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GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p G_α subunit and functions in a Ras-independent pathway

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The yeast *RAS1* and *RAS2* genes appear to be involved in control of cell growth in response to nutrients. Here we show that this growth control also involves a signal mediated by the heterotrimeric G protein α subunit homolog encoded by *GPA2*. A *GPA2* null allele conferred a severe growth defect on cells containing a null allele of *RAS2*, although either mutation alone had little effect on growth rate. A constitutive allele of *GPA2* could stimulate growth of a strain lacking both *RAS* genes. Constitutive *GPA2* conferred heat shock sensitivity on both wild-type cells and cells lacking *RAS* function, but had no effect in a strain containing a null allele of *SCH9*, which encodes a kinase related to protein kinase A. The *GPR1* gene was isolated and was found to encode a protein with the characteristics of a G protein-coupled receptor. Double Δ*gpr1* Δ*ras2* mutants displayed a severe growth defect that was suppressed by expression of the constitutive allele of *GPA2*, confirming that *GPR1* acts upstream of *GPA2*. Gpr1p is expressed on the cell surface and requires sequences in the membrane-proximal region of its third cytoplasmic loop for function, as expected for a G protein-coupled receptor. *GPR1* RNA was induced when cells were starved for nitrogen and amino acids. These results are consistent with a model in which the *GPR1/GPA2* pathway activates the Sch9p kinase to generate a response that acts in parallel with that generated by the Ras/cAMP pathway, resulting in the integration of nutrient signals.

Keywords: *GPA2*/*GPR1*/G protein/Ras/signal transduction/yeast

Introduction

Signal transduction pathways that regulate growth in response to nutrients are essential for cell viability; these pathways, however, are not well understood at the molecular level. In yeast, one aspect of nutrient-induced growth control is thought to be mediated by the *RAS*/cAMP pathway, which responds to the addition of a fermentable sugar by a transient increase in cAMP concentration (Broach, 1991; Thevelein, 1994). The presence of the sugar generates a signal that has not been defined but appears to impinge on the Ras proteins. Once activated, the Ras proteins stimulate adenylyl cyclase to produce cAMP (Kataoka *et al.*, 1985; Toda *et al.*, 1985). cAMP binds the regulatory subunit of protein kinase A (PKA), which results in the release of active catalytic kinase subunits that then phosphorylate targets involved in energy metabolism and cell growth (Matsumoto *et al.*, 1982; Toda *et al.*, 1987a,b). This pathway is essential, because deletion of both of the genes that encode the Ras proteins, *RAS1* and *RAS2*, or of the single gene that encodes adenylyl cyclase, *CYR1*, results in inviable cells. The inviability of cells containing deletions of the *RAS* genes or of *CYR1* is suppressed by overexpression of the *SCH9* gene, which encodes a protein kinase related to PKA (Toda *et al.*, 1988). However, it is not clear whether the *SCH9* gene product functions directly in the *RAS*/cAMP pathway or whether it functions in a parallel pathway.

Yeast cells express many different classes of signaling molecules in addition to guanine nucleotide-binding proteins of the Ras family. One such class is composed of heterotrimeric G proteins made up of α, β and γ subunits. The identification of G protein α subunits in yeast was accomplished originally by screening a yeast genomic library with a probe made from cDNA clones encoding mammalian $G_{\alpha i2}$ and $G_{\alpha o}$ (Nakafuku *et al.*, 1987, 1988). Genes encoding two G_α subunits, *GPA1* and *GPA2*, were isolated by this procedure. Sequencing of the entire *Saccharomyces cerevisiae* genome has now established that *GPA1* and *GPA2* are the only two G_α subunit genes encoded in this genome, indicating that there are two pathways in yeast that signal through G_α subunits. *GPA1* subsequently was shown to be involved in mediating the pheromone response signal transduction pathway (Dietzel and Kurjan, 1987; Miyajima *et al.*, 1987). Whereas Gpa1p is capable of productively coupling to the α-factor receptor, Gpa2p is not (Blumer and Thorner, 1990).

GPA2 was proposed to function in the regulation of cAMP levels, based on the finding that its overexpression causes a 2-fold increase in the level of cAMP induced by glucose (Nakafuku *et al.*, 1988). Overexpression of *GPA2* also restores the cAMP response to a strain containing a temperature-sensitive mutation in the *RAS2* gene and suppresses the growth defect of this strain. These results support the idea that the Gpa2p protein is involved in regulating the production of cAMP by adenylyl cyclase, in the same way that mammalian $G_{\alpha s}$ stimulates adenylyl cyclase activity (Simon *et al.*, 1991). However, it was shown that deletion of the *GPA2* gene had no effect on cAMP levels (Nakafuku *et al.*, 1988), which made it difficult to establish whether *GPA2* is involved in this process. The phenotype of the *GPA2* deletion is inconclusive because deletion of genes that are known to be involved in cAMP regulation, such as the phosphodiesterase genes, has little effect on cAMP levels. This phenomenon is due to feedback control on cAMP levels by the Ras proteins over many orders of magnitude (Nikawa *et al.*, 1987).

Therefore, changes in cAMP levels are difficult to document in cells with intact *RAS* genes.

In the study described here, we show that the *GPA2* and *RAS* pathways have partially redundant functions that act in parallel, and that cells with defects in both pathways are impaired for growth. We have also isolated a gene encoding a putative G protein-coupled receptor that appears to provide the upstream signal that activates Gpa2p, which will ultimately allow one of the ligands that initiates the nutrient signaling pathway to be identified.

Results

Activated Gpa2p confers phenotypes similar to those produced by high cAMP levels

Overexpression of the G protein α subunit encoded by *GPA2* increases the level of cAMP in cells and suppresses the phenotype of a *ras2*^{ts} mutant (Nakafuku *et al.*, 1988). One interpretation of these observations is that the normal function of Gpa2p is to regulate the concentration of cAMP. Activated alleles of *RAS2*, which are known to increase cAMP levels, confer characteristic phenotypes such as decreases in sporulation efficiency and accumulation of storage carbohydrates (Toda *et al.*, 1985). To test whether activation of Gpa2p has similar effects on cell physiology, a constitutive allele of *GPA2* was constructed by replacing the arginine at position 273 with an alanine. The same change in the corresponding residue of mammalian $G_{\alpha s}$, which is also the site modified by cholera toxin, results in a protein that constitutively activates adenylyl cyclase and displays a 100-fold decreased rate of GTP hydrolysis (Freissmuth and Gilman, 1989). Diploid cells transformed with the constitutive *GPA2*^{R273A} allele or with an activated allele of *RAS2* were incubated in liquid sporulation medium for 3 days, and the degree of sporulation was determined. Whereas the percentage sporulation of control cells was 42.1 ± 4.0, that of cells containing the constitutive *GPA2*^{R273A} allele was 11.1 ± 0.9 (Figure 1A). The percentage sporulation of cells containing the activated *RAS2* allele was 18.8 ± 0.4, slightly higher than the value obtained for cells containing constitutive *GPA2*. These results indicate that activation of Gpa2p causes a significant sporulation defect that is comparable with the defect conferred by activated Ras2p.

Another aspect of cell physiology that is affected by elevated cAMP levels is the ability to survive heat shock (Toda *et al.*, 1987a). To test the effect of constitutive *GPA2* on thermotolerance, stationary phase cultures of haploid cells transformed with the constitutive *GPA2*^{R273A} allele or with the activated allele of *RAS2* were exposed to a heat shock, and the percentage of surviving cells was determined. Cells containing constitutive *GPA2* were ~60-fold more sensitive to heat shock than wild-type cells (Figure 1B). An even larger effect was seen with activated *RAS2*, which caused a 10-fold increase in heat shock sensitivity compared with constitutive *GPA2*. Thus, in contrast to the sporulation results, the degree of heat shock sensitivity is substantially greater in cells containing activated Ras2p than in cells containing activated Gpa2p.

In summary, the constitutive *GPA2* allele caused changes in cell physiology that are consistent with a role for Gpa2p either in cAMP regulation or in a pathway that is redundant with the cAMP/PKA pathway. These experiments do not distinguish between cAMP-dependent and cAMP-independent mechanisms because the cellular functions that were measured can be regulated by both mechanisms (Cameron *et al.*, 1988).

