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Yeast pseudohyphal growth is regulated by GPA2, a G protein α homolog

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Pseudohyphal differentiation, a filamentous growth form of the budding yeast *Saccharomyces cerevisiae*, is induced by nitrogen starvation. The mechanisms by which nitrogen limitation regulates this process are currently unknown. We have found that GPA2, one of the two heterotrimeric G protein α subunit homologs in yeast, regulates pseudohyphal differentiation. Δ*gpa2*/Δ*gpa2* mutant strains have a defect in pseudohyphal growth. In contrast, a constitutively active allele of *GPA2* stimulates filamentation, even on nitrogen-rich media. Moreover, a dominant negative *GPA2* allele inhibits filamentation of wild-type strains. Several findings, including epistasis analysis and reporter gene studies, indicate that GPA2 does not regulate the MAP kinase cascade known to regulate filamentous growth. Previous studies have implicated GPA2 in the control of intracellular cAMP levels; we find that expression of the dominant *RAS2*^{Gly19Val} mutant or exogenous cAMP suppresses the Δ*gpa2* pseudohyphal defect. cAMP also stimulates filamentation in strains lacking the cAMP phosphodiesterase PDE2, even in the absence of nitrogen starvation. Our findings suggest that GPA2 is an element of the nitrogen sensing machinery that regulates pseudohyphal differentiation by modulating cAMP levels.

Keywords: cAMP/dimorphism/G proteins/pseudohyphal differentiation/yeast

Introduction

In response to severe nitrogen starvation, diploid cells of the budding yeast *Saccharomyces cerevisiae* undergo a dimorphic transition known as pseudohyphal differentiation (Gimeno *et al.*, 1992). During pseudohyphal growth, cells adopt a unipolar budding pattern, elongate and can invade the growth substrate. In addition, cells remain attached following cytokinesis, resulting in chains of cells reminiscent of fungal hyphae. It has been suggested that this mode of growth may allow this non-motile species to forage for nutrients under adverse conditions (Gimeno *et al.*, 1992; Kron *et al.*, 1994).

Pseudohyphal differentiation is regulated, in part, by elements of the MAP kinase cascade which also controls mating, namely the protein kinases STE20, STE11 and STE7 and the transcription factor STE12 (Liu *et al.*, 1993). A heterodimer composed of STE12 and TEC1 binds to a filamentation response element (FRE), which may regulate expression of genes necessary for pseudohyphal differentiation (Laloux *et al.*, 1994; Mösch *et al.*, 1996; Madhani and Fink, 1997). A reporter gene under the control of an FRE [FG(TyA)::*lacZ*] is induced under nitrogen starvation conditions in a STE12- and TEC1-dependent manner (Gavrias *et al.*, 1996; Mösch *et al.*, 1996; Madhani and Fink, 1997). The proximal elements that regulate mating, the pheromone receptors (STE2 and STE3) and the heterotrimeric guanine nucleotide binding protein (GPA1, STE4 and STE18), are not expressed in diploids and play no role in pseudohyphal differentiation (Liu *et al.*, 1993). The mechanisms by which nitrogen starvation activates the MAP kinase cascade or triggers filamentous growth are not known, but the parallels between the signalling pathways in haploid and diploid cells led us to consider whether pseudohyphal growth might also be G protein regulated.

Heterotrimeric G proteins mediate a vast array of signalling processes in all eukaryotes, including mating response and development in fungi, vision in mammals and metazoans, and neurotransmitter and hormone action in mammals (reviewed in Neer, 1995; Borkovich, 1996). These regulatory complexes typically serve as a bridge between transmembrane receptors and effectors, including adenylate cyclase, phospholipases or protein kinases. A ligand-bound receptor can either activate or inhibit effectors via the G protein. The activity of the G protein is controlled by a guanine nucleotide cycle in which an activated receptor promotes GDP to GTP exchange on the α subunit of the G protein. GTP binding stimulates dissociation of the α subunit from the βγ complex, and either the free α or free βγ dimer, or in some cases both, regulate downstream effectors. The intrinsic GTPase activity of the α subunit hydrolyzes GTP to GDP, promoting reassociation of the heterotrimer and attenuation of signalling.

The best characterized Gα subunit in *S.cerevisiae*, GPA1 (also known as SCG1), regulates mating response by binding to the pheromone receptors STE2 (α-factor receptor) and STE3 (**a**-factor receptor; reviewed in Kurjan, 1993; Bardwell *et al.*, 1994). In this pathway, the free βγ subunit transmits the signal to a conserved MAP kinase cascade through the interaction of a large multimeric complex (Leeuw *et al.*, 1995; Akada *et al.*, 1996). Haploid Δ*gpa1* mutants are inviable due to constitutive activation of the pheromone response pathway and subsequent cell cycle arrest (Miyajima *et al.*, 1987). G proteins also mediate mating response in the fission yeast *Schizosaccharomyces pombe*. In this organism two distinct Gγ subunits regulate the two environmental signals required for mating: the presence of pheromone is signalled by Gpa1 (Obara *et al.*, 1991) and nitrogen starvation is signalled by Gpa2 (Isshiki *et al.*, 1992). Morphogenesis triggered by environmental stimuli is regulated by

G protein action in the fungi *Coprinus congregatus* (Kozak *et al.*, 1995) and *Neurospora crassa* (Ivey *et al.*, 1996).

The yeast genome project has identified only one protein other than GPA1 likely to encode a Gα subunit, GPA2, which was first isolated on the basis of its homology to a rat brain Gαs isoform (Nakafuku *et al.*, 1988). Addition of glucose to glucose-starved yeast cells induces a rapid but transient increase in cAMP levels. Expression of *GPA2* from a high copy plasmid enhances this rise in cAMP (Nakafuku *et al.*, 1988; Papasavvas *et al.*, 1992). The glucose-stimulated cAMP pulse is partially inhibited in the presence of mating pheromone; this inhibition requires GPA2 and appears to involve interactions between GPA2 and RAS2 (Arkinstall *et al.*, 1991; Papasavvas *et al.*, 1992). Yeast Ras modulates adenylate cyclase activity and cAMP levels (Toda *et al.*, 1985; Field *et al.*, 1988), and overexpression of *GPA2* suppresses the growth defect of a temperature-sensitive *ras2* mutant (Nakafuku *et al.*, 1988). High levels of cAMP activate the cAMP-dependent protein kinase (protein kinase A, PKA); among its many functions, PKA mediates cellular responses to nutrient stress, heat shock and oxidative damage (reviewed in Broach and Deschenes, 1990). GPA2 and *S.pombe* Gpa2 are highly homologous (42% identity). The fission yeast Gpa2 protein is known to regulate mating in response to nitrogen starvation; *S.pombe gpa2*$^{-}$ mutant cells mate under nutrient-rich conditions unlike wild-type *gpa2*$^{+}$ cells (Isshiki *et al.*, 1992). This phenotype is shared by *S.pombe* mutants lacking adenylate cyclase (Maeda *et al.*, 1990), and Gpa2 regulates cAMP levels in fission yeast (Isshiki *et al.*, 1992).

Ras also regulates filamentous growth in budding yeast, and activated mutants of yeast *RAS2* stimulate pseudohyphal differentiation (Gimeno *et al.*, 1992). It was initially proposed that RAS2 would regulate pseudohyphal differentiation by modulating cAMP levels (Gimeno *et al.*, 1992), a suggestion supported by the finding that overproduction of the cAMP phosphodiesterase PDE2 inhibits pseudohyphal growth in wild-type strains and blocks hyperfilamentation induced by the dominant active RAS2^{Gly19Val} mutant (Ward *et al.*, 1995). Other work, however, has suggested that Ras acts upstream of the MAP kinase cascade (Mösch *et al.*, 1996; Madhani and Fink, 1997). The interactions observed between RAS2, GPA2 and cAMP led us to test whether GPA2 is involved in filamentous growth. We report here that Δ*gpa2*/Δ*gpa2* mutant strains have a defect in filamentous growth. Moreover, a dominant active *GPA2* allele stimulates pseudohyphal differentiation, even on nitrogen-rich media. Epistasis analysis and reporter gene experiments, however, indicate that GPA2 does not act upstream of the MAP kinase cascade. We also find that cAMP stimulates filamentation, and both RAS2^{Gly19Val} and cAMP suppress the pseudohyphal defect conferred by the Δ*gpa2* mutation. We propose that GPA2 is an element of the nitrogen sensing machinery which signals pseudohyphal differentiation under starvation conditions via a signalling pathway involving cAMP.

