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Regulation of G Protein-Coupled Receptor Signaling in Yeast

By

Douglas Paul Gladue

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Abstract of the Dissertation

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G-protein Coupled Receptors (GPCRs) comprise a large family of medically important receptors, which respond to a variety of signals, including hormones, neurotransmitters, ions and light. Proper regulation of GPCR function requires that they interact with many different proteins. Studying the proteins that interact with GPCRs has been difficult, in part, due to the hydrophobic nature of these membrane proteins. Therefore, I took advantage of the genetic accessibility of the yeast *Saccharomyces cerevisiae* to study proteins that interact with the alpha factor receptor, Ste2p, a GPCR responsible for the induction of the mating pathway. By implementing a two-hybrid system specifically designed for membrane proteins, I identified proteins that interact with Ste2p. Dimerization of

Ste2p, as well as interaction of Ste2p with the regulatory proteins Afr1p and Sst2p was detected. Further analysis using this assay helped to demonstrate that some mutant forms of Afr1p and Sst2p were defective due to failure to bind to Ste2p. A second approach involved studying the mechanism by which GPCRs activate the next component in the signaling pathway, known as a heterotrimeric G protein. Residues in six regions of the alpha subunit of the G protein (Gpa1) predicted to be in close proximity to Ste2p were targeted for mutagenesis. Thirty-three different amino acid substitutions displayed a range of phenotypes from non-signaling to constitutive activation of the pheromone pathway, indicating many regions of Gα contribute to the interaction with the receptor. Altogether, these studies provide new insight into the function of the proteins that regulate GPCR signaling and to the ability of GPCRs to activate heterotrimeric G proteins.

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List of Abbreviations

Ade	Adenine
AFR1	Alpha factor Resistance gene 1
CGRP	calcitonin-gene-related peptide
CRLR	calcitonin receptor like receptor
CuB	C terminal half of ubiquitin
Cys	Cysteine
DNA	Deoxyribonucleic acid
FRET	Fluorescence resonance energy transfer
GABA	Gamma-aminobutyric acid
GAP	GTPase accelerating protein
GDP	Guanosine diphosphate
GFP	Green florescent protein
GPCR	G protein-coupled receptor
GRK	GPCR protein kinase
GTP	Guanosine triphosphate
G Protein	Guanine-nucleotide-binding regulatory protein
His	Histidine
Hr	Hour
JAK2	Janus kinase 2
KanR	Kanamycin resistance
LacZ	E.coli gene for beta galactosidase

LEU	Leucine
Lys	Lysine
M	Molar
MAPK	Mitogen activated protein kinase
MATa	Mating type a
Min	Minute
mM	Millimolar
nm	nanometer
NTR	Neurotensin receptor
NuB	N terminal half of ubiquitin
ONPG	o-Nitrophenyl-β-D-galactopyranoside
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PLV	Protein A, Lex A, VP16 protein fusions
RAMP	receptor activity modifying protein
RGS	Regulator of G protein signaling
S. cerevisiae	*Saccharomyces cerevisiae*
SD	synthetic complete medium plus dextrose
SDS	Sodium dodecyl sulfate
STAT1	signal transducer and activator of transcription 1
STE2	Receptor for α-factor pheromone
Trp	Tryptophan

Tyr	Tryosine
UBP	Ubiquitin specific protease
µl	Microliter
URA	Uracil
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
Yck1	Yeast Casein kinase I isoform

Chapter 1: Introduction

GPCRs mediate the cellular response to extracellular signals, such as nutrients, hormones, neurotransmitters, or light, inducing a wide range of responses including: stimulation of cell growth, migration, polarization, gene expression and secretion of proteins [1-4]. This broad range of functions, together with the fact that GPCRs have proven to be good targets for small molecule drugs, have made GPCRs an important focus in the search for new therapeutic agents. In fact, about 50% of all known drugs target GPCRs [5, 6]. The current sales for drugs targeting GPCRs exceeds $30 billion [6, 7]. However, many of these drugs lose efficacy via various mechanisms, such as receptor endocytosis and down regulation. As such, a better understanding of GPCRs and their interactions with proteins that change their affinity or signaling activity will be important for the identification of new drugs to treat disease.

G protein coupled receptors comprise a large and diverse family of transmembrane proteins [8]. Following ligand binding, the receptor undergoes a conformational change causing activation of the heterotrimeric guanine nucleotide binding protein (G protein) by exchanging GDP for GTP in the Gα subunit. This causes disassociation of the Gβγ subunits from the Gα subunit, allowing the Gα and Gβγ to induce a signaling cascade through their interaction with downstream effector molecules (Figure 1).

Model for GPCR Function

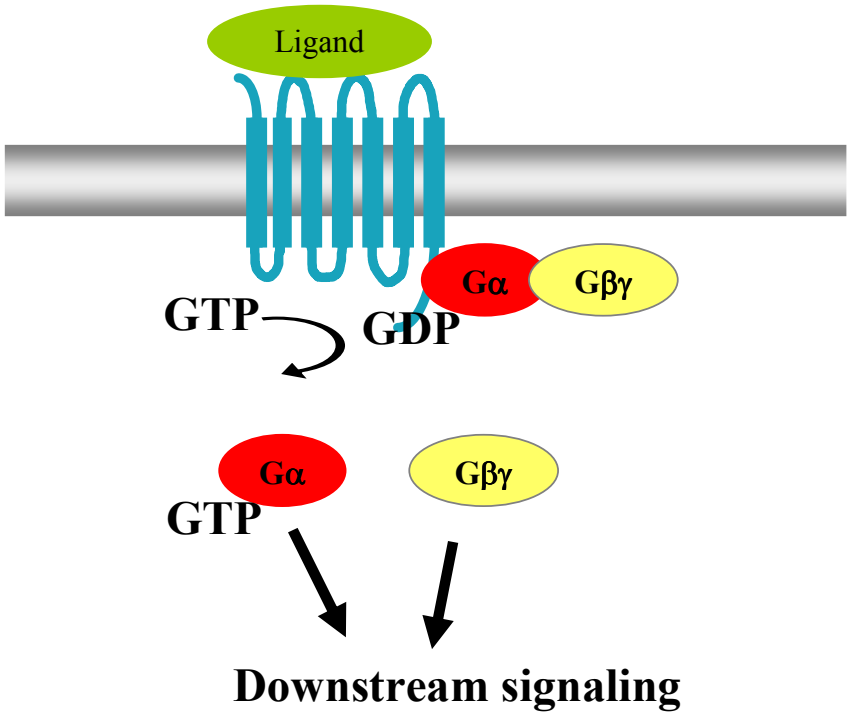

Figure 1 : Following receptor ligation, a heterotrimeric guanine nucleotide binding protein (G protein) is activated resulting in the exchange of GDP for GTP in the Gα subunit. This causes disassociation of the Gβγ subunits from the Gα subunit. Either the Gα or Gβγ can then transduce signal through their interaction with downstream effector molecules

Receptor G protein interface

It is not well understood how receptors activate G proteins, in part because of the technical difficulties in working with membrane-bound receptor proteins. In addition, it has been surprisingly difficult to identify conserved sequence motifs in the intracellular domains of GPCRs that might be involved in G protein activation. For example, mammalian GPCRs can be divided into at least three different families that lack obvious sequence similarity, even though they all activate a common set of G proteins [9]. Similarly, the yeast mating pheromone receptors for α-factor and **a**-factor, Ste2 and Ste3, do not display obvious sequence similarity even though they activate the same G protein [10]. Although mutational analyses of a wide range of GPCRs have identified regions in the intracellular loops that are important for signaling, the specific residues that have been identified often vary, even between closely related GPCRs [11, 12]. Residues which have been implicated in ligand binding, constitutive activity or dominant negative effects have been identified [13-16] (Figure 2). However, mutagenesis studies of the intracellular loops of the receptor have yielded very few mutants that have shown changes in receptor signaling (Figure 2). Studies involving the receptor G protein interface have mainly focused on the N-termini and C-termini, implicating both of them to be involved in receptor signaling.

Thus, the interface region between the G protein and the receptor is not well understood

Genetic analysis of α–factor receptor (Ste2) signaling

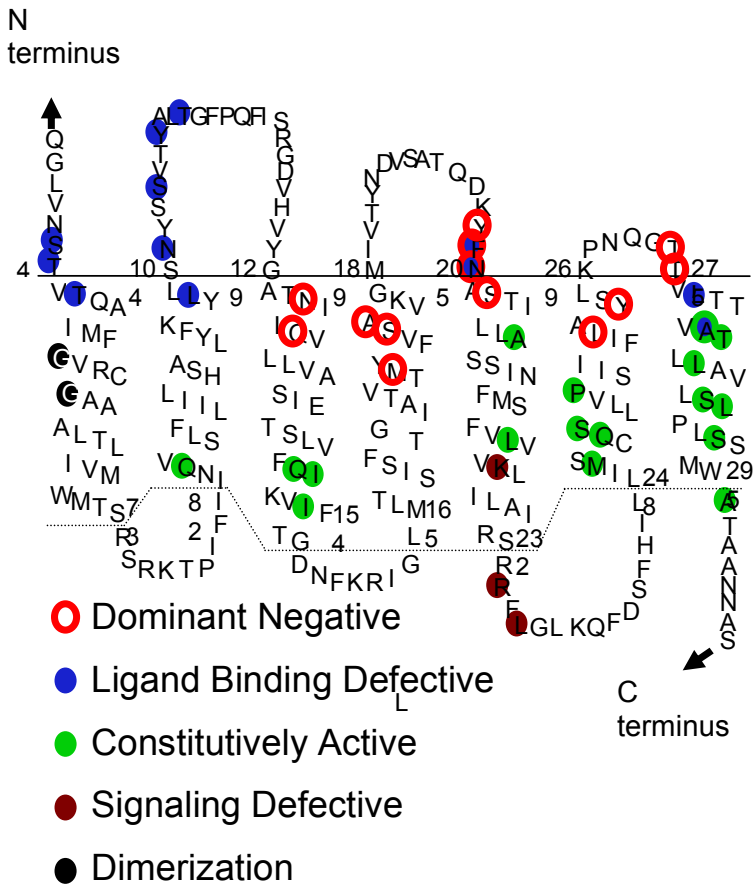

Figure 2: Genetic analysis of Ste2 signaling. Residues found to cause a phenotype when mutated are highlighted in various colors as indicated below the receptor figure.

Roles of GPCRs in human diseases

GPCRs can contribute to human diseases in several ways. One mechanism is malfunction due to a mutation that causes a defect in GPCR structure. Mutations affecting as little as one amino acid can change the conformation of the receptor, making it either unable to signal or to signal independent of ligand interaction. Retinitis pigmentosum is caused by mutations in the G protein-coupled photoreceptor rhodopsin, which result in a loss of function or a constitutively active receptor [17, 18]. In addition to receptor mutations, other diseases can occur by changes in the levels of receptor interacting proteins. For example, when the bradykinin receptor is over expressed, heterodimerization occurs between it and the angiotensin receptor, causing cells to become supersensitive to angiotensin, leading to preeclampsia (hypertension) in pregnant women [19]. Furthermore, heart failure has been linked to a block in downstream signaling of the beta-adrenergic receptor due to its phosphorylation by GPCR protein kinases (GRKs) [20]. Many serious neurological diseases including Parkinson's, Alzheimer's and epilepsy are linked to the histamine H3 receptor. Although the mechanism for pathogenesis is not clear, H3 receptor antagonists have shown potential for treatment of these diseases [21].

Known protein-protein interactions involving GPCRs

Proteins that interact with GPCRs can be divided into two basic groups: those involved in receptor signaling and those involved in receptor trafficking. Perhaps the most interesting are those thought to be necessary for signaling, such as GPCR oligomerization. For example, the GABA receptor has been found to only be functional when it is in a heterodimeric form consisting of GABA1 and GABA2 [22]. The case is quite different when it comes to the angiotensin and bradykinin receptors. Whereas both of these receptors can function as homodimers, signaling is enhanced when these two receptors form a heterodimer [19]. The neurotensin receptor (NTR) is comprised of three separate components: NTR1 and NTR2 are seven transmembrane GPCRs, and NTR3 is a single transmembrane protein. It has been shown that NTR1 is required for NTR3 to internalize after ligand stimulation [23].