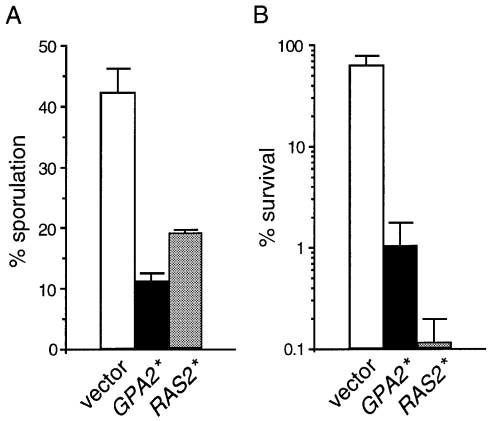

Fig. 1. Physiological effects of constitutively active *GPA2*. (**A**) A wild-type diploid strain (W303) transformed with either plasmid pG2CT-112.2, which contains a constitutive *GPA2*^{R273A} allele (*GPA2**), plasmid YCp50-RAS2^{ala18val19}, which contains an activated *RAS2* allele (*RAS2**), or vector YEplac112 (vector) was incubated in sporulation medium for 3 days and the percentage sporulation was determined by visual inspection. The value for pG2CT-112.2 is represented by the filled bar (n = 4), that for YCp50-RAS2^{ala18val19} by the shaded bar (n = 3) and that for YEplac112 by the open bar (n = 4). Values shown are the mean and standard deviation from independent experiments. (**B**) A wild-type haploid strain (W3031A) transformed with either plasmid pG2CT-112.2 (*GPA2**), plasmid YCp50-RAS2^{ala18val19} (*RAS2**) or vector YEplac112 (vector) was grown to saturation for 2 days, incubated at 50°C for 20 min, and diluted and plated to determine the percentage survival. The value for pG2CT-112.2 is represented by the filled bar (n = 4), that for YCp50-RAS2^{ala18val19} by the shaded bar (n = 3) and that for YEplac112 by the open bar (n = 4). Values shown are the mean and standard deviation from independent experiments.

GPA2 and RAS2 are functionally related

Previous results have shown that overexpression of *GPA2* suppresses the growth phenotype of a *ras2*^{ts} mutant (Nakafuku *et al.*, 1988), suggesting that there is a functional relationship between *GPA2* and the *RAS* genes. The potential relationship between these genes was explored further by investigating whether null alleles of *GPA2* and *RAS* genes display genetic interactions. To determine the phenotype of Δ*gpa2* Δ*ras2* double mutants, a diploid strain heterozygous for *GPA2* and *RAS2* deletion alleles was sporulated and tetrads were dissected. Although strains containing single Δ*gpa2* or Δ*ras2* mutations grew normally, strains containing both Δ*gpa2* and Δ*ras2* mutations displayed a severe growth defect (Figure 2A). Colonies of cells containing double Δ*gpa2* Δ*ras2* mutations were barely visible after 2 days of growth, and were still quite small after 3 days of growth. Therefore, the phenotype of the double mutant uncovers a requirement for a growth function that can be supplied by either *GPA2* or *RAS2*. This function is specific to *RAS2*, because strains containing double Δ*gpa2* Δ*ras1* mutations displayed little or no growth defect (data not shown). The synthetic growth defect of Δ*gpa2* Δ*ras2* strains has also been seen by investigators studying the involvement of *GPA2* in pseudohyphal development (Kübler *et al.*, 1997; Lorenz and Heitman, 1997).

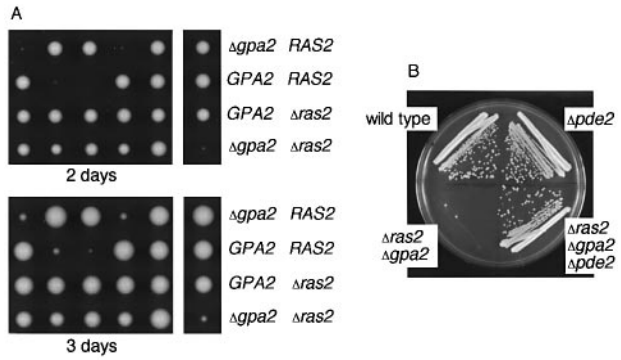

Fig. 2. Phenotype of *gpa2* and *ras2* mutants. (**A**) A diploid heterozygous for *gpa2::TRP1* and *ras2::LEU2* mutations (H91) was sporulated and tetrads were dissected. Left: representative sample of tetrads after growth for 2 or 3 days, as indicated. Right: tetrad labeled with genotype of spore colonies. (**B**) Strains with the following genotypes were streaked out for single colonies: wild-type; *RAS2 GPA2 PDE2* (W3031A); *RAS2 GPA2 pde2::HIS3* (YX4); *ras2::LEU2 gpa2::TRP1 PDE2* (YX8); and *ras2::LEU2 gpa2::TRP1 pde2::HIS3* (H95-3D).

In *S.cerevisiae*, *RAS2* plays a role in at least two different processes, nutrient signaling and completion of mitosis. Both of these processes are involved in regulating cell growth, but the mitotic function of *RAS* does not act through cAMP generation (Morishita *et al.*, 1995). To determine whether the redundant function of *GPA2* and *RAS2* involves cAMP regulation, a triple mutant was constructed that contained deletion alleles of *GPA2*, *RAS2* and *PDE2*, which encodes a high affinity phosphodiesterase (Wilson and Tatchell, 1988). Deletion of *PDE2* restored normal growth to a Δ*gpa2* Δ*ras2* strain (Figure 2B), indicating that elevation of the *in vivo* cAMP concentration compensated for the lack of *GPA2* and *RAS2*. It is therefore possible that the redundant function of these genes involves positive control of cAMP levels or that the function of *GPA2* controls a pathway that is redundant with the Ras/cAMP pathway.

GPA2 acts in parallel to the RAS function

The synthetic slow growth phenotype observed in a Δ*gpa2* Δ*ras2* strain is consistent with two models of signaling by Ras proteins and Gpa2p. One possible model is that Gpa2p acts upstream of Ras1p and Ras2p in the same signaling pathway. Upstream activation of this pathway would stimulate Gpa2p to transmit the signal to the Ras proteins by increasing their activity. If this were the case, the Δ*gpa2* Δ*ras2* phenotype would be due to the low basal activity of Ras1p, which would be insufficient for full stimulation of adenylyl cyclase. An alternative model is that Gpa2p acts through a pathway that is independent of the Ras proteins. In this case, the Δ*gpa2* Δ*ras2* phenotype would be due to the lack of stimulatory inputs from both of the parallel pathways. These two models can be distinguished by testing whether the effects of constitutive *GPA2* occur in a strain that lacks all Ras proteins.

The constitutive *GPA2* allele did not suppress the inviability of a Δ*ras1* Δ*ras2* strain (data not shown). The finding that activated Gpa2p cannot compensate for the lack of Ras function can be interpreted in a manner consistent with either of the models described above. If Gpa2p acts upstream of the Ras proteins, then activated Gpa2p would have no effect in the absence of Ras. Alternatively, if the Gpa2p and Ras pathways act independently, activation of Gpa2p alone could fail to maintain viability.

To determine the effect of constitutive Gpa2p in a *RAS* null strain, $GPA2^{R273A}$ was overexpressed in a strain containing deletions of the *RAS1*, *RAS2* and *PDE2* genes. In this strain, the lethal phenotype associated with *RAS* null alleles is suppressed by a deletion of the phosphodiesterase gene (Wilson and Tatchell, 1988). Overexpression of $GPA2^{R273A}$ conferred a growth advantage in a Δ*ras1* Δ*ras2* Δ*pde2* background when compared with vector alone (Figure 3A). Measurement of growth rates indicated that the strain containing the vector had a doubling time of ~225 min, whereas the strain containing $GPA2^{R273A}$ had a doubling time of ~102 min, a difference of ~2-fold. This result is consistent with the idea that *GPA2* and *RAS* act in independent pathways. However, because the $GPA2^{R273A}$ allele did not have a very dramatic effect on the growth rate of a *RAS* null strain, another experiment was performed to confirm this result.

In wild-type cells, the $GPA2^{R273A}$ allele conferred a significant decrease in heat shock resistance (Figure 1B), demonstrating that this parameter is a sensitive measure of *GPA2* function. The effect of $GPA2^{R273A}$ on thermotolerance was therefore tested in a *RAS* null strain. Whereas Δ*ras1* Δ*ras2* Δ*pde2* cells containing vector displayed 85% survival after heat shock, the same cells overexpressing $GPA2^{R273A}$ displayed only 3% survival (Figure 3B). These results indicate that *RAS* function is not required for the physiological effects of constitutive *GPA2*, and strongly suggest that the *GPA2* and *RAS* genes act in independent pathways.

GPA2 function requires SCH9

The results presented above demonstrate that, although constitutive *GPA2* confers phenotypes similar to those produced by high cAMP levels, it does not act through the *RAS* genes. These characteristics are similar to those of the *SCH9* gene, which also acts in parallel to *RAS* (Toda *et al.*, 1988). *SCH9* encodes a kinase related to cAMP-dependent kinase, and its overexpression compensates for the lack of *RAS* function. *SCH9* has been shown recently to be required for the response of nitrogen-starved cells to the re-addition of nitrogen (Crauwels *et al.*, 1997). To investigate whether the *GPA2* function requires *SCH9*, the effect of $GPA2^{R273A}$ on heat shock resistance was determined in a Δ*sch9* strain. In contrast to the result obtained with a strain lacking *RAS* function, expression of $GPA2^{R273A}$ in a Δ*sch9* strain had no effect on heat shock resistance (Figure 3B). This finding suggests that *SCH9* is required for the function of *GPA2*, and is consistent with a model in which the Sch9p kinase acts downstream of Gpa2p in a signaling pathway that does not include Ras. To test whether *RAS2* function also requires *SCH9*, the effect of an activated allele of *RAS2* on heat shock resistance was determined in a Δ*sch9* strain. Expression of activated *RAS2* in either a wild-type or Δ*sch9* strain conferred the same degree of heat shock sensitivity (Figure 3C), suggesting that *RAS2* function is independent of *SCH9*.

