concentrations (Kübler *et al.* 1997; Lorenz and Heitman 1997; Xue *et al.* 1998). In addition, either addition of exogenous cAMP or expression of a dominant-active *GPA2* allele induces filamentous growth in nitrogen-rich conditions that normally repress this differentiation pathway (Lorenz and Heitman 1997). Activated *RAS2* alleles, elevated cAMP levels, or expression of a dominant-active *GPA2* allele confers sensitivity to heat shock and alters glycogen accumulation (Toda *et al.* 1985; Xue *et al.* 1998). Genetic evidence suggests that some of these phenotypes are mediated by Sch9p, a protein kinase related to protein kinase A (PKA; Xue *et al.* 1998). Overexpression of *SCH9* can suppress the lethality of deletions of both *RAS* genes or of the sole adenylyl cyclase gene *CYR1* (Toda *et al.* 1988), and Sch9p has been proposed to act in a pathway that is parallel to PKA. The role of Sch9p in filamentous growth is presently unknown.

A second signaling pathway regulating pseudohyphal differentiation includes elements of the haploid pheromone-responsive MAP kinase cascade, including the protein kinases Ste20p, Ste11p, Ste7p, and Kss1p and the transcriptional activator Ste12p (Liu *et al.* 1993; Cook *et al.* 1997; Madhani *et al.* 1997). Addition of exogenous cAMP or expression of the dominant *GPA2-2* allele suppresses the pseudohyphal defect of strains lacking MAP kinase elements (Lorenz and Heitman 1997). How this pathway is activated to effect filamentous

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growth is not understood, though the pheromone receptors and the Gpa1p/Ste4p/Ste18p heterotrimeric G protein are not involved (Liu *et al.* 1993). Ras2p likely plays a dual role in regulating both the MAP kinase pathway and cAMP metabolism (Gimeno *et al.* 1992; Mösch *et al.* 1996; Roberts *et al.* 1997).

A characteristic of nitrogen-starvation-induced filamentation is the ability of cells to invade the agar substrate. A similar phenomenon has been described in haploid cells grown on rich medium (Roberts and Fink 1994). Haploid-invasive growth is not a response to nitrogen deprivation, though it shares many similarities with pseudohyphal differentiation, including changes in cell polarity and filament formation (Roberts and Fink 1994). In addition, the same MAP kinase elements that regulate diploid filamentation also regulate haploid invasion (Roberts and Fink 1994; Cook *et al.* 1997; Madhani *et al.* 1997). The mechanism by which signal specificity between invasion and pheromone response is achieved in haploid cells is not fully understood, but it involves the Ste12p-binding proteins Dig1p and Dig2p (Cook *et al.* 1996) and specialization of the MAP kinases Fus3p and Kss1p for mating and filamentous/invasive growth (Cook *et al.* 1997; Madhani *et al.* 1997).

The high-affinity ammonium permease Mep2p is required for pseudohyphal differentiation in response to ammonium limitation and may serve as a sensor of nitrogen starvation (Lorenz and Heitman 1998). Δ*mep2*/Δ*mep2* mutant strains do not exhibit a filamentation defect in the presence of other nitrogen sources, such as glutamine or proline, indicating that the function of Mep2p is ligand specific. Mutations in the lower-affinity ammonium permeases Mep1p and Mep3p do not confer defects in pseudohyphal differentiation. Because Δ*mep2*/Δ*mep2* strains have no discernible alterations in ammonium uptake or growth rates, we have proposed that Mep2p has a signaling role to regulate filamentation (Lorenz and Heitman 1998). Activation of the Gpa2p/cAMP-signaling pathway restores pseudohyphal growth in Δ*mep2*/Δ*mep2* strains (Lorenz and Heitman 1998), suggesting that Mep2p may function upstream in this signaling cascade.

To address the mechanisms by which Mep2p senses ammonium, we further examined the role of the three ammonium permeases and uncovered a potential role for Mep1p in the regulation of pseudohyphal differentiation. Activation of the MAP kinase pathway is more effective in restoring filamentation to Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* and Δ*mep1*/Δ*mep1* Δ*gpa2*/Δ*gpa2* strains than in the corresponding *MEP1*$^+$ parent strains. Thus, the Mep1p ammonium permease inhibits the filamentous growth response under some conditions.

We took advantage of the unique phenotype of the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* double-mutant strain to isolate high copy suppressors of the filamentation defect. Overexpression of 17 genes suppressed either the growth or pseudohyphal defects of the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* strain on low-ammonium media. Among these are the known pseudohyphal regulators Phd1p, Phd2p (Mss10p, Msn1p, and Fup4p), and Tec1p (Gimeno and Fink 1994; Gavrias *et al.* 1996; Lambrechts *et al.* 1996), as well as proteins not previously known to affect filamentous growth, including Cdc6p, Mss11p, Mga1p, Skn7p, Msn5p, Dot6p, and two uncharacterized open reading frames that we have named *HMS1* and *HMS2* (for *h*igh-copy *M*EP suppressor). Several of these proteins have been previously implicated in the cellular response to changes in carbon source (Phd2p, Mss11p, and Mga1p) or oxidative damage (Skn7p), suggesting that nitrogen limitation may not be the only environmental signal that can trigger pseudohyphal differentiation. Epistasis analysis between these suppressors and previously characterized pseudohyphal regulators illustrates the complexity of filamentous growth and suggests that the cell integrates multiple signals via several pathways to control differentiation.

MATERIALS AND METHODS

Yeast strains and genetic methods: Standard yeast media and genetic methods were used (Sherman 1991). SLAD media, for induction of filamentous growth, contains 0.17% yeast nitrogen base, 50 μm $(NH_4)_2SO_4$, 2% Bacto-agar, and 2% glucose (Gimeno *et al.* 1992; Lorenz and Heitman 1997). SLADG, to induce the pGAL-*STE12* allele, contains 2% galactose and 0.13% glucose (Liu *et al.* 1993). SLARG, to induce the pGAL-*GPA2* alleles, contains 2% raffinose and 0.5% galactose (Lorenz and Heitman 1997). Epistasis experiments with Δ*mep2* and Δ*mep1* Δ*mep2* strains (Figure 1) were performed on media containing 100 μm ammonium sulfate and 3% Noble agar, as Bacto-agar contains low concentrations of nonammonium nitrogen sources that can complicate analysis of Mep2p function.

Yeast strains are listed in Table 1 and plasmids in Table 2. Strains deleted for each of the multicopy suppressors were constructed in strains MLY40 and MLY41 by replacing the coding sequences with a gene encoding G418 resistance using the PCR-mediated disruption technique of Wach *et al.* (1994). MLY40 and MLY41 were derived from strains Σ1278b and 3962c (Bechet *et al.* 1970), respectively. Candidate disruptants in MLY40 and MLY41 were confirmed by PCR and crossed to construct the homozygous diploid strains.

Multicopy suppressor screen: The Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* strain (MLY115a/α) was transformed with a library made from strain CAY1 in the 2μ-*URA3* vector pRS426 (provided by C. Alarcon). Transformants were selected on SD-Ura medium, pooled, diluted, and replated to SLAD at a density of 1000 cells per plate. Plates were screened microscopically after 4–6 days at 30° for filamentous colonies. Plasmid loss and rescue/retransformation tests demonstrated that the filamentous phenotype was plasmid dependent. Twenty-seven unique plasmids were characterized via sequencing and restriction mapping to identify the genomic region carried by the plasmid. Where necessary, plasmids were subcloned to identify the specific open reading frame responsible for the phenotype.

