Interaction Between G-Protein-Coupled Receptor Signaling Pathways in *Saccharomyces cerevisiae*

an Honors Project submitted by

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TABLE OF CONTENTS

ABSTRACT

The purpose of this study was to investigate possible interactions between the two G-protein-coupled receptor (GPCR) signaling pathways in baker's yeast, *Saccharomyces cerevisiae. S. cerevisiae* is an important and widely used model organism in biological research, and GPCRs are popular drug targets, comprising about one third of all pharmaceuticals. Since *S. cerevisiae* possesses only two GPCR-regulated pathways, the mating and glucose-sensing pathways, it serves as an excellent model for exploring the function of this class of proteins. Activity of the mating pathway was measured in strains with gene deletions from the glucose-sensing pathway. Activity of the Ste2 mating pathway was inhibited with deletion of the glucose-sensing receptor, Gpr1, and/or its Gα protein, Gpa2, but the Ste3 mating pathway was not affected. Because the activity of the glucose-sensing pathway is difficult to measure, a chimeric protein fusing the C-terminus of the glucose-sensing GPCR, Gpr1, to the N-terminus of the pheromone-sensing Gα protein, Gpa1, was constructed. The chimera did not provide effective signal transduction and also yielded petite mutants. From the results it may be implied that the detection of glucose as an energy and carbon source has a regulatory effect on activation of the pheromone-sensing mating pathway.

INTRODUCTION

Saccharomyces cerevisiae, commonly known as baker's or brewer's yeast, is a widely used model organism for molecular and cell biology research. *S. cerevisiae* is an easily grown and manipulated eukaryote with a genome that has been completely sequenced. Its proteins share many structural characteristics with those in human cells. Among these shared characteristics are G-protein-coupled receptors (GPCRs), a group of proteins found "in just about every organ system" in the human body as well as many other types of eukaryotic cells (Filmore, 2004).

Most G-protein-coupled receptors function by sensing and binding to a ligand molecule outside the cell. Binding of the ligand results in a change in the protein's conformation, which then activates the receptor's coupled G-protein (guanine nucleotide-binding protein). The G-protein acts as a sort of "molecular switch" that alternates between active and inactive states by exchanging GDP for GTP (Figure 1). In its activated GTP-bound state, the G-protein in turn activates a signaling cascade that mediates responses in the cell.

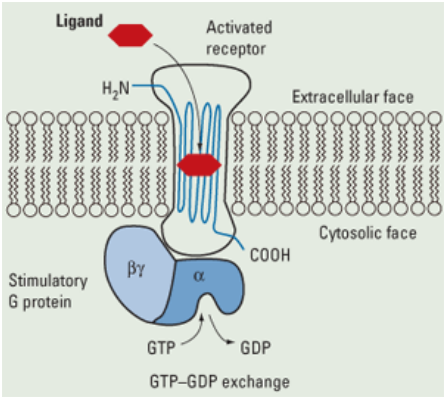


Fig. 1. A sample GPCR and its coupled G-protein (Filmore, 2004).

One to two percent of the human genome is reported to encode five to six hundred different GPCRs in addition to 26 different G-proteins (Paila and Chattopadhyay, 2008; Versele et al., 2001). About half of these receptors are thought to mediate a variety of sensory events

including vision, taste, and olfaction (Wise et al., 2004). GPCRs also bind a wide variety of stimuli including "light, protons, Ca^{2+}, odorants, amino acids, nucleotides, proteins, peptides, steroids, and fatty acids" (Xue et al., 2008).

Due to their abundance and variety of functions, GPCRs have become a popular target for the development of new drug therapies. According to Wise and colleagues (2004),

> drugs active at G-protein-coupled receptors (GPCRs) have therapeutic benefit across a broad spectrum of human diseases as diverse as pain, cognitive dysfunction, hypertension, peptic ulcers, rhinitis, and asthma. Of the approximately 500 clinically marketed drugs, greater than 30% are modulators of GPCR function, representing approximately 9% of global pharmaceutical sales, making GPCRs the most successful of any target class in terms of drug discovery.

This information illustrates the potential benefit inherent in a more complete understanding of this group of proteins.

Though humans contain hundreds of types of GPCRs, yeast cells have only two types. Each of the yeast mating types contains a unique pheromone receptor, Ste2p (mating type MATa) or Ste3p (mating type MATα), each of which associates with the G-protein Gpa1 (Nakayama et al., 1988). The second receptor is Gpr1p, a glucose-sensing GPCR that associates with the G-protein Gpa2 (Xue et al., 1998). The smaller variety of GPCRs in *S. cerevisiae* lends itself as an excellent model to study GPCR activity and regulation.

Ste2 and the Mating Pathway

Ste2p is a class D (fungal mating pheromone receptor) GPCR with characteristic seven transmembrane domains, but little sequence similarity to other GPCRs. However, this receptor demonstrates ligand binding and activation mechanisms similar to other GPCRs and can even couple to mammalian G-proteins (Brown et al., 2000).

Upon binding its ligand pheromone, α-factor, Ste2p undergoes a conformational change and activates its G-protein by GDP-GTP exchange. The type of G-protein associated with Ste2 is heterotrimeric, meaning it is composed of alpha, beta, and gamma subunits. Exchange of GDP for GTP causes the G-protein alpha subunit, Gpa1, to dissociate from the beta-gamma dimer subunit, which activates a signaling cascade of mitogen-activated protein kinases (MAPK) (Bahn et al., 2007). Activation of the MAPK pathway mediates cellular responses including growth arrest, formation of mating projections (shmoos), and expression of proteins involved in cell fusion.