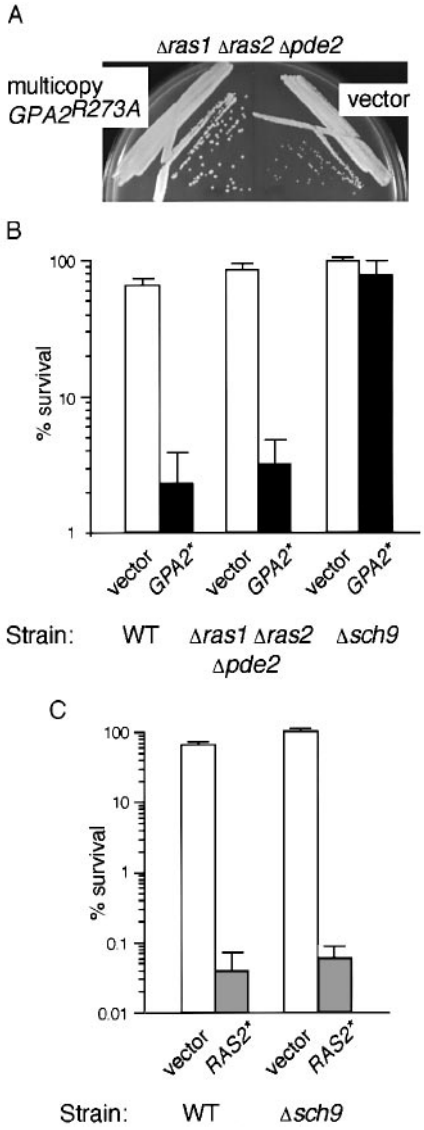

Fig. 3. Effect of constitutively active *GPA2* in strains lacking *RAS* or *SCH9* genes. (**A**) A strain with the genotype *ras1::URA3 ras2::LEU2 pde2::HIS3* (H97-36D) was transformed with a multicopy $GPA2^{R273A}$ plasmid (pG2CT-112.2) or vector (YEplac112) and streaked out for single colonies on selective medium. (**B**) Heat shock sensitivity of the following strains was assayed as described in the legend to Figure 1: a wild-type strain (B2-3C) transformed with YEplac112 (vector), a wild-type strain (B2-1B) transformed with plasmid pG2CT-112.2 (*GPA2**), a strain with the genotype *ras1::URA3 ras2::LEU2 pde2::HIS3* (H97-36D) transformed with either YEplac112 or pG2CT-112.2, a strain with the genotype *sch9::URA3* (B2-3A) transformed with YEplac112 and a strain with the genotype *sch9::URA3* (B2-1A) transformed with pG2CT-112.2. Values for pG2CT-112.2 are represented by the filled bars ($n = 3$) and those for YEplac112 by the open bars ($n = 3$). (**C**) Heat shock sensitivity of the following strains was assayed as described in the legend to Figure 1: a wild-type strain (B2-3C) transformed with YEplac112 (vector), a wild-type strain (B2-2D) transformed with plasmid YCp50-$RAS2^{ala18val19}$ (*RAS2**), a strain with the genotype *sch9::URA3* (B2-3A) transformed with YEplac112 and a strain with the genotype *sch9::TRP1* (B2-1A.T) transformed with plasmid YCp50-$RAS2^{ala18val19}$ (*RAS2**). Values for YCp50-$RAS2^{ala18val19}$ are represented by the shaded bars ($n = 3$) and those for YEplac112 by the open bars ($n = 3$).

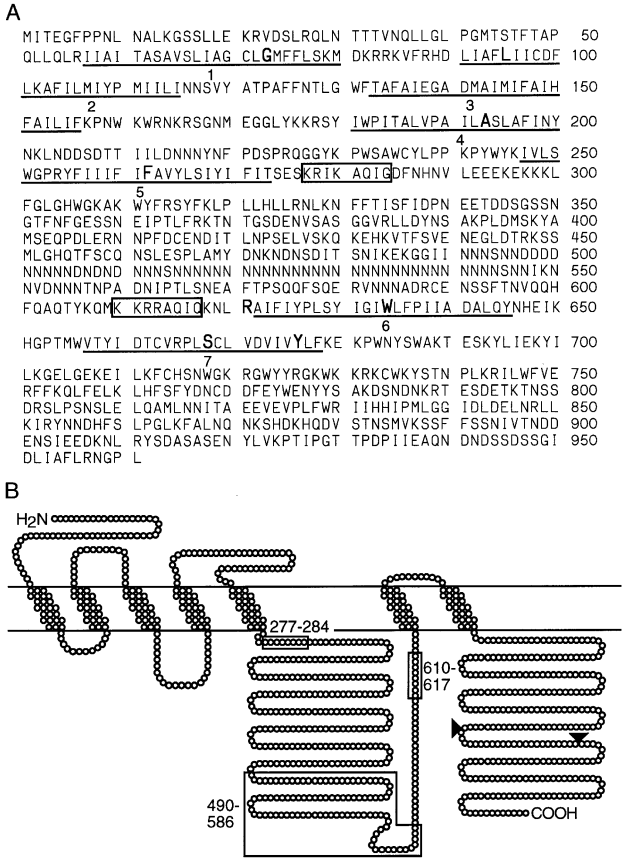

Fig. 4. *GPR1* encodes a protein that associates with Gpa2p and has seven transmembrane domains. (**A**) The sequence of Gpr1p with potential transmembrane domains underlined. Residues shown in bold are conserved in almost all G protein-coupled receptors. Boxed regions show sequence motifs in the third cytoplasmic loop that are related to sequences found in the third cytoplasmic loops of the pheromone receptors. (**B**) Predicted topology of Gpr1p in the membrane. Arrowheads indicate junction sites in plasmids obtained from the two-hybrid screen. Boxed regions show sequence motifs related to sequences in the third cytoplasmic loops of the pheromone receptors and an asparagine-rich region in the third cytoplasmic loop.

Isolation of GPR1, a putative receptor gene

To isolate other components of the *GPA2* signaling pathway, a two-hybrid protein interaction screen (Fields and Song, 1989) was performed using *GPA2* as the bait. Screening of a yeast genomic library with a *GPA2* fusion construct resulted in the isolation of plasmids containing short segments of an uncharacterized gene that was given the name *GPR1*. The full-length *GPR1* gene encodes a protein of 961 amino acids (DDBJ/EMBL/GenBank accession No. Z74083) that is predicted to contain seven membrane-spanning domains, a feature characteristic of G protein-coupled receptors (Figure 4A). The putative structure of this protein indicates that it would contain a very large third cytoplasmic loop of ~346 amino acids, and a large cytoplasmic tail of ~281 amino acids. The third cytoplasmic loop contains two copies of a short, basic sequence; one copy is present at the N-terminal end of the loop and the other copy is present at the C-terminal end (Figure 4A and B, boxed). The third cytoplasmic loop also contains a polyasparagine stretch of unknown function.

In contrast to the pheromone receptors, which have no homology to other receptors of this class, Gpr1p can be aligned with the G protein-coupled receptor superfamily (Baldwin, 1993). In particular, Gpr1p contains several amino acids in its transmembrane domains that are con-

served within this superfamily. Of these, the most highly conserved residues are the alanine at position 193 in transmembrane domain 4, the phenylalanine at position 262 in transmembrane domain 5, the tryptophan at position 634 in transmembrane domain 6, and the tyrosine at position 676 in transmembrane domain 7 (Figure 4A). When these residues are positioned with respect to the predicted arrangement of the receptor transmembrane α-helices (Baldwin, 1993), they all face away from the surrounding membrane lipid and toward the center of the molecule or the other helices. Intramolecular interactions between these transmembrane α-helices are thought to maintain the structure of the receptor in the membrane and allow it to bind the G protein.

Two *GPR1*-containing plasmids were isolated in the two-hybrid screen; one contained the coding region for the C-terminal 122 amino acids and the other contained the coding region for the C-terminal 99 amino acids (Figure 4B). The cytoplasmic tail regions of several mammalian G protein-coupled receptors have also been shown to interact with G_α subunits, although in these cases the membrane-proximal region of the cytoplasmic tail contains the G_α-binding activity (O'Dowd *et al.*, 1988; König *et al.*, 1989; Münch *et al.*, 1991; Ohyama *et al.*, 1992; Hawes *et al.*, 1994). The finding that the C-terminal end of the Gpr1p cytoplasmic tail interacts with Gpa2p suggests that other G_α subunits may also interact with this region of their associated receptors. If the assays used previously to measure α-subunit–receptor binding are less sensitive than the two-hybrid assay, this area of contact could have been overlooked.