Phenotypic analysis of suppressor deletion strains: Strains deleted for each of the suppressor genes were analyzed for their effect on filamentous growth. We assayed the morpholog-

TABLE 1

Yeast strains

Strain	Genotype	Reference
MLY40	*ura3-52 MAT*α	Lorenz and Heitman (1997)
MLY41	*ura3-52 MAT***a**	Lorenz and Heitman (1997)
MLY42	*ura3-52* Δ*leu2::hisG MAT*α	Lorenz and Heitman (1997)
MLY43	*ura3-52* Δ*leu2::hisG MAT***a**	Lorenz and Heitman (1997)
MLY61	*ura3-52/ura3-52 MAT***a**/α	Lorenz and Heitman (1997)
MLY104a/α	Δ*mep1::LEU2*/Δ*mep1::LEU2 ura3-52/ura3-52* Δ*leu2::hisG*/Δ*leu2::hisG MAT***a**/α	Lorenz and Heitman (1997)
MLY108a/α	Δ*mep2::LEU2*/Δ*mep2::LEU2 ura3-52/ura3/52* Δ*leu2::hisG*/Δ*leu2::hisG MAT***a**/α	Lorenz and Heitman (1997)
MLY115a/α	Δ*mep1::LEU2*/Δ*mep1::LEU2* Δ*mep2::LEU2*/Δ*mep2::LEU2 ura3-52/ura3-52* Δ*leu2::hisG*/Δ*leu2::hisG MAT***a**/α	Lorenz and Heitman (1997)
MLY132a/α	Δ*gpa2::G418*/Δ*gpa2::G418 ura3-52/ura3-52 MAT***a**/α	Lorenz and Heitman (1997)
MLY135a/α	Δ*gpa2::G418*/Δ*gpa2::G418* Δ*mep1::LEU2*/Δ*mep1::LEU2* Δ*leu2::hisG*/Δ*leu2::hisG ura3-52/ura3-52* MAT**a**/α	This study
MLY140a/α	Δ*ure2::G418*/Δ*ure2::G418 ura3-52/ura3-52 MAT***a**/α	Lorenz and Heitman (1997)
MLY168a/α	Δ*hms1::G418*/Δ*hms1::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY169a/α	Δ*hms2::G418*/Δ*hms2::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY170a/α	Δ*msn5::G418*/Δ*msn5::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY172a/α	Δ*skn7::G418*/Δ*skn7::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY179a/α	Δ*mga1::G418*/Δ*mga1::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY180a/α	Δ*phd2::G418*/Δ*phd2::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY181a/α	Δ*mss11::G418*/Δ*mss11::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY182a/α	Δ*phd1::G418*/Δ*phd1::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY183a/α	Δ*tec1::G418*/Δ*tec1::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY191a/α	Δ*dot6::G418*/Δ*dot6::G418 ura3-52/ura3-52 MAT***a**/α	This study
MLY224a/α	Δ*dal80::G418*/Δ*dal80::G418 ura3-52/ura3-52 MAT***a**/α	This study

ical response (see Figure 3) and the three activities that comprise pseudohyphal differentiation, cell polarity, cell elongation, and invasive growth (see Table 7), as defined by Mösch and Fink (1997). Previous work had found that *CDC6* is essential (Hartwell 1976; Zhou *et al.* 1989), and it was not analyzed in these experiments. In addition, *SRK1* is polymorphic in various yeast strains, has pleiotropic effects, and, as such, was excluded from further analysis.

Invasive growth assays: Nitrogen-starvation-induced invasive growth was assayed as described by Mösch and Fink (1997) by streaking diploid strains to SLAD medium and incubating at 30° for 3 days. The colony positions were marked, and the surface cells were washed off the plate. Invasive colonies were identified by microscopic analysis. Invasive growth was quantified using the system of Mösch and Fink (1997): ++++, >90% of colonies invaded; +++, 70–90%; ++, 30–70%; +, 5–30%; −, <5%. At least 50 colonies were assayed for each strain.

Haploid-invasive growth was assayed as described (Roberts and Fink 1994; Cook *et al.* 1997). Haploid strains (*MAT***a**) were grown on YPD medium at 30° for 3 days. Surface cells were washed off, and the plate was incubated for 30 hr at 30° to better visualize invaded cells.

Cell morphology assays: Cell elongation was assayed as described by Mösch and Fink (1997). Diploid strains were grown on SLAD medium for 3 days at 30°. Surface cells were washed off the plate as in the invasion assay, and cells that had invaded the substrate were scraped out of the agar and analyzed microscopically for the proportion of elongated pseudohyphal cells *vs.* ovate yeast form cells. At least 200 cells were counted for each strain.

Bud site determination assays: Cell polarity and budding pattern were analyzed as described by Mösch and Fink (1997). Diploid strains were grown in YPD medium to an OD_{600} ~0.4–0.6 at 30° and then fixed for 2 hr in 3.7% formaldehyde at room temperature. Cultures were washed three times in water

TABLE 2

Plasmids

Construct	Description	Reference
pRS426	*URA3* 2μ	Christianson *et al.* (1992)
YEplac195	*URA3* 2μ	Gietz and Sugino (1988)
pCG38	*PHD1 URA3* 2μ	Gimeno and Fink (1994)
pNC252	p*GAL::STE12 URA3* CEN	Liu *et al.* (1993)
pSL1509	*STE11-4 URA3* CEN	Stevenson *et al.* (1992)
pML160	p*GAL::GPA2-2 URA3* CEN	Lorenz and Heitman (1997)
pML180	p*GAL::GPA2 URA3* CEN	Lorenz and Heitman (1997)
p2.5-2	*TEC1 URA3* 2μ	This study

and then incubated in 1 mg/ml Calcofluor (Fluorescent Brightener 28; Sigma, St. Louis) for 10 min in the dark. Cells were washed twice in water and spotted to polylysine-coated microscope slides. Fields of cells were photographed at ×100, and these photos were used to identify cells with two or more bud scars as being bipolar (scars on both ends), unipolar/axial (scars on only one end), or random (scars not localized to the ends). At least 100 cells were counted for each strain.

Photomicroscopy: Whole-colony photographs were taken at ×25 using a 35-mm camera connected to the trinocular objective of a Nikon Axiophot-2 microscope. To control the density-dependent variability of filamentous growth, strains were streaked to plates at six sectors per plate. We endeavored to photograph colonies whose phenotype was representative of the strain being assayed.

RESULTS

A role for Mep1p as an inhibitor of pseudohyphal differentiation: Our previous studies revealed that the ammonium permease Mep2p and the Gα protein Gpa2p are required for pseudohyphal growth (Lorenz and Heitman 1997, 1998). In previous epistasis experiments, the filamentation defect of Δ*mep2*/Δ*mep2* and Δ*gpa2*/Δ*gpa2* mutant strains could be readily suppressed by dominant-active *GPA2* or *RAS2* mutants and by cAMP, but not by activation of the MAP kinase cascade via the dominant *STE11-4* allele. Because of the complexity of the signaling pathways regulating pseudohyphal differentiation, as well as the presence of both multiple branchpoints and parallel signaling pathways, we have further analyzed the epistatic relationships between the Mep2p and Mep1p permeases, Gpa2p, and the MAP kinase cascade. Two important observations emerge from this analysis.

First, as shown in Figure 1, modest activation of the MAP kinase cascade by the dominant-active *STE11-4* allele does not suppress the filamentation defect of either Δ*mep2*/Δ*mep2* or Δ*gpa2*/Δ*gpa2* mutant strains. We now find that more marked activation of the MAP kinase cascade by overexpression of *STE12* can suppress the pseudohyphal differentiation defects of Δ*mep2*/Δ*mep2* and Δ*gpa2*/Δ*gpa2* mutant strains. Expression of the *STE11-4* allele does restore filamentation in mutant strains lacking the upstream MAP kinase component Ste20p (Liu *et al.* 1993); thus, this allele can suppress a mutation in the same pathway, but it cannot suppress mutations in genes in the parallel Mep2p/Gpa2/cAMP pathway. Instead, the relatively stronger phenotype of *STE12* overexpression is required to cross-suppress Δ*mep2*/Δ*mep2* or Δ*gpa2*/Δ*gpa2* mutations.