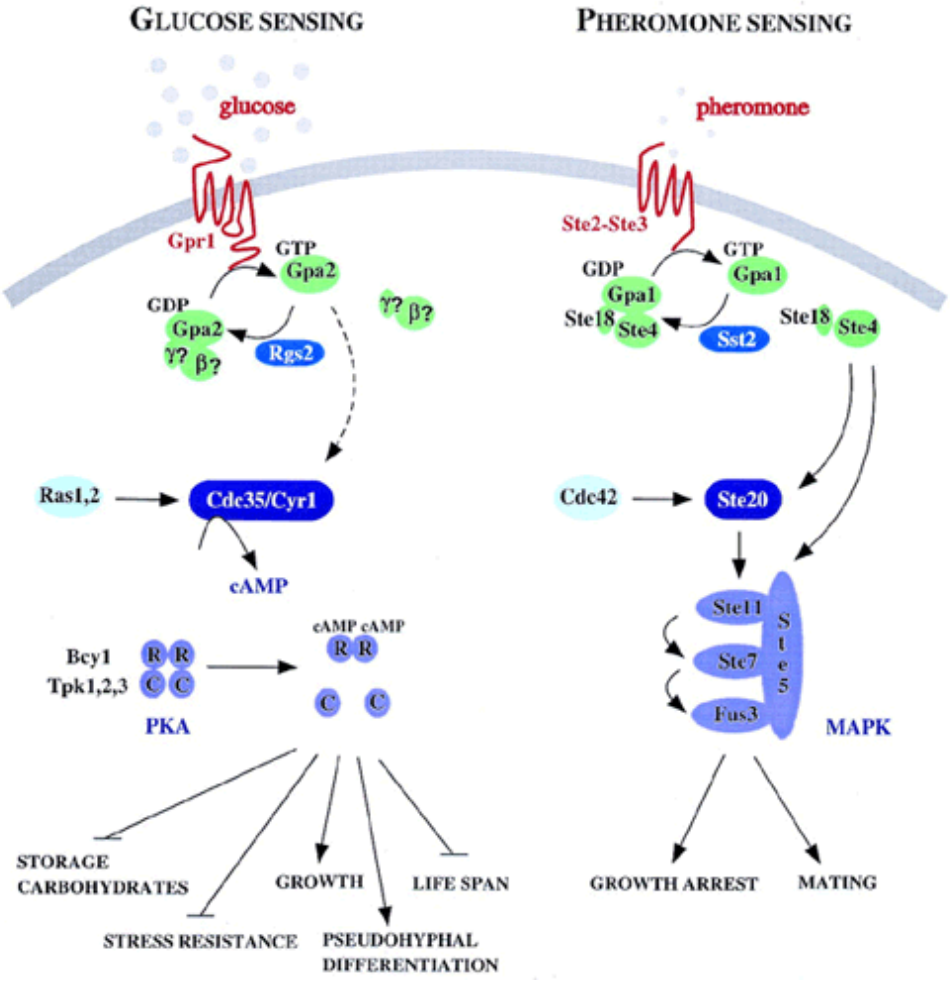


Fig. 2. Overview of glucose and pheromone sensing in *S. cerevisiae* (Versele et al., 2001).

Gpr1 and the Glucose-Sensing Pathway

The relatively recently discovered second GPCR in yeast is the glucose receptor Gpr1 (Kraakman et al., 1999). Gpr1 interacts with its G-protein, Gpa2, which unlike Gpa1, does not appear to have beta-gamma subunits. Glucose sensing is responsible for cAMP pathway activation, which converts the cell from utilizing gluconeogenesis to utilizing fermentation for energy production. This pathway has also "been shown to regulate morphogenesis," causing cells to undergo pseudohyphal differentiation (Tamaki, 2007). In addition, it has been shown that the Gpr1 pathway "regulates cell size by affecting both growth rate and cell division," allowing cells to adapt to starvation periods by altering metabolic activity (Tamaki et al., 2005; Rolland et al., 2002).

It has been shown that certain GPCRs do interact with each other and in some cases form homo- or heterodimers (Milligan, 2001). Ste2 forms a homodimer, but it is unknown whether Gpr1 forms either homo- or heterodimers (Kim et al., 2009). It has also been shown that the G-protein of the glucose-sensing pathway, Gpa2, is not capable of functionally coupling to the mating GPCR Ste2 (Blumer and Thorner, 1990). However, it is unknown whether the G-protein Gpa1 can couple to the receptor Gpr1.

In preliminary research on the Ste2p pathway conducted in the summer of 2008, an *S. cerevisiae* strain lacking Gpr1 (Gpr1Δ) exhibited decreased levels of pheromone-induced cell cycle arrest compared with other mutant and wild type strains. In other words, the cells continued to grow, possibly indicating decreased activity of the mating pathway. This unexpected finding raises the question of whether Gpr1 and/or its pathway components are in some way connected to the Ste2 pathway. As seen in Figure 2, there is currently no known mechanism by which these two pathways overlap. By exploring these pathways, it is possible to

gain more insight into the function of these GPCRs, particularly Gpr1, about which less is known.

The activity of the Ste2 pathway is relatively easy to monitor and measure, using different procedures to observe growth arrest, shmoo formation, and mating gene activation, which are either directly or, through the use of reporter genes, indirectly measurable. However, the Gpr1 pathway causes cellular responses that are not easily quantified.