GPR1 acts upstream of GPA2

To determine if *GPR1* acts in the same signaling pathway as *GPA2*, a diploid strain heterozygous for *GPR1* and *RAS2* deletion alleles was sporulated and tetrads were dissected. Strains containing single Δ*gpr1* or Δ*ras2* mutations grew normally, but strains containing both Δ*gpr1* and Δ*ras2* mutations displayed a severe growth defect (Figure 5A). The slow growth rate of Δ*gpr1* Δ*ras2* strains was essentially identical to that of Δ*gpa2* Δ*ras2* strains (Figure 2A), suggesting that *GPR1* and *GPA2* function in the same process. An experiment was therefore performed to determine the effect of different *GPA2* alleles on the growth rate of a Δ*gpr1* Δ*ras2* strain. A single copy plasmid containing *GPA2* had no effect on the growth rate of the Δ*gpr1* Δ*ras2* strain; however, multicopy *GPA2* partially suppressed the growth defect of this strain (Figure 5B). Moreover, the constitutive $GPA2^{R273A}$ allele in single copy completely suppressed the growth phenotype of the Δ*gpr1* Δ*ras2* strain. The most straightforward interpretation of these results is that Gpa2p acts downstream of Gpr1p in the same signaling pathway, as would be expected for a G_α subunit and its associated receptor. In addition, the finding that Δ*gpa2* and Δ*gpr1* mutations produce the same degree of growth inhibition in a Δ*ras2* strain suggests that Gpr1p is the only receptor that is coupled to Gpa2p. The idea that Gpr1p and Gpa2p act in the same signaling pathway is also supported by the finding that the growth defect of a Δ*gpa2* Δ*gpr1* Δ*ras2* strain is no more severe than that of a Δ*gpa2* Δ*ras2* strain (data not shown).

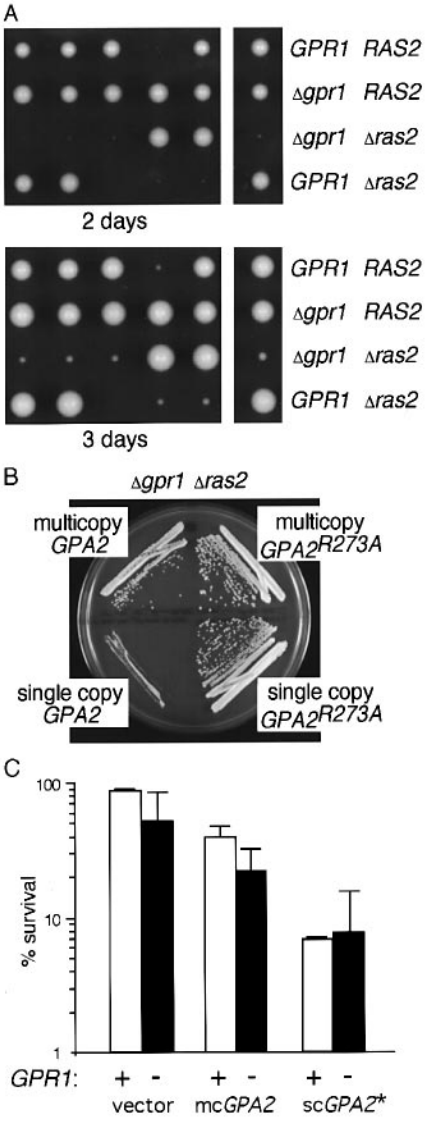

Fig. 5. Phenotype of *gpr1* and *ras2* mutants. (**A**) A diploid heterozygous for *gpr1::HIS3* and *ras2::LEU2* mutations (H96) was sporulated and tetrads were dissected. Left: representative sample of tetrads after growth for 2 or 3 days, as indicated. Right: tetrad labeled with the genotype of spore colonies. (**B**) A strain with the genotype *gpr1::HIS3 ras2::LEU2* (YX12) was transformed with either a single copy *GPA2* plasmid (pGPA2-33.1), a multicopy *GPA2* plasmid (pGPA2-112.1), a single copy $GPA2^{R273A}$ plasmid (pG2CT-33.2) or a multicopy $GPA2^{R273A}$ plasmid (pG2CT-112.2) and streaked out for single colonies on selective medium. (**C**) A wild-type strain (W3031B) and a strain with the genotype *gpr1::HIS3* (YX6B) transformed with either multicopy *GPA2* plasmid pGPA2-112.1 (mc*GPA2*), single copy $GPA2^{R273A}$ plasmid pG2CT-33.2 (sc*GPA2**) or vector YEplac112 (vector) were grown to saturation for 2 days, incubated at 50°C for 20 min, and diluted and plated to determine the percentage survival. Values for *GPR1* strains are represented by the open bars ($n = 3$) and those for *gpr1::HIS3* strains by the filled bars ($n = 3$).

GPR1 is not required for GPA2 expression or basal activity

The genetic experiment that places the function of *GPR1* upstream of *GPA2* is consistent with more than one possible relationship of their gene products. As mentioned above, a likely possibility is that *GPR1* encodes the receptor that couples to Gpa2p. However, alternative possibilities are that the *GPR1* gene product is required for the expression of the *GPA2* gene or that it is required

to maintain the stability or activity of the Gpa2p protein. To test these possibilities, the effect of *GPA2* on heat shock sensitivity was determined in cells lacking *GPR1* function.

Wild-type cells carrying a single copy plasmid with the constitutive $GPA2^{R273A}$ allele under its own promoter (sc*GPA2**, Figure 5C) were 13-fold more sensitive to heat shock than cells carrying vector alone. Expression of $GPA2^{R273A}$ conferred a similar increase in heat shock sensitivity on Δ*gpr1* cells. If the function of the *GPR1* gene product was to promote efficient expression of the *GPA2* gene, then a null allele of *GPR1* would be expected to decrease the expression of $GPA2^{R273A}$ and thus decrease its ability to confer heat shock sensitivity. These results therefore demonstrate that *GPR1* is not required for efficient expression of *GPA2*.

Overexpression of the wild-type *GPA2* gene by expressing it from the GAPDH promoter on a multicopy plasmid conferred a modest 2-fold increase in heat shock sensitivity on wild-type cells (mc*GPA2*, Figure 5C). Overexpression of *GPA2* also conferred an ~2-fold increase in heat shock sensitivity on Δ*gpr1* cells when compared with vector alone in the same cells. Therefore, the basal activity of Gpa2p is maintained in the absence of a functional *GPR1* gene, suggesting that *GPR1* is not required for the stability or activity of the Gpa2p protein.

Strains containing the Δ*gpr1* mutation and wild-type *GPA2* were slightly more sensitive to heat shock than the corresponding *GPR1* strains, suggesting that the *GPA2* pathway is activated to a low level in Δ*gpr1* strains. This phenotype can be compared with deletion of the pheromone receptor gene *STE3*, which confers an ~2-fold increase in the basal activity of the pheromone response pathway (Boone *et al.*, 1993). Therefore, these results are entirely consistent with the assignment of Gpr1p as the receptor that couples to Gpa2p.

Gpr1p is localized to the cell surface

If Gpr1p is a member of the G protein-coupled receptor family, then it should be located at the cell surface. To determine the subcellular location of Gpr1p, the *GPR1* gene was fused with the coding sequence of green fluorescent protein (GFP; Chalfie *et al.*, 1994) and transformed into wild-type cells. The *GPR1–GFP* construct complemented the growth defect of a Δ*gpr1* Δ*ras2* strain, demonstrating that the fusion gene is fully active (data not shown). Cells expressing *GPR1–GFP* showed a cell surface staining pattern, demonstrating that Gpr1p is localized at the plasma membrane (Figure 6). In addition to cell surface staining, a portion of the signal appeared in discrete foci within cells, suggesting that Gpr1p may also be located on intracellular vesicles.

Membrane-proximal regions of the Gpr1p third cytoplasmic loop are required for function

A number of studies have demonstrated that G protein-coupled receptors contain sequences in the membrane-proximal regions of their third cytoplasmic loops that are required for coupling to the G protein (Baldwin, 1994). The Gpr1p third cytoplasmic loop contains the sequences KRIKAQIG near its N-terminal end and KKRRAQIQ near its C-terminal end (Figure 4A and B, boxed). A related sequence is present in the third cytoplasmic loop of the *S.cerevisiae* pheromone receptors, which have very

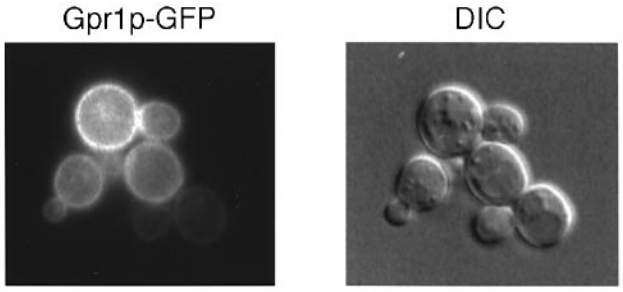

Fig. 6. Gpr1p is localized to the cell surface. A wild-type strain (W3031A) was transformed with a multicopy plasmid containing a *GPR1–GFP* fusion construct (pGPR1-GFP.1) and viewed by fluorescence microscopy with an FITC filter (Gpr1p-GFP) or with differential interference contrast (DIC) optics.

short third cytoplasmic loops. Mutation of some these residues in the α-factor receptor affects its ability to couple to the Gpa1p G_α subunit without affecting its ability to bind ligand (Clark *et al.*, 1994). Likewise, the pheromone receptors from *Schizosaccharomyces pombe* have a related sequence in their third cytoplasmic loops (Kitamura and Shimoda, 1991; Tanaka *et al.*, 1993). An alignment of these sequences is shown in Figure 7A.