Results

The GPA2 Gα protein regulates pseudohyphal growth

Elements upstream of the pheromone responsive MAP kinase cascade which sense nitrogen starvation during pseudohyphal growth have not yet been identified. In fission yeast, the Gα subunit Gpa2 regulates mating in response to nitrogen limitation; this G protein is distinct from the pheromone-stimulated Gpa1 protein (Obara *et al.*, 1991; Isshiki *et al.*, 1992). These studies led us to test whether nitrogen sensing in budding yeast might also be G protein-mediated. Previous work found no role for *S.cerevisiae* GPA1 in pseudohyphal differentiation (Liu *et al.*, 1993), thus we focused on GPA2, which has been implicated in the regulation of cAMP levels and Ras functions (Nakafuku *et al.*, 1988).

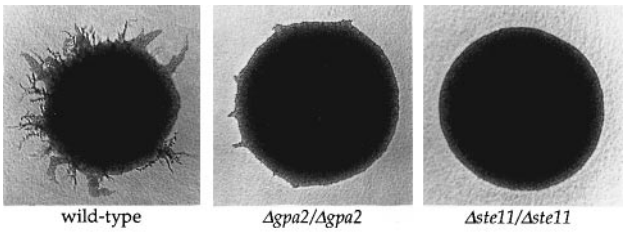

Fig. 1. GPA2 regulates pseudohyphal growth. Diploid strains of the Σ1278b background with the indicated genotypes (wild-type, MLY61; Δ*gpa2*/Δ*gpa2*, MLY132**a**/α; Δ*ste11*/Δ*ste11*, HLY506) were incubated on nitrogen limiting SLAD medium for 4 days at 30°C.

We replaced the *GPA2* open reading frame with a gene encoding resistance to G418 in the Σ1278b background (Grenson *et al.*, 1966) commonly used for analysis of filamentous growth (Gimeno *et al.*, 1992). Cells lacking GPA2 (*gpa2*-Δ*1*) were viable and had no obvious defects in growth, mating or sporulation (data not shown). A homozygous Δ*gpa2*/Δ*gpa2* diploid strain, however, exhibited a defect in pseudohyphal differentiation when grown on low nitrogen (50 μM ammonium sulfate) SLAD medium (Figure 1). Δ*gpa2*/Δ*gpa2* mutant strains filament weakly, as do strains with mutations of MAP kinase elements (with the exception of Δ*ste20* mutant strains, which do not filament at all; Liu *et al.*, 1993). Expression of wild-type *GPA2* complemented and restored pseudohyphal growth in Δ*gpa2*/Δ*gpa2* mutant strains (data not shown). The pseudohyphal defect conferred by the Δ*gpa2* mutation was also observed when cells were grown in the presence of limiting concentrations of proline, glutamine or urea (data not shown), indicating that GPA2 is a general regulator of filamentation.

A dominant active GPA2 allele enhances pseudohyphal growth

Several mutations have been described in other Gα subunits that perturb proper function of the G protein and have been invaluable in dissecting G protein function. One such mutation, in which valine replaces the second glycine in the highly conserved GXGXXG motif, has been shown to reduce the GTPase activity of Gαs and the small G protein Ras (Graziano and Gilman, 1989; Masters *et al.*, 1989), thus promoting the GTP-bound and active forms of these proteins. The human H-ras^{Gly12Val} mutation confers a transforming phenotype (reviewed in Lowy and Willumsen, 1993). In yeast, RAS2^{Gly19Val} constitutively activates adenylate cyclase, raises intracellular cAMP levels (Toda *et al.*, 1985) and promotes pseudohyphal differentiation (Gimeno *et al.*, 1992). The corresponding mutation in GPA1, Gly50Val, increases basal activation of the pheromone response pathway, leading to a growth

defect and impaired adaptation to prolonged pheromone exposure (Kurjan *et al.*, 1991).

To characterize further the function of GPA2, we constructed an allele with the analogous mutation, Gly132-Val (*GPA2-2*), under the control of a galactose-inducible promoter in a low-copy, centromeric plasmid. Expression of the *GPA2*^{Gly132Val} allele on nitrogen limiting medium containing galactose greatly enhanced pseudohyphal differentiation (Figure 2A). This stimulation was not observed with expression of the wild-type allele or when expression was repressed with glucose (Figure 2A). In addition, this activated allele did not promote filamentous growth when expressed in a haploid strain (data not shown). Strains expressing the *GPA2*^{Gly132Val} allele grew more slowly than wild-type strains, and formed smaller colonies. This is not unusual in hyperfilamentous strains, as overexpression of *STE12* confers a similar phenotype (Liu *et al.*, 1993, and data not shown).

If GPA2 is indeed a component of the nitrogen sensor, the *GPA2*^{Gly132Val} allele might induce filamentation under nitrogen-rich conditions in which this developmental pathway is normally repressed. Consistent with this hypothesis, expression of the *GPA2*^{Gly132Val} mutation stimulated filamentous growth on media containing 5 mM ammonium sulfate (Figure 2B), a 100-fold excess over standard pseudohyphal media (SLAD; 50 μM ammonium sulfate). Filamentation also occured, albeit to a lesser extent, on the synthetic medium YNB, which contains an even higher nitrogen concentration (38 mM ammonium sulfate; data not shown). Thus, constitutive activation of *GPA2* relieves the requirement for nitrogen starvation to induce pseudohyphal growth.

A dominant negative GPA2 allele inhibits pseudohyphal growth

We next introduced a mutation in the hinge region of GPA2 which would be predicted to result in a dominant negative allele. The corresponding mutation in other Gα subunits prevents conformational changes normally induced by GTP binding that are required for βγ release and signalling (Miller *et al.*, 1988), resulting in a dominant negative phenotype. This mutation in yeast *GPA1* complements the lethality of the Δ*gpa1* mutation, but confers a semidominant sterile phenotype (Kurjan *et al.*, 1991). The corresponding mutant allele, *GPA2*^{Gly299Ala} (*GPA2-3*), again under the control of a galactose-inducible promoter, did not complement the pseudohyphal defect of Δ*gpa2* mutant strains, as expected (data not shown). Expression of *GPA2*^{Gly299Ala} inhibited pseudohyphal differentiation in wild-type diploid strains (Figure 3A). Thus, two mutations predicted to alter GPA2 function both have significant effects on filamentous growth and demonstrate that pseudohyphal differentiation is G protein regulated.