Some GPCRs recruit other proteins, for example, the angiotensin receptor recruits Jak2 [24], which in turn phosphorylates STAT1, initiating the signaling cascade. RAMPs, or receptor activity modifying proteins, are single transmembrane proteins that change receptor specificity. This has been described for the calcitonin receptor like receptor (CRLR). One RAMP allows CRLR to bind calcitonin-gene-related peptide (CGRP), whereas another RAMP allows the CRLR to bind adrenomedullin [25]. Understanding how these GPCR interacting

proteins affect specificity and signaling is important for designing new drugs to combat diseases.

Many proteins are involved in the trafficking of GPCRs to the plasma membrane, or in endocytic pathways that bring activated receptors back into the cell. RAMPs have been shown to promote proper transport of CRLR to the plasma membrane [25]. For the mouse pheromone receptor V1R and V2R, MHC Class Ib molecules need to be coexpressed in order for the receptor to be localized to the plasma membrane [26]. Additionally, beta-arrestins direct receptors to clathrin coated pits for endocytosis, as described for the adrenergic and angiotensin receptors [27]. Chaperones are also good candidates for assisting GPCR trafficking and folding, as shown with rhodopsin [28]. As a signal for endocytosis, some GPCRs are ubiquitinated, indicating ubiquitin conjugating enzymes most likely interact with GPCRs [29, 30]. Although receptor families share many structural and functional similarities, it is not clear if all GPCRs undergo the same type of regulation and trafficking. Therefore, a better understanding of these GPCR-interacting proteins is needed to determine mechanisms of disease and to improve therapy.

Yeast as a genetic model system for analysis of GPCR function

Because*, Saccharomyces cerevisiae* is a genetically accessible organism, it can be used as a tool to study how GPCRs function. Unlike mammalian cells, yeast allows easy manipulation of the genome to delete genes or to use

sophisticated plasmid technology to express genes under the control of various promoters. This allows us to explore functions associated with various components much more easily than with mammalian cells. Special strategies for determining protein-protein interactions have been developed for use in yeast, such as many biochemical approaches and the yeast two-hybrid assay. The pheromone pathway in yeast is well defined and Ste2p is not an essential GPCR, since it is only required for mating, allowing for easy manipulation of the *STE2* gene. In addition, discoveries on GPCR signaling in yeast will likely be applicable to higher organisms. Despite diverse sequences among GPCRs, all GPCRs are thought to share a conserved 3D structure and function, and Ste2p shows overall similarity to mammalian GPCRs [31, 32]. Thus, studies on Ste2p could have an impact similar to the studies on other aspects of the pheromone pathway, such as the MAP kinase signaling cascade, that have contributed greatly to elucidating similar signal pathways in mammalian cells (Figure 3).

In addition to its accessibility to yeast genetic approaches, the pheromone pathway is advantageous to study since activation of Ste2p results in strong induction of mating responses, which contrasts with the relativity small amplitude of induced signaling promoted by many GPCRs in mammalian cells. Pheromone-induced responses, such as induction of mating genes and cell division arrest, can be easily quantified. Furthermore, yeast lack endogenous β-galactosidase activity, which permits very rapid and sensitive analysis of pheromone signaling by measuring β-galactosidase activity that results from induction of a pheromone-responsive reporter gene (*Fus1-lacZ)* that can be engineered into yeast. The β-

galactosidase activity can easily be measured using samples in 96 well plate format, which permits rapid and sensitive tests of Ste2p function in vivo that would not be possible with many types of mammalian cells.

Mating in *Saccharomyces cerevisiae*

In *Saccharomyces cerevisiae* there are two mating types: α and **a**. Cells of each mating type releases its corresponding pheromone, α cells release α-factor and **a**-cells release a-factor. Each mating type also has receptors for the opposite pheromone. Studies in this thesis will make use of **a** cells that express the α-factor receptor (Ste2p) on the surface. This receptor responds to α-factor to induce the mating pathway, causing cell division arrest, polarized growth of the cell, and eventual cell fusion to an α-cell. A modified strain will be used in my studies that has a mutation in the *FAR1* gene, which prevents pheromone-induced cell division arrest, but not pheromone-induced gene expression. Therefore, this strain contains a *FUS1-LacZ* reporter gene allowing for quantification of the signaling response by using a β-galactosidase assay. As various receptor mutants and ligands are readily available for Ste2p, this is a good model system for studying GPCRs [13, 16].

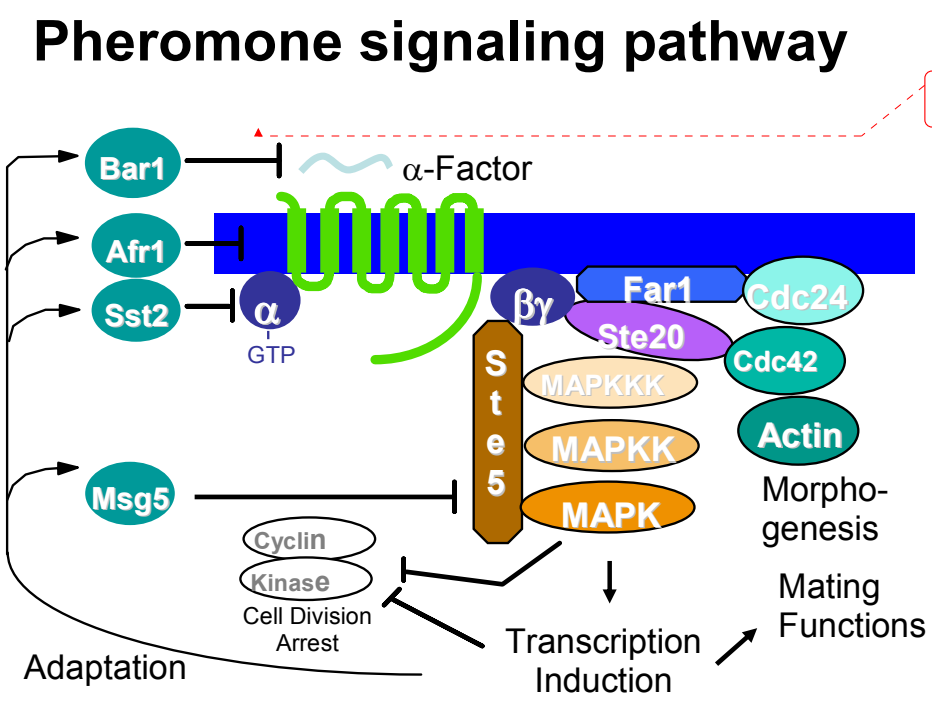

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Figure 3: Schematic representation of the pheromone response pathway in *Saccharomyces cerevisiae*. Upon ligand (α-factor) binding to the receptor (Ste2p) the receptor undergoes a conformational change resulting in the exchange of GDP for GTP in the Gα subunit of the heterotrimeric G protein. This results in recruitment of a MAP kinase cascade, and upregulation of various regulatory proteins, including Afr1 and Sst2.

DEP domains and signal transduction

DEP domains are conserved domains often found in proteins that are involved in signal transduction, and often linked to membrane association. DEP domains were initially described in Disheveled, a *D. melanogaster* protein [33], in a regulator of G protein signaling (RGS) protein EGL-10 in *C. elegans*, and in mammalian Pleckstrin. Interestingly, the yeast RGS protein Sst2p contains a DEP domain. The function of this DEP domain will be highlighted in Chapter 4 of this thesis, as it was shown to mediate the binding to Ste2p [34].

Ste2p *in vivo* interactions

Many proteins that interact with GPCRs have been identified. However, because of the limitations of traditional biochemical methods, there is no systematic way of identifying new protein interactions. Genetic analysis has predicted several proteins that interact with Ste2p (Figure 3). These can be divided into proteins involved in signaling and proteins involved in trafficking. Those involved in signaling are the focus of this thesis. Oligomerization of Ste2p can be assessed by FRET (fluorescence resonance energy transfer) analysis. However, FRET was only able to show oligomerization using truncated receptors, which lack almost all the C-terminal cytoplasmic tail [35]. Receptors with shortened tails are defective in endocytosis. Potentially, FRET was only observed

due to an increased receptor number at the plasma membrane. However, FRET was specific when tested against other mammalian receptors [34]. Gα or Gβγ also interact with GPCRs, as they are the first components that initiate signaling upon GPCR stimulation [36]. Afr1p, a cytoplasmic protein that causes resistance to α-factor in a manner independent of destabilizing Ste2p or causing Ste2p endocytosis, is also a likely candidate for interacting with Ste2p *in vivo* [37-39]. In addition, Sst2p, which acts as a GAP (GTPase Activating Protein) for Gα, has also been proposed to interact with receptors based on genetic evidence [40]. Yck1p and Yck2p, members of the protein casein kinase family shown to phosphorylate Ste2p, localize to the plasma membrane as expected for proteins that should interact with Ste2p [41]. The second class of protein interactions includes those involved in receptor trafficking, such as those ranging from the initial membrane insertion of Ste2p in the ER to trafficking to the plasma membrane and ultimately to the vacuole for degradation. As Ste2p has been shown to be ubiquitinated in order to be endocytosed [30], ubiquitin conjugating enzymes are also likely to interact with Ste2p. In addition, Ste2p is predicted to interact with other proteins, such as chaperones and sorting proteins (Figure 4).

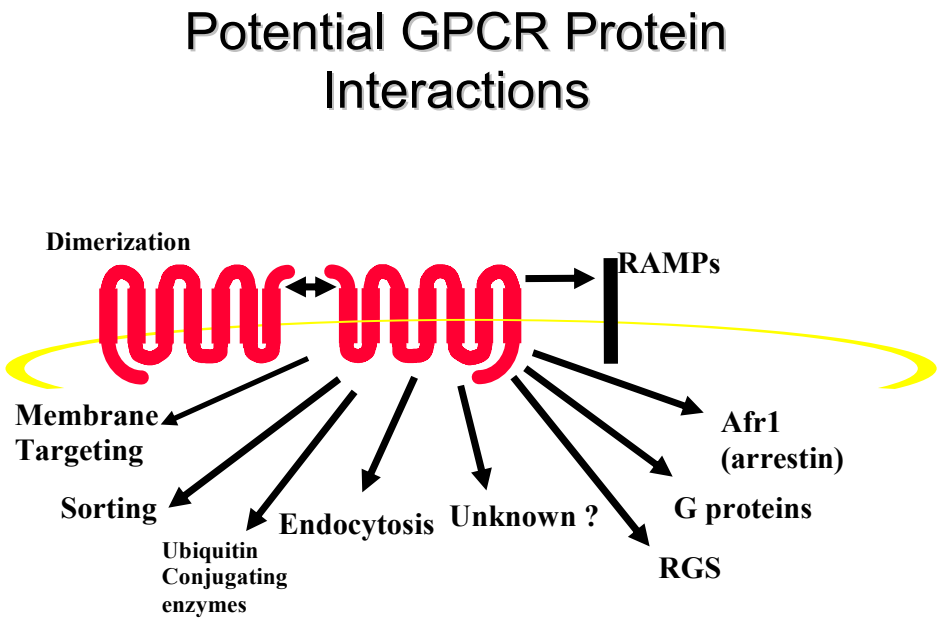

Figure 4: Diagram of protein interactions with GPCRs involving signaling and trafficking functions. A representative GPCR is shown in Red on the plasma membrane, indicated in yellow.