In hopes of monitoring the activity of the GPCR pathways, a fused Gpr1-Gpa1 protein was engineered. This construct would theoretically allow the mating pathway to be activated by the binding of glucose to Gpr1. Then certain assays could be used to quantify Gpr1's activity through the pathways' response using the chimera protein cells as well as other mutant strains of *S. cerevisiae.*

MATERIALS AND METHODS

Preparation of plasmids

Plasmid pBUGf containing GPA1 DNA, provided by George Umanah of the University of Tennessee, was transformed into competent JM109 bacterial cells and grown on selective media following the *E. coli* competent cells protocol (Promega). The plasmid was then purified using the PureYield Plasmid Midiprep kit (Promega).

The same procedure was used to prepare a GPR1 plasmid, using ultra-competent bacterial cells (Stratagene Solopack Gold) in place of the competent JM109 cells to ensure higher transformation efficiency.

PCR and Restriction Digest

In order to create a chimeric protein, the genes coding for both units must be attached for the genes to be expressed simultaneously. The polymerase chain reaction (PCR) uses single-stranded DNA and primers with specifically designed complementary base sequences to amplify a certain portion of DNA.

First, primers that included bases from the beginning and end of GPR1 and from each side of the insertion site in the Gpa1 plasmid were designed and ordered. Primers PRPA-FOR: 5'-CGACGGATCTAGAACTAGTGGATCCATAATGATAACTGAGGGATTTCCCC-3' and prpa-rev: 5'-GCGTACTCACTGTACACCCCATTAATGGTCCATTTCTTAAGAAGGC -3' were used to perform the polymerase chain reaction on the Gpr1 plasmid (underlined portions indicate GPR1 sequences). This procedure created multiple copies of the GPR1 gene with "sticky ends" to correlate with the corresponding ends in the GPA1 plasmid for GPR1 insertion.

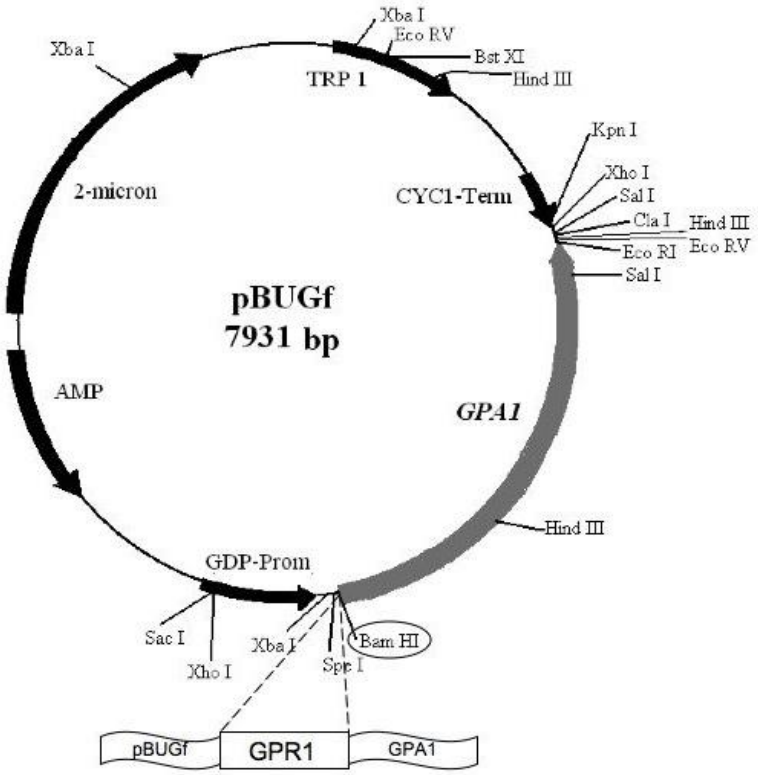


Fig. 3. Restriction map of pBUGf (Gpa1 plasmid), shown with PCR product at the insertion site.

The GPA1 plasmid was digested with BamHI restriction enzyme, cutting open the circular DNA at a specific site engineered into the plasmid (see Fig. 3), thus providing an insertion site for the PCR product. The cut plasmid and the PCR product were then transformed into TM 5117 (Far1Δ Gpa1Δ Ste2Δ) *S. cerevisiae* cells to recombine by in vivo ligation (Gietz and Woods, 2002).

Western Blot

In order to confirm the phenotype of the fused protein, a Western blot was performed. Western blotting is a technique used to detect the presence of certain proteins in a sample. Using

gel electrophoresis, proteins were separated along an 8-16% Tris-Glycine gel according to size, and then electro-transferred to Immobilon-P membrane paper. The membrane was blocked in Tris-buffered saline (TBS) buffer with 5% milk for approximately one hour, after which it was incubated with anti-FLAG polyclonal rabbit antibody at a dilution of 1:4000 in TBS-Tween for 90 minutes at room temperature. The membrane was then washed twice for five minutes in TBS/milk, once for five minutes in TBS plus 0.1% Tween (TBST), once for five minutes in TBS/milk, and briefly rinsed in TBST. Goat anti-rabbit HRP secondary antibody (Biorad) at a dilution of 1:3000 in TBST was added and incubated for approximately three hours at room temperature.

During this process, the antibodies bind to specific proteins, allowing the proteins to be detected. To rinse off unbound secondary antibodies, the membrane was washed three times for five minutes in TBS, twice for five minutes in TBST, and twice for five minutes in TBS. The samples were amplified and detected using an Opti-4CN substrate kit (Biorad).