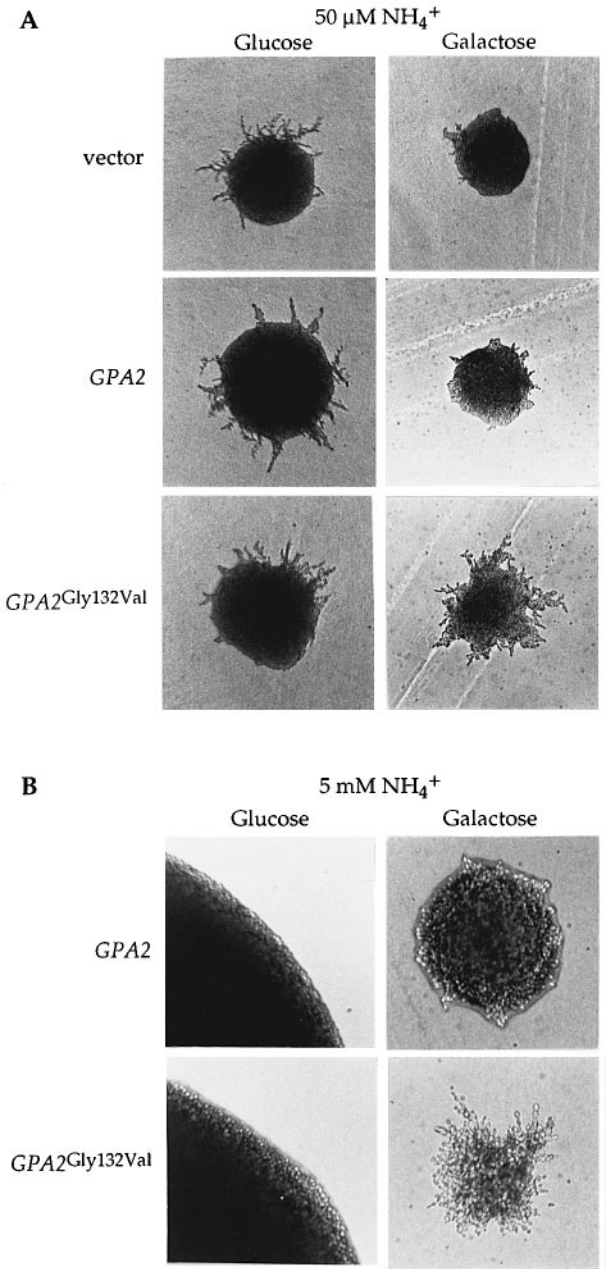

Fig. 2. A dominant *GPA2* allele stimulates pseudohyphal growth. (**A**) Wild-type diploid strain MLY61 with a control plasmid (vector), or plasmids expressing *GPA2* (pML180) or *GPA2*^{Gly132Val} (pML160) under repressing (glucose, left) or inducing (galactose, right) conditions were incubated on nitrogen limiting medium (SLAD or SLARG) for 4 days at 30°C. The colonies were photographed at 25× magnification. (**B**) The strains and *GPA2* plasmids indicated in (A) were incubated on nitrogen-rich medium containing 5 mM ammonium sulfate for 2 days at 30°C under repressing (glucose, left) or inducing (galactose, right) conditions. The colonies were photographed at 50× magnification.

β and γ subunits remain to be identified

In several G protein mediated signalling pathways, both the α and the βγ subunits positively contribute to signalling. The residual filamentation observed in Δ*gpa2* mutants could be indicative of a role for a βγ complex in signalling, although it is clear that the pheromone-responsive βγ subunits (STE4 and STE18) do not regulate pseudohyphal growth (Liu *et al.*, 1993). Using the fungal β proteins STE4 (*S.cerevisiae*) and Gpb1 (*S.pombe*) in BLAST searches, we identified eight candidate β genes in the *S.cerevisiae* genome; gene disruption experiments did not reveal a role for any of these genes in pseudohyphal differentiation (see Materials and methods). STE18 is the only known fungal γ subunit (Whiteway *et al.*, 1989); none of the three candidate γ subunits we identified affected pseudohyphal growth (see Materials and methods). Other methods will be necessary to identify these components.

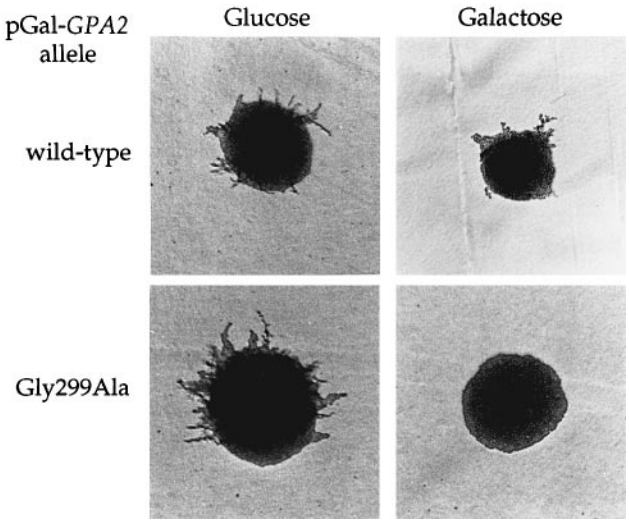

Fig. 3. A dominant negative *GPA2* allele inhibits pseudohyphal growth. Wild-type diploid strain MLY61 expressing *GPA2* (pML180) or *GPA2*^{Gly299Ala} (pML179) under repressing (glucose, left) or inducing (galactose, right) conditions on nitrogen limiting media was incubated for 4 days at 30°C.

GPA2 does not regulate the MAP kinase cascade

Previous work has identified several genes and mutant alleles that promote filamentous growth (Gimeno *et al.*, 1992; Liu *et al.*, 1993; Gimeno and Fink, 1994), and these allowed us to test the point at which GPA2 acts by epistasis analysis. Both the dominant *STE11-4* allele and overexpression of *STE12* stimulate pseudohyphal differentiation when expressed in wild-type strains and suppress mutations of upstream components (Liu *et al.*, 1993). If GPA2 were to act solely upstream of the MAP kinase cascade, expression of these alleles should suppress the pseudohyphal defect conferred by the Δ*gpa2* mutation. To our surprise, neither *STE11-4* nor increased *STE12* expression resulted in filamentation in a Δ*gpa2*/Δ*gpa2* mutant strain (Figure 4A). These observations suggest that GPA2 does not act upstream of the MAP kinase elements that regulate filamentous growth. Likewise, overexpression of *PHD1*, which induces hyperfilamentation in other strains (Gimeno *et al.*, 1992; Gimeno and Fink, 1994), had no effect in Δ*gpa2*/Δ*gpa2* strains. In contrast, expression of the dominant *RAS2*^{Gly12Val} mutant did suppress the pseudohyphal defects conferred by the Δ*gpa2* mutation. This finding is consistent with a role for GPA2 in regulating cAMP levels, as has been suggested (Nakafuku *et al.*, 1988). Others have proposed that RAS2 lies solely upstream of the MAP kinase elements (Mösch *et al.*, 1996); however, we also find that *RAS2*^{Gly12Val} suppresses the pseudohyphal defects of the Δ*ste7*, Δ*ste11* and Δ*ste12* mutants, findings which do not support this model. RAS2 may instead have multiple roles in regulating filamentous growth, as has recently been suggested (Mösch and Fink, 1997).

The dominant active *GPA2*^{Gly132Val} allele allowed us to address the relationship between GPA2 and the MAP kinase elements by another means. The *GPA2*^{Gly132Val} allele was expressed in diploid strains carrying homozygous deletions of *STE20*, *STE11*, *STE7* and *STE12*. None of the Δ*ste* mutations blocked the ability of *GPA2*^{Gly132Val} to stimulate pseudohyphal differentiation (Figure 4B).

