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GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p G alpha subunit and regulates cell growth in parallel to *RAS* genes

By

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GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p G alpha subunit and regulates cell growth in parallel to *RAS* genes

Abstract

Advisor: Professor Jeanne Hirsch

The studies described here aim to understand the function of *GPA2*, which encodes a heterotrimeric G protein alpha subunit in *Saccharomyces cerevisiae*. The gene was cloned based on its homology to mammalian G alpha subunit genes, but had no demonstrated functions other than that *GPA2* might be involved in the Ras/cAMP pathway in *Saccharomyces cerevisiae* (60). In my thesis studies, Gpa2p was shown to be localized to the plasma membrane. A *gpa2* disruption was found to cause synthetic lethality with a *ras2* disruption, and overexpression of Gpa2p or a constitutively active allele of *GPA2* showed similar physiological effects as activation of the Ras/cAMP pathway. Further studies also suggested that *GPA2* signaling is independent of *RAS* genes.

In a two-hybrid screen, *GPR1* was isolated as an interacting protein for

GPA2. *GPR1* is a previously uncharacterized open reading frame. It shows limited sequence homology to members of the G-protein coupled receptor (GPCR) family and also has seven transmembrane domains characteristic of this receptor family. Further genetic studies confirmed that *GPA2* signals downstream of *GPR1* in the same pathway. A Gpr1p-GFP fusion protein was also localized to the plasma membrane of yeast. Based on these findings and other evidence, we therefore propose that *GPR1* is a novel G protein coupled receptor for *GPA2*.

Based on the expression pattern of *GPR1* and the role of*GPA2* and *GPR1* in pseudohyphal differentiation, we also propose that the main function of the *GPR1/GPA2* pathway is to sense the availability of nutrients, especially nitrogen sources, and to initiate appropriate cellular response. This growth regulation function of *GPA2* acts independently of the yeast *RAS* genes which have well established roles in nutrient signaling. Finally, I have also obtained preliminary evidence suggesting that *GPR1* may have *GPA2* independent signaling functions.

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General Background

Signal transduction by heterotrimeric G proteins

Cells encounter all kinds of extracellular stimuli, both chemical ones and physical ones. Some of these chemicals, such as steroid hormones, can enter cells freely through the plasma membrane and carry out the signaling function intracellularly. Another class of extracellular signals, however, do not enter the cell directly. They transduce their signals by binding to receptors on the cell surface. These receptors thus serve as the link between the extracellular stimulus and the intracellular signaling cascade. One family of these cell surface receptors is coupled to heterotrimeric G proteins. The signaling by these heterotrimeric G proteins is the focus of my thesis study.

Heterotrimeric G proteins are a family of GTP-binding proteins. They are known to play very important roles in initiating cell responses to a variety of extracellular signals (64). These signals range from light and odor to hormones and neurotransmitter (64). The effectors, which are the proteins directly regulated by active G proteins, have also been studied extensively.

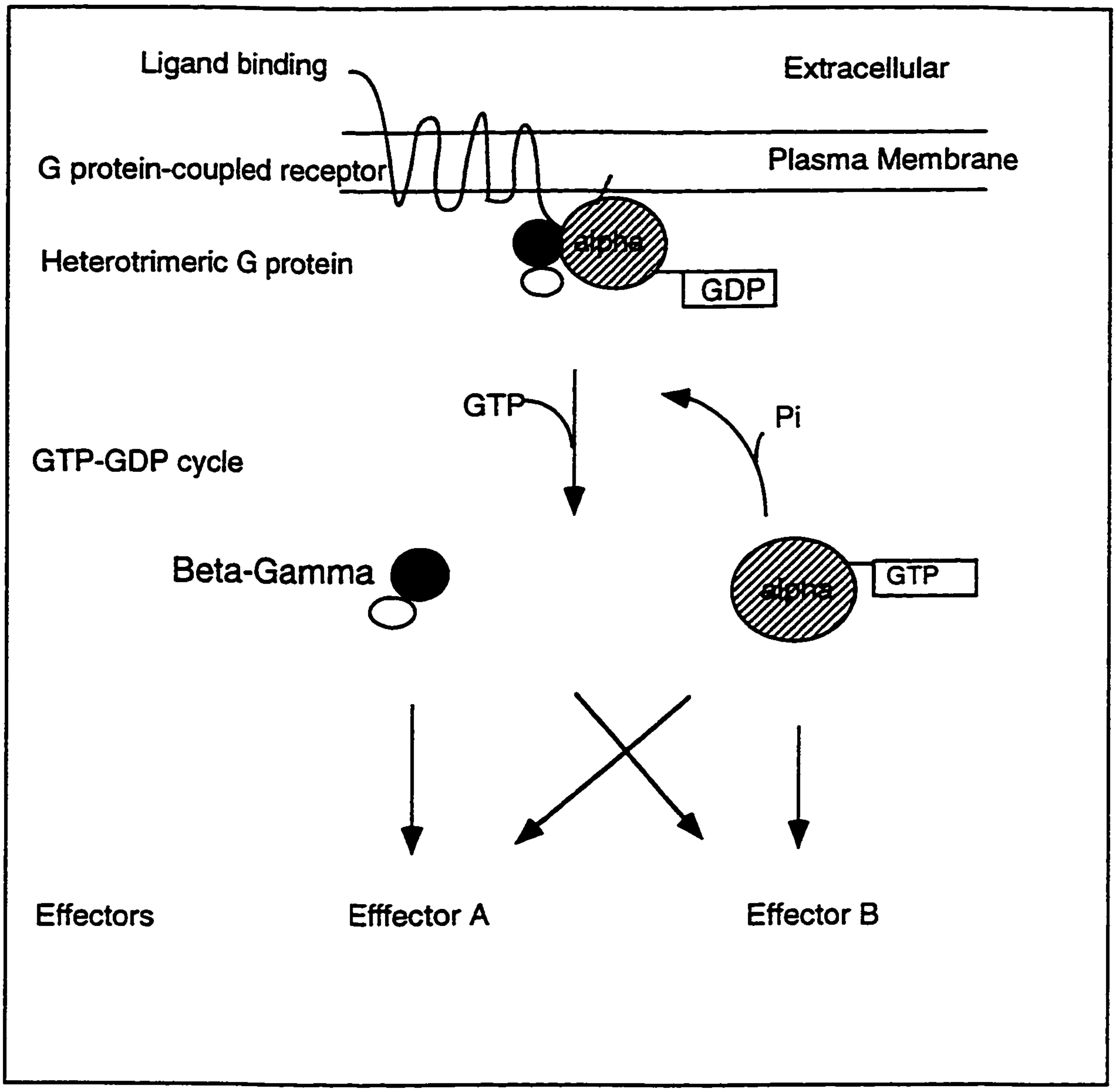
These effectors include adenylate cyclase, the first and most well studied, cGMP phosphodiesterase (cGMP PDE), ion channels, and phospholipase C (PLC) (64). Defects in the G proteins, their coupled receptors, or other components in the signaling pathway can cause a number of diseases in humans. For instance, a constitutively active Gs alpha subunit mutation causes pituitary tumors (45). Therefore, the study of G protein pathways has significant importance in clinical as well as in basic science.

Heterotrimeric G proteins consist of three subunits called alpha, beta and gamma. Different G proteins are named after their alpha subunit, which is the most conserved subunit. G alpha is also the guanine nucleotide binding subunit of the complex. Conformational change in G alpha is critical for G protein function. Beta and gamma subunits generally form a tight complex which can only be separated under denaturing conditions. The crystallographic structure of a G protein heterotrimeric complex has recently been determined (86) (44). Together with other biochemical and structural studies, this provides us with vital information regarding the mechanism of G protein signaling.

1. Mechanism of G protein signaling

Figure 1 Mechanism of signaling by heterotrimeric G proteins. Upon ligand binding, the receptor transduces a conformational change to the coupled G protein alpha subunit. The function of G protein alpha subunit is dependent on a GDP/GTP cycle. Both alpha and beta-gamma subunits can activate effectors.

Figure 1


Ligand binding
Extracellular
G protein-coupled receptor
Plasma Membrane
Heterotrimeric G protein
alpha
GDP
GTP
Pi
GTP-GDP cycle
Beta-Gamma
alpha
GTP
Effectors
Efffector A
Effector B

The mechanism of G protein activation is conserved among all G proteins found. A diagram for the mechanism is shown in Figure 1. It involves a GDP-GTP cycle on the alpha subunit. The G protein, when in its ground state, is coupled to a cell surface receptor, which with no exception is a transmembrane protein with seven membrane-spanning domains. The C-terminal part of G alpha is mainly responsible for the strength and specificity of this interaction. Although a monomeric G alpha subunit alone is capable of binding to the receptor, the presence of beta-gamma subunits greatly strengthens the G protein-receptor coupling. Recent studies also suggest that beta-gamma subunits can bind to the receptors directly, but the significance of this binding is not well understood(39).

When the receptor is activated by an extracellular signal, a conformational change in the receptor is relayed to the coupled G protein. The G protein alpha subunit, through a conformational alteration, release its bound GDP. Since the concentration of GTP is much higher in the cell than that of GDP, GTP becomes bound to G alpha and the beta-gamma subunits of the G protein dissociate from the alpha subunit. Originally, it was believed that only the GTP-bound alpha was a positive regulator of downstream events. Now, it is clear that the beta-gamma complex can also be a direct regulator, either alone or in combination with a G alpha subunit (76) (78).

For instance, adenylyl cyclase can both be activated and inhibited by G alpha and beta gamma subunit. All G α subunits have intrinsic GTPase activity. After this activity hydrolyzed GTP to GDP, the beta-gamma subunits reassociate with the alpha subunit and signaling is turned off. It is important to note that this GTP hydrolysis step is the rate limiting step of G protein signaling. Recently, a group of protein termed RGS (regulators of G protein signaling) have been found to act as stimulators of the GTPase activity of G alpha subunits. These proteins in essence are the negative regulators of G protein signaling(41).

2. Components of G protein signaling

The major players of the G protein signaling pathway have been well conserved during evolution. These include the following:

1. G protein coupled receptors (GPCR)

These receptors serve as the junction between an extracellular stimulus and the intracellular G protein signaling cascade. Over one thousand of GPCRs have been found to date. Without exception, they all have seven transmembrane domains. The intracellular loops linking the transmembrane helices form the G protein binding regions.

2. G alpha subunits

There are about 20 different mammalian G alpha subunits that have been found. The overall amino acid similarity among them is 56%-95%. According to their sequence similarity as well as their functional similarity, they are further grouped into several subclasses: the Gs subfamily mainly activates adenylate cyclase and regulates Ca^{2+} channels; the Gi subfamily inhibits adenylate cyclase as well as regulates K^+ and Ca^{2+} channels and activates cGMP phosphodiesterase; the Gq subfamily mainly regulates phospholipase C (PLC); the function of G12 subfamily is the least understood, but there are ample evidence that at least some of its members regulate a Na^+/K^+ ion channel (64).

All G alpha subunits have two domains. One domain, called the G domain, has binding sites for the guanine nucleotide. This domain is almost structurally identical to that found in small-GTP binding proteins, such as Ras and elongation factors. The other domain of G alpha consists mostly of helices. Unlike the G domain, this domain is unique to the hetreotrimeric G proteins.

3. Beta-gamma subunits

At least 6 beta and 12 gamma subunits have been discovered to date(39). Although in theory they can form over seventy dimers, in reality the number of combinations is much smaller. Beta and gamma subunits usually form a dimer that can only be separated in denaturing conditions. Thus, functionally, they are treated as one molecule. Beta and gamma subunits have an extended interaction surface. Repeated WD motifs in the beta subunit form a circular sevenfold propeller. The interface of the alpha and beta subunits lies in the switch II region of the alpha subunit where guanine nucleotide binding induces a major conformational change. Although both G alpha and beta-gamma subunits can activate effectors, there is one important difference: while G alpha changes its conformation after activation, beta-gamma subunits assume the identical structure after their dissociation from the alpha subunit and during the activation of effectors(13). This is inferred by comparing the structure of the free beta-gamma subunits and that of beta-gamma subunits in the heterotrimer.

4. Effectors

Effectors are the proteins that are activated directly by the activated G proteins. They range from enzymes to ion channels. Both alpha and beta-gamma subunits can regulate similar or even identical effectors. They are capable of this because they can act either independently, cooperatively or antagonistically. The number of effectors for alpha and beta gamma subunits is expanding rapidly and the pattern of their activation by G proteins is getting more and more complicated. For instance, adenylyl cyclase, probably the most well studied effector for G proteins, can both be activated and inhibited by G alpha and beta gamma subunit. The outcome not only depends on which alpha or beta-gamma subunit is involved, but also on the isoform of the enzyme itself(78)(26)(80).

Signal Transduction in Saccharomyces cerevisiae

In multicellular organisms, the goals of many extracellular molecules are to coordinate the proliferation and differentiation of target cells in relation to other cells. In unicellular organisms such as budding yeast, however, the majority of extracellular signaling molecules are nutrients that regulate the metabolism, proliferation and development of the organism.

1. Nutrient sensing and signaling in *Saccharomyces cerevisiae*

For yeast cells to grow, they need many kinds of nutrients. These include nitrogen, phosphate, sulfate and carbon sources. Expression of many genes is controlled by nutrient availability and examination of gene expression patterns for the whole genome is underway (19). How yeast senses the presence of nutrients and responds to them is a fundamental question, but is largely unknown. The study of nutrient signaling in yeast has long been hampered by an essential problem: nutrients can have dual functions. Besides regulatory signaling functions, they also serve as sources of energy or substrates for the organism. It is very hard to distinguish between these two functions.

Conceivably, yeast does not need to have extracellular receptors to detect nutrient availability. Many nutrients can be transported into cells and metabolized. Therefore, it has been proposed for some time that the transporter proteins and metabolic enzymes integrate signals from the availability of different nutrients. Many experimental results have supported this notion. For instance, some genes believed to sense glucose concentration are homologues of known glucose transporters but do not have the transporting functions themselves(81).

Among the signaling molecules, GTP-binding proteins have been long suspected to be candidates for nutrient monitoring. Ras proteins have well established roles in responding to environmental stress and nutrient availability. The evidence mainly come from the phenotype of strains carrying mutant *RAS* alleles. An overactive Ras pathway causes sensitivity to heat shock, a lower level of the storage carbohydrate, inability to grow on non-fermentable carbon sources and failure to arrest in G1 phase in nutrient limiting conditions. Reduced activity of the Ras pathway has the opposite effects on yeast cells. It is important to note that other signaling molecules such as *SNF1* and *SLK1* can also monitor nutrient availability in a manner independent of the Ras/cAMP pathway (33) (23). In none of these cases is the primary input to these signaling molecules known.

During the course of my thesis studies, I have cloned the gene encoding a putative G-protein coupled receptor, *GPR1*. Several observations suggest that *GPR1*, together with *GPA2*, is involved in nutrient monitoring and signaling in yeast. Heterotrimeric G proteins, unlike other nutrient monitoring molecules identified so far, almost always act as the transducers for signals outside the cell. The *GPR1/GPA2* pathway therefore provides a distinctive system to study the signaling role of nutrients without the complications caused by their housekeeping functions.

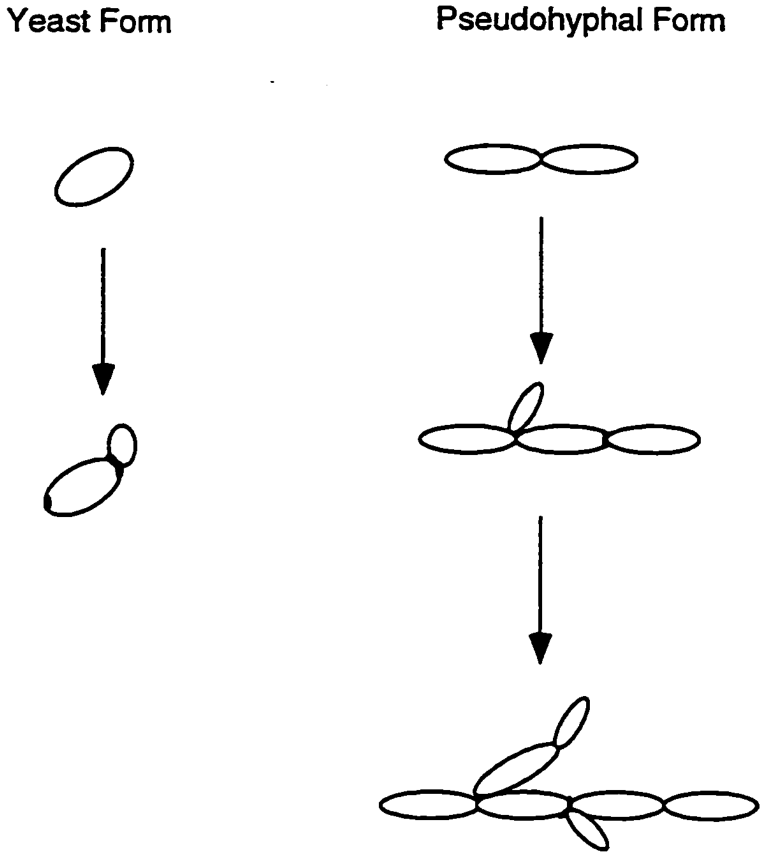
2. Pseudohyphal differentiation of *Saccharomyces cerevisiae* - an example of a nutrient response

Pseudohyphal differentiation is a very typical response to nutrient limitation. During the course of my thesis studies, both *GPA2* and *GPR1* were found to be essential for pseudohyphal growth. This section describe pseudohyphal growth as an example of nutrient signaling in yeast.

Pseudohyphal growth has been documented in the literature for several decades, but it was only in 1992 that it was rediscovered and redefined (29). When certain strains of *Saccharomyces cerevisiae*, especially those from the Σ1278b background, are starved for nitrogen, they change from a yeast form to a filamentous growth form (29) (73). The standard lab strain S288C, on the other hand, can not form good pseudohyphae due to mutation of *PHD10* gene. This circumstance thus hampered the study of pseudohyphal growth for many years. The pseudohyphal (PH) form and the yeast form of yeast differ in many aspects of cell physiology such as budding pattern, cell cycle control and cell shape. While yeast form cells use bipolar budding for cell division, pseudohyphal form cells adopt a unicellular budding pattern, i.e. always bud at the opposite of the mother-daughter junction. Cell cycle control in yeast form is mainly in the G1/S transition while in the PH form cell cycle regulation is at cytokinesis. In the pH form, cells are elongated and attached to each other in contrast to the oval shape of yeast form cells. A schematic diagram showing the

Figure 2 Pseudohyphal differentiation of *Saccharomyces cerevisiae*. Cells in yeast form are round and bud in a bipolar pattern. Cells in pseudohyphal form are more elongated and divide in a unipolar pattern. The protrusions on the cell surfaces are budding scars.