More importantly, this epistasis analysis indicated that the Mep1p ammonium permease can, under some conditions, function to inhibit pseudohyphal differentiation. For example, whereas expression of *STE11-4* fails

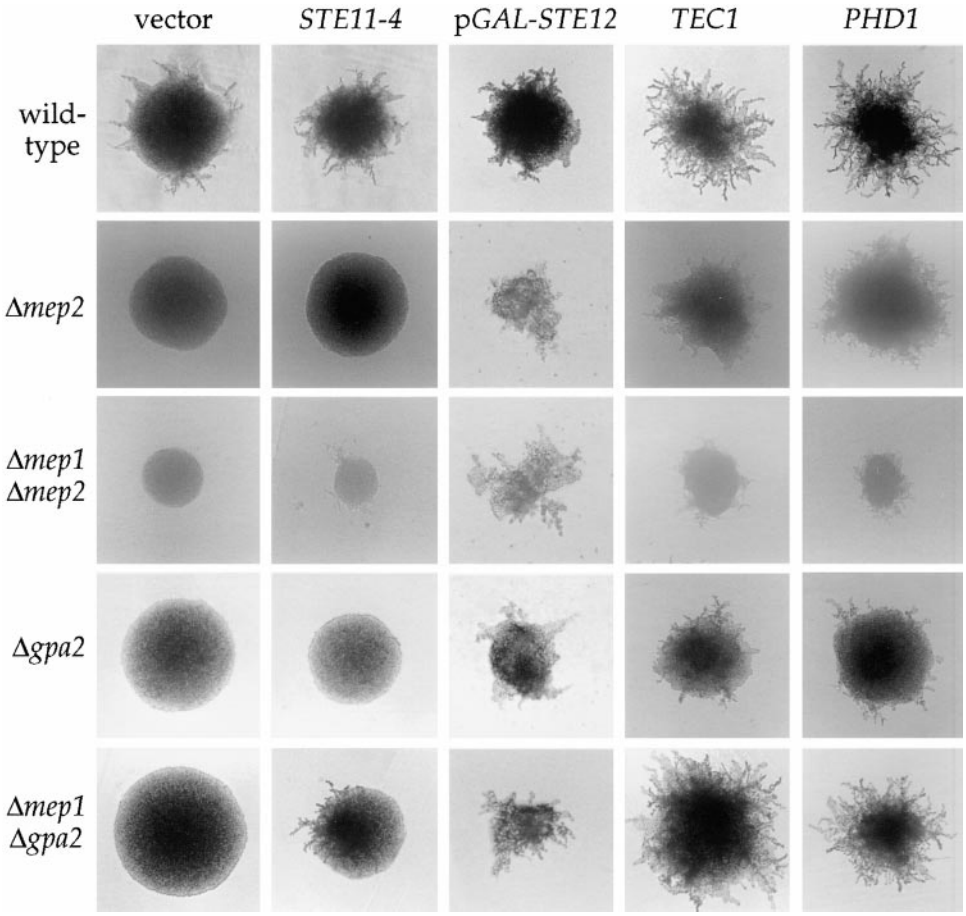

Figure 1.—Δ*mep1* mutations alter epistasis of Δ*mep2* or Δ*gpa2* mutations with the MAP kinase cascade. Homozygous diploids of the indicated genotype (wild type, MLY61; Δ*gpa2*/Δ*gpa2*, MLY132a/α; Δ*mep1*/Δ*mep1* Δ*gpa2*/Δ*gpa2*, MLY135a/α) expressing the indicated alleles (vector, YEplac195; *STE11-4*, pSL1509; *TEC1*, p2.5-2; *PHD1*, pCG38) were incubated on SLAD medium for 4 days at 30°. Strains expressing the pGAL-*STE12* (pNC252) allele were incubated on inducing SLADG medium for 6 days at 30°. Δ*mep2*/Δ*mep2* (MLY108a/α) and Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* (MLY115a/α) strains were incubated on media containing 3% Noble agar instead of Bacto-agar (see materials and methods) for 4 days (6 days for the pGal-*STE12* allele) at 30°.

to suppress the filamentation defect of either Δ*mep2*/Δ*mep2* or Δ*gpa2*/Δ*gpa2* strains, *STE11-4* does suppress Δ*mep2*/Δ*mep2* Δ*mep1*/Δ*mep1* and Δ*gpa2*/Δ*gpa2* Δ*mep1*/Δ*mep1* double-mutant strains (Figure 1). Deletion of the *MEP1* gene in an otherwise wild-type background does not reduce or enhance filamentous growth, even in response to a range of ion concentrations from 5 μm to 5 mm (Lorenz and Heitman 1998; data not shown); thus, the Mep1p ammonium permease inhibits pseudohyphal differentiation only under certain conditions.

One explanation for the difference in epistasis between Δ*mep1* Δ*mep2* and *MEP1* Δ*mep2* strains is that the growth defect of the Δ*mep1* Δ*mep2* strain, resulting from impaired ammonium uptake, predisposes the cell to nitrogen-dependent differentiation. Two lines of evidence indicate that this is not the case. First, lowering the ammonium concentration in the medium to a level below that in standard pseudohyphal media (5 μm in this experiment *vs.* 50 μm standard) does not alter the epistasis behavior of the Δ*mep2*/Δ*mep2* strain. Second, the Δ*gpa2*/Δ*gpa2* Δ*mep1*/Δ*mep1* mutant does not have a growth defect, indicating that ammonium import is not the basis of these differences (data not shown).

In the epistasis experiment shown in Figure 1, we also expressed the transcription factors Phd1p and Tec1p, which strongly enhance pseudohyphal growth when expressed from a multicopy vector (Gimeno and Fink 1994; Gavrias *et al.* 1996). As with *STE11-4* or *STE12* overexpression, induction of filamentation by high-copy expression of *PHD1* or *TEC1* was more dramatic in the Δ*mep1* strains relative to the *MEP1*$^+$ strains. This provides further evidence for an inhibitory function of Mep1p in response to genetic alterations in the regulation of pseudohyphal differentiation.

Multicopy suppressor analysis of Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* mutants: To address the role of Mep1p and Mep2p in the regulation of pseudohyphal differentiation, we screened for genes that suppress the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* pseudohyphal defect when present in high copy number. The Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* strain (MLY115a/α) was transformed with a yeast genomic DNA library in the high-copy (2μ) *URA3* vector pRS426. Transformants were selected on synthetic medium lacking uracil, pooled, diluted, and replated at ~1000 cells per plate on SLAD media. Colonies were screened after 4–6 days for filament formation or for significantly enhanced growth under these conditions. After plasmid loss and plasmid rescue/retransformation controls, 27 unique plasmids (with distinct restriction patterns) representing 17 genomic loci, which suppressed the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* phenotype were identified.

TABLE 3

Identities of the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* suppressors

ORF	Gene	Suppression of Δ*mep1* Δ*mep2*	Comments	Reference
YNL142w[a]	*MEP2*	+	High-affinity NH_4^+ permease	Marini *et al.* (1997)
YKL043w	*PHD1*	+	TF[b] promotes filamentation	Gimeno and Fink (1994)
YBR083w[a]	*TEC1*	±	TF, regulates FRE/Ty1	Gavrias *et al.* (1996)
YOL116w	*PHD2*[b]	±	TF, interacts with SNF1	Lambrechts *et al.* (1996)[c]
YMR164c	*MSS11*	±	Similar to SSN6 repressor	Webber *et al.* (1997)
YGR249w	*MGA1*	+	Heat shock TF homolog	Zhang *et al.* (1997)
YHR206w[a]	*SKN7*	±	Two-component TF homolog	Brown *et al.* (1993)
YDR335w	*MSN5*	±	*snf1* suppressor	Estruch and Carlson (1990)
YJL194w	*CDC6*	+	ORC component	Zhou *et al.* (1989)
YER088c	*DOT6*	±	myb-like TF	This study
YOR032c	*HMS1*	±	myc-like TF	This study
YJR147w	*HMS2*	±	Heat shock TF homolog	This study
Growth suppressors				
YGR121[a]	*MEP1*	±	Medium-affinity NH_4^+ permease	Marini *et al.* (1994)
YPR138c	*MEP3*	±	Low-affinity NH_4^+ permease	Marini *et al.* (1997)
YDR239c	*SRK1*	±	Pleiotropic suppressor	Wilson *et al.* (1991)
YKR034w	*DAL80*	±	Nitrogen regulatory TF	Cunningham and Cooper (1991)
YNL229c	*URE2*	±	Nitrogen regulatory protein	Coschigano and Magasanik (1991)

ORC, origin recognition complex; TF, transcription factor; FRE, filamentation response element.

[a] Genes isolated multiple times include *MEP2* (four times), *MEP1* (five times), *TEC1* (twice), and *SKN7* (three times).

[b] Also identified as *MSS10* (Lambrechts *et al.* 1996), *MSN1* (Estruch and Carlson 1990), and *FUP4* (Eide and Guarente 1992).

[c] See also Gimeno and Fink (1994).