ß-galactosidase Assay

This assay involves using a reporter gene to monitor the activity of the pheromone mating pathway. FUS1 is a gene activated by the pheromone pathway that causes the yeast cell to change shape for fusion with another cell. The TM strains are engineered so that FUS1 is attached to the lacZ gene, which codes for the enzyme beta-galactosidase. Measuring the amount of product released after beta-galactosidase reacts with certain substrates can determine the amount of beta-galactosidase produced. The product released gives off measurable fluorescence proportional to the amount of enzyme present, and thus to the level of FUS1-lacZ gene induction.

Overnight cultures of cells were centrifuged, resuspended in PBS buffer, and incubated at 30°C for one hour. Cells were resuspended in varying concentrations of dextrose solution and 150 μl of each were added to a 96-well plate. 30 μl of 5% Triton-X 100 50mM PIPES buffer with the fluorescent substrate fluorescein di-β-D-galactopyranoside (FDG) was added to each well. The plate was incubated at 37°C for 30 minutes and then read on a SpectraMax Gemini XS spectrofluorometer.

Mating Assay

Cultures of wild type, Gpr1Δ, and Gpa2Δ cells of both mating type MATa (BY4741) and MATα (BY4742) were grown overnight, centrifuged, washed three times in sterile water, and resuspended in 6 ml of sterile water. Cells were counted using a hemocytometer. Each possible combination of MATa and MATα cells was mixed in 100 μl YPD media, maintaining a 1:5 ratio of MATa to MATα cells. Mixtures were incubated at 30°C for five hours. Cells were harvested by centrifugation, washed three times with sterile water, and resuspended in 2 ml sterile water. Cells were diluted 1:20 in water, and then 75 μl was plated onto MLMK, a selective media. Plates were incubated for two days at 30°C, and then the number of colonies formed was counted. From this data, mating efficiency was calculated using the formula:

$$\text{mating efficiency (\%)} = (100 \times X)/W$$

where X=the number of colonies formed between mutant strains, and W=the number of colonies formed between wild type strains.

Growth Arrest Assay

This procedure measures the amount of growth arrest by incubating serial dilutions of the ligand with a certain number of cells in a 96-well plate. Overnight cultures of BY4741 wild type, Gpr1Δ, and Ste5Δ cells were diluted to 100 cells per 50 μl. 50 μl of 0, 20, 40, or 60 μM α-factor was added to 50 μl of cells in a 96-well plate to yield final concentrations of 0, 10, 20, or 30 μM α-factor. The plate was incubated for two days at 30°C. The degree of growth arrest was then determined by spectrophotometry, since the amount of light that a sample scatters or appears to absorb is proportional to the number of cells present in the growth media, i.e., the more growth arrest, the fewer cells, the lower light absorbance.

RESULTS

Growth Arrest Assay

When pheromone activates a yeast cell's mating pathway, one result is arrest of the cell cycle in G1 phase in preparation for fusion with another cell. Using spectrophotometry, the growth arrest assay measures the amount of growth arrest that has or has not occurred as a result of pheromone addition.

After addition of α-factor pheromone, Gpr1Δ cells showed less pheromone-induced growth arrest than did wild type cells. In fact, absorbance values for the Gpr1Δ strain were comparable to values for Ste5Δ, a sterile strain of *S. cerevisiae* in which the Ste2 pathway is not functional (Figure 4). This data indicates that without Gpr1 present, the binding of pheromone does not activate the Ste2 pathway.

Mating Assay

This assay was used to determine the degree of mating efficiency between the two mating types, MATa and MATα. Strains with either the glucose-sensing receptor (Gpr1) or its G-protein (Gpa2) deleted were used to investigate whether disruption of the Gpr1 pathway would affect mating efficiency.

Results of the mating assay showed no significant difference in mating efficiency between wild type MATa and either Gpr1Δ or Gpa2Δ MATα cells as compared to mating between both wild type strains (Figure 5). However, the student's t-test showed a significant decrease in mating efficiency compared to wild type when either Gpr1Δ or Gpa2Δ MATa cells were crossed with each of the MATα strains. From this information, one can infer that the

activity of the pheromone receptor Ste2 (MATa) is inhibited by disruption of the Gpr1 glucose-sensing pathway, whereas the activity of Ste3 (MATα) is not affected.