Figure 2



difference between the PH form and yeast form is shown in Figure 2. All these phenotypic changes have been proposed to cause cells to form better pseudohyphae. Presumably, filaments have the advantage of being able to forage for nutrients and invade the agar. This is especially beneficial for yeast that need to survive in nutrient limiting conditions (29). Pseudohyphal growth of *Saccharomyces cerevisiae* is very similar to hyphal growth in the human pathogen, *Candida albicans*. In that yeast, the dimorphic change from yeast form to hyphal form has recently been shown to be essential for its virulence(48). The true hyphae in *Candida albicans* are characterized by a continuous envelope of cell wall along the entire length of the filament. The individual cells are separated by septae and can not be dissociated. In contrast, the pseudohyphal filaments in *Saccharomyces cerevisiae* are formed by chains of elongated yeast cells. The individual cells are separated by structures similar to septae and can be easily separated by mechanical means. The regulation of pseudohyphal and hyphal growth thus has been under intensive investigation due to its apparent clinical as well as basic science importance.

The signaling events leading to the dimorphic change are still not clear, but several mediators have been identified during the past several years (47) (4). Both Ras and the MAP kinase cascade of the pheromone pathway have been shown to be required for pseudophyphal growth(17). Ras has been proposed to act through the MAP kinase cascade as well as through adenylyl cyclase (58) (71). The specificity of the MAP kinase pathway in pseudohyphal growth is achieved through the combinational control of different transcription factors(53). During the course of this study, *GPA2* has also been shown to play a role in this primitive developmental program(50).

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 BamHI site. This fragment was cloned into the BamHI site of YCplac33 (28) to create pGPA2-33.1. To construct a disruption of *GPA2* by *TRP1* marker, a 0.9kb NruI-SmaI fragment containing *TRP1* gene was cloned into the MluI-BssHII 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 1.4kb 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 BamHI site. This fragment was cloned into the BamHI site of YEplac112 (28) under the control of the GAPDH promoter to produce pGPA2-112.1. The GAPDH promoter was subcloned into YEplac112 from vector pAB23BXN as a 0.4kb BamHI-BglII 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 1.4kb BamHI 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 1kb MluI-BssHII 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 1.4kb fragment (without the promoter elements of *GPA2*) from yeast genomic DNA by PCR using primers oGPHYB, 5'-CCGGATCCTGGGTCTCTGCGCATCTTCA-3' and oGPA2-4, each of which contains a flanking BamHI site. This fragment was cloned into the

BamHI site of vector pGBT9 (Clontech) to produce pGBT9-GPA2.

To construct a *pde2::HIS3* allele, a 1.6kb fragment containing the *PDE2* gene (encodes the high-affinity phosphodiesterase) was amplified from yeast genomic DNA by PCR using primers 5-PDE2, 5'-CGTCTAGAGATCACTACTACTTAATTG-3' and 3-PDE2, 5'-CGGTCGACACAATGAATGGTACAAGA-3', that contain an XbaI site or a SalI site. This fragment was cloned into XbaI-SalI-digested pUC19 to create pUC19-PDE2. The disruption construct was made by cloning a 1.8kb HincII-SmaI fragment from pUC18-HIS3 into the HpaI-EcoRV sites of pUC19-PDE2, to produce ppde2-1::HIS3.

To construct a *gpr1::HIS3* allele, a 1.4kb 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 PstI, which cuts within the primer, and with XbaI, which cuts within the *GPR1* insert. The PstI-XbaI fragment was cloned into PstI-XbaI-digested pUC19 to create pUC19-GPR1N. The pgpr1-1::HIS3 disruption construct was made by cloning a 1.8kb HincII-SmaI fragment from pUC18-HIS3 into the HpaI-BstBI 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 (35) with the ^{32}P-labeled 1.4kb XbaI-PstI fragment from pUC19-GPR1N. A 2.5kb SacI-SalI fragment containing the *GPR1* promoter and an N-terminal portion of the coding sequence was subcloned from pGPR1-50.1 into SacI-SalI-digested YEplac112 to produce pGPR1N-112.1. The 3' end of the *GPR1* gene from the unique SalI site to the end of the coding region was amplified by PCR using primer 5-GPR1C, 5'-AGTTGTCTCGTCGACGTCATT-3', which includes this SalI site, and primer 3-GPR1C, 5'-CGCTGCAGGCGGCCGCATAATGGTCCATTTCTTAAGAAG-3', which contains PstI and NotI sites. The product was digested with SalI and PstI and subcloned into the SalI-PstI sites of pGPR1N-112.1 to produce pGPR1-112.2, which reconstructs the entire *GPR1* gene. The same fragments were used to construct a centrimeric plasmid containing the 3.4kb SacI-PstI fragment that includes *GPR1*, which was called pGPR1-22.2. To construct an in-frame fusion between the *GPR1* and GFP coding regions, a 0.7kb NotI fragment containing the GFP gene was cloned into the NotI site at the end of the *GPR1* coding region in pGPR1-112.2 to create pGPR1-GFP.1. The 3.4kb SacI-PstI fragment from pGPR1-112.2 was cloned into the SacI-PstI 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'-TTCATTACCAGTGAAAGTGACTTTAACCATAACGTA-3', and its reverse complement; for *GPR1*$^{d490-586}$, primer oGPR1DEL2, 5' -AAGGAAAAAGGAGGCATCGACAGATGCGAAAATTCA-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'-TGGGCAAAAACAGAATCAAAATTCTTAAGAAATGGACCA-3' using pGPR1-19.2 as the template. The resulting deletion construct was cloned into the SacI-PstI sites of YCplac22 and YEplac112 to create pGPR1$^{d694-954-22.2}$ and pGPR1$^{d694-954-112.2}$, respectively. A 0.7kb NotI fragment containing the GFP gene was cloned into the NotI 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'-ATTCCAATGCTTGGCGGATTCTTAAGAAATGGACCATTA-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 1. The *GPA2* null allele was made by transformation of cells with the 1.4kb BamHI fragment from pgpa2-1::TRP1. The *RAS2* null allele was made using the *ras2::LEU2* construct from p530 as described (79). *RAS1* null alleles were made either using the *ras1::URA3* construct from p545 as described (79) or by transformation of cells with the 4kb HindIII fragment from pras1-1::LEU2. The *PDE2* null allele was made by transformation of cells with the 3kb SphI-SacI fragment from ppde2-1::HIS3. The *GPR1* null allele was made by transformation of cells with the 2.2kb SphI-SacI 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 (31)

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 (38). HF7C strain contains both *HIS3* and beta-galactosidase genes under the control of Gal4p responsive promoters. Transformation mixtures were plated on medium lacking histidine, and positive transformants were retested for beta-galactosidase expression by incubation in the presence of 0.3mg/ml X-gal. *GPR1*-containing plasmids TH110 and TH112 were both isolated from library Y2HL-C1. Controls for nonspecific 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 beta-galactosidase activity.

Yeast cells were sporulated by resuspending 0.1ml of a saturated culture into 2.5ml of sporulation medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 0.1mM tryptophan, 0.2mM leucine, 0.03mM histidine, 0.05mM uracil and 0.07mM adenine) and incubating them at 30^{0}C with shaking for 3days.

Heat shock assays were performed by diluting an overnight saturated culture 1:20 into fresh medium and incubating it at 30^{0}C with shaking for 2 days. Then 1ml of this culture was removed into a glass tube which was placed in a 50^{0}C water bath for 30 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, 0.26mM adenine and 1.7% Difco yeast nitrogen base without amino acids and ammonium sulfate for 24 hours, as described (33). Addition of nitrogen to starved cells was performed by adding asparagine and essential amino acids to the following final concentrations: 10mM asparagine, 0.4mM tryptophan, 0.9mM leucine and 0.13mM histidine.

Yeast transformations were performed by the lithium acetate method (37) modified as described previously (35). Yeast RNA was extracted from cells as described previously (21).

Immunoblots

Cell lysates were prepared by harvesting 12ml of log phase cells, washing once with cold TE and resuspending in 150ul of lysis buffer [50mM Tris-HCl (pH 8.0), 1% SDS, 1mM phenylmethylsulfonyl fluoride (PMSF), 1ug of (apoprotin, leupeptin, chymostatin and pepstatin) per ml]. The mixture was added to acid-washed glass beads(0.5mm) and shaken at high speed for 10min. 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 1:10000, 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 (46). The membranes were UV cross-linked using a Stratalinker UV box. Pre-hybridization and hybridization were done at 65^0C in a buffer containing 0.9M NaCl, 0.09M sodium citrate, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 33mM sodium pyrophosphate and 50mM 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 1.4kb XbaI-MluI fragment from plasmid TH1-10 and a 0.5kb BamHI-XbaI 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 camera via 100x objective.

Pseudohyphal growth conditions

A yeast strain of Σ1278 background with a disrupted *TRP1* gene was transformed with a multi-copy plasmid containing Gpr1-GFP. Transformants were streaked onto slides covered with thin layer of SLAD medium (Synthetic Low Ammonium, Dextrose) and grew overnight. SLAD medium contains 2% glucose and 50 uM ammonium sulfate as described(50). The cells were 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 camera via a 100x objective.

Table 1

Strain	Genotype	Source
HF7c	*MATa 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	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R.Rothstein
W3031B[a]	*MATa*	R.Rothstein
W303[a]	*MATa/a*	R.Rothstein
YX1B[a]	*MATa gpa2::TRP1*	this study
YX2[a]	*MATa ras2::LEU2*	this study
YX4a	*MATa pde2::HIS3*	this study
YX6B[a]	*MATa gpr1::HIS3*	this study
YX8[a]	*MATa ras2::LEU2 gpa2::TRP1*	this study
YX12[a]	*MATa ras2::LEU2 gpr1::HIS3*	this study
H95-3Da	*MATa ras2::LEU2 gpa2::TRP1 pde2::HIS3*	this study

Table 1 continued

H97-36D[a]	*MATa ras1::URA3 ras2::LEU2 pde2::HIS3*	this study
H91[a]	*MATa/a ras2::LEU2/RAS2 GPA2/gpa2::TRP1*	this study
H96a	*MATa/a ras2::LEU2/RAS2 GPR1/gpr1::HIS3*	this study
W3031B.TLH[a]	*MATa leu2-3,112::LEU2 trp1-1::TRP1 his3-11,15::HIS3*	this study
MLY 185[b]	*MATa/a trp1:HisG/trp1::HisG ura3-52/ura3-52*	J. Heitman

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a. All these strains are isogenic to W3031A

b. This strain is isogenic to Σ1278b.

Part I *GPA2* functions in parallel to the Ras/cAMP pathway

Background

1. Gpa2p, a heterotrimeric G protein alpha subunit of previously unknown function

GPA2, which is the focus of my study, was first cloned by Yoshito Kaziro's group by low stringency hybridization using rat G_0 alpha as a probe to search a *Saccharomyces cerevisiae* genomic library (60). The deduced primary sequence of Gpa2p identifies it as a member of the heterotrimeric G protein alpha subunit family. It is about 60% homologous and 38% identical to the other yeast G protein alpha subunit, Gpa1p/Scg1p. In the three most conserved regions which define the G protein alpha subunit family, the homology is especially remarkable. In the GTP hydrolysis region (residues 125-140), the guanine-nucleotide binding region (residues 359-374), and in the region common to all members of the family (residues 96-312), most of the amino acids in Gpa2p are identical to its mammalian counterparts while the rest are all conservative substitutions (60). Since G protein alpha subunits are such a conserved family in sequence, judging from its primary sequence, Gpa2p is without doubt a heterotrimeric G protein alpha subunit.

G proteins have been well conserved during evolution not only in sequence but also in their functional mechanism as well. Studies in lower organisms often shed light on the function of their mammalian counterparts and studies on the *Saccharomyces cerevisiae* pheromone pathway have revealed a strikingly similar signaling pathway to mammalian ones. Ste2p and Ste3p, which are the G protein coupled receptors, bind to pheromone and transduce the signal to their coupled G protein, Gpa1p. During activation, Gpa1p undergoes a conformational change, exchanges its bound GDP for GTP and causes the release of the beta-gamma subunits (Ste4p and Ste18p). Then, the free beta-gamma complex positively regulates downstream events to initiate the mating response (61) (88) (89). This positive signaling role of beta-gamma subunits in yeast, considered an exception at first, is now well established for mammalian G proteins as well. Now that the whole genome of *Saccharomyces cerevisiae* has been sequenced, we can be certain that there are only two heterotrimeric G protein alpha subunit in budding yeast because G alpha subunits are highly conserved in sequence. Although in the mammalian system it has been reported, in many instances, that different G protein alpha subunits can interact with the same beta-gamma subunit to form a functional trimer (75), this is highly unlikely to be the case in *Saccharomyces cerevisiae*. Recent studies have shown that *GPA2* is required for pseudohyphal growth while the beta and gamma subunit for the pheromone pathway *STE4* and *STE18* are not involved in the process(47).

Disruption of *GPA2* has no observable phenotype in vegetative growth conditions (60). This suggests that either *GPA2* is required only in special conditions such as nutrient limiting conditions, or that Gpa2p is redundant with other proteins with similar functions in nutrient rich conditions. It has also been reported that overexpression of Gpa2p can suppress the growth defect of a *ras2* mutant (60). Since mammalian Gi and Gs are important in regulating the cAMP level and in yeast the cAMP level is regulated by Ras, a study was carried out to test the possible involvement of *GPA2* in the Ras/cAMP pathway. The Ras/cAMP pathway is summarized in the next section which is unique to *Saccharomyces cerevisiae*.

2. The *RAS*/cAMP pathway in budding yeast: molecular machinery and functions

a. Components of the Ras/cAMP pathway

Ras belongs to a family of small GTP binding proteins with molecular weights of 20-25 KD. These proteins play very important roles in a number of signaling events, such as cell transformation and cell differentiation (51). In mammalian cells, many extracellular signals are transduced through tyrosine receptor kinases to activate Ras. One function of activated Ras is to recruit Raf to the plasma membrane where the latter is possibly activated by a mechanism independent of Ras. Then, Raf activates a MAP kinase cascade. Like the heterotrimeric G protein alpha subunits, Ras performs its function through its GDP-GTP cycle: only the GTP-bound form can activate its effector. Since the intrinsic GTPase activity of Ras is very low, Ras activity *in vivo* is actually regulated through two proteins that balance its catalytic activity. One protein activates the GTPase activity of Ras and is called GTPase activating protein or GAP. The other protein, GNEF, facilitates GTP exchange for GDP. In other words, GNEF activates Ras while GAP inactivates Ras.

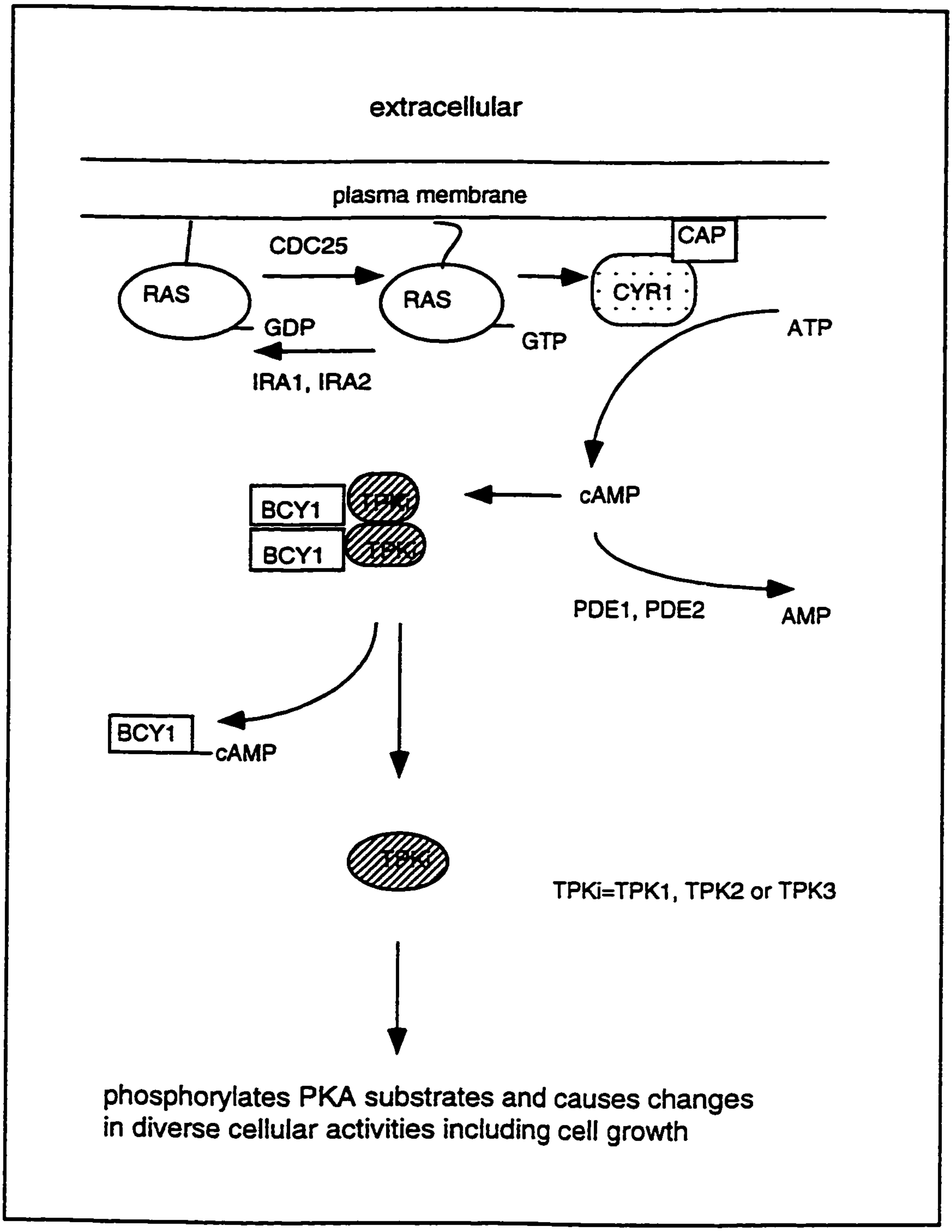
In the budding yeast *Saccharomyces cerevisiae*, there are two *RAS* related genes: *RAS1* and *RAS2*. They are redundant, which means that either of them is able to provide their function, but disruption of both genes is lethal to cells (79) Yeast Ras and mammalian Ras proteins share significant sequence homology. They can even functionally compensate for each other

: mammalian Ras can restore growth of the otherwise inviable yeast strain with both *RAS* genes disrupted, while a constitutively active yeast *RAS2* allele can transform mammalian cultured cells (22). But unlike the mammalian Ras, which functions to regulate Raf, the yeast Ras proteins are mainly responsible for activating adenylate cyclase(83).