The identity and phenotypes of these suppressors are shown in Table 3 and Figure 2. The *MEP2* gene itself was identified four times. In addition, we isolated both *MEP1* (five times) and *MEP3* (once); overexpression of either *MEP1* or *MEP3* mostly suppresses the growth defect of Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* strains, although a few filaments were present in each case. Several other primarily growth suppressors were identified, including *URE2*, *DAL80*, and *SRK1*. In the presence of favored nitrogen sources, such as ammonium or glutamine, Ure2p inhibits Gln3p, a GATA family transcription factor that regulates the expression of many genes necessary for the assimilation of alternative nitrogen sources (such as proline or urea). Ure2p has weak homology to glutathione *S*-transferases and is a prion analog, but its mechanism of action is not known (Coschigano and Magasanik 1991; Wickner 1994; Xu *et al.* 1995). Δ*ure2*/Δ*ure2* (and Δ*gln3*/Δ*gln3*) mutants also have a filamentation defect (Lorenz and Heitman 1998). Dal80p is a DNA-binding protein that negatively regulates many of the same pathways as Gln3p and Ure2p (Cunningham and Cooper 1991; Daugherty *et al.* 1993). Srk1p is a protein of unknown function that has pleiotropic effects on many cellular processes, including RNA polymerase function (Chiannilkulchai *et al.* 1992; Stettler *et al.* 1993), chromosome stability (Uesono *et al.* 1994), phosphatase function (Sutton *et al.* 1991; Wilson *et al.* 1991), and polarized cell growth and morphology (Costigan and Snyder 1994; Costigan *et al.* 1992; Kim *et al.* 1994).

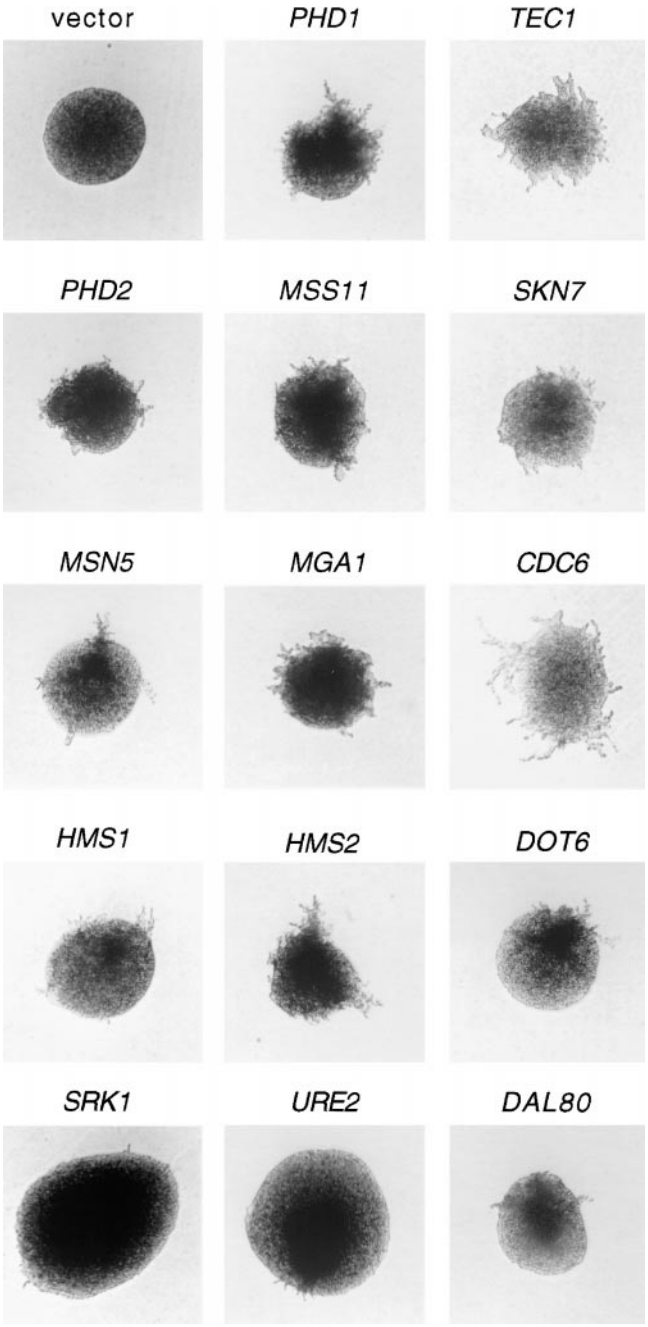

Figure 2.—Pseudohyphal phenotypes conferred by the Δ*mep1* Δ*mep2* multicopy suppressors. The Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* strain MLY115a/α expressing each of the genes listed from a multicopy plasmid were incubated on SLAD medium for 6 days at 30°.

The other class of genes identified in this screen did not significantly affect the growth rate of the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* parent strain, but did suppress the filamentation defects (Table 3). We identified *PHD1*, *PHD2* (also known as *MSS10*, *MSN1*, *FUP4*), and *TEC1*, each of which are predicted to be transcription factors and are known to regulate filamentous growth (Gimeno and Fink 1994; Gavrias *et al.* 1996; Lambrechts *et al.* 1996). In addition, several genes not previously implicated in pseudohyphal growth were identified. These include *MSS11*, whose product, like Mss10p/Phd2p, regulates the *STA1*, *STA2*, and *STA3* glucoamylase genes on the basis of the carbon source; overexpression of *MSS11* can rescue expression of *STA2* in Δ*mss10* strains (Webber *et al.* 1997). *MSS11* encodes a protein very similar to the Ssn6p transcriptional repressor. Ssn6p and Tup1p form a heterodimer that mediates glucose response in conjunction with Snf1p signaling (Schultz and Carlson 1987; Williams *et al.* 1991; Keleher *et al.* 1992). Tup1p has also been implicated in filamentous growth in both *S. cerevisiae* and *C. albicans* (Braun and Johnson 1997). *MGA1* encodes a heat shock transcription factor homolog of unknown function that was identified as a multicopy suppressor of a *snf2*/*gam1* mutation (Zhang *et al.* 1997). *MSN5* was isolated in the same *snf1* suppressor screen that identified *MSN1* (*PHD2*; Estruch and Carlson 1990); the function of Msn5p is not known.

Other genes identified include *SKN7*, which encodes a homolog of response regulator proteins of bacterial two-component systems that appears to mediate several cellular processes, including cell wall biosynthesis, cell cycle control, and response to oxidative stress (Moye-Rawley *et al.* 1989; Brown *et al.* 1993; Brown and Bussey 1993; Kuge and Jones 1994; Morgan *et al.* 1995). Overexpression of *CDC6*, which encodes an es-

sential component of the origin recognition complex, also suppresses the Δ*mep1* Δ*mep2* pseudohyphal defect. *DOT6* was recently identified as a mutation that affects telomeric silencing.

We also identified two genes that had only systematic ORF designations from the yeast genome project, which we have renamed *HMS*. Both are predicted to be DNA-binding proteins; Hms1p is in the myc family, while Hms2p is similar to heat shock trascription factors.

The rationale behind this screen was to identify genes that may mediate the signaling function of Mep2p, hopefully including direct effectors. We identified each of the *MEP* genes, as well as the known pseudohyphal regulators *PHD1* and *TEC1*, demonstrating that the screen worked successfully. However, almost all the genes we identified are likely to be general regulators of pseudohyphal differentiation, an idea confirmed by the experiments described below, and are not specific effectors of Mep2p function. The multiplicity of predicted DNA-binding proteins (11 in this screen) was quite surprising and reaffirms the complexity of this differentiation pathway.

Epistasis with other pseudohyphal-deficient mutants: We expressed each of the genes identified here in a wild-type strain (MLY61) to test if this would enhance filamentation (*e.g.*, overexpression of *PHD1*, *PHD2*, or *TEC1* is known to stimulate pseudohyphal differentiation in wild-type cells; Gavrias *et al.* 1996; Gimeno and Fink 1994). As shown in Figure 3 and Table 4, most of these genes conferred a hyperfilamentous phenotype, including *MGA1*, *CDC6*, *SKN7*, *MSN5*, and *HMS1*. Strains were classified as hyperfilamentous on the basis of two criteria: first, colony morphology (more vigorous filamentation than wild type on limiting ammonium SLAD medium; see Figure 3) and, second, induction of filamentation on higher ammonium media that normally repress differentiation (10-fold higher than in SLAD; see Table 4). None of these inducers, however, are as potent as the $GPA2^{Val132}$ allele or cAMP, both of which allow filamentous growth, even on nitrogen-rich media with 5 mm ammonium (Lorenz and Heitman 1997). Filamentation was also assayed on medium containing 100 μm glutamine (Mgln; Table 4). The pseudohyphal phenotypes are similar between cells grown in the presence of ammonium or glutamine, indicating that these suppressors are not specific effectors of Mep2p action (which might be ammonium specific), but instead are general regulators of dimorphism. As expected, the growth suppressors (*MEP1*, *MEP3*, *URE2*, *DAL80*, and *SRK1*) had no affect on filamentation when expressed in wild-type strains.

