Activation of G Protein Signaling by a Non-Receptor Exchange Factor

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ABSTRACT

MICHAEL J. LEE: Activation of G Protein Signaling by a Non-Receptor Exchange Factor
(Under the direction of Dr. Henrik G. Dohlman)

G proteins are biological signaling switches, which typically exist in a multi-protein complex at the cell periphery. Included in these systems is a seven-transmembrane spanning G protein coupled receptor (GPCR), a guanine nucleotide binding protein G alpha subunit (Gα) and the obligate dimer Gβγ. GPCRs bind extracellular ligands, and transmit signals to intracellular G proteins. Ligand binding causes the Gα subunit to exchange GDP for GTP. GTP-bound Gα no longer interacts with Gβγ, and both entities are free to interact with downstream effector proteins. Gα regulates the duration of the signal because Gα is a GTPase, an activity that can be accelerated by GTPase Accelerating Proteins (GAPs) such as RGS proteins.

The standard G protein signaling model, in its basic tenets, has remained largely unchanged for decades. This paradigm, however, fails to take into account recent findings that G proteins are not restricted to the cell periphery. A number of reports suggest that Gα subunits in particular exist at, and even signal at, intracellular locations. These findings open up the possibility that Gα subunits regulate novel signaling pathways, away from the cell periphery, and potentially independent of the traditional “Gα-Gβγ-GPCR” paradigm. One particular example comes from the yeast model system, where the G protein Gpa1 regulates a cell fusion process called mating. It was recently identified that Gpa1 regulates a Gβγ-mediated signal at the cell periphery, and PI3K-mediated signal from the cell interior.
Studies presented in this thesis focus on: 1) the regulation of Gpa1 signaling by post-translational modifications such as palmitoylation and ubiquitination, and 2) the activation of Gpa1 by non-receptor activators. Specifically, we detail the discovery that Arr4 functions as an exchange factor for Gpa1, and may contribute to the activation of Gpa1 at the endosome. Also, we find that Gpa1 localization to the plasma membrane and endosome is regulated by multiple forms of ubiquitination and also by dynamic palmitoylation. Finally, we create a novel method to be used for the identification of proteins involved in palmitoylation. Collectively, these discoveries further the growing understanding that G proteins function at intracellular locations.
This thesis is dedicated to my parents,

for allowing me to follow my own path.
ACKNOWLEDGEMENTS

I owe a great deal of gratitude to a great number of people. First of all, I would like to thank my thesis mentor, Henrik, as well as the other members of my committee for being overwhelmingly supportive during my graduate career. Without exception, each member of my committee made critical contributions to this work, through their comments, their praise, and sometimes their criticism. The ideas I pursued were inspired both directly and indirectly by the members of my committee, and in many ways, I think this thesis reflects their influence. I would also like to thank a number of people who, at some point, let me know that they felt I could succeed as a scientist. In addition to Henrik and each of my committee members (Ken Harden, David Siderovski, Klaus Hahn, and Pat Brennwald), this list includes Sharon Milgram, Gil White, Adrienne Cox, Gary Johnson, JoAnn Trejo, John Sondek, Rob Nicholas, Lee Graves, Tom Kunkel, and Janeen Vanhooke. Sometimes their comments came from something I did or said in class, but more often than not, they were unprovoked. Graduate school can be filled with so much doubt, and so much failure, the knowledge that others whom I respect believe in my ability was something in which I often took comfort.

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<th>Definition</th>
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<tr>
<td>6XHIS</td>
<td>Six Histidine residues (used as an affinity tag)</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>AGS</td>
<td>Activator of G protein Signaling</td>
</tr>
<tr>
<td>AKR1</td>
<td>AnKyrin Repeat-containing protein</td>
</tr>
<tr>
<td>Ala or A</td>
<td>Alanine</td>
</tr>
<tr>
<td>AlF4-</td>
<td>Aluminum Tetra-fluoride</td>
</tr>
<tr>
<td>ARR4</td>
<td>Arsenicals Resistance Related</td>
</tr>
<tr>
<td>Asn or N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Asp or D</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>C. elegans</td>
<td><em>Caenorhabditis elegans</em> (worm)</td>
</tr>
<tr>
<td>Ca++</td>
<td>(ionic) Calcium</td>
</tr>
<tr>
<td>CIM3</td>
<td>Co-lethal In Mitosis</td>
</tr>
<tr>
<td>CuSO4</td>
<td>Copper Sulfate</td>
</tr>
<tr>
<td>Cys or C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Δ</td>
<td>Deletion</td>
</tr>
<tr>
<td>DHHC-CRD</td>
<td>Aspartic Acid-Histidine-Histidine-Cysteine, within a Cysteine Rich Domain</td>
</tr>
<tr>
<td><em>Drosophila</em></td>
<td><em>Drosophila melanogaster</em> (fly)</td>
</tr>
<tr>
<td>E1</td>
<td>ubiquitin activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>ubiquitin ligase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluoro-Di-d-Galactoside</td>
</tr>
<tr>
<td>FLAG</td>
<td>Flag epitope tag (DYKDDDDK)</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence Recovery After Photo bleaching</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>Gα</td>
<td>G protein alpha subunit</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Accelerating Protein</td>
</tr>
<tr>
<td>Gβ</td>
<td>G protein beta subunit</td>
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<td>GDP</td>
<td>Guanine Di-Phosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine-nucleotide Exchange Factor</td>
</tr>
<tr>
<td>GET</td>
<td>Golgi-to ER-Trafficking</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>Gγ</td>
<td>G protein gamma subunit</td>
</tr>
<tr>
<td>GIV</td>
<td>G alpha Interacting Vesicle protein</td>
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<tr>
<td>Gln or Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Gly or G</td>
<td>Glycine</td>
</tr>
<tr>
<td>GPA1</td>
<td>G Protein Alpha subunit</td>
</tr>
<tr>
<td>GPCR</td>
<td>G Protein Coupled Receptor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-Transferase</td>
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<tr>
<td>GTP</td>
<td>Guanine-Tri-Phosphate</td>
</tr>
<tr>
<td>IP</td>
<td>ImmunoPrecipitate</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo-Dalton</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Leu or L</td>
<td>Leucine</td>
</tr>
<tr>
<td>Lys or K</td>
<td>Lysine</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>Mitogen Activated Protein Kinase Kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen Activated Protein Kinase Kinase Kinase</td>
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<td>Met or M</td>
<td>Methionine</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MP</td>
<td>Mastoparan</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MYC</td>
<td>c-Myc epitope tag (EQKLISEEDL)</td>
</tr>
<tr>
<td>PAT</td>
<td>Protein Acyl-Transferase</td>
</tr>
<tr>
<td>PEP4</td>
<td>carboxy-PEPtidase Y-deficient</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase</td>
</tr>
<tr>
<td>REG1</td>
<td>REsistance to Glucose repression</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulator of G protein Signaling</td>
</tr>
<tr>
<td>Ric-8a</td>
<td>Resistance to Inhibitors of Cholinesterase</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces Cerevisiae (yeast)</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Ser or S</td>
<td>Serine</td>
</tr>
<tr>
<td>SST2</td>
<td>Super-SensiTive to pheromone</td>
</tr>
<tr>
<td>STE</td>
<td>STErile</td>
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<tr>
<td>TEV</td>
<td>Tobacco Etch Virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>-----------------------------</td>
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<tr>
<td>Thr or T</td>
<td>Threonine</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UBP</td>
<td>Ubiquitin Protease</td>
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<tr>
<td>VPS</td>
<td>Vacuolar Protein Sorting</td>
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CHAPTER I

Introduction

All Figures contributed by Michael J. Lee
The ability for an organism or cell to interpret and respond to changes in its immediate environment is one of the critical processes that allow for sustained life. Environmental cues are processed at the cellular level by a number of different signaling systems, but throughout biology, no system is more widely used than those coupled to heterotrimeric G proteins. The receptors of these systems alone encode over 1% of the human genome, and account for the single largest family of genes (55, 149). Heterotrimeric G proteins and their associated receptors respond to a wide array of signals, including neurotransmitters, hormones, tastes, odors and light (5, 20, 23). In accordance with the fact that G proteins regulate many aspects of human physiology, defects in these systems are known to contribute to a number of human diseases (51). To date, it has been estimated that approximately 30% of all drugs currently on market target GPCRs, and this number is increased to approximately 50% if we include signaling components targeted downstream or upstream of GPCRs (169). Thus, it is self-evident that a more complete understanding of G protein signaling will help in the identification of new drug targets and the generation of new drug therapeutics.

HETEROTRIMERIC G PROTEIN SIGNALING

Heterotrimeric G proteins are a biological signaling switch, which typically exist in a multi-protein complex at the cell periphery (61). Included in these systems is a seven-transmembrane spanning G protein coupled receptor (GPCR), a guanine nucleotide binding protein (G protein) alpha subunit (Gα), and the obligate dimer comprised of Gβ and Gγ (often written as Gβγ). Signaling through these systems is controlled by the differential binding of GDP/GTP by Gα, and the signaling cycle can be considered as four distinct steps.
(Figure 1.1). In the first step, the basal state, Gα is bound to GDP, is considered inactive, and in this conformation also interacts with Gβγ. Ligand binding to the GPCR (step 2) causes the receptor to function as a guanine nucleotide exchange factor (GEF). The active receptor stabilizes the nucleotide-free form of Gα, allowing for release of GDP, which is replaced with the more abundant molecule GTP. Nucleotide release is the rate-limiting step in the signaling cycle (53). GTP-bound Gα is considered active, and no longer interacts with Gβγ. Both entities are then free to regulate downstream signals (step 3). The Gα subunit is a GTP hydrolase, allowing for inactivation of the pathway (step 4). Hydrolysis is often aided by members of the regulator of G protein signaling (RGS) family of proteins (121, 133), which function as GTPase accelerating proteins (GAPs) by stabilizing the transition state of hydrolysis (13). Once GTP is hydrolyzed to GDP, the inactive Gα-Gβγ heterotrimer is reformed.

This standard model, in its basic tenets, has remained largely unchanged for decades. This paradigm, however, fails to take into account recent findings that G proteins are not restricted to the cell periphery. A number of reports suggest that Gα subunits in particular exist at, and even signal at intracellular locations (7, 136, 146, 157, 175). These findings open up the possibility that Gα subunits regulate novel signaling pathways, away from the cell periphery, and potentially independent of the traditional “Gα-Gβγ-GPCR” paradigm. This thesis will focus on two aspects of Gα biology that are related to novel functions of intracellular Gα subunits: Gα activation and Gα trafficking. In this introductory chapter, special attention will be paid to the yeast Gα subunit Gpa1, the focus of this thesis.

*G alpha subunits.* Gα subunits are part of a larger superfamily of GTPases, which also includes “small” G proteins such as Ras and its homologs (141). All proteins in this
superfamily contain a conserved structural motif, the Ras-like domain, suggesting a common evolutionary origin of these proteins. Heterotrimeric (or “large”) G proteins contain two distinct domains: the Ras-like domain that is conserved among all GTPases, and an independently folded six-helix bundle, often referred to as the all-helical domain (170). Based on the resolved crystal structures of GDP and GTP bound $G_\alpha$ subunits, it is clear that activation results in movement in three flexible loops, called switch regions (designated I-III). For small GTPases, it is known that GEFs function by making direct contact with these switch regions (141). Although similar movements must occur in heterotrimeric G proteins, it is not clear how receptors (or non-receptor GEFs) are able to cause these rearrangements. This continues to be one of the unresolved mysteries in heterotrimeric G protein biology.

MODEL SYSTEMS FOR STUDYING G PROTEIN SIGNALING: THE YEAST PHEROMONE-INDUCED MATING RESPONSE

Because of the complexity of G protein mediated signal networks in mammalian cells, the G protein field has benefited greatly from studies performed in simpler eukaryotic organisms such as $C.\ elegans$ and $S.\ cerevisiae$. Yeast in particular has been an invaluable resource because of: 1) the advantages of genetically manipulating an organism that can exist stably as a haploid or diploid, 2) the efficiency of homologous recombination in yeast, 3) the existence of a fully sequenced genome, and 4) the availability of a number of genomic tools such as knock out libraries, GFP-tagged libraries, and epitope-tagged libraries.

With regard to heterotrimeric G protein signaling, the yeast system has been very well characterized (Figure 1.2) (41). In yeast, G proteins control the fusion of two haploid cells to create a diploid cell. Yeast can exist as haploid cells of either the $a$- or $\alpha$- mating type and
secrete mating pheromones, a-factor and α-factor, respectively. Binding of pheromone to receptors on adjacent cells induces growth arrest in G1, polarized growth towards the mating partner, and new gene transcription in preparation for cell fusion. Most of the components of this signaling cascade were identified genetically as deletions that resulted in a mating-deficient (sterile) phenotype. Included are genes that encode a GPCR (STE2), Gβ and Gγ subunit (STE4 and STE18), but not a Gα. Deletion of Gpa1, the Gα of the mating pathway, does not result in a sterile phenotype, but rather a constitutively active signal, due to the uncontrolled activation of Gβγ (37, 105). Subsequent data showing that Gβγ was both necessary and sufficient to activate the mating signal left the conclusion that Gα played no active role in this process. Recent evidence, however, suggests that Gpa1 may play an active roll in signaling. Investigating this possibility is a central focus of this thesis.

HETEROTRIMERIC G ALPHA SUBUNIT: ACTIVATION BY RECEPTORS AND NON-RECEPTOR EXCHANGE FACTORS

Much of the recent focus in heterotrimeric G protein research has been on the activation of the Gα subunit. G protein activation is the least understood of the various stages of G protein signaling (78). This lack of understanding stems, in large part, from the absence of a resolved crystal structure of either the empty Gα subunit or Gα/Gβγ in complex with a GPCR. The main barriers are the inherent instability of nucleotide-free Gα subunits (53), and the difficulty in crystallizing GPCRs alone, let alone in complex with other proteins.
Heterotrimeric G proteins

Figure 1.1
Figure 1.1 The heterotrimeric G protein signaling cycle. G protein coupled receptors bind extracellular ligands, and transmit signals to intracellular G proteins. Ligand binding causes the Gα subunit to exchange GDP for GTP. GTP-bound Gα no longer interacts with Gβγ, and both entities are free to interact with downstream effector proteins. Gα regulates the duration of the signal because Gα is a GTPase, an activity that can be aided by GAPs such as RGS proteins.
**Figure 1.2** The yeast mating response pathway. In yeast, mating is controlled by a typical heterotrimeric G protein coupled system. Gpa1 interaction with Ste4/Ste18, and activation of a MAPK effector signaling cascade, is regulated by pheromone binding to the receptor Ste2. Downstream MAPKs are activated directly by Ste4/Ste18. Gpa1 was thought to play only a regulatory role in signaling; however, we now know Gpa1 activates Vps34, and endosomal PI3K. The mechanism of Vps34 activation is currently unknown.
**Receptor-mediated activation**

The central mystery with regard to receptor-mediated activation of G proteins is that all modeled structures predict a distance of at least 30 Å between receptor-Go contact sites and the Go switch regions (78). This distance, which is predicted to be too large to allow for direct contact of switch regions by the receptor, suggests that heterotrimeric Go subunits and small G proteins do not share a common mechanism of activation. Various models have been proposed to account for this discrepancy, either proposing usage of Gβγ as a “lever,” or proposing allosteric modulation of switches through the C-terminal α5 helix of Go (114, 119). Evidence exists in favor of both of these mechanisms, and recent crystal structures have suggested the possibility that both models could be correct (78, 79). Further complicating the mystery of activation, however, is the fact that GPCRs are not the only proteins that are known to activate Go subunits. Although a handful of non-receptor GEFs have been characterized, a sequence, domain, or structural motif has not been identified to account for the similar functions of seemingly dissimilar GEFs.

**Activation by non-receptor exchange factors**

*Mastoparan.* The standard—and without question most common—mechanism of heterotrimeric G protein activation is through agonist activated GPCRs. Although the notion that G proteins could be activated by non-receptor GEFs is currently being revisited (65), it was first addressed 20 years ago by a number of groups—notably by Elliott Ross and colleagues—when they discovered the non-receptor GEF mastoparan. The critical connection came in 1988 when it was identified that wasp venom mastoparan (MP) mediated histamine release through mobilization of Ca++ and production of the second messenger
inositol-1,4,5-triphosphate (IP$_3$) (113). This fact did not go unnoticed by Ross and colleagues, as this process was already known to be G protein mediated (61). Work by Tsutomu Higashijima (while in the Ross lab) identified that, like GPCRs, MP bound directly to the G protein, accelerated guanine nucleotide exchange, did not alter hydrolysis of bound GTP (single-turnover), was affected by Mg$^{++}$ concentration, and was blocked by pertussis toxin-catalyzed ADP-ribosylation of G$\alpha$ (72, 73). Subsequent studies identified that MP is a cationic amphipathic helix, as are neuropeptide substance P and a synthetic polyamine compound 48/80. Both substance P and 48/80 were subsequently identified to be GEFs for G$\alpha$ subunits (106). Based on the characteristics shared among these peptide GEFs, as well as the activity they share with receptors, an analogy was drawn to the intracellular loops of GPCRs. Ross and others began the argument that mastoparan and the other peptide GEFs were true “receptor mimics,” suggesting that cationic amphipathic structure must form in one or more of the intracellular loops of the receptor (72).

A number of facts have since arisen that call into question the idea that MP is a true receptor mimic. For example, in the initial discovery, it was identified that while potent, MP was not at all selective amongst G$\alpha$ subtypes. Although not completely incongruent with being a G$\alpha$ GEF, this is certainly different than what is typically seen for receptors. Also, binding studies show that MP makes contacts near the extreme N-terminus of G$\alpha$, a region thought to be important for G$\beta\gamma$ binding but not for receptor interaction. It is not inconceivable that a GEF could bind at the N-terminus and simultaneously contact critical residues in the C-terminus of G$\alpha$, but this too was nonetheless different from receptors. Another critical difference was the dependence on G$\beta\gamma$ for activity. Studies from Bernard Fung have shown that GPCRs absolutely require G$\beta\gamma$ to function as GEFs (56, 57). MP was
found to interact with and activate monomeric Gα subunits in the absence of Gβγ, and both the Gα binding and the GEF activity was enhanced by the presence of Gβγ. This subtle difference in Gβγ dependence likely suggests that GPCRs and MP activate Gα subunits using different mechanisms.

Two decades after the initial discovery that mastoparan was a Gα GEF, very little has been done to develop this idea. MP is a wasp venom known to be toxic to cells; however, research focused on MP as a GEF has suffered from questions about whether the GEF function was responsible for the toxicity. Some data exists to suggest that MP toxicity does not depend on G protein signaling (81). In addition, even amongst those that consider the in vivo target to be Gα subunits, there is debate about the mechanism of action. Some reports suggest that MP binds to and activates nucleoside diphosphate kinases, allowing the conversion of GDP-GTP (82), and others suggest that MP toxicity results only from membrane disruption (91). To date, it is far from a certainty that MP and other cationic, amphiphilic peptides are truly receptor mimics in vivo, however, their biochemical function in vitro suggests that these peptides could still be valuable tools for studying Gα subunits.

**Activation by synthetic peptides.** It was recognized early on that the ability to crystallize GPCRs would likely be the limiting step in attempts to understand the G protein activation process. Even from the early studies on mastoparan, attempts were made to draw comparisons between MP and GPCRs. Subsequently, a number of groups created synthetic peptides as a means of better understanding receptor-mediated activation. Prominent examples included MP-S and Galparin, both of which were designed from MP (85, 148). By the early 90’s Neubig and others began using receptor-derived peptides as tools for studying Gα activation (152). The fact that some of these peptides retained GEF function gave hope
that studying peptides could give insight into the mechanism of receptor-mediated activation. Recently, Siderovski and colleagues used a combination of peptides derived from receptors and peptides identified using phage display to provide the first structural evidence for how receptors catalyze GDP release (79, 80).

**Non-receptor activation—Ric-8a.** Approximately five years ago, research on receptor-independent activation of Gα subunits was reinvigorated with the discovery of the non-receptor GEF Ric-8a. This protein was identified by Kenneth Miller in a genetic screen in *C. elegans*, designed to identify mutations that suppressed the neurotoxic effects of acetylcholinesterase inhibitors (102). The 21 genes identified in this screen were named *RIC*, for *resistance to inhibitors of cholinesterase*. The connection to Gα came seven years later, when Al Gilman and colleagues performed a yeast-2-hybrid screen intending to identify novel G protein signaling factors. Using Gαo and Gαs as bait, they identified the mammalian homolog of Ric-8a (151). The first clue to the function of this novel regulator was the binding profile; authors found that Ric-8a bound to a subset of Gα subunits in a GDP selective manner. The critical discovery, however, was that Ric-8a formed a stable complex only with nucleotide-free Gα. This was determined using radiolabelled nucleotides and purified proteins separated by size exclusion chromatography. Considering that GEFs are known to stabilize the nucleotide-free form of G proteins, this data all but assured that Ric-8a was a GEF. Using various *in vitro* assays to measure the rate of exchange, Tall et al. found that Ric-8a was a GEF for Gαi, o, and q but not Gs.

One of the subtle discoveries in Tall and Gilman’s initial report was that the GEF activity of Ric-8a is *inhibited* by Gβγ. As mentioned previously, GPCRs are known to *require* Gβγ for activity, while peptide GEFs like mastoparan are *enhanced* by Gβγ. The
implication of this finding is that Ric-8a is not a “receptor mimic” but rather a new type of GEF. Furthermore, this finding raises the possibility that Gα could function in a signaling system that was completely independent from the traditional Gα-βγ-GPCR paradigm.

By autumn of 2004, the Ric-8a story reached new heights. In a period of one month, at least five articles were published detailing that during asymmetric cell divisions, Gα proteins regulate differential microtubule force generation in a process that requires Ric-8a (1, 30), RGS proteins (69, 99), GoLoco domain containing proteins like LGN (43), microtubule binding protein NuMA (43), and of course a Gα subunit. Comparisons between these publications revealed a mechanism that is conserved between C. elegans, Drosophila, and even mammals (65, 167). These findings, which in many ways depart from the established G protein paradigms, marked the beginning of an exciting new time in Gα research.

Non-receptor activation—life after Ric-8a. In the four years since the Ric-8a discovery, the focus has shifted to determining: 1) how (or maybe if) Ric-8a fits into the traditional paradigm of G protein signaling, and also, 2) how prevalent are non-receptor GEFs like Ric-8a. Addressing the first of these questions, the prevailing theory is that Ric-8a activation requires prior stimulation of Gα by another GEF or in some instances possibly a GPCR. This model seems likely given the inability of Ric-8a to activate G protein heterotrimeric in vitro. In this model, activated Gα is stabilized in its “Gβγ-free” form and presented to Ric-8a by a guanine nucleotide dissociation inhibitor (GDI). Indeed, some evidence exists in mammalian cells for the modulation of known GPCR-dependent signals by Ric-8a (95). These data are consistent with Ric-8a functioning as a GEF in vivo, even in pathways that contain a known receptor. One question with this model is why another round
of nucleotide exchange would be necessary considering that \( G\alpha \) is already liberated and free to signal. Of course, one explanation is that activation by Ric-8a alters the timing, localization or duration of the signal. Some, however, have questioned the notion that promoting nucleotide exchange is truly the function of Ric-8a. A series of reports have identified that Ric-8a is required for cortical localization of \( G\alpha \) subunits (32, 64, 160), and some have postulated that membrane targeting, not \( G \) protein activation, is the function of Ric-8a and its homologues. Another possibility is that Ric-8a may be an important regulator of both the localization and activation of \( G\alpha \) subunits. This question may remain unanswered until mutants are developed that maintain \( G\alpha \) binding function but have lost GEF activity in vitro.

The second question that has arisen from the Ric-8a findings is how prevalent are non-receptor GEFs. To this end, many have performed screens with the intent of identifying novel activators. Baranski and colleagues used a screening approach in yeast to identify non-receptor activators of heterotrimeric \( G \) proteins in a human adipocyte cDNA library (63), and Lanier and colleagues performed a similar functional screen using an NG108-15 cDNA library (150). Both screens utilized the yeast pheromone response pathway, in cells that lack a receptor. This is possible because of the high degree of conservation among the various \( G \) protein subunits between yeast and mammals. In the latter screen, a handful of proteins were identified and renamed as activators of \( G \) protein signaling (AGS). It is worth noting that these proteins were named due to functional similarity and do not comprise a protein “family,” in the sense that AGS proteins are not evolutionarily or structurally related proteins. Also, the term AGS does not connote a mechanism. AGS proteins are divided into three categories depending on the proposed mechanism of action: Group 1) nucleotide
exchange dependent mechanisms (i.e. functioning as GEFs), Group 2) exchange-independent mechanisms (i.e. functioning as GDIs), or Group 3) direct interaction with Gβγ (15). Only one protein—the Ras-related small G protein Dexras/AGS1—has been proposed to be a GEF, and for the most part, more data is necessary in order to determine the extent to which AGS proteins function as G protein activators in vivo.