Difficulties in studying proteins that interact with Ste2p

Despite the genetic accessibility of yeast, there are still some limitations to studying proteins that interact with Ste2p. For one, the hydrophobic nature of GPCRs makes it difficult to employ traditional biochemical approaches. Also, the conventional yeast two-hybrid system requires that the protein is able to enter the nucleus; however, receptors normally traffic to the plasma membrane, not the nucleus. This limits the yeast two-hybrid assay to soluble domains or sub-domains of the receptor [42]. The best way to screen for proteins that interact with GPCRs is when the receptor is in its native state, and this can be done most confidently *in vivo*. Therefore, one aspect of my thesis studies involved the use of a modified yeast two-hybrid system to identify proteins that interact with Ste2p in the plasma membrane [42]. As this two-hybrid system does not require the receptor to enter the nucleus, protein interactions can be studied with Ste2p in its natural plasma membrane environment, as will be described in more detail below.

The membrane yeast two-hybrid system

The basis of this membrane-compatible two-hybrid approach is that ubiquitin can be split into two halves, which can reassociate *in vivo* and thus be recognized by a ubiquitin specific protease (UBPs). Typically, these UBPs recognize newly ubiquitinated proteins, and cleave the ubiquitin off. The UBPs

don’t recognize specific sequences, instead they recognize the conformation of the folded form of ubiquitin [43, 44] (Figure 5).

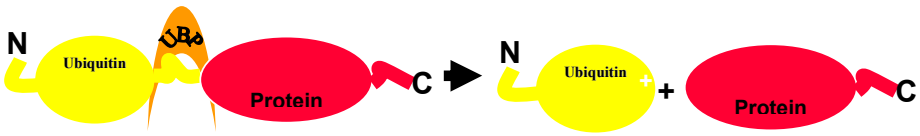

Figure 5: Ubiquitin Specific Proteases (UBP's) cleave newly formed ubiquitin fusions. This results in free ubiquitin and protein free of ubiquitin fusions.

For this version of the yeast two-hybrid system, ubiquitin is expressed as a C-terminal half (Cub) and an N-terminal half (Nub), preventing its recognition as fully folded ubiquitin by the ubiquitin specific protease. Cub is fused to a transcription factor, PLV (further explained in Chapters 2 and 3). This Cub-PLV is added to the end of one of the proteins to be studied (bait). Nub is then fused to another protein (prey). If the two proteins interact, Cub and Nub will be close enough to correctly fold into a mature ubiquitin that is recognized by UBPs. The UBPs will then cleave at the C terminus of ubiquitin, thereby releasing PLV and allowing it to enter the nucleus and activate the reporter genes. Typically *lexA-HIS3* and *lexA-LacZ* reporter genes are used to allow for selection of positive clones based on His3 production or β-galactosidase activity. This provides a system to examine membrane protein-protein interactions outside the nucleus in vivo, unlike the traditional yeast two-hybrid system.

Overview of thesis

This thesis will describe two different approaches for examining proteins that interact with GPCRs, which is important in order to gain a better understanding of how GPCR signaling is regulated. Chapter 3 will describe results using the membrane yeast two-hybrid assay to analyze proteins that interact with Ste2p. These studies include receptor dimerization and interaction with regulatory proteins Afr1p and Sst2p. The importance of DEP domains in receptor binding will also be addressed. Chapter 4 will contain the results of

cysteine scanning mutagenesis of Gpa1p in the predicted interface region between Gpa1p and Ste2p. The phenotypes resulting from mutagenesis of Gpa1p ranged from constitutive activity to complete loss of function. The results of these studies and the prospects for future research on GPCRs will be summarized in Chapter 5.

Chapter 2: Experimental Procedures

Strains and media

Yeast strains used for the analysis of *gpa1* mutants were Pmy3.2 (*MATa bar1::hisG far1Δ his3::FUS1-HIS3 mfa1Δ::FUS1-LacZ, ade2-1^o, leu2^o trp1^o ura3^o can1-100 ste2Δ gpa1::kanR) and DJ11-5-3 (MATa ade2-1 bar1-1 cry1 his4-580^a leu2 lys2^o trp1^a tyr1^o ura3 SUP4-3ts*), or the membrane yeast two-hybrid assay was carried out using strain L40 (*MATa trp1 leu2 his3 LYS2::lexAO-HIS3 URA3::lexAOlacZ*) [45]. Plasmids were introduced into yeast using the lithium acetate method [46]. Cells carrying plasmids were grown in synthetic medium containing adenine and amino acid additives but lacking uracil and leucine to select for plasmid maintenance [46].

The membrane-yeast-two-hybrid system

The membrane yeast two-hybrid assay for assessing interaction between membrane proteins was carried out using gene fusions to the N-terminus of ubiquitin (Nub) and to a module containing the C-terminus of ubiquitin in-frame with the synthetic transcription factor, LexA-VP16 (Cub-PLV), essentially as described by Stagljar and coworkers [44, 47, 48]. Nub was fused to the C-terminus of Ste2p carried on the *TRP1*-marked plasmid YEplac112 [49], and a derivative containing an I13G mutation to weaken intrinsic interaction with Cub

[44] introduced by site-directed mutagenesis. The Sst2-NubG fusion or *AFR1-NubG* was then created by replacing the *STE2* coding region in the mutant *STE2-NubG* vector with the *SST2* or *AFR1* open reading frame. The same was done for all *AFR1* point mutations [37]. The Q304L, Q304E, and Q304N substitutions were then introduced into SST2-NUbG by swapping in the corresponding restriction fragments from the appropriate plasmid DNAs. *STE2-Cub*-PLV was created by fusing *Cub-PLV* to the C-terminal coding sequence of *STE2* on *LEU*2 marked plasmid, YCplac111 [49]. Combinations of plasmids expressing Nub-G alone, *AFR1-Nub-G*, *SST2-Nub-G* and the derived mutants, and *STE2-NUb* were introduced into L40 cells along with the plasmid expressing *STE2-Cub-PLV*, and transformants were selected on -Leu-Trp medium. Transformants were tested by imprinting cells on filter paper, and frozen in liquid nitrogen and thawed three times, and incubated with Z-buffer. (500ug/mil X-gal and 0.05% [v/v] 2-mercaptoethanol), until the blue color fully developed (4 hrs to 3 days).

In 96 well plate assays, representative Leu+ Trp+ transformants were grown to a density of OD_{600nm} = 0.8 – 1.0, collected by centrifugation, and the cell pellet (corresponding to ~1 OD_{600nm} unit) was lysed by two freeze thaw cycles in liquid nitrogen and resuspended in a 96-well microtiter plate with 100 µl PBS buffer, pH 7.4, containing 500 µg/ml X-gal, 0.5% (w/v) agarose, and 0.05% (v/v) 2-mercaptoethanol. The plates were incubated at room temperature to allow β-galactosidase activity produced from expression of the *lexA-lacZ* reporter gene to act on the colorimetric substrate X-gal. Development of blue color was recorded using a digital image scanner.

Cysteine scanning mutagenesis

A set of Cys substitution mutants was constructed in plasmid pPK22 (YCp-LEU2-GPA1-myc) that was based on the vector YCplac111 [49]. This plasmid carries a modified *GPA1* with a c-myc epitope tag introduced at residue 125 that does not affect signaling, as described previously [50]. Site-directed mutagenesis was carried out using PCR with mutagenic oligonucleotides to introduce mutations followed by digestion with Dpn I to destroy template DNA. Mutagenic oligonucleotides were designed to be complementary to the *GPA1* sequence except for the substitution required to change the indicated codon to TGT to code for cysteine. DNA sequence analysis to confirm the mutations was carried out at the Stony Brook University DNA sequencing facility using the Big Dye cycle sequencing reagents (Applied Biosystems Inc.).

α-factor-induced responses

PMY3.2 cells were transformed with a plasmid carrying *STE2*, plasmid pPD225 [16] and with plasmid pPK22 carrying wild-type *GPA1* or a cysteine-substituted version. Overnight cultures were diluted 1:5000 in selective media. Cells were grown at 30˚C for one hr and then transferred to a 96-well flat bottom plate along with the indicated concentration of α-factor and incubated for two hrs. The cells were permeabilized by incubation at 30˚C with 0.10% SDS, 0.1% Triton

X-100, and 45 mM 2-mercaptoethanol, and then incubated for one hr with o-nitrophenyl β-D-galactopyranoside (ONPG; Sigma) as a substrate [51]. 1M $NaCO_3$ was added to stop the reactions and then the absorbance of the samples at 420 nm was determined using a 96-well plate reader (Molecular Devices, Sunnyvale, CA). β-Galactosidase units were determined using the following equation: $(1000 \times OD_{420}) \div (T \times V \times OD_{660})$ [52]. OD_{420} is the optical density of the assays after incubation, T is the time of incubation, V is the volume of cell culture analyzed, and OD_{660} is the optical density of the cell culture. The assays were normalized to an induction level of 100% for cells carrying the wild-type *GPA1* plasmid, and the results represent the average of three independent transformants that were analyzed in at least two independent experiments.

The dominant phenotypes of the *GPA1* mutants were examined by introducing the plasmids into strain DJ211-5-3, which carries a wild-type copy of GPA1 in the genome. Cells were grown overnight and then ~10^6 cells were spread on the surface of a solid medium agar plate. Filter discs containing different amounts of α-factor were placed on the lawn of cells and then the plates were incubated for 2 days at 30˚C. Cells that are susceptible to α-factor-induced cell division arrest form a clear zone (halo) surrounding the discs that is proportional in size to the amount of α-factor.

Quantitative western blot analysis

Approximately 2×10^8 logarithmic-phase cells were harvested by centrifugation and lysed by agitation with glass beads in 200 µl of buffer containing 50 mM Tris (pH 7.5) and the following concentrations of protease inhibitors: 3.6 mM Aprotinin, 2.1 mM Leupeptin, 15 µM Pepstatin A, 10 mM benzamidine, 5 mM phenylmethanesulfonyl fluoride. The lysate was cleared twice by centrifugation at 2,000 × g for 1 min. The membrane fraction was harvested by centrifugation at 15,000 × g for 20 min, and was then resuspended in 20 µl of Gel Sample Buffer (4% SDS, 0.1M Tris pH 6.8, 8 M urea, 10% 2-mercaptoethanol). Samples were boiled for 10 min prior to loading, separated by electrophoresis on a 12% SDS-polyacrylamide gel, and then electrophoretically transferred to a 0.4 µm nitrocellulose membrane (Whatman, Florham Park NJ). To detect the c-myc-tagged Gpa1, the blots were probed with mouse anti-myc 9E10 antibody (Upstate, Temecula, CA, #05-419). As a control for sample loading, the blots were also probed with rabbit anti-Ras2 (Santa Cruz Biotechnology, Santa Cruz, CA #sc-28549). Immunoreactive proteins were detected by using goat anti-mouse IRDye 800cw or goat anti-rabbit IRDye 680 (Li-Cor Biosciences, Lincoln, NE, USA). Quantitative analysis was carried out using a Li-Cor Biosciences Odyssey Infrared Imaging system. Results presented represent the average of two independent western blots, and were consistent with other independent assays.

Chapter 3: Identification of Proteins that Interact with Ste2p

Summary

GPCRs have been difficult to study, in part because of their hydrophobic nature, making traditional biochemical approaches difficult. This study utilized a membrane yeast two-hybrid assay (MYTH). Unlike the traditional yeast two-hybrid assays, the MYTH assay does not require membrane proteins to enter the nucleus. Thus, proteins that interact with GPCRs can be examined at the plasma membrane. In *Saccharomyces cerevisiae,* the G protein-coupled α-factor receptor (Ste2p), responsible for initiating the mating response in response to α-factor, is an ideal candidate for studying GPCR interactions. The signaling pathway has many conserved features, such as a MAP kinase cascade, Afr1p which is similar in function to mammalian arrestins, and regulators of G protein signaling (RGS) proteins, like Sst2p that act on the Gα protein Gpa1.

Studying Ste2p interaction with other proteins using the membrane yeast two-hybrid assay

Since the traditional yeast two-hybrid system requires tagged proteins to enter the nucleus. This limits the system to studying only soluble domains or subdomains, making it not well suited for detecting membrane protein interactions. However, the membrane yeast two-hybrid assay (MYTH) allows membrane protein interactions to be assayed on the plasma membrane. MYTH takes advantage of ubiquitin specific proteases (UBPs), which efficiently cleave

new fusions between proteins and ubiquitin. It has been shown that UBPs do not recognize the target sequence, but instead recognize the conformation of a fully folded ubiquitin.