We next expressed the suppressor genes in strains lacking *MEP2*, *GPA2*, or *STE11* to examine the epistasis relationships between these mutations and suppressor function (Table 5). As with the MAP kinase epistasis experiment (Figure 1), filamentous growth induced by most of these suppressors was more vigorous in the

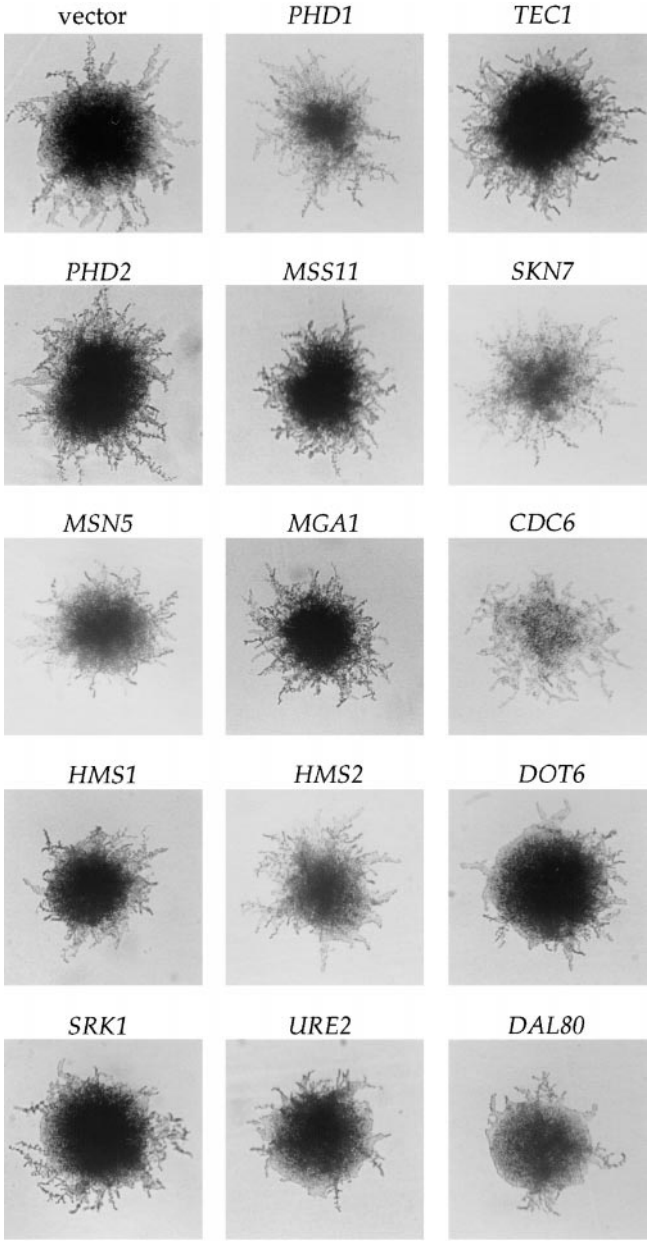

Figure 3.—Pseudohyphal phenotypes of the suppressors in wild-type strains. Wild-type strains (MLY61) expressing each of the multicopy suppressors indicated were incubated on SLAD medium for 4 days at 30°.

Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* double-mutant strain than in the Δ*mep2*/Δ*mep2* single mutant (Table 5). Similarly, the phenotype conferred by expression of these suppressor genes was weaker in the Δ*gpa2*/Δ*gpa2* single-mutant strain than in the Δ*gpa2*/Δ*gpa2* Δ*mep1*/Δ*mep1* strain (Table 5). Thus, these findings again indicate that Mep1p functions to inhibit filamentous growth. In contrast, all these genes, except for *MGA1*, suppressed the filamentation defect conferred by the Δ*ste11* mutation, and most did this relatively well (Table 5). The finding that multicopy expression of *TEC1* suppresses the defect of a Δ*ste* strain (Δ*ste11*/Δ*ste11* in this case)

TABLE 4

Nitrogen regulation of filamentous growth induced by expression of 2μ suppressors

	Pseudohyphal formation			
	$(NH_4)_2SO_4$			100 μm
Gene	50 μm	500 μm	5 mm	Gln
None	+	±	−	+
MEP2	+	±	−	+
PHD1	++	+	−	++
TEC1	++	±	−	++
PHD2	++	±	−	++
MSS11	++	±	−	++
MGA1	++	++	−	++
SKN7	++	±	−	++
MSN5	++	±	−	+
CDC6	++	+	−	++
HMS1	++	+	−	++
HMS2	+	±	−	+
DOT6	+	±	−	+

Wild-type diploid (MLY61) strains expressing each of the genes listed above were incubated on the indicated media for 2 days [500 μm and 5 mm $(NH_4)_2SO_4$] or 4 days [50 μm $(NH_4)_2SO_4$ and 100 μm glutamine] at 30°.

is in contrast to an earlier report in which *TEC1* overexpression did not restore filamentation in a Δ*ste12*/Δ*ste12* strain (Gavrias *et al.* 1996). The presence of Ste12p, even in its basal, unactivated state (resulting from the MAP kinase pathway mutation), may be required for suppression of Δ*ste11* by *TEC1*, as Ste12p and Tec1p form a heterodimer thought to be critical for the filamentation response (Gavrias *et al.* 1996; Madhani and Fink 1997). In summary, most of the genes identified as suppressors of the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* pseudohyphal growth defect appear not to be specific effectors of Mep2p action, but rather, general downstream elements that likely regulate transcriptional responses necessary for pseudohyphal differentiation.

Deletion analysis of the multicopy suppressors: We constructed an isogenic series of strains lacking each gene through a PCR-mediated disruption protocol (Wach *et al.* 1994). Homozygous diploids were analyzed for pseudohyphal phenotypes, as shown in Figure 4 and Table 6. Several of these mutations were known to confer pseudohyphal defects, including Δ*tec1* (Gavrias *et al.* 1996), Δ*phd2* (Lambrechts *et al.* 1996), and Δ*ure2* (Lorenz and Heitman 1998). In addition to these genes, Δ*mss11* and Δ*mga1* mutations confer strong pseudohyphal defects; Δ*msn5*/Δ*msn5* mutant strains have a moderate defect in filamentous growth (Figure 4; Table 6). Strains lacking *PHD1*, *SKN7*, *DAL80*, *DOT6*, *HMS1*, or *HMS2* do not have significant filamentation defects (Figure 4, Table 6; the Δ*phd1* phenotype has been reported previously; Gimeno and Fink 1994).

We next tested if alleles known to stimulate filamentation, such as $GPA2^{Val132}$ (Lorenz and Heitman 1997), $RAS2^{Val19}$ (Gimeno *et al.* 1992), *STE11-4* (Liu *et al.* 1993), or overexpression of *PHD1* (Gimeno and Fink 1994) or *TEC1* (Gavrias *et al.* 1996), would continue

TABLE 5

Phenotypes of multicopy suppressors in pseudohyphal-deficient strains

	Pseudohyphal phenotype when expressed in:					
Gene	Δ*mep1* Δ*mep2*	wild-type	Δ*mep2*	Δ*gpa2*	Δ*gpa2* Δ*mep1*	Δ*ste11*
None	−	+	−	±	±	−
MEP2	+	+	+	±	±	−
PHD1	±	++	±	±	+	+
TEC1	+	++	±	+	+	±
PHD2	±	++	+	±	+	+
MSS11	±	++	±	±	+	±
MGA1	+	++	++	±	++	±
SKN7	±	++	±	±	+	+
MSN5	±	++	±	±	±	±
CDC6	+	++	±	+	+	+
HMS1	+	++	±	±	+	+
HMS2	±	++	±	±	++	++
DOT6	±	+	±	±	±	±
MEP1	±	+	−	±	±	−
MEP3	±	+	−	±	±	−
URE2	±	+	−	±	±	±
DAL80	±	+	±	±	±	±
SRK1	±	+	−	±	±	±

Library plasmids with each of the above genes were used to transform wild-type (MLY61), Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* (MLY115a/α), Δ*mep2*/Δ*mep2* (MLY108a/α), Δ*gpa2*/Δ*gpa2* (MLY132a/α), Δ*gpa2*/Δ*gpa2* Δ*mep1*/Δ*mep1* (MLY135a/α), or Δ*ste11*/Δ*ste11* (HLY351) diploid strains, and were incubated for 4 days at 30°.