Thus far, the functional screens involving the yeast mating system (described in detail below) have utilized the yeast signaling backbone, but have not addressed the existence of endogenous non-receptor activators. Considering the high degree of conservation between signaling components in yeast and in higher organisms, the existence of a non-receptor activator in yeast would imply a greater relevance of these proteins than is currently understood. Furthermore, recent studies in the yeast pheromone response have identified that the Gα protein Gpa1 is involved in regulating multiple signaling pathways, with only one known GEF (135). It is unclear whether both Gpa1-mediated signals are regulated by the same GEF, or if other non-receptor GEFs exist in yeast. These data will be revisited in greater detail in the next section of this introduction. As a part of this thesis, we screened for non-receptor GEFs. Rather than using a structural criterion (as was commonly done when identifying GPCRs) or a purely functional criterion (as was done in previous yeast-based screens), our screen was designed around the physical binding profile exhibited by most GEFs. The results of the screen as well as the characterization of the proteins are detailed in Chapter IV of this thesis as well as in the future directions, Chapter V.
TRAFFICKING: POST-TRANSLATIONAL MODIFICATIONS AND IMPLICATIONS ON SIGNALING

It is self-evident that proper signaling requires proper protein trafficking. Historically, however, trafficking was rarely considered a true signaling step, because of a lack of evidence that the trafficking of proteins was modulated in a signal-dependent fashion akin to G protein activation or protein phosphorylation. Recently, many have shown that signals can be amplified from, or sometimes even initiated from intracellular sites (153). This type of regulation allows for more refined spatiotemporal signals, and it has been proposed that “coincidence detection” on endomembranes would allow for reduced signal crosstalk and increased specificity (118). Two prominent examples are the trafficking of TGF-β and EGFR. Both receptors are trafficked in multiple mechanisms (clatherin-mediated and raft/caveolar-mediated), with different trafficking patterns resulting in the activation of different pathways (36). Another well-publicized example is the discovery by Mark Phillips and colleagues that Ha-Ras is activated specifically at Golgi membranes (14), a finding that was likened to “life on Mars, cellularly speaking.” (35). Similar to the reports cited above, evidence exists for direct GPCR-mediated signals from intracellular locations. One specific example is the recruitment of mitogen activated protein kinases (MAPKs) to the endosome by β-arrestin (93). In this report, Lefkowitz and colleagues found that β-arrestin functions as a MAPK scaffold, directing signaling to subcellular compartments. These findings have forced the signaling community to begin thinking about signaling more three-dimensionally. The remainder of this chapter will focus on the current state of our understanding of intracellular signaling, as well as how post-translational modifications regulate protein
localization. The focus will be primarily on Gα proteins and primarily on modifications that are regulated during the course of signaling.

**Intracellular signaling by Gα**

_Translocation of Gα._ Gα subunits have long been known to exist at intracellular locations. Over the past 20 years, it has become increasingly evident that Gα subunits are present at Golgi apparatus (49), endoplasmic reticulum (7), secretory granules (157), endosomes (2), and even the nucleus (31). In these early examples, Gα appeared to be present alone, without a receptor or other components of the heterotrimer. Thus, it was unclear how—or if—Gα is a functional signaling protein at these varied intracellular locations. One recent piece of evidence in favor of Gα signaling from intracellular sites is the stimulus-dependent translocation of Gα and Gβγ subunits to endomembranes. Gautam and colleagues have used a variety of live cell imaging techniques to visualize the dynamics of G protein localization. Using a combination of FRET and FRAP analysis of fluorescently tagged G proteins, they found that G proteins shuttle rapidly between the plasma membrane and intracellular endomembranes (25). The kinetics of this translocation suggested that the movement was not vesicle mediated, but rather diffusion based. Furthermore, the translocation was dependent on palmitoylation, potentially foreshadowing the use of this lipid modification as a regulated signaling switch (this idea will be further discussed later in this chapter, as well as in Chapter III and Chapter V of this thesis). Finally using Gα-Gβγ FRET, the authors found heterotrimer interaction at both the plasma membrane and at the Golgi. This suggests the existence of a functional signaling complex at an intracellular
location. Without question, the phenomenon of intracellular signaling by G protein heterotrimeric needs to be more rigorously addressed before these results are widely accepted.

*Gα effectors at intracellular sites.* One of the pieces missing from the work by Gautam and colleagues is the identification of an actual signal or a unique intracellular effector protein. Others, however, have found Gα binding proteins that exist specifically at intracellular sites. Of particular note are a series of findings by Marilyn Farquhar’s group. Farquhar and colleagues performed a yeast-2-hybrid screen to identify Gαi binding partners, and have identified a number of interesting interactions. Their findings included GIV (G alpha Interacting Vesicle associate protein), a protein localized to ER-Golgi transport vesicles (86), and calnuc, a homolog of calreticulin that is associated with Golgi (90). The discovery of calnuc is particularly interesting as this is the only protein to date that is known to simultaneously associate with mobilized calcium and Gα subunits. In both cases, these discoveries are in their most preliminary stages, and it is too early to gauge their full impact.

*Endosomal signaling by Gα.* Perhaps the best evidence for intracellular signaling by a Gα subunit is a discovery by Slessareva et al, detailing signaling at the endosome by the yeast Gα, Gpa1 (136). In yeast, the belief was that Gβγ produced the active signal, while Gα only served to regulate Gβγ. Surprisingly, however, the authors identified that a constitutively active Gα could activate some, but not all, aspects of the pathway. In an effort to identify components involved in signaling by Gpa1, they systematically screened deletions of all non-essential genes in yeast, looking for those that blocked signaling. Two of the seven genes identified were Vps15 and Vps34, the regulatory and catalytic subunits of sole PI3K in yeast. What made this discovery even more surprising is the fact that Vps15/34 are believed to exist only at endosomes, where they control protein trafficking (129, 142). The
authors went on to find that activated Gpa1 localizes to endosomes, binds directly to Vps34, and activates the kinase activity of Vps34. Another critical finding in this work was that the inactive form of Gpa1 bound directly to the regulatory subunit Vps15. This was made all the more interesting because Vps15 shares some structural characteristics with Gβ proteins, in that both are predicted to form seven-bladed propeller structures, and both bind selectively to the GDP-bound form of Gα. The modeled structure of Vps15 suggests that it lacks critical residues necessary for interaction with Gα in the manner of typical Gβ subunits; thus, the question of whether Vps15 is truly functioning as a “Gβ” remains unanswered.

Post-translational modifications of Gα subunits

Gα proteins are peripheral membrane associated proteins. They attach to lipid bilayers through the use of post-translational N-terminal lipid-acylation (24). The pattern of lipidation differs depending on subtype: for example, Gαs members are palmitoylated; Gαi members are both myristoylated and palmitoylated; Gαi is only myristoylated; and Gαq members are palmitoylated and some are also thought to use an N-terminal poly-basic stretch in addition to lipidation (166). In general, Gα subunits adhere to the “two signal hypothesis,” which suggests that a protein requires two membrane localization signals to associate to the plasma membrane. Evidence in support of this principle is that Gαi, whose membrane signals are myristoylation and Gβγ binding, dissociates from the plasma membrane upon GTP binding (120).

Proper protein localization is a necessary precursor to proper protein function. As evidence grows for protein translocation and re-localization in the course of signaling, it is becoming increasingly clear that post-translational regulation is a key factor. This regulation
can take the shape of phosphorylation, ubiquitination, or reversible lipid-acylation, and much of the work presented in this thesis will test the hypothesis that diverse combinations of modifications may account for different subcellular localization of various Gα subunits. Here, we will review what is known for post-translational regulation of Gα subunits. Because Gα subunits vary so widely in the precise details of their regulation, we will focus on the yeast Gαi homolog, Gpa1.

**Ubiquitination.** Ubiquitination is the covalent attachment of an 8.5 kilo-Dalton (kDa) protein called ubiquitin (68). This modification occurs on lysine residues and generally marks a protein for degradation. In the canonical method, the ubiquitin signal is actually that of a poly-ubiquitin chain, which is added to the substrate protein through a three step process involving a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3). Substrate recognition generally occurs at the level of the E3, and there are usually hundreds of E3’s in mammalian cells. Poly-ubiquitination occurs either through the sequential addition of 4 or more ubiquitin monomers to the substrate (creating a ubiquitin chain) or the direct transfer of a poly-ubiquitin chain from the E2 to the substrate (with the aid of an E3). In both cases, poly-ubiquitination typically results in the destruction of a protein by the 26S proteasome. Another, less common form of ubiquitination is mono-ubiquitination. As the name suggests, this differs from the canonical modification in that only a single ubiquitin monomer is added to the substrate. This modification was first demonstrated to occur for histones, and later on the yeast pheromone receptor Sterile-2 (Ste2) (71). In this report, Hicke et al identified that Ste2 was mono-ubiquitinated in response to stimulus and trafficked to the vacuole (the yeast lysosome) to be destroyed. Although poly-ubiquitination and Ste2 mono-ubiquitination both result in destruction, there is evidence for
ubiquitin-mediated signal activation. Work by James Chen and colleagues has uncovered that poly-ubiquitination of TRAF6 is necessary for TAK1-TAB-TRAF6 complex formation, IKK phosphorylation, and NF-kB signal activation (34). The key observation of Chen’s group was that ubiquitination of TRAF6 is through Lys-63 (on ubiquitin) rather than the traditional Lys-48. Other examples have since emerged—including work by Bill Tansey’s group detailing the role of ubiquitination in transcriptional activation (127)—showing that ubiquitination can be involved in signal activation as well as signal desensitization.

Gpa1 was recently found to be ubiquitinated (94, 98). Marotti et al recognized that in a proteasomal protease-deficient yeast strain, Gpa1 accumulated high molecular weight species. When separated using SDS-PAGE and probed for Gpa1, the characteristic poly-ubiquitin associated “laddering” was visible. Using a mass-spectrometry based approach, they identified that Gpa1 is specifically ubiquitinated on K165. The initial report did not address the outcome of Gpa1-ubiquitination, so it remains to be seen whether this modification is being used for protein turnover, or more directly for signaling. Gpa1 was the first Gα known to be ubiquitinated, although there is also a report that Gαs is modified by ubiquitin (109). Furthermore, alignments of Gα subunits show sequence conservation of the region around the ubiquitinated residue on Gpa1, including K165 (145). Thus, it is possible that other Gα subunits are also modified in this manner.

Myristoylation. Myristoylation is the addition of a saturated 14-carbon fatty acid (C14:0) to a glycine residue at amino acid position two of the substrate protein (consensus sequence is always M-G-X-X-(S/T)-X-X-X, with “M” being the translated ATG start codon, which is subsequently cleaved off) (17). Myristoylation is believed to be cotranslational and irreversible. Myristoylation is also unique in that not all myristoylated
proteins are membrane bound (Figure 1.3) (97). This raises the possibility that myristate may not simply be inserting into lipid bilayers, as is commonly thought (166). Regardless of the mechanism of action, myristoylation of Go subunits is known to promote their membrane attachment (96). In accordance with the “2 signal hypothesis” it has been found that the hydrophobicity of myristate is not sufficient for stable association with the plasma membrane. The working theory in the field is that myristoylation of Go delivers the protein to the plasma membrane where palmitoylation can occur (presumably because of the existence of specifically localized protein acyl-transferases, or PATs). Gpa1 is known to exist as both myristoylated and unmyristoylated protein (144). Myristoylation of Gpa1 is regulated, in that it is increased upon pheromone treatment (40). Given the understanding, however, that the modification must be co-translational, it is likely pheromone-stimulated myristoylation occurs during Gpa1 production, and not subsequently as a direct modification of unmyristoylated protein.

Palmitoylation. Palmitoylation is the attachment of the fatty acid palmitate (C16:0) to a cysteine residue using a thioester bond (137). Compared to myristoylation, palmitoylation results in a much more stable association with lipid membranes and can occur post-translationally; however, the mechanism by which palmitoylation occurs is not nearly as well understood. The first clues came from the lab of Bob Deschenes, who screened yeast for genes involved in the palmitoylation of yeast Ras (11). Shortly thereafter, Nick Davis’ group identified the yeast gene Akr1 as having a role in the palmitoylation of type I casein kinases (52). It quickly became apparent that Erf2 and Akr1 share a common element: a conserved 50 residue, cysteine rich DHHC-CRD domain. To date, all identified PATs (only 7 have been identified to date) contain this DHHC motif (104). Determining how
palmitoylation occurs and identifying novel PATs is still an active area of research. Many Gα subunits are palmitoylated, including Gpa1, although it is unknown which PATs are responsible for this modification.

Signaling researchers have focused on palmitoylation because evidence exists to suggest it is a reversible—and thus a regulatable—modification. The first evidence for this came from studies on Gαs. Various researchers found that the turnover of Gαs palmitoylation was dramatically accelerated by receptor activation. Palmitate turnover on the inactive G protein exhibited a half-life of approximately 90 minutes, however, β-adrenergic receptor activation resulted in palmitate turnover with a t1/2 of ~ 2 minutes (165). Furthermore, mutationally activated G protein exhibited a turnover rate similar to that of receptor activated G protein (107). These data raise the possibility that depalmitoylation is involved in signal desensitization or potentially in G protein translocation. More evidence for this came from Gilman and colleagues, who identified a family of conserved Gα-specific de-palmitoylating enzymes (45). Although the in vitro biochemistry characterizing these enzymes is very compelling, it is still unclear what role these proteins play in vivo. One of these family members, Apt1, exists in yeast (44), although it too has not been implicated in any G protein-specific pathway. Determining if Apt1 contributes to the regulation of Gpa1 will be addressed in Chapter V of this thesis.
Figure 1.3

Potential roles of myristate. Like other lipid modifications, myristoylation generally causes membrane association. However, the hydophobicity of myristate is below the threshold necessary for stable membrane binding, and there is some question regarding the mechanism of myristate-dependent membrane attachment. Possible roles for myristoylation include (A) direct insertion into the lipid bilayer, (B) direct interaction with a transmembrane protein, (C) myristate-dependent protein-protein interaction, (D) myristate-dependent conformational change of the substrate protein.
THESIS SUMMARY

When research for this thesis began, intracellular signaling by Gα subunits was just beginning to be understood. It had already been established that Gα subunits existed at many intracellular locations, but lacking was any evidence that these alternatively localized proteins were relevant as signaling proteins. Borrowing from the precedent set by other signaling molecules—small GTPases, receptor and non-receptor tyrosine kinases—many, including our group, began to address the function of Gα subunits away from the plasma membrane. The major unanswered questions were very basic in nature: do signals emanate from non-plasma membrane sites? How do G proteins traffick to various cellular locations? How are intracellular G proteins activated? Using the very basic yeast mating system, our lab determined that the answer the first of these questions, finding that activated Gpa1 binds to and activates an endosomal PI3K (136). For my thesis research, I intended to answer the latter two of the basic questions, specifically I set out too identify other activators of the yeast mating pathway, and determining what signals Gpa1 uses to localize at the plasma membrane and at intracellular locations.

The remainder of this thesis will be presented as four chapters. In Chapter II, “Determinants of Gpa1 poly- versus mono-ubiquitination,” I will present work performed by myself as well as others from the Dohlman lab, in which we: 1) detail the discovery that Gpa1 is both poly- and mono-ubiquitinated, 2) test the hypothesis that poly-ubiquitination is reserved for misfolded protein, and 3) determine that myristoylation is necessary for Gpa1 mono-ubiquitination. One of the critical components in this chapter is the creation and validation of a mutant form of Gpa1 that is not myristoylated but nonetheless localizes correctly to the plasma membrane. In Chapter III, “Development of a Cell Viability Assay
to Monitor Palmitoylation of Gpal," I will describe the creation of a novel mutant form of Gpal that facilitates the screening of Gpal palmitoylation. In addition, this chapter will contain a validation of the screening method, as well as preliminary data detailing attempts to determine how Gpal palmitoylation is regulated. In Chapter IV: "Identification and characterization of a non-receptor exchange factor for yeast Ga subunit, Gpal," I will describe a screen directed at identifying candidate activators of Gpal, as well as the characterization of a novel GEF, Arr4. Finally, in Chapter V, "Conclusions and Future Directions," I will discuss the big picture findings associated with this thesis, as well as preliminary data associated with future directions for each of the chapters.
CHAPTER II

Determinants of Mono- versus Poly-Ubiquitination of Gpa1

Elements of the work referenced in this chapter has been published in:


Figure contributed by:

Michael Lee: 2.1A; 2.2; 2.3C; 2.4A; 2.7-2.11
Matthew Torres: 2.5; 2.6; 2.12; 2.13
Yuqi Wang: 2.1B; 2.3A, B; 2.4B, C
Feng Ding: Table 2.1
Summary

Previously we used mass spectrometry to show that the yeast G protein α subunit Gpa1 is ubiquitinated at Lys-165, located within a sub-domain not fully conserved with other Gα proteins. Here we describe the functional role of Gpa1 ubiquitination. We find that Gpa1 is both mono- and poly-ubiquitinated. Proteasomal protease-defective *rpt6/cim3* mutants accumulate poly-ubiquitinated Gpa1, and much of the protein exhibits cytoplasmic localization. In contrast, vacuolar protease *pep4* mutants accumulate mono-ubiquitinated Gpa1, and in this case the protein is localized within the vacuolar compartment. Cells that lack *Ubp12* ubiquitin-processing protease activity accumulate both mono- and poly-ubiquitinated forms of Gpa1. In this case, Gpa1 accumulates in both the cytoplasm and vacuole. All of these ubiquitin-mediated phenotypes are blocked by a Gpa1<sup>Δ128-236</sup>, a mutant lacking the ubiquitinated sub-domain. We show that poly-ubiquitination specifically clears misfolded protein, whereas mono-ubiquitination regulates plasma membrane targeted, fully mature Gpa1. Mutations that decrease the fold stability of Gpa1 result in a corresponding increase the poly-ubiquitination, but do not alter mono-ubiquitination. Mutations that decrease myristoylation but not plasma membrane targeting diminish mono-ubiquitination but do not alter poly-ubiquitination, suggesting a direct role of myristate in the recognition of Gpa1 by the mono-ubiquitination machinery. Findings reported herein describe the ubiquitin-mediated regulation of Gpa1, and provide evidence for multiple non-redundant mechanisms for ubiquitin-mediated degradation.
INTRODUCTION

Many sensory and chemical stimuli act via cell surface receptors and intracellular G proteins. In yeast, G protein-coupled receptors initiate a signaling cascade that leads to morphological changes, new gene transcription, cell cycle arrest, and eventually mating. Mating, the process by which a and α haploid cells fuse to form the a/α diploid, is initiated by cell-type-specific peptide pheromones. Haploid a-type cells secrete a-factor pheromone, which binds to specific receptors found only on α-type cells, while α-type cells secrete α-factor that acts exclusively on a-cells (41).

Most components of the pheromone-signaling cascade in yeast have been identified through the isolation of gene mutations that produce an unresponsive sterile (ste) phenotype. Genes required for mating include the receptors for α-factor (STE2) and a-factor (STE3), the G protein β (STE4) and γ (STE18) subunits, G protein effectors (STE5, STE20, CDC24), downstream protein kinases (STE20, STE11, STE7, FUS3, KSS1), as well as a transcription factor (STE12). The G protein α subunit serves primarily to regulate the levels of free Gβγ. Cells lacking the Gα subunit gene (GPA1) cannot sequester Gβγ, and so are permanently activated (37, 105). The Gα subunit can also modulate signaling through direct interaction with the phosphatidyl-inositol-3-kinase (PI3K) Vps34 (136).

As with other G protein systems, pheromone binding to its receptor promotes the exchange of GTP for GDP on the Gα subunit, followed by dissociation of Gα from the Gβγ subunit complex (38). The dissociated subunits in turn transmit and amplify the signal to effector proteins that produce an intracellular response. Signaling persists until GTP is hydrolyzed to GDP and the subunits reassemble. Given their position as intermediaries between signal detectors (receptors) and signal transmitters (effectors), G proteins are well
positioned to serve as targets of regulation. Particularly important in this role is the RGS (“Regulator of G protein Signaling”) protein Sst2, which attenuates the pheromone signal by accelerating Gpa1 GTP hydrolysis and thereby reducing the lifetime of the activated G protein (4, 39).

There is growing evidence that G protein signaling components are also regulated by post-translational modifications (24). Recent attention has focused on ubiquitination, the process by which a ubiquitin polypeptide is covalently attached to specific target proteins (117). The typical ubiquitin signal is actually comprised of a chain of four or more ubiquitin monomers covalently linked through lysine-48. Poly-ubiquitinated substrates are then captured by the proteasome protease complex and rapidly degraded (159). Of the pheromone signaling components in yeast, ubiquitination has been reported for the Gα protein Gpa1 (94, 98), the RGS protein Sst2 (66), and the effector kinase Ste7 (162, 163). Ubiquitination of both Ste7 and Sst2 are induced by pheromone, and these modifications are thought to represent feedback loops leading to pheromone desensitization and resensitization, respectively (66, 162, 163).

Ubiquitination has also been described for the pheromone receptors Ste2 and Ste3 (71, 123). However in this instance the proteins are mono-ubiquitinated instead of poly-ubiquitinated, and degraded by the vacuole (the yeast counterpart to the lysosome) instead of by the proteasome (33, 71, 123, 128). This discovery led to the hypothesis that ubiquitination is primarily a protein trafficking signal, and mono-ubiquitination promotes degradation only indirectly by allowing the substrate to undergo internalization and delivery to the vacuole compartment (70).
Recently we used mass spectrometry to identify the ubiquitination site of Gpa1 in vivo (98). The site of modification (Lys-165) lies within the all-helical domain of the G protein, and more specifically within a 110-residue sub-domain not fully conserved in other G\textalpha subunits. Here we show that the Gpa1 sub-domain undergoes both mono- and poly-ubiquitination: mono-ubiquitinated Gpa1 is targeted to the vacuole while poly-ubiquitinated Gpa1 is delivered to the proteasome. Furthermore, we demonstrate that Ubp12 is a ubiquitin-processing enzyme that acts on both species of ubiquitinated Gpa1. Finally, we demonstrate that poly-ubiquitinated Gpa1 targets misfolded/unstable protein for destruction, whereas mono-ubiquitinated Gpa1 is a trafficking signal for fully mature plasma membrane localized Gpa1. While testing the latter of these two hypotheses, we uncovered that myristoylation is directly required for mono-ubiquitination of Gpa1. Our findings show for the first time that a single protein can be both mono- and poly-ubiquitinated, and we suggest a rationale for the existence of two separate ubiquitination systems.

RESULTS

Gpa1 is both mono- and poly-ubiquitinated in yeast

Many cellular proteins are poly-ubiquitinated and degraded by the proteasome. However, a handful of proteins are instead mono-ubiquitinated and delivered to the vacuole. This alternative pathway was first documented for the yeast G protein-coupled receptors Ste2 and Ste3 (33, 71, 123). Both of these receptors are mono-ubiquitinated in response to prolonged treatment with pheromone, and once modified they are rapidly endocytosed and degraded within the vacuole compartment (71, 123, 128). We and others have previously found Gpa1 to be mono-ubiquitinated (94, 98), however, the possibility that Gpa1 could also
be poly-ubiquitinated has never been addressed. To test this possibility, we monitored Gpa1 expression in a temperature sensitive \textit{cim3-1} strain, a yeast strain deficient for proteasomal activity (60) and \textit{pep4}Δ, a strain deficient for vacuolar protease activity(126). Cim3 (also known as Rpt6) is a subunit of the proteasome 19S regulatory particle, which confers specificity for ubiquitinated proteins for presentation to the proteasome proteolytic subunits (54), and Pep4, also known as Proteinase A, is a saccharopepsin aspartyl protease required for activation of degradative enzymes within the vacuole. Gpa1 normally migrates as a doublet of 54 kDa and 56 kDa (representing myristoylated and non-myristoylated species, respectively) (40, 144). As shown in Figure 2.1 A, steady state levels of Gpa1 were ~2-fold higher in the vacuolar protease-deficient \textit{pep4} mutant strain. Expression of Ste4 (Gβ) was also elevated in these mutants. Surprisingly, however, Gpa1 (but not Ste4) expression was also enriched in the \textit{cim3-1} mutant, suggesting that the Gα protein can also be degraded by the proteasome (Figure 2.1 A) (98). These findings suggest that Gpa1 can be degraded either by the vacuole or the proteasome, while Ste4 is degraded primarily by the vacuole.