For this assay, ubiquitin is expressed in two separate halves, a C-terminal half (Cub) and a N-terminal half (Nub). The transcription factor, PLV, which contains Protein A, LexA and Vp16 is attached to the end of the C-terminal half of ubiquitin. The protein A sequence can be used to detect PLV fusions on a Western blot. The LexA and Vp16 sequences are the binding and activation domains required for transcription of *lexA-HIS3* and *lexA-LacZ* reporter genes. Neither of these constructs, Nub or Cub-PLV, is recognized by the UBPs alone. However when both are expressed in cells, the two halves spontaneously refold to form a complete ubiquitin, and the PLV is cleaved. In order to prevent this, isoleucine 13 in Nub was mutated to glycine, weakening the interaction between Cub and Nub (Figure 6). To carry out the MYTH assay, the Cub-PLV is attached to the bait protein and the Nub is attached to the prey protein (Figure 7).

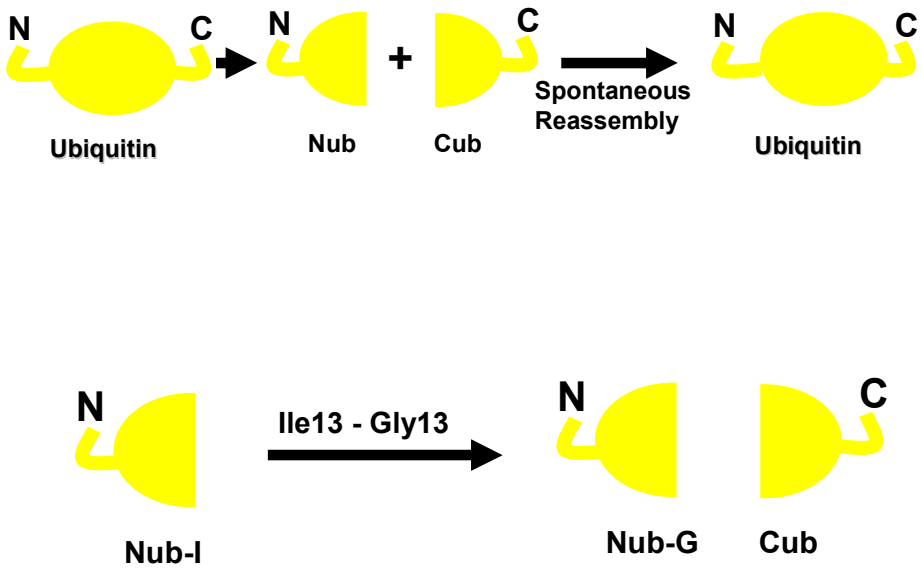

Figure 6: The MYTH system relies on splitting ubiquitin into two halves. The N-terminal half (Nub) and the C-terminal half (Cub). Initially the two halves of ubiquitin had high affinity for each other and spontaneously reassembled. To decrease the affinity of the two halves of ubiquitin Nub was modified by mutating Ile13 to Gly13, preventing spontaneous reassembly.

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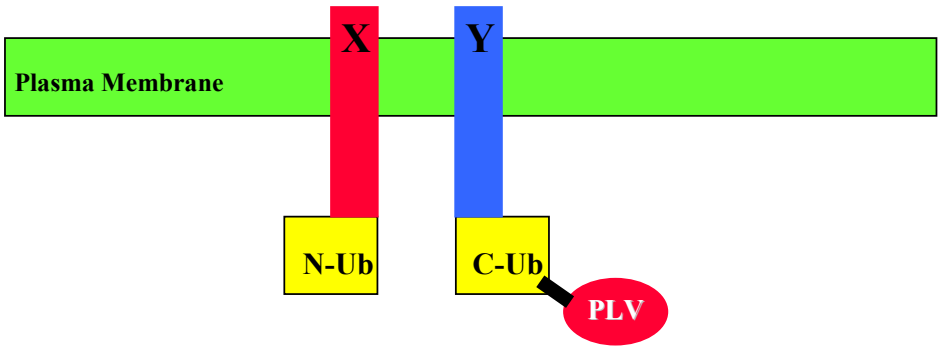


Figure 7: If the bait and prey proteins interact (X and Y), the Nub and Cub come together and form a complete ubiquitin. This will be recognized by the ubiquitin specific protease and cleaved. After release, the PLV enters the nucleus and activates the reporter genes *lexA-HIS3* and *lexA-LacZ*.

Positive and negative controls for membrane yeast two-hybrid assay

Because the Nub-I construct forms a strong interaction with Cub, it gives a strong false-positive signal. I used Nub-I constructs to show that a particular protein was being produced. If produced, the fusion protein will interact strongly with the Cub-PLV constructs and will be positive for reporter gene activation. I used Bradykinin-CubPLV and Wbp1-CubPLV as negative controls, since they were available from previous studies that I did not continue. To observe an interaction, I performed standard β-galactosidase filter lift assays. Strong results are visualized in a few hours, whereas weak results may take a few days, depending on the size of the colonies and strength of the signal.

Strain and plasmid construction

The strain used in the traditional yeast two-hybrid system, L40, had to be modified for use in studying *STE2*. The chromosomal copy of *STE2* was deleted by replacing it with a kanamycin resistance cassette to prevent endogenous Ste2p from interfering with this assay. The *ste2::kan*^{r} mutation was confirmed by showing that the strain could no longer mate or respond to α-factor. This yeast strain also contains the reporter genes *lexA*-HIS3 and *lexA*-LACZ, but lacks the PLV transcription factors.

To make the *STE2* plasmids for the MYTH system, I constructed a bait plasmid by fusing the coding sequence for Ste2p and Cub-PLV. LexA and VP16

are the transcription factor and activation domain used in the traditional YTH assay; both of which are required for transcription of the reporter genes. Utilizing a low copy plasmid allowed lower background by limiting the amount of PLV present in the cell. This plasmid also carries *LEU2* for selection in yeast

The prey plasmid was made by fusing coding regions for Ste2p to Nub (the N-terminal half of ubiquitin). This plasmid was constructed using a high copy vector to ensure that enough Ste2p-Nub would be made to interact with Ste2p-CubPLV. As Ste2p-Nub contains no transcription factor, their was no concern for this plasmid to give any background signal. This plasmid also contains the yeast selectable marker *TRP1*.

Both of the resulting plasmids: Ste2p-Cub-PLV (*LEU2*) and Ste2p-Nub (*TRP1*) were shown to express a functional Ste2p receptor, since they complemented the mating defects. Thus, both copies of Ste2p were functional despite the Cub-PLV or Nub fusions. I used these constructs to then examine Ste2p protein interactions that had previously been predicted by genetic analysis, including receptor dimerization, as well as interaction with negative regulators Afr1p and Sst2p (Figure 8).

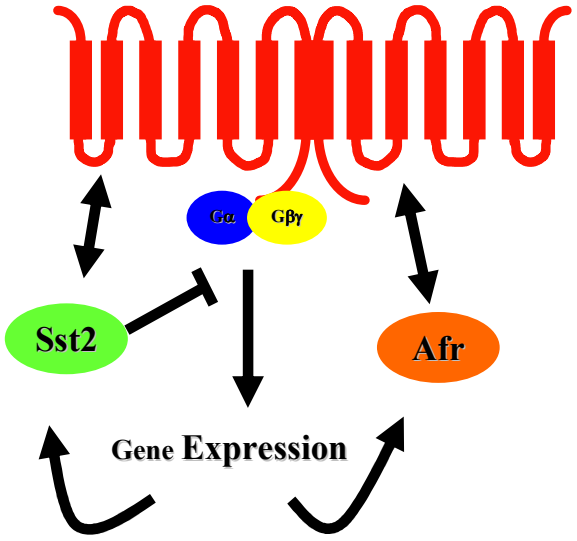


Figure 8: Ste2p protein interactions predicted by genetic analysis: In red is Ste2p forming a dimer. Ste2p also interacts with Gαβγ, along with negative regulators Afr1p and Sst2p.

Since Ste2p was shown to dimerize by FRET analysis, I predicted it should show a positive interaction in the MYTH assay. I co-expressed both constructs and determined that Ste2p-Ste2p interaction was weak but detectable in this system. However, since the FRET studies showed that truncating the cytoplasmic tail improved the detection of dimerization, I examined a shortened version of Ste2p in the MYTH assay to attempt to gain an increase in MYTH signal. When using shortened Ste2p-Nub and full length Ste2p-Cub receptors, a very strong interaction was detected. However this interaction proved to be non-specific when tested with the negative control Bradykinin-Cub. This could have been due to an increase in Ste2p-Nub receptors present at the membrane, since they lack the C terminus that promotes endocytosis [34] (Figure 9).

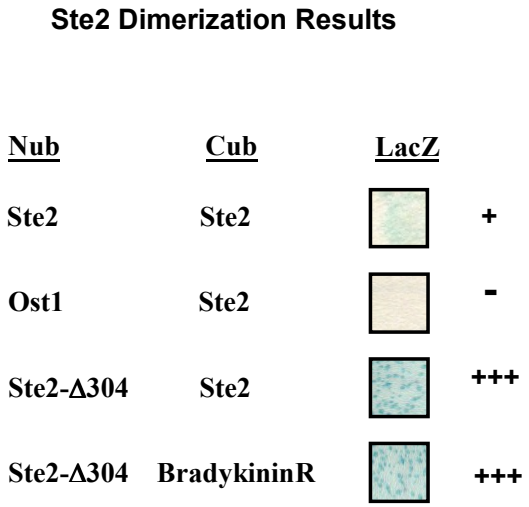
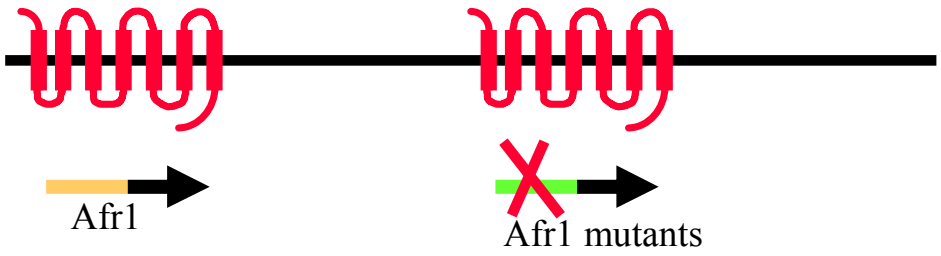


Figure 9: Ste2 dimerization results. Assays were performed using filter lifts of colonies containing the indicated Nub and Cub Plasmids. This was repeated >10 times with similar results. Above depicts representative samples of the filter lift assays. Filters were developed for 72 hrs.

Ste2p interacts with Afr1p

Afr1p was analyzed since genetic evidence indicates it can interact with Ste2p [37]. The traditional yeast two-hybrid system failed to detect an interaction between Ste2p and Afr1p (C. Davis & J. Konopka unpublished). One possibility was that Ste2p was unable to enter the nucleus. To test the interaction using MYTH, Nub was fused to the N-terminus of *AFR1*. The interaction observed between Ste2p-CubPLV and Afr1p-Nub was strong. A set of previously made point mutations in *AFR1* that block its function were also tested. Previous studies suggested that these point mutations were located in a predicted binding domain, located in the N-terminal half of Afr1p [53]. However because of the insolubility of Afr1p, it was not possible to do a Co-IP. Thus, at the time, there was no readily available experimental procedures to test whether or not these point mutants were incapable of binding Ste2p [37]. Therefore, Nub-G was fused to the previously isolated *afr1* alleles. I found these Afr1p mutants were unable to bind Ste2p. However, as a control, and to further prove lack of binding of these point mutants, Nub-I was fused to the mutant *afr1* alleles. The false positive Nub-I showed that all of the point mutants of *afr1* gave a false positive result, confirming that the Nub-G *afr1* mutants were defective in binding to Ste2p without additional protein-protein contacts. This suggests the inability of the *afr1* point mutations to cause α-factor resistance is due to their inability to bind Ste2p (Figure 10).