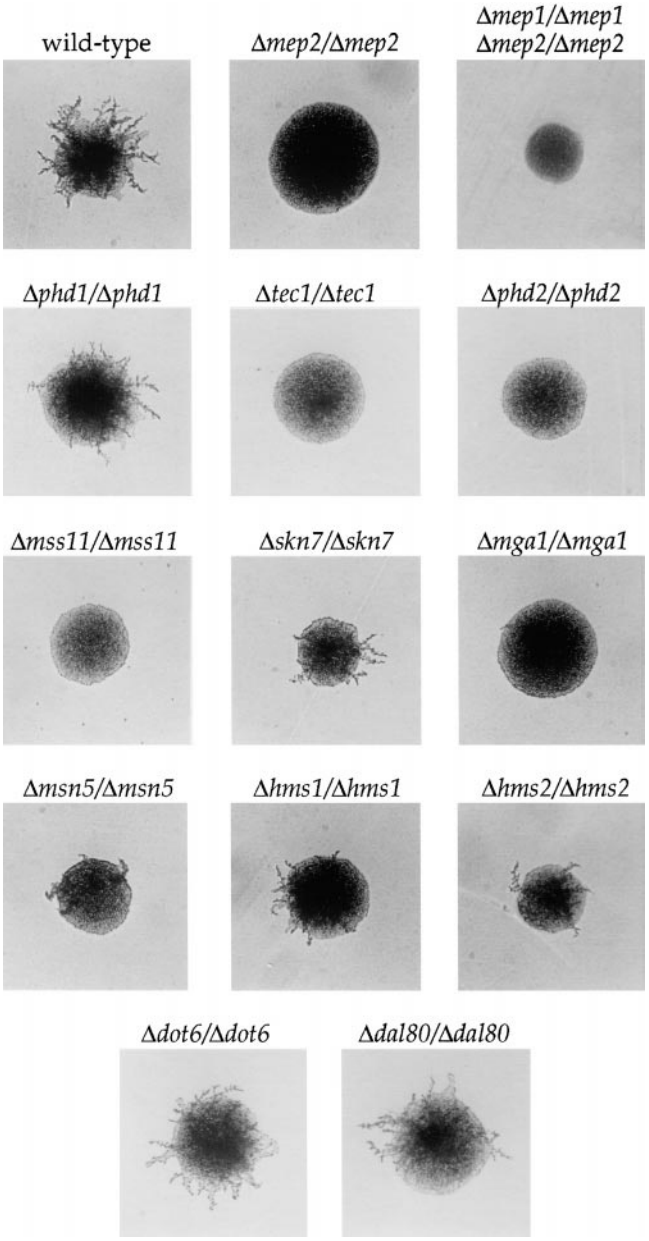

Figure 4.—Pseudohyphal phenotypes of the suppressor deletion strains. An isogenic strain series lacking each of the multicopy suppressor genes was constructed as detailed in materials and methods (see also Table 7). These strains were incubated on SLAD medium for 4 days at 30°.

to do so in the context of these deletion strains. The dominant active *GPA2*^{Val132} allele suppressed each of these deletions, with the exception of Δ*tec1*/Δ*tec1* strains (Table 6). This finding was unexpected, as *GPA2*^{Val132} suppresses Δ*ste* mutations. Because Tec1p is thought to act as a dimer with Ste12p (Madhani and Fink 1997), we anticipated that the phenotype of the activated *GPA2* allele would be similar in both Δ*ste12*/Δ*ste12* and Δ*tec1*/Δ*tec1* strains. In combination with the finding presented above that *TEC1* overexpression suppresses the filamentous growth defect of Δ*ste11*/Δ*ste11* (Table 4) but not Δ*ste12*/Δ*ste12* mutant strains (Gavrias *et al.* 1996), these results indicate that Tec1p may act at multiple steps or that it may have a function that is not shared with Ste12p in the regulation of pseudohyphal differentiation.

The epistasis data summarized in Table 6 presents a complicated picture. Each of the mutants tested had a pseudohyphal defect, indicating that they are all required for full activation of filamentous growth. With the exception of Δ*tec1*/Δ*tec1*, all these mutations were suppressed by at least one of the pseudohyphal stimulatory alleles (or by galactose as a carbon source); thus, it appears that none of these pathways are absolutely essential for the dimorphic transition and each can be bypassed under some conditions. This analysis also indicates a central role for the transcription factor Tec1p in the regulation of filamentous growth. While the role of nitrogen starvation in inducing pseudohyphal differentiation is quite clear, combinations of environmental stresses may also serve to activate this developmental fate, similar to the initiation of meiosis in response to the combination of nitrogen starvation and a nonfermentable carbon source. Several parallel pathways may then be coordinately regulated to properly control dimorphism.

Phenotypic analysis of suppressor deleletion strains: Mösch and Fink (1997) separated the phenomenon of pseudohyphal differentiation into its constituent activities: cell elongation, budding pattern (cell polarity) changes, and substrate invasion. This analysis identified mutations that affect all these behaviors, as well as mutations that affect only a subset, *e.g.*, blocking the polarity change without affecting morphology or invasion. We examined each of the strains deleted for a given suppressor gene to determine which properties might be altered.

We assayed substrate invasion associated both with nitrogen starvation in diploid cells and on rich media in haploid cells (Gimeno *et al.* 1992; Roberts and Fink 1994), as described in materials and methods. Δ*tec1*, Δ*phd2*, and Δ*mss11* strains have significant defects in both haploid and diploid invasion (Table 7); Δ*mep2*, Δ*mep1* Δ*mep2*, and Δ*msn5* mutations affect only diploid (starvation-induced) invasion.

To assess the ability of the various mutant strains to adopt an elongated shape, we analyzed the morphology of nitrogen-deprived cells, as described by Mösch and Fink (1997) and in materials and methods. Of wild-type cells that have invaded the agar substrate, 12.8% have an elongated morphology (Table 7). This is reduced to 2.9% in Δ*mep2*/Δ*mep2* mutants, though interestingly, the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* double-mutant strain does not have a defect in cell elongation. This finding may, in part, explain the epistasis difference between the single- and double-mutant strains. Δ*tec1*/Δ*tec1*, Δ*mss11*/Δ*mss11*, and Δ*ure2*/Δ*ure2* strains each have severe defects in cell elongation, but Δ*mga1*/

TABLE 6

Suppressor deletion strains: phenotypes and epistasis with previously characterized alleles

Strain	Genotype	Vector	*RAS2*^{Val19}	*GPA2*^{Val132}	*STE11-4*	*STE12*	*PHD1*	*TEC1*
MLY61	Wild type	+	++	++	+	++	++	++
MLY132a/a	Δ*gpa2*	±	++	++	±	±	±	±
MLY108a/α	Δ*mep2*	−	+	+	±	+	±	+
MLY115a/α	Δ*mep1* Δ*mep2*	−	+	+	+	+	+	+
MLY183a/α	Δ*tec1*	−	−	±	−	−	−	+
MLY180a/α	Δ*phd2*	−	±	+	±	±	++	±
MLY181a/α	Δ*mss11*	−	±	+[a]	±	+[a]	±	±
MLY179a/α	Δ*mga1*	±	++	+[a]	+	+[a]	++	+
MLY170a/α	Δ*msn5*	±	++	+	+	+	++	+
MLY182a/α	Δ*phd1*	+						
MLY172a/α	Δ*skn7*	+						
MLY168a/α	Δ*hms1*	+						
MLY169a/α	Δ*hms2*	+						
MLY191a/α	Δ*dot6*	+						
MLY224a/α	Δ*dal80*	+						

Homozygous diploid strains of the indicated genotypes were incubated on SLAD medium (SLARG medium to induce *GPA2*^{Val132} and SLADG medium to induce pGAL-*STE12*) for 4 days (6 days for *GPA2*^{Val132} and pGAL-*STE12*).