The *RAS*/cAMP pathway has been investigated intensively over the years. Most, if not all, of the components have been identified and characterized(9) (8) (6). Components of this pathway are shown in Figure 3. Like mammalian Ras, yeast Ras is lipid modified and thus attached to the plasma membrane. In budding yeast, the GDP-GTP exchange factor (GNEF)is encoded by *CDC25* and the GTPase activating protein (GAP) is encode by two redundant genes: *IRA1* and *IRA2*. Ras1 and Ras2 activate the adenylate cyclase-CAP complex, which in turn generates the second messenger, cAMP. As seen in other organisms, cAMP regulates cAMP-dependent protein kinase (PKA), which is a protein complex composed of two regulatory subunits and two catalytic subunits. When bound by cAMP, the regulatory subunit dissociates from the catalytic subunit and the free catalytic subunit is then able to phosphorylate a number of substrates in the cytoplasm and in the nucleus. In *Saccharomyces cerevisiae*, the regulatory subunit of PKA is encoded by

Figure 3 The Ras/cAMP pathway in *Saccharomyces cerevisiae*. The function of both *RAS* genes is to activate adenylyl cyclase. cAMP then bind to the regulatory subunit of cAMP-dependent protein kinase and free the catalytic subunit of the kinase to phosphorylate substrates. The overall function of the Ras/cAMP pathway is in growth control.

Figure 3


extracellular
plasma membrane
CDC25
CAP
RAS
RAS
CYR1
GDP
GTP
ATP
IRA1, IRA2
BCY1
BCY1
TPKi
TPKi
cAMP
PDE1, PDE2
AMP
BCY1
cAMP
TPKi
TPKi=TPK1, TPK2 or TPK3
phosphorylates PKA substrates and causes changes in diverse cellular activities including cell growth

BCY1 and the catalytic subunit of PKA is encoded by three redundant genes *TPK1, TPK2* and *TPK3*. cAMP can also be degraded by cAMP phosphodiesterase (PDE). In budding yeast, PDE is encoded by two redundant genes *PDE1* and *PDE2/SRA5*. All the components in the Ras/cAMP pathway are subject to feedback control from cAMP, and PKA activity is delicately regulated(65).

The function of the *RAS*/cAMP pathway in yeast

First of all, *RAS* genes in yeast have housekeeping functions. cAMP is required under all growth conditions. Depletion of intracellular cAMP causes cell death. Secondly, the Ras/cAMP pathway has complex signaling roles in yeast. For the purpose of this discussion, the signaling function of the Ras/cAMP pathway can be divided into three areas. The first signaling function of Ras is to regulate pseudohyphal differentiation, as described earlier. The second signaling role of Ras pathway is to monitor nutrient availability and withdraw from the cell cycle under starvation conditions. This pathway is able to sense the depletion of carbon and nitrogen in the medium and initiate specific responses, which include heat shock resistance, accumulation of a storage carbon source, and withdrawal from the active cell cycle to enter the G_0 state (9)(8). The molecular mechanism of the starvation signal input to Ras is still unknown, but this function of Ras seems not to need plasma membrane attachment(9). The third signaling function of the Ras/cAMP pathway is that it can sense the presence of a fermentable sugar, e.g., glucose, when cells are starved for it or growing on a non-fermentable carbon source. In response to glucose addition, the intracellular cAMP level displays a dramatic increase within minutes and then decreases quickly to the basal level typical for cells growing in glucose. This dynamic change of cAMP level in response to glucose has been shown to inhibit the synthesis of G1 cyclins and therefore delay the Start transition (84). Cells are thus able to adjust to a longer G1 phase, a larger cell size, and more active growth. Therefore, the Ras/cAMP pathway provides the link between cell growth and cell division (84). This part of its function seems to require membrane attachment of Ras, because the non-membrane associated Ras mutant is unable to carry out the glucose signaling function (6).

RESULTS

1. Gpa2p locates at the plasma membrane, consistent with its

suggested role as an extracellular signal transducer

Although it is traditionally believed that G proteins function as extracellular signal transducers, recent research on heterotrimeric G proteins has also shown their potential roles in regulating intracellular membrane traffic in addition to their classical roles as transducers for extracellular signals. In that case, G proteins could reside at the Golgi membrane as well as at the plasma membrane (69) (25). This newly discovered function has been hypothesized to have arisen earliest in G protein evolution (25). Therefore, it was of great interest to test if *GPA2* could play a role in regulating membrane traffic in *Saccharomyces cerevisiae.*

In order to carry out biochemical studies on Gpa2p, an influenza virus hemagglutinin antigen (HA) epitope tag (12CA5 epitope) was inserted in frame into the *GPA2* gene. Because the C-terminal part of a G protein alpha subunit is critical for its interaction with the receptor and the N-terminal part is very important for its interaction with the beta subunit, it was necessary to insert the HA tag into an internal part of the coding region of the gene. This altemation could create the problem of disrupting the three-dimensional structure of the protein. Fortunately, G protein alpha subunits are very conserved both in terms of sequence and structure, and G protein structure has been studied very extensively. The HA tag was inserted into the most variable part of the gene (middle of L2 loop) which presumably is not in contact with any other proteins. Coincidentally, Dr. A.Gilman's group put a hexahistidine tag in the same region of a mammalian G protein alpha subunit in an effort to purify the G protein (42), and this construct was fully functional.

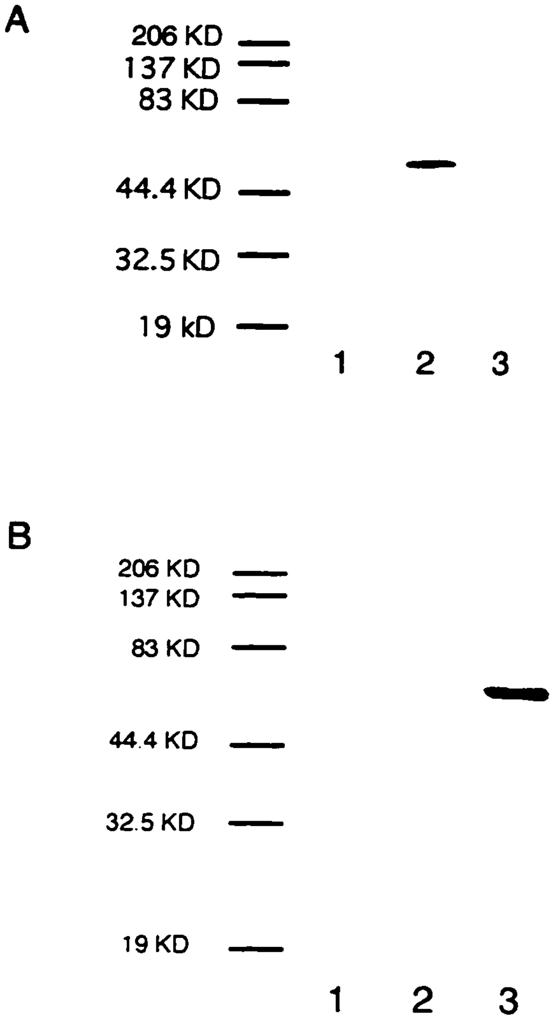
To identify HA-tagged Gpa2p, an experiment was done to extract proteins from a log-phase yeast culture under denaturing conditions. The protein extract was then loaded onto an SDS-PAGE gel and transferred to a nitrocellulose filter. HA tagged proteins were detected by Western blot using the anti-HA antibody. Wild type yeast transformed with *GPA2* under the control of the *GAL1* promoter were used in the study. The *GAL1* promoter is induced when yeast are grown in galatose-containing medium but repressed when glucose is present. As shown in Figure 4a, HA-Gpa2p was detected in the cell only when it was overexpressed under the *GAL1* promoter. With its own promoter in single copy, HA-tagged Gpa2p did not reach a detectable level. The species present at a molecular weight of approximately 50 KD (lane 2) represents Gpa2p because it can only be detected in the induced condition (in galatose-containing medium) (lane 2) but not in the repressed condition (in glucose-containing medium) (Lane

3).

To test if Gpa2p resides at the plasma membrane, cell fractionation study was performed. An experiment was done to purify the plasma membrane fraction of a yeast strain with *GPA2-HA* under the control of the *GAL1* promoter. The protocol was adapted from that described in a paper by Zinser and Daum (94). The method is extremely reproducible and numerous biochemical studies have been carried out to prove that the final plasma membrane fraction is virtually free of contamination from other intracellular membranes. Log phase yeast culture was harvested, and yeast cells were broken with glass beads. After a first centrifugation to remove cell debris, the supernatant was further subjected to 20,000g centrifugation. Then the pellet, which included most of the cell membrane fractions, was loaded onto a sucrose gradient and subjected to ultracentrifugation. The plasma membrane fraction was collected at the 43%-53% sucrose interface. After it was washed with water, the plasma membrane fraction was dissolved in SDS loading buffer and loaded onto an SDS - PAGE gel. Western blot analysis was performed using the anti-HA antibody. As shown in Figure 4b, Gpa2p is very highly enriched in the plasma membrane fraction (about 10-100 fold enrichment comparing lane 1 and

Figure 4 (a) Detection of Gpa2p-HA in yeast. A wild-type strain (W3031A) was transformed with a plasmid containing either *GPA2* (lane 1) or *GPA2 -HA* (lanes 2, 3) under the control of the *GAL1* promoter. Cell extracts were prepared from cells grown in galactose (lanes 1, 2) or in glucose (lane 3). A Western blot containing these samples was probed with anti-HA monoclonal antibody. An equal amount of protein was loaded in each lane. (b) Gpa2p is highly enriched in the plasma membrane. A wild-type strain (W3031A) was transformed with a plasmid containing *GPA2-HA* under the control of the *GAL1* promoter. An equal amount of protein from homogenized cell fraction (lane 1), 20,000g pellets fraction (lane 2) and plasma membrane fraction (lane 3) was loaded in each lane. A Western blot containing these samples was probed with anti-HA monoclonal antibody.

Figure 4


A
206 KD
137 KD
83 KD
44.4 KD
32.5 KD
19 kD
1
2
3
B
206 KD
137 KD
83 KD
44.4 KD
32.5 KD
19 KD
1
2
3

lane 3). An equal amount of protein was loaded in each lane. This result is consistent with the fact that plasma membrane proteins account for about 1-2 percent of all the protein in the cell. Although further biochemical marker studies are needed to test the purity of the plasma membrane fraction, the result not only provides a good starting point for further biochemical analysis, but also strongly suggests that Gpa2p functions as an extracellular signal transducer rather than a regulator of intracellular membrane traffic.

2. Construction of constitutively active *GPA2*

For many signaling molecules, the constitutively active alleles have much greater effects than their wild type counterparts in performing their physiological functions. A constitutively active allele of *GPA2* would be particularly useful if we want to study the physiological effects of *GPA2* on yeast cells. Therefore, a constitutively active allele of *GPA2* was constructed.

As mentioned previously, G protein alpha subunit function depends on its GDP-GTP cycle. Only the GTP-bound form binds to its effector and performs its regulatory function. When the intrinsic GTPase activity of the alpha subunit hydrolyses GTP to GDP, the protein is no longer active. In many G protein alpha subunits, mutations that block their intrinsic GTPase activity can cause them to be constitutively active (45) (52). Strict conservation in the GTP hydrolysis region in this gene family makes construction of a constitutively active *GPA2* gene very feasible.

The corresponding arginine to Gs protein in Gpa2p was changed to Ala using an *in vitro* mutagenesis procedure. Sequencing was performed to determine if the codon for Arg had indeed been changed to that for Ala in *GPA2*. One of the mutagenized *GPA2* genes was selected for all further studies and called *GPA2-CT* for clarity.

3. Activated Gpa2p confers phenotypes similar to those produced by increased Ras activity

A previous report has shown that overexpression of Gpa2p increases the level of cAMP in cells and suppresses the phenotype of a *ras2*^{ts} mutant (60) One explanation for this observation is that Gpa2p functions to regulate the intracellular cAMP level, similar to Ras. If this were the case, an activated allele of *GPA2* would be expected to have similar effects on cell physiology as activated *RAS*. Phenotypes caused by activated alleles of *RAS2* or a high level of cAMP have been well documented. Two of these are a decrease in sporulation efficiency of diploid yeast and an increased sensitivity to heat shock (83). Yeast cells transformed with the wild type*GPA2* allele and a constitutively active *GPA2* allele can be used to test if activated Gpa2p can cause similar phenotypic changes.

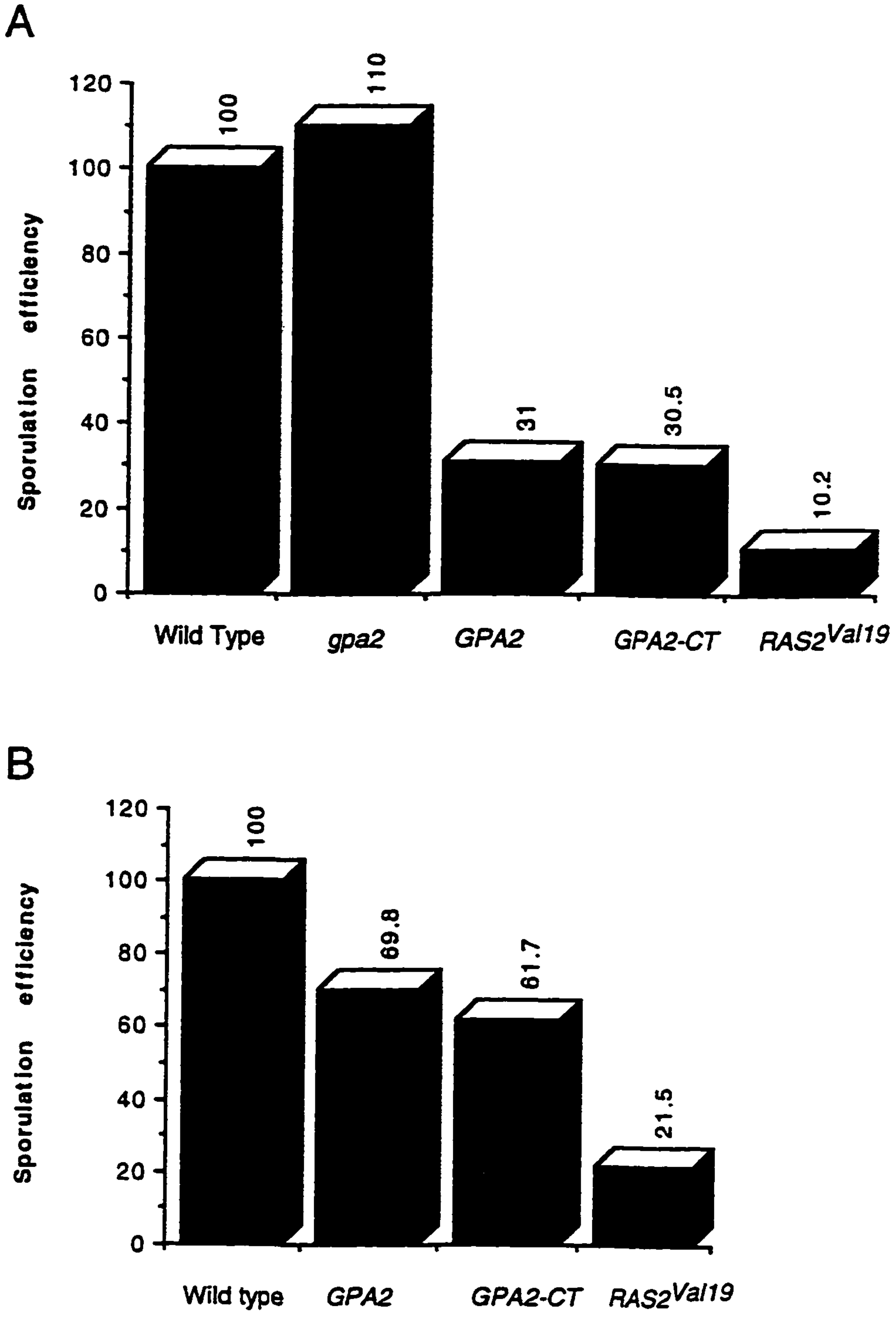
Wild type diploid yeast were transformed with a single copy plasmid bearing *GPA2* under the *GAL1* promoter. The strain was grown in synthetic medium plus galactose for 30 hrs to saturation, and the cells were washed with water and transferred to sporulation medium at a 1:25 dilution. The cells were starved for nitrogen in the presence of a non-fermentable carbon source to induce sporulation. Three days later, the sporulation efficiency was determined as the ratio between sporulated asci and total cells. Sporulated asci were defined as having more than one spore. The wild type yeast strain transformed with vector alone was used as a negative control. The wild type strain transformed with a centromeric plasmid containing a *RAS2* constitutive mutant (a missense mutation at Val 19) under the control of its own promoter was also included as a positive control. The latter strain has previously been shown to be sporulation deficient with a sporulation efficiency of 10 percent. As shown in Figure 5a, overexpression of Gpa2p decreased the sporulation efficiency to 30 percent. Overexpression of constitutively active Gpa2p decreased the sporulation efficiency to the same extent. Two independent experiments were done and the average is shown in the figure.

A similar experiment was done with *GPA2* under control of an constitutively active *GAPDH* promoter in a multicopy plasmid and similar results was obtained, as shown in Figure 5b. These results indicate that activation of Gpa2p causes a significant sporulation defect that is comparable with the defect conferred by activated Ras2p. To support this observation, other members in our research group have found that the degree of heat shock sensitivity is substantially greater in cells containing activated Ras2p or cells containing activated Gpa2p than wild type cells although Ras2p seems to have a greater effect.

From the above evidence, it can be concluded that activated Gpa2p can cause changes in cell physiology similar to those caused by activated Ras proteins. It is important to note, however, that a number of genes can also cause similar cell physiology changes although they function independently of *RAS* genes. For instance, *SLK1* and *SNF1* can both monitor nutrient

Figure 5 Activated Gpa2p causes a sporulation deficiency. (a) A wild type diploid yeast strain (W303) was transformed with a plasmid containing either *GPA2* or *GPA2-CT* under the *GAL1* promoter. As a control, the same strain was also transformed with a plasmid containing a $RAS2^{Val19}$ allele. A diploid strain homozygous for *gpa2::TRP1* was also used. (b) A wild type diploid yeast strain (W303) was transformed with a plasmid containing either *GPA2* or *GPA2-CT* under the *GAPDH* promoter. As a control, the same strain was also transformed with a plasmid containing a $RAS2^{Val19}$ allele. All strains were sporulated in sporulation medium and the sporulation efficiency was determined.