Cub	Nub	Nub-G	Nub-I
Ste2	Afr1 wt	+++	+++
Ste2	Afr1 I259K	–	+++
Ste2	Afr1 V257A	–	+++
Ste2	Afr1 N254S	–	+++

Figure 10: Ste2p-Afr1p Results**:** Ste2-CubPLV and the indicated Nub-G or Nub-I plasmids. Filter spots were performed with $1X10^8$ cells per spot. Filters were incubated for 48 hrs. This was repeated >10 times and above is a representation of the results.

Sst2p interaction with Ste2p

Sst2p is an RGS protein that regulates pheromone-responsive Gα protein, Gpa1p that is activated by Ste2p. It is likely that Sst2p could bind Ste2p as many signaling proteins form complexes. Also, some genetic evidence has suggested that direct interaction with Ste2p may occur [39, 40]. To test Sst2p using MYTH, I fused Nub to the C terminus of Sst2p. The interaction observed between Ste2p-CubPLV and Sst2p-Nub was stronger than Ste2p dimerization, but much weaker than the Afr1p-Ste2p interaction (Figure 11). Several point mutations in the DEP domain of Sst2p were also tested for interaction with Ste2p. None of the Sst2p point mutations gave a significant MYTH assay signal with Ste2p, suggesting that the DEP domain in Sst2p is responsible for binding to Ste2p (Figure 11).

Other work done by collaborators determined DEP domains in Sst2p were essential for Sst2p function, as deletion of these domains resulted in increased sensitivity to pheromone similar to strains containing a complete deletion of the *SST2* gene [54]. It was also shown that the DEP domain function was more than just membrane targeting, as substitution of other membrane targeting sequences did not restore Sst2p function. Overexpression of Ste2p resulted in partial suppression of the s*st2-Q304N* mutant phenotypes and was allele specific, having no effect on the *sst2-Q304E* or *sst2-Q304L* mutants, suggesting that the restoration by overexpression of Ste2p was due to direct protein-protein interaction. It was further shown that phosphorylation of Ste2p displaces Sst2p,

and that Sst2p is recycled to other receptors lacking phosphorylation. In conclusion, the DEP domain of Sst2p is responsible for binding Ste2p, and therefore other DEP domains may be important in specifying which other GPCR pathways are targeted for desensitization via RGS proteins.

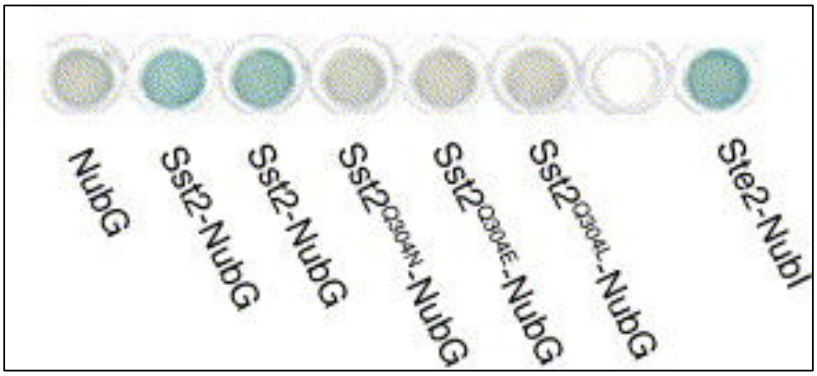


Figure 11: Sst2p-Ste2p β-galactosidase assay for the MYTH in 96 well plate format, all wells contain Ste2p-Cub-PLV and the indicated Nub plasmid. Nub G was used as a negative control, and Ste2-Nub-I as the positive control.

MYTH summary

The MYTH assay was able to detect Ste2p oligomerization. Interestingly, although the signal was weak, oligomerization could be detected with full-length receptors. Previous FRET studies were unable to detect dimerization using full-length receptors, and required the truncation of Ste2p cytoplasmic tail [35]. Truncating the C-terminal tail of Ste2 in the Ste2-Nub construct increased the dimerization signal, but it appeared to be non-specific as interaction was also detected with a negative control, the mammalian Bradykinin receptor. FRET was still specific with the truncated receptors and did not show interaction between Ste2p and other GPCRs [35]. The ability of the MYTH system, but not FRET, to detect oligomerization with full-length receptors could be due to MYTH being more sensitive than FRET. Thus, upon increasing the receptor production by truncating the tails, the higher sensitivity led to a higher background signal, perhaps because truncated receptors are more stable at the plasma membrane. The other possibility is that the Bradykinin receptor forms a heterodimer with Ste2p when there is an abundance of Ste2p at the plasma membrane. Although these two receptors do not occur in the same organism, perhaps there is a low affinity when they are both expressed and one of them is in abundance.

Ste2p interaction with negative regulator Afr1p was also detected by MYTH. Afr1p was initially discovered in a screen for proteins which when over expressed conferred resistance to α-factor [38]. Deletion mutagenesis studies

indicated that the first half of Afr1p was thought to be the Ste2p-binding domain, and the second half of Afr1p was thought to be an anchoring domain that binds to septins [55]. Several point mutations in *afr1* that were defective in causing resistance to α-factor were found to map to this first domain, which was presumed to be the binding domain for Ste2p [53]. However, the insolubility of Afr1p in mild detergents made this difficult to prove. In the MYTH system, all of these point mutations were shown to be defective in binding Ste2p, consisted with interpretations of genetic studies which showed that these point mutations were defective in α-factor resistance but not in binding to Cdc12p [53].

The RGS protein Sst2p was also tested for binding to Ste2p, and shown to bind Ste2p (Figure 11). Sst2p negatively regulates signaling, by acting as a GTPase for GPA1. Also, Sst2p contains a DEP domain, which was predicted to be responsible for specific recognition of GPCRs [54]. Sst2p mutations were made in the DEP domain, and were shown to be defective in binding Ste2p. These results are in agreement with results from the Thorner lab, showing that Sst2p is tethered to Ste2p via the DEP domain, and is responsible for dictating the duration and intensity of the signaling response [54].

Discussion of MYTH system

The MYTH system was able to detect targeted protein interactions with Ste2p. This suggests that the MYTH is capable of detecting targeted protein interactions with mammalian GPCRs. I was able to show receptor dimerization

using full length receptors, although shortening the cytoplasmic tail did increase detectable dimerization, this led to a more non-specific signal. However I cannot disprove that the non-specific signal was due to the possibility of Ste2p being able to interact with the Bradykinin receptor, as they both are GPCRs and may share structural similarities that allow for heterodimerization. Unpublished results have shown that GPCR dimerization with the MYTH could be extended to mammalian GPCRs as homodimerization and heterodimerization was shown with the Arginine Vasopressin receptor 1 and 2. Homodimerization with the Bradykinin receptor and heterodimerization with Bradykinin receptor and Angiotensin receptor was also shown; however, only if the assay was performed at 15^{o}C (unpublished data). Presumably, the lower temperature may lead to the upregulation of protein folding chaperones that help GPCRs to fold properly. Due to this low temperature, the growth rate was hard to control, mostly due to variations in temperature caused by shaking the cells, and such the function of these receptors at this temperature is still unknown and was not studied further. I also detected protein interactions between the Calcatonin receptor and Ramp1 and Ramp3 (Gladue D.P. and Konopka J.B unpublished data). This was expected as previous studies have shown that the Calcitonin receptor requires co-expression of Ramps to signal. Varying Ramp expression was also shown to change ligand specificity of the Calcitonin receptor [25]. However, due to the high cost and multitude of the ligands, whether or not the Calcitonin receptor signaled differently in *Saccharomyces cerevisiae* with Ramp1 and Ramp3 was not tested.

Identification of protein binding domains

DEP domains are widely found in proteins involved in signaling, and consist of a ~90 aa sequence motif that was first found in fly Dishevelled [56], EGL 10 in *C. elegans* and mammalian Pleckstrin [33]. In spite of the extensive studying of signaling proteins, it is surprising that the role for DEP domains hasn't been determined [57-60]. In collaboration with the Thorner lab, I utilized the MYTH to look at point mutations in the DEP domain of Sst2p. I determined that Sst2 was able to bind Ste2p; however, Sst2p with point mutations in the DEP domain showed no detectable binding. This, along with results shown in the Thorner lab, determined that DEP domains were necessary and sufficient for Sst2p binding to Ste2p [54]. The binding domain of Afr1 was also shown using various point mutations. The results of these two studies suggest that the identification of binding domains could be tested with mammalian GPCRs. The Arginine Vasopressin Receptors were tested and both homo-and heterodimerization could be detected, I utilized a reverse yeast two-hybrid to try to identify the residues responsible for dimerization. However, the reverse yeast two-hybrid strain was not sensitive enough to easily distinguish basal signaling from dimerization of the receptors. Future studies could test other receptors with lower levels of background signaling, or perhaps identify the binding domain of RAMPs.

Library Screening

Two Nub plasmid libraries containing *S. cerevisiae* genomic inserts were used to screen for proteins that interact with Ste2p-Cub. Approximately 100,000 colonies of each library were screened for potential interactions. However, upon sequencing the potential clones, most were false positives, containing various sequences of mitochondrial DNA or out of frame proteins. These results were similar to those of other groups using MYTH and the same libraries. However, I was able to recover Ste2p-Nub from the library screening, indicating that screening could work if the method could be optimized. Due to the false positive rate of up to 10% using the genomic DNA libraries, a cDNA library was made in the laboratory of Dr. Stagljar to eliminate mitochondrial DNA contamination in the library. However, this cDNA library was enriched for a subdomain of *DCW1* that caused a false positive. Because of the high number of false positives in all the libraries, library screening was not continued.

CHAPTER 4: Cysteine Scanning Mutagenesis of Gpa1

Fungal pheromone receptors belong to the large medically important family of G protein coupled receptors that transduce signals for a variety of stimuli and are also implicated in a broad range of diseases, such as retinitis pigmentosa, inflammation, and hypertension [61]. GPCR signaling also regulates important pathways in a wide range of eukaryotes from yeast to humans [62]. Receptors in this family are composed of seven transmembrane domains (TMDs) and function by activating the Gα subunit of a heterotrimeric G protein to bind GTP. Ligand binding induces receptors to undergo a conformational change, which enables them to stimulate Gα to exchange GDP for GTP. The GTP bound Gα releases Gβγ and then either Gα or Gβγ can activate downstream effectors.

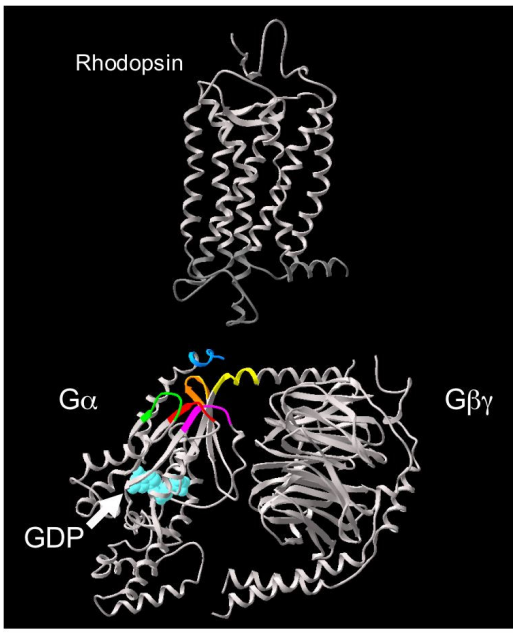
Several general models have been proposed for the mechanisms of G protein activation. Some models propose a direct role for the C terminus in GTP exchange, because it is directly connected via the α5 helix to the residues that bind the guanine ring [63, 64]. Other regions are also thought to contribute to GTP exchange (Figure 12). For example, the N terminus of Gα has been proposed to act as a lever to open up the guanine nucleotide binding pocket [65], since it connects via a short β-sheet sequence to residues that contact the phosphates on GDP. An alternative "Gear Shift Model" proposes that the N terminus acts as a lever to force the Gβ subunit into the guanine nucleotide binding site (switch regions) in Gα, analogous to the manner in which exchange

factors promote guanine nucleotide exchange on the small Ras-like proteins [66]. Interestingly, there are at least three other regions of Gα predicted to form part of the interface region that have not been well studied including the β2-β3 loop, α2-β4 loop, and the α3-β5 loop.