To test whether the membrane-proximal regions of the third cytoplasmic loop of Gpr1p are required for its function, each of these regions was deleted individually from the *GPR1* coding sequence. *GPR1* mutations in which the coding region contained a deletion of eight amino acids at the N- (residues 277–284) or C-terminal region (residues 610–617) of the third cytoplasmic loop were unable to complement the growth defect of a Δ*gpr1* Δ*ras2* strain (Figure 7B). The third cytoplasmic loop of Gpr1p also contains a long stretch of polyasparagine residues (Figure 4B, boxed). To determine whether this asparagine-rich sequence is required for Gpr1p function, a *GPR1* mutation containing a deletion of this region (residues 490–586) was also constructed. The $GPR1^{d490–596}$ gene was able to complement the growth defect of a Δ*gpr1* Δ*ras2* strain (Figure 7B).

The abundance and localization of the mutant Gpr1p proteins was investigated by tagging each construct with GFP. To determine the relative abundance of the mutated versions of Gpr1p, an immunoblot containing cell extracts from strains expressing each of the *GPR1* deletions was probed with anti-GFP antiserum. The $GPR1^{d277–284}$, $GPR1^{d490–596}$ and $GPR1^{d610–617}$ constructs all expressed proteins at a level equal to or higher than the wild-type expression level (Figure 7C, lanes 2–5). Localization of the mutated versions of Gpr1p was determined by observing cells expressing the GFP-tagged versions of the proteins by fluorescence microscopy. The $GPR1^{d277–284}$, $GPR1^{d490–596}$ and $GPR1^{d610–617}$ constructs all expressed proteins that were localized to the cell surface (Figure 7D). These results demonstrate that deletion of an internal region of the Gpr1p third cytoplasmic loop that encompasses 97 amino acids has no effect on the function of Gpr1p, but that deletion of the membrane-proximal regions of this loop abolishes the function of Gpr1p. This evidence supports the idea that Gpr1p is a member of the G protein-coupled receptor family.

It was also of interest to determine whether the region of *GPR1* that encodes the cytoplasmic tail of the protein

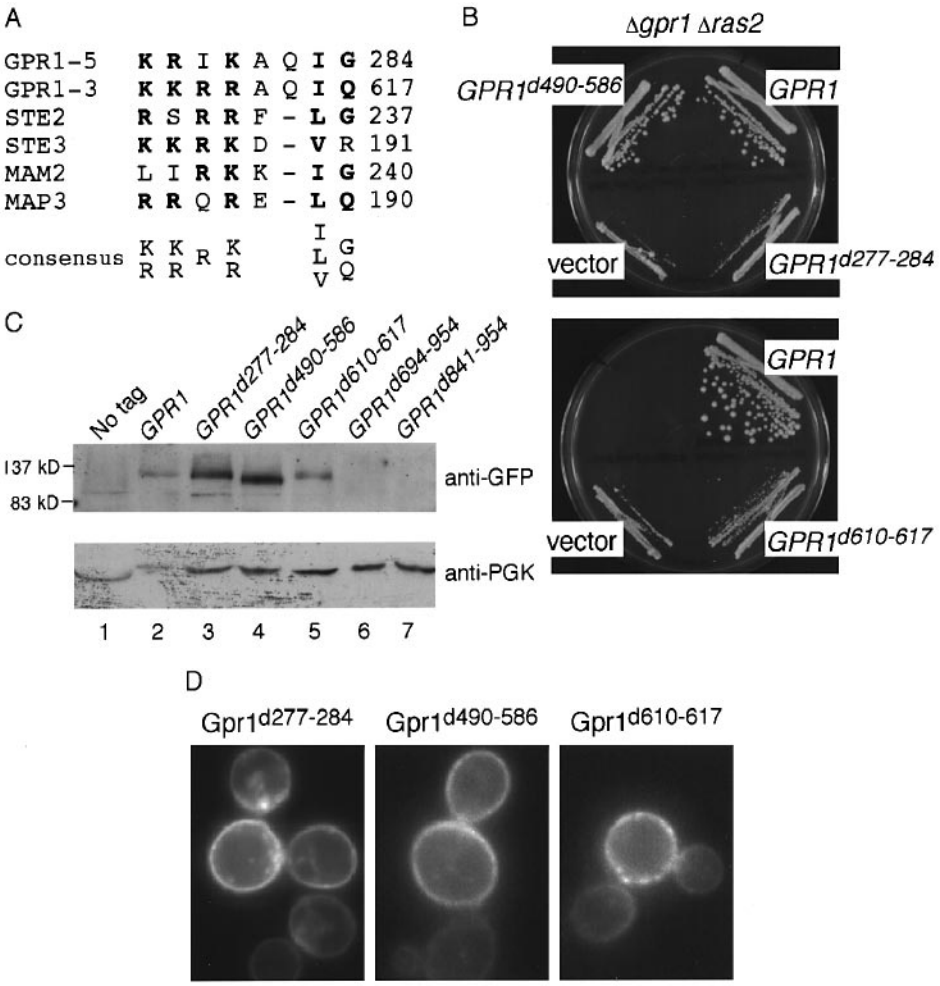

Fig. 7. Effect of deleting sequences in the third loop and cytoplasmic tail of Gpr1p. (**A**) Alignment of sequences in the third cytoplasmic loops of Gpr1p, the α-factor receptor Ste3p, the **a**-factor receptor Ste2p, the *S.pombe* P-factor receptor mam2 and the *S.pombe* M-factor receptor map3. (**B**) A strain with the genotype *gpr1::HIS3 ras2::LEU2* (YX12) carrying either pGPR1-22.2, pGPR1$^{d490–586}$-22.2, pGPR1$^{d277–284}$-22.2, pGPR1$^{d610–617}$-22.2 or YCplac 22 (vector) was streaked out for single colonies. (**C**) Cell extracts were prepared from a wild-type strain (W3031A) containing vector YEplac 112 (lane 1), pGPR1-GFP.1 (lane 2), pGPR1$^{d277–284}$-GFP.1 (lane 3), pGPR1$^{d490–586}$-GFP.1 (lane 4), pGPR1$^{d610–617}$-GFP.1 (lane 5), pGPR1$^{d694–954}$-GFP.1 (lane 6) and pGPR1$^{d841–954}$-GFP.1 (lane 7). A Western blot containing these samples was probed with anti-GFP polyclonal antiserum. The blot was reprobed with anti-PGK polyclonal antiserum. (**D**) A wild-type strain (W3031A) transformed with pGPR1$^{d277–284}$-GFP.1, pGPR1$^{d490–586}$-GFP.1 or pGPR1$^{d610–617}$-GFP.1 was viewed by fluorescence microscopy with an FITC filter.

is required for its function because this portion of *GPR1* was isolated in the two-hybrid screen based on its interaction with Gpa2p. However, constructs that deleted most of the Gpr1p cytoplasmic tail (residues 694–954) or the smallest region that was isolated in the two-hybrid screen (residues 841–954) did not produce a protein product that was detectable by immunoblot (Figure 7C, lanes 6 and 7), so this region appears to be important for some step in the production or stabilization of Gpr1p.

GPR1 RNA is induced in response to starvation for nitrogen and amino acids

To test whether the *GPR1* gene is regulated by the availability of nutrients, the effect of nitrogen starvation on the abundance of *GPR1* RNA was determined. RNA samples were isolated from cells in log phase, from cells that had been starved for nitrogen and essential amino acids for 24 h, and from starved cells to which asparagine and essential amino acids had been added back for 2 h. The abundance of *GPR1* RNA increased to a very high level in cells starved for nitrogen and amino acids compared with its abundance in cells growing in log phase (Figure 8, lanes 1 and 2). Addition of essential amino acids and asparagine, an efficient nitrogen source, to starved cells caused a decrease in the abundance of *GPR1* RNA (Figure 8, lane 3). Cells starved for a carbon source did not display induction of *GPR1* RNA (data not shown), suggesting that this induction is not a general response to growth arrest. In addition, there was no difference in the abundance of *GPR1* RNA in cells growing on a fermentable carbon source compared with cells growing on a non-fermentable carbon source (data not shown). To determine whether induction of *GPR1* RNA requires amino acid starvation, a strain that is prototrophic for all amino acids was starved solely for nitrogen. Likewise, an auxotrophic strain was starved for nitrogen in the presence of essential amino acids. In both of these cases, *GPR1* RNA was not induced (Figure 8, lanes 4–9), indicating that amino acid starvation is necessary for this response. The induction of *GPR1* RNA therefore appears to be a specific response to nitrogen and amino acid deprivation.