Figure 5


A
Sporulation efficiency
0
20
40
60
80
100
120
100
110
31
30.5
10.2
Wild Type
gpa2
GPA2
GPA2-CT
RAS2^{Val19}
B
Sporulation efficiency
0
20
40
60
80
100
120
100
69.8
61.7
21.5
Wild type
GPA2
GPA2-CT
RAS2^{Val19}

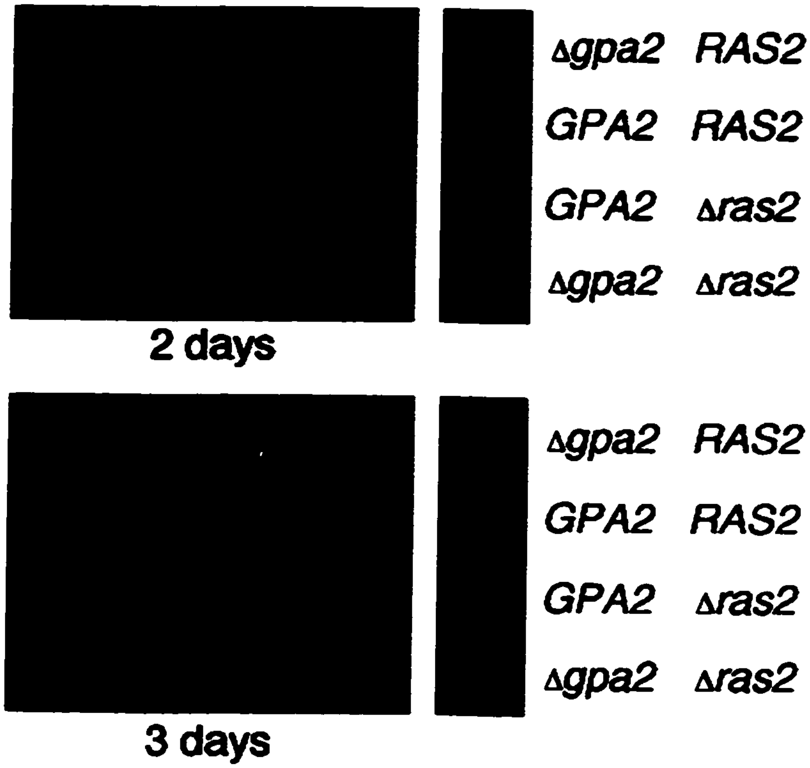
availability and cause similar phenotypic changes in a fashion that is independent of both Ras and protein kinase A(18) (10). Therefore, these experiments with *GPA2* do not distinguish between cAMP-dependent and cAMP-independent mechanisms in the signaling pathway of this molecule.

5. *GPA2* and *RAS2* share common functions in vegetative growth conditions

Previous results suggest that there is a functional similarity between *GPA2* and the *RAS* genes. In yeast, it is very common to have redundant genes that share common functions. Redundancy between *RAS1* and *RAS2* is one such example. To explore further the potential relationship between Gpa2p and Ras2p, null alleles of *GPA2* and *RAS* genes were examined for genetic interactions. To determine the phenotype of *gpa2 ras2* double mutants, a diploid strain heterozygous for *GPA2* and *RAS2* deletion alleles was sporulated and tetrads were dissected. As shown in Figure 6, although strains containing single *gpa2* or *ras2* mutations grew at a rate comparable to that of wild type yeast, strains containing both *gpa2* and *ras2* mutations displayed a severe growth defect. Colonies of cells containing double *gpa2 ras2* mutations were barely visible after 2 days of growth on the dissection plate, and were still quite small after 3 days of growth. This slow growth

Figure 6 Phenotypes of *ras2* and *gpa2* mutants. A diploid heterozygous for *gpa2::TRP1* and *ras2::LEU2* mutations 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.

Figure 6


Δgpa2 RAS2
GPA2 RAS2
GPA2 Δras2
Δgpa2 Δras2
2 days
Δgpa2 RAS2
GPA2 RAS2
GPA2 Δras2
Δgpa2 Δras2
3 days

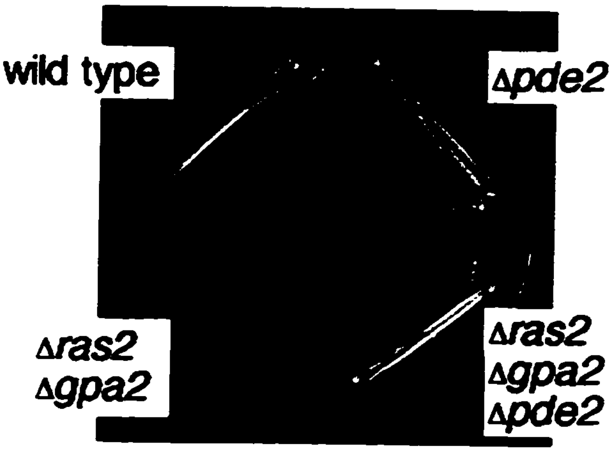
phenotype of the double mutant suggests a common growth function that is shared by both *GPA2* and *RAS2*. A similar experiment was done to test the genetic interaction between *RAS1* and *GPA2*. In contrast to the *ras2 gpa2* strain, a strain containing deletion of both *GPA2* and *RAS1* displayed little or no growth defect. This observation can be explained in at least two ways. One possibility is that the function of *GPA2* in growth control is specific to *RAS2* but not *RAS1* although both *RAS* genes have essentially the same function - to stimulate the adenylyl cyclase. Alternatively, it could be explained by the fact that *RAS1* generally contribute much less in activating adenylyl cyclase(8). During the course of my thesis study, the synthetic growth defect of *gpa2 ras*2 strains has also been seen by investigators studying the involvement of *GPA2* in pseudohyphal development (43) (50).

One recent study on *RAS2* uncovered a surprising role of the gene. Besides its function in nutrient signaling, *RAS2* is also required for completion of mitosis in conjunction with *RSR1*. Both of these processes are involved in regulating cell growth, but the mitotic function of *RAS2* does not act through cAMP generation and the mitotic defect of a *ras2* null allele can not be rescued by an increase in the cAMP level(57). To determine whether or not the redundant function of *GPA2* and *RAS2* involves cAMP regulation, a triple mutant was constructed that contained deletion alleles of *GPA2*, *RAS2* and *PDE2*. *PDE2* encodes a high affinity phosphodiesterase that degrades cAMP (90). As shown in Figure 7, deletion of *PDE2* restored normal growth to a *gpa2 ras2* strain. This indicates that elevation of the in vivo cAMP concentration can compensate for the lack of *GPA2* and *RAS2*. It therefore suggests that the redundant function of *RAS2* and *GPA2* genes involves positive control of cAMP levels or that the function of *GPA2* controls a pathway that is redundant with the Ras/cAMP pathway.

The synthetic slow growth phenotype observed in a *gpa2 ras2* strain can be explained by either of two mechanisms of signaling by Ras proteins and Gpa2p. In the first model, Gpa2p acts upstream of Ras1p and Ras2p in the same signaling pathway. Upstream activation of this pathway, presumably from an extracellular signal, would stimulate Gpa2p to transmit the signal to the Ras proteins by increasing their activity. The slow growth phenotype of a *gpa2 ras2* strain would therefore be due to the low basal activity of Ras1p, which would be insufficient for full stimulation of adenylyl cyclase in the absence of the major player Ras2p. In the second model, Gpa2p acts through a pathway that is independent of the Ras proteins. The slow growth phenotype of a *gpa2 ras2* strain would be due to the lack of sufficient

Figure 7 *PDE2* disruption rescues the growth defect of a *ras2 gpa2* strain. 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).

Figure 7



stimulatory inputs from both of the parallel pathways. To distinguish between these two models, the effects of constitutive *GPA2* can be examined in a strain that lacks all Ras proteins. Studies done by a member of our research group have shown that overexpression of Gpa2p-CT conferred a growth rate increase in a *ras1 ras2 pde2* background when compared with vector alone. This observation therefore indicates that *GPA2* regulates growth independent of *RAS* genes.

Since *GPA2* acts in parallel to *RAS* genes, one possible mechanism for Gpa2p function is to activate a kinase other than protein kinase A that has a similar set of substrates. Sch9p is one such candidate effector. *SCH9* encodes a kinase similar to cAMP-dependent kinase in sequence and its overexpression can suppress a *ras2* mutant (82) (11). Members of our research group have shown that the *sch9* deletion completely abolished the heat shock effects of Gpa2p-CT on yeast cells (91). This finding suggests that *SCH9* is required for the function of *GPA2*, at least in its control of heat shock sensitivity effects on yeast. The same study also showed that Sch9p functions downstream of Gpa2p but not downstream of Ras2p, because the *sch9* deletion had no effects on the heat shock sensitivity phenotype conferred by the activated *RAS2* allele.

PART II *GPR1* encodes a novel G-protein coupled receptor for *GPA2* and senses nutrient availability

INTRODUCTION

My thesis studies resulted in the cloning of *GPR1*, a gene that encodes a novel G-protein coupled receptor. *GPR1* belongs to a the G-protein coupled receptor family, which has been under extensive study. A more detailed introduction to this protein family is given below.

Overview of the G-protein coupled receptor family

To date, G-protein coupled receptor family consists over 1000 members (70). In mammalian cells, this is a very conserved protein family. Almost all members share significant sequence homology and have the characteristic seven transmembrane domains. Almost without exception, the function of the receptors is to transduce extracellular signals, which include light, odors and neurotransmitter. Upon binding of these ligands, the receptors change their conformation and transduce that conformational change to the coupled G protein alpha subunits.

a. Functional domains of G protein-coupled receptors

So far, there is no high resolution structure of a G-protein coupled receptor, but a low resolution electron diffraction structure for rhodopsin is available(39). Information gathered from this and other mutagenesis and biochemical studies have established the arrangement of the seven transmembrane segments in the receptor. Receptor activation by ligand binding involves a change in orientation of transmembrane segments 3 and 6. These changes, in turn, are transduced to the coupled G protein alpha subunit and cause GDP release. The C-terminal part of G alpha subunits, especially the last 7 amino acids, are critical for the specificity of receptor-G protein coupling (36)(68). In receptor, the transmembrane proximal regions of the third cytoplasmic loop are the major regions that contact the G alpha subunit. The role of beta-gamma subunits in G protein coupling has long been thought to be maintenance of the G alpha subunit in a GDP binding state, thus aiding coupling. Recent studies, however, have shown that beta and gamma subunits can contact a G protein coupled receptor directly. The specific role of this interaction is still under investigation.

The roles of individual amino acids and domains in this family of receptors have been studied extensively using a number of techniques, such as domain swapping and antibody mapping. Some regions of the G-protein coupled receptors are well conserved among different members (2) (3). The third cytoplasmic loop and cytoplasmic tail have been shown to be important for G protein coupling. The ligand binding sites on the receptor are more variable and depend on the ligand type. For instance, the N-terminal region upstream of the first transmembrane domain of some receptors has been shown to be the binding site for large ligands, such as glycoproteins, while the transmembrane core is usually the binding site for small ligands, such as nucleosides (3) (32) (59). The cytoplasmic tail of the receptor is believed to be important in determining G-protein binding specificity, as alteration in this region can change the identity of the G protein that binds to the receptor(16)(63).

In *Saccharomyces cerevisiae*, *STE2* and *STE3* encode the two G-protein coupled receptors that couple to Gpa1p and function in the pheromone response (55) (34). They encode proteins of 431 and 470 amino acids, respectively (62). In sequence, they do not show extensive homology to mammalian G-protein coupled receptors. In fact, they do not show significant homology to each other, even though they are coupled to the same G protein Gpa1p. Many studies have demonstrated that Ste2p and

Ste3p are indeed the receptors that couple to Gpa1p. These receptors can bind to pheromone and couple to Gpa1p, and many domains essential for their function have been defined (7). As mentioned earlier, neither Ste2p nor Ste3p can couple to Gpa2p. Therefore, one or more G-protein coupled receptors that have not yet been identified are expected to couple to Gpa2p.

b. Signaling through G-protein coupled receptors

It is traditionally believed that the signaling function of G-protein coupled receptors is carried out exclusively through the coupled heterotrimeric G proteins. However, recent studies have generated a much more complex picture. In addition to the G alpha subunits, a number of proteins have been shown to physically interact with G-protein coupled receptors and transduce some of their signals. These molecules include Jak2 kinase, protein kinase C and phospholipase C (85) (54). In a well studied example, angiotensin II AT1 receptor, a member of G protein-coupled receptor, was shown to activate the *JAK/STAT* pathway directly after binding to the ligand angiotensin (54). The binding site of Jak2 kinase on the receptor has been mapped to the intracellular carboxyl tail. However, the role of the heterotrimeric G protein in this signaling process is still not clear (1).

Results:

To study the function of a largely uncharacterized gene such as *GPA2*, one approach to take is to search for proteins that interact with Gpa2p. If one or more of the Gpa2p binding proteins has a known function, it could provide clues to the cellular functions of *GPA2*. To isolate other components of the *GPA2* signaling pathway, a two-hybrid protein interaction screen (27) was performed. The strategy for the two-hybrid screen is shown in Figure 8. As mentioned above, the function of all G protein alpha subunits depends strictly on the GDP/GTP cycle. The ground state Gpa2p and the activated Gpa2p-CT are expected to bind to signaling molecules with different affinities. For this reason, two baits were used in the two-hybrid system. Either the *GPA2* wild type allele or the *GPA2-CT* allele was fused with Gal4 binding domain coding region producing two fusion constructs. The first fusion protein is expected to bind preferably to the receptor, beta-gamma subunits or other proteins that bind the G alpha subunit in its inactive state, while the constitutively active Gpa2p-CT fusion protein is expected to bind to effectors with higher affinity. The results of the two-hybrid screens are shown in Table 2.

Figure 8 Two-hybrid screen to identify proteins that bind to Gpa2p. Either the *GPA2* wild type allele or the *GPA2-CT* allele was fused with *GAL4* binding domain coding region to producing two fusion constructs. After two rounds of selection, colonies were further tested for true positives. In the screen with pGBT9-GPA2, 6 millions colonies were estimated to contain both fusion constructs. In the screen with pGBT9-GPA2CT, 4 million colonies were estimated to contain the two fusion construct.

Figure 8

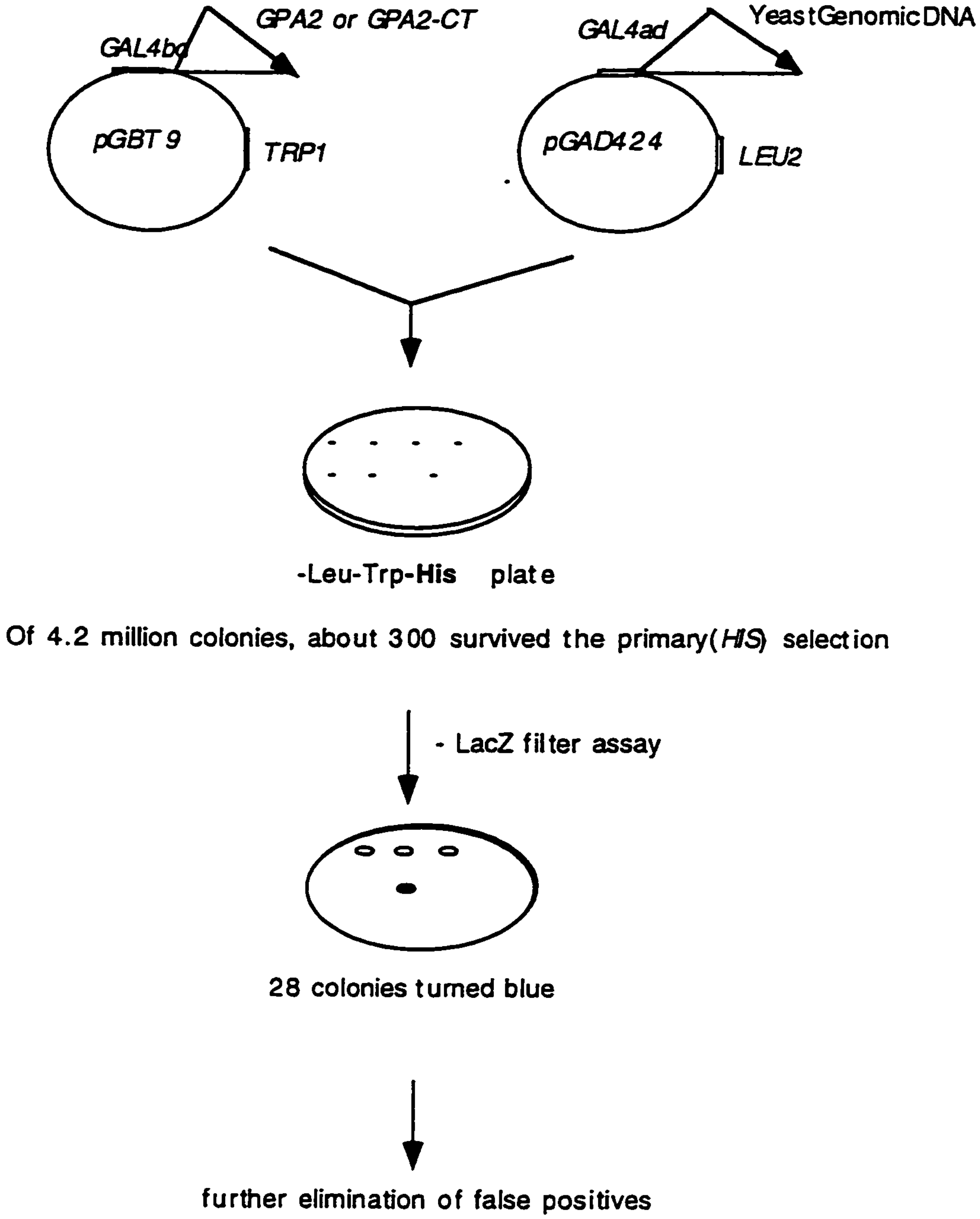

GAL4bd
GPA2 or GPA2-CT
pGBT 9
TRP1
GAL4ad
YeastGenomicDNA
pGAD424
LEU2
-Leu-Trp-**His** plate
Of 4.2 million colonies, about 300 survived the primary(*HIS*) selection
- LacZ filter assay
28 colonies turned blue
further elimination of false positives

Table 2 Putative Gpa2p binding proteins isolated in the two-hybrid screen. Beta-galactosidase activity was used to measure the binding affinity between fusion proteins: -, no detectable level of activity; +, low level of activity; ++, high level of activity. In the screen with pGBT-GPA2 as bait, *IME2*-containing plasmid was isolated 5 times with 2 different fusion junctions with *GAL4*, Th16-containing plasmid was isolated twice with same fusion junction with *GAL4*, *GPR1*-containing plasmid was isolated twice with 2 different fusion junctions with *GAL4*. In the screen with pGBT9-GPA2CT, *IME2*-containing plasmid was isolated 3 times with the same fusion junction with *GAL4* as in the screen using pGBT9-GPA2. TH14-containing plasmid was isolated twice with the same fusion junction with *GAL4*.