[a] Δ*mga1*/Δ*mga1* and Δ*mss11*/Δ*mss11* strains filament on media containing galactose as the carbon source. As a result, the suppression by the indicated alleles is probably an artifact of the carbon source.

Δ*mga1* and Δ*msn5*/Δ*msn5* strains have little or no alteration in the number of elongated cells after growth on nitrogen-starvation media (Table 7). Δ*phd2*/Δ*phd2* strains, despite the defect in filamentous growth assayed by colony morphology, actually have an increase in the number of elongated cells (approximately twofold more than wild type, see Table 7). Among mutants with wild-type colony morphology, Δ*phd1*/Δ*phd1*, Δ*dot6*/Δ*dot6*, and Δ*dal80*/Δ*dal80* strains do have moderate defects in cell elongation. Thus, the colony morphology of these strains does not necessarily reflect the cellular morphology accurately.

To form filaments, cells must be able to adopt a unipolar budding pattern, which is an alternate form of the

TABLE 7

Phenotypic analysis of suppressor deletion strains

Genotype	Filamentous growth	Invasive growth		Cell morphology		Budding pattern		
		Haploid	Diploid	% YF	% PH	% Bipolar	% Axial/unipolar	% Random
Wildtype	+	+	++++	87.2	12.8	59.3	27.8	13.0
Δ*mep2*	−	+	++	97.1	2.9	51.8	26.4	21.8
Δ*mep1* Δ*mep2*	−	+	+	86.1	13.9	52.0	24.0	24.0
Δ*tec1*	−	−	+	97.1	2.9	54.4	24.8	20.8
Δ*phd2*	−	±	−	75.7	24.3	25.2	25.2	49.6
Δ*mss11*	−	−	−	100	<1	44.2	16.8	38.9
Δ*mga1*	±	±	++	92.2	7.8	41.5	17.8	40.7
Δ*msn5*	±	+	+++	86.1	13.9	73.2	25.0	19.6
Δ*ure2*	−	±	+	100	<1	46.7	11.7	41.7
Δ*phd1*	+	+	+++	97.1	2.9	64.4	19.2	16.3
Δ*skn7*	+	±	++	86.8	13.2	ND[a]	ND	ND
Δ*hms1*	+	+	+++	78.4	21.6	56.2	23.8	20.0
Δ*hms2*	+	+	++	83.4	16.6	58.2	20.9	20.9
Δ*dot6*	+	±	+++	96.0	4.0	55.9	29.4	14.7
Δ*dal80*	+	+	+++	96.6	3.4	58.2	30.0	11.8

Strains of the indicated genotype were analysed for invasive growth, cellular morphology, and budding pattern as described in materials and methods. PH, pseudohyphal form; YF, yeast form.

[a] Δ*skn7*/Δ*skn7* mutant strains form aggregates with diffuse staining with Calcofluor, obscuring the budding pattern.

standard diploid bipolar pattern in which buds can emerge from either end of the cell. In unipolar division, the buds emerge predominantly from the end of the cell opposite its "birth end," the end at which that cell was attached to its mother. Haploid cells bud in an axial pattern, in which all buds form at the birth end, adjacent to previous bud sites. We analyzed budding patterns using the fluorescent dye Calcofluor (Fluorescent Brightener 28; Sigma) to stain the chitinous scars that mark previous bud sites (see materials and methods). These assays were performed in diploid cells grown in rich (YPD) media; thus, we expected predominantly bipolar budding patterns. In the wild-type strain MLY61, 59.3% of the cells show the bipolar pattern, 27.8% axial or unipolar, and 13.0% random (Table 7). Both Δ*mep2*/Δ*mep2* and Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* mutants increase the percentage of random bud patterns (to 21.8 and 24.0%, respectively). Likewise, the number of cells budding randomly was significantly increased in Δ*phd2*/Δ*phd2* (49.6%), Δ*mss11*/Δ*mss11* (38.9%), Δ*mga1*/Δ*mga1* (40.7%), and Δ*ure2*/Δ*ure2* (41.7%) mutants; Δ*tec1*/Δ*tec1* strains had only a minor effect on budding pattern, although this strain does have defects in elongation and invasion. Genes that did not confer a colony morphology defect did not alter budding pattern significantly (Table 7).

DISCUSSION

Cross talk between distinct signaling pathways regulates filamentous growth: Previous work has demonstrated that filamentous growth in yeast is regulated by at least two distinct signaling pathways, one including elements of the pheromone-responsive MAP kinase cascade and the other involving cAMP signaling (Liu *et al.* 1994; Kübler *et al.* 1997; Lorenz and Heitman 1997). Genetic evidence is consistent with a model in which the cAMP signaling branch could be activated via a Mep2p-dependent mechanism upon ammonium starvation (Lorenz and Heitman 1997, 1998). Several lines of evidence indicate that there may be some cross talk between these two pathways. First, activation of the cAMP signaling pathway, by dominant mutations in *RAS2* or *GPA2*, or by cAMP, suppresses the morphological defects associated with MAP kinase mutant strains (Lorenz and Heitman 1997). Second, as shown here, the dominant *GPA2-2* allele strongly suppresses the pseudohyphal defect of a Δ*ste12*/Δ*ste12* mutant strain, but only weakly suppresses a Δ*tec1*/Δ*tec1* mutant strain. Ste12p and Tec1p heterodimerize to regulate expression of the filamentation response element found in the FG(TyA)::*lacZ* reporter and in the *TEC1* promoter (Madhani and Fink 1997); thus, this difference in epistasis with *GPA2* was not expected. Finally, Ras2p has been implicated in both pathways; in addition to its well-characterized role in regulating adenylyl cyclase activity, the $RAS2^{Val19}$ mutant stimulates expression of the FG(TyA)::*lacZ* reporter via the Ste MAP kinase cascade (Mösch *et al.* 1996; Madhani and Fink 1997).

In this work, we have identified a third example of cross talk between signaling cascades that regulate filamentous growth. The ability of activating alleles of MAP kinase components (particularly *STE11-4*) to suppress the pseudohyphal defect of Δ*mep2*/Δ*mep2* or Δ*gpa2*/Δ*gpa2* strains is enhanced by deletion of *MEP1*, implicating an inhibitory role for Mep1p. Ammonium import or growth defects associated with the Δ*mep1* Δ*mep2* double mutant are not likely to be responsible for this difference in phenotype, as lowering the ammonium concentration 10-fold did not alter the epistasis behavior of the Δ*mep2* single-mutant strain. Furthermore, we also observed enhanced suppression by *STE11-4* expression in Δ*gpa2* Δ*mep1* strains, which have no growth defects; thus, this role of Mep1p does not appear to be directly related to its ammonium transport function. It is possible that Mep2p senses low ammonium concentration and Mep1p senses high ammonium, similar to the glucose sensors Rgt2p and Snf3p, which are homologous to glucose transporters. In the presence of low concentrations of glucose, Snf3p induces the transcription of the genes encoding the high-affinity sugar transporters Hxt2p and Hxt6p; in the presense of high concentrations of glucose, Rgt2p induces expression of the gene encoding the low-affinity transporter Hxt1p (Liang and Gaber 1996; Özcan *et al.* 1996).

Identification of high-copy suppressors of the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* mutant: We used the findings summarized above as a starting point for a genetic screen designed to identify effectors of the signaling function of Mep2p using a high-copy-suppression approach. We chose to use the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* strain, as the ability to restore filamentous growth in this strain appeared more permissive than in the Δ*mep2*/Δ*mep2* single mutant. In addition, using the double mutant allowed us to identify genes that improved the growth of this strain under ammonium-limiting conditions. We identified 12 genes whose overexpression suppressed the filamentation defect of the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* strain and 5 genes that suppressed the growth defect of this strain on ammonium-limiting media (Table 3). Among this set are several genes that are required for filamentous growth, including *MEP2*, *TEC1*, *PHD2*, *MSS11*, *MGA1*, and *URE2* (see Table 6; Gavrias *et al.* 1996; Lambrechts *et al.* 1996; Lorenz and Heitman 1998). Epistasis tests between strains lacking the suppressor genes and alleles previously found to regulate filamentous growth confirm that the genetic control of this phenomenon is complex, involving several pathways coordinating multiple signals. No single gene was absolutely required, as conditions that restore filamentation were found for each mutant strain.