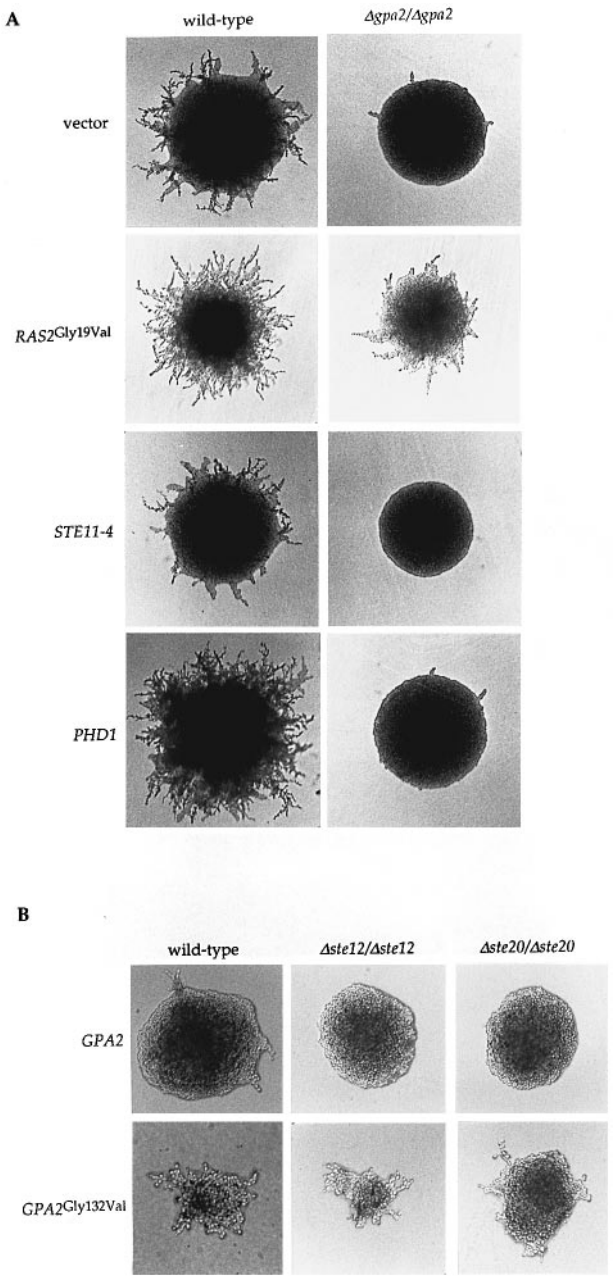

Fig. 4. GPA2 functions independently of the MAP kinase pathway. (**A**) Diploid wild-type (MLY61) or Δ*gpa2*/Δ*gpa2* (MLY132a/α) strains expressing *RAS2*^{Gly19Val}, *STE11-4* or *PHD1* were incubated on SLAD medium for 4 days at 30°C. The colonies were photographed at 25× magnification. (**B**) Diploid wild-type (L3566), Δ*ste12*/Δ*ste12* (HLY506) or Δ*ste20*/Δ*ste20* (HLY492) strains expressing *GPA2* (pML180) or *GPA2*^{Gly132Val} (pML160) were incubated on SLARG medium for 4 days at 30°C. The colonies were photographed at 50× magnification.

Moreover, as in wild-type *STE*$^{+}$ strains, *GPA2*^{Gly132Val} also allowed pseudohyphal growth of Δ*ste* mutant strains on nitrogen-rich medium (data not shown). Filamentation in the Δ*ste20*/Δ*ste20* strain expressing *GPA2*^{Gly132Val} was somewhat reduced compared with wild-type or other Δ*ste* mutants (Figure 4B), which is consistent with previous observations that the Δ*ste20* mutant phenotype is more severe than that of other Δ*ste* mutants (Liu *et al.*, 1993). These observations again support the conclusion that

Table I. Activation of a FG(TyA)::*lacZ* reporter by *GPA2* alleles

Plasmid	Relative β-galactosidase activity	
	50 μM NH_4^+	5 mM NH_4^+
Vector	6.2	1.0
GPA2^{Gly132Val}	4.9	0.5
GPA2^{Gly299Ala}	4.7	2.6

FG(TyA)::*lacZ* expression was assayed by monitoring β-galactosidase activity in wild-type cells (MLY97) expressing pIL30-*LEU2* and the indicated *GPA2* allele after incubation on solid SLARG medium for 48 h at 30°C essentially as described (Mösch *et al.*, 1996). Activities were normalized to protein concentration and are reported as relative to the vector control on high nitrogen media. Values are the average of two independent transformants, each tested in duplicate.

GPA2 does not regulate pseudohyphal differentiation via the MAP kinase cascade.

GPA2 alleles do not affect expression of a MAP kinase-regulated reporter gene

To address an alternate explanation for these epistasis results, namely that GPA2 might regulate both the MAP kinase cascade and another pathway, we employed a reporter gene previously found to respond to MAP kinase activation under nitrogen starvation conditions (Laloux *et al.*, 1994; Mösch *et al.*, 1996). This reporter, FG(TyA)::*lacZ*, was first identified as a control element for the transposable element Ty1, and includes binding sites for TEC1 and STE12. It is induced upon nitrogen starvation but not in response to mating pheromone (Gavrias *et al.*, 1996; Mösch *et al.*, 1996; Madhani and Fink, 1997). Expression of the dominant active *GPA2* allele had no affect on the activity of this reporter gene (Table I), and pseudohyphal growth induced by *GPA2*^{Gly132Val} on nitrogen-rich media (see Figure 2C) was not accompanied by an increase in reporter activity (Table I). Conversely, the dominant-negative *GPA2*^{Gly299Ala} allele did not prevent induction of FG(TyA)::*lacZ* under low nitrogen conditions, despite inhibiting pseudohyphal growth (Table I). In addition, alleles which induce expression of this reporter, such as *STE11-4* (Mösch *et al.*, 1996), were not affected by deletion of *GPA2* (Table II), even though *STE11-4* does not suppress the pseudohyphal defects of Δ*gpa2* mutant strains. Furthermore, the *GPA2*^{Gly132Val} allele, while it suppressed the filamentation defect of Δ*ste* mutants, did not rescue reporter activity in these strains (Table III). Thus, the phenotypes conferred by mutant *GPA2* alleles occur in the absence of any detectable difference in activation of the MAP kinase cascade, indicating that GPA2 does not regulate this pathway. Moreover, the activity of the FG(TyA)::*lacZ* reporter gene and the morphological response of filamentation can be separated under some conditions.

Pseudohyphal growth is regulated by cAMP

Earlier reports have suggested a connection between GPA2, Ras and cAMP metabolism (Nakafuku *et al.*, 1988; Papasavvas *et al.*, 1992). Our findings that GPA2 regulates filamentation and that the Δ*gpa2* mutation is suppressed by *RAS2*^{Gly19Val} led us to test whether cAMP might also regulate this dimorphic transition. The efficacy of

Table II. FG(TyA)::*lacZ* induction in *GPA2* versus Δ*gpa2* strains

Construct	Relative β-galactosidase activity			
	GPA2/GPA2		Δ*gpa2/*Δ*gpa2*	
	50 μM NH_4^+	5 mM NH_4^+	50 μM NH_4^+	5 mM NH_4^+
Control	1.9	1.0	2.3	0.5
RAS2^{wt}	2.1	0.7	2.9	0.7
RAS2^{Gly19Val}	6.8	0.4	5.0	0.7
STE11-4	26.7	4.0	10.3	1.5
PHD1	3.6	1.3	3.2	1.1

FG(TyA)::*lacZ* expression was assayed in wild-type (MLY97) or Δ*gpa2/*Δ*gpa2* (MLY212a/α) cells by coexpressing pIL30-*LEU2* with the indicated allele. After 48 h on solid media containing 50 μM or 5 mM ammonium sulfate, β-galactosidase activity was measured as described (see Materials and methods). Activities were normalized to protein concentration and are reported as relative to the vector control in wild-type cells on high nitrogen media. Values are the average of two independent transformants assayed in duplicate.

Table III. Effects of *GPA2* alleles on FG(TyA)::*lacZ* expression in Δ*ste* strains

GPA2 allele	Relative β-galactosidase activity					
	Wild-type		Δ*ste20/*Δ*ste20*		Δ*ste12/*Δ*ste12*	
	50 μM	5 mM	50 μM	5 mM	50 μM	5 mM
None	11.8	1.0	2.8	0.2	3.1	0.1
GPA2^{wt}	20.0	0.7	6.2	0.1	3.4	0.2
GPA2^{Gly132Val}	13.3	1.8	2.1	0.2	1.5	<0.1

FG(TyA)::*lacZ* expression was assayed in wild-type (MLY97), Δ*ste20/*Δ*ste20* (MLY219a/α) and Δ*ste12/*Δ*ste12 (*MLY216a/α) strains coexpressing pIL30-*LEU2* and the indicated *GPA2* allele. Cells were incubated for 48 h at 30°C on solid media containing 50 μM or 5 mM ammonium sulfate with 0.5% galactose and 2% raffinose. Activities were normalized to protein concentrations and are reported as relative to the vector control in wild-type cells on high nitrogen media. Values are the average of two independent transformants, each assayed in duplicate.

exogenous cAMP in yeast is greatly enhanced by mutations in the high affinity cAMP phosphodiesterase PDE2. Addition of 1 mM cAMP to nitrogen-limiting media stimulated filamentation of diploid Δ*pde2/*Δ*pde2* mutant strains, and filamentation was even more dramatic at 10 mM cAMP (Figure 5A). At 10 mM cAMP, even wild-type (*PDE2*$^+$) strains exhibit enhanced pseudohyphal growth (Figure 5A), and pseudohyphal differentiation also occured on nitrogen-rich media (5 mM NH_4^+; Figure 6). These effects of cAMP were not observed with either AMP or cGMP (data not shown), indicating a specific role for the second messenger cAMP in regulating pseudohyphal differentiation.