Table 2

ORF name	*IME2*	*GPR1*	*YAL056*	*YGL121*
Library Plasmid	–	–	–	–
Library plasmid *GAL4*-bd	–	–	–	–
Library plasmid *GAL4-P53*	–	–	–	–
Library plasmid *GAL4bd-GPA2*	++	++	+	++
Library plasmid *GAL4-GPA2CT*	+	+	++	+

1. *IME2* was isolated in the two-hybrid screen for Gpa2p interacting proteins.

Ime2p is a protein kinase required for the initiation of sporulation in diploid yeast. It is expressed only during sporulation in the diploid form of yeast (92) (74). Since *GPA2* is expressed in all of the yeast life cycle phases, it is not obvious if the interaction of *GPA2* and *IME2* is physiologically relevant. However, it is possible that the interaction with Ime2p is only important for one of many functions of Gpa2p. In any case, since Ime2p is specifically involved in sporulation, one might expect that Gpa2p should also have a role in the initiation of sporulation if the interaction is significant *in vivo*. As shown previously, overexpression of Gpa2p does cause a sporulation deficiency in diploid yeast. However, it is hard to establish that the effects of Gpa2p in sporulation are due to an interaction with Ime2p rather than to activation of a pathway with a similar function to the Ras/cAMP pathway. Further detailed studies are still needed to clarify the role of Ime2p in Gpa2p signaling.

2. The roles of putative Gpa2 interacting proteins encoded by *YAL056* and *YGL121* in Gpa2p signaling are inconclusive

Previously uncharacterized open reading frames *YAL056* and *YGL121* were also identified as encoding proteins that bind to the Gpa2p fusion protein in the two-hybrid screen.

Yal056 shows limited sequence homology to rat adenylyl cyclase but the degree of sequence homology is too low in the residues that are conserved in the adenylyl cyclase family for this finding to be conclusive. As a candidate for an effector, this protein would be expected to carry out the signaling function of Gpa2p and disruption of the gene would be expected to have the same physiological effects as a *GPA2* disruption. To test if *YAL056* is involved in the *GPA2* pathway, the null allele strain of *YAL056* was crossed to a *RAS2* null strain and the diploid was sporulated. Haploids containing double knockouts of both genes were obtained. In contrast to the *ras2 gpa2* double knockout strain, the *yal056 ras2* strain had no observable growth defect compared to the wild type strain (not shown). Further studies by Dr. Heitman's research group did not show any effect of a *yal056* disruption on pseudohyphal formation. Thus, whether *YAl056* is a component of the *GPA2* pathway still remains unclear.

In the two-hybrid screen, *YGL121*, an open reading frame of unknown function, was also isolated based on the ability of its encoded protein to interact with Gpa2p. It shows very limited homology to RGS proteins in a blast search. Since RGS proteins are negative regulators of G protein signaling, overexpression of Ygl121p would be expected to inhibit Gpa2p signaling if it functions as a RGS for Gpa2p. However, overexpression of Ygl121p in a *ras2* null strain failed to produce any observable growth defect. Moreover, disruption of *YAL121* in a pseudohyphal strain did not yield any observable effects(49). Therefore, the role of *YGL121* in Gpa2p pathway is also not clear.

3. Isolation of *GPR1*, a putative receptor for *GPA2* by a two-hybrid screen

Screening of a yeast genomic library cloned into a GAL4 activation domain vector (38) with the *GPA2* fusion constructs resulted in the isolation of plasmids containing short segments of an uncharacterized gene that was named *GPR1*. The full-length *GPR1* gene encodes a protein of 961 amino acids (DDBJ/EMBL/GenBank accession No. Z74083). A hydrophobicity plot of the protein predicts that Gpr1p contains seven membrane-spanning domains, a feature characteristic of G-protein coupled receptors. As shown in Figure 9, Gpr1p is predicted to have a very large third cytoplasmic loop of about 346 amino acids and a large cytoplasmic tail of about 281 amino acids. The third cytoplasmic loop contains two copies of a short, basic

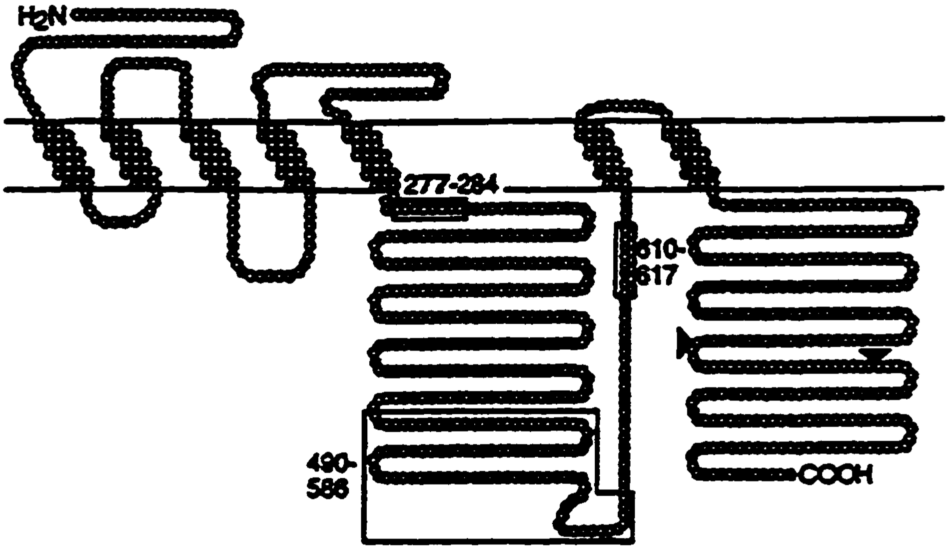
Figure 9 *GPR1* encodes a protein that associates with Gpa2p and has seven transmembrane domains. (a) The sequence of Gpr1p with potential transmembrane domains underlined. Boxed regions show sequence motifs in the third cytoplasmic loop that are related to sequences found in the third cytoplasmic loops of the yeast pheromone receptors. (b) Predicted topology of Gpr1p in the membrane. Arrowheads indicate junction sites in plasmids obtained from the two-hybrid screen. They are in positions 840 and 863 of Gpr1p, respectively. 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.

Figure 9

A

```
MITEGFPPNL NALKGSSLLE KRYDSLRQLN TTTVNQLLGL PGMTSTFTAP  50
QLLQLRIIAI TASAVSLIAG CLGMFFLSKM DKRRKYFRHD LIAFLIICDF 100
                  1
LKAFILMIYP MIILINNSVY ATPAFFNTLG WFTAFAIEGA DMAIMIFAIH 150
    2                                         3
FAILIFKPNW KWRNKRSGNM EGGLYKKRSY IWPITALVPA ILASLAFINY 200
                                            4
NKLNDDSDTT IILDNNNYNF PDSPRQGGYK PWSAWCYLPP KPYWYKIVLS 250
WGPRYFIIIF IFAVYLSIYI FITSESKRIK AQIGDFNHNY LEEEKEKKKL 300
            5
FGLGHWGKAK WYFRSYFKLP LLHLLRNLKN FFTISFIDPN EETDDSGSSN 350
GTFNFGESSN EIPTLFRKTN TGSDENVSAS GGVRLLDYNS AKPLDMSKYA 400
MSEQPDLERN NPFDCENDIT LNPSELVSKQ KEHKYTFSVE NEGLDTRKSS 450
MLGHQTFSCQ NSLESPLAMY DNKNDNSDIT SNIKEKGGII NNNSNNDDDD 500
NNNNNDNDND NNNSNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNSNNIKN 550
NVDNNNTNPA DNIPTLSNEA FTPSQQFSQE RYNNNADRCE NSSFTNVQQH 600
FQAQTYKQMK KRRAQIQKNL RAIFIYPLSY IGIWLFPIIA DALQYNHEIK 650
                                       6
HGPTMWVTYI DTCVRPLSCL VDVIVYLFKE KPWNYSWAKT ESKYLIEKYI 700
                  7
LKGELGEKEI LKFCHSNWGK RGWYYRGKWK KRKCWKYSTN PLKRILWFVE 750
RFFKQLFELK LHFSFYDNCD DFEYWENYYS AKDSNDNKRT ESDETKTNSS 800
DRSLPSNSLE LQAMLNNITA EEVEVPLFWR IIHHIPMLGG IQLDELNRLL 850
KIRYNNDHFS LPGLKFALNQ NKSHDKHQDV STNSMVKSSF FSSNIVTNDD 900
ENSIEEDKNL RYSDASASEN YLVKPTIPGT TPDPIIEAQN DNDSSDSSGI 950
DLIAFLRNGP L
```

B



sequence. One copy is present at the N-terminal end of the loop and the other copy is present at the C-terminal end. These two short sequences are very similar to short segments of the pheromone receptors both in sequence and relative positions. In its third cytoplasmic loop, *GPR1* also encodes a unique region of polyasparagine which encompasses over 100 amino acids.

Unlike the pheromone receptors, Gpr1p shares limited homology with other G-protein-coupled receptors. The homology is about 20% identity in the transmembrane regions. Gpr1p can be aligned within the G-protein coupled receptor family (2). In particular, Gpr1p contains several amino acids in its transmembrane domains that are conserved within the GPCR family. These 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. Interaction between these transmembrane domains are thought to maintain the structure of the receptor in the membrane and to enable efficient conformational change and signal transduction to coupled G proteins. In the two-hybrid assay, beta-galactosidase activity is used as a measurement of the binding affinity of the interacting fusion proteins. As estimated by the level of beta-galactosidase activity seen in the filter assay, it was found that the Gpr1p binds with higher affinity to Gpa2p than to the constitutively active Gpa2p. This is also consistent with the suggestion that Gpr1p is the G-protein coupled receptor for Gpa2p.

4. *GPR1* signals upstream of *GPA2* in the same pathway

If Gpr1p is indeed the receptor for Gpa2p, one would expect that deletion of *GPR1* would have the same phenotype as deletion of *GPA2*, and that defects conferred by a *gpr1* null allele would be suppressed by activating Gpa2p. To investigate whether if Gpr1p acts in the same signaling pathway as Gpa2p, a diploid strain heterozygous for *GPR1* and *RAS2* deletion alleles was sporulated and tetrads were dissected. As shown in Figure 10, strains containing single *gpr1* or *ras2* mutations grew normally, but strains containing both *gpr1* and *ras2* mutations displayed a severe growth defect. The slow growth rate of *gpr1 ras2* strains was similar to that of *gpa2 ras2* strains, suggesting that *GPR1* and *GPA2* may function in the same process.

To test whether Gpa2p acts downstream of Gpr1p, an experiment was performed to determine the effect of different *GPA2* alleles on the growth rate of a *gpr1 ras2* strain. As shown in Figure 11, a single copy plasmid containing the *GPA2* gene had no distinguishable effect on the growth rate

Figure 10 Phenotype of *gpr1* and *ras2* mutants. A diploid heterozygous for *gpr1::HIS3* and *ras2::LEU2* mutations 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.

Figure 10

2 days

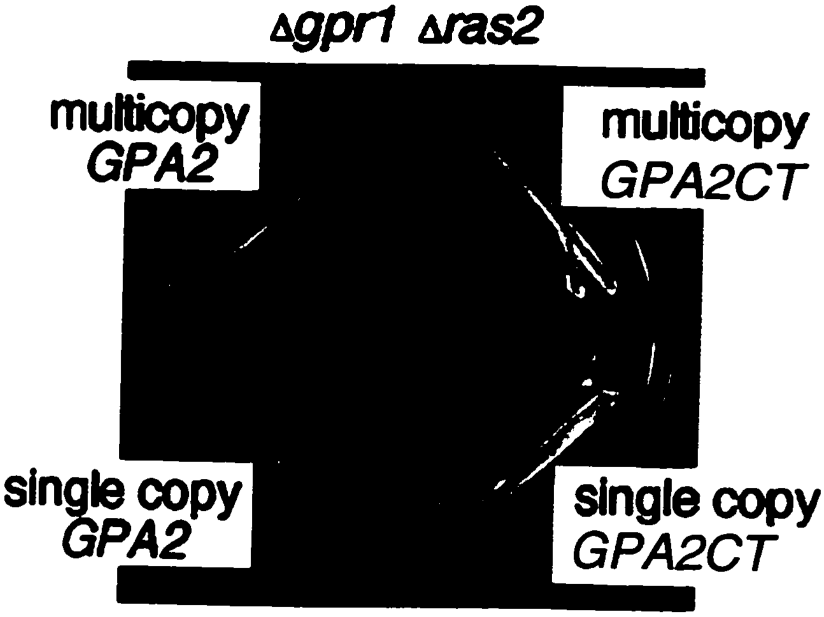
3 days

of the *gpr1 ras2* strain. However, when multi-copy *GPA2* was introduced into the strain, the growth rate increased significantly. A constitutive *GPA2-CT* allele in single copy had an even bigger effect, and restored the growth rate of the *gpr1 ras2* strain to that of a wild type strain. These genetic results suggest that Gpa2p acts downstream of Gpr1p in the same signaling pathway, as would be expected for a G alpha subunit and its associated receptor. Furthermore, the growth defect of a *gpa2 gpr1 ras2* triple deletion strain is no more severe than that of a *gpa2 ras2* strain (not shown). This finding indicates further that Gpr1p is likely to be the only receptor for Gpa2p. Taken together, these results indicate that *GPR1* and *GPA2* are more likely to function in the same signaling process than in parallel pathways with redundant functions.

The previous experiment suggested that Gpa2p acts downstream of Gpr1p. Therefore, it seems likely that Gpr1p is the receptor for Gpa2p. However, other explanations do exist. For example, the *GPR1* gene product could be required for the expression of the *GPA2* gene or to maintain the stability or activity of the Gpa2p protein. A similar situation has been discovered for the *RAS* genes, for instance. Some genes have been assigned upstream of *RAS* by genetic studies, but further biochemical investigations have shown that they are required for the post-translational modification of Ras

Figure 11 Gpa2p functions downstream of Gpr1p in growth control. A strain with the genotype *gpr1::HIS3 ras2::LEU2* was transformed with either a single copy *GPA2* plasmid (pGPA2-33.1), a multicopy *GPA2* plasmid (pGPA2-112.1), a single copy *GPA2CT* plasmid (pG2CT-33.2) or a multicopy *GPA2CT* plasmid (pG2CT-112.2) and streaked out for single colonies on selective medium.

Figure 11


Δgpr1 Δras2
multicopy GPA2
multicopy GPA2CT
single copy GPA2
single copy GPA2CT

rather than for upstream signaling (30). However, studies done by other members of our research group have shown that overexpression of *GPA2* and*GPA2-CT* conferred a similar degree of increase in heat shock sensitivity on *gpr1* cells compared to wild type cells (91). These results therefore demonstrate that *GPR1* is not required for efficient expression of *GPA2*.

5. Gpr1p is localized at the cell surface

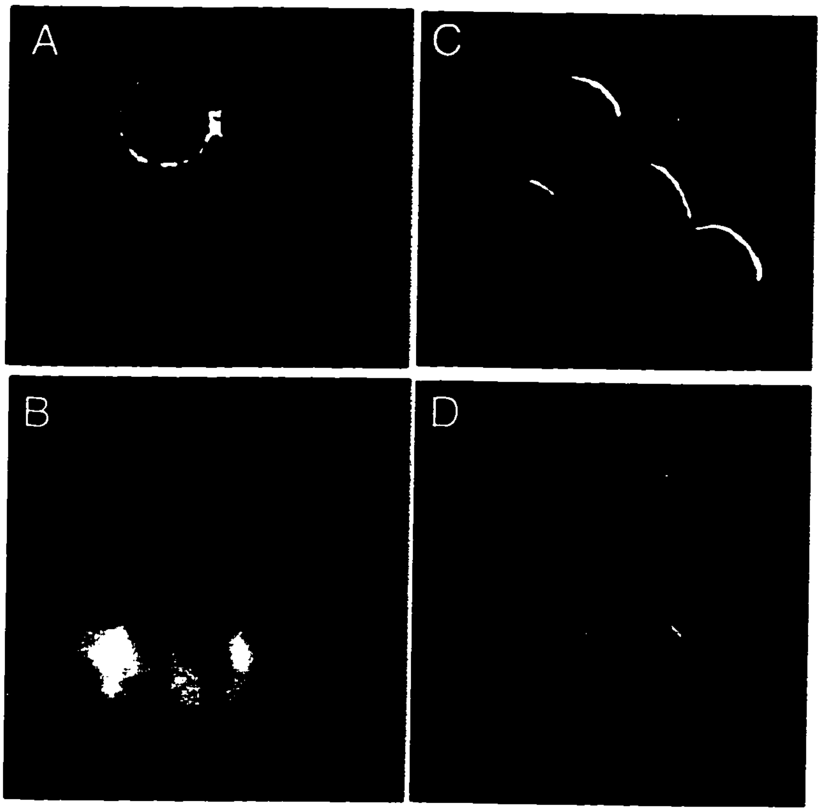
In order to function as signal transducers for extracellular signals, members of the G-protein coupled receptor family are located at the cell surface. To determine the subcellular location of Gpr1p, the coding sequence of green fluorescent protein (GFP) (12)was fused with *GPR1* at the C terminus and transformed into wild-type cells. Previous studies have shown that fusing GFP protein to the C-teminus of the beta-adrenergic receptor does not affect its ability to bind to ligand or couple to G protein (5). As expected, the Gpr1-GFP construct can complement the growth defect of a *gpr1 ras2* strain, demonstrating that the fusion gene is fully functional (not shown). A fluorescence signal is only detectable in yeast transformed with *GPR1-GFP* in multi-copy plasmid but not in cells transformed with a single copy plasmid containing *GPR1-GFP*. This is presumably due to the low level expression of Gpr1p. As shown in Figure 12, cells expressing *GPR1-GFP* from a multi-copy plasmid showed a cell surface staining pattern, demonstrating that Gpr1p is localized at the plasma membrane. As a control, cells expressing a construct containing only the *GFP* gene showed a diffuse fluorescence signal in the cytoplasm. 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. However, detailed cell fractionation studies are needed to confirm this observation.

6. Domain functions of Gpr1p

As mentioned previously, domains of G-protein coupled receptors have been studied extensively and a significant amount of information has been gathered. Many of these domains have been shown to be well conserved. For instance, sequences in the membrane-proximal regions of the third cytoplasmic loops are usually required for coupling to the G proteins (3). Preliminary studies on the function of specific regions of Gpr1p were carried out to serve two purposes. First,these studies can identify the essential domains of the receptor. Second, they can provide further evidence for Gpr1p as a member of the G-protein coupled receptor family

Figure 12 Gpr1p-GFP fusion protein is localized to the cell surface. A wild-type strain (W3031A) was transformed with a multicopy plasmid containing either a *GPR1*-GFP fusion construct (pGPR1-GFP.1) (panels A, C) or a GFP construct (panels B, D) and viewed by fluorescence microscopy.