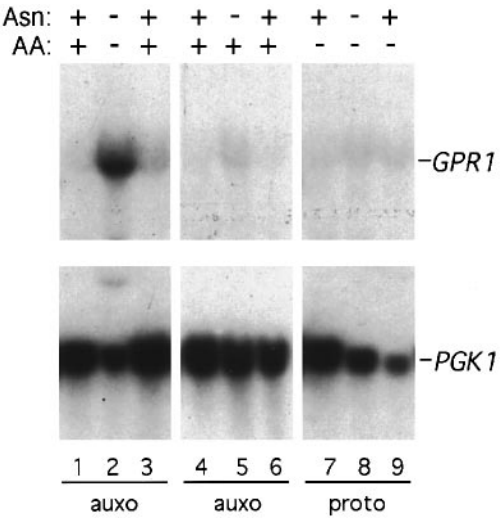

Fig. 8. *GPR1* RNA levels in cells starved for nitrogen and amino acids. RNA was isolated from wild-type auxotrophic (W3031A, lanes 1–6) and prototrophic (W3031B.TLH, lanes 7–9) strains under the following conditions: growing in log phase in the presence (lanes 1 and 4) or absence (lane 7) of amino acids, incubated in the absence of nitrogen and amino acids for 24 h (lanes 2 and 8), incubated in the absence of nitrogen for 24 h with essential amino acids present (lane 5), 2 h after the addition of 10 mM asparagine and essential amino acids to starved cells (lanes 3 and 6) or 2 h after the addition of 10 mM asparagine to starved cells (lane 9). A Northern blot prepared from the RNA was hybridized with a *GPR1* probe and then rehybridized with a *PGK1* probe as a loading control.

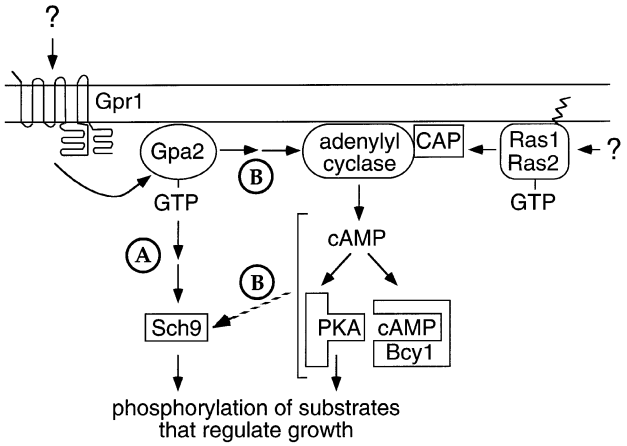

Fig. 9. Model for the Ras and Gpr1p/Gpa2p signaling pathways. For clarity, some of the known interactions between the Ras and Gpa2p pathways have not been shown.

Discussion

The Ras/cAMP pathway has long been thought to play a role in detecting and responding to nutrients, although the connection between pathway activation and nutrient availability has remained obscure. This work shows that the G protein α subunit Gpa2p functions in a signaling pathway that acts parallel to Ras and upstream of the Sch9p kinase. It also describes the isolation of the *GPR1* gene, which encodes a putative G protein-coupled receptor that is proposed to initiate the Gpa2p signaling pathway. The following observations support the idea that Gpr1p is a G protein-coupled receptor and that it couples to Gpa2p: (i) Gpr1p shares sequence homology with members of the G protein-coupled receptor family and is predicted to contain seven transmembrane domains; (ii) Gpr1p was shown to be located on the cell surface; (iii) Gpr1p physically associated with Gpa2p in the yeast two-hybrid assay; (iv) the Δ*gpa2* and Δ*gpr1* mutations caused essentially identical severe growth defects in a Δ*ras2* strain; (v) *GPA2* acts downstream of *GPR1* by genetic criteria because an activated allele of *GPA2* compensated for the loss of *GPR1*; (vi) *GPR1* was not required for the efficient expression of the *GPA2* gene or for the basal activity of the Gpa2p protein; and (vii) Gpr1p contains short stretches of amino acids in the membrane-proximal region of its third cytoplasmic loop that are homologous to sequences in other yeast G protein-coupled receptors and that are required for its function. These results make a strong case for the assignment of Gpr1p as the receptor that couples to Gpa2p, and suggest that an extracellular ligand binds to Gpr1p and causes the activation of Gpa2p.

The results described here are consistent with two alternative models for the role of Gpa2p in growth control (Figure 9). In both cases, binding of ligand to the Gpr1p receptor is expected to stimulate guanine nucleotide exchange on Gpa2p, resulting in its activation. Both models require that Gpa2p does not act through Ras because the effects of a constitutive allele of *GPA2* are not affected by deletion of both *RAS* genes. Therefore, it is likely that Gpa2p and the Ras proteins act independently and in parallel to elicit common responses. The two models propose different pathways for transmission of the signal from Gpa2p to downstream components. One possibility is that Gpa2p acts in a cAMP-independent pathway that leads to activation of the Sch9p kinase (Figure 9, labeled A). The other possibility is that Gpa2p directly stimulates adenylyl cyclase, as is seen for $G_{\alpha s}$ activation of mammalian adenylyl cyclase (Figure 9, labeled B). In the latter case, it is necessary to propose that the function of Sch9p is at least partially required downstream of adenylyl cyclase activation (Figure 9B, dotted arrow) to explain the observation that deletion of *SCH9* blocks the effect of activated Gpa2p on heat shock sensitivity. A regulatory link between *SCH9* and the PKA pathway has been demonstrated by the finding that deletion of *SCH9* causes an increase in PKA activity (Crauwels *et al.*, 1997). Direct activation of adenylyl cyclase by Gpa2p is also consistent with the previous finding that overexpression of *GPA2* causes a small increase in the level of cAMP induced by glucose (Nakafuku *et al.*, 1988). In addition, activated Gpa2p causes changes in cell physiology that are similar to the changes caused by elevated PKA activity, such as increased heat shock sensitivity and reduced sporulation efficiency. However, we strongly favor the possibility that Gpa2p acts in a cAMP-independent pathway that activates Sch9p (Figure 9). This possibility is supported by the following observations. First, deletion of *GPA2* has no effect on the level of cAMP (Nakafuku *et al.*, 1988). Second, the physiological parameters affected by constitutive Gpa2p can be regulated in a cAMP-independent manner (Cameron *et al.*, 1988), in addition to their known regulation through the cAMP/PKA pathway. Finally, all the genetic evidence indicates that *GPA2* and *RAS2* function in different pathways. For example, deletion of *SCH9* completely blocks the heat shock sensitivity of strains expressing a constitu-

tive allele of *GPA2*, but has no effect on the heat shock sensitivity of strains expressing a constitutive allele of *RAS2*. This result suggests that *SCH9* functions downstream of *GPA2* but does not function downstream of *RAS2*. Furthermore, constitutive *GPA2* does not affect the growth rate of a Δ*sch9* strain (data not shown), although it does increase the growth rate of a Δ*ras1* Δ*ras2* Δ*pde2* strain. In contrast, a constitutive allele of *RAS2* suppresses the growth defect of a Δ*sch9* strain (Toda *et al.*, 1988), suggesting that *SCH9* is not downstream of *RAS2*. Sch9p is most closely related to yeast and mammalian PKA, so it would not be surprising if the substrate specificity of these two kinases overlaps. Common substrates of these downstream kinases could account for the finding that mutational activation of *GPA2* and *RAS2* has similar physiological effects. The severe growth defect of strains that contain a Δ*ras2* mutation in combination with a Δ*gpa2* or Δ*gpr1* mutation could be due to the fact that both the Ras and Gpa2p pathways are compromised in their ability to activate downstream kinases in the double mutant strains.

The model for the *RAS* and *GPA2* pathways proposes that they are partially redundant. One question raised by this model is why *RAS1* and *RAS2* can compensate completely for the lack of *GPA2* function, but *GPA2* cannot compensate for the lack of all *RAS* function. A rationale for this observation is that the *RAS* pathway detects one type of nutrient, such as carbon, and the *GPA2* pathway detects another type of nutrient, such as nitrogen. If cells can maintain a slow growth rate in the presence of a carbon source by using internal stores of nitrogen, it might be advantageous to have both an essential carbon detection pathway and a non-essential nitrogen detection pathway. The nitrogen pathway would then contribute to growth control when the essential requirement for carbon is met. This mechanism is consistent with the finding that the presence of glucose is required for the response of nitrogen-starved cells to the addition of nitrogen, as measured by trehalase activation (Thevelein, 1994). Nutrient-mediated growth control in yeast has been proposed to involve the integration of signals from several different sensing pathways (Broach, 1991; Thevelein, 1994). The uncovering of a signaling pathway that acts parallel to the Ras pathway and is partially redundant with it suggests a way in which the cell could sum up the input from different sensors. If each signal activates kinases that have some common substrates, cell cycle progression could occur when a critical level of substrate phosphorylation is reached.