To examine the function of Gα residues that are implicated in interfacing with GPCRs, we carried out a scanning mutagenesis of residues in 6 different regions of the pheromone-responsive Gα protein Gpa1 in the yeast *S. cerevisiae*. In cells of mating type **a**, the pheromone pathway is activated when α-factor pheromone binds to its receptor (Ste2), leading to GTP exchange on Gpa1 [62, 67]. This causes the release of the Gβγ subunits (Ste4 and Ste18), which recruit the Ste5 scaffold protein to the plasma membrane to activate a MAP kinase cascade [68, 69]. The pheromone pathway is well suited for mutational analysis of *GPA1* because of the genetic accessibility of yeast, and because the pheromone pathway only involves one G protein, in contrast to more complex multicellular organisms, which typically encode multiple Gα subtypes [62]. Scanning mutagenesis of 33 residues in *GPA1* identified a range of phenotypes from constitutive pheromone pathway activation to loss of signaling. These results implicate multiple Gα regions in transducing the signal that promotes GTP exchange.

Prediction of Gpa1 interface residues to target for scanning mutagenesis based on structures of rhodopsin and transducin

To identify regions of Gα (Gpa1) that would be likely to interact with the α-factor receptor (Ste2), the crystal structures of rhodopsin and transducin were juxtaposed. As described previously, the interface regions of rhodopsin are defined as the cytoplasmic loops and C terminal tail that are determined by the membrane insertion of the seven TMDs [70, 71]. The G protein was oriented so that the lipid modifications of the Gα and Gγ proteins faced the plasma membrane [63, 72]. Juxtaposing rhodopsin and transducin in this manner revealed that the N and C termini, as well as four loop regions (β2-β3, α2-β4, α3-β5, α4-β6) of the Gα protein, were likely to face the receptor (Figure 12).



Gα regions	(proximal region)
1 N terminus	(PO_4 of GDP)
2 β2-β3 loop	(Switch 1 and 2)
3 α2-β4 loop	(Swtch 2)
4 α3-β5 loop	(Switch 3; GDP)
5 α4-β6 loop	(Guanine ring)
6 C terminus	(Guanine ring)

Figure 12: Juxtaposition of the crystal structures of rhodopsin and transducin.

The crystal structures of the photoreceptor rhodopsin [73] and the G protein transducin [63] are displayed. The regions in Gα that can potentially interface with the receptor are colored as indicated. The GDP is shown in turquoise. Indicated below is the proximity of these interface regions the switch regions that change conformation upon GTP binding and the residues that contact the GDP [74]. The intracellular loops of rhodopsin are presented in a darker shade of grey. The last seven residues of the C terminus of Gtα were not ordered in the crystal structure [63] so they were modeled as an α-helix based on an NMR structure of a C-terminal peptide complexed with rhodopsin [75] using Swiss PDB Viewer [76].

The corresponding interface regions in the yeast Gα protein, Gpa1, were identified by comparing an amino acid alignment of transducin Gα with Gpa1. The interface regions were easily identified because these Gα subunits share ~45% amino acid identity. In addition, these interface domains were flanked by highly conserved residues. Thirty-three substitution mutations were created in *GPA1* in which one of the predicted interface residues was substituted with cysteine (see Materials and Methods). Residues were substituted with cysteine rather than the more widely used alanine, so that future studies could take advantage of the thiol side chain of cysteine to study Gpa1 function by chemical modification or crosslinking approaches [77]. The cysteine substitution mutations were introduced into *GPA1* carried on the low copy yeast vector YCplac111 (*ARS1 CEN4 LEU2*) [49]. The version of Gpa1 encoded on this plasmid contains the myc epitope. This Gpa1-myc is functional in vivo and can be readily detected on Western blots [78]. The myc epitope was introduced at residue 125, which is located on the opposite side of Gpa1 from the plasma membrane, and therefore does not interfere with signaling.

Phenotypes of cysteine substitution mutants

The phenotypes of the *GPA1* substitution mutations were tested by introducing the plasmids into a genetically modified yeast strain in which signaling could be measured by activation of a pheromone response *FUS1-lacZ*

reporter gene [79]. Cells also contained a deletion of the endogenous *GPA1* gene, and a *far1* mutation to allow for survival in absence of Gpa1 by preventing pheromone-induced cell division arrest [80]. Cells carrying the mutant *GPA1* alleles were initially tested for basal and pheromone-stimulated levels of *FUS1-lacZ*. Mutations affecting distinct regions of Gα caused a wide range of phenotypes (Figure 13).

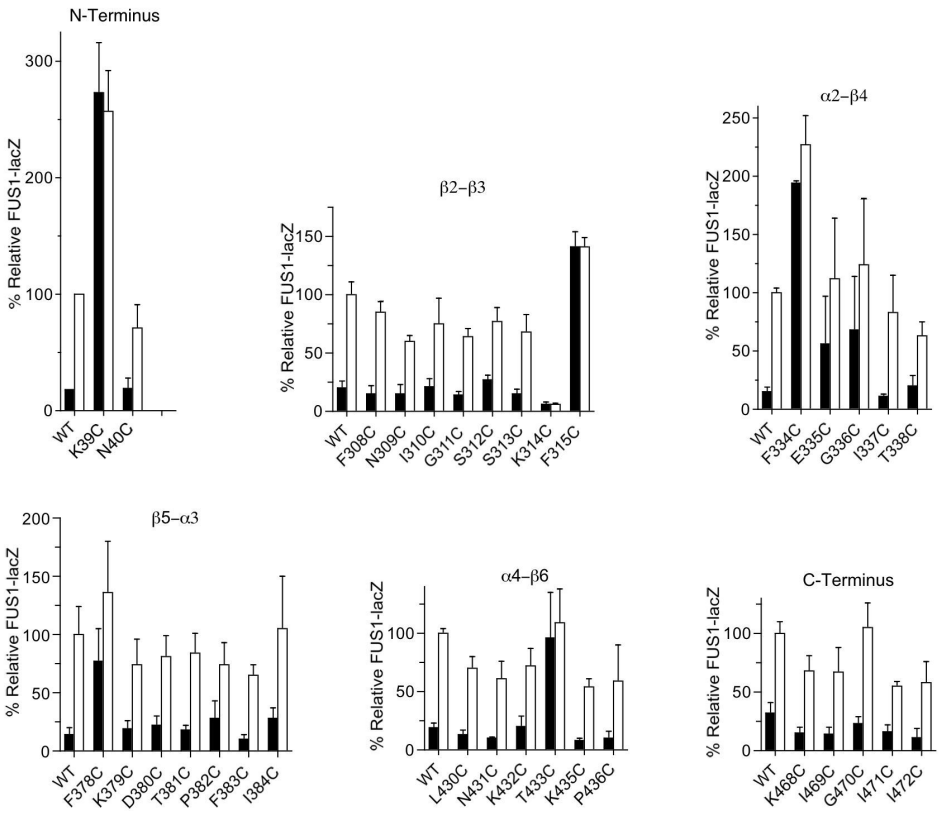


Figure 13: Pheromone pathway activation by *GPA1* mutants.
Plasmids carrying the indicated wild-type or cysteine-substituted version of *GPA1* were introduced into strain PMY3.2, which lacks the genomic copy of *GPA1*. Strains were incubated in the absence (black bars) or presence (white bars) of 1×10^{-4} M α-factor and then activation of the *FUS1-LacZ* reporter gene was assessed by assaying β-galactosidase activity. The results were normalized to the maximum value of wild-type cells. Error bars indicate SD

The mutants with the strongest phenotypes could be placed into three groups that will be described in detail below. One set of mutants displayed very high basal signals (K39C, G315C, F334C and T433C). Another group showed increased basal signaling, but were still inducible with α-factor (E335C, G336C and F378C). The third group included a strong loss of function substitution mutant (K314C) and several substitution mutants that displayed a partial loss of function (K435C, I471C, and I472C). Some of the other mutants displayed weaker effects on basal or induced signaling and were not studied further.

GPA1 mutants that display high constitutive activation of pheromone pathway

The *GPA1* substitution mutants that showed high constitutive activity in the absence of α-factor included K39C, which affected a residue in the N-terminus, F315C the β2-β3 loop, F334C the α2-β4 loop, and T433C the α4-β6 loop. *FUS1-lacZ* activity was examined in dose-response assays to see if these mutants could be further induced by α-factor. Both F334C and T433C showed a slight increase in *FUS1-lacZ* activity with increasing α-factor concentration, suggesting at least partial function was retained (Figure 14A).

The K39C and F315C mutants did not appear to respond to α-factor. Since the Gβγ subunits are primarily responsible for activating the downstream steps in the pathway [62, 81], there are three possibilities that are likely to cause

constitutive signaling by mutation of *GPA1*. One is that the constitutive phenotype is caused by decreased protein production, allowing Gβγ to signal in an unregulated manner. Alternatively, Ste2 may activate these Gpa1 mutants in the absence of ligand. The third possibility is that the mutant Gpa1 proteins are defective in sequestering Gβγ.

To determine if constitutive activity was due to decreased levels of the mutant Gpa1 proteins, a quantitative Western blot was performed.(Figure 14B) Gpa1-K39C and Gpa1-F315C were not detected, indicating that these proteins were present at <10% of the level of wild-type Gpa1. The Gpa1-T433C protein was produced at only 28% of wild-type Gpa1 levels. Thus, low levels of Gpa1 can explain the constitutive activity of these mutants. In contrast, Gpa1-F334C was detected at a slightly higher level than the wild-type Gpa1 protein. The increased levels of this mutant protein may be due to in part to the fact that this strain displays constitutive activity and the *GPA1* gene is slightly induced by the pheromone pathway [82]. In order to determine if Gpa1-F443C could be activated by Ste2 in the absence of ligand, *FUS1-lacZ* levels were analyzed in a strain lacking the *STE2* gene (Figure 14C). The constitutive activity of Gpa1-F334C was still high in the absence of *STE2*, indicating that the constitutive activity is independent of the α-factor receptor. These results indicate that Gpa1-F344C is defective in sequestering Gβγ.

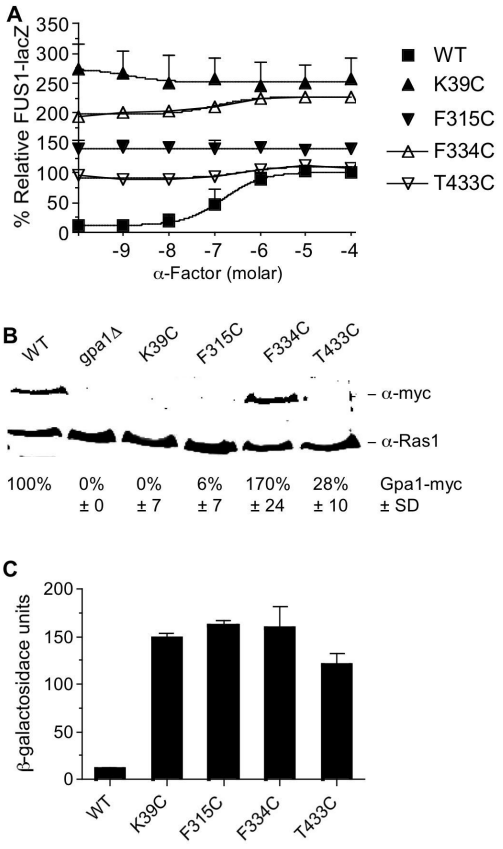


Figure 14. Mutants with high basal activation of pheromone pathway.