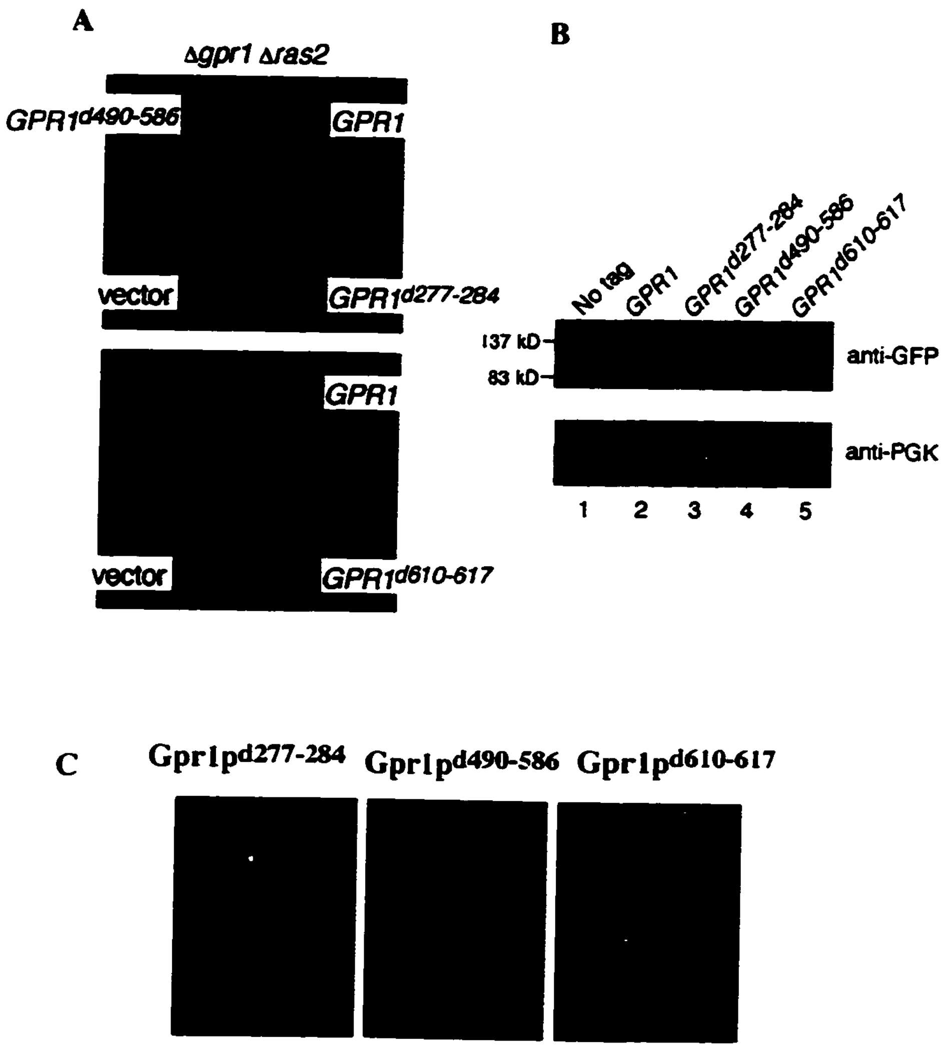
Figure 12



by showing that conserved regions among G-protein coupled receptors are also essential for Gpr1p function. The third cytoplasmic loop of Gpr1p contains the sequence KRIKAQIG near its N-terminal end and the sequence KKRRAQIQ near its C-terminal end. Similar sequences are present in the third cytoplasmic loops of the *Saccharomyces cerevisiae* pheromone receptors Ste2p and Ste3p. Site-directed mutagenesis studies have shown that these residues in one of the pheromone receptors is important for coupling to the Gpa1p alpha subunit (14). Furthermore, the pheromone receptors from *Schizosaccharomyces pombe* also have a related sequence in their third cytoplasmic loops (40) (77). 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. The resulting mutated genes were introduced into a *ras2 gpr1* strain to see if they restore cell growth in a manner to wild type *GPR1*. As shown in Figure 13, expression of altered Gpr1p containing deletions of eight amino acids at the N-terminal (residues 277-284) or C-terminal (residues 610-617) regions of the third cytoplasmic loop was unable to complement the growth defect of a *gpr1 ras2* strain. This result suggests that these regions are essential for the function of Gpr1p. The third cytoplasmic loop of Gpr1p also contains a long stretch of polyasparagine residues with unknown function. To

Figure 13 Effect of deleting sequences in the third cytoplasmic loop on the function of Gpr1p. (A) A strain with the genotype *gpr1::HIS3 ras2::LEU2* (YX12) carrying either pGPR1-22.2, pGPR1d$^{490-586}$-22.2, pGPR1$^{d277-284}$-22.2, pGPR1$^{d610-617}$-22.2 or YCplac 22 (vector) was streaked out for single colonies. (B) Cell extracts were prepared from a wild-type strain (W3031A) containing vector YEplac112 (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). A Western blot containing these samples was probed with anti-GFP polyclonal antiserum. The blot was reprobed with anti-PGK polyclonal antiserum. (C) 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.

Figure 13


A
Δgpr1 Δras2
GPR1d490-586
GPR1
vector
GPR1d277-284
GPR1
vector
GPR1d610-617
B
No tag
GPR1
GPR1d277-284
GPR1d490-586
GPR1d610-617
137 kD
83 kD
anti-GFP
anti-PGK
1 2 3 4 5
C
Gpr1pd277-284
Gpr1pd490-586
Gpr1pd610-617

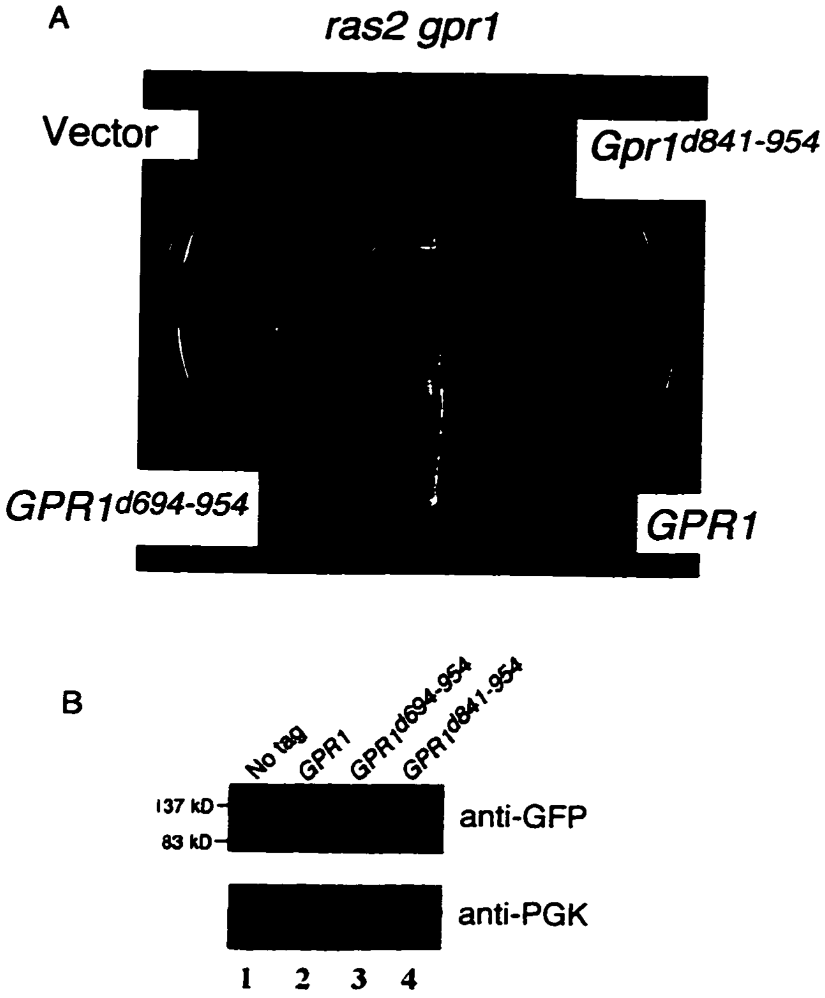
determine whether this unique asparagine-rich sequence is required for Gpr1p function, a *GPR1* mutation containing a deletion of this region (residues 490-586) was also constructed. Unexpectedly, the altered protein containing a deletion of the asparagine-rich region fully complemented the growth defect of a *gpr1 ras2* strain. This indicates that this asparagine-rich region of Gpr1p is not required for the function of Gpr1p that is redundant with Ras function, to regulate growth in nutrient rich conditions.

Since we are interested in the regions of Gpr1p that are important for signaling, it was necessary to rule out the possibilities that the essential regions in the third cytoplasmic loop are required for the expression or localization of the receptor. For each altered Gpr1p protein, construct was made in which GFP was fused at the C-terminus just before the stop codon. To determine the relative abundance of the altered Gpr1p proteins, an immunoblot containing cell extracts from strains expressing each of the altered versions of Gpr1p protein was probed with anti-GFP antiserum. As shown in Figure 13, the $GPR1^{d277\text{-}284}$, $GPR1^{d490\text{-}596}$ and $GPR1^{d610\text{-}617}$ constructs all expressed proteins at a level comparable to the wild-type expression level. The species representing wild type Gpr1p migrated at the predicted molecular weight of 110 KD. The Gpr1p species that contains a deletion of the asparagine-rich region migrated faster on the gel, presumably due to the removal of a region encoding about 100 amino acids. Localization of the altered versions of Gpr1p was determined by observing cells expressing the GFP-tagged Gpr1p proteins by fluorescence microscopy. The $GPR1^{d277-284}$, $GPR1^{d490-596}$ and $GPR1^{d610-617}$ constructs all expressed proteins that were mainly localized to the plasma membrane. These patterns were indistinguishable from that of the wild type protein. Therefore, it can be concluded that deletion of the two regions of sequence in the third cytoplasmic loop does not affect in the expression or localization of Gpr1p. Thus, these deletions most likely affect G protein coupling or other signaling events carried out by Gpr1p. Since this region has been shown previously to be conserved in G-protein coupled receptors and to be required for G protein coupling, this result further supports the idea that Gpr1p is a member of the G-protein coupled receptor family.

Since the cytoplasmic tail of Gpr1p was shown to interact with Gpa2p in the two-hybrid assay, it was also of interest to determine whether this region is important for the function of Gpr1p. As shown in Figure 14, constructs that deleted most of the Gpr1p cytoplasmic tail (residues

Figure 14. Effect of deleting sequences in the cytoplasmic tail on the function of Gpr1p. (A) A strain with the genotype *gpr1::HIS3 ras2::LEU2* (YX12) carrying either pGPR1-22.2, pGPR1$^{d694-954}$-22.2, pGPR1$^{d841-954}$-22.2 or YCplac 22 (vector) was streaked out for single colonies. (B) Cell extracts were prepared from a wild-type strain (W3031A) containing vector YEplac112 (lane 1), pGPR1-GFP.1 (lane 2), pGPR1$^{d841-954}$-GFP.1 (lane 3) and pGPR1$^{d694-954}$-GFP.1 (lane 4). A Western blot containing these samples was probed with anti-GFP polyclonal antiserum. The blot was reprobed with anti-PGK polyclonal antiserum. (C) A wild-type strain (W3031A) transformed with pGPR1$^{d841-954}$-GFP.1 or pGPR1$^{d694-954}$-GFP.1 was viewed by fluorescence microscopy with an FITC filter.

Figure 14


A
ras2 gpr1
Vector
Gpr1d841-954
GPR1d694-954
GPR1
B
No tag
GPR1
GPR1d694-954
GPR1d841-954
137 kD
83 kD
anti-GFP
anti-PGK
1 2 3 4

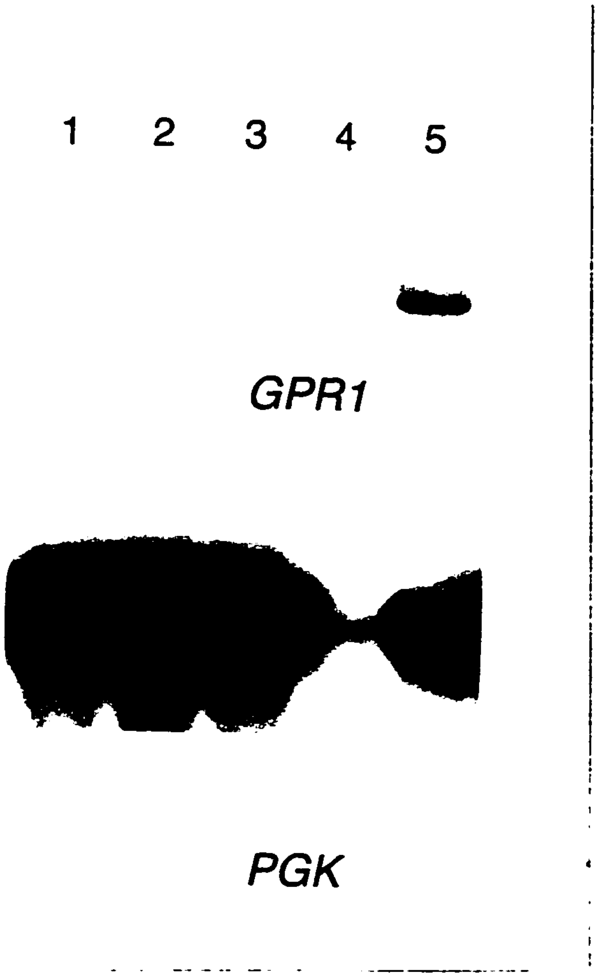
694-954) or the smallest region that was isolated in the two-hybrid screen (residues 841-954) were not able to complement the *ras2 gpr1* growth defect. However, immunoblot analysis showed that these constructs did not express detectable levels of proteins. Therefore, the cytoplasmic tail of Gpr1p appears to be important for Gpr1p function, but it may be required for stabilization of Gpr1p .

7. *GPR1* function is related to nitrogen availability

As mentioned earlier, for a unicellular organism such as yeast, regulation of cell proliferation and differentiation depends mostly on nutrients in the environment. Since Gpr1p is proposed to sense extracellular signals, it is possible that Gpr1p is responsive to nutrient conditions. To test whether the *GPR1* gene is regulated by the availability of nutrients, the effect of nutrient availability on the abundance of *GPR*1 RNA was determined. RNA samples were isolated from cells in log phase, from cells that had been starved for glucose, phosphate, sulfate, and nitrogen. As shown in Figure 15, in vegetative growth condition, the abundance of *GPR1* RNA was very low and barely detectable. Cells starved for a carbon source did not display induction of *GPR1* RNA. In addition, there was no difference in the abundance of *GPR1* RNA in cells growing in glucose compared with cells growing on a week fermentable carbon source galactose. As shown in Figure 15, only in cells starved for nitrogen and essential amino acids, the *GPR1* mRNA level was greatly increased compared to the level in log phase cells. Further experiments were performed to confirm this result. RNA samples were isolated from cells in log phase, from cells that had been starved for nitrogen and amino acids for 24 hr, and from starved cells to which asparagine and essential amino acids had been added back for 2 hr. Compared to the level in log phase cells, the abundance of *GPR1 RNA* increased to a very high level in cells starved for nitrogen and amino acids, as shown in Figure 16. Addition of essential amino acids and asparagine, an efficient nitrogen source, to starved cells resulted in a decrease in the *GPR1* RNA level to that of wild type. The strain used in this experiment was auxotrophic, which means that it needs to be supplied with certain amino acids to grow. To determine whether induction of *GPR1* RNA requires amino acid starvation, a strain that is prototrophic was also starved for nitrogen. In a parallel experiment, an auxotrophic strain was starved for nitrogen in the presence of the essential amino acids that it requires. In both of these cases, *GPR1* RNA was not induced, indicating that amino acid starvation is necessary for the induction of *GPR*1 RNA. Therefore, the induction of *GPR1* RNA appears to be a specific response to nitrogen and amino acid deprivation. These results are particularly

Figure 15 *GPR1* RNA levels in cells grown in different nutrient conditions. RNA was isolated from wild-type auxotrophic strains W303lA (lanes 2, 3) or W303 (lanes 1, 4, 5) under the following conditions: growing in log phase in the presence of glucose(lanes 1, 2), galactose (lane 3), incubated in the absence of carbon source (lane 4), incubated in the absence of nitrogen and amino acids for 24 h (lane 5). A Northern blot prepared from the RNA was hybridized with a *GPR1* probe and then rehybridized with a *PGK1* probe as a loading control.

Figure 15



informative because they provide a link between the *GPR1* gene and availability of nitrogen in the medium. Since pseudohyphal growth is a very well characterized response to nitrogen starvation, it was necessary to investigate the role of *GPR1* in pseudohyphal differentiation.

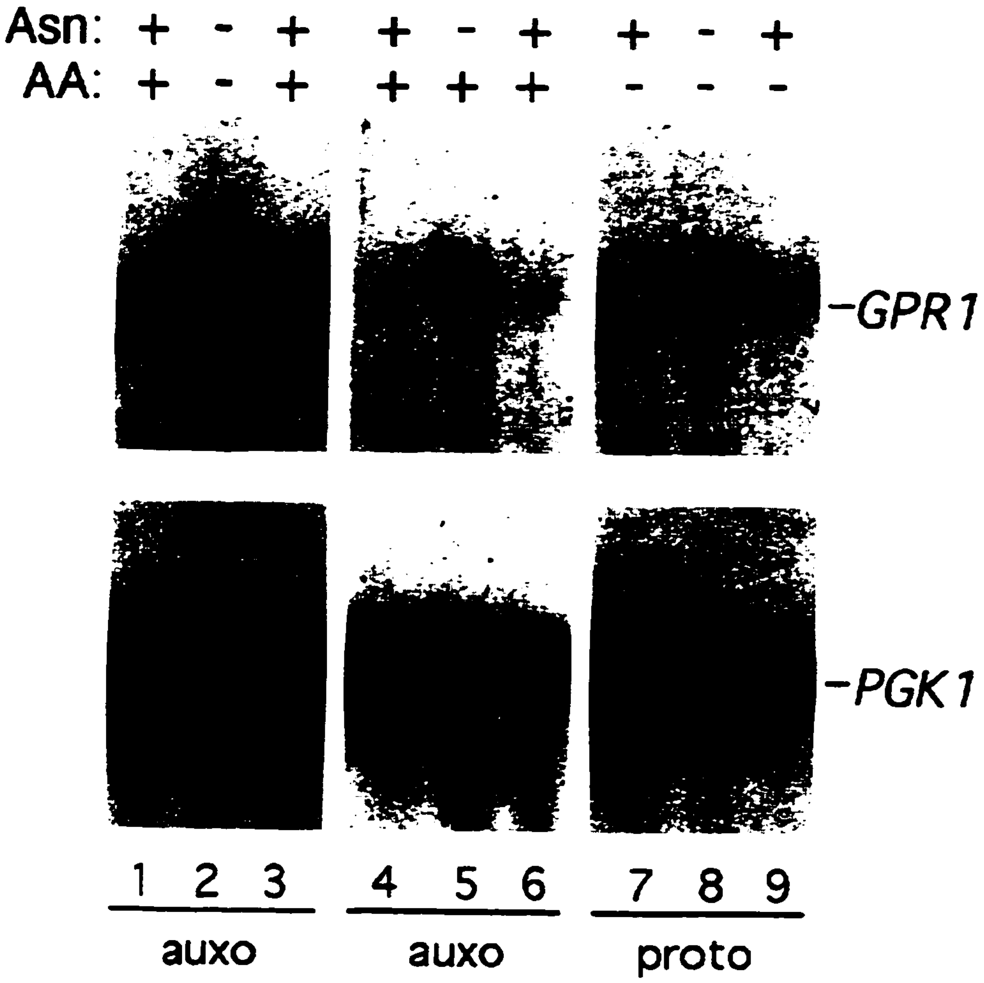
To test whether *GPR1* is involved in pseudohyphal differentiation, the*GPR1* gene was disrupted in a yeast strain capable of pseudohyphal differentiation. Cells were streaked onto medium containing a low level of nitrogen to induce pseudohyphal differentiation. After incubation for several days, the *GPR1* null strain had significantly less pseudoyhyphae than the isogenic wild type strain (49). This work was done by Dr. Joseph Heitman's research group at Duke University in collaboration with our group.