Whereas degradation by the proteasome typically requires substrate poly-ubiquitination, vacuolar degradation of Ste2 is triggered by mono-ubiquitination (71). If Gpa1 is degraded by the vacuole, defects in this pathway should result in an accumulation of the mono-ubiquitinated form of the protein. Conversely, if Gpa1 is degraded by the proteasome, defects in that pathway should result in an accumulation of poly-ubiquitinated Gpa1. To test this we over-expressed Gpa1 in mutant strains deficient in either vacuolar protease or proteasome activity. Over-expression was necessary for this experiment in order to detect the minor ubiquitinated species. In wild-type cells, Gpa1 migrated at 54 and 56 kDa
Figure 2.1

A

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Figure 2.1 Gpa1 is poly- and mono-ubiquitinated in yeast. (A) Whole cell extracts were prepared from a vacuolar protease mutant (pep4::KanMX), a temperature-sensitive proteasomal protease defective mutant (cim3-1) grown at the restrictive temperature of 37 °C for 4 h, and isogenic wild-type strains. Samples were resolved by 7.5% SDS-PAGE and detected by immunoblotting with antibodies against Gpa1 (Gα), Ste4 (Gβ), or Pgk1 (loading control), as indicated. (B) left, A vacuolar protease-deficient (pep4::KanMX) mutant and the isogenic wild-type strain were transformed with a plasmid containing wild-type GPA1 (pAD4M-GPA1). Plasmid-borne over-expression of Gpa1 was required in order to detect the minor ubiquitinated species. Whole cell extracts were resolved by 7.5% SDS-PAGE and immunoblotting with anti-Gpa1 antibodies. center, A proteasomal protease defective (cim3-1) mutant and the isogenic wild-type strain were transformed with plasmid pAD4M-GPA1, grown at the restrictive temperature of 37 °C for 4 h, and analyzed by immunoblotting with anti-Gpa1 antibodies as described above. right, The same strains as in the left panel except lacking the α-factor receptor gene STE2. Arrows indicate the mobility of unmodified Gpa1, mono-ubiquitinated Gpa1 (“ubi-Gpa1”), and poly-ubiquitinated Gpa1 (“ubi^n-Gpa1”).
It was shown previously that the pheromone receptor accumulates in the vacuole of cells deficient in vacuolar protease activity (71, 128). To determine if Gpa1 is likewise delivered to the vacuole we compared the distribution of the protein (expressed as a chromosomally-integrated GFP fusion) in wild-type and pep4Δ mutant strains. As shown in Figure 2.2, Gpa1 in wild-type cells was detected at the plasma membrane. In contrast, Gpa1 in the pep4Δ mutant was visible at the plasma membrane and also within the large vacuolar compartment, which is easily identified as the most prominent organelle within the cell. Taken together these data indicate that Gpa1, like Ste2, is mono-ubiquitinated and degraded by the vacuole. Whereas the receptor is delivered to the vacuole upon pheromone stimulation, the G protein is delivered in a constitutive or pheromone-independent manner.

Most cytoplasmic proteins are poly-ubiquitinated and delivered to the proteasome complex. Thus we anticipated that poly-ubiquitinated Gpa1 would not be targeted to the vacuole. To test this prediction we examined the subcellular distribution of Gpa1-GFP in the proteasome-defective cim3-1 mutant strain, which accumulates the poly-ubiquitinated form of the substrate. As shown in Figure 2.2, Gpa1 in the cim3-1 mutant was predominantly localized in the cytoplasm, and in contrast to pep4Δ was largely excluded from the vacuole. We presume that the cytoplasmic staining represents Gpa1 associated with, or en route to, the proteasome complex. Taken together these data suggest that Gpa1 is delivered to the proteasome when poly-ubiquitinated but not when mono-ubiquitinated. Conversely, Gpa1 is delivered to the vacuole when mono-ubiquitinated but not when poly-ubiquitinated.
Figure 2.2  Ubiquitin-dependent trafficking of Gpa1. Vacuolar protease-deficient (pep4::KanMX), proteasomal protease-deficient (cim3-1), and the isogenic wild-type strains were transformed with an integrating plasmid (pRS406-GPA1-GFP) containing the native GPA1 promoter and gene fused to the gene encoding yeast-enhanced green fluorescent protein. Cells were grown to mid-log phase at 30°C and visualized by DIC and fluorescence microscopy. Cells expressing Gpa1 alone exhibited negligible background autofluorescence (data not shown).
Ubp12 is a ubiquitin-processing protease for Gpa1

We next aimed to determine how Gpa1 ubiquitination was regulated. Yeast have 16 ubiquitin-processing proteases (UBPs), otherwise known as de-ubiquitinating enzymes (DUBs), or ubiquitin-specific proteases (USPs). By screening mutants that lack each of 16 UBPs we previously had found one (ubp3Δ) that resulted in the accumulation of poly-ubiquitinated Ste7 (162, 163). We conducted a similar screen of the UBP deletion mutants and found another, ubp12Δ, that specifically accumulated ubiquitinated Gpa1 (Figure 2.3 A and data not shown). Moreover, immunoprecipitation of Ubp12 resulted in the copurification of Gpa1, further suggesting that Gpa1 ubiquitination is regulated directly by Ubp12 (Figure 2.3 B). To further corroborate these results, we also monitored Gpa1 expression and localization in the ubp12Δ background. To facilitate detection of ubiquitin (mono-ubiquitin in particular) we also over-expressed myc-tagged ubiquitin under the control of a copper-inducible promoter. Consistent with our previous observations, we found that the ubp12Δ caused accumulation of both mono- and poly-ubiquitinated Gpa1 (Figure 2.3 C). Also, in ubp12Δ cells—under conditions where Gpa1 is poly-ubiquitinated and mono-ubiquitinated in the same cell—the protein is directed to both the vacuolar compartment and the cytoplasm. Stated differently, the distribution of Gpa1 in this case is an amalgamation of the distribution seen in pep4Δ and cim3-1 cells.

Deletion of the ubiquitinated sub-domain eliminates Gpa1 ubiquitination

We then wished to determine the fate of Gpa1 that is not ubiquitinated. Initially we examined the localization of Gpa1K165R, which lacks the primary site of in vivo ubiquitination (98). However we found the subcellular distribution of Gpa1K165R was similar to that of the
Figure 2.3

A

Wild type  ubp12Δ  Wild type  ubp12Δ

−  −  +  +

Cu²⁺

ubi^n-Gpa1

ubi-Gpa1

Gpa1

B

Ubp12-FLAG

Vector

−  +  −

IP: Flag

WCE

IB: Gpa1

IB: Flag

IB: Gpa1

C

Wild type  ubp12Δ


Figure 2.3 Regulation of Gpa1 by Ubp12. (A) A ubiquitin-processing protease deficient (ubp12::KanMX) mutant and isogenic wild-type strain were co-transformed with plasmids containing Gpa1 (pAD4M-GPA1) and Myc-ubiquitin under the control of the copper-inducible CUP1 promoter (pND747). Mid-log phase cells were treated with 100 µM CuSO₄ for 4 h, as indicated, and then collected and resolved by 7.5% SDS-PAGE and immunoblotting using anti-Gpa1 antibodies. Arrows indicate the mobility of unmodified Gpa1, as well as the mono-ubiquitinated (“ubi-Gpa1”) and poly-ubiquitinated (“ubiⁿ-Gpa1”) species. (B) Wild-type cells were co-transformed with plasmids containing Gpa1 (pAD4M-GPA1) and Flag-epitope-tagged Ubp12 (pYES-UBP12-Flag) or the empty parent vector. Flag-Ubp12 was immunoprecipitated (“IP”) with M2 anti-Flag resin and the copurification of Gpa1 with Ubp12 was detected by immunoblotting (“IB”) with anti-Gpa1 and anti-Flag antibodies, respectively. An immunoblot of whole cell extracts (“WCE”) is also shown to confirm equal expression of Gpa1. (C) A ubiquitin-processing protease deficient (ubp12::KanMX) mutant and isogenic wild-type strain were transformed with an integrating plasmid (pRS406-GPA1-GFP) encoding Gpa1 fused to GFP and a plasmid containing Myc-ubiquitin under the control of the copper-inducible CUP1 promoter (pND747). Cells were treated with CuSO₄ for 4 h and visualized at mid-log phase by DIC and fluorescence microscopy. Cells expressing Gpa1 alone exhibited negligible background autofluorescence (data not shown).
In the vacuolar-protease deficient \textit{pep4}Δ strain an additional mono-ubiquitinated species migrated near 63 kDa (Figure 2.1 B, \textit{left}) (94, 98). In contrast, the proteasome-deficient \textit{cim3}-1 strain accumulated a ladder of high molecular weight bands representing poly-ubiquitinated Gpa1 (Figure 2.1 B, \textit{center}) (94, 98). These results support the suggestion that Gpa1 is degraded by two routes; one entails mono-ubiquitination and delivery to the vacuole while the second requires poly-ubiquitination and delivery to the proteasome.

**Mono-ubiquitinated Gpa1 trafficks to the vacuole, whereas poly-ubiquitinated Gpa1 is degraded by the proteasome**

The vacuolar degradation pathway is used turnover of the pheromone receptor and, as shown here, the G protein \(\alpha\) subunit Gpa1. Mono-ubiquitination of the receptor is enhanced by pheromone. Since the receptor and G protein bind to one another, we investigated whether mono-ubiquitination and/or degradation of both substrates occur in a coordinated manner. We first examined whether mono-ubiquitination of Gpa1 was affected by prolonged exposure to pheromone, and consistently found no difference (data not shown) (98). Second, we tested whether mono-ubiquitination of Gpa1 was affected by deletion of the receptor gene \textit{STE2}, and again found no difference (compare \textit{pep4}Δ and \textit{ste2}Δ/\textit{pep4}Δ mutants, Figure 2.1 B, \textit{right}). These data indicate that Gpa1 mono-ubiquitination as well as vacuolar sorting occurs independently of receptor binding or pheromone occupancy.
wild-type protein (data not shown). We presume that one or more secondary sites are modified when Lys-165 is altered, and this accounts for the residual ubiquitination as well as the unaltered subcellular localization of the mutant protein. We also reasoned that any secondary sites are likely to be clustered within the same 110-residue sub-domain that contains Lys-165. To test this possibility we constructed and analyzed a mutant form of the protein that lacks the ubiquitinated sub-domain altogether (Gpa1^{Δ128-236}). The position of the internal deletion was chosen based on a sequence alignment of Gpa1 with Gαi and Gαt, two mammalian proteins that lack the sub-domain and for which crystal structures are available (16, 141). We first compared the ubiquitination of wild type Gpa1 and Gpa1^{Δ128-236}. Using a Gpa1-specific antibody (as in Figure 2.1 B) we found that Gpa1^{Δ128-236} was neither mono- nor poly-ubiquitinated (data not shown). These results suggest that ubiquitination is largely blocked by deletion of the 110 residue sub-domain in Gpa1.

Since either mono- or poly-ubiquitination promotes a redistribution of Gpa1 away from the plasma membrane, the absence of ubiquitination would most likely favor Gpa1 localization to the plasma membrane. Indeed, Gpa1^{Δ128-236} was present almost exclusively at the plasma membrane even when expressed in the protease-deficient pep4Δ and ubp12Δ mutants (Figure 2.4 A). Since removal of the ubiquitinated sub-domain results in increased localization to the plasma membrane, we then asked whether Gpa1^{Δ128-236} diminishes Gβγ-mediated signaling. Over-expression of Gpa1 is known to cause slight signal inhibition, because more Gα exists to sequester Gβγ. Initially we measured pheromone sensitivity using a standard growth arrest assay. In this method a nascent lawn of cells is exposed to a point source of α-factor. As the lawn develops a zone of growth inhibition appears, the size of which reflects the sensitivity of the cells to pheromone-induced growth arrest. As shown in
Figure 2.4

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**pep4Δ**

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**ubp12Δ**

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C

![Graph](image13)
Figure 2.4  Blocking Gpa1 ubiquitination enhances plasma membrane localization and Gpa1 function. (A) A pep4Δ or ubp12Δ strain was transformed with an integrating plasmid encoding Gpa1 fused to GFP (pRS406-GPA1-GFP) or the corresponding mutant lacking the ubiquitination sub-domain (pRS406-GPA1\textsuperscript{128-236}-GFP). Cells were grown to mid-log phase and visualized by DIC and fluorescence microscopy. Cells expressing Gpa1 alone exhibited negligible background autofluorescence (data not shown). (B) and (C) Strain YGS5 (gpa1\textsuperscript{stell\textsuperscript{ts}}) was transformed with a single-copy plasmid (pRS316) containing either the wild-type GPA1 promoter and gene (“Gpa1”) or a mutant lacking the ubiquitination sub-domain (“Gpa1\textsuperscript{128-236}”), as well as a plasmid (pRS425-FUS1-lacZ) containing the pheromone-induced FUS1 promoter and lacZ reporter. (B) Cells were plated onto solid medium and exposed to paper discs containing -factor pheromone (clockwise from bottom right: 1, 5, 15, and 45 µg) for 48 h. (C) Cells in mid-log phase of growth were treated with the indicated concentrations of -factor, and the resulting \beta-galactosidase activity was measured spectrofluorimetrically. Data shown are representative of three independent experiments performed in triplicate. Error bars, ± SEM.
Figure 2.4 B, cells expressing Gpa1<sup>Δ128-236</sup> exhibited a smaller and more turbid zone of growth inhibition compared with the wild-type protein, indicating a diminished pheromone response. We also compared Gpa1<sup>Δ128-236</sup> and Gpa1 activity using a reporter-transcription assay consisting of the pheromone-inducible FUS1 promoter driving expression of lacZ (β-galactosidase) (74). As shown in Figure 2.4 C, cells expressing Gpa1<sup>Δ128-236</sup> exhibited a ~30% reduction in the maximum pheromone response, consistent with the reduced growth arrest response described above. Thus deletion of the ubiquitination sub-domain appears to enhance the ability of Gpa1 to block the Gβγ-mediated signal, resulting in diminished growth arrest and transcription-induction responses. Taken together, these data suggest that intracellular targeting of Gpa1 depends on the extent of ubiquitination. Whereas non-ubiquitinated Gpa1 is retained at the plasma membrane, mono-ubiquitinated Gpa1 is delivered to the vacuole and poly-ubiquitinated Gpa1 is delivered to the proteasome.

**Decreasing the fold stability of Gpa1 increases poly- but not mono-ubiquitination of Gpa1**

Here, we’ve found that Gpa1 is targeted for degradation by mono- and poly-ubiquitination, both of which affect Gpa1 steady state levels in vivo as well as pheromone-dependent signaling in yeast. One possible explanation for the existence of two separate ubiquitin-mediated degradation mechanisms is that mono- and poly-ubiquitination are non-redundant protein degradation signals that target distinct cellular pools of Gpa1: where poly-ubiquitination signals the degradation of Gpa1 that is structurally unstable or misfolded, and mono-ubiquitination signals the degradation of properly folded Gpa1 as part of a regulated protein-clearing mechanism. Indeed it has been estimated that as much as 30% of newly
synthesized proteins are incorrectly made and quickly degraded (130). To test this hypothesis, we used the protein modeling software *Medusa* to identify point mutations in the known crystal structure of human $\beta\alpha_i$ (PDB code: 1AGR) that were conserved in yeast and predicted to create a gradient of protein fold stabilities relative to the wild type protein (Table 2.1) (PMID 16839198). Benchmarked on a large set of experimentally characterized mutations, Medusa has been used to recapitulate changes in the structural stability of proteins upon amino acid substitution, and to do so with high accuracy (PMID 18073107; PMID 17538626). Five point mutations (F383A, F369A, L385A, V340A, and F406A) were selected that were predicted not to interfere with the catalytic, switch, or RGS binding regions of Gpa1 so as to avoid altering protein function. To evaluate their effect on Gpa1 poly-ubiquitination, each mutation was introduced separately into *GPA1* contained on high expression vector (pAD4M), and expressed in yeast harboring the *cim3-1* temperature sensitive mutation or an isogenic wild type strain. As shown in Figure 2.5, Gpa1 poly-ubiquitination appeared as a ladder of high molecular weight western blot bands visible in extracts prepared from the *cim3-1* strains but not from the isogenic wild type strain (Figure 2.5 A, left two lanes). Laddering was not observed in extracts from cells transformed with empty vector, indicating that the high molecular weight western blot signal was specific to Gpa1 (data not shown). In addition, poly-ubiquitination increased in proportion to the predicted destabilization for each Gpa1 point mutation. These data suggest that misfolded Gpa1 is recognized by the poly-ubiquitination machinery (Figure 2.5 A, C).

We next asked if destabilization alters mono-ubiquitination. We expressed each of the Gpa1 destabilizing point mutants in *pep4Δ* cells or the isogenic wild type strain and conducted western blot analyses of whole cell extracts using anti-Gpa1 antibodies.
Table 2.1

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*Relative to WT

Table 2.1 Relative free energies of Gpa1 protein fold-destabilizing mutations. Medusa protein modeling software was used in combination with the high resolution crystal structure of human G\(_{\alpha i}\) (PDB code: 1agr) to identify a set of five point mutations that are conserved in the yeast G alpha protein Gpa1 and predicted to create a gradient of protein fold stabilities relative to the wild type protein (see methods). The structural stability of the mutant protein is computed using the relaxed structure and the mutation-induced stability change (\(\Delta \Delta G\)) is obtained by subtracting the wild type stability. Mutations with higher \(\Delta \Delta G\) values are predicted to create the greatest negative effect on protein fold stability.
Figure 2.5 Gpa1 polyubiquitination, but not monoubiquitination, increases with increasing fold destabilization of Gpa1. (A) A proteosomal protease defective mutant (cim3-1) and the isogenic wild type strain were transformed with plasmid pAD4M-GPA1 (WT) or the indicated point mutant, grown at the restrictive temperature of 37°C for 5 hours. Whole cell extracts were resolved by 10% SDS-PAGE and immunoblotting with anti-Gpa1 antibodies. (B) A vacuolar protease-deficient (pep4Δ) mutant and the isogenic wild type strain were transformed with plasmid pAD4M-GPA1 or the indicated point mutant and grown to mid-log phase at 30°C. Whole cell extracts were examined as described above. The difference in film exposures between cim3-1 and pep4Δ experiments is required to ensure visualization of either the monoubiquitin band (which is in close proximity to the Gpa1 bands) or polyubiquitin laddering. (C) Densitometry of polyubiquitin laddering (from A) or the monoubiquitin band (from B) of Gpa1 relative to the wild type protein expressed in the cim3-1 or pep4Δ strain, respectively.
The presence of Gpa1 mono-ubiquitination was evident as a single band at ~63 kDa that was enhanced in the pep4Δ strains (Figure 2.5 B, first two lanes). In contrast to Gpa1 poly-ubiquitination, Gpa1 mono-ubiquitination did not change with respect to the predicted increase in protein fold destabilization (Figure 2.5 B, C). We determined that the lack of mono-ubiquitination of the Gpa1 mutants was not due to gross mutation-dependent functional defects in Gpa1, since Gpa1 mutant isoforms complemented a gpa1Δ mutation, responded normally to pheromone in both halo growth arrest and FUS1-lacZ transcriptional assays, and exhibited no difference in steady state levels when expressed from the native GPA1 promoter (data not shown). Taken together, these data indicate that poly-ubiquitination, but not mono-ubiquitination, is used to signal the degradation of misfolded Gpa1 in vivo.

Mono-ubiquitination of Gpa1 requires Gpa1 myristoylation

Poly-ubiquitination and proteasomal degradation of proteins occurs primarily in cytoplasmic, nuclear, and rough endoplasmic reticulum regions of the cell (18, 19, 48), and is a principal mechanism for the elimination of misfolded proteins in vivo (130). In contrast, mono-ubiquitination has been shown to serve as an endocytosis signal that initiates trafficking of plasma membrane-bound proteins such as Ste2 and Ste3 to the vacuole, where they are degraded (71, 123, 128). Since Gpa1 simultaneously exists as two different lipid modified isoforms at two different locations—an N-myristoylated form at the plasma membrane and a non-myristoylated form that is incapable of localizing to the plasma membrane—we investigated whether mono-ubiquitination was specific for the plasma membrane targeted for of Gpa1. We compared mono-ubiquitination (in pep4Δ cells) or poly-
ubiquitination (in cim3-1 cells) of three specific forms of Gpa1: wild-type Gpa1 (plasma membrane and non-plasma membrane), Gpa1$^{G2A}$ (only non-plasma membrane), Gpa1$^{S200A}$ (only plasma membrane). Gpa1$^{G2A}$ cannot be myristoylated and is localized in the cytoplasm, yet still retains wild type GDP-dependent binding affinity for G$\beta\gamma$ (139). Gpa1$^{S200A}$ is a point mutant of the Serine-200 phosphorylation site (89), which we found exists only in the myristoylated state (Figure 2.6 A) (155). We determined that the S200A mutation did not significantly alter Gpa1 function as cells transformed with the mutant exhibited wild type plasma membrane association and pheromone responsiveness as determined by fluorescence microscopy and a halo growth arrest assay, respectively (Figure 2.6 B). We found that Gpa1$^{S200A}$ was mono- and poly-ubiquitinated to the same degree as wild type Gpa1 (Figure 2.6 C). In contrast, mono-ubiquitination was undetectable for the unmyristoylated Gpa1$^{G2A}$ and poly-ubiquitination was significantly reduced but not absent compared to wild type Gpa1 (Figure 2.6 D). These data suggest that plasma membrane localization and/or N-myristoylation of Gpa1 is required for its mono-ubiquitination and critical, although not required, for poly-ubiquitination of Gpa1.