(A) Dose response assays for the *FUS1-lacZ* reporter gene. PMY3.2 cells that carried the indicated version of *GPA1* on a plasmid were incubated in the presence of different concentrations of α-factor and then assayed for β-galactosidase activity. Values were normalized to the maximum value for cells carrying a wild-type *GPA1* plasmid. Error bars indicate SD and are only shown in one direction to avoid obscuring other data points.

(B) Quantitative Western blot analysis of cells described in panel A was performed as described in the Materials and Methods using anti-myc antibody followed by secondary goat anti-mouse antibody (IRDye 800cw, Li-Cor Biosciences) to detect myc-tagged Gpa1 proteins. The Gpa1 levels were normalized to the level of Ras2 protein. Quantitative analysis was carried out using a Li-Cor Biosciences Odyssey Infrared Imaging system

(C) Quantification of basal activity in the absence of Ste2

GPA1 mutants that display intermediate basal activation of pheromone pathway

Another group of *GPA1* mutants showed moderately elevated basal signaling but were still readily induced with pheromone to essentially the same maximum as the wild type. These mutants carried the following substitutions: E335C (α2-β4 loop), G336C (α2-β4 loop), and F378C (α5-β3). Quantitative western blotting analysis showed that both Gpa1-E335C and Gpa1-F378C were produced at approximately wild-type levels, suggesting that the high basal activity was not due to a problem with protein stability. In contrast, Gpa1-G336C was produced at lower levels than wild-type Gpa1 (43%) (Figure 15).

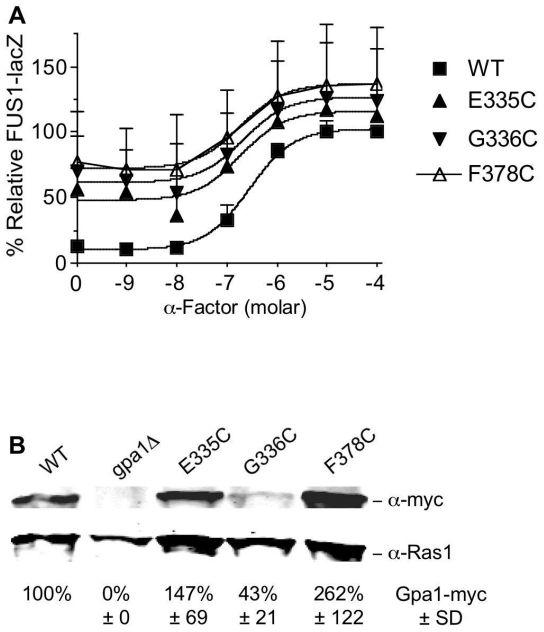


Figure 15: Mutants with intermediate basal activation of pheromone pathway.

(A) Dose response assays for the *FUS1-lacZ* reporter gene. PMY3.2 cells carrying the indicated version of *GPA1* on a plasmid were incubated in the presence of different concentrations of α-factor and then assayed for β-galactosidase activity. Values were normalized to the maximum value for cells carrying a wild-type *GPA1* plasmid. Error bars indicate SD and are only shown in one direction to avoid obscuring other data points.

(B) Quantitative Western blot analysis of cells described in panel A was performed as described in the Materials and Methods using anti-myc antibody followed by secondary goat anti-mouse antibody (IRDye 800cw, Li-Cor Biosciences) to detect myc-tagged Gpa1 proteins. The Gpa1 levels were normalized to the level of Ras2 protein. Quantitative analysis was carried out using a Li-Cor Biosciences Odyssey Infrared Imaging system.

***GPA1* mutants with decreased response to pheromone**

GPA1-K314C (β2-β3 loop) caused the only strong loss of function phenotype among the collection of mutants (Figure 16A). It displayed low basal activity and was not detectably inducible by α-factor. Gpa1-K314C was only produced at 60% of the wild-type Gpa1 protein level. This slight decrease in the level of Gpa1-K314C level relative to wild-type Gpa1 may be due to lower basal signaling, which causes slightly reduced expression of *GPA1* (Figure 16B). More importantly, these results demonstrate that the defect in signaling is not due to overproduction of Gpa1, which is known to inhibit signaling [62]. Instead, these results suggest that Gpa1-K314C is able to bind Gβγ but cannot be activated by Ste2p. To further confirm the defect in signaling, *GPA1-K314C* was coexpressed with wild-type *GPA1* to determine if it caused a dominant interfering phenotype. Cells carrying a *GPA1-K314C* plasmid displayed a much stronger interfering effect on α-factor-induced cell division arrest than did the introduction of the wild-type *GPA1* gene on the same plasmid vector (Figure 16C). This further indicates that Gpa1-K314C is able to sequester Gβγ.

Several other *GPA1* mutants showed a partial defect in α-factor signaling. Substitutions K435C (α4-β6 loop), I471C (C terminus), and I472C (C terminus) caused reduced basal and pheromone-induced signaling in α-factor dose response

assays. Gpa1-I471C and Gpa1-I472C protein levels were 80% and 102% of wild-type Gpa1, respectively. This demonstrates that they are not overproduced and suggests they are defective in signaling, consistent with previous studies implicating the Gpa1 C terminus in receptor coupling [83]. The Gpa1-K435C protein was present at elevated levels compared to wild-type Gpa1 (170%), indicating that reduced signaling by this mutant may be due in part to increased levels of Gα.

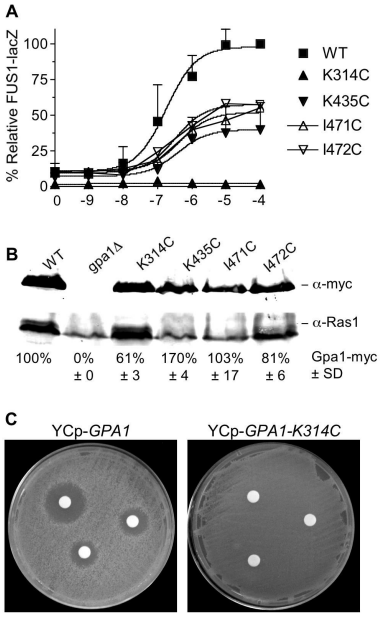


Figure 16: Loss of function mutants

(A) Dose response assays for the *FUS1-lacZ* reporter gene. PMY3.2 cells carrying the indicated version of *GPA1* on a plasmid were incubated in the presence of different concentrations of α-factor and then assayed for β-galactosidase activity. Values were normalized to the maximum induction for cells carrying a wild-type *GPA1* plasmid. Error bars indicate SD and are only shown in one direction to avoid obscuring other data points.
(B) Quantitative Western blot analysis of cells described in panel A was performed as described in the Materials and Methods using anti-myc antibody followed by secondary goat anti-mouse antibody (IRDye 800cw, Li-Cor Biosciences) to detect myc-tagged Gpa1 proteins. The Gpa1 levels were normalized to the level of Ras2 protein. Quantitative analysis was carried out using a Li-Cor Biosciences Odyssey Infrared Imaging system.
(C) Dominant effects of *GPA1-K314C* on pheromone-induced cell division arrest. DJ211-5-3 cells, which carry a wild-type copy of *GPA1* in the genome, were transformed with a plasmid carrying either *GPA1* or *GPA1-K314C*. The cells were tested for ability to undergo α-factor induced cell division arrest in a halo assay. Cells were spread on a solid medium plate and then filter discs containing 500, 250, or 125 ng α-factor were placed on the surface to promote a zone of clearing (halo) due to cell division arrest. The plates were incubated for 48 h at 30˚ and then photographed.

DISCUSSION:

Cysteine scanning mutagenesis of *GPA1*

Juxtaposing the structures for rhodopsin and its cognate G protein transducin predicted that at least six regions of Gα are in close proximity to the receptor. Therefore, the genetic accessibility of *Saccharomyces cerevisiae* was used to examine the function of four loop regions that are predicted to face the receptor (β2-β3, α2-β4, α3-β5, and α4-β6) as well as the N and C termini of the pheromone-responsive Gα protein Gpa1. Previous mutational studies identified *GPA1* mutations affecting the N and C termini [78, 83, 84], but not in the loop regions. Scanning mutagenesis of 33 different residues in these regions revealed a range of phenotypes that fell into three groups: (i) high constitutive signaling, (ii) intermediate basal signaling but still highly induced by α-factor, and (iii) decreased pheromone-induced signaling. As described below, these results suggest that many different residues in the predicted interface regions contribute to activation of Gα.

Mutants with elevated basal signaling.

The seven mutants that displayed high or intermediate basal activity of the *FUS1-lacZ* reporter gene were analyzed on Western blots to determine if this phenotype was caused by decreased production of the mutant Gpa1 proteins.

Four mutants showed decreased Gpa1 levels (F315C, K39C, G336C and T433C). Since these residues are predicted to face the plasma membrane, it seems unlikely that they would all be accessible to proteases. One possibility is that these substitutions cause general defects in protein folding. Phe-315 and Lys-39 are partially conserved in the Gα family and may be important for Gα structure. Gly-336 and Thr-433 are not conserved.

Three mutants that showed elevated basal signaling contained Gpa1 levels similar to or higher than the wild type (F334C, E335C, F378C). The high basal signaling activity of these mutants was independent of the α-factor receptor Ste2, suggesting that these forms of Gpa1 are defective in sequestering Gβγ. In agreement, the homologous residues in the transducin crystal structure (Phe-211, Glu-212, and Phe-255) are closely clustered on the side of Gtα that faces Gtβ. Phe-334 in Gpa1 may directly contact Gβ, since Phe-211 in Gtα appears to interact with Trp-99 in Gtβ [63]. Consistent with these residues forming an important interaction, mutation of the Ste4 residue that is homologous to Trp-99 (W136R) prevented binding to Gpa1 and caused constitutive signaling [85]. Glu-335 of Gpa1 may also directly interact with Gβ, since Gtα Glu-212 appears to contact Lys-57 of Gtβ. Furthermore, both the Lys-57 and Trp-99 residues in Gtβ are highly conserved in other Gβ proteins, including the yeast Gβ protein Ste4. Interestingly, Phe-334 and Glu-335 in Gpa1 are predicted to be near the boundary of the Gα Switch 2 region that changes conformation upon GTP binding [74]. Phe-378 in Gpa1 does not appear to make a direct contact with Gβ. The effects of

mutating this residue may therefore alter Gβ interaction indirectly, such as by affecting the switch regions or by altering GTP hydrolysis. However, Phe-378 is in close proximity to Phe-334 and Glu-335, suggesting this cluster of residues Gpa1 is important in interacting with Gβ (Figure 17).

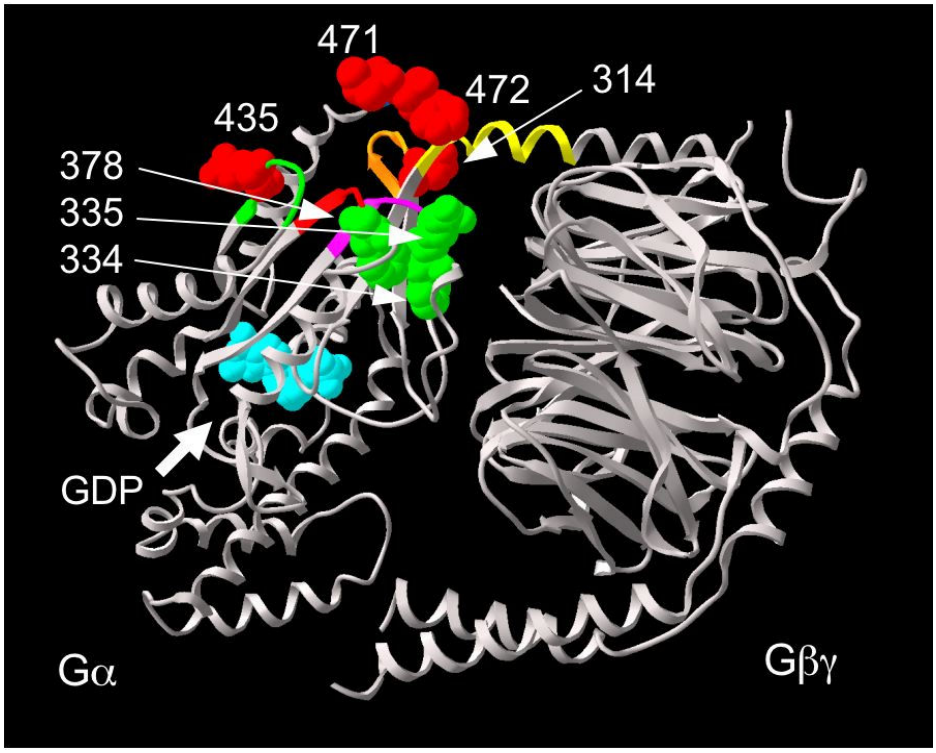


Figure 17. Relative position of Gpa1 mutations mapped onto the transducin structure.