8. In pseudohyphal differentiated cells, Gpr1p-GFP localization is not polarized

One possible reason for the requirement for *GPR1* in pseudohyphal growth is that *GPR1* may be needed for the polarization event required for the dimorphic change. In that case, the localization of Gpr1p could change during the formation of pseudohyphae. To test this hypothesis, a

Figure 16 *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.

Figure 16



multi-copy plasmid containing *GPR1-GFP* was transformed into a yeast strain capable of pseudohyphal differentiation.

As shown in Figure 17 (panels C, F), the intensity of the immunofluorescence signal for the Gpr1p-GFP protein appears lower in pseudohyphal cells than in the surrounding cells that are in yeast form. However, due to variable expression in multi-copy plasmids, this observation needs to be confirmed by further studies with more quantitative techniques. The results in Figure 17 do show that in all pseudohyphal form cells examined, the signals do not appear to localize to the cell-cell junction or any particular sites, but are evenly distributed at the plasma membrane.

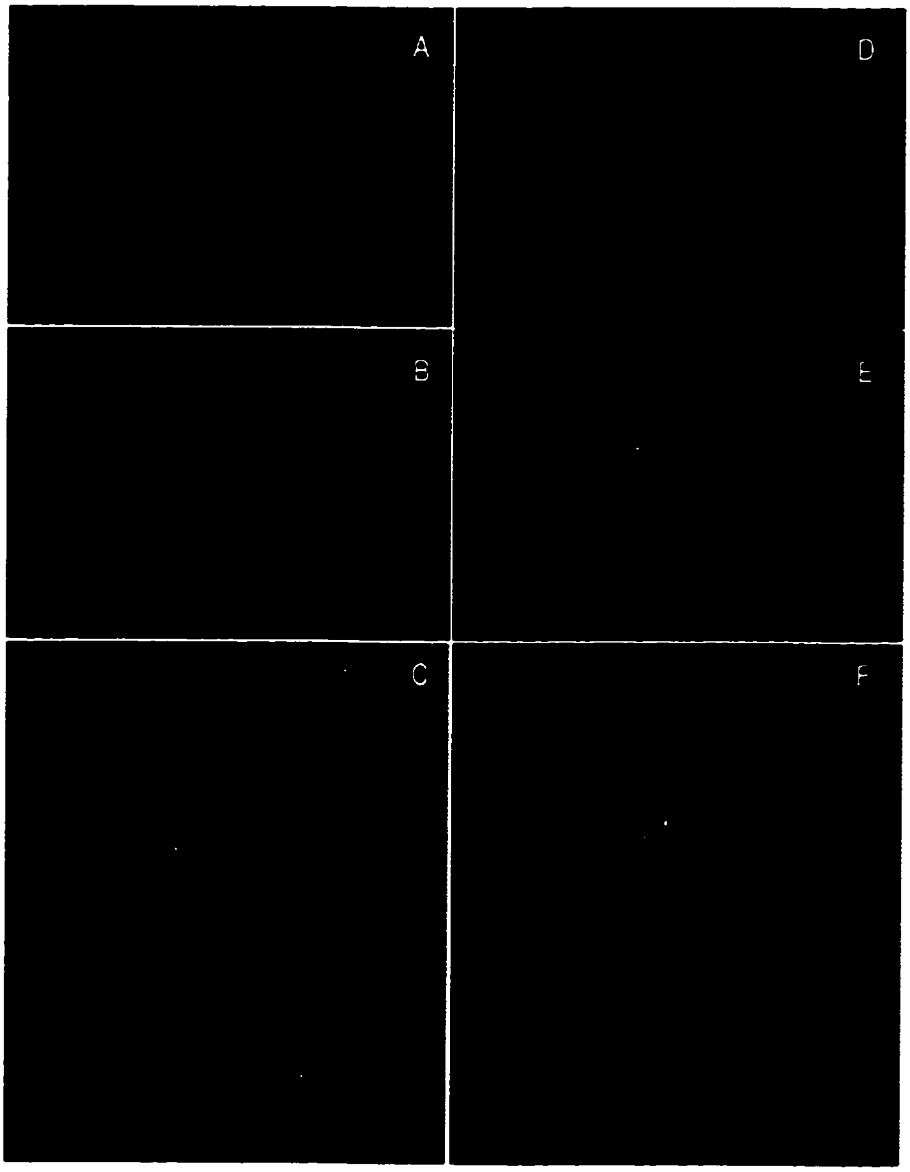
9. *GPR1* has *GPA2*-independent signaling functions

a. Deletion of *GPR1* has greater effects on thermotolerance of yeast than deletion of *GPA2*

Since the constitutively active allele of *GPA*2 causes yeast to become heat shock sensitive, it is expected that a *GPA2* null strain would be more heat shock resistant than a wild type strain. Moreover, because Gpr1p is the

Figure 17 Gpr1p localization in pseudohyphal differentiation. A strain capable of pseudohyphal differentiation was transformed with either a Yeplac112 (vector) (Panels A, D) or a multicopy plasmid containing a *GPR1*-GFP fusion construct (pGPR1-GFP.1).(Panels B, C, E, F) and viewed using either Nomarski optics for differential interference contrast microscopy on a Zeiss Axiophot microscope (Panels A, B, C) or the fluorescein isothiocyanate (FITC) filter for fluorescence microscopy (Panels D, E, F). The cells shown in panels A, B, D,E were grown in nutrient rich conditions while the cells shown in panels C, F were grown in SLAD medium to induce pseudohyphal differentiation.

Figure 17



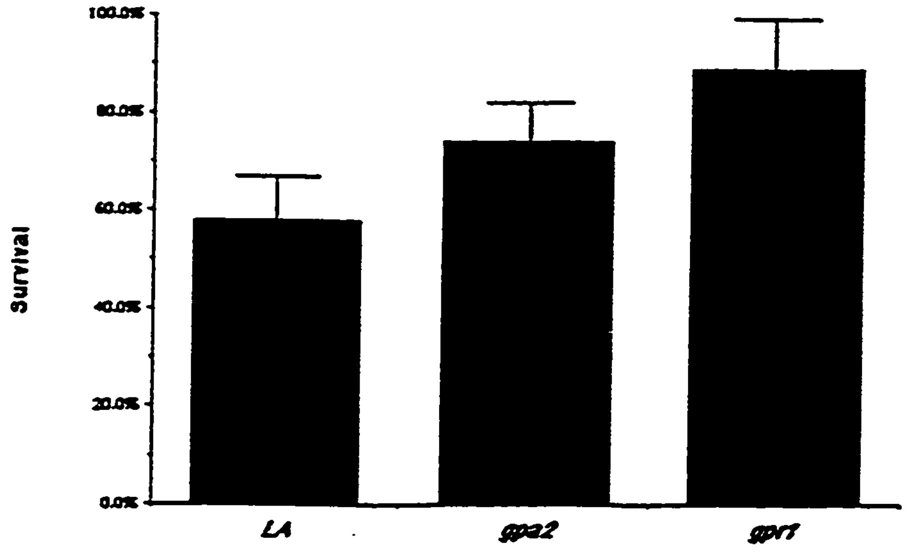
putative receptor for Gpa2p, a *GPR1* null strain would also be expected to have a similar level of heat shock resistance as a *GPA2* null strain. To test this hypothesis, a *GPR1* disruption strain and a *GPA2* disruption strain were grown to stationary phase in rich medium and were subjected to heat shock treatment. Various temperatures and durations of the heat shock were tested, and treatment at 50^0C for 30 min was found to be optimal. Under these conditions, 60% of the wild type cells survived the heat shock. As shown in Figure 18, in each experiment, the *GPR1* null strain was found to be consistently more heat shock resistant than the *GPA2* null strain. This indicates that *GPR1* disruption has more severe effects on yeast cells than *GPA2* disruption. Therefore, it raises the possibility that Gpr1p could signal through other proteins in addition to Gpa2p. For instance, it is possible that Gpr1p can function through both Gpa2p and Ras proteins.

b. *GPA2* and *GPR1* null strains behave differently in regulation of *SSA3* expression during nitrogen starvation.

When yeast cells are starved for nitrogen, expression of certain genes is induced. *SSA3* is one such example (19). *SSA3* encodes a member of the heat shock protein family, and it has a stress response element (STRE) in

Figure 18 *GPR1* null strain is more heat shock resistance than *GPA2* null strain. Yeast strains with disrupted alleles of either *GPA2* or *GPR1* were subjected to heat shock at 50^0C. The survival rates shown were averages of three independent experiments.

Figure 18



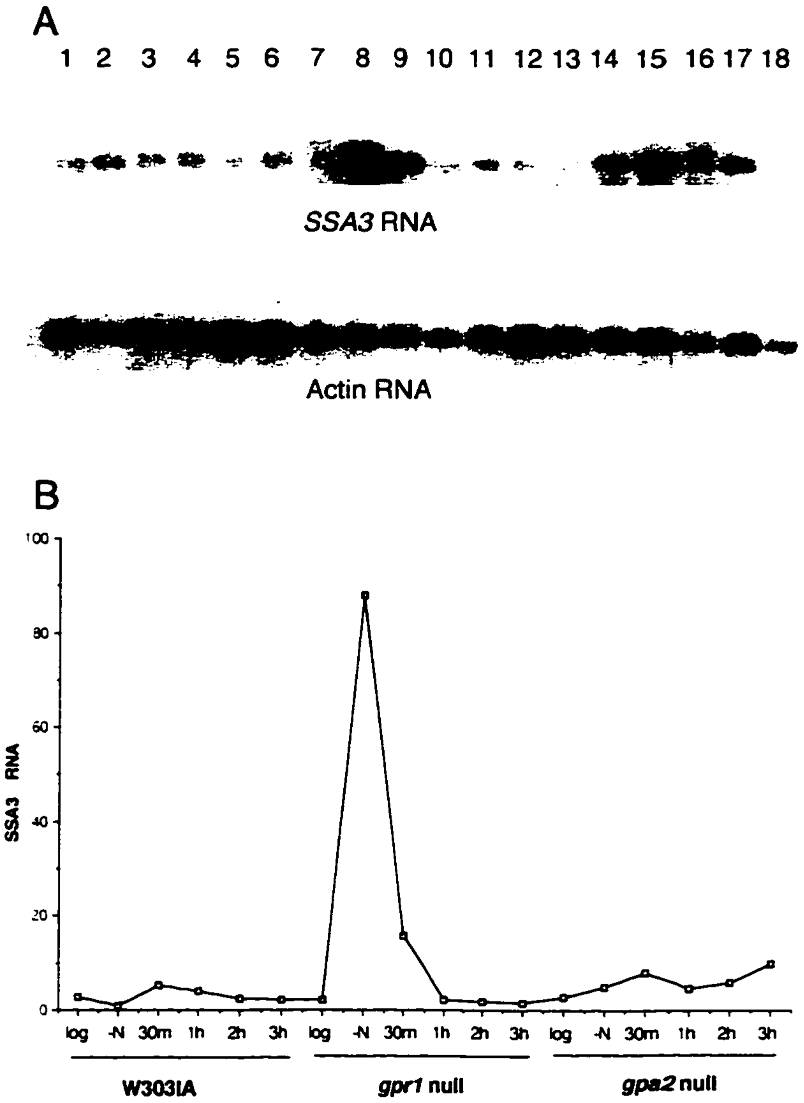
its promoter. *SCH9* has been reported to play a role in the regulation of *SSA3* expression during nitrogen starvation and induction(19)(24). Since *SCH9* is believed to be a downstream component of the *GPR1/GPA2* pathway, we decided to test if *GPR1* and *GPA2* have a similar function to *SCH9* in the control of *SSA3* expression. As shown in Figure 19, *SSA3* expression was not induced during nitrogen starvation in a wild type yeast strain. In a *GPR1* deletion strain, *SSA3* expression was highly induced during nitrogen starvation and the expression level decreased to basal level upon addition of nitrogen source, asparagine. But, unexpectedly, in a *GPA2* null strain, *SSA3* gene expression was not induced during nitrogen starvation. Therefore, repression of *SSA3* gene expression during nitrogen starvation requires *GPR1* but does not require *GPA2*. These results further confirm the hypothesis that Gpr1p could signal through other proteins in addition to Gpa2p.

DISCUSSION

A number of genes have been implicated in cell growth control in budding yeast. The Ras/cAMP pathway is widely believed to play a major role in detecting and responding to nutrients, although the connection between activation of the pathway and nutrient availability is still unknown. The work described here shows that the G protein alpha subunit Gpa2p

Figure 19 The role of Gpr1p and Gpa2p in regulating expression of *SSA3* . (a) RNA were prepared from yeast strains disrupted of either*GPA2* allele or *GPR1* allele growing in the following conditions: in log phase (Lanes 1, 7, 13), incubated in the absence of nitrogen and amino acids for 24 h (lanes 2, 8 ,14), 30 min after the addition of 10 mM asparagine and essential amino acids to starved cells (lanes 3, 9, 15), 1 h after the addition of 10 mM asparagine to starved cells (lane 4, 10, 16), 2 h after the addition of 10 mM asparagine to starved cells (lane 5, 11, 17), 3 h after the addition of 10 mM asparagine to starved cells (lane 6, 12, 18). A Northern blot prepared from the RNA was hybridized with a *GPR1* probe and then rehybridized with a actin probe as a loading control. (b) The abundance of *SSA3* RNA shown in the Northern blot was quantitated.

Figure 19


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
SSA3 RNA
Actin RNA
B
SSA3 RNA
100
80
60
40
20
0
log -N 30m 1h 2h 3h log -N 30m 1h 2h 3h log -N 30m 1h 2h 3h
W303IA
gpr1 null
gpa2 null

functions in a signaling pathway that is independent of *RAS* genes. It also describes the isolation of the *GPR1* gene, which encodes a putative G protein-coupled receptor for *GPA2* .

1. *GPR1* encodes a novel G-protein coupled receptor for *GPA2*

To confirm that Gpr1p is indeed a G protein-coupled receptor, further biochemical evidence is needed. Ultimately, it will be necessary to identify the ligand. However, based on the following evidence, we conclude that Gpr1p is a G-protein coupled receptor that is coupled to Gpa2p. First of all, Gpr1p shares sequence homology with members of the G-protein coupled receptor family and is predicted to contain the characteristic seven transmembrane domains. Conserved regions in the third cytoplasmic loop of G-protein coupled receptors are also essential for Gpr1p function. Secondly, Gpr1p is located on the cell surface and Gpr1p interacts with Gpa2p in the yeast two-hybrid assay. Lastly, Gpr1p and Gpa2p function in the same signaling pathway. The *gpa2* and *gpr1* mutations cause essentially identical severe growth defects in a *ras2* strain and both *GPA2* and *GPR1* are required for pseudohyphal differentiation. For both phenotypes, activated Gpa2p suppresses a *GPR1* deletion, which implies that *GPA2* functions in the same process as *GPR1* and acts downstream (91)(49).

Taken together, all these results strongly suggest that Gpr1p is the G-protein coupled receptor for Gpa2p.

Preliminary studies have identified some essential regions in Gpr1p, but further characterization still needs to be done. For instance, it will be of great interest to determine the minimum essential region of the third cytoplasmic loop of Gpr1p since this loop in Gpr1p is unusually large for a G-protein coupled receptor. Also, many proteins have an asparagine-rich region, but the role of this domain is not clear. Interestingly, in *Dictyostelium*, the cAMP receptors, members of the G-protein-coupled receptor family, also contain an asparagine-rich region in their cytoplasmic tails. As we know, the asparagine is a desirable nitrogen source for yeast. Although we have shown that it is not essential for the function of Gpr1p in nutrient rich conditions, further studies are needed to test if this region can play an important role in pseudohyphal differentiation, in which the sensing of nitrogen availability is critical.

To my knowledge, this is the first time that a G-protein coupled receptor has been cloned by the two-hybrid system. The two-hybrid system has not been considered a suitable system to identify G-protein coupled receptors for the obvious reason that the assay requires the interaction partners to be translocated to the nucleus. However, G-protein-coupled receptors almost certainly need to be at the plasma membrane to be functional. In the case of Gpr1p, 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. The cytoplasmic tail regions of several mammalian G protein-coupled receptors have also been previously shown to interact with G subunits and to determine the specificity of the coupled G protein (59) (66) (32). Therefore, the two-hybrid system may provide an alternative way to clone novel G-protein coupled receptors for the so-called orphan G proteins alpha subunits which have no receptors assigned to them, such as gustducin (56).