**Membrane localization alone is not sufficient to restore mono-**

Having determined that mono-ubiquitination occurs only if Gpa1 is properly myristoylated and plasma membrane targeted, we next questioned whether the lack of mono-ubiquitination resulted simply from mislocalization or directly from the lack of myristoylation of the Gpa1$^{G2A}$ mutant. In order to separate membrane attachment from other possible functions of myristate, we created a mutant form of Gpa1 that was not myristoylated but nevertheless properly localized at the plasma membrane.
Figure 2.6

A

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Gpa1
Gpa1<sub>myr</sub>

B

WT
S200A

GFP
DIC

C

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Gpa1-<sub>(UD)<sub>n</sub>
Gpa1
Gpa1

D

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Gpa1-<sub>(Ub)<sub>n</sub>
Gpa1
Gpa1
Figure 2.6  N-myristoylation is critical for polyubiquitination but required for monoubiquitination of Gpa1. (A) Anti-Gpa1 western blot of BY4741 yeast expressing pAD4M-GPA1 or the indicated point mutant. (B) DIC and fluorescence microscopy images of BY4741 yeast transformed with an integrating plasmid (pRS406-GPA1-GFP) containing the native GPA1 promoter and either the native or a S200A mutant open reading frame fused to the gene encoding yeast-enhanced green fluorescent protein (GFP) (left panel). Strain ste7Δ gpa1Δ was transformed with single copy plasmids pRS315-STE7 and either pRS316-GPA1 (WT) or pRS316-GPA1S200A (S200A), with each gene under the control of its native promoter. Transformed cells were plated onto solid medium and exposed to paper discs containing α-factor pheromone (clockwise from top: 1.5, 4.5, 15, and 45 µg) (right panel). (C) pAD4M-gpa1S200A was expressed in either cim3-1, pep4Δ, or the corresponding isogenic wild type strains and analyzed by western blotting with anti-Gpa1 antibodies as described in Figure 2.5. Note that all lanes shown within a single panel are from a single gel. (D) pAD4M-gpa1G2A was expressed in either cim3-1, pep4Δ, or the corresponding isogenic wild type strains and analyzed by western blotting with anti-Gpa1 antibodies as described in Figure 2.5. Note that all lanes shown within a single panel are from a single gel. Polyubiquitinated Gpa1 (Gpa1-(Ub)n), monoubiquitinated Gpa1 (Gpa1-Ub), and myristoylated Gpa1 (Gpa1myr) are indicated.
Figure 2.7

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B

Gpa1

Gpa1\(^{4K}\)

Gpa1 minutes post pheromone

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Slit2
Kss1
Fus3

C

RT

34°
Figure 2.7 \textbf{Gpa1}^{G2A/4K} \textit{rescues inviability of the unmyristoylated mutant.} (A) Sequence alignments of Gpa1 myristoylation and poly-basic mutants (shown are the first 14 amino acids) (B) \textit{ste7Δ gpa1Δ} cells were transformed with pRS315-STE7 and pRS316-GPA1 or the 4K mutant. \textit{Top} Cell extracts were resolved using 7.5\% SDS-PAGE, and immunoblotting using anti-Gpa1 antibody. \textit{Bottom} Cells were grown to mid-log phase, treated with 3 \textmu M \textalpha{} mating factor, and aliquots removed at timed intervals. Cell extracts were resolved using 12\% SDS-PAGE and immunoblotting using anti-phospho-p44/42 MAPK antibody. (C) YGS5 strain was transformed with pRS316-GPA1 or the indicated mutant. Cells were grown to saturation, and 3 \textmu l saturated culture was spotted in serial 2-fold dilutions onto selective media agar plates.
Our method in creating such a mutant was inspired from findings by Deschenes and others that a poly-basic stretch of amino acids can substitute for lipid-acylation and restore membrane localization (103). Gpa1 is known to contain two N-terminal lipid-modifications—a myristoylated Gly at position 2, and a palmitoylated Cys at position 3—and we designed the poly-basic stretch to be close to the N-terminus near myristoylation/palmitoylation sites. The resulting mutant will from here forward be referred to as Gpa1G2A/4K, indicating that it lacks the ability to be myristoylated contains 4 Lys residues at amino acid positions 7-10 in place of a Thr-Gln-Thr-Ile (Figure 2.7 A). We found that the 4 lysine residues were not disruptive to protein stability or protein function., given that Gpa14K (which can be myristoylated but also has the poly-lysine stretch) was expressed at similar levels to wild-type Gpa1 and was still capable of regulating pheromone-dependent phosphorylation of downstream MAPKs (Figure 2.7 B). Thus, replacing residues 7-10 with lysines does not appear to hamper Gpa1 function.

Cells that express unmyristoylated Gpa1G2A as the only form of Gpa1 are not viable, as Gpa1 cannot localize to the plasma membrane, and Ste4 signals constitutively resulting in growth arrest. To determine if Gpa1G2A/4K could rescue the inviability associated with unmyristoylated Gpa1, we expressed Gpa1G2A and Gpa1G2A/4K in the YGS5 strain (gpa1Δ ste11ts). Serial dilutions show that Gpa1G2A/4K, but not Gpa1G2A, conferred viability in signaling-competent cells (Figure 2.7 C). At the permissive temperature where signaling is restricted, cells expressing Gpa1G2A and Gpa1G2A/4K were equally viable. Thus, the addition of the poly-lysine stretch rescued the inviability of the Gpa1G2A mutation.
Figure 2.8

Gpa1 ΔG2A/4K-GFP

Gpa1 ΔG2A-GFP

Gpa1-GFP

DIC

GFP
Figure 2.8  Gpa1^{G2A/4K} is localized to the plasma membrane. ste7Δ gpa1Δ strains were transformed with an integrating plasmid (pRS406-GPA1-GFP, or indicated mutant in the same plasmid) containing the native GPA1 promoter and gene fused to the gene encoding yeast-enhanced green fluorescent protein. Cells were grown to mid-log phase at 30 °C and visualized by DIC and fluorescence microscopy. Cells expressing Gpa1 alone exhibited negligible background autofluorescence (data not shown).
Figure 2.9 Gpa1<sup>G2A/4K</sup> retains wild-type signaling function when over-expressed. BY4741 strain was transformed with a high copy plasmid (pAD4M) containing either the wild-type GPA1 or indicated mutants, as well as a plasmid (pRS425-FUS1-lacZ) containing the pheromone-induced FUS1 promoter and lacZ reporter. Cells in mid-log phase of growth were treated with the indicated concentrations of α-factor, and the resulting β-galactosidase activity was measured spectrofluorimetrically. Data shown are representative of three independent experiments performed in triplicate. Error bars, ± SEM. (A) Basal activity (B) pheromone-induced activity.
We next wanted to determine if the restored viability was due to proper localization at the plasma membrane. To answer this question, we created a GFP-tagged version of Gpa1^{G2A/4K} and monitored the localization of this protein in live cells using fluorescent microscopy. We found that Gpa1^{G2A/4K}-GFP localized to the plasma membrane, as did wild-type Gpa1-GFP. In contrast, Gpa1^{G2A}-GFP localized diffusely throughout the cytoplasm (Figure 2.8). Quantization of the fluorescence revealed that the membrane localization of the G2A/4K mutant was similar to that of wild-type Gpa1 (data not shown).

Although Gpa1^{G2A/4K} is localized correctly at the plasma membrane, it remained a possibility that the mutant was not a functional signaling protein. To determine whether Gpa1^{G2A/4K} was competent to signal, first we tested the effect of Gpa1 over-expression on the expression of the FUS1-LacZ transcription reporter. Over-expression of wild-type Gpa1 causes dampening of both basal and pheromone-induced pathway activation, by providing more Gpa1 to sequester free Gβγ at the plasma membrane. We found that over-expression of Gpa1^{G2A/4K} dampened basal pathway activation similarly to over-expression of wild-type Gpa1 (Figure 2.9 A). Over-expression of Gpa1^{G2A} had no effect, relative to empty vector, consistent with its inability to target the plasma membrane and sequester Gβγ. Similar results were seen for pheromone-induced transcriptional activation (Figure 2.9 B).

As another measure of signaling competence, we also monitored sensitivity to pheromone using the standard growth arrest assay. When compared to cells expressing wild-type Gpa1, Gpa1^{G2A} expressing cells were not viable (data not shown), however, the addition of the poly-lysine stretch restored the ability to regulate pheromone signaling (Figure 2.10). To be certain that the poly-lysine stretch itself was responsible for restoring the signaling lost in the Gpa1^{G2A} mutant, we also created a series of mutants containing varied lysine stretches.
(1K, 2K, 5K, and 6K, in addition to 4K). We found that pheromone sensitivity was inversely proportional to the basic nature of the poly-lysine stretch, with the least basic (1K) being the most sensitive, and the most basic (6K) being the least sensitive (Figure 2.10). We noticed that 6K cells were almost indistinguishable from wild-type, whereas 4K cells were slightly super-sensitive to pheromone. For further analysis, however, we decided to use 4K cells because: 1) we desired to keep mutations to a minimum considering that the N-terminal region is involved in other critical functions such as Gβγ binding, and 2) the addition of 4K fully rescued plasma membrane localization of Gpa1G2A, suggesting the gain of function provided by 6K was not mediated by increased membrane localization. These functional data suggest that poly-lysine Gpa1 is able to restore localization and signaling in the absence of myristoylation.

We were also interested in determining if Gpa1G2A/4K was modified correctly. The wild-type G protein is known to be palmitoylated in addition to being myristoylated, and palmitoylation is also known to be dependent on myristoylation (96). It is believed that this is true simply because myristoylation delivers Gpa1 to the plasma membrane, where palmitoylation likely occurs (46). Thus, we anticipated that Gpa1G2A/4K should also be correctly palmitoylated. Loss of palmitoylation is known to cause increased sensitivity to pheromone, which can be measured using the pheromone-induced growth arrest assay (Figure 2.11 A). We found that Gpa1G2A/C3S/4K—the unmyristoylated, unpalmitoylated, poly-lysine containing mutant of Gpa1—showed a 15% increase in the zone of pheromone induced growth inhibition when compared to Gpa1G2A/4K (Figure 2.11 B). The magnitude of the increased sensitivity was equal to that seen when the C3S mutation is added to Gpa1.
Figure 2.10

Gpa1 G2A/4K

Gpa1 G2A/2K

Gpa1 G2A/1K

Gpa1 G2A/1K

Gpa1 G2A/6K

Gpa1 G2A/5K
Figure 2.10  Pheromone-sensitivity of unmyristoylated Gpa1 is recovered by the addition of a poly-basic stretch.  ste7Δ gpa1Δ strain was transformed with pRS315-STE7 and pRS316-GPA1, or the indicated mutant. Cells were grown to saturation overnight in selective media, plated onto solid medium, and exposed to paper discs containing α-factor pheromone (clockwise from right: 1, 5, 15, and 45 μg) for 48 h.
These data suggest that in the absence of myristoylation, restoring membrane localization using Gpa1G2A/4K is sufficient to restore Gpa1 palmitoylation. Taken together, these assays for cell viability, protein localization, and G protein signaling confirm that Gpa1G2A/4K is a functional, properly localized, but not myristoylated form of Gpa1.

We then determined if localization to the plasma membrane was sufficient to restore mono-ubiquitination of unmyristoylated G protein. We found that mono-ubiquitination of Gpa1G2A/4K was undetectable in comparison to Gpa1 in pep4Δ and in wild type cells (Figure 2.12 A). Since mono-ubiquitination was observed for Gpa14K, which contains Gly-2 and is myristoylated, we conclude that the lack of mono-ubiquitination of Gpa1G2A/4K was not caused by the poly-basic stretch. When we analyzed the same Gpa1 mutants in the cim3-1 strain, we found that poly-ubiquitination of Gpa1G2A/4K was slightly elevated relative to Gpa1G2A, yet not to the same level as wild type Gpa1 (Figure 3.12 B, right panel). Taken together, these data suggest that N-myristoylation itself, and not plasma membrane localization, is required for Gpa1 monoubiquitination.
Figure 2.11

A

Gpa1   Gpa1

Gpa1G2A/4K   Gpa1

Gpa1G2A/C3S/4K   Gpa1

B

% increase

Gpa1   Gpa1

Gpa1G2A/4K   Gpa1

Gpa1G2A/C3S/4K   Gpa1
Figure 2.11  Poly-basic Gpa1 mutants require the palmitoylated Cys residue for full sensitivity to pheromone.  (A) Performed as in Figure 2.10.  (B) Diameter of growth restriction was measured for 4 different alpha factor concentrations on 3 different plates. Data are mean ± SEM.
Figure 2.12  Establishing plasma membrane localization of Gpa1^{G2A} through an N-terminal polybasic stretch does not re-establish Gpa1 monoubiquitination. (A) pAD4M-GPA1 (WT) or the indicated point mutant were expressed in pep4Δ or the corresponding isogenic wild type strain and analyzed by western blotting with anti-Gpa1 antibodies as described in figure 2.1. (B)pAD4M-GPA1 (WT) or the indicated point mutant were expressed in cim3-1 or the corresponding isogenic wild type strain and analyzed by western blotting with anti-Gpa1 antibodies as described in Figure 2.5. Polyubiquitinated Gpa1 (Gpa1-(Ub)_n) and monoubiquitinated Gpa1 (Gpa1-Ub) are indicated.
DISCUSSION

Two modes of ubiquitin-mediated destruction of Gpa1

Ubiquitination is a well-known trafficking/degradation signal. Many proteins are poly-ubiquitinated and destroyed by the proteasome, while others are mono-ubiquitinated and typically trafficked to the lysosome/vacuole. In an effort to characterize the ubiquitination of Gpa1, we found that Gpa1 can be both mono- and poly-ubiquitinated: mono-ubiquitinated Gpa1 is trafficked to the vacuole and poly-ubiquitinated Gpa1 is trafficked to the proteasome. These results further validate the hypothesis that ubiquitination is a trafficking signal first, and a degradation signal second (70). We found that the two modes of Gpa1 ubiquitination are not redundant: poly-ubiquitin-mediated degradation appears to regulate misfolded protein, whereas mono-ubiquitin-mediated degradation regulates the turnover of functional Gpa1. These data provide an explanation as to why two forms of ubiquitination exist for a single protein.

Our analysis benefited greatly from several unique features of the experimental system. First, our previous mass spectrometry analysis had revealed that Gpa1 is ubiquitinated at Lys-165 in vivo (98). This allowed us to design a mutant form of Gpa1 (Gpa1128-236) that does not undergo ubiquitination. Another important advantage was the available crystal structure of mammalian Gαi and Gαs proteins, which closely resemble yeast Gpa1 in both sequence and function (16, 141). Thus our destabilizing mutants and Gpa1128-236 mutant could be designed in a rational manner based on the predicted folded structure of the protein, without altering known sites of GTP binding, subunit interaction, receptor coupling, or membrane association.
Figure 2.13  

Figure 2.13  Gpa1 mono- and poly-ubiquitination requires N-myristoylation. Data presented herein suggest that N-myristoylation is necessary for Gpa1 ubiquitination. Shown here is a simple model depicting the relationship between phosphorylation, myristoylation and ubiquitination of Gpa1.
Another interesting finding in this study is that mono-ubiquitination of Gpa1 requires Gpa1 myristoylation, but not simply plasma membrane localization. This finding required the creation of Gpa1\textsuperscript{G2A/4K}, a mutant form of Gpa1 that is localized correctly but not myristoylated, and also Gpa1\textsuperscript{S200A}, a mutant that is fully myristoylated. Here, we presented validation that the G2A/4K mutant was properly localized, competent to transmit pheromone signals, and correctly modified by palmitate.

Another unique feature of our experimental system is the ability to manipulate the extent of ubiquitination, through the use of mutants that selectively stabilize mono- or poly-ubiquitinated substrates. Myc-ubiquitinated proteins are poor substrates for proteolysis and therefore accumulate to higher-than-normal levels within the cell (47). \textit{pep4} mutants disrupt vacuolar protease function, and have been successfully used to enrich mono-ubiquitinated substrates (71, 123, 126). \textit{cim3} mutants disable proteasome protease activity and therefore result in the accumulation of poly-ubiquitinated substrates (28, 29, 60, 98, 162). Although less commonly applied, \textit{ubp} mutants can also be very effective in preserving short-lived changes in protein ubiquitination. This approach is analogous to using specific phosphatase inhibitors to preserve transient increases in protein phosphorylation.

**Potential contribution to intracellular signaling**

Of the pheromone signaling proteins shown to undergo ubiquitination, nearly all are modified in response to pheromone treatment. Documented examples include the pheromone receptors Ste2 and Ste3, the RGS protein Sst2, and the effector MAP kinase kinase Ste7. In contrast, Gpa1 is ubiquitinated in a constitutive manner, independent of pheromone stimulation, thus it seems unlikely that the purpose of Gpa1 mono-ubiquitination is signal
desensitization. It is not clear why constitutive ubiquitination would be beneficial, however, one possibility is that the true function of Gpa1 ubiquitination is to create a steady state population of internalized Gpa1. We have previously shown that Gpa1 regulates the endosomal PI3K Vps34, however, we have not determined the modification status of endosomal Gpa1. Considering that mono-ubiquitinated Gpa1 must traffick through endosomes before arriving at the vacuole, it is possible that the function of mono-ubiquitination is to localize Gpa1 to endosomes.

**Unique functions of myristate**

Our findings suggest that Gpa1\textsuperscript{G2A/4K} mimics the wild-type protein but is not myristoylated and surprisingly, is also not mono-ubiquitinated. Potential explanations for this finding are that the mono-ubiquitination machinery requires direct recognition of the myristate moiety or recognition of a myristate-dependent conformational change in Gpa1. Another possibility is that Gpa1\textsuperscript{G2A/4K} somehow interferes with mono-ubiquitination. Considering that this mutant can still be poly-ubiquitinated and is otherwise modified correctly (i.e. palmitoylated) it seems unlikely that the lysine string itself is negatively contributing to mono-ubiquitination.

In conclusion, we have exploited the unique advantages of our experimental system to determine how the functional status of Gpa1 can influence the extent of ubiquitination. Based on our data we can propose a model in which Gpa1 is degraded by two pathways. The first pathway, used by the fully mature G protein, involves mono-ubiquitination, endocytosis, and delivery to the vacuole. The second pathway, used by misfolded Gpa1, involves poly-ubiquitination and delivery to the proteasome. This strong relationship between the activity,
the extent of ubiquitination, and the trafficking of the G protein α subunit to its site of degradation establishes a new mechanism of G protein regulation. Identifying proteins involved in Gpa1 ubiquitination as well as determining how ubiquitination regulates Gpa1 signaling will be the focus of future studies.

**EXPERIMENTAL PROCEDURES**

*Strains and plasmids* - Standard methods for the growth, maintenance, and transformation of yeast and bacteria, and for the manipulation of DNA, were used throughout (9). Yeast *Saccharomyces cerevisiae* strains used in this study were BY4741 (*MATα* leu2Δ *met15Δ* ura3Δ), BY4741-derived mutants lacking *PEP4* or *UBP12* (disrupted using the KanMX G418-resistance marker, from Research Genetics, Huntsville, AL), BY4741-derived mutant lacking both *STE7* and *GPA1* (GPA1 disrupted using *gpa1::hisG* and STE7 disrupted using KanMX G418-resistance), MHY753 (*MATα* his3Δ200 *leu2Δ1* ura3-52 lys2-801 *trp1Δ63* ade2-101), MHY754 (MHY753, *cim3*-1), CRY1 (*MATα* ura3-1 *leu2,3-112* his3-11 *trp1-l* ade2-1*α* *can1-100*), CB007-1D (CRY1, *pep4-2::HIS3 prb1::LEU2*), LHY488 (*MATα* ura3-1 *leu2,3-112* *his3-11* *trp1-l* ade2-1*α* *can1-100*), LHY489 (LHY488, *pep4-2::HIS3 prb1::LEU2*) (provided by Linda Hicke, Northwestern University) (60), and YGS5 (*MATα* ura3-52 lys2 *ade2α* *trp1* leu2-1 *gpa1::hisG ste11α*) (139). *STE2* was disrupted in BY4741 and BY4741-derived *pep4Δ* mutant strains by single-step gene replacement with *ste2::HIS3* (this work).

Yeast shuttle plasmids used here were pRS316 (*CEN*, ampR, *URA3*), pRS406 (ampR, *URA3*) (134), pRS316-GPA1 which contains *GPA1* under the control of its native promoter (139), pAD4M (2 μm, ampR, *LEU2*, *ADH1* promoter/terminator, from Peter McCabe, Onyx
Pharmaceutical), pAD4M-GPA1 (139), pND747 (2 µm, ampR, \textit{URA3}, \textit{MYC}-tagged ubiquitin under the control of the \textit{CUP1} promoter, from Nicholas Davis, Wayne State University) (124) and pRS425-FUS1-lacZ (74). pRS316-GPA1\textsuperscript{Δ128-236} was constructed by replacing the 1.5 kbp HindIII-HindIII fragment with an 1170 bp PCR product, generated using \textit{GPA1} as template. The forward primer 5'-CCC AAG CTT TAA TTC ACG AAG ACA TTG CTA AGG CAA TAA AGC AAC TTT GG- 3' disrupts and regenerates the \textit{GPA1} HindIII site; the reverse primer 5'-AGG TCG ACG GTA TCG ATA AGC-3' flanks the multiple cloning site HindIII upstream of the gene insert within pRS316. pAD4M-GPA1\textsuperscript{Δ128-236} was constructed by PCR amplification of \textit{GPA1}\textsuperscript{Δ128-236} using primers 5'-ACG CGT CGA CAT GGG GTG TAC AGT GAG TAC GCA AAC AAT A-3' and 5'-CGA GCT CTC ATA TAA TAC CAA TTT TTT TAA GGT TTT GCT-3', engineered with SalI and SacI sites, and subcloning into the SalI/SacI sites within the multiple cloning site of the pAD4M vector. Note that positions 127 and 236 both encode Leu. A triple-Flag epitope tag was placed at the C-terminus of Ubp12 (UBP12-Flag) by PCR amplification and subcloning into pYES2.1/V5-His-TOPO (2 µm, \textit{URA3}, \textit{GAL1} promoter, \textit{CYC1} terminator) (Invitrogen, Carlsbad, CA), to yield plasmid pYES-UBP12-Flag. PCR primers were 5'-CCC AAG CTT CCA GAA TGG GTT CTT CAG ATG TTT CAA GTC-3' and 5'-G TTA CTT GTC ATC GTC ATC TTT ATA ATC AAG CTT TTC TGG CGA TTC TAG TGT CAC-3'.

The Gpa1-GFP (green fluorescent protein) fusion was constructed by PCR amplification of the \textit{GPA1} gene, digestion with XbaI and Clal, and subcloning into the corresponding sites of yeast-enhanced GFP-containing plasmid pUG35 (\textit{CEN}, \textit{URA3}, \textit{MET25} promoter, \textit{CYC1} terminator) (112). PCR primers used were 5'-CCT GCA GCC CGG CGG GGG
ATC CAC TAG TCT AGA-3’ (forward) and 5’-ACA TCG ATT ATA ATA CCA ATT TTT TTA AGG TTT TGC TGG ATC-3’ (reverse), and include unique XbaI and ClaI restriction sites. A cassette containing the promoter, GPA1-GFP, and terminator was digested with SacI and KpnI and subcloned into the corresponding sites of integrating vector pRS406 to yield pRS406-GPA1-GFP. The resulting plasmid was linearized with HindIII (a site unique to GPA1) to direct gene integration. GPA1Δ128-236-GFP was constructed as described for GPA1-GFP except for the use of a different forward primer (5’-GGT CTA GAC ATG GGG TGT ACA GTG AGT ACG-3’) and GPA1Δ128-236 as the template.

The G2A, C3S, 4K, 1K, 2K, 5K, 6K, and 8K mutations were added to the various constructs using site directed mutagenesis. The templates used for G2A, C3S, 1K, and 2K were wild-type Gpa1. The poly-lysine mutants were created sequentially such that each additional lysine was added using the previous lysine mutant as a template (4K uses 2K as a template, 5K uses 4K as a template, etc.).

**Growth, transcription, degradation and ubiquitination bioassays** - The pheromone-dependent growth inhibition (halo) and reporter-transcription assays were conducted as described previously (74). Gpa1 expression and ubiquitination was monitored as described previously (98). To monitor the loss of Gpa1, cultures were treated with cycloheximide (10 mg/ml in 0.1 % ethanol, final concentrations) for up to 120 min, as described previously (58). Growth was stopped at mid-log phase (A600nm ~ 1) by the addition of 10 mM NaN3 and transfer to an ice bath. Cells were centrifuged, washed with 10 mM NaN3, and the cell pellet was resuspended directly in boiling SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.0005% bromphenol blue) for 10 min. The samples were then subjected to glass bead homogenization, clarified by microcentrifugation,
and resolved by SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose. The membrane was probed with antibodies to Gpa1 at 1:1,000 (40), Ste4 at 1:2,000 (from Duane Jenness, Univ. Massachusetts), or Pgk1 at 1:75,000 (from Jeremy Thorner, Univ. California Berkeley). Immunoreactive species were visualized by enhanced chemiluminescence detection (Pierce) of horse radish peroxidase-conjugated anti-rabbit IgG (BioRad).

**Immunoprecipitation.** The association of Gpa1 and Ubp12 was examined by immunoprecipitation of Flag-tagged Ubp12 and immunoblotting with anti-Gpa1 antibodies. Cells (50 ml) co-transformed with plasmids pAD4M-GPA1 and pYES-UBP12-Flag were grown to $A_{600\text{nm}} \sim 1$, harvested and resuspended in 550 ml of lysis buffer (50 mM NaPO$_4$ pH 7.5, 400 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.5 mM dithiothreitol, 25 mM NaF, 25 mM glycerophosphate, 1 mM sodium orthovanadate, 10 mM N-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, and one pellet of complete EDTA-free protease inhibitor cocktail (Roche)). This and all subsequent manipulations were carried out at 4 °C. Cells were subjected to glass bead vortex homogenization for 30 s, repeated eight times, and centrifuged twice at 6,000 x $g$ for 5 min and 25 min. Lysates were incubated for 2 h with a bead volume of 10 ml of anti-Flag M2 affinity resin (Sigma) equilibrated in lysis buffer. Immunoprecipitates were collected by centrifugation at 1,000 x $g$ for 30 s, and pellets were washed with 1 ml of lysis buffer for 3 min, repeated four times before final resuspension in 30 ml of 2x SDS-PAGE sample buffer. Each sample was resolved by 7.5% polyacrylamide gel electrophoresis and immunoblotting with anti-Gpa1 polyclonal antibodies at 1:1,000 or anti-Flag monoclonal antibodies at 1:2,000.