If the role of GPA2 is to regulate intracellular cAMP levels, as has been previously suggested (Nakafuku *et al.*, 1988; Papasavvas *et al.*, 1992), the pseudohyphal defects of Δ*gpa2* mutant strains might be suppressed by exogenous cAMP. To test this hypothesis, we constructed a homozygous diploid strain lacking both GPA2 and PDE2. As shown in Figure 5A, 100 μM cAMP restored filamentation to approximately wild-type levels in a Δ*gpa2/*Δ*gpa2*

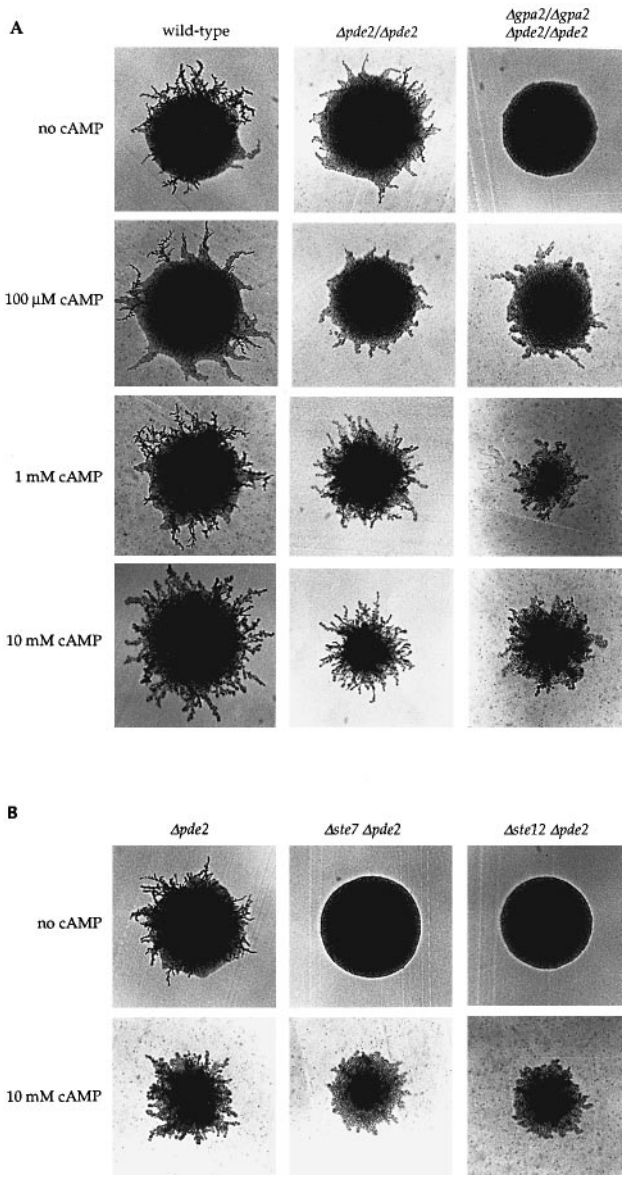

Fig. 5. cAMP stimulates pseudohyphal differentiation. (**A**) Homozygous diploid strains with genotypes: wild-type (MLY61), Δ*pde2*/Δ*pde2* (MLY162**a**/α) or Δ*gpa2*/Δ*gpa2* Δ*pde2*/Δ*pde2* (MLY171a/α) were incubated on SLAD medium containing the indicated concentrations of cAMP for 4 days at 30°C. (**B**) Strains of genotypes Δ*pde2 MAT*α (MLY173), Δ*ste7* Δ*pde2 MAT***a** (MLY174) and Δ*ste12* Δ*pde2 MAT***a** (MLY175) expressing a plasmid-borne copy of the opposite *MAT* locus were incubated on SLAD medium with or without 10 mM cAMP for 4 days at 30°C.

Δ*pde2*/Δ*pde2* mutant strain. The effects of cAMP are not mediated by the MAP kinase pathway, as cAMP also stimulated filamentation in Δ*ste7* Δ*pde2* and Δ*ste12* Δ*pde2* mutant strains (Figure 5B), as did expression of the dominant active *GPA2*^{Gly132Val} allele (Figure 4B and data not shown). Surprisingly, cAMP actually repressed expression of the FG(TyA)::*lacZ* reporter under nitrogen limiting conditions (Table IV). This is another example in which increased filamentous growth, as assayed morphologically, was not accompanied by increased expression of this reporter. These findings provide support for a model in which GPA2 regulates pseudohyphal differentiation by stimulating cAMP production and regulating a signalling pathway independent of the MAP kinase cascade.

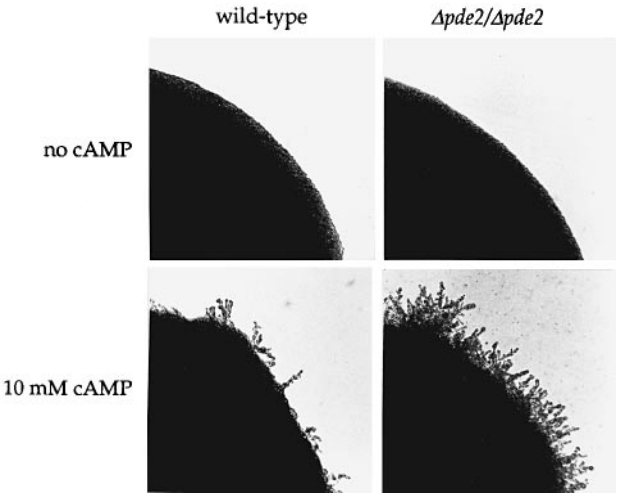

Fig. 6. cAMP allows pseudohyphal growth on nitrogen-rich media. Homozygous diploid wild-type (MLY61) and Δ*pde2*/Δ*pde2* strains (MLY162**a**/α) were incubated on media containing 5 mM ammonium sulfate without (top) or with (bottom) 10 mM cAMP for 8 days at 30°C.

Table IV. Effects of cAMP on FG(TyA)::*lacZ* expression

Genotype	cAMP (mM)	Relative β-galactosidase activity	
		50 μM NH_4^+	5 mM NH_4^+
Δ*pde2 STE*$^+$	0	4.8	1.0
	1	0.6	1.2
	10	<0.1	1.1
Δ*pde2* Δ*ste7*	0	1.8	0.5
	1	0.1	1.2
	10	<0.1	0.7
Δ*pde2* Δ*ste12*	0	0.8	0.4
	1	0.2	0.5
	10	<0.1	<0.1

Δ*pde2* (MLY213), Δ*pde2* Δ*ste7* (MLY214) and Δ*pde2* Δ*ste12* (MLY215) strains were incubated on solid media containing 50 μM or 5 mM ammonium sulfate and the indicated concentration of cAMP for 48 h at 30°C. Values are reported as relative to the Δ*pde2 STE*$^+$ strain on high nitrogen media lacking cAMP. Each determination was the average of two independent transformants, each assayed in duplicate.