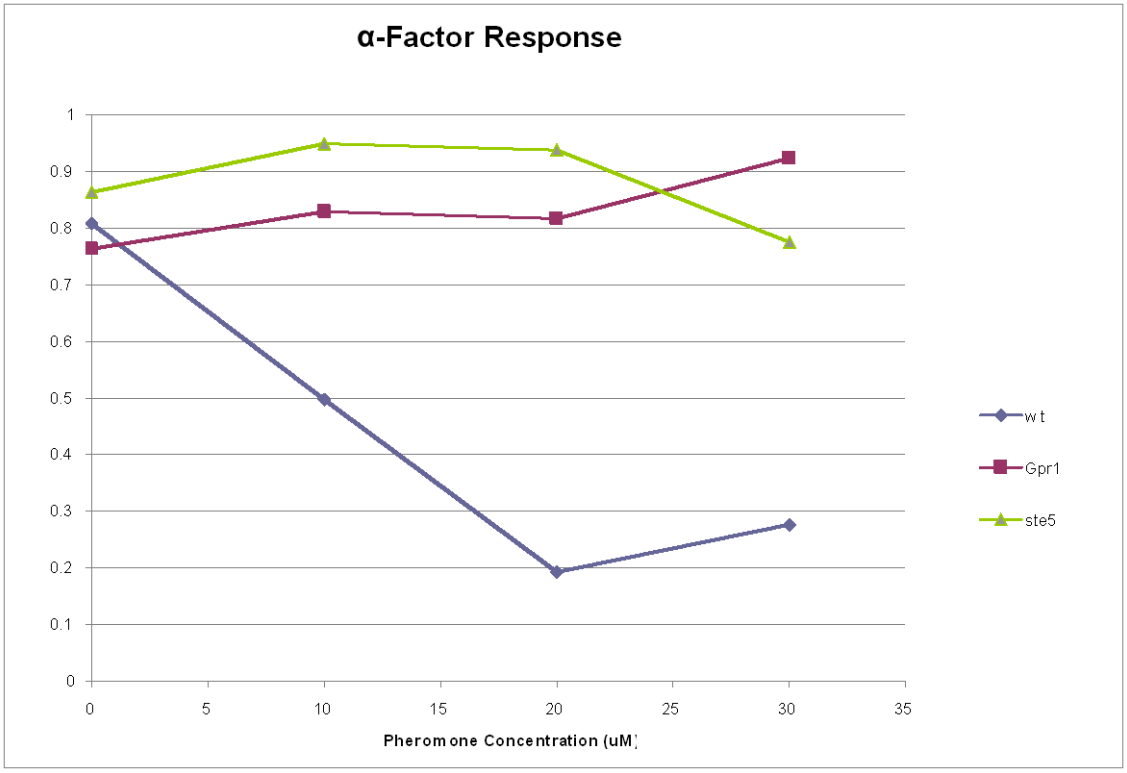


Fig. 4. Growth arrest data for BY4741 wt, Gpr1Δ, and Ste5Δ strains. High absorbance values for Gpr1Δ and Ste5Δ indicate little to no cell cycle arrest with addition of pheromone, while absorbance values for wild type cells show increased cell cycle arrest with increased pheromone concentration.

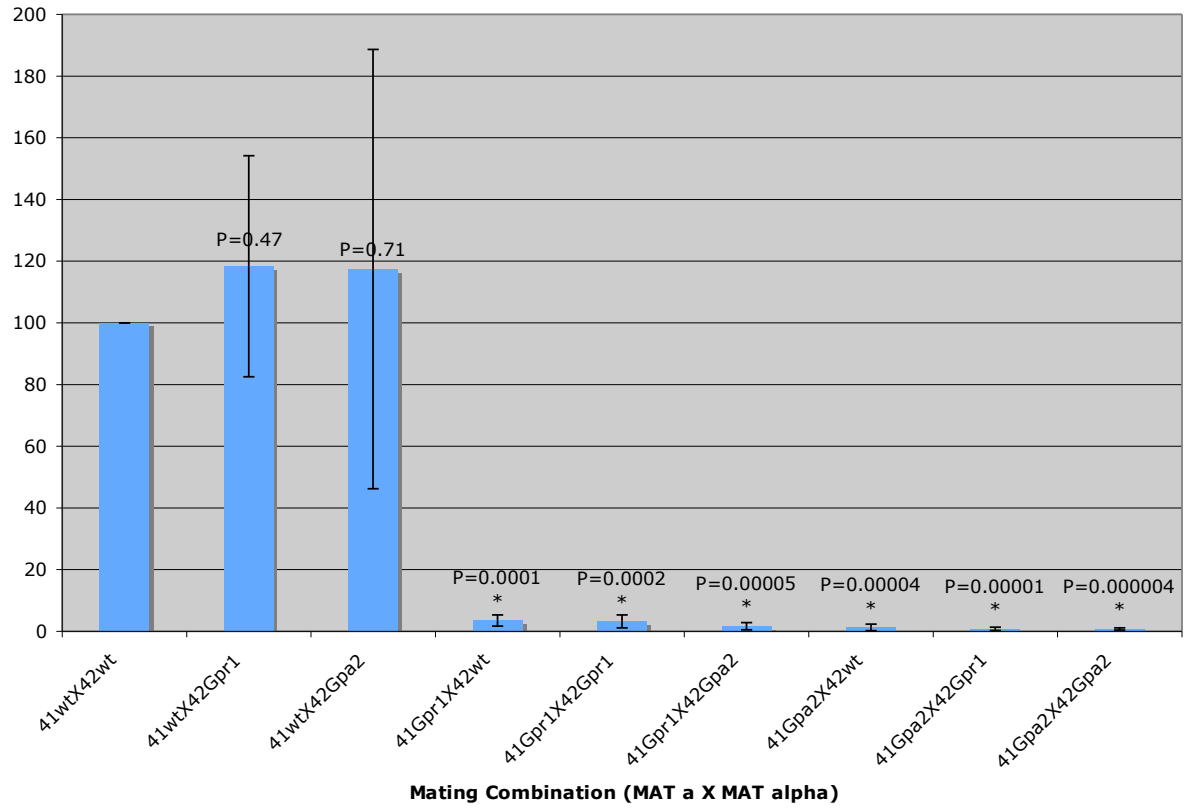


Figure 5. Effect of GPR1 or GPA2 deletion on mating efficiency between BY4741 (MATa) and BY4742 (MATα) cells. Asterisks indicate a significant difference in mating efficiency compared to wild type. (n=3)

Dot Blot, Protein Assay, and Western Blot

After much trial and error with the transformation protocol, transformant colonies were produced using 6 µl of BamHI-digested pBUGf and 2 or 6 µl of PCR product, following the transformation protocol (Gietz and Woods, 2002). Twenty-one distinct colonies were selected, two from the 6 µl / 6 µl plate, and 19 from the 2 µl / 6 µl plate. A dot blot was performed with anti-FLAG polyclonal rabbit antibody in order to gain a rough idea of the amount of protein being expressed in each colony (see Figure 6), which was subsequently quantified using the BioRad protein assay (Figure 7).