Residues affected by cysteine substitution mutations are shown in space-filling mode on a ribbon model of the transducin structure as described in Figure 12. Residues corresponding to sites of mutations that cause high basal activity are shown in blue and the loss of function residues are shown in red. Residues corresponding to mutations that caused reduced Gpa1 protein production are not shown.

Loss of function mutants

The strongest defects in signaling were caused by K314C (β2-β3), K435C (α4-β6), and the I471C and I472C substitutions that affect the most distal residues in the C terminus of Gpa1. The K314C substitution caused a very strong phenotype; no response was detected even at the highest doses of pheromone tested. Introduction of *GPA1-K314C* on a plasmid in a wild-type *GPA1* strain caused a dominant interfering effect that prevented pheromone-induced cell division arrest (Figure 16), further suggesting that Gpa1-F314C was able to tightly bind Gβγ but was unable to be activated. These results implicate the β2-β3 loop in G protein activation. Interestingly, this domain is located between the Switch 1 and Switch 2 regions that change conformation upon GTP binding [86]. Mutations in Gsα within this region have been reported to influence the repression of adipogenesis, but not adenylyl cyclase activation [87]. The K435C, I471C and I472C substitutions in *GPA1* also caused significant defects in signaling, consistent with previous studies that have implicated the α4-β6 domain [12] and the C terminus in G protein activation [83].

Receptor - Gα interface

It was surprising that only one out of the 33 substitutions in the interface region caused a severe defect in signaling. However, scanning mutagenesis of the

intracellular loops of the α-factor receptor Ste2 also identified very few mutants with strong signaling defects [88, 89]. This suggests the possibility that many residues contribute to the interface between receptors and G proteins, such that mutation of one residue may not strongly affect overall signaling efficiency. Consistent with this, mutations that caused partial defects in signaling were not clustered and were found in different regions of Gpa1. The cysteine substitutions created in this study could be used in future studies to better define the receptor-Gα interface. One possibility is to assay the ability of disulfide bonds to crosslink between cysteines substituted into Gpa1 and Ste2. Preliminary crosslinking studies failed to detect efficient disulfide bond formation between the cysteines in the third loop of Ste2 and Gpa1 (D.P.G. and J.B.K., unpublished data).

Further studies will also be needed to understand how receptors interface with the entire Gαβγ heterotrimer, since Gβγ is also thought to contribute to receptor coupling [90]. In addition, recent studies indicate that GPCRs function as dimers or higher order multimers, and that two or more receptors may interface with a single G protein [70, 91], although this is controversial [92]. A further complication is that mammalian cells contain multiple G protein subunits and it is unclear why some receptors are specific for one G protein but other receptors can activate multiple types of G proteins [72, 90]. Altogether, these findings together with the results for scanning mutagenesis of *GPA1* indicate that the receptor-G protein interface is likely to involve concerted action of many different contact regions.

Chapter 5: Discussion

The ubiquitous distribution of GPCRs, their importance in various physiological functions, and their role in many diseases makes it crucial to gain a better understanding of GPCR function. Given that 50% of all pharmaceutical drugs interact with GPCR's, identification of proteins interacting with GPCR's may provide a new mechanism to modulate GPCR function and treat disease. Therefore, Ste2p was studied as a model to develop methods for analyzing proteins that interact with GPCRs.

In one approach, MYTH was used to identify proteins that interact with Ste2p. Once proteins that interact with GPCRs are identified, the mechanisms and functions of these protein interactions must be defined to allow better understanding of these signal pathways. For example, the mechanisms by which Gα subunits are stimulated to bind GTP are not well understood. Insight to this mechanism may allow for development of drugs that specifically target the interaction with receptor and G protein. Therefore, cysteine scanning mutagenesis of Gpa1p was used to study the Receptor – G protein interface in order to develop insights into how these two proteins interact. In this chapter I will place these results in context by discussing future studies for discovering proteins that interact with GPCRs as well as identifying the specific regions of proteins that mediate the interaction.

Relevance of results in this thesis to mammalian GPCRs

Studies performed using the yeast GPCR (Ste2p) in this thesis have implications for GPCRs in mammalian cells. First, MYTH assays can be extended to examine proteins that interact with mammalian GPCRs. Many mammalian GPCRs have been shown to retain their signaling function in yeast, and therefore it is likely that they will also retain their ability to bind other proteins. The results that the Sst2p-DEP domain interacts with Ste2p are also important, as many RGS proteins in mammalian cells contain DEP domains, indicating that these other RGS proteins may bind to the appropriate GPCRs via their DEP domains. Future studies in this area may determine how mammalian RGS proteins are selectively directed to act on G proteins activated by specific GPCRs. However some RGS proteins are very small and lack DEP domains, suggesting they may not bind GPCRs. Nonetheless, the ability to disrupt the specific binding of some RGS proteins to their cognate receptors could be useful in developing new strategies for therapeutic intervention.

GPCR crystal structures

The crystal structure of rhodopsin was a landmark discovery [93]. However, it has been seven years since the crystal structure of the inactive state was reported and the crystal structure of Rhodopsin in its activated state or coupled to the G protein is still unknown. Crystallization of additional GPCRs

has also not been reported. This is most likely due to complexity in creating stable crystals of GPCRs outside of their membrane environment. Therefore, it is important to develop alternative techniques to examine GPCRs in the activated state in order to determine how the G protein interacts and how it is activated by the receptor. Due to the complexity and technical difficulties in determining the crystal structure of other GPCRs, cysteine scanning mutagenesis and crosslinking the G protein to the receptor is an alternative method for understanding how the G protein and receptor interact with each other.

Co-immunoprecipation for identifying new protein interactions

One method for identifying new interacting proteins has been the use of co-immunoprecipation or GST pull down assays. One protein is pulled out of solution by antibodies recognizing a specific protein or tag on the protein, and proteins that also come down are identified. This has had some limited success with membrane proteins, and seems to work best if the protein binds to the soluble C-terminal tail of the GPCR. Such techniques have been used to study the interaction of GPCRs with RGS proteins [54, 94]. However, these techniques have not worked well for the seven-transmembrane core that requires harsh detergents to extract from the membrane. Thus, it is difficult to identify proteins that interact with GPCRs in the native state, or to identify proteins that interact with the hydrophobic transmembrane domains.

Identification of novel proteins via FRET

Fluorescence Resonance Energy Transfer or FRET involves the absorption by a fluorophore of light of one wavelength, and the emission of light at another wavelength that activates a secondary fluorophore. FRET is possible for examining specific proteins that interact with GPCRs, such as Gα or GPCR dimerization partners. However, for screening purposes to identify new proteins, it would be difficult in part due to the lack of selection for cells that exhibit FRET vs. cells that do not exhibit FRET. It would take careful stimulation and microscopy techniques for hundreds of thousands of individual cells, and would be extremely time consuming, as a selection scheme isn't available. Each library transformant would have to be examined individually.

MYTH

The membrane yeast two-hybrid worked well in targeted studies with Ste2p (see Chapter 3). However, screening proved difficult, in part due to the high false positive rate with the yeast libraries. MYTH has also been used for library screening with mammalian proteins such as ERB3b [95]. Recent developments in this system have shown that using a new variation called iMYTH, which integrates the bait plasmid, permitted identification of new proteins that interact with yeast ABC transporter Ycf1p by library screening [96].

Perhaps new libraries and use of the iMYTH will allow for improved screening with GPCRs.

Alternative approaches for screening

One alternative approach to identify protein-protein interactions is Bi-molecular Fluorescence, or BiFT. GFP is split into two halves, and works in a similar manner as the MYTH. Both halves of GFP are fused to proteins, and if the proteins interact with each other, GFP refolds and can be detected under a fluorescent microscope [97, 98]. This has been used primarily to study targeted protein-protein interactions, and for reverse screening to identify mutations that disrupt of protein-protein complexes [99]. It would be difficult to identify new protein interactions via library screening, as there is no selection technique and every colony would have to be examined individually or by FACS.

Alternatively, β-galactosidase has also been split into the α and Ω subunits, and works in the same way as the split GFP system. However, the difference is that the split β-galactosidase system would allow for similar blue/white screening on X-gal plates. The limitations of this system are β-galactosidase assays have a high false positive rate for screening purposes, due to variation in growth rates of individual colonies expressing different library plasmids, unlike the traditional yeast two-hybrid which allows for additional selection on plates lacking Histidine. To date there are no reports where this assay has been used for screening

Defining how proteins interact with GPCRs

To understand exactly how pharmaceutical drugs affect GPCR signaling, it is important to understand specifically how various proteins interact with GPCRs. Determining exactly how these proteins interact may lead to a better understanding of why some drugs lose effectiveness for various diseases due to adaptation and receptor down regulation. In my thesis I tried to better define how the Gα protein interacts with the receptor, using cysteine scanning mutagenesis described in (Chapter 4). I chose to create cysteine substitutions so that I could attempt to crosslink the Gα protein to the receptor. Previous studies, such as those carried out by the Khorana lab, crosslinked Rhodopsin to Transducin using bifunctional chemical and light activated crosslinkers. However, these long crosslinking arms have only been able to determine that certain regions of Gα interact with the receptor [100, 101]. In spite of this limitation, these studies have proven useful in determining the relative orientation of the Gα and the receptor in the membrane. In contrast, the advantages of using cysteine crosslinking is that it would allow for a 2Å resolution of the receptor-Gα interface, and the specificity of the thiol side chain would ensure the correct identification of residues involved in the crosslink.

Site-directed spin labeling

Monitoring how proteins change confirmation has been very difficult; however, many studies have used site directed spin labeling (SDSL). In this approach, an organic molecule has an unpaired electron with ability to pair with another atom. This is monitored by the electron paramagnetic resonance (EPR) spectrum of the spin label. In this spectrum, changing of the magnetic field constant allows for measurement of the unpaired electron state of either absorbing or emitting electromagnetic radiation. The EPR spectrum is then used to identify the position of the spin labels within a protein. Recent studies by the Hamm laboratory have shown allosteric interactions of the GTP binding pocket in Gα [12, 63, 72]. However, spin labeling is only able to map protein conformational changes within small regions of a protein, and would require thousands of assays to determine exactly how two proteins interact.

Comment [DPG1]: Rewrite and look up spin labeling

Future implications for drug discovery and human health

The goal of this thesis was to develop new ways to study proteins that interact with GPCRs. New techniques will be required to gain a high degree of resolution of how the GPCR interacts with Gα and other proteins. These discoveries will provide insight into the complex activation and regulation of GPCR signaling. This understanding is required to be able to determine the

mechanism for how drugs target GPCRs and perhaps allow for the design of better drugs. For example, the design of drugs that are more specific would decrease side effects. First, a higher resolution of how proteins interact to mediate receptor signaling and down regulation is required. In addition, understanding how GPCRs reside in the plasma membrane and interact with various lipids will be required. This adds yet another layer of complexity for how GPCRs signal in their native environment. Perhaps future studies will extend beyond the study of proteins that interact with GPCRs and will identify drugs affecting protein-lipid interactions that could be used to regulate GPCR signaling.

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