**Microscopy analysis.** Cells expressing single-copy, integrated GFP-tagged gene fusions were visualized by differential interference contrast (DIC) and fluorescence
microscopy using a Nikon eclipse E600EN, photographed with a Hamamatsu digital camera, and analyzed with MetaMorph Version 5.0 software.

**MAPK phosphorylation assays**—Yeast strains were grown with shaking at 30°C in selective media to A_{600 nm} \sim 0.8 and treated with 3 \mu M \alpha-factor pheromone. Samples were removed at timed intervals, collected by centrifugation, and stored at -70°C. To prepare extracts, cell pellets were thawed on ice and resuspended in 250 \mu l of ice cold TCA buffer (10 mM Tris pH 8.0, 10% trichloroacetic acid, 25 mM NH_4OAc, 1 mM EDTA). Cells were disrupted by vortexing with 100 \mu l of glass beads in 5 x 1 min bursts with chilling on ice in between. Lysates were transferred to new tubes and centrifuged for 10 min at 16,000 \times g at 4°C. Pellets were resuspended in 0.1 M Tris pH 11.0, 3% SDS, and boiled for 5 min, then centrifuged at 16,000 \times g. The resulting supernatant was separated and protein concentration was determined using the DC protein assay (Bio-Rad Laboratories). 20 \mu g of protein in 2x SDS-PAGE sample buffer was used per time point. MAPK phosphorylation was determined by 12% SDS-PAGE and immunoblotting with p44/42 MAPK antibody at 1:500 (Cell Signaling Technology). Densitometry of developed blots was determined using ImageJ.

**Protein Folding Simulation Algorithms** – Identification of destabilizing mutations in yeast Gpa1 was accomplished using Medusa protein modeling software (PMID 16839198). Briefly, Medusa models a protein in atomic resolution and features a physical force field as well as a rapid amino acid sidechain packing algorithm to recapitulate changes in protein fold stability upon amino acid substitution with high accuracy (PMID 18073107; 17538626). Since the structure of yeast Gpa1 is unknown, we used its mammalian homolog, G\alpha_i, as the modeling system. G\alpha_i exhibits 67% sequence similarity with Gpa1, and its structure has been solved by high resolution x-ray crystallography (PDB code: 1agr). Using the Medusa
software, we conducted an alanine scan to systematically evaluate the stability change due to structural perturbation upon substituting the native amino acid with alanine along the entire Gαi sequence. The stability of the mutant is computed with the relaxed structure and mutation-induced stability change (ΔΔG) is obtained by subtracting the wild type stability. Finally, the destabilizing mutations are mapped onto the yeast Gpa1 sequence and only evolutionarily-conserved residues are selected as the putative destabilizing mutant. To minimize disruption of the G protein function, we avoided mutating residues associated with nucleotide catalysis, switch regions, or other protein binding regions.
CHAPTER III

Development of a Cell Viability Assay to Monitor for Palmitoylation of Gpa1

All Figures contributed by Michael J. Lee
Summary

Protein palmitoylation is the post-translational addition of a 16-carbon lipid to a substrate protein, via a thioester linkage. Modifications of this type are known to be critical factors in anchoring peripheral membrane proteins to lipid bilayers, and recent evidence suggests that reversible palmitoylation may play a role in signal regulation. However, our understanding of how proteins are palmitoylated is underdeveloped. Progress has been hampered by the lack of sensitive assays for monitoring protein palmitoylation in vivo.

Gpa1, the Gα subunit in the yeast mating response pathway, is palmitoylated. Blocking palmitoylation—which can be achieved by mutating the palmitate-acceptor cysteine residue—results in only minor signaling defects. Here we describe a variant of Gpa1 that is functional only if palmitoylated. By replacing the myristoylated glycine-2 with a short stretch of basic amino acids, palmitoylation becomes absolutely required for Gpa1 targeting to the plasma membrane, and sequestration of Gβγ subunits. The availability of this mutant makes it possible to screen for Gpa1 palmitoylation in vivo, using cell viability as a reporter. Furthermore, we provide proof of the screening principle, and preliminary data suggesting redundancy among a subset of known protein acyl-transferases (PATs).
INTRODUCTION

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are a large and diverse family of signal regulators (61). These G protein signaling systems regulate responses to a wide variety of chemical and environmental signals, including hormones, neurotransmitters, odors and light. The signaling complex is comprised of three subunits, which signal as two entities: Gα and Gβγ. These subunits, through their coupling to transmembrane receptors, function as signal mediators, connecting signals from outside the cell with effectors inside the cell. Gα functions as a signaling switch; it binds to either GDP or GTP, and differentially to effector proteins depending on its nucleotide bound state.

G proteins exist in essentially all eukaryotic organisms, including the yeast Saccharomyces cerevisiae. In yeast, G proteins mediate mating, the process by which two haploid cells fuse to create a single diploid cell. The mating response is characterized by growth arrest, polarized cell morphology and the transcription of a new subset of genes required for the cell fusion process. All of these responses are regulated by G protein activation (41).

In recent years, it has become increasingly clear that G proteins exist at multiple locations inside the cell, in addition to their well-established location at the plasma membrane (35). Furthermore, it has become evident that some G proteins re-localize in response to activation, opening up the possibility that signaling is occurring from multiple cellular compartments (118). Thus, understanding the mechanisms by which G proteins localize to various cellular locations is vital to understanding how G protein function is regulated. One such example comes from yeast, where the Gα subunit Gpa1 is known to exist at the cell periphery and at endosomes (135). At the plasma membrane, Gpa1 regulates
the function of the Gβγ subunit Ste4/Ste18, and at the endosome, Gpa1 regulates the function of the phosphatidyl inositol 3-kinase (PI3K) Vps34 (136). As with other Gα subunits, it is not fully understood what signals regulate Gpa1 localization to the plasma membrane or to the endosome.

G proteins are anchored to the inner leaflet of lipid bilayers through the post-translational addition of a variety of lipid modifications. For Gα subunits, principle among these modifications are myristoylation and palmitoylation (24). All Gα subunits are either myristoylated, palmitoylated, or modified by both lipids. Myristoylation of Gα subunits is well understood at the biochemical level; the consensus sequences have been identified, and the enzymes responsible for N-terminal myristoylation are known (17). Gα subunit palmitoylation is less well understood. It is not clear what constitutes a “palmitoylation site,” although it is known that the lipid is attached to cysteine residues, and the enzymes responsible for palmitoylation of most proteins have not been identified.

The identification of enzymes involved in palmitoylation is in its infancy. Recently, the DHHC motif was identified because of its presence in both Erf2 and Akr1—the first two proteins shown to function as protein acyl-transferases (PATs). Erf2 regulates the palmitoylation of Ras2, whereas Akr1 palmitoylates type I casein kinases (11, 52). To date, 7 DHHC proteins have been identified, all in yeast, and all are now known to function as PATs (104). Even so, it is unlikely that these seven DHHC proteins truly represent all PATs in existence, but rather those of a single PAT sub-family. A comprehensive genome-level screen was recently performed to identify palmitoylated proteins in yeast, as well as to determine PAT-substrate pairs (125). After these efforts, 35 new palmitoylated proteins were
identified, but PATs have still not been identified for the vast majority of palmitoylated proteins.

The yeast Gα subunit Gpa1, and many other Gα subunits, is known to be palmitoylated (92), however, it is still not clear what proteins are involved in the addition of palmitate to Gpa1 or to any other Gα. One difficulty in identifying a PAT for Gpa1 is that palmitoylation in this case has only very mild effects on activity (96, 138). In yeast—an organism whose value often rests in its utility for genetic screens—the lack of a strong palmitoylation phenotype complicates the ability to perform high throughput genetic screening for palmitoylation-defective mutants. Here, we characterize Gpa1G2A/2K, a novel mutant form of Gpa1 that can be used to screen for defects in palmitoylation. Unlike the wild-type form of Gpa1, cells that express Gpa1G2A/2K are only viable if properly palmitoylated. Furthermore, using this mutant, we provide data suggesting that no currently known PAT is necessary for Gpa1 palmitoylation. These findings suggest either redundancy or another as yet unidentified PAT for Gpa1.

RESULTS

Mis-palmitoylated Gpa1 displays only minor defects in signaling and localization

Gpa1 is palmitoylated on cysteine-3, however, it is not clear how this modification is regulated. Understanding this process is particularly relevant because Gpa1 is known to localize to multiple intracellular membranes, and palmitoylation is one of the few membrane anchors known to be reversible (45). In order to identify proteins involved in palmitoylating Gpa1, we first needed to identify a robust phenotype that could be used for high-throughput genetic screening. Because palmitoylation is known to regulate Gpa1 localization to the
plasma membrane, we began by monitoring protein localization in cells expressing Gpa1-GFP or Gpa1\(^{C3S}\)-GFP. We found that introducing the C3S substitution did not significantly alter the localization of Gpa1 (Figure 3.1 B). Likewise, adding C3S did not alter the localization of Gpa1 harboring the G2A/4K substitution, which replaces myristoylation with a poly-basic stretch (Figure 3.1 C, see *Chapter II* for validation of mutant). As another measure, we monitored pheromone sensitivity in wild-type cells and in cells that express a palmitoylation-deficient Gpa1, Gpa1\(^{C3S}\). The addition of mating pheromone to a nascent lawn of cells is known to cause a zone of growth inhibition due to G\(\beta\gamma\) signaling; the size of the restricted growth zone is proportional to the cell’s sensitivity to pheromone. When compared to wild-type yeast, cells expressing Gpa1\(^{C3S}\) were more sensitive to pheromone (Figure 3.2 A) (96, 138), presumably because the lack of palmitoylation resulted in less Gpa1 at the plasma membrane to sequester G\(\beta\gamma\). Although the Gpa1\(^{C3S}\) cells were more sensitive to pheromone, the difference in sensitivity (halo size) was only 15%, a phenotype that is likely too subtle to be useful in high throughput genetic screening. Monitoring the pathway at the level of mating gene transcription showed a similarly subtle defect in Gpa1\(^{C3S}\) expressing cells (138).

The lack of difference between wild-type and Gpa1\(^{C3S}\) cells was surprising. Other G\(\alpha\) subunits show severe localization and signaling defects when not palmitoylated (116). We noticed, however, that as opposed to some other G\(\alpha\) subunits, Gpa1 has two N-terminal lipid modifications: myristoylation and palmitoylation. When G\(\beta\gamma\) is considered as one of the membrane anchors, wild-type Gpa1 actually has three membrane-localization signals, leaving the Gpa1\(^{C3S}\) mutant with two intact membrane anchors. Thus, we next wanted to test the hypothesis that Gpa1 is resistant to defects when not palmitoylated because of the
presence of two other membrane-localization signals. To do so, we needed a form of Gpa1 that is absolutely dependent on being palmitoylated.

Blocking Gpa1 myristoylation (using the G2A substitution), results in protein that is also not palmitoylated, not membrane bound, and not functional. We have previously determined that Gpa1^{G2A/4K}—a mutant form that cannot be myristoylated but has a poly-lysine stretch in its place—is membrane bound and functional (see Chapter II) (156). Poly-basic stretches have been used to rescue membrane localization of Ras and even Gαs in the absence of lipid modification (103, 154). Also, it is believed that naturally occurring poly-basic stretches are utilized by some Gα subunits to aid in membrane attachment (116). We reasoned that the Gpa1^{G2A/4K} mutant, or some variant of this mutant, could be used to confer an absolute dependence on palmitoylation for Gpa1 activity. Palmitoylation is known to occur in a myristoylation-dependent manner, but it is believed that myristate only serves to localize the protein to the plasma membrane, where palmitoylation can occur (46, 96). If this is the case, Gpa1^{G2A/4K} should also be palmitoylated, despite the lack of myristoylation. To test if this is true, we again compared pheromone sensitivity in cells expressing Gpa1^{G2A/4K} to cells expressing Gpa1^{G2A/C3S/4K}. The “C3S” mutation should block palmitoylation (138). We found that Gpa1^{G2A/C3S/4K} cells were approximately 15% more sensitive to mating pheromone as compared with cells expressing Gpa1^{G2A/4K} (Figure 3.2). A similar difference was observed when comparing Gpa1 and Gpa1^{C3S}, suggesting that Gpa1^{G2A/4K} is indeed palmitoylated.
Figure 3.1

A

<table>
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<tr>
<th></th>
<th>Gpa1</th>
<th>Gpa1$^{C3S}$</th>
<th>Gpa1$^{G2A/1K}$</th>
<th>Gpa1$^{G2A/2K}$</th>
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B

C

Gpa1

Gpa1$^{C3S}$
Figure 3.1 Gpa1 lacking the palmitoylated Cys residue remains membrane bound. (A) Alignment of N-terminal amino-acids of various palmitoylation mutants used in this study. (B) ste7Δ gpa1Δ cells were transformed with an integrating plasmid (pRS406-GPA1-GFP, or indicated mutant in pRS406-GPA1-GFP plasmid) containing the native GPA1 promoter and gene fused to the gene encoding yeast-enhanced green fluorescent protein. Cells were grown to mid-log phase at 30 °C and visualized by DIC and fluorescence microscopy. Cells expressing Gpa1 alone exhibited negligible background autofluorescence (data not shown).
Figure 3.2

A

WT       WT+C3S
G2A/4K   G2A/4K+C3S

B

\[
\begin{array}{cccc}
\text{WT} & \text{WT+C3S} & \text{G2A/4K} & \text{G2A/4K+C3S} \\
\end{array}
\]

\% increase

\[
\begin{array}{cccc}
\text{WT} & \text{WT+C3S} & \text{G2A/4K} & \text{G2A/4K+C3S} \\
\end{array}
\]
Figure 3.2  

Gpa1$^{G2A/4K}$ requires the palmitoylated Cys residue to retain full pheromone-sensitivity. ste7Δ gpa1Δ was transformed with single copy plasmids pRS315-STE7 and either pRS316-GPA1 (WT), pRS316-GPA1$^{G2A/4K}$ (G2A/4K), pRS316-GPA1$^{C3S}$ (WT+C3S), or pRS316-GPA1$^{G2A/C3S/4K}$ (G2A/4K+C3S), with each gene under the control of its native promoter. Transformed cells were plated onto solid medium and exposed to paper discs containing α-factor pheromone (clockwise from bottom right: 1.5, 4.5, 15, and 45 µg).
Gpa1<sub>G2A/1K</sub> and Gpa1<sub>G2A/2K</sub>, but not Gpa1<sub>G2A/4K</sub> or wild-type Gpa1, must be palmitoylated to maintain viability

Having found evidence that the unmyristoylated Gpa1<sub>G2A/4K</sub> also requires the palmitoylated Cys for full function, we next determined if a shorter lysine stretch could confer a dependence on palmitoylation. Our rationale was that a short poly-lysine stretch represented a “weaker” membrane localization signal than myristate or palmitate, and might not support membrane localization alone. In order to test this hypothesis, we altered the length of the lysine stretch from 4 residues to 1 residue (4K to 1K), and monitored both viability and pheromone sensitivity (Figure 3.1). We found that all lysine mutants were viable (Figure 3.3 A), suggesting that even a single lysine placed near the N-terminus of Gpa1 was sufficient to rescue the inviability associated with the Gpa1<sub>G2A</sub> (unmyristoylated) mutant. Furthermore, we found that pheromone sensitivity was inversely proportional to the length of the lysine stretch (Figure 3.3 B). Gβγ is known to facilitate the membrane localization of Gα (50); the addition of mating pheromone should decrease interaction between Gα and Gβγ, placing a greater burden on Gα to sustain its own membrane binding. In this way, the increased pheromone sensitivity supports our hypothesis that Gpa1 mutants with a shorter lysine-stretch will depend more greatly on palmitoylation.

To determine if Gpa1 mutants with short lysine-stretches require the palmitoylation site Cys for viability, we introduced the C3S substitution into each of our lysine mutants. Each mutant was expressed behind the endogenous GPA1 promoter in the YGS5 strain (ste11ts gpa1Δ). As shown above, we found that all lysine mutants were viable if the palmitoylation site was left intact (Figure 3.4 A). However, we also found that mutants of Gpa1 with one lysine or two lysines were not viable if palmitoylation was blocked (Figure
3.4 A). Mutants with 4 or more lysines were viable with or without the palmitoylated Cys, as was wild-type Gpa1 (Figure 3.4 A and data not shown). At 34°C, where the ste11 MAPKKK is not functional and signaling does not occur, all cells were equally viable showing that the defects were limited to membrane binding/Gβγ sequestering capability of the Gpa1 mutants (Figure 3.4 B). These data suggest that Gpa1^{G2A/1K} and Gpa1^{G2A/2K} could be used in a screen for proteins involved in Gpa1 palmitoylation.

None of the known PATs are alone necessary for palmitoylation of Gpa1

We have developed the first cell viability screen for Gpa1 palmitoylation. Given the genomic tools available in yeast, it now should be feasible to identify proteins involved in this process. We began by asking whether any of the known PATs was alone necessary for the palmitoylation of Gpa1. We would expect that the deletion of a critical PAT would result in inviability of the cells expressing Gpa1^{G2A/2K}. Using the YGS5 strain as above, we found that Gpa1^{G2A/2K} conferred viability in each of the known PAT deletions (Figure 3.5). Although it is difficult to make any broad conclusions from these data, our findings suggest that none of the known PATs are *alone* necessary for palmitoylation of Gpa1.
Figure 3.3

The palmitoylated Cys residue is necessary for viability of cells expressing Gpa1$^{G2A/1K}$ or Gpa1$^{G2A/2K}$, but not Gpa1$^{G2A/4K}$ or wild-type Gpa1. YGS5 strain cells were transformed with single copy plasmids pRS316-GPA1 or the indicated mutant in the pRS316-GPA1 plasmid, each containing the native GPA1 promoter region. Cells were grown at 34° overnight to saturation. 3µl of each saturated culture was spotted in serial 2-fold dilutions on selective media agar plates, and incubated either at 34° or room-temperature until colonies began to form (3-5 days).
Figure 3.4  No known PAT is necessary for Gpa1 palmitoylation. PAT genes disrupted with the KanMX resistance marker were amplified from BY4741-derived strains harboring the gene deletions and transformed into the YGS5 strain. The resulting strains were then transformed with single copy GPA1 plasmids and spotted as in Figure 3.3.
DISCUSSION

Screening for palmitoylation using poly-lysine mutants of Gpa1

The principle finding of this study is that mutants of Gpa1 that are not myristoylated but have a short poly-lysine stretch near the N-terminus can be used to screen for proper palmitoylation. The value of this new screening approach is that strains can be screened for viability rather than for subtle differences in the mating response. Using this new tool, we tested whether deletion of any of the known DHHC family PATs affected the palmitoylation status of Gpa1. We were unable to identify any single gene deletion that results in mis-palmitoylation of Gpa1. These findings represent only a preliminary investigation of Gpa1 palmitoylation, and are presented mainly to show the feasibility of this new screening method. In order to more fully understand the role played by the known DHHC family members in Gpa1 palmitoylation, we will need to repeat these experiments in cells in which various PATs are deleted in combination.

One of the interesting findings from this study is that a single lysine near the N-terminus of Gpa1 was sufficient to rescue the inviability associated with the lack of Gpa1 myristoylation. Addition of this single lysine (at amino acid position 9) was not sufficient, however, to fully rescue membrane localization, as monitored using a GFP-tagged version of the protein (data not shown). Our presumption is that the membrane-cytoplasm equilibrium of the Gpa1G2A/1K mutant is heavily in favor of cytoplasmic localization, however, the small amount of protein at the plasma membrane is sufficient to decrease Gβγ signaling to below the threshold required for growth arrest. Poly-basic stretches have been used previously to rescue the membrane localization of signaling proteins, such as Ras (103). The fact, however, that Gpa1G2A/1K was at all viable did come as a surprise, as there are no reports of a
single basic charge being sufficient for membrane attachment. In the N-terminal region, two separate small basic patches do exist (K21, R22 and K35, R37). It is likely that the single basic charge adds to a general basic nature already in existence in that area of the G protein, raising the charge above the threshold required to function as a membrane anchor.

**Redundancy among the PATs for Gpa1**

One potential explanation for the lack of phenotype in the PAT deletion strains is that redundancy exists among these proteins in their ability to palmitoylate Gpa1. This question was recently addressed in a genomic effort to identify all palmitoylated proteins in yeast (125). The authors used a three-step extraction protocol called acyl-biotinyl exchange (ABE), which allows for specific biotinylation of palmitoylated proteins. Using this procedure, Roth et al found that six of the seven known PATs must be deleted before Gpa1 is no longer palmitoylated.

Another possibility is that other, as yet unidentified, proteins have palmitoyl-transferase activity. Seven DHHC family members exist in yeast, however, it is possible that other non-DHHC proteins also have PAT function. Using Gpa1<sup>G2A/2K</sup> in a genome-wide screen would identify other PATs, and could potentially identify additional proteins required for G protein palmitoylation. Furthermore, the idea of using a short lysine stretch to replace a lipid modification could be used on any protein that contains multiple lipid modifications. Using our findings as a model, proteins other than Gα subunits could feasibly be made amenable for similar screens.
Acknowledgements

A special thanks to Bob Deschenes, Maurine Linder, and Cathy Berlot for their helpful discussion and willingness to lend their expertise.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—standard methods for growth, maintenance, and transformation were used throughout (9). Yeast strains used in this study were BY4741 (MATα leu2Δ met15Δ his3Δ ura3Δ), BY4741-derived mutants lacking GPA1 and STE7 (GPA1 was disrupted using a gpa1::hisG deletion plasmid that allows for deletion of Gpa1 and recovery of the URA3 marker; STE7 was disrupted using the KanMX G418-resistence marker; Yeast Deletion Clones, Invitrogen, Carlsbad, CA), YGS5 (MATα ura3-52 lys2 ade2Δ trp1 leu2-1 gpa1::hisG ste1Δts), and YGS5-derived strains lacking either APT1, AKR1, AKR2, SWF1, ERF2, PFA3, PFA4, or PFA5. Each gene was deleted using the KanMX G418-resistence marker.

Yeast shuttle plasmids used here were pRS316 (CEN, ampR, URA3), pRS315 (CEN, ampR, LEU2), pRS316-GPA1 which contains GPA1 under the control of its native promoter (139), pRS315-STE7 which contains STE7 under the control of its native promoter (161), and pRS316-GPA1C3S (138). pRS316-GPA1G2A/1K was created by site directed mutagenesis using pRS316-GPA1G2A (139) as a template and mutagenic primer 5’- CA GTG AGT ACG CAA AAA ATA GGA GAC GAA AGT GAT CC – 3’ and its complement. pRS-GPA1G2A/2K and pRS-GPA1G2A/4K were created using pRS316-GPA1G2A/1K as a template and the mutagenic primers 5’- GCG TGT ACA GTG AGT ACG AAA AAA ATA GGA GAC GAA AGT GAT CC – 3’ (for 2K) and 5’- GCA TGT ACA GTG AGT ACG AAA AAA AAA
AAA GGA GAC GAA AGT GAT CC – 3' (for 4K), and their complements, respectively. The “C3S” mutation was added to each of the pRS316-GPA1 constructs described above using site directed mutagenesis and the various plasmids described above as templates. Mutagenic primers were 5’- AGG TAG GAA ATA ATG GGG TCT ACA GTG AGT ACG CAA – 3’ and its complement.

*Pheromone sensitivity/growth arrest assay (Halo Assay)*—halo assays were performed as described previously (74). Briefly, cells were grown to saturation in selective media and 100 µl of the culture was added to a 4 ml of liquid 0.5% agar solution (melted and cooled to 55°C). Agar-cell solution was spread onto selective plates and allowed to solidify. Various concentrations of α mating factor (pheromone) were added to paper discs, which were then applied to the solidified agar. Cells were allowed to grow for 48 hours, and zones of growth inhibition measured.