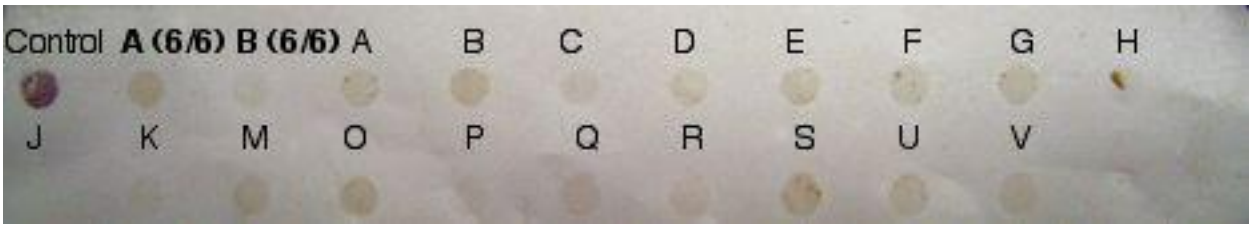
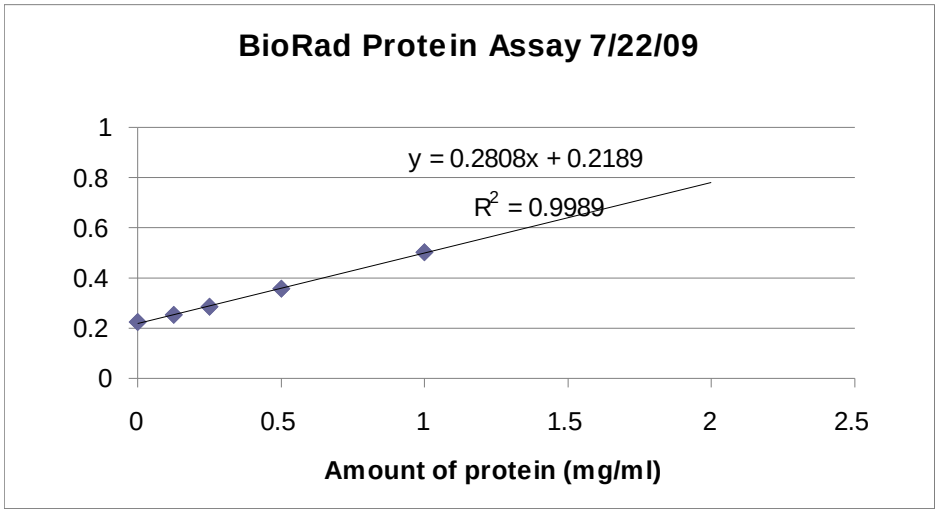


Fig. 6. Dot blot with transformant colonies. Anti-FLAG antibody showed the presence of FLAG-tagged proteins in the darker circles.

The samples producing the highest amounts of protein were colony A from the 6 μl / 6 μl plate, and colonies B, F, O, Q, and S from the 2 μl / 6 μl plate. These samples contained approximately 0.5 to 1 mg of protein per ml of solution (Figure 7b).

a)



b)

Sample	Absorbance (595 nm)	Concentration (mg/ml)
A	0.342857	0.441442308
B	0.467857	0.886599003
F	0.396429	0.632225783
O	0.382143	0.581349715
Q	0.539286	1.140975783
S	0.557143	1.204569088

Fig. 7. BioRad protein assay BSA standard curve (a) and most concentrated samples (b). Sample A (bold font) was from the 6 μl / 6 μl plate, and colonies B, F, O, Q, and S were from the 2 μl / 6 μl plate.

A Western blot was performed using samples from colonies listed in Figure 7. The chimeric protein, consisting of Gpa1 fused to Gpr1, if present in the cell, would have a molecular weight of roughly 350 kilodaltons. Samples A and O showed relatively small bands on the blot corresponding to this high molecular weight (Figure 8; O not shown). Although faint, these bands suggest that the chimeric protein was successfully inserted and expressed in each strain.

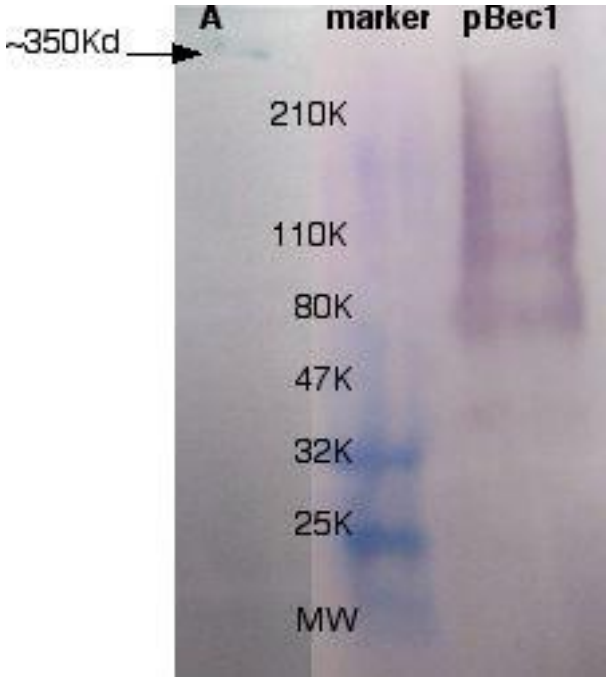


Fig. 8. Western blot. Sample A showed a faint band around 350 Kd.