Several observations suggest that the *GPR1/GPA2* pathway has a potential role in nutrient sensing, either in nitrogen detection or in a broader function of detecting nutrients other than carbon (Thevelein, 1994). First, the abundance of *GPR1* RNA was found to increase under conditions of nitrogen and amino acid starvation, suggesting that the *GPR1/GPA2* pathway is involved in regulating growth in response to the presence of these nutrients. The response of nitrogen-starved cells to the addition of nitrogen and amino acids is independent of functional Ras proteins (Durnez *et al.*, 1994), consistent with the involvement of the *GPR1/GPA2* pathway. Secondly, the nitrogen response is defective in strains containing a null allele of *SCH9* (Crauwels *et al.*, 1997), which acts downstream of *GPA2*. Finally, it has been shown recently that *GPA2* is required for pseudohyphal growth, which occurs in response to nitrogen starvation (Kübler *et al.*, 1997; Lorenz and Heitman, 1997). However, it should be noted that the response to nitrogen also requires PKA (Durnez *et al.*, 1994), although it is not associated with an increase in cAMP levels (Hirimburegama *et al.*, 1992). Therefore, this response appears to involve both the PKA and the Sch9p kinases, and is probably not the result of a simple, linear signaling pathway, but rather involves cross-pathway interactions. Interactions between the two pathways is one explanation for the observation that overexpression of *GPA2* causes a small increase in the level of cAMP induced by glucose (Nakafuku *et al.*, 1988). Although the PKA and Sch9p kinases are involved in the response to nitrogen, strains containing null alleles of these genes are defective for growth in the presence of all nutrients. Therefore, these kinases must also play a role during normal growth in complete medium. Similarly, Δ*ras2* strains containing null alleles of *GPR1* or *GPA2* are defective for growth in the presence of all nutrients, suggesting that Gpr1p and Gpa2p also play a role in normal growth.

Previous work aimed at identifying specific molecules that act as growth control signals has been complicated by the metabolic requirement for nutrients. Thus the signal that ultimately causes Ras to be activated could be initiated by a molecule that functions both outside the cell as a ligand and inside the cell as a substrate for metabolic processes. Similarly, the ligand that binds the Gpr1p receptor could function both extracellularly and intracellularly. The isolation of a cell surface receptor that is likely to be involved in growth control by nutrients will allow a definitive approach for identifying an extracellular signal of this class.

Materials and methods

Plasmid construction

The *GPA2* gene was cloned by amplifying a 1.6 kb fragment from yeast genomic DNA by polymerase chain reaction (PCR) using primers oGPA2-1, 5′-CCGGATCCCAGCTGCGCCCAAATGATTC-3′ and oGPA2-4, 5′-CCGGATCCGCTGTGCATTCATTGTAACAC-3′ (genomic sequences are underlined in all primers), each of which contains a flanking *Bam*HI site. This fragment was cloned into the *Bam*HI site of YCplac33 (Gietz and Sugino, 1988) to create pGPA2-33.1. To construct a *TRP1* disruption of *GPA2*, a 0.9 kb *Nru*I–*Sma*I fragment from D759 was cloned into the *Mlu*I–*Bss*HII sites of pGPA2-33.1, which had been blunt-ended using Klenow fragment, to produce pgpa2-1::TRP1. To construct a multicopy plasmid with *GPA2* under the control of the GAPDH promoter, a 1.4 kb fragment was amplified from yeast genomic DNA by PCR using primers oGPA2-3, 5′-CCGGATCCGCGAGCCTTATTGTTACAGC-3′ and oGPA2-4, each of which contains a flanking *Bam*HI site. This fragment was cloned into the *Bam*HI site of YEplac112 (Gietz and Sugino, 1988) under the control of the GAPDH promoter to produce pGPA2-112.1. The GAPDH promoter was subcloned into YEplac112 from vector pAB23BXN as a 0.4 kb *Bam*HI–*Bgl*II fragment. Site-directed mutagenesis with primer oCTGPA-2, 5′-CTGACGTCATCTGT**GC**CGATCT-3′ (changed nucleotides are in bold) was used to change the arginine at position 273 in *GPA2* to an alanine (Transformer kit, Clontech). The altered gene was subcloned as a 1.4 kb *Bam*HI fragment into vector YEplac112 under the control of the GAPDH promoter to produce pG2CT-112.2. A single copy $GPA2^{R273A}$ plasmid was constructed by replacing the 1 kb *Mlu*I–*Bss*HII fragment in pGPA2-33.1 with the corresponding fragment containing the $GPA2^{R273A}$ allele to produce pG2CT-33.2. The *GPA2* construct used in the two-hybrid screen was made by amplifying a 1.4 kb fragment from yeast genomic DNA by PCR using primers oGPHYB, 5′-CCGGAT-

Table I. Strains used in this study

Strain	Genotype	Source
HF7c	*MAT***a** *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4)$_3$-CYC1-lacZ*	Clontech
W3031A	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R.Rothstein
W3031B[a]	*MAT*α	R.Rothstein
W303[a]	*MAT***a**/α	R.Rothstein
YX1B[a]	*MAT*α *gpa2::TRP1*	this study
YX2[a]	*MAT***a** *ras2::LEU2*	this study
YX4[a]	*MAT***a** *pde2::HIS3*	this study
YX6B[a]	*MAT*α *gpr1::HIS3*	this study
YX8[a]	*MAT***a** *ras2::LEU2 gpa2::TRP1*	this study
YX12[a]	*MAT***a** *ras2::LEU2 gpr1::HIS3*	this study
H95-3D[a]	*MAT***a** *ras2::LEU2 gpa2::TRP1 pde2::HIS3*	this study
H97-36D[a]	*MAT***a** *ras1::URA3 ras2::LEU2 pde2::HIS3*	this study
H91[a]	*MAT***a**/α *ras2::LEU2/RAS2 GPA2/gpa2::TRP1*	this study
H96[a]	*MAT***a**/α *ras2::LEU2/RAS2 GPR1/gpr1::HIS3*	this study
B2-1A[a]	*MAT*α *sch9::URA3* [pG2CT-112.2]	this study
B2-1A.T[a]	*MAT***a** *sch9::TRP1* [YCp50-RAS2^{ala18val19}]	this study
B2-1B[a]	*MAT***a** [pG2CT-112.2]	this study
B2-2D[a]	*MAT***a** [YCp50-RAS2^{ala18val19}]	this study
B2-3A[a]	*MAT*α *sch9::URA3* [YEplac112]	this study
B2-3C[a]	*MAT*α [YEplac112]	this study
W3031B.TLH[a]	*MAT*α *leu2-3,112::LEU2 trp1-1::TRP1 his3-11,15::HIS3*	this study

[a]All these strains are isogenic to W3031A.

CCTGGGTCTCTGCGCATCTTCA-3′ and oGPA2-4, each of which contains a flanking *Bam*HI site. This fragment was cloned into the *Bam*HI site of vector pGBT9 (Clontech) to produce pGBT9-GPA2.

To construct a *pde2::HIS3* allele, a 1.6 kb fragment containing the *PDE2* gene was amplified from yeast genomic DNA by PCR using primers 5-PDE2, 5′-CGTCTAGAGATCACTACTACTTAATTG-3′ and 3-PDE2, 5′-CGGTCGACACAATGAATGGTACAAGA-3′, that contain an *Xba*I site or a *Sal*I site. This fragment was cloned into *Xba*I–*Sal*I-digested pUC19 to create pUC19-PDE2. The disruption construct was made by cloning a 1.8 kb *Hin*cII–*Sma*I fragment from pUC18-HIS3 into the *Hpa*I–*Eco*RV sites of pUC19-PDE2, to produce ppde2-1::HIS3. To construct a *sch9::URA3* allele, a 1.4 kb fragment containing the *SCH9* gene was amplified from yeast genomic DNA by PCR using primers o5SCH9, 5′-CCGGATCCGAATAACATCAGAAAATGCC-3′ and o3SCH9, 5′-GGGGATCCAATCGCAAAGAGCGATGTTA-3′, that contain *Bam*HI sites. This fragment was cloned into *Bam*HI-digested pUC19 to create psch9.19. The disruption construct was made by cloning a 1.2 kb *Xba*I–*Bam*HI fragment from pura3.BS into the *Nhe*I–*Bgl*II sites of psch9.19, to produce psch9.19::URA3.