*Viability Screen (serial dilutions)*—viability of cells was determined by growing cells to saturation at the permissive temperature (34°C). Saturated cells were diluted in sterile water in 1:2 serial dilutions; and 3µl of each dilution was spotted onto a selective media-agar plate and allowed to dry. Plates were spotted in duplicate, and one plate was incubated at 34°C while the other was incubated at room temperature. After 4 days, cells plates were scored for viability (+/- growth and # of colony forming units).
CHAPTER IV

Identification and Characterization of a Non-Receptor Exchange Factor for the Yeast

Gα Subunit Gpa1

Elements of the work referenced in this chapter has been accepted for publication:


All figures contributed by Michael J. Lee
SUMMARY

G protein-coupled receptors (GPCRs) mediate responses to a broad range of chemical and environmental signals. In yeast a pheromone-binding GPCR triggers events leading to the fusion of haploid cells. In general, GPCRs function as guanine nucleotide exchange factors (GEFs); upon agonist binding the receptor induces a conformational change in the G protein α subunit, resulting in exchange of GDP for GTP and in signal initiation. Signaling is terminated when GTP is hydrolyzed to GDP (141). This well-established paradigm has in recent years been revised to include new components that alter the rates of GDP release, GTP binding (1, 15, 27, 43, 69, 151, 168), and GTP hydrolysis (41, 111). Here we report the discovery of a non-receptor GEF, Arr4. Like receptors, Arr4 binds directly to the G protein, accelerates guanine nucleotide exchange, and stabilizes the nucleotide-free state of the α subunit. Moreover, Arr4 promotes G protein-dependent cellular responses including mitogen-activated protein kinase (MAPK) phosphorylation, new gene transcription and mating. In contrast to known GPCRs, however, Arr4 is not a transmembrane receptor, but rather a soluble intracellular protein. Our data suggest that intracellular proteins function in cooperation with mating pheromones to amplify G protein signaling, thereby leading to full pathway activation.
INTRODUCTION

Heterotrimeric G proteins respond to a wide variety of chemical and sensory signals and are highly conserved in eukaryotic organisms, including yeast and humans. In the well-established paradigm of G protein signaling (61) inactive Gα-GDP associates with both a seven-transmembrane spanning G protein coupled receptor (GPCR) and the Gβγ dimer. Upon ligand binding, GPCRs act as guanine nucleotide exchange factors (GEFs), inducing conformational changes in the three flexible “switch” regions in Gα, resulting in GDP release, and subsequent GTP binding. GTP-bound Gα no longer interacts with Gβγ, and both entities are free to activate downstream effectors. The signal is terminated when Gα hydrolyzes GTP to GDP, and the inactive heterotrimer is reformed.

This paradigm, although still largely true, has been expanded to account for the discovery of additional proteins involved in the G protein signaling cycle. Proteins in the Regulator of G protein Signaling (RGS) family were discovered 10 years ago to function as GTPase Accelerating Proteins, or GAPs, for Gα subunits (110). More recently, proteins in the GoLoco family were discovered to function as Guanine-nucleotide Dissociation Inhibitors, or GDIs, for Gα subunits (168). Both RGS and GoLoco proteins have since proven to be critical components of G protein signaling.

In the yeast Saccharomyces cerevisiae, a single heterotrimeric G protein regulates a cell fusion event called mating. Yeast can exist as haploid cells of either the a- or α- mating type and secrete mating pheromones, a-factor and α-factor, respectively. Binding of pheromone to receptors on adjacent cells induces growth arrest in G1, polarized growth towards the mating partner, and new gene transcription in preparation for cell fusion. Most of the components of this signaling cascade were identified genetically as deletions that
resulted in a mating-deficient (sterile) phenotype. Included are genes that encode a GPCR (STE2), Gβ and Gγ subunit (STE4 and STE18), but not a Gα. Deletion of Gpa1, the Gα of the mating pathway, does not result in a sterile phenotype, but rather a constitutively active signal, due to the uncontrolled activation of Gβγ. Subsequent data showing that Gβγ was both necessary and sufficient to activate the mating signal left the conclusion that Gα played no active role in this process (as reviewed in (42).

We recently reported, however, that a constitutively active (GTPase-deficient) mutant Gpa1 alone activates some components of the mating response (136). We were surprised to find that this required both the regulatory and catalytic components of the sole yeast phosphatidyl inositol-3 kinase (PI3K), Vps15 and Vps34. This was particularly interesting because Vps15/Vps34 are known to exist primarily at the late endosome in yeast. Subsequently, we determined that activated Gpa1 also exists at late endosomes, binds to Vps34, and activates Vps34 kinase activity. Thus, our conclusion was that Gpa1 at late endosomes activates a unique signal through the PI3K Vps34. Although G proteins have long been known to exist at intracellular sites, this was the first evidence in any system of an active signaling function of a G protein away from the cell periphery.

Further investigation also revealed that Vps15 is structurally similar to known Gβ subunits. In fact, Vps15 is also functionally similar to known Gβ subunits, in that it: 1) interacts directly with Gpa1 in a GDP dependent manner, and 2) is required for the proper localization of Gpa1. Thus our findings suggest that Vps15 is an atypical Gβ subunit for Gpa1 at the late endosome. Having established the existence of atypical effectors (endosomal Vps34) and atypical Gβs (Vps15), we next chose to determine whether atypical activators also exist in yeast. Activation of Gα subunits by non-receptor proteins has been
shown recently for Ric-8a and some of the AGS proteins (15, 151). Using a candidate gene approach, we screened for genes that activate Gpa1. Here, we report the identification of a novel GEF for Gpa1, a protein called Arr4. Arr4 was previously identified in a yeast-2-hybrid screen as a Gpa1 interacting protein (75). Using biochemical, genetic, and cellular analyses, we determine that Arr4 acts as a GEF for Gpa1 in vitro and is an activator of Gpa1 signaling in vivo. Furthermore, we find that activation of Gpa1 by Arr4 is necessary for normal pheromone-induced signaling. Cells that lack Arr4 show lower levels of pheromone-induced transcription, and lower levels of mating. Our findings contribute to the growing understanding that non-receptor GEFs contribute to signaling through heterotrimeric G proteins.

RESULTS

Screen for a novel GEF in yeast

Heterotrimeric G proteins are well known to function as signal transducers, coupling receptors at the cell surface to specific enzymes inside the cell. Recent research has uncovered the existence of non-receptor activators of G protein signaling, including a non-receptor GEF Ric-8a (1, 15, 27, 43, 69, 151). It is not known however whether such factors contribute to GPCR signaling. A number of screens have been performed in yeast, specifically to identify non-receptor activators, but none have attempted to identify endogenous yeast proteins.

To address this question, we investigated if the yeast mating-response pathway makes use of a non-receptor GEF. When designing our screen, we first considered that there is no common domain, or signature sequence, or predictive fold that is indicative of GEF activity.
~30 candidate Gpa1-associated proteins

7 bind Gpa1-GDP > Gpa1-GTP
2 bind Gpa1<sup>ND</sup> > Gpa1-GDP
Figure 4.1 Screen for a novel GEF in yeast. The screen for unidentified GEFs focused on two Gα binding characteristics: nucleotide-dependent interaction, and selective binding to the dominant negative mutant Gpa1$^{N385D}$. Our screen covered 27 proteins, all previously shown to interact with Gpa1. All experiments were performed using TAP-tagged versions of candidate GEFs. TAP purified cell extracts were probed with anti-Gpa1 antibodies to monitor Gpa1 binding.
Table 4.1 Nucleotide-dependent Gpa1-binding proteins. Of 27 proteins tested, seven were found to bind to Gpa1 in a nucleotide-dependent manner, similar to other GEFs. Two of these seven proteins also bound preferentially to the Gpa1<sup>N388D</sup> mutant. Binding was determined by TAP purification in cells expressing Gpa1, Gpa1<sup>Q323L</sup> or Gpa1<sup>N388D</sup>. Data are from 3 separate experiments.

<table>
<thead>
<tr>
<th>Systematic Name</th>
<th>Standard Name</th>
<th>Function</th>
<th>GDP-dependent</th>
<th>N388D dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>YDL100C</td>
<td>ARR4</td>
<td>Possible ATPase, subunit of GET complex; Golgi-to-ER transport</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>YBL016W</td>
<td>FUS3</td>
<td>Mating MAPK; activated by Ste7; regulates specificity of mating versus invasive growth</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>YHR194W</td>
<td>MDM31</td>
<td>Mitochondrial inner membrane protein; unknown function</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>YMR069W</td>
<td>NAT4</td>
<td>N-acetyltransferase; acetylates histones H4 and H2A</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>YNL119W</td>
<td>NCS2</td>
<td>Uncharacterized ORF; role in invasive growth</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>YDR028C</td>
<td>REG1</td>
<td>Regulatory subunit of protein phosphatase; regulation of glucose repressible genes</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>YDR356W</td>
<td>SPC110</td>
<td>Inner plaque spindle pole body component; connects nuclear MT to SPB</td>
<td>Y</td>
<td>N</td>
</tr>
</tbody>
</table>
In the absence of these physical features, we chose nucleotide-dependent binding for our screening criteria. Accordingly, we considered 27 proteins reported to bind to the yeast Gα subunit Gpa1 (Table 4.1). Of the proteins we tested, seven exhibited preferential interaction with GDP-bound Gpa1 compared to the constitutively-active GTP-bound Gpa1^{Q323L} (Figure 4.1). GDP-dependent binding is a common characteristic of GEFs for small GTPases (122), as well as for Ric-8a (151). Since Gβ subunits and other GDIs also bind preferentially to Gα in the GDP-bound state, we next determined which of the candidate proteins preferentially interact with the Gα mutant Gpa1^{N388D}, previously determined to form a stable but non-productive complex with a known GPCR and GEF Ste2 (binding was observed even in the absence of pheromone) (171). Our rationale was that the known GEF bound preferentially to this mutant, so we anticipated that the mutant G protein might bind preferentially to other proteins having exchange factor activity as well. Of the seven candidates tested, only Arr4 and Reg1 bound preferentially to Gpa1^{N388D}. Notably, Arr4 is expressed as a dimer, as are many GPCRs including Ste2 (101, 115). Relative to total expression Arr4 co-purified 8-fold more Gpa1^{N388D} than Gpa1, and 3-fold more Gpa1 than Gpa1^{Q323L} (Figure 4.2 A and B). A similar preference was shown previously for binding to Ste2 (171).

**Direct, nucleotide-dependent, subtype-selective interaction between Arr4 and Gpa1**

We next investigated whether Arr4 over-expression activates G protein signaling in vivo. Over-expression of a GEF, even in the absence of any stimulus, often increases basal activation of the G protein. For example, over-expression of Ric-8a leads to increased basal phospho-ERK production in CHO cells (95). Similarly, we found that over-expression of *ARR4* induced a 3.5-fold increase in the basal transcription of Gpa1-dependent mating genes.
Figure 4.2  

**Arr4 as a GEF candidate.** (A) Cells expressing Arr4-TAP were transformed with vector (pAD4M) containing either no insert, GPA1, GPA1^{Q323L} or GPA1^{N388D}. TAP fusion protein was purified (IP) using Calmodulin-Sepharose resin, eluted in SDS-PAGE sample buffer, and resolved by immunoblotting (IB) with Gpa1 antibody. Gpa1 typically migrates as a doublet of 52 and 54 kDa, representing the myristoylated and unmyristoylated forms of the protein, respectively. (B) Densitometry of data in panel A expressed as Gpa1 bound relative to total Gpa1 expressed. Data are mean ± SD of 3 separate experiments. (C) BY4741 wild-type cells co-expressing FUS1-lacZ and vector (pAD4M) containing either no insert, ARR4, or dimerization-deficient mutant arr4^{CCTT} were monitored for β-galactosidase activity. (D) Effect of known mating components on Arr4 activity. Experiment performed as in (C), except that receptor (ste2) or MAPK kinase (ste7) deletion strains were used when noted. Results for (C) and (D) are the mean ± SEM for 3 individual experiments each performed in triplicate.
Over-expression of the receptor Ste2 yields a comparable (~ 3-fold) increase in basal \textit{FUS1-lacZ} expression (data not shown), while pheromone binding produces an even larger (~50-fold) increase in activity. This phenotype was dependent on the function of Arr4, as over-expression of a dimerization-deficient mutant Arr4\textsuperscript{CCTT} had no effect (101). Furthermore, Arr4-stimulated transcription required mating pathway effectors, such as Ste7, but not the pheromone receptor Ste2 (Figure 4.2 D). Basal activation by Arr4 was equivalent in the presence and absence of receptor; however the baseline activation was higher in the absence of receptor, as noted previously (67). The mechanism by which Ste2 dampens basal activity is unknown, but may result from sequestration of the G protein heterotrimer and dampening of spontaneous activation in the absence of a pheromone signal. These data suggest that Arr4 functions as a GEF for Gpa1 \textit{in vivo}.

To determine whether the interaction between Arr4 and Gpa1 is direct or facilitated by another protein, we purified 6xHIS-Gpa1 and GST-Arr4 from \textit{E. coli} and reconstituted these proteins in the presence of GDP or GDP plus AlF\textsubscript{4} (a transition state mimic that induces the activated conformation of G\(\alpha\)). Consistent with our \textit{in vivo} observations, purified Arr4 co-precipitated more efficiently with the GDP-bound form of Gpa1 than with GDP-AlF\textsubscript{4}-bound Gpa1 (Figure 4.3 B, lanes 1-6). Arr4 did not interact with Gpa2, the only other G\(\alpha\) subunit in yeast (Figure 4.3 B, lanes 7-12).

Arr4 was shown to be a copper-binding protein, and copper induces Arr4 dimer formation (101). As noted above Arr4 dimerization is required for Gpa1-mediated signaling \textit{in vivo} (Figure 4.2 C). Dimerization is likewise required for strong binding to G\(\alpha\), since Arr4 in the absence of copper bound poorly to Gpa1 \textit{in vitro} (Figure 4.3 A). Copper did not
Figure 4.3  

**A**

| 6xHIS-Gpa1 | + | + | + | + | + | + | + | + | + | + | + |
| GST-Arr4<sub>CTT</sub> | - | - | - | - | - | - | - | - | - | - | - |
| GST-Arr4 | - | + | + | - | + | - | - | - | - | - | - |
| GST | + | + | + | + | + | + | + | + | + | + | + |
| CuSO<sub>4</sub> | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

**IP:** GST  
**IB:** Gpa1  

Applied

---

**B**

| 6xHIS-Gpa1 | + | + | + | + | + | + | + | + | + | + | + |
| 6xHIS-Gpa2 | - | - | - | - | - | - | - | - | - | - | - |
| GST-Ar14 | - | + | - | - | + | - | - | - | - | - | - |
| GST | + | + | + | + | + | + | + | + | + | + | + |
| AlF<sub>4</sub> | - | - | - | + | + | - | - | - | + | + | + |

**IP:** GST  
**IB:** 6xHIS

---

**Figure 4.3  Arr4 binds directly to Gpa1.** (A) and (B) Experiments performed using recombinant purified components (*E. coli*). 100 nM of each protein was combined, purified with GST-Sepharose resin (IP), resolved by SDS-PAGE and probed with penta-HIS, Gpa1, or GST antibodies (IB). Note that the same protein preparations were used in the functional assays presented in Fig. 3. (A) Binding with or without 150 nM CuSO<sub>4</sub> (Cu) when noted. (B) Arr4 binding to Gpa1 *versus* Gpa2 was performed as in panel A except that CuSO<sub>4</sub> was present in all lanes.
stimulate binding between Gpa1 and the dimerization deficient Arr4\textsuperscript{CCTT} mutant (Figure 4.3A), consistent with the established evidence that copper binding induces dimerization. These data together show that dimerized Arr4 activates Gpa1-mediated signaling, and interacts with Gpa1 in a direct, subtype-selective, and GDP-dependent manner consistent with known GEFs.

**Arr4 stabilizes nucleotide-free Gpa1**

GPCRs are known to bind to and stabilize the nucleotide-free form of G\textalpha{} proteins. To determine if Arr4 can perform this function, we resolved purified Arr4 and Gpa1 by steric-exclusion chromatography. Proteins were detected by absorbance, by immunoblotting, and by protein staining (Figure 4.4, and data not shown). In the presence of copper, Arr4 and Gpa1 co-eluted with a mass corresponding to that predicted for the Arr4-dimer/Gpa1 complex at a 2:1 stoichiometry. This complex was disrupted by the addition of excess GDP, yielding two distinct peaks: one at the predicted mass of Gpa1 and another corresponding to dimerized Arr4. The Arr4 dimer was further dissociated by the removal of copper (Figure 4.4). These results demonstrate that Arr4 dimer binds stably to the nucleotide-free form of G\textalpha{}.

**Arr4 promotes nucleotide exchange on Gpa1 in vitro**

In the absence of a GEF, the rate-limiting step in the G protein nucleotide cycle is the release of GDP (53). A GEF accelerates GTP binding by stabilizing the nucleotide-free state of G\textalpha{}, thereby promoting GDP release. Using purified components, we found that a 2:1 molar ratio of Arr4 to Gpa1 accelerates the rate of GTP binding, from 0.085 pmol to 0.36
Figure 4.4  

Arr4 forms a stable complex with Gpa1 that can be disrupted with excess nucleotide. 4 mg of 6xHIS-Arr4 and 2 mg of 6xHIS-Gpa1 were combined and resolved by steric-exclusion chromatography in the presence or absence of excess GDP and CuSO₄, as indicated. Note that the void volume elutes 200 minutes after sample loading. Top panel, A₂₆₀nm chromatogram. The peak of UV absorbance in the void volume is evidently due to a non-protein buffer component, as determined by Coomassie staining as well as by immunoblotting with penta-His antibodies. Bottom panels, immunoblots using penta-HIS antibody. 20 ml of each 7 ml elution fraction were loaded and resolved by SDS-PAGE. All data are representative of 3 separate experiments.
pmol GTPγS·pmol Gpa1⁻¹·min⁻¹ (Figure 4.5 A). This is comparable to the GEF activity reported for Ric-8a (1, 151) (the GEF activity of the receptor Ste2 has never been quantified). In the absence of copper Arr4 had almost no effect on GTPγS binding to Gpa1 (Figure 4.5 B). Arr4 alone, with or without copper, could not itself bind GTPγS (Figure 4.5 A and B).

Because GEFs function at the rate-limiting step in the GTPase cycle, accelerating nucleotide exchange should also increase the rate of GTP hydrolysis. As a second measure of Arr4 GEF activity, we showed that Arr4 accelerates the rate of $[^{32}P]GTP$ hydrolysis, from 0.009 to 0.021 pmol P·pmol Gpa1⁻¹·min⁻¹ (Figure 4.5 C). Arr4 itself, with or without copper, could not hydrolyze GTP. We also purified a related human protein (hAsna-I) (84), but detected no GEF activity towards Gpa1, Gαi or Gαs (data not shown).

**Arr4 promotes pheromone signaling in vivo**

To determine the role of Arr4 in GPCR signaling, we used three measures of pathway activity: MAPK activation, gene transcription, and mating. Pheromone is known to stimulate a downstream kinase cascade consisting of Ste11, Ste7, and the partially redundant MAPKs Fus3 and Kss1. To determine if Arr4 modulates this response pathway, we first measured phosphorylation of Fus3 and Kss1 using a phospho-MAPK specific antibody. As shown in Figure 4.6 A, basal and pheromone-induced MAPK activation was substantially diminished in arr4 mutant cells. We also tested salt stress-dependent activation of another MAPK, Hog1. In this case we found no difference between wild-type cells and mutant cells that lack ARR4 (Figure 4.6 B).

Activation of the MAPKs Fus3/Kss1 results in selective induction of genes, such as *FUS1*. To further confirm that Arr4 is a component of the mating-response pathway, we
Figure 4.5
Figure 4.5  **Arr4 is a GEF for Gpa1.** (A) and (B) Time-course of $[^{35}S]GTP\gamma S$ binding to 100 nM purified 6xHIS-Gpa1 in the presence of 200 nM GST-Arr4. Results are the mean ± SEM of duplicate samples, and are presented as percent of maximum bound (saturated binding occurred between 50-75%). (A) with 500 nM CuSO$_4$ added. (B) Same as in (A), but no copper added to the reaction. (C) Time-course of P$_i$ released from $[^{32}P]GTP$ in the presence or absence of 6xHIS-Gpa1 (250 nM), GST-Arr4 (500 nM), and copper (500 nM). Results are the mean ± SEM of duplicate samples.
measured transcription of mating-specific genes using the highly selective FUS1-lacZ transcription reporter. Compared to wild-type, deletion of ARR4 caused a ~50% decrease in pheromone-induced transcription (Figure 4.6 C). Finally, to determine directly if Arr4 contributes to cell fusion, we measured mating efficiency of MATα cells with wild-type MATa cells or MATa cells that lack ARR4. When compared to wild-type, arr4 mutant cells were ~40% less likely to mate successfully (Figure 4.6 D). The observed decrease in mating efficiency indicates that Arr4 contributes to mating, but is not essential for this activity. Deletion of other pathway modulators that bind Gpa1, such as the effector protein Vps34 and the RGS protein Sst2, exhibit a comparable degree of mating impairment (21, 22, 136). The observed reduction in mating mirrors the reduction in MAPK and transcription-reporter activities, and further establishes Arr4 as a positive modulator of the mating pathway.

DISCUSSION

For more than 20 years the yeast pheromone-response pathway has served as a model system for G protein signaling (41). For the last decade, our understanding of the proteins involved in this pathway has remained relatively unchanged. Here, we report the discovery of a novel activator of yeast mating, one operating upstream of the G protein in conjunction with cell surface pheromone receptors. Specifically, we find that Arr4 binds directly to the Gα subunit Gpa1 and induces nucleotide exchange. Arr4 binds as a dimer, since the stoichiometry of the Arr4-Gpa1 complex is 2:1. Binding is selective, since Arr4 co-precipitates with Gpa1 but not Gpa2. Binding is stable, since the complex can be resolved by steric-exclusion chromatography. Binding appears to be ligand-dependent, since the complex is dissociated by removal of copper. Most of these behaviors are characteristic of known
Figure 4.6

A

<table>
<thead>
<tr>
<th></th>
<th>Vector</th>
<th>arr4Δ</th>
<th>minutes post pheromone</th>
<th>Kss1 (MAPK)</th>
<th>Fus3 (MAPK)</th>
<th>Pgk1 (loading control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0  5  15 30  60  90</td>
<td>0  5  15 30  60  90</td>
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</table>

B

![Graph](image3.png)

C

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>MATING EFFICIENCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100 ± 12.06%</td>
</tr>
<tr>
<td>arr4Δ</td>
<td>61 ± 8.32%</td>
</tr>
<tr>
<td>Ste7Δ</td>
<td>0 ± 0.00%</td>
</tr>
</tbody>
</table>
Figure 4.6 Arr4 is necessary for maximal transmission of the mating signal. (A) Top BY4741 or BY4741-derived cells lacking the ARR4 gene were treated with 3 μM α-factor mating pheromone and samples were removed at times indicated. MAPK activation was determined by immunoblotting using a phospho-p44/42 MAPK antibody. Pkg1, loading control. Bottom As above, however, cells were treated with 500 mM KCl, and MAPK activation was determined using a phospho-p38 MAPK antibody. (B) Cells as in (A) were transformed with pRS423-FUS1-lacZ, and treated with the indicated concentrations of mating pheromone for 90 mins. Results are the mean ± SEM for 3 individual experiments each performed in triplicate. (C) DC17 MATα cells were mixed with BY4741 (wild-type MATa cells), arr4 deletion, or ste7 deletion as a control. Mating was performed by co-incubation of cells on nitrocellulose filters. Maximum mating efficiency of the wild-type cells was approximately 75%. Percent mating efficiency was calculated as number of diploids/total number of MATa cells. Data are mean ± SD of 3 separate experiments.
GPCRs, including the pheromone receptor Ste2. Finally, using complementary assays of mating pathway activation, we find that Arr4 promotes Fus3 (but not Hog1) MAPK activation, promotes Fus3-mediated gene induction, and enhances mating efficiency. Again, all of these behaviors are characteristic of pheromone receptor activation. Taken together, our results suggest that Arr4 functions as a GEF for Gpa1, and this GEF activity serves to amplify the pheromone-response pathway.

It is worth emphasizing that all of the bioassays used here are highly specific for the mating pathway. Fus3 and the transcription reporter *FUS1-lacZ* are activated only in mating-competent haploid cells, and only upon stimulation by mating pheromones. Neither response occurs in the absence of a functional G protein. While it is conceivable that Arr4 has additional functions in the cell, Arr4 clearly binds selectively and directly to Gpa1, and loss of this binding has significant consequences for the pheromone response. Moreover, comparable differences were observed for MAP kinase activity, transcription induction, and mating efficiency. Such differences are likely to be especially important in non-ideal (non-laboratory) growth conditions.