Petite Mutation

Upon microscopic examination of the transformant cells, they were discovered to be much smaller than normal yeast, and were in fact mistaken for bacterial contamination for several days (Figure 9). This phenomenon is known as a petite mutation, which is most often the result of a mutation in mitochondrial DNA (Goldring et al., 1971). It is hard to say what specifically caused this mutation, but it could be cause for further study. It is also uncertain how

this mutation might have affected the results of experiments involving these cells, due to their small size and slow growth.

When the petite cells were grown on YPD (nonselective) media, a mixture of normal-sized and petite cells was observed (Figure 10). This might indicate that the normal-sized cells had lost the plasmid and reverted to normal size/growth conditions.

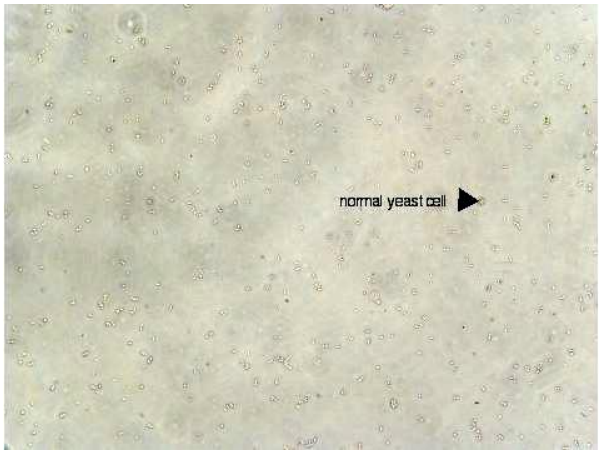


Fig. 9. Petite mutation in Gpr1-Gpa1 transformant cells.

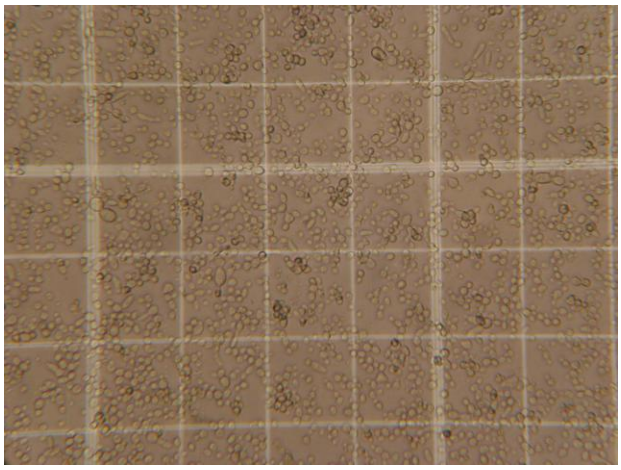


Fig. 10. Revertants grown on YPD showed both petite and normal-sized cells.

ß-galactosidase Assay

The ß-galactosidase assay, using the transformant colonies with glucose as a substrate, showed that the mating pathway was not activated by glucose binding to Gpr1. This result indicates that the receptor was not functionally coupling with Gpa1 to activate the mating pathway. However, the Ste2 pathway in the TM5117 strain (Far1Δ Gpa1Δ Ste2Δ) is normally constitutively active, as there is no Gpa1 present to hold back the beta-gamma subunits. Because of this, a ß-galactosidase assay with TM5117 cells would normally show high levels of fluorescence or pathway activity. However, the fluorescence levels in the transformant cells were brought down to low levels (Figure 11). This would suggest that Gpa1 was present in the cell, sequestering the beta-gamma subunits and inactivating the pathway. While the chimeric protein may not have been functional, this data further suggests that it was successfully introduced and expressed in the cells.