Discussion

Pseudohyphal differentiation is regulated by the Gα subunit GPA2

We find that cells lacking the Gα homolog GPA2 have a defect in pseudohyphal development. A constitutively active mutant allele, *GPA2*^{Gly132Val}, which is predicted to have decreased GTPase activity, stimulates pseudohyphal growth; remarkably, this occurs even under nitrogen-rich conditions. These findings suggest that GPA2 is a component of the nitrogen sensing machinery. A second mutation, *GPA2*^{Gly299Ala}, which is predicted to prevent βγ release and interaction with signalling effectors, results in a dominant-negative *GPA2* allele that inhibits filamentation in wild-type strains. We propose that GPA2 detects nitrogen starvation conditions through its interaction with an as yet unknown receptor (see below) and stimulates filamentous growth. Our data also indicate that GPA2 plays a positive role in signalling, in contrast to the pheromone response pathway in which the βγ complex

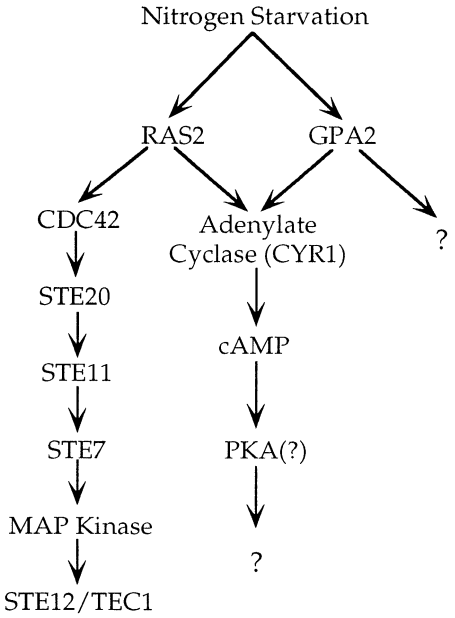

Fig. 7. A model for regulation of pseudohyphal differentiation. We propose that at least two pathways, one GPA2-dependent and one MAP kinase-dependent, regulate pseudohyphal differentiation in response to nitrogen starvation. In this model, Ras may participate in both signalling pathways. See text for more details.

transduces the signal and the GPA1 α-subunit serves an inhibitory role.

GPA2 does not regulate the MAP kinase cascade

Our initial model was that GPA2 might act analogously to the G protein-containing GPA1 and regulate the MAP kinase cascade in response to the appropriate stimuli. Epistasis analysis between *GPA2* mutations and alleles of the MAP kinase cascade indicates that this is not the case. The Δ*gpa2* pseudohyphal defect is not suppressed by constitutive alleles of MAP kinase components, whereas the dominant active *GPA2*^{Gly132Val} allele suppresses the filamentation defect in mutants lacking *STE20*, *STE11*, *STE7* or *STE12*. Moreover, despite the dramatic morphological phenotypes, mutant *GPA2* alleles had no effect on the expression of a filamentation response element regulated by STE12 and TEC1. Thus, the mechanism of activation of the MAP kinase cascade remains unknown. Nitrogen starvation does induce this reporter gene, even in the absence of GPA2, so it is likely that nutrient limitation regulates this pathway as well (Mösch *et al.*, 1996; Madhani and Fink, 1997). Bypass of the MAP kinase pathway has been demonstrated in *Candida albicans*, in which single mutant strains lacking homologs of STE20, STE7 and STE12 (CST20, HST7 and CPH1, respectively) do undergo filamentous growth in response to some, but not all, environmental signals (Liu *et al.*, 1994; Kohler and Fink, 1996; Leberer *et al.*, 1996). As outlined in Figure 7, we propose that at least two signalling pathways, either parallel or partially interconnected, regulate pseudohyphal growth; one comprised by the MAP kinase cascade and the other including GPA2. Our findings, and the studies of others (Mösch *et al.*, 1996; Madhani and Fink, 1997), suggest that both of these pathways may be nitrogen-responsive; whether this occurs by a common mechanism is yet to be determined.

A role for cAMP in filamentous growth

Our studies have also demonstrated that exogenous cAMP dramatically stimulates pseudohyphal differentiation, particularly in mutants lacking the cAMP phosphodiesterase PDE2. cAMP promotes filamentous growth on nitrogen-rich media and suppresses the pseudohyphal defects of strains lacking STE7 or STE12, phenotypes shared with the dominant active *GPA2* allele. In addition, cAMP restores filamentation to Δ*gpa2* Δ*pde2* mutant strains. The activity of cAMP provides one possible explanation for the hyperfilamentous phenotype of *RAS2*^{Gly19Val} mutant strains, which hyperactivate adenylate cyclase, thus raising intracellular cAMP (Toda *et al.*, 1985; Field *et al.*, 1988). In addition, we find that *RAS2*^{Gly19Val} suppresses the pseudohyphal defect of Δ*gpa2* mutant strains, while expression of the dominant *STE11-4* does not. Thus, the well-established role for Ras in regulation of cAMP levels appears to also be operative in filamentous growth. This suggestion, made previously (Gimeno *et al.*, 1992), is supported by the finding that overexpression of *PDE2* suppresses the enhanced filamentation phenotype conferred by activated *RAS2*^{Gly19Val} (Ward *et al.*, 1995). However, RAS2 has also been implicated as an upstream regulator of the MAP kinase cascade (Mösch *et al.*, 1996), as it is in signalling pathways in some multicellular organisms. Because cAMP suppresses the pseudohyphal defect of Δ*ste* mutant strains, the effects of cAMP are not mediated by the MAP kinase cascade. Interestingly, cAMP actually represses the FG(TyA)::*lacZ* reporter, which responds to MAP kinase activation. Further study will be necessary to clarify the role of the target of cAMP, PKA, in dimorphism in yeast. It seems likely that Ras regulates filamentous growth through multiple pathways, as has been recently proposed (Mösch and Fink, 1997), possibly one branch via cAMP and another via the MAP kinase cascade (see Figure 7).

Conserved role of G proteins and cAMP in fungal development

Regulation of fungal morphogenesis and development by G proteins and cAMP is not limited to *S.cerevisiae*. The requirement for nitrogen starvation for mating in *S.pombe* is bypassed by null mutations in Gpa2 (Isshiki *et al.*, 1992), the fission yeast homolog of GPA2. Mating in rich media is also a phenotype of adenylate cyclase (*cyr1*) mutants in fission yeast, and this phenotype is suppressed by exogenous cAMP (Maeda *et al.*, 1990). Fission yeast strains carrying dominant active *Gpa2* mutations have elevated cAMP levels (Isshiki *et al.*, 1992). In the corn pathogen *Ustilago maydis*, filamentous growth and pathogenicity are regulated by the mating type loci and result from conjugation of compatible cell types (Hartmann *et al.*, 1996). The *U.maydis* Gα subunit Gpa3 has recently been shown to regulate both virulence and mating response (Regenfelder *et al.*, 1997). G proteins also regulate development in *N.crassa* as mutants lacking the Gα homolog Gna1 are female-sterile and defective in macroconidia formation (Ivey *et al.*, 1996).

Changes in cAMP levels have been implicated in dimorphic transitions, particularly between the yeast form and the filamentous or mycelial form, in a number of fungi. In *U.maydis*, adenylate cyclase mutants have a constitutively filamentous phenotype which can be sup-

pressed by exogenous cAMP or by a mutation in the protein kinase A regulatory subunit Ubc1 (Gold *et al.*, 1994). In *N.crassa*, a PKA homolog, cot-1, is required for hyphal elongation (Yarden *et al.*, 1992). Elevated cAMP levels have been associated with mycelial growth or germ-tube formation in the dimorphic species *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *C.albicans* (Niimi *et al.*, 1980; Medoff *et al.*, 1986; Sabie and Gadd, 1992). The stimulation of filamentous growth by elevated cAMP in these organisms is similar to our findings in *S.cerevisiae*, whereas in *U.maydis* and *Mucor racemosus*, lower cAMP levels are found in the hyphal state (Paznokas and Sypherd, 1975; Gold *et al.*, 1994). Thus, regulation of cAMP metabolism is closely linked to dimorphism in a diverse group of fungi.

Nitrogen sensing: GPA2-coupled receptors?

While this work implicates GPA2 in the recognition of nitrogen starvation, we do not yet know the mechanism by which this occurs. Heterotrimeric G proteins typically bind directly to a transmembrane receptor; no such receptor has been identified that may sense nitrogen limitation. As a wide variety of structurally dissimilar compounds can satisfy cellular nitrogen requirements (reviewed in Magasanik, 1992), this signalling is potentially complex. The cell may sense the concentration of a common intracellular metabolite, in which case there may be a cytoplasmic binding protein that serves this role. Alternatively, the cell may have receptors for each potential nitrogen source, as it has multiple transmembrane permeases. Activity or inactivity of these permeases could transduce a signal through GPA2 to indicate the relative nitrogen abundance. Our recent findings support the latter model, as we find that an ammonia permease is required to promote pseudohyphal differentiation in response to limiting concentrations of ammonia (M.C.Lorenz and J.Heitman, manuscript in preparation). In carbon source metabolism, recent work also supports the model of permeases as receptors. Two glucose permease homologs participate in signalling glucose repression. The varying affinity of these permeases allows the cell to regulate the transcription of metabolic enzymes and other permeases based on the extracellular glucose concentration (Liang and Gaber, 1996; Ozcan *et al.*, 1996).