A potential role for other signaling events in inducing filamentous growth: Several of the suppressors have been previously identified to regulate cellular response

to changes in carbon source, namely *PHD2* (also *MSN1*, *MSS10*, and *FUP4*), *MSS11*, *MSN5*, and *MGA1*. *PHD2* (*MSN1*), *MSN5*, and *MGA1* were each identified as multicopy suppressors of the *s*ucrose *n*on-*f*ermenting (*SNF*) phenotype of either *snf1* or *snf2*/*gam1* mutations (Estruch and Carlson 1990; Gimeno and Fink 1994; Zhang *et al.* 1997); Phd2p (Mss10p) and Mss11p regulate expression of the genes that encode the starch-degrading glucoamylases Sta1p, Sta2p, and Sta3p on the basis of carbon availability (Lambrechts *et al.* 1996; Webber *et al.* 1997). Poorly used carbon sources, such as the starch amylopectin, have been found to induce filamentous growth, even in nitrogen-rich conditions; Δ*phd2* (Δ*mss10*) mutations block this response (Lambrechts *et al.* 1996). With the exception of *MSN5*, each of these genes has significant similarity to transcriptional regulatory proteins.

The connection between carbon signaling and pseudohyphal differentiation is intriguing. Diploid cells have two mutually exclusive developmental fates upon nitrogen starvation: pseudohyphal growth in the presence of carbon abundance and meiosis in the presence of a nonfermentable carbon source. The mechanism by which the cell chooses between these two fates (*i.e.*, how the cell recognizes the carbon source) is not understood. These carbon-regulatory proteins may participate in such signaling to control the pseudohyphal/meiosis decision; none of these mutants, however, affect meiotic competence (M. C. Lorenz and J. Heitman, unpublished results). The finding that nitrogen-rich, carbon-poor media can also induce filamentous growth (Lambrechts *et al.* 1996) suggests that a reciprocal signaling event may also be present.

Another of the suppressor genes described here, *SKN7*, is critical for the cellular response to oxidative stress. Δ*skn7* strains are sensitive to several oxidizing agents, including hydrogen peroxide and cadmium, a phenotype shared with mutations in *YAP1* (Yeast AP-1, a homolog of *c-jun*), another transcriptional activator (Moye-Rawley *et al.* 1989; Kuge and Jones 1994). Skn7p and Yap1p coordinately regulate expression of *TRX2*, encoding thioredoxin (Morgan *et al.* 1997). Yap1p has also been proposed as a downstream effector of PKA activity (Gounalakai and Thireos 1994). Thus, oxidative damage may be another stress that can stimulate differentiation via a Skn7p-dependent pathway. Skn7p also has a role in cell wall biosynthesis and cell cycle progression (Brown and Bussey 1993; Brown *et al.* 1993; Morgan *et al.* 1995).

We found that high-copy expression of *CDC6* strongly suppresses the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* pseudohyphal defect. Cdc6p is a component of the origin recognition complex and is essential for cells to initiate DNA synthesis. Thus, Cdc6p may be part of a checkpoint sensing DNA damage or blocks to DNA synthesis, another potential stress-responsive pathway that can trigger filamentous growth. However, we favor an alternate explanation: expression of *CDC6* is limited to G1, and when this temporal control is distorted by expressing *CDC6* from a constitutive promoter, nuclear division is delayed and cells develop highly elongated buds (Bueno and Russell 1992; Piatti *et al.* 1995). Because the elongated buds produced after *CDC6* overexpression are reminiscent of pseudohyphae, it is possible that this phenotype is responsible for the Δ*mep1*/Δ*mep1* Δ*mep2*/Δ*mep2* suppression. Recent findings have identified an N-terminal region of Cdc6p that is necessary for its degradation; deletion of this region allows the protein to persist into G2 (Drury *et al.* 1997). The *CDC6* construct described here lacks ~300 bp of coding sequence from the 3′ end. It is possible that this region of the protein may also be a stability determinant.

Ironically, though nitrogen starvation is the best-characterized environmental stress that induces pseudohyphal differentiation, the nitrogen regulatory genes (*URE2* and *DAL80*) have among the weakest phenotypes when overexpressed. Other elements of nitrogen regulatory networks are required for pseudohyphal differentiation, including the protein kinase Npr1p and the ubiquitin ligase Npi1p/Rsp5p (Lorenz and Heitman 1998), but, again, the only characterized role for these proteins is the regulation of the stability of plasma membrane permeases such as Mep2p. Other connections between these proteins and the filamentation response have yet to be elucidated.

Comparisons to development in other fungi: The study of differentiation pathways, both in yeast and in other fungi, has been motivated by the connection of such events to pathogenicity. Mating and conjugation in *U. maydis* leads to a filamentous growth state that is essential for infection of the maize host (Banuett 1991). *C. albicans* mutant strains that are unable to filament are avirulent *in vivo* (Lo *et al.* 1997). Differentiation events have been correlated with infection of plant or animal hosts in many other species, including *Uromyces appendilaticus* and *Cryptococcus neoformans* (Zhou *et al.* 1991; Wickes *et al.* 1996; Alspaugh *et al.* 1997). Moreover, differentiation events in various fungi have regulatory similarities to pseudohyphal development in *S. cerevisiae*. MAP kinase elements with roles in dimorphism have been identified in *C. albicans*, *Schizosaccharomyces pombe*, *U. maydis*, and several others (Banuett and Herskowitz 1994; Liu *et al.* 1994; Kohler and Fink 1996; Leberer *et al.* 1996). PKA or cAMP signaling have been shown to regulate development in many organisms, including *C. neoformans*, *S. pombe*, *Neurospora crassa*, and *U. maydis* (Sabie and Gadd 1992; Yarden *et al.* 1992; Gold *et al.* 1994; Maeda *et al.* 1994; Alspaugh *et al.* 1997).

The implication that stimuli other than nitrogen starvation may trigger pseudohyphal growth in yeast is reminiscent of *C. albicans*, in which many signals, including serum, temperature, CO_2 levels, and nutrient stresses have been shown to induce filamentation. Mutations in

the *C. albicans* MAP kinase pathway inhibit filamentation in response to some but not all of these stimuli (Liu *et al.* 1994; Kohler and Fink 1996; Leberer *et al.* 1996). Subsequent deletion of the *EFG1* gene, which encodes a transcriptional regulator similar to Phd1p (Stoldt *et al.* 1997), eliminates this residual filamentation (Lo *et al.* 1997). This is analogous to yeast in which Δ*ste* mutant strains have a severe but not absolute filamentation defect (Liu *et al.* 1993). Deletion of *PHD1*, as for *EFG1*, eliminates this remaining filamentous growth (Lo *et al.* 1997). In addition, Tup1p is a global transcriptional repressor in yeast and regulates filamentous growth in both *S. cerevisiae* and *C. albicans*, although the *tup1* mutant phenotype is opposite: constitutive filamentation in *C. albicans* and a pseudohyphal deficiency in *S. cerevisiae* (Braun and Johnson 1997). In *S. cerevisiae*, Tup1p physically interacts with Ssn6p (Williams *et al.* 1991), a protein closely related to Mss11p. Understanding filamentous growth control in yeast, therefore, has broad implications for pathogenicity in many fungi.

Complexity of filamentous growth revealed in transcriptional regulatory proteins: The other surprising finding from this screen, in addition to the potential involvement of other stress-responsive pathways, is the multiplicity of predicted transcriptional regulators that have been identified to affect pseudohyphal differentiation in some manner. Our studies have identified Phd1p, Tec1p, Phd2p, Mss11p, Mga1p, Skn7p, Dot6p, Hms1p, Hms2p, and Dal80p (see also Gimeno and Fink 1994; Gavrias *et al.* 1996; Lambrechts *et al.* 1996); other studies have found a role for Ste12p, Ash1p, and Gln3p (Liu *et al.* 1993; Chandarlapaty and Errede 1998; Lorenz and Heitman 1998). Although many laboratory strains of *S. cerevisiae* are not competent to undergo pseudohyphal growth, the complexity of transcriptional responses and the reglatory conservation between filamentous growth in yeast and differentiation events in diverse fungi highlights the evolutionary importance of this stress-responsive developmental pathway.

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