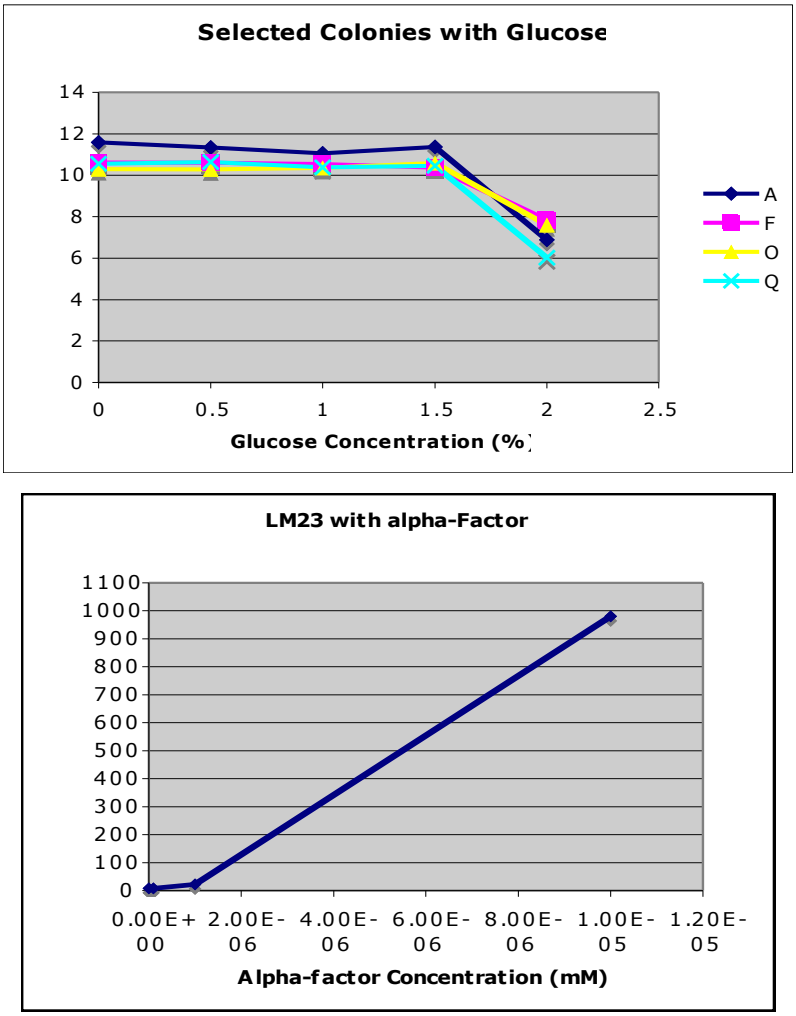


Fig. 11. ß-galactosidase Assay. Bottom graph shows regular active (~1000 units) and inactive (~10 units) levels of fluorescence.

DISCUSSION

Gpr1-Gpa1 Chimera Protein

The preliminary focus of this project was the creation and implementation of a chimeric protein consisting of the glucose-sensing GPCR, Gpr1, and the Gα subunit of the mating pathway, Gpa1, in *Saccharomyces cerevisiae.* The data shows that the chimera was successfully constructed and inserted into the TM5117 strain (Figures 8 and 11). However, results of the β-galactosidase assay showed that binding of glucose to the Gpr1-Gpa1 protein did not result in signal transduction to the Ste2 mating pathway (Figure 11). This finding suggests that the C-terminus of Gpr1 and the N-terminus of Gpa1 do not interact to regulate their respective pathways. However, this does not rule out other possible interactions between the two pathways.

Petite Mutation

Disruptions in mitochondrial DNA result in a phenomenon known as the petite mutation, in which cells become markedly smaller than wild type cells (hence the name "petite"). Yeasts such as *S. cerevisiae* are facultative anaerobes, meaning they can survive with or without the presence of oxygen. Although aerobic respiration produces more ATP, yeasts prefer alcoholic fermentation, even in the presence of oxygen, because it enables them to produce energy more quickly and also produces ethanol, which can inhibit the growth of competing organisms (Rolland et al., 2002). In yeasts, petite mutants "are characterized by an inability to utilize nonfermentable substrates such as glycerol for growth" (Goldring et al., 1971).

Many varied factors have been shown to induce petite colonies in yeast including chemicals such as ethidium bromide, as well as a large number of genes that contribute to the stability of mitochondrial DNA (Contamine and Picard, 2000). Currently among these genes are

a small number of cell division cycle, or CDC, genes. In particular, mutations in CDC8 or CDC21 genes have been shown to disrupt replication of mitochondrial DNA (Newlon et al., 1979). While two CDC genes, CDC42 and CDC35, are downstream regulators of the pheromone and glucose-sensing pathways, respectively, it is unclear whether they could play a role in mitochondrial DNA stability. According to Contamine and Picard (2000), the effects of most CDC genes on mitochondrial DNA are "either quite trivial or still very puzzling," so it is likely the petite mutations seen in this experiment stemmed from another source.

Perhaps the actual procedure used to create the chimeric protein caused some sort of mutation leading to the petite phenotype. Transformation procedures have been shown to be "highly mutagenic," so it is likely that the petite mutation was a residual effect of transforming the TM5117 strain with the chimeric protein plasmid (Contamine and Picard, 2000). Since petite mutants grown on nonselective media reverted back to normal size (Figure 10), the petite mutation might be further linked to the transformation with the fused protein. It is possible that the normal-sized cells seen here resulted from petite cells losing the fused protein-containing plasmid when grown in nonselective media.

Effect of Gpr1 Deletion on Ste2 Pathway

Although the Gpr1-Gpa1 chimeric protein appeared not to be functional, results of the growth arrest and mating assays still reveal the possibility of a link between the pheromone and glucose-sensing pathways. If some sort of regulatory connection between the two pathways exists, it is likely further downstream as opposed to occurring between the glucose receptor and pheromone Gα subunit, or perhaps also involves the pheromone receptor. If the latter is the case, data from the mating assay (Figure 5), indicates that only Ste2 is affected by inactivation of

the glucose pathway, whereas the pheromone pathway controlled by Ste3 remains fully functional. A possible explanation might be that Ste3 is has a slightly different structure than Ste2, which somehow allows it to be immune to any possible cues from the glucose-sensing pathway.

While the exact mechanism has yet to be elucidated, it follows logically that the cell would curb "nonessential" activities—such as mating—in times when energy sources are scarce. It has been shown that when glucose or other fermentable carbon sources are unavailable and the cell is using non-fermentable carbon sources, genes involved in resistance to stress are highly active (Rolland, et al. 2002). Perhaps a nonfunctional glucose-sensing pathway, as in Gpr1Δ or Gpa2Δ, induces a stress response in the cell which includes inactivation of the mating pathway.

According to Rolland et al. (2002), the "dramatic effects of glucose on growth and metabolism clearly support a hormone-like function for this sugar in yeast cells," the presence or absence of which regulates many important processes in the cell. Additionally, "nutrient-sensing and –signalling mechanisms must have evolved early in evolution and might be at the origin of the sophisticated hormone- and growth factor-induced signal transduction pathways" (Rolland et al., 2002). Experimental evidence from this study supports such an evolutionary link between the nutrient-sensing Gpr1 pathway and pheromone-sensing Ste2 pathway. The exact mechanism of this purported link, however, remains to be discovered.

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