Arr4 binds preferentially to the nucleotide-free form of Gpa1, although binding can still be detected for the GDP-bound form of the protein. Other cellular proteins, such as Gβγ, may further modulate the nucleotide-dependence of binding. Our working model, fully supported by the data, is that Arr4 acts after dissociation of G protein subunits, and thereby sustains the signal. Alternatively, Arr4 could function like Ric-8a to promote GTP binding to Gpa1 directly, in the absence of bound Gβγ. This model seems plausible considering that Gα expression was estimated to be ~2-fold higher than that of Gβγ (59). Moreover, we have shown that Arr4 binds stably to Gα in the absence of Gβγ (Fig. 2). Other possibilities are that
Arr4 activates the G protein heterotrimer in cooperation with pheromone receptors, or competes with Gβγ for binding to Gα. Testing these more complex models will be challenging, since they require the ability to express and purify functional receptor and Gβγ.

Another open question is how Arr4 is itself regulated, and whether Arr4 is activated by any internal or external stimulus. Arr4 was originally named because of its resemblance to bacterial ARsenicals Resistance proteins. More recently, the ARR4 gene was implicated through hierarchical clustering analysis to be involved in ER/Golgi trafficking, and renamed GET3 (131). ARR4/GET3 was also shown to function as an extragenic suppressor of npl4, encoding a component in the ubiquitin-proteasome system (8). Gpa1 is ubiquitinated, but a role for Gpa1 activation in its ubiquitination has not been established (94). Another possibility is that Arr4 is dynamically regulated by copper. In fact, Arr4 has been reported to localize to the cytoplasm but then translocate to punctate intracellular structures when cells are stressed with heat and/or metal ions (132). The effect of these stresses on copper homeostasis is not known however. Regardless, Gpa1 is expressed on the cytoplasmic face of the plasma membrane and would therefore be fully accessible to Arr4 in vivo.

Identification of non-receptor exchange factors has been difficult, as no signature sequence or domain exists that accurately predicts activity. Indeed, there is no sequence similarity and no common predicted fold among receptor and non-receptor GEFs. Here we have identified a novel GEF based on its preferential binding to the Gpa1N388D mutant. Our data suggest that analogous mutations in other Gα proteins could be used to identify GEFs in other systems as well. Our genetic studies in yeast reveal a critical role for Arr4 signaling in vivo, and suggest further that non-receptor GEFs may be more common and have a broader physiological function than previously appreciated.
ACKNOWLEDGMENTS

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EXPERIMENTAL PROCEDURES

Strains and plasmids—Standard methods for growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout. Yeast *Saccharomyces cerevisiae* strains used in this study were BY4741 (*MATa* *leu2Δ* *met15Δ* *his3Δ* *ura3Δ*), BY4741-derived mutants constructed using the KanMX G418-resistance marker (Yeast Deletion Clones, Invitrogen; originally purchased from Research Genetics), BY4741-derived strains containing a C-terminal tandem affinity purification (TAP) tag (Yeast TAP-Fusion Library, Open Biosystems), and DC17 (*MATa* *his1*). The arr4Δ strain from Research Genetics did not produce a phenotype that could be rescued by addition of a plasmid-borne copy of *ARR4*, so the strain was remade by PCR amplification of the original deletion cassette and transformation of the parent strain.

Yeast shuttle plasmids used were pRS305 (amp\(^R\), *LEU2*) and pAD4M (2\(\mu\m,\) amp\(^R\), *LEU2*, *ADH1* promoter and terminator). Expression plasmids described previously were pAD4M-GPA1 (139), pAD4M-GPA1\(^{Q323L}\) (62), pRS423-FUS1-lacZ (74). The pAD4M-GPA1\(^{N388D}\) plasmid was created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and mutagenic primer 5’-CG TTC AGA ACG CAG GCC TCC AGC GTC GA-
3’ and its complement. pAD4M-ARR4 was constructed by PCR amplification of BY4741 genomic DNA, followed by XmaI and SalI digestion, and ligation into the corresponding sites of pAD4M. Primers used were: forward 5’-TCC CCC GGG GGA CGT ACG ACA AGA ACA AGA AGA TC-3’ and reverse 5’-CGC GTC GAC GTC CTG AGC TGG GCT TAT TAC TTC GTT GC-3’. The pRS305-ARR4 plasmid was created using a similar method; however, the forward primer amplified a region beginning approximately 600 bp upstream of the start ATG (forward primer sequence: 5’-TCC CCC GGG GGA GCA TCA TCC TTT CCC ACA TTT GAT G-3’). The arr4\textsuperscript{CCTT} mutations were created using QuikChange and mutagenic primer 5’-GAT CAA GAG CAC AAC ACT AAG AGA ACT CAG GCA AGA TGG AAG ATG-3’ and its complement.

Screen for candidate Gpa1-binding partners from yeast—To identify candidate GEFs, we considered 27 proteins reported to bind to the yeast Gα subunit Gpa1. This list was compiled from published genome-wide yeast-2-hybrid screens, protein co-purification screens, as well as unpublished data from our laboratory (76, 83). The first criterion for candidate GEFs was nucleotide-dependent interaction with the G protein. We have shown previously that the GPCR Ste2 (even in the absence of pheromone) binds more tightly to Gpa1\textsuperscript{N388D} than to the wild-type form of the G protein (171). Plasmids pAD4M, pAD4M-GPA1, pAD4M-GPA1\textsuperscript{Q323L}, or pAD4M-GPA1\textsuperscript{N388D} were transformed into yeast strains expressing TAP-tagged fusions of candidate Gpa1-binding proteins. Purification of TAP fusions was performed as described previously (136). Briefly, cells were grown to mid-log phase in selective media, and harvested by centrifugation at 3,000 x g. The remaining steps were performed at 4° C. Cell pellets were resuspended in lysis buffer (50 mM NaPO₄ pH 7.5, 400 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.5 mM DTT, 25 mM NaF, 25 mM 2-
glycerophosphate, 1 mM NaVO₃, 5 mM PMSF, and one pellet per 50 ml buffer of complete EDTA-free protease inhibitor cocktail (Roche Applied Science)). (buffer composition indicates final concentrations). Cells were disrupted by vortexing with glass beads 8 x 30 s with 1 min of cooling on ice in between. Lysates were centrifuged at 6,000 x g for 5 min and again for 25 min, and the resulting supernatant was mixed with Calmodulin Affinity Resin (Stratagene). Cells were rotated at 4°C for 2 h, and washed 4 times with 1 ml of lysis buffer. Bound protein was eluted in 2x SDS-PAGE sample buffer at 65°C for 5 min. Samples were resolved by 8% SDS-PAGE and immunoblotting with anti-Gpa1 polyclonal antibodies at 1:1000 for 2 h, and visualized using horse radish peroxidase-conjugated secondary antibodies and chemiluminescence detection.

_**E. coli expression of GST- and 6xHIS fusion proteins**—6xHIS-Gpa1 expression plasmid was described previously (4). Plasmids for bacterial expression of GST-Arr4 and 6xHIS-Arr4 fusion protein were generated by ligation-independent cloning as described previously (143). _**ARR4**_ was PCR-amplified from genomic DNA (Forward primer: 5’—TAC TTC CAA TCC AAT CGC ATG GAT TTA ACC GTG GAA CC—3’; reverse primer: 5’—TTA TCC ACT TCC AAT GCG CTA CTA TTC TTT ATC TTC TAA CTC—3’), annealed to the gapped 6XHIS vector pMCSG7 or a version of pMCSG7 modified to contain a GST tag (from Jason Snyder and John Sondek, Univ. North Carolina), and transformed into BL21 (DE3) _**E. coli**_. Overnight cultures from single colonies grown at 37°C in Luria Broth (LB) supplemented with 100 µg/ml carbenicillin were diluted 1:100 into fresh media and grown to A₆₀₀ nm ~ 0.7. 6xHIS-Gpa1 and 6xHIS-Gpa2 expression was induced by addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside and incubation at room temperature for 5 h with shaking. GST-Arr4 was induced by addition of 0.1 mM isopropyl β-D-1-
thiogalactopyranoside and incubation at 18°C for 14 h. Cells were harvested by centrifugation, resuspended in Buffer A (20 mM Tris pH 8.0, 200 mM NaCl, 5% glycerol, 1 mM DTT), and homogenized using an Emulsiflex-C5 Homogenizer (Avestin Inc.). Lysates were clarified by centrifugation at 12,000 x g for 30 min and the resulting supernatant was mixed with Glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech AB) equilibrated with Buffer A, and incubated for 2 h at 4°C with gentle rotation. Resin was collected by centrifugation at 500 x g for 5 min and washed 3 times in Buffer A, 10 mM reduced glutathione. Elution was concentrated using Vivaspin concentrators (Vivascience AG).

In vitro co-precipitation of GST fusion proteins—6xHIS-Gpa1 was equilibrated in binding buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.1% Triton, 1 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 10 μM GDP) for 20 min at 16°C. GST-Arr4 was added, and allowed to incubate at 16°C for 20 min with gentle mixing. Samples were then mixed with 50 ml of a 50% slurry of Glutathione-Sepharose 4B resin equilibrated with binding buffer. The total volume of the reaction was 500 μl. Protein was incubated in the presence of GST resin for 1 h, then centrifuged at 500 x g, and washed three times with binding buffer for 10 min each. Bound proteins were eluted with 10 mM reduced glutathione in binding buffer and resolved by 10% SDS-PAGE, and immunoblotting with penta-HIS antibody (Qiagen), Gpa1 polyclonal antibody or GST antibody (from Joan Steitz, Yale University) (all at 1:1000).

Steric-exclusion chromatography—Purified protein was subject to steric-exclusion chromatography using an Akta FPLC system and a Sephacryl 26/60 S200 column (GE Healthcare). Protein was equilibrated in 20 mM Tris pH 8.0, 100 mM NaCl, 5% glycerol, 1 mM DTT, 2 mM MgCl₂, 20 μM GDP (when added), and 150 nM CuSO₄ (when added). Protein was separated at 0.5 ml/min, and collected in 7 ml fractions. UV absorbance of each
fraction (A_{280nm}) was recorded and data analyzed using the UNICORN program associated with the FPLC system. A 20 µl sample from each fraction was separated by SDS-PAGE and either probed with penta-HIS antibody or stained with Coomassie Brilliant Blue R250 (Bio-Rad).

**GTP binding assay**—GTPγS binding was measured using a filter binding method, as previously described (4). Briefly, purified proteins were diluted to desired concentrations in assay buffer (50 mM HEPES pH 8.0, 1 mM EDTA, 0.1% C_{12}E_{10}, 1 mM DTT, 2 mM MgCl_{2}, 2 µM GTPγS), and reactions initiated by addition of [^{35}S]GTPγS at 20°C (2,000 cpm/fmol; Perkin Elmer). Duplicate aliquots were removed at timed intervals, and quenched by addition of ice-cold stop buffer (20 mM Tris pH 7.4, 125 mM NaCl, 25 mM MgCl_{2}). Quenched reactions were passed through nitrocellulose membranes using vacuum and washed 4 times with ice-cold stop buffer. Non-specific binding was determined in parallel reactions by addition of 100-fold excess of unlabelled GTPγS to the reactions. Binding was quantified using liquid scintillation counting.

**Steady state GTP hydrolysis assay**—Steady state GTPase reactions were performed as described previously (4). Briefly, purified proteins were diluted as in the GTP binding assay and reactions initiated by addition of an equal volume of assay buffer containing [γ^{32}P]GTP (2,000 cpm/fmol; Perkin Elmer). Duplicate aliquots were removed at timed intervals and quenched with ice-cold activated charcoal in 20 mM phosphoric acid. Charcoal was separated by centrifugation, and the supernatant subjected to scintillation counting to quantify released [^{32}P]P_i. Background was determined in parallel reactions in the absence of Gpa1, and background values were subtracted to determine specific P_i produced.
**MAPK phosphorylation assays**—Yeast strains were grown with shaking at 30°C in selective media to A₆₀₀ nm ~0.8 and treated with either 3 µM α-factor pheromone or 500 mM KCl. Samples were removed at timed intervals, collected by centrifugation, and stored at -70°C. To prepare extracts, cell pellets were thawed on ice and resuspended in 250 µl of ice cold TCA buffer (10 mM Tris pH 8.0, 10% trichloroacetic acid, 25 mM NH₄OAc, 1 mM EDTA). Cells were disrupted by vortexing with 100 µl of glass beads in 5 x 1 min bursts with chilling on ice in between. Lysates were transferred to new tubes and centrifuged for 10 min at 16,000 x g at 4°C. Pellets were resuspended in 0.1 M Tris pH 11.0, 3% SDS, and boiled for 5 min, then centrifuged at 16,000 x g. The resulting supernatant was separated and protein concentration was determined using the DC protein assay (Bio-Rad Laboratories). 20 µg of protein in 2x SDS-PAGE sample buffer was used per time point. MAPK phosphorylation was determined by 12% SDS-PAGE and immunoblotting with p44/42 MAPK antibody or p38 MAPK antibody at 1:500 (Cell Signaling Technology). Densitometry of developed blots was determined using ImageJ. The MAPK signaling defect in arr4 cells was rescued by a plasmid-borne copy of ARR4 (data not shown).

**Transcription reporter assay**—Cells were transformed with pRS423-FUS1-lacZ, and β-galactosidase activity was determined as described previously (74). Briefly, cells were grown to A₆₀₀ nm ~ 0.8, and treated with α-factor mating pheromone for 90 min. 1 mM fluoro-di-D-galactopyranoside (FDG) diluted in 25 mM PIPES pH 7.2 was added, and cells were incubated at 37°C in the dark for 45 min. Reaction was quenched by addition of Na₂CO₃ to a final concentration of 200 µM and fluorescence at 485-530 nm was measured using a VersaMax optical plate reader. The transcription-reporter defect in arr4 cells was rescued by a plasmid-borne copy of ARR4 (data not shown).
Mating assay—Yeast mating efficiency was determined using the filter method, as described previously (140). Briefly, cells were grown in YPD to $A_{600 \text{ nm}} \sim 0.8$. $2 \times 10^6$ MATa cells (counted using a hemacytometer) were mixed with $2 \times 10^7$ MATa cells in a volume of 15 ml, and the mixture was passed through a nitrocellulose filter (Millipore). Filters were incubated cell side up on YPD agar plates for 5 h, and resuspended into water. Serial dilutions were spread onto selective media plates, and diploids counted after 3 days. The mating defect in arr4 cells was rescued by a plasmid-borne copy of ARR4 (data not shown).
CHAPTER V

Conclusions and Future Directions

Elements of the work referenced in this chapter have been accepted for publication:


Figures contributed by:

Michael Lee: Table 5.1; Table 5.2; 5.1; 5.2; 5.3; 5.4; 5.5; 5.6; 5.7; 5.9
Yu-Lin Wu: 5.8
Heterotrimeric G proteins and their associated receptors are arguably the most medically relevant signaling proteins in human physiology. Without question, targeting these systems for the creation of new drugs has been among the biomedical community’s most common—and most successful—efforts. It is self evident then, that an increased understanding of how heterotrimeric G protein signaling is regulated will result in an increased ability to target these systems for therapeutic benefit.

In the last half-decade, one emerging trend in the field is the possibility that G proteins signal at intracellular sites. Before we can fully appreciate how the cell utilizes intracellular signaling by G proteins, a number of basic questions need to be addressed. For example: are intracellular signals being transmitted by activated (and subsequently translocated) pools of plasma membrane G protein or by intracellular pools of G protein? How does the G protein traffic and specifically localize in the cell? How is localization regulated? What role is played in intracellular signaling by GPCRs? What role is played by non-receptor GEFs? What is the benefit to spatially distinct signaling? In an effort to answer some of the basic questions related to intracellular signaling, I have addressed two questions: 1) what are the factors that regulate the membrane localization and internalization of Gpa1 (Chapter II and III), and 2) does a non-receptor activator exist in yeast to activate Gpa1 (Chapter IV)? In this chapter, I will briefly review the major findings presented in this thesis, discuss the implications of these findings, present some unfinished data for each of the major aims, and discuss the future directions of this work. Of particular interest will be the crystallization trials for Gpa1, Arr4, and the two-protein complex; preliminary investigation of the role of the de-palmitoylating enzyme Apt1; and a detailed discussion about the value of the dominant negative mutant Gpa1N388D.
REGULATION OF GPA1 PALMITOYLATION

Presented in Chapter III of this thesis is the characterization of a novel tool for screening proteins involved in Gpa1 palmitoylation. Considering that the unpalmitoylated mutant of Gpa1 has only minor signaling phenotypes, the genetic advantages of using the yeast model system are not easily exploited. Using the Gpa1$^{G2A/2K}$ mutant—a form of the G protein that is unmyristoylated but has a short lysine stretch in its place—Gpa1 palmitoylation now is necessary for viability.

In addition to the screening potential of Gpa1$^{G2A/2K}$, these findings also provide a validation of the “two signal hypothesis” for membrane attachment. It had previously been proposed that stable association with the plasma membrane required two membrane anchoring signals (some combination of lipid modifications, interaction with a transmembrane protein, basic patch of amino acids, etc.). For the most part, G$\alpha$ subunits adhere to the two signal theory: subunits with only 1 signal fall-off of the membrane when activated (G$i$ for example), subunits with 2 signals remain stably associated with the membrane. In accordance with this theory, it was hypothesized that G$\alpha$ subunits that have only 1 lipid modification must use a poly-basic patch as a second signal in order to reach the plasma membrane, and this was recently found to be true (116). Here by combining G2A and a short poly-lysine mutation, we crippled one membrane anchor of Gpa1, creating a protein that requires palmitoylation and G$\beta\gamma$ for membrane binding.

Screening for PATs

The future directions of this project are to first test the known DHHC family members for redundancy in activity towards Gpa1. This will require that the genes be deleted in
various combinations until inviable cells are found. It is known that mispalmitoylation requires deletion of 6 of the 7 known PATs (all except Swf1) (125). It remains a possibility, however, that some specificity exists among these six PATs, because all permutations of the remaining 6 genes have not been tested. The next step will be to test the entire yeast genome deletion collection for defects in Gpa1 palmitoylation. This screen is likely to uncover proteins involved in the trafficking of Gpa1 to relevant PATs, and potentially also new non-DHHC PATs. Both of these findings would be met with interest. The identification of PATs is in its infancy, so screening for these enzymes using functional criteria, rather than simply by homology is a reasonable endeavor.

**Regulation of Gpa1 localization and function by the de-palmitoylating enzyme Apt1.**

Acyl-Protein Thioesterase 1 (APT1) is a gene identified by Duncan and Gilman because of its homology to a rat protein they had previously determined to function as a de-palmitoylating enzyme (45). Gilman’s group thought to look for an enzyme of this type because of the increase in palmitate turnover following activation of many Gα subunits. Furthermore, it had been known for some time that, unlike N-myristoylation or isoprenylation, palmitoylation is metabolically reversible. The authors found that yeast Apt1 had the same function as rat Apt1 in vitro: it contained low lysophospholipase activity and acyl-protein thioesterase activity (Apt1 preferred palmitoyl-Gα over lysophospholipids by several hundred fold). Yeast then became a suitable host for answering the fundamental question surrounding APT1: what is the purpose of regulated deacylation? Unfortunately, although the authors were able to show that Apt1 is exquisitely specific for Gα subunits, they found no phenotypes of the apt1Δ in the Gα controlled yeast mating pathway (44).
Figure 5.1

A  

wild-type  apt1Δ

B  

\[ \log_{10}[\alpha \text{ factor}] \]

\[ \begin{array}{c|cccccccc}
\text{wild-type} & \text{0} & 5 & 15 & 30 & 60 & 90 & \text{minutes} \\
\hline
\text{apt1Δ} & \text{0} & 5 & 15 & 30 & 60 & 90 &
\end{array} \]

C  

\begin{align*}
\text{wild-type} & \quad \text{apt1Δ} \\
0 & \quad 0 \\
5 & \quad 5 \\
15 & \quad 15 \\
30 & \quad 30 \\
60 & \quad 60 \\
90 & \quad 90 \\
\end{align*}
Figure 5.1 *APT1* deletion does not result in defects in the mating response. (A) BY4741 wild-type cells, and BY4741-derived cells lacking *APT1* were grown to saturation, plated, and exposed to paper discs containing various amounts of α-factor pheromone (*clockwise from top right*: 1.5, 4.5, 15, and 45 µg). (B) Cells as in (A) were transformed with pRS423-FUS1-lacZ, grown to mid-log phase and exposed to various concentrations of α-factor for 90 minutes. Results are the mean ± SEM for 3 individual experiments each performed in triplicate. (C) Cells as in (A) were grown to mid-log phase and exposed to 3 µM α-factor. Cells were removed at timed intervals. Extracts were resolved using 12% SDS-PAGE and immunoblotting using phospho-specific p44/42 MAPK antibodies.
As an extension of my work to identify PATs for Gpa1, I also wanted to revisit the regulation of Gpa1 localization and/or function by Apt1. In the five years since the studies by Duncan and Gilman, the tools available for assaying mating pathway activation have improved greatly both in sensitivity and in number. So, we monitored mating pathway activation at the level of pheromone-sensitivity (using the classic halo assay), MAPK phosphorylation (using a phospho-specific p44/42 MAPK antibody), mating gene transcription (using a FUS1-LacZ transcription reporter construct and the fluorescent reagent FDG), and localization (using a Gpa1-GFP reporter in live cells). Beginning with the pheromone-sensitivity assay, as did Duncan and Gilman, we found no difference between wild-type yeast cells and cells that lack apt1 (Figure 5.1 A). In addition, using our more refined assays for pathway activation, MAPK phosphorylation and gene transcription, we also found no significant differences in apt1 cells (Figure 5.1 B, C). The first clue of the function of Apt1 came when we monitored Gpa1 localization using our Gpa1-GFP reporter protein. We found that in apt1Δ cells, Gpa1-GFP accumulated in intracellular puncta rather then the plasma membrane (Figure 5.2 A). This finding was unexpected, especially considering that Gpa1C35-GFP (a fluorescently tagged mutant that 0% palmitoylated, as opposed to apt1Δ which is presumably 100% palmitoylated), is localized normally with respect to wild-type (data not shown).

Our interpretation of Gpa1 localization in apt1Δ cells is that de-palmitoylation is necessary either for delivery to the plasma membrane (during protein maturation) or for clearance, following endocytosis. If Gpa1 is being endocytosed, we would expect that the mono-ubiquitinated form of Gpa1 would accumulate in apt1Δ cells. To test this, we over-expressed Gpa1 (to increase visibility of the mono-ubiquitinated form of the G protein) and
probed cell lysates with a Gpa1-specific antibody. This method was previously successful in identifying both poly- and mono-ubiquitinated forms of Gpa1 (see Chapter II) (164). We found no accumulation of mono-ubiquitinated Gpa1 in apt1Δ cells, suggesting the puncta are not en route to being degraded in the vacuole (Figure 5.2 B).