2. The *GPR1/GPA2* pathway regulates growth independent of *RAS* genes

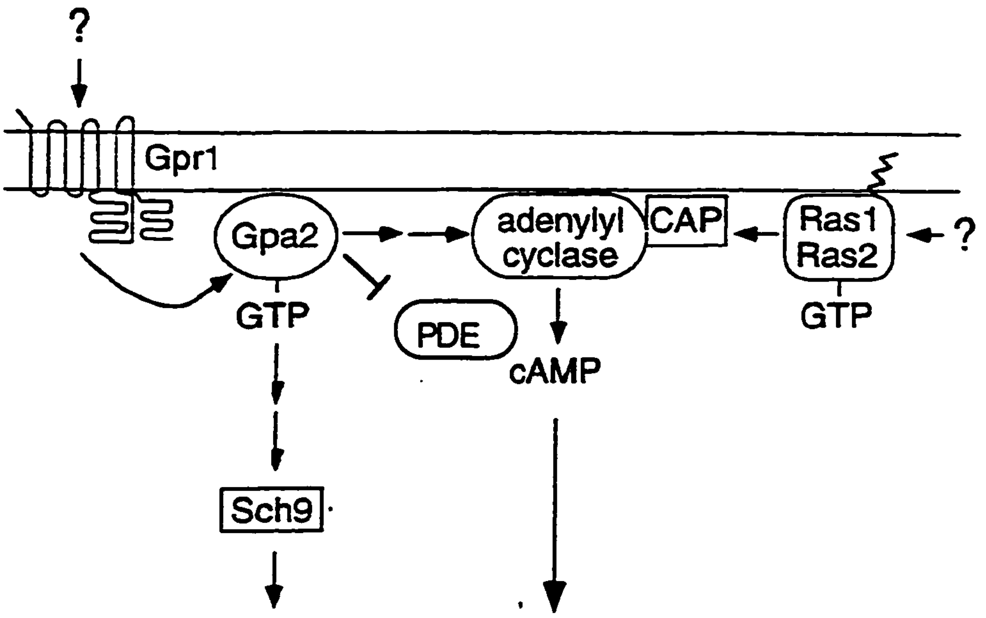
It has long been suggested that, in addition to the Ras pathway, cAMP-independent mechanisms may exist for nutrient monitoring and growth regulation (10) (67). The results described in my thesis studies suggest that the function of Gpa2p in growth control is independent of both *RAS* genes. However, these studies do not address whether the function of Gpa2p is cAMP-independent. Binding of ligand to the Gpr1p receptor is expected to result in the activation of Gpa2p. Gpa2p then transduces a signal that is independent of both *RAS* genes because the effects of a constitutive allele of *GPA2* on growth and heat shock sensitivity are not affected by deletion of both *RAS* genes. Signaling events downstream of Gpa2p are, however, not well understood. One possibility is that Gpa2p acts in a cAMP-independent pathway. The effects of Gpa2p on heat shock sensitivity of cells are carried out at least partly through the Sch9p kinase, but we can not rule out the possibility that Gpa2p can also regulate the intracellular cAMP level directly. Gpa2p could either stimulate adenylyl cyclase directly or it could inhibit phosphodiesterase proteins. Some experimental results do support the notion that Gpa2p can increase the cAMP level. First, overexpression of Gpa2p causes a small increase in the level of cAMP induced by glucose (60). Second, the changes in cell physiology caused by activated Gpa2p are very similar to those caused by elevated protein kinase A activity. Also, an increase of cAMP level overcomes the pseudohyphal defect of *GPA2* null strain (50). Finally, recent studies also show that disruption of *GPA2* decreases the basal level of intracellular cAMP, although this result is controversial due to the experimental conditions and technical problems(15).

In the model shown in Figure 20, we propose that the *RAS* and *GPA2*

Figure 20 Model for *GPR1/GPA2* signaling pathway. Gpa2p can transduce signals through Sch9p. However, it is also likely that Gpa2p can regulate intracellular cAMP level by activating adenylyl cyclase or inhibiting phosphodiesterase.

Figure 20

GPR1/GPA2 pathway



phosphorylation of substrates that regulate growth pathways are partially redundant. It is quite interesting to speculate on why yeast needs such a system. One hypothesis is that both Ras and Gpa2p function in cell growth control in response to different nutrient conditions. However, Ras and Gpa2p may have slightly different roles. Glucose appears to have the most influence on cell physiology and gene expression in yeast. The established role of Ras in transmitting the response to the addition of a fermentable sugar suggests that Ras has a role in monitoring glucose availability. Yeast can maintain a slow growth rate in the presence of a carbon source by using internal stores of nitrogen. It may be advantageous for yeast to have other pathways similar to Ras responding to other less vital nutrients such as nitrogen or phosphate. Cell growth would then be determined by the combinatorial input from different pathways monitoring carbon and other nutrients. The *GPR1/GPA2* pathway may be one of the mechanisms involved in detecting nitrogen availability. This hypothesis is consistent with the finding that the presence of glucose is required for the response of nitrogen-starved cells to the addition of nitrogen (81).

3. Function of the *GPR1/GPA2* pathway is related to nitrogen availability

The results from these studies and studies by others have suggested that the function of *GPR1/GPA2* pathway is related to nitrogen availability, most likely as a sensor for nitrogen availability (81). First, in my thesis studies, it was found that the *GPR1* RNA level is only induced under conditions of nitrogen and amino acid starvation, suggesting that the *GPR1/GPA2* pathway is regulated in response to nitrogen level in the medium. Secondly, *SCH9* has been reported to have a role in the nitrogen response (19) and Sch9p is thought to be at least one of the downstream effectors activated by Gpa2p. Finally, during the course of my thesis studies, both *GPR1* and *GPA2* have been shown to be essential for pseudohyphal differentiation, which is a well characterized response to nitrogen starvation (43)(50)(49).

It should also be noted that the severe growth defect caused by disruption of *GPA2* or *GPR1* in a *ras2* null strain occurs under nutrient rich conditions. This suggests that both *GPR1* and *GPA2* also play some role in growth control when the level of nitrogen source is high.

4. Gpa2p-independent signaling by the Gpr1p receptor

These studies showed that Gpr1p had greater effects than Gpa2p on thermotolerance and on regulation of gene expression during nitrogen starvation,. These observations suggest that Gpr1p may have functions that are carried out by a Gpa2p-independent mechanism. Recently, another study reported that Gpr1p is required for the glucose-mediated induction of cAMP which does not require Gpa2p (93). This Gpa2p-independent function of Gpr1p, however, requires the Gpa2p binding domains of Gpr1p. One explanation for this apparent contradiction is that an unidentified beta-gamma complex is responsible for the residual function of Gpr1p that is Gpa2p-independent. Since the beta-gamma subunits of the pheromone pathway are not coupled to Gpa2p and are not required for pseudohyphal differentiation, it is very unlikely that they are components of the Gpr1p pathway. Therefore, it is reasonable to propose that there is a novel beta-gamma complex that functions in parallel to Gpa2p in the Gpr1p pathway. The genome of *Saccharomyces cerevisiae* has been sequenced and a number of uncharacterized open reading frames have been shown to contain WD40 repeats similar to those possessed by beta subunits of heterotrimeric G proteins. Unfortunately, disruptions of these genes have not provided us with a clear answer. These genes are either essential or are not required for pseudohyphal differentiation (50). These two criteria make them very unlikely to be the beta subunit that binds to Gpa2p because Gpa2p is required for pseudohyphal growth and is not essential for the normal growth of yeast. However, it is still possible that one of the essential genes with WD40 repeats is indeed is the beta subunit that bind to Gpa2p, but that the beta subunit has other essential functions. Further studies are needed to identify the potential beta-gamma subunits that function in the *GPR1/GPA2* pathway.

One other possible explanation for Gpa2p-independent signaling of Gpr1p is that Gpr1p may transduce a signal through signaling molecules other than heterotrimeric G protein subunits. During the past few years, an increasing number of signaling molecules have been found to physically interact with G-protein coupled receptors and to be responsible for transmitting at least part of the signal from the receptors. These include Jak2 kinase, phospholipase C and protein kinase C. It is therefore not inconceivable that another signaling molecule such as Ras1p or Ras2p transmits part of the signal from Gpr1p and acts in parallel to Gpa2p.

6. Speculation on *GPR1* ligands

Since Gpr1p is postulated to sense the presence of nitrogen source, at first glance it is obvious to suggest that the ligand for Gpr1p is one or more of nitrogen source. However, this appears to be an unlikely possibility. Many kinds of nitrogen sources can be utilized by yeast. Since Gpr1p is required for pseudohyphal differentiation when cells are starved for nitrogen (49), it is hard to imagine that Gpr1p can detect so many different nitrogen sources. An alternative model is that Gpr1p is in close association with a complex that includes many proteins for nitrogen metabolism and transport. In that case, Gpr1p could act as a integration point for inputs from a variety of nitrogen source signals. The ligand for Gpr1p could be a common nitrogen metabolite. There is no evidence, however, to suggest that this kind of nutrient sensor complex exists in yeast. The third and more attractive model is that the ligand for Gpr1p is a molecule secreted by yeast. The secretion of this molecule would be greatly enhanced when yeast cells are starved for nitrogen. In this model, Gpr1p would be able to respond to a general nitrogen starvation condition. Yeast cells secrete many kinds of molecules and nutrient availability and metabolic level directly affect the amount of secretion of these molecules (87). For instance, some alcohols have been found to increase secretion from yeast during nitrogen starvation. One or more of these molecules could be a candidate for the Gpr1p ligand.

7. Concluding Remarks

Heterotrimeric G protein and Ras pathways have been under intensive study during the past few years due to their crucial roles in cell proliferation and differentiation. Increasing evidence has suggested that there is crosstalk between these two signaling pathways in mammalian cells (20) (72). Understanding how cells integrate signals from different pathways is now a major area in the field of signal transduction. I believe that the interaction between Gpa2p and the Ras/cAMP pathway in yeast provides a excellent system in which to study this problem. Yeast has the advantages of a simple system with powerful genetics. Past experience has proven that the information gained from the study of lower organisms such as yeast is very informative for understanding similar mammalian systems. Therefore, the interaction between Gpa2p and the Ras pathway in growth control merits further thorough investigation. Besides this apparent similarity to mammalian signaling systems, the study of the *GPR1/GPA2* pathway could be very rewarding in its own right. The function of Gpa2p as a transducer of an extracellular signal in a unicellular organism is extremely interesting. It is also the first proposal of the idea that extracellular signaling molecules could play a role in nutrient signaling in yeast. The *GPR1/GPA2* pathway therefore provides a distinctive system in which to study nutrient sensing and signaling processes in *Saccharomyces cerevisiae* as separated from the housekeeping functions of nutrients.

Appendix

1. To find other proteins that have similar functions as Gpa2p, a screen was carried out to search for genes whose deletion can cause lethal in yeast deleted of *GPA2* allele. A strain disrupted of *GPA2* allele was transformed with a plasmid containing *GPA2* under the control of *GAL1* inducible promoter. The *GAL1* promoter enabled that the expression of Gpa2p was turned on in galactose containing medium while being shut off in glucose containing medium. After been mutagenized using UV radiation, yeast cells were spread onto galactose containing medium. After the colonies grown to visible size, they were replicated onto glucose containing medium. No mutated yeast colonies were found to dependent on *GPA2* allele for its survival. In retrospect, it is surprising because double disruption of *RAS2* and *GPA2* has been shown to cause severe growth defect. It is possible that the small colonies of *gpa2 ras2* on glucose medium is hard to distinguish among other colonies in the screen. This deficiency is further augmented by one major weakness in the experimental strategy. Even though the expression of Gpa2p was turned off in the glucose containing medium, it take several cell divisions for the yeast cells to be completely depleted of Gpa2p and these residual Gpa2p protein may contribute to a higher growth

rate.

2. Since Ime2p is shown to interact with Gpa2p in the two-hybrid system and Ime2p is specifically involved in the initiation of sporulation, it is possible that Gpa2p is regulated by Ime2p during sporulation, mostly likely being phosphorylated by Ime2p. A preliminary study was carried out to test if Gpa2p can be phosphorylated during sporulation. A diploid yeast was transformed with a *GPA2-HA* containing plasmid and sporulated under nitrogen limiting condition. Proteins were extracted from the cells 30min, 1hr, 2hr, 3hr, 8hr, and 3days after transfer to sporulation medium and equal amounts of proteins were loaded into PAGE. A western blot was probed with anti-HA antibody and later reprobed with anti-PGK antibody as a control. We were not able to observe any significant change of protein level of Gpa2p. There was also no mobility change of the species representing Gpa2p-HA. Therefore, whether Gpa2p is regulated by Ime2p during sporulation needs further investigation.

3. It is of our great interest to find the heterotrimeric G protein beta subunit that can associate with Gpa2p. The yeast genome database was searched for any open reading frames homologous to G beta subunit in sequence. Deduced amino acid sequence of *YCR072C*, a uncharacterized

ORF, shows limited homology to members of the transducin beta subunit family. To test if Ycr072p can interact with Gpa2p in the two-hybrid system, a plasmid containing *YCR072C* fused with activation domain of *GAL4* was transformed into the two-hybrid strain HF7C with a plasmid containing *GPA2-GAL4bd* fusion allele. No detectable beta-galactosidase activity was observed in this yeast strain. This result makes it unlikely for Ycr072p to be the beta subunit bound to Gpa2p because previous study has shown that Gpa1p and its beta subunit Ste4p can interact in the two-hybrid system.

4. An constitutively active allele of *GPR1* allele will be very helpful for further epistasis studies on *GPR1* pathway as well as for understanding the structure of Gpr1p. In the effort to make a constitutively active *GPR1* allele, an in vitro mutagenesis experiment was carried out to change the proline of position of 637 to leucine. The proline is in the 6th transmembrane domain. Similar change in other G-protein coupled receptors results either in constitutively active receptors or unstable proteins. In the case of *GPR1*, altered version of Gpa2p was not able to localized to the plasma membrane. Transformation of a plasmid containing *GPR1P637L* was unable to suppress the growth defect of *gpr1 ras2*. Therefore, *GPR1P637L* is not an constitutively active allele of *GPR1*.

Further studies are needed to find constitutively active alleles of *GPR1*.

5. For most of G-protein coupled receptors, both the cytoplasmic tail and the 3rd cytoplasmic loop can interact with their coupled G proteins. Cytoplasmic tail of Gpr1p was shown to bind to Gpa2p in the two-hybrid assay. It is of our interest to determine if the 3rd cytoplasmic loop of Gpr1p can also interact with Gpa2p. A large part of the coding region of 3rd loop of Gpr1p was fused with Gal4p activation domain and expressed in a strain containing a plasmid with *GPA2* allele fused with *GAL4* binding domain. No detectable level of beta-galactosidase activity was observed in this strain. This suggest that this region of Gpr1p may not be important in the interaction with Gpa2p. But we also have to keep in mind that the two-hybrid system is an artificial system. It may not represent faithfully to physiological situations inside cells. For instance, if the surrounding transmembrane regions of Gpr1p are required to maintain the proper structure of 3rd cytoplasmic loop and thus important for its interaction with Gpa2p, the interaction between Gpa2p and the loop region of Gpr1p can not be detected in the two-hybrid system.

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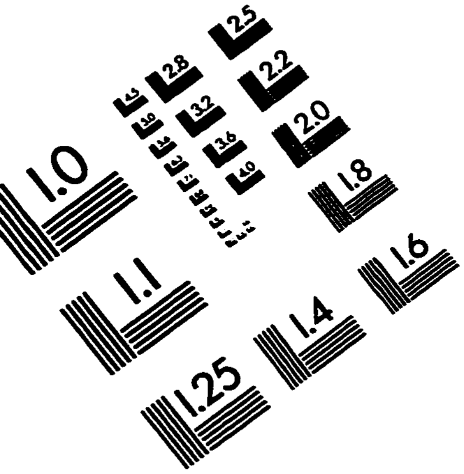

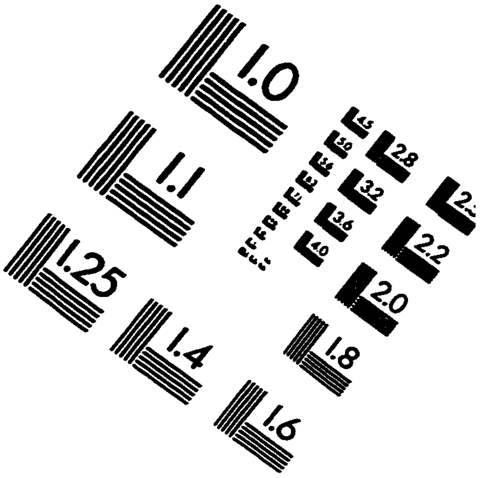

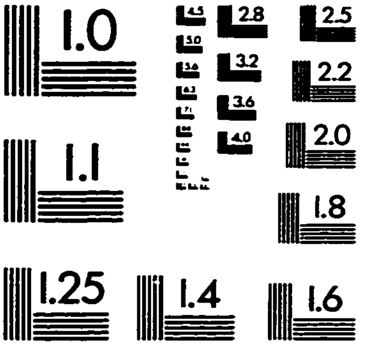

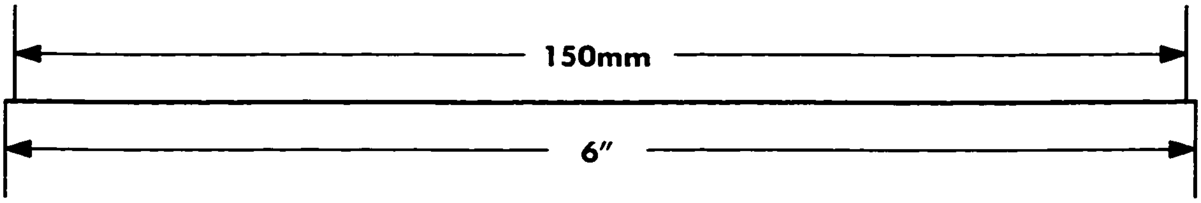

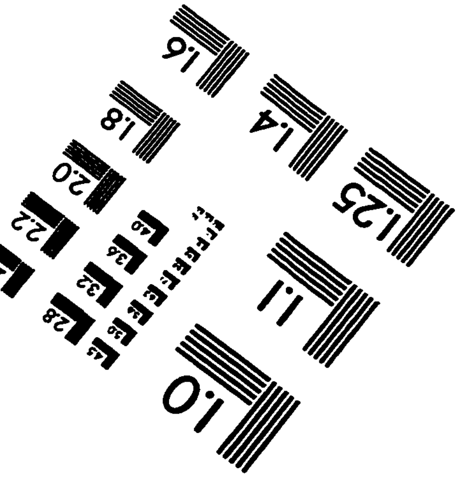

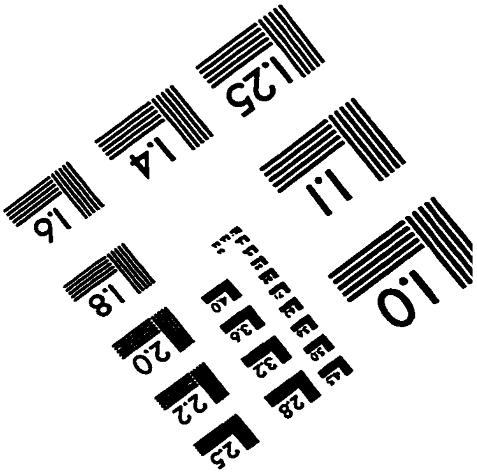

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