To construct a *gpr1::HIS3* allele, a 1.4 kb fragment containing the 5′ end of the *GPR1* gene was amplified from yeast genomic DNA by PCR using primers 5TH110, 5′-CGCTGCAGATGATAACTGAGGGATTT-3′ and 3TH110, 5′-GTCGCTGTTATCGTTCTT-3′. This fragment was digested with *Pst*I, which cuts within the primer, and with *Xba*I, which cuts within the *GPR1* insert. The *Pst*I–*Xba*I fragment was cloned into *Pst*I–*Xba*I-digested pUC19 to create pUC19-GPR1N. The pgpr1-1::HIS3 disruption construct was made by cloning a 1.8 kb *Hin*cII–*Sma*I fragment from pUC18-HIS3 into the *Hpa*I–*Bst*BI sites of pUC19-GPR1N, which had been blunt-ended using Klenow fragment. The *GPR1–GFP* fusion gene was constructed using a *GPR1* genomic clone (pGPR1-50.1) that was obtained by screening bacterial colonies containing DNA from the YCp50 yeast library 3JDAF2 (Hirsch and Cross, 1993) with the ^{32}P-labeled 1.4 kb *Xba*I–*Pst*I fragment from pUC19-GPR1N. A 2.5 kb *Sac*I–*Sal*I fragment containing the *GPR1* promoter and an N-terminal portion of the coding sequence was subcloned from pGPR1-50.1 into *Sac*I–*Sal*I-digested YEplac112 to produce pGPR1N-112.1. The 3′ end of the *GPR1* gene from the unique *Sal*I site to the end of the coding region was amplified by PCR using primer 5-GPR1C, 5′-AGTTGTCTC-**GTCGAC**GTCATT-3′, which includes this *Sal*I site, and primer 3-GPR1C, 5′-CGCTGCAGGCGGCCGCATAATGGTCCATTTCTTAA-GAAG-3′, which contains *Pst*I and *Not*I sites. The product was digested with *Sal*I and *Pst*I and subcloned into the *Sal*I–*Pst*I sites of pGPR1N-112.1 to produce pGPR1-112.2, which reconstructs the entire *GPR1* gene. The same fragments were used to construct a *CEN* plasmid containing the 3.4 kb *Sac*I–*Pst*I fragment that includes *GPR1*, which was called pGPR1-22.2. To construct an in-frame fusion between the *GPR1* and *GFP* coding regions, a 0.7 kb *Not*I fragment containing the *GFP* gene was cloned into the *Not*I site at the end of the *GPR1* coding region in pGPR1-112.2 to create pGPR1-GFP.1. The 3.4 kb *Sac*I–*Pst*I fragment from pGPR1-112.2 was cloned into the *Sac*I–*Pst*I sites of pUC19 to create pGPR1-19.2.

GPR1 deletions were made with the QuikChange kit (Stratagene) using either pGPR1-GFP.1 or pGPR1-22.2 as the template with the following primers: for *GPR1$^{d277–284}$*, primer oGPR1DEL1, 5′-TT-CATTACCAGTGAAAGTGACTTTAACCATAACGTA-3′, and its reverse complement; for *GPR1$^{d490–586}$*, primer oGPR1DEL2, 5′-AAGG-AAAAAGGAGGCATCGACAGATGCGAAAATTCA-3′, and its reverse complement; and for *GPR1$^{d610–617}$*, primer oGPR1DEL3, 5′-CAAACCTACAAACAAATGAAGAATCTAAGGGCAATA-3′, and its reverse complement. *GPR1$^{d694–954}$* was made using the Transformer kit (Clontech) with primer oGPR1DEL4, 5′-TGGGCAAAAACAGAAT-CAAAATTCTTAAGAAATGGACCA-3′ using pGPR1-19.2 as the template. The resulting deletion construct was cloned into the *Sac*I–*Pst*I sites of YCplac22 and YEplac112 to create pGPR1$^{d694–954}$-22.2 and pGPR1$^{d694–954}$-112.2, respectively. A 0.7 kb *Not*I fragment containing the *GFP* gene was cloned into the *Not*I site of pGPR1$^{d694–954}$-GFP.1 112.2 to create pGPR1-GFP.1. *GPR1$^{d841–954}$* was made using the Transformer kit (Clontech) with primer oGPR1DEL5, 5′-ATT-CCAATGCTTGGCGGATTCTTAAGAAATGGACCATTA-3′ using pGPR1-19.2 as the template. pGPR1$^{d841–954}$-22.2, pGPR1$^{d841–954}$-112.2 and pGPR1$^{d841–954}$-GFP.1 were made as described for the GPR1$^{d694–954}$ constructs.

Strain construction and media

Strains used in this study are listed in Table I. The *GPA2* null allele was made by transformation of cells with the 1.4 kb *Bam*HI fragment from pgpa2-1::TRP1. The *RAS2* null allele was made using the *ras2::LEU2* construct from p530 as described (Tatchell *et al.*, 1984). *RAS1* null alleles were made either using the *ras1::URA3* construct from p545 as described (Tatchell *et al.*, 1984) or by transformation of cells with the 4 kb *Hin*dIII fragment from pras1-1::LEU2. The *PDE2* null allele was made by transformation of cells with the 3 kb *Sph*I–*Sac*I fragment from ppde2-1::HIS3. The *SCH9* null allele was made by transformation of cells with the 1.9 kb *Bam*HI fragment from psch9.19::URA3. The *sch9::TRP1*null allele was made by transformation of an *sch9::URA3* strain with a 3.8 kb *Sma*I fragment from marker swap plasmid pUT11 (Cross, 1997). The *GPR1* null allele was made by transformation of cells with the 2.2 kb *Sph*I–*Sac*I fragment from pgpr1-1::HIS3. Diploid H91 was made by crossing strain YX1B to strain YX2. Diploid H96 was made by crossing strain YX6B to strain YX2. Prototrophic strain W3031B.TLH was made by transforming strain W3031B with plasmids containing the *TRP1*, *LEU2* and *HIS3* genes.

Strains were grown on YEPD (2% glucose) or YEP-Gal (3% galactose),

and strains under selection were grown on synthetic dropout media, as described (Guthrie and Fink, 1991).

Two-hybrid screen and yeast methods

pGBT9-GPA2 was transformed into reporter strain HF7c (Clontech) and the resulting strain was transformed individually with each of three yeast genomic DNA fusion libraries, Y2HL-C1, Y2HL-C2 and Y2HL-C3 (James *et al.*, 1996). Transformation mixtures were plated on medium lacking histidine, and positive transformants were retested for β-galactosidase expression by incubation in the presence of 0.3 mg/ml X-gal. *GPR1*-containing plasmids TH110 and TH112 were both isolated from library Y2HL-C1. Controls for non-specific protein interactions included co-expression of pGBT-GPA2 with a plasmid expressing a *GAL4* activation domain fusion with SV40 large T-antigen and co-expression of TH1-10 with a plasmid expressing a *GAL4*-binding domain fusion with p53, both of which gave background levels of β-galactosidase activity.

Yeast cells were sporulated by resuspending 0.1 ml of a saturated culture into 2.5 ml of sporulation medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 0.1 mM tryptophan, 0.2 mM leucine, 0.03 mM histidine, 0.05 mM uracil and 0.07 mM adenine) and incubating them at 30°C with shaking for 3 days.

Heat shock assays were performed by diluting an overnight saturated culture 1:20 into fresh medium and incubating it at 30°C with shaking for 2 days. Then 1 ml of this culture was removed into a glass tube which was placed in a 50°C water bath for 20 min. Heat-shocked and non-heat-shocked cultures were then diluted and plated for counting.

Yeast cells were starved for nitrogen by growing them to log phase in YEPD and transferring them to medium containing 4% glucose, 0.26 mM adenine and 1.7% Difco yeast nitrogen base without amino acids and ammonium sulfate for 24 h, as described (Hirimburegama *et al.*, 1992). Addition of nitrogen to starved cells was performed by adding asparagine and essential amino acids to the following final concentrations: 10 mM asparagine, 0.4 mM tryptophan, 0.9 mM leucine and 0.13 mM histidine.

Yeast transformations were performed by the lithium acetate method (Ito *et al.*, 1983) modified as described previously (Hirsch and Cross, 1993). Yeast RNA was extracted from cells as described previously (Cross and Tinkelenberg, 1991).

Immunoblots

Cell lysates were prepared by harvesting 12 ml of log phase cells, washing once with cold TE and resuspending in 150 µl of lysis buffer [50 mM Tris–HCl (pH 8.0), 1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg of (apoprotin, leupeptin, chymostatin and pepstatin) per ml]. The mixture was added to acid-washed glass beads (0.5 mm) and shaken at high speed for 10 min. Glass beads and cell debris were separated from the lysate by centrifugation in a microfuge for 2 min. The protein concentration of the samples was determined using a bicinchoninic acid protein assay kit (Pierce) and equal amounts were loaded onto SDS–polyacrylamide gels (10% polyacrylamide). Separated proteins were transferred to nitrocellulose and the blot was probed with anti-GFP rabbit polyclonal antiserum at a dilution of 1:1000 or with anti-phosphoglycerate kinase (PGK) rabbit polyclonal antiserum at a dilution of 1:300 000. Donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Amersham) was used at a dilution of 1:10 000, and immune complexes were detected with an enhanced chemiluminescence kit (Amersham).

Northern blots

RNA was transferred to a nitrocellulose membrane after formaldehyde–agarose gel electrophoresis as described (Lehrach *et al.*, 1977). The membranes were UV cross-linked using a Stratalinker UV box. Prehybridization and hybridization were done at 65°C in a buffer containing 0.9 M NaCl, 0.09 M sodium citrate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 33 mM sodium pyrophosphate and 50 mM sodium phosphate monobasic. The probes used were gel-purified DNA restriction fragments ^{32}P-labeled by random primer labeling using a Prime-It kit (Stratagene). The fragments used were a 1.4 kb *Xba*I–*Mlu*I fragment from plasmid TH1-10 and a 0.5 kb *Bam*HI–*Xba*I fragment from pPGK1, which encodes phosphoglycerate kinase.

Microscopy

Cells containing the Gpr1p–GFP fusion protein were grown at room temperature and viewed using either the fluorescein isothiocyanate (FITC) filter for fluorescence microscopy or Nomarski optics for differential interference contrast microscopy on a Zeiss Axiophot microscope. They were photographed with a 100× objective.

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