Origins of the dual function of MAP kinase cascades

The extensive connections between mating, morphogenesis and nitrogen deprivation may provide an explanation for the dual role of the MAP kinase pathway in regulating both mating and filamentous growth in yeast. As *S.cerevisiae* exists in the environment primarily as diploids, this species may have evolved a cell type specificity to mating and differentiation, where responses to nutrient stress, such as sporulation and pseudohyphal differentiation, are confined to the more common diploid cell type. In contrast, in other fungi, including the fission yeast *S.pombe* and the basidiomycetous pathogen *Cryptococcus neoformans*, which are haploid in the environment, both pheromone and nitrogen starvation are required for mating. In these organisms, the two signalling pathways function coordinately in a single cell type to integrate nutrient responses with mating and conjugation. We hypothesize that these two signalling processes evolved to function coordinately in the evolutionary precursors of these diverse fungi and that this role has been maintained in some descendents (e.g. *S.pombe* and *C.neoformans*), but developed cell type specificity in others (e.g. *S.cerevisiae*).

Materials and methods

Yeast strains and media

Standard yeast media and microbiological techniques were used (Sherman, 1991). SLAD media contains 50 μM ammonium sulfate, 2% glucose and 2% Bacto-agar (Gimeno *et al.*, 1992). Our formulation of SLAD differs slightly from previous reports (Gimeno *et al.*, 1992), and contains 0.17% yeast nitrogen base minus amino acids minus ammonium sulfate as opposed to the previously described 0.67% YNB. We have found no significant differences in pseudohyphal growth with these two formulations. Media used to induce the pGal-*GPA2* constructs (SLARG) contained 2% raffinose and 0.5% galactose.

Yeast strains are listed in Table V and are all derived from the Σ1278b background (obtained from M.Grenson). The Δ*gpa2::G418* mutant was created by the PCR mediated disruption technique of Wach *et al.* (1994), using the G418 resistance cassette from plasmid pFA6-KanMX2 and bifunctional disruption primers (homology to the G418-resistance cassette underlined): 5′-TGTTACAGCACAAATCACGCGTATTTTCAAGCAAATATCATGGGTCAGCTGAAGCTTCGTACGC-3′ and 5′-GCATGCAGTTTTGTCTCTGTTTTAGCTGTGCATTCATGGTAACACGCATAGGCCACTAG TGGATCTG-3′. Independently derived haploid strains (created in strains MLY40 and MLY41; see Table V) were mated to produce the homozygous diploid MLY132a/α. The Δ*pde2::G418* mutant strain was derived in the same manner to produce strain MLY162a/α using primers: 5′-TTTGAGATCACTACTTAATTGAAGAAAACATAACCTATTGATCAGCTGAAGCTTCGTACGC-3′ and 5′-CAATGAATGGTACAAGAAATTTTGATATTCTTGCTATTGTGGGCATAGGCCACTAGTGGATCTG-3′.

The Δ*gpa2::G418* Δ*pde2:G418* double mutant strain MLY171 was created through a cross between MLY132α and MLY162a. Δ*pde2::G418* Δ*ste* strains were constructed by disrupting *PDE2* in strains L3566 (wt, *MAT***a**/α), HLY351 (Δ*ste7*/Δ*ste7*) and HLY352 (Δ*ste12*/Δ*ste12*). Heterozygous Δ*pde2/PDE2* strains were sporulated and dissected; pseudohyphal growth was assayed in strains MLY173 (*STE*$^+$ Δ*pde2 MAT*α), MLY174 (Δ*ste7* Δ*pde2 MAT***a**) and MLY175 (Δ*ste12* Δ*pde2 MAT***a**) by expressing a plasmid containing either the *MAT***a** or *MAT*α locus; such pseudodiploid strains are competent to undergo pseudohyphal differentiation (Gimeno *et al.*, 1992).

Strains used for the FG(TyA)::*lacZ* reporter assays were constructed to allow selection for the *LEU2*-marked reporter plasmid pIL30-*LEU2*. Haploid Δ*gpa2* strains MLY132a and MLY132α were made *leu2*$^-$ using the Δ*leu2::hisG* disruption cassette from plasmid pNKY85 (Alani *et al.*, 1987), then crossed to create MLY212a/α (see Table V). Strains lacking Δ*ste20* and Δ*ste12* were constructed through G418-disruption in haploid strains MLY42 and MLY43. Oligonucleotides used to disrupt *STE20* were 5′-CACCCCATCCTAAATATCCCACAAGATCCTCGACTAATACAAGAACAGCTGAAGCTTCGTACGC-3′ and 5′-GTACCCTGCTTGCTACGTTTACTTTTGTTTATCATCTTCAGTGCATAGGCCACTAGTGGATCTG-3′ and for *STE12* were 5′-ATAGCGGAACCGCTTTCTTTATTTGAATTGTCTTGTTCACCAAGGCAGCTGAAGCTTCGTACGC-3′ and 5′-AAAAATTATATTATATCAGGTTGCATCTGGAAGGTTTTTATCGCATAGGCCACTAGTGGATCTG-3′ (homology to G418 cassette underlined). After transformation with complementing plasmids to allow mating, these strains were crossed, forming MLY216a/α (Δ*ste12::G418*/Δ*ste12::G418*) and MLY219a/α (Δ*ste20::G418*/Δ*ste20::G418*). Strains MLY173 (Δ*pde2*), MLY174 (Δ*ste7* Δ*pde2*) and MLY175 (Δ*ste12* Δ*pde2*) were converted to *leu2*$^-$ using pNKY85, resulting in MLY213, MLY214 and MLY215, respectively.

Plasmids

Yeast plasmids are listed in Table VI. The pGal-*GPA2* construct was created by PCR amplification of *GPA2* from genomic DNA using primers: 5′-TCCTCTAGAGCAAATATCATGGGTCTCTGC-3′ and 5′-CCCAAGCTTCTCAGAATGGTGCAAGTC-3′. The resulting PCR product was cloned under the control of the *GAL1,10* promoter in the *Xba*I–*Hin*dIII sites of pSEYC68 (CEN *URA3*). The pGal-*GPA2*^{Gly132Val} and pGal-*GPA2*^{Gly299Ala} alleles were created through PCR-mediated overlap site-directed mutagenesis (Ho *et al.*, 1989) using outer primers (above) and mutagenic primers (mutation in bold), for Gly132Val:

Table V. Yeast strains

Strain	Genotype	Reference
MLY40	*ura3-52 MAT*α	this study
MLY41	*ura3-52 MAT***a**	this study
MLY42	*ura3-52* Δ*leu2::hisG MAT*α	this study
MLY43	*ura3-52* Δ*leu2::hisG MAT***a**	this study
MLY61	*ura3-52/ura3-52 MAT***a**/α	this study
MLY97	*ura3-52/ura3-52* Δ*leu2::hisG/*Δ*leu2::hisG MAT***a**/α	this study
MLY132a	Δ*gpa2::G418 ura3-52 MAT***a**	this study
MLY132α	Δ*gpa2::G418 ura3-52 MAT*α	this study
MLY132a/α	Δ*gpa2::G418/*Δ*gpa2::G418 ura3-52/ura3-52 MAT***a**/α	this study
MLY162a	Δ*pde2::G418 ura3-52 MAT***a**	this study
MLY162α	Δ*pde2::G418 ura3-52 MAT*α	this study
MLY162a/α	Δ*pde2::G418/*Δ*pde2::G418 ura3-52/ura3-52 MAT***a**/α	this study
MLY171a	Δ*gpa2::G418* Δ*pde2::G418 ura3-52 MAT***a**	this study
MLY171α	Δ*gpa2::G418* Δ*pde2::G418 ura3-52 MAT*α	this study
MLY171a/α	Δ*gpa2::G418/*Δ*gpa2::G418* Δ*pde2::G418/*Δ*pde2::G418 ura3-52/ura3-52 MAT***a**/α	this study
L3566	*ura3-52/ura3-52 MAT***a**/α	Liu *et al.* (1993)
HLY351	Δ*ste7::LEU2/*Δ*ste7::LEU2 ura3-52/ura3-52 MAT***a**/α	Liu *et al.* (1993)
HLY352	Δ*ste12::LEU2/*Δ*ste12::LEU2 ura3-52/ura3-52 MAT***a**/α	Liu *et al.* (1993)
HLY492	Δ*ste20::LEU2/*Δ*ste20::LEU2 ura3-52/ura3-52 MAT***a**/α	Liu *et al.* (1993)
HLY506	Δ*ste11::LEU2/*Δ*ste11::LEU2 ura3-52/ura3-52 MAT***a**/α	Liu *et al.* (1993)
MLY173	Δ*pde2::G418 ura3-52 MAT*α	this study
MLY174	Δ*pde2::G418* Δ*ste7::LEU2* Δ*leu2::hisG ura3-52 MAT***a**	this study
MLY175	Δ*pde2::G418* Δ*ste12::LEU2* Δ*leu2::hisG ura3-52 MAT***a**	this study
MLY212a/α	Δ*gpa2::G418/*Δ*gpa2::G418* Δ*leu2::hisG/*Δ*leu2::hisG ura3-52/ura3-52 MAT***a**/α	this study
MLY213	Δ*pde2::G418* Δ*leu2::hisG ura3-52 MAT*α	this study
MLY214	Δ*pde2::G418* Δ*ste7::leu2::hisG* Δ*leu2::hisG ura3-52 MAT***a**	this study
MLY215	Δ*pde2::G418* Δ*ste12::leu2::hisG* Δ*leu2::hisG ura3-52 MAT***a**	this study
MLY216a/α	Δ*ste12::G418/*Δ*ste12::G418* Δ*leu2::hisG/*Δ*leu2::hisG ura3-52/ura3-52 MAT***a**/α	this study
MLY219a/α	Δ*ste20::G418/*Δ*ste20::G418* Δ*leu2::hisG/*Δ*leu2::hisG ura3-52/ura3-52 MAT***a**/α	this study

All strains congenic with Σ1278b.

5′-CTACTGCTGGGTGCCG**T**TGAAAGTGGTAAGTCC-3′ and 5′-GGACTTACCACTTTCA**A**CGGCACCCAGCAGTAG-3′, and for Gly-299Ala: 5′-ATATACGACGTGGGTG**C**ACAGCGTTCCGAAAGA-3′ and 5′-TCTTTCGGAACGCTGT**G**CACCCACGTCGTATAT-3′.

Assay for FG(TyA)::lacZ activity

Plasmid pIL30-*LEU2*, encoding the *lacZ* gene under the transcriptional control of the filamentation response element from Ty1 (Laloux *et al.*, 1994; Mösch *et al.*, 1996), was used for the reporter assays. To examine the effects of various *GPA2* alleles, pIL30-*LEU2* was coexpressed in wild-type (MLY97), Δ*ste20/*Δ*ste20* (MLY219**a**/α) or Δ*ste12/*Δ*ste12* (MLY216**a**/α) strains with control vector (pSEYC68), *GPA2*^{wt} (pML180), *GPA2*^{Gly132Val} (pML160) or *GPA2*^{Gly299Ala} (pML179). To examine the effects of deletion of *GPA2*, pIL30-*LEU2* was coexpressed in wild-type (MLY97) or Δ*gpa2/*Δ*gpa2* (MLY212**a**/α) strains with control vector (Yeplac195), *RAS2*^{wt} (pMW1), *RAS2*^{Gly19Val} (pMW2), *STE11-4* (pSL1509) or *PHD1* (pCG38). For the cAMP experiment, pIL30-*LEU2* was coexpressed with plasmids bearing the appropriate *MAT* locus in haploid strains MLY213 (Δ*pde2*), MLY214 (Δ*ste12* Δ*pde2*) and MLY215 (Δ*ste7* Δ*pde2*). The assays were performed essentially as described (Mösch *et al.*, 1996). Strains were grown in liquid selective media to mid-logarithmic phase, collected by centrifugation and washed with water. The cells were plated to media containing 50 μM or 5 mM ammonium sulfate and 2% glucose (0.5% galactose and 2% raffinose were used when necessary to induce expression of the *GPA2* alleles). After 48 h at 30°C, the cells were pooled and split; standard β-galactosidase assays were performed on half of the pool, using chlorophenolred-β-D-galactopyranoside (CPRG; Calbiochem) as substrate. The other half of the pool was disrupted by agitation with glass beads in lysis buffer (100 mM Tris–Cl, pH 8.0, 20% glycerol, 1 mM DTT, 100 U/ml aprotinin and 0.5 μM PMSF). Protein concentrations from the clarified extracts were determined using a Bradford assay (Bio-Rad), with BSA as a standard. β-galactosidase activities were normalized to protein concentration.

Photomicroscopy

Representative colonies were photographed directly on agar plates using a Nikon Axiophot-2 microscope with a 10× objective and 2.5× trinocular camera adaptor, except in Figure 2B and Figure 4B where a 20× objective and 2.5× trinocular adaptor were used.

Table VI. Plasmids

Plasmid	Description	Reference
pSEYC68	*CEN URA3* pGal1,10	S.Elledge
pML160	*GPA2-2* in pSEYC68 (Gly132Val)	this study
pML179	*GPA2-3* in pSEYC68 (Gly299Ala)	this study
pML180	*GPA2* in pSEYC68	this study
YEplac195	2μ *URA3*	Gietz and Sugino (1988)
pMW1	*CEN URA3 RAS2*	Ward *et al.* (1995)
pMW2	*CEN URA3 RAS2* (Gly19Val)	Ward *et al.* (1995)
pSL1509	*CEN URA3 STE11-4*	Stevenson *et al.* (1992)
pNC272	2μ *URA3* pGal-*STE12*	Liu *et al.* (1993)
pCG38	2μ *URA3 PHD1*	Gimeno and Fink (1994)
MATa	*CEN URA3 MAT***a**	M.Hall
MATα	*CEN URA3 MAT*α	M.Hall
pIL30-LEU2	*CEN LEU2* FG(TyA)::*lacZ*	Mösch *et al.* (1996)
pNKY85	Δ*leu2::hisG-URA3-hisG*	Alani *et al.* (1987)

Identification of Gβ and Gγ homologues

To find potential β subunits, BLAST searches of the yeast database were performed using *S.cerevisiae* STE4 (accession number Z75120; Whiteway *et al.*, 1989) and *S.pombe* Gpb1 (L28061; Kim *et al.*, 1996) sequences. Candidate genes, listed below (Table VII), were disrupted through the G418/PCR disruption protocol described above in diploid strain MLY61. Gγ candidates were identified based on small size (less than 150 amino acids) and a C-terminal lipid modification motif (CAAX box) through the Yeast Protein Database (http://www.YPDhome.html).

Table VII.

Gene	β/γ	Acc. No.	Deletion phenotype
DIP2	β	Z73301	essential
YCR072	β	X59720	essential
PWP2	β	X78964	essential
YNL006	β	Z71282	essential
YNL317	β	Z71593	essential
YMR116	β	Z49702	no effect
YJL112	β	Z49387	no effect
YLR122	β	U19027	no effect
YDL009	γ	Z74059	no effect
YBL048	γ	Z35809	no effect
YOL014	γ	Z74756	no effect

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Note added in proof

We have recently found that the Δ*gpa2* and Δ*ras2* mutations exhibit a near synthetic lethal growth defect that is suppressed by a Δ*pde2* mutation, which increases intracellular cAMP levels (data not shown). Similar observations have recently been reported by Y.Xue and J.Hirsch (personal communication, manuscript submitted) and by Kubler *et al.*, who also reported that GPA2 is required for pseudohyphal differentiation [Kubler *et al.* (1997) *J. Biol. Chem.*, **272**, 20321–20323].