A number of possibilities need to be addressed before we can state with any certainty what role Apt1 is playing in Gpa1 function. Using co-localization with known compartment markers in yeast, we will attempt to identify where Gpa1 is accumulating. Also, a question that has not been addressed is if Apt1 regulates Gpa2, the other Gα subunit in yeast (responsible for regulating responses to glucose) (108, 173). Finally, using cell fractionation, we can determine quantitatively how much G protein is present (if any at all) at the cell periphery. One of the interesting implications of Gpa1 localization in apt1Δ cells is that pheromone dependent signals can transmit directly from intracellular sites. In apt1Δ cells, the vast majority of Gpa1 appears to be localized away from the plasma membrane, but signaling remains normal. If Gβγ were localized at the plasma membrane without Gpa1, we would expect aberrant signal activation. Since signaling remains normal, either Gβγ expression has been compromised or that Gβγ is localized along with Gpa1 at the intracellular compartments; both of these possibilities will be tested. Also, the fact that pheromone signals can still be transmitted in apt1Δ cells suggests that the internalized pheromone receptor is competent to transmit a signal. Studies using fluorescent α factor (pheromone) show that the ligand remains bound to the receptor throughout the internalization process (10), making signaling by the internalized receptor a formal possibility.
Figure 5.2

A

wild-type

apt1Δ

Gpa1-GFP

DIC

B

Strain

pAD4M-GPA1

WT  WT  pep4Δ  apt1Δ

-  +  +  +

mub-Gpa1

unmyr-Gpa1

myr-Gpa1
Figure 5.2 *APT1* deletion cells mislocalize Gpa1-GFP. (A) BY4741 wild-type cells and BY4741-derived cells lacking *APT1* were transformed with the integrating plasmid pRS406-GPA1-GFP. Cells were grown to mid-log phase, and visualized using a fluorescent microscope. (B) A high-copy over-expression plasmid (pAD4M) containing *GPA1* was transformed into BY4741, or cells lacking *PEP4* or *APT1*. Cell extracts were resolved by 7.5% SDS-PAGE and immunoblotting using anti-Gpa1 antibodies. The presence of myristoylated (myr-Gpa1), unmyristoylated (unmyr-Gpa1), and mono-ubiquitinated (mub-Gpa1) species of Gpa1 can be seen as in Figure 2.1.
GPA1 UBIQUITINATION: THE 110 AMINO ACID UBIQUITINATED SUB-DOMAIN

One of the main findings in this thesis is that Gpa1 ubiquitination can result in trafficking to the vacuole or degradation by the proteasome (see Chapter II). Furthermore, I provided evidence that the proteasomal degradation pathway regulates turnover of misfolded protein, while mono-ubiquitination regulates vacuolar trafficking of functional, plasma membrane localized, and myristoylated Gpa1. The implication of this finding is that the myristate moiety is recognized by the ubiquitination machinery and is necessary either for stable ubiquitination or internalization, however, more work needs to be done to directly test this hypothesis.

For future studies, we are interested in resolving the structure of Gpa1. Gpa1 is unique in that it contains a 110 amino acid extension in the all-helical domain that is not conserved among other Gα subunits. As detailed in this thesis, the 110-residue sub-domain contains all residues necessary for ubiquitination of Gpa1. Considering that this sub-domain contains important regulatory elements, yet the structure remains unresolved, one of our present and future efforts is to crystallize full-length Gpa1. We have initiated Gpa1 crystallization trials in parallel with trials for Arr4, and for the Arr4-Gpa1 complex. All of these trials will be described below.

CRYSTALLIZATION TRIALS: GPA1, ARR4, AND THE ARR4-GPA1 COMPLEX

In terms of receptor-mediated activation, the question remains: how do receptors act at a distance to manipulate switch regions of Gα subunits? Our understanding of this process will remain incomplete until the structure of a receptor in complex with G protein
<table>
<thead>
<tr>
<th>Screen</th>
<th>Description</th>
<th>Conc.</th>
<th>Temp</th>
<th>Total Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peg Suite</td>
<td>96 total conditions; range of PEG MW and concentrations; range of low salt additives</td>
<td>12 mg/ml</td>
<td>20 C</td>
<td>Arr4 - 384</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mg/ml</td>
<td>4 C</td>
<td>Gpa1 - 192</td>
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<td></td>
<td></td>
<td>7.5 mg/ml</td>
<td>4 C</td>
<td>Complex - 0</td>
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<td></td>
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<td>6 mg/ml</td>
<td>4 C</td>
<td></td>
</tr>
<tr>
<td>Crystal Screen 1</td>
<td>Sparse matrix of buffers, pH, organic compounds, PEGs, salts</td>
<td>12 mg/ml</td>
<td>20 C</td>
<td>Arr4 - 288</td>
</tr>
<tr>
<td></td>
<td>48 total conditions</td>
<td>10 mg/ml</td>
<td>4 C</td>
<td>Gpa1 - 192</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5 mg/ml</td>
<td>4 C</td>
<td>Complex - 0</td>
</tr>
<tr>
<td>Crystal Screen 2</td>
<td>Extension of the original Crystal Screen, 48 conditions</td>
<td>12 mg/ml</td>
<td>20 C</td>
<td>Arr4 - 288</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mg/ml</td>
<td>4 C</td>
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<tr>
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<td></td>
<td>7.5 mg/ml</td>
<td>4 C</td>
<td>Complex - 0</td>
</tr>
<tr>
<td>Salt RX</td>
<td>Sparse matrix of 22 salts and pH (4.6-8.5), 96 total conditions</td>
<td>12 mg/ml</td>
<td>20 C</td>
<td>Arr4 - 288</td>
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<td>10 mg/ml</td>
<td>4 C</td>
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<td>7.5 mg/ml</td>
<td>4 C</td>
<td>Complex - 0</td>
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<td>96 conditions created by the Joint Center for Structural Genomics; optimized based on over half a million trials</td>
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<td>20 C</td>
<td>Arr4 - 192</td>
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<td></td>
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<td>7.5 mg/ml</td>
<td>4 C</td>
<td>Gpa1 - 96</td>
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<td></td>
<td></td>
<td>6 mg/ml</td>
<td>4 C</td>
<td>Complex - 96</td>
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<tr>
<td>HWI</td>
<td>I. Matrix of 36 different salts (3 different concentrations) and 8 buffers</td>
<td>10 mg/ml</td>
<td>RT</td>
<td>Arr4 - 1596</td>
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<td>II. As in I, but with the addition of PEG (3 different concentrations of 5 different PEG polymers</td>
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<td>Gpa1 - 1596</td>
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<td></td>
<td>III. 9 different commercial screens from Hampton Research</td>
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<td>Complex - 1596</td>
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<td>1596 conditions total</td>
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<td>Rigaku (UNC)</td>
<td>Crystal Screen HT (96 conditions); PEG Ion (96 conditions); SaltRx (96 conditions)</td>
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<td>20 C</td>
<td>Arr4 - 0</td>
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Table 5.1 Crystallization trials for Gpa1, Arr4, and Gpa1/Arr4 complex. To date, crystallization screening trials have focused on sparse matrix commercial screening kits from Hampton Research and NexTal (Qiagen). Some of the trials were performed robotically, either at the Hauptman-Woodward Institute (HWI) or at the UNC Crystallization Core Facility (Rigaku UNC). Trials at HWI were performed using the microbatch under oil technique; trials performed at the UNC core facility were performed using a Rigaku robot utilizing the sitting drop technique. The rest of the trials were performed manually using hanging drops.
heterotrimer is resolved. Although many lines of evidence suggest that activation by non-receptor GEFs is likely by a different mechanism than that of receptors, understanding activation by non-receptor GEFs is also an important question. Considering that we recently identified a non-receptor GEF for the yeast Gα subunit Gpa1, one of our highest priorities is to solve the co-crystal structure of the GEF/G protein in complex. As mentioned above, we are also interested in solving the structure of Gpa1 alone, because of the functional importance of 110 amino acid sub-domain in ubiquitination and trafficking.

Our first task when attempting to crystallize Arr4 and Gpa1 was to purify both proteins to greater than 95% purity. Using 6xHIS-tagged proteins, we were able to obtain pure protein through a procedure involving nickel-affinity purification in conditions of high salt (400 mM NaCl), tobacco etch virus (TEV) protease cleavage of the 6xHIS tag (and subsequent binding of cleaved 6xHIS tag to Ni- affinity resin), and finally size exclusion chromatography (S200) (Figure 5.3). Each of these steps was critical to protein purity, and the final protocol evolved after a number of trials.

Once we obtained high-purity protein, we next needed to identify conditions that support stable Arr4 dimerization and stable Arr4-Gpa1 complex formation. We and others identified that Arr4 functions as a dimer, and that copper facilitates dimerization (101). Using size exclusion chromatography, we found that the vast majority Arr4 protein migrates as a dimer in the presence of copper (Figure 5.4). Furthermore, as shown in Chapter IV, incubating Arr4 and Gpa1 in the absence of GDP (and presence of copper) allowed for stable complex formation between the Arr4 dimer and Gpa1 (see Chapter 4).
Figure 5.3

A

B

C

Gpa1

Arr4
Figure 5.3 Purification of Gpa1 and Arr4 for crystallization. (A) Gpa1 and Arr4 were purified for crystallography using affinity purification using 6xHIS tagged protein and Ni-NTA resin; cleavage of the affinity tag followed by separation of uncleaved protein (as well as non-specific binding proteins); size-exclusion chromatography using an S200 gel filtration column (Amersham). (B) Coomassie stained gel showing various stages of Gpa1 purification. Gpa1 was purified to single band purity using the method described in (A). *lane 1* 6xHIS Gpa1 before TEV cleavage; *lane 2* Gpa1 following TEV cleavage and binding to Ni-NTA resin; *lanes 3-11* S200 fractions. Fractions 8-10 were collected and concentrated. 6-10 mg of Gpa1 was purified from 4 liters of bacterial culture. (C) Coomassie stained gel showing various stages of Arr4 purification. Gel loaded as in (B). Fractions 7-9 were collected and concentrated. 25-35 mg of Arr4 was purified from 4 liters of bacterial culture.
Having established conditions for the expression and purification of Gpa1 and Arr4, we then focused on screening to identify conditions that support crystal formation. For our initial screening trials, we utilized four different commercial kits, with the goal of testing a wide array of salts, buffers, and precipitants (Table 5.1). We also varied temperature and protein concentration, all while using the hanging drop method of crystallization (12). In this initial screening, we were unable to identify any crystal forming conditions for Arr4 alone, or Arr4-Gpa1 complex, but did find a condition that supported crystal formation of Gpa1 alone (data not shown). The crystals formed were thin, long, rods. Upon manipulation, they appeared to be protein, not salt, however, “defraction quality” crystals were not obtained. Attempts to screen around this condition were not successful.

_Hauptman-Woodward Institute, Buffalo NY_—We have also utilized a fee-for-service high-throughput crystallization screening facility at the Hauptmann-Woodward Institute (HWI) in Buffalo, NY. We were not limited by protein abundance, and felt the trials performed by HWI would complement those we were performing manually. HWI uses a robotic crystal-screening device to set up 1600 separate conditions of a particular test protein using the microbatch under oil crystallization method. This method is significantly different than other popular methods that rely on vapor diffusion, such as hanging drop and sitting drop. The 1600 conditions tested by HWI represent a wide array of salts, buffers, precipitants, PEGs, and 9 commercial kits produced by Hampton Research (Table 5.1). All trays are set up at room temperature, and protein is typically used at a concentration of 10 mg/ml.

Our crystallization trial yielded a number of potentially interesting hits for Arr4 alone, but not Gpa1 alone or Arr4-Gpa1 complex. We found 4 different conditions that
Figure 5.4 Efficient dimerization of Arr4 in the presence of copper. Purified Arr4 protein was separated using an S200 gel filtration column in the presence of CuSO₄. 3 peaks are visible (as labeled): void (likely containing aggregated or insoluble protein), Arr4 dimers, and Arr4 monomers (column size calibration not shown).
Figure 5.5  Preliminary Arr4 crystals. Crystallization trials were performed using purified Arr4 (10 mg/ml) at the HWI high-throughput crystallization screening facility in Buffalo, NY. All trials were performed using the microbatch under oil method. Pictures were taken 24 hours after drop set-up. Conditions were (A) 0.1 M Ammonium Nitrate; 0.1 M HEPES (pH 7.5); 40 % PEG 4000; (B) 1.0 M Lithium Chloride; 0.1 M HEPES (pH 7.0); 30 % PEG 6000; (C) 0.2 M Sodium Chloride; 0.1 M bis-Tris (pH 6.5); 25 % PEG 3350; (D) 0.2 M Ammonium Acetate; 0.1 M bis-Tris (pH 5.5); 25 % PEG 3350.
supported crystal formation of Arr4 (Figure 5.5). All crystals formed between 0-24 hours. All conditions contained PEG as a precipitant, and all conditions were neutral in pH (5.5-7.5). Our initial attempts to reproduce these crystals using hanging drops were unsuccessful (data not shown). Subsequent attempts using the microbatch technique also did not yield crystals, but these trials were set up with a different preparation of protein, so minor differences in protein composition may have led to these differences.

*Rigaku robot trials, Crystallization Core Facility, UNC*—Our most recent crystallization trials were conducted at the Crystallization Core Facility at the UNC at Chapel Hill with assistance from Dr. Laurie Betts and Dr. Janeen Vanhooke. These trials were robotically performed utilizing a sitting drop technique, and we began by screening three different commercial kits across two different temperatures and two different protein concentrations (Table 5.1). These trials were begun very recently, and to date, no crystals have formed.

**LOCALIZATION OF ARR4 IN PHEROMONE TREATED CELLS**

Presented in this thesis is the discovery that Arr4 functions as a non-receptor GEF for Gpa1 (see *Chapter IV*). We found using purified components that the Arr4 functions in a manner that is similar to that of GPCRs, and also that Arr4 activity promotes the yeast mating response. Our conclusion is that Arr4 does not initiate the signal, and cannot functionally replace the receptor, but is necessary for maximum signal amplification. It is still unclear, however, where Arr4 functions, particularly given our previous findings that Gpa1 exists at multiple cellular compartments and signals from both the plasma membrane and endosomes. At least three possibilities exist: Arr4 could function immediately
downstream of the GPCR Ste2 at the plasma membrane, Arr4 could activate Gpa1 at endosomes, or Arr4 could function at a yet unidentified compartment. Here, I will present preliminary data addressing these possibilities.

**Vps34 is required for Arr4-mediated activation of Gpa1**

Following our discovery that Arr4 activates Gpa1, we first wanted to determine if Arr4 contributes to endosomal signaling. Arr4 is generally considered to exist at the endoplasmic reticulum (ER), but also has been shown to exist at late endosome (132). We have previously determined that over-expressed **ARR4** results in mating pathway activation through Gpa1 (see Chapter IV) (88). To determine if Arr4-induced pathway activation occurs at the cell periphery or at the cell interior, we tested whether pathway activation requires Vps34, the endosomal effector of Gpa1. As shown previously, in the wild-type genetic background, **ARR4** over-expression caused a nearly 4-fold increase in basal pathway activation. Using a **FUS1-LacZ** transcription reporter, we found that **ARR4** over-expression had no effect on pathway activation in **vps34Δ** cells (Figure 5.6 A). These data suggest that Arr4 signaling requires Vps34, and potentially, that Arr4 functions as an intracellular GEF for Gpa1.

**Arr4 translocates to intracellular puncta upon treatment with mating pheromone**

Having found evidence that Arr4 contributes to endosomal signaling, we next wanted to determine if Arr4 exists at the endosome. To answer this question, we created a GFP-tagged version of Arr4 and monitored its localization in cells treated with and without mating pheromone. We found, as reported by others, that Arr4 appears throughout the cytoplasm,
with slight enrichment in ER regions (Figure 5.6 B). However, when treated with mating pheromone, Arr4 became localized to very distinct, very bright intracellular puncta (Figure 5.6 B). These puncta were not morphologically consistent with what we had previously seen as endosomes, and did not co-localize with a marker for the endosomal compartment Snf7-RFP (Figure 5.6 B). Although Arr4 does not reside at the endosome, it is still possible that Arr4 contributes to endosomal signaling. In addition, these data provide further evidence that Arr4 is responsive to pheromone, and provide the first evidence that Arr4 functions from an intracellular location.

**Identification of Arr4 localization using mass spectrometry**

Using microscopy of live cells, we were able to determine that Arr4 resides at an intracellular location upon treatment with pheromone, but we were not able to identify that location. Rather than attempting co-localization with a larger panel of compartment markers, we decided to purify Arr4 from yeast and identify all co-purifying bands using mass spectrometry. Identification of pheromone-dependent binding partners could give us clues to the localization of Arr4 and also give insights into Arr4 regulation and function.

Using a FLAG-tagged version of Arr4, expressed at endogenous levels behind its native promoter, we purified Arr4 in the presence and absence of pheromone. Arr4 containing a myc tag, rather than FLAG tag, was used as a negative control. A small portion of the eluant was analyzed by silver stain and also immunoblotted with anti-FLAG antibody, and the rest analyzed by Orbitrap liquid chromatography/mass spectrometry (LC/MS). Silver stain analysis showed that Arr4 purification was relatively clean (not many co-purifying bands), however, at least one pheromone-dependent interaction was detected (Figure 5.7 A).
Figure 5.6

A

Relative β-galactosidase Activity

WT  WT+Arr4  vps34Δ  vps34Δ ARR4

B

DIC  GFP-Arr4  Snf7-RFP

0 min

90 min
Figure 5.6  Evidence that Arr4 regulates Gpa1 from an intracellular location.  (A) BY4741 or BY4741-derived cells lacking *VPS34* were transformed with a high copy vector (pAD4M) containing *ARR4* (or empty vector). Cells were also transformed with the reporter plasmid pRS423-FUS1-lacZ. Data are basal β-galactosidase activity; Results are the mean ± SEM for 3 individual experiments each performed in triplicate.  (B) BY4741 cells were co-transformed with integrating plasmids pRS406-GFP-ARR4 and pRS403-SNF7-RFP (endosomal marker protein). Cells were treated with 3 μM α-factor for 90 minutes and visualized using a fluorescent microscope.
Figure 5.7

A

B

Alpha-Factor

kDa

75 50 37 25

FLAG-Arr4 MYC-Arr4
Figure 5.7 Identification of Arr4-interacting proteins in yeast. (A) and (B) BY4741 cells were transformed with either pRS316-FLAG-ARR4 or pRS316-MYC-ARR4 (each plasmid is single copy and expression is driven by the endogenous ARR4 promoter). 6 liters of cells were grown to mid-log phase and treated with water or 3 μM α-factor and harvested. Extracts were purified using FLAG-M2 antibody resin (Sigma), and eluted using 3x-FLAG peptide. Eluant was resolved by 12 % SDS-PAGE and either (A) staining with Silver stain (Bio-Rad), (B) immunoblotting with anti-FLAG antibody, Orbitrap LC/MS analysis (data not shown).
To test whether this was a pheromone-dependent breakdown product of Arr4, we also probed with anti-FLAG antibodies. Although some breakdown did appear to be pheromone dependent, the 25 kDa band observed in the silver stained gel did not appear in the gel probed for FLAG (Figure 5.7 B). Since Arr4 was only tagged with FLAG on its N-terminus, this could still be a C-terminal breakdown product.

The Orbitrap LC/MS has been completed, but the extensive data analysis is just underway. To date, we have simply analyzed each sample (FLAG-Arr4 with pheromone, FLAG-Arr4 without pheromone, and MYC-Arr4 with pheromone) using a very stringent 95% confidence interval for peptide presence or absence. It is not uncommon to vary the confidence interval threshold of detection. A better understanding of the data will result from a detailed analysis of the data set across confidence intervals, and also upon further ratiometric analysis between the samples to define quantitatively how protein presence varies between conditions. These analyses will be performed in the very near future.

From the existing data set (Table 5.2) some interesting information has arisen. Only a few proteins were identified that bound specifically to Arr4 in the presence of pheromone. Two of these proteins, Tdh1 and Tdh3, are proteins that are known to be resident in the yeast lipid particle. The lipid particle is an intracellular compartment thought to function as a reserve center for cellular lipids. It is thought to be comprised mostly of triglycerols and sterol esters, and only a few proteins (6). Some lipid particle proteins, like Erg1, are also resident in ER, establishing a relationship between these two compartments (87). As mentioned previously, Arr4 is known to exist in the ER, and is known genetically known to play a role in Golgi-to-ER trafficking (hence the name Get3, an alias for Arr4) (131). These findings suggest that in the presence of pheromone, Arr4 also exists at the lipid particle. It is
not clear how Arr4 at the lipid particle would regulate Gpa1 signaling, but at least one other lipid particle protein, Erg6, is known to be required for proper mating (77). In future studies, we will first confirm pheromone dependent interaction between Arr4 and lipid particle subunits, as well as other proteins that are uncovered after our detailed analysis. Following these studies, we will test the effect of gene deletions on Gpa1/Arr4 localization, signaling, and mating.

The studies presented in Chapter IV of this thesis and in this section provide evidence that Arr4 is a bona fide component of the yeast mating pathway. In addition to the finding that arr4 deletion results in hampered signaling through the mating pathway, here we present evidence that Arr4 translocates in response to pheromone treatment and physically interacts with a new subset of proteins. Evident both in our microscopy analysis as well as in our mass spectrometry analysis is the fact that less than 100% of Arr4 translocates to intracellular puncta in response to pheromone. Thus, we anticipate that a more detailed analysis of this data set will result in a better understanding of pheromone dependent changes in Arr4 localization and function. Nonetheless, our preliminary findings suggest that Arr4 translocates to lipid particles in response to pheromone treatment. These data provide the first preliminary evidence that Gpa1 can be activated from an intracellular location, and in general, these findings further develop the idea that G proteins can function from intracellular locations.
Table 5.2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Cellular Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pdc1</td>
<td>Pyruvate decarboxylase</td>
<td>cytoplasm/nucleus</td>
</tr>
<tr>
<td>Get2</td>
<td>Subunit of the GET Complex, Golgi-ER trafficking; required for nuclear division; may be involved in cell wall function</td>
<td>ER</td>
</tr>
<tr>
<td>Get1</td>
<td>Subunit of the GET Complex, Golgi-ER trafficking</td>
<td>ER</td>
</tr>
<tr>
<td>Tdh3</td>
<td>Glycericlicid 3 phosphate dehydrogenase isozenyme 1</td>
<td>lipid particle</td>
</tr>
<tr>
<td>Tdh1</td>
<td>Glycericlicid 3 phosphate dehydrogenase isozenyme 3</td>
<td>lipid particle</td>
</tr>
<tr>
<td>Pho88</td>
<td>Membrane protein involved in phosphate transport</td>
<td>mitochondria</td>
</tr>
<tr>
<td>Rtn1</td>
<td>Protein of unknown function; interacts with Sec6, Yip3, Sbh1</td>
<td>ER/Golgi</td>
</tr>
<tr>
<td>Thr3</td>
<td>Thiamine biosynthesis</td>
<td>cytoplasm/nucleus</td>
</tr>
<tr>
<td>Gpa1*</td>
<td>GTPase; alpha subunit of heterotrimeric G protein</td>
<td>plasma membrane/endosome</td>
</tr>
<tr>
<td>Ypt6*</td>
<td>GTPase; required for endosome-Golgi trafficking; Rab6 homolog required for signalling by constitutively active Gpa1</td>
<td>Golgi</td>
</tr>
<tr>
<td>Gyp6*</td>
<td>GAP for Ypt6; involved in vesicle transport</td>
<td>endosome</td>
</tr>
</tbody>
</table>

Table 5.2 Proteins interacting specifically with FLAG-Arr4 in the presence of pheromone. This table shows preliminary analysis of LC/MS (Orbitrap) identified proteins that interact with Arr4 in the presence of pheromone. Excluded from this list were a large number of proteins involved in ribosomal function, as well as some heat shock proteins. Proteins on this list were identified above a 95% confidence threshold.

* Preliminary analysis suggests that some specific binding proteins were obscured by co-migration of more abundant proteins of similar mass (personal communication from Dr. Carol Parker and Dr. Matthew Torres). Closer analysis of peptides identified reveals a number of proteins that fell below the threshold of identification solely because of other more abundant proteins of similar mass. Included in this list are Gpa1, Ypt6, and Gyp6.
GPA1\textsuperscript{N388D}: A NOVEL TOOL FOR THE IDENTIFICATION OF GEFS FOR G\alpha SUBUNITS

Summary

G protein coupled receptors (GPCRs) are the canonical activators of heterotrimeric G protein signaling pathways. Since the identification that GPCRs were heptahelical membrane spanning receptors, the search for other activators of G\alpha subunits has typically involved identifying proteins that were structurally related to GPCRs. Screening in this way has caused the field to overlook proteins that are functional, but not structural, GPCR analogs. In recent years, many in the field have begun to search for G protein activators using functional criteria, and as a result, a number of non-receptor activators have been identified. Here, we will discuss a novel tool for the identification of G\alpha activators: a dominant negative G\alpha mutant.

Non-receptor activators of heterotrimeric G\alpha subunits

G protein coupled receptors (GPCRs) regulate a wide variety of physiologically relevant pathways and are the most commonly targeted proteins in all of medicine (61, 169). When ligand bound, the receptor functions as a guanine nucleotide exchange factor (GEF), stabilizing the nucleotide-free form of the G protein, thus allowing exchange of GDP for GTP (141). The process by which receptors are able to activate G\alpha subunits has been the subject of intense investigation, as it is still unclear exactly how this occurs. The elusive mechanism of G\alpha activation is further complicated by the existence of non-receptor activators that bear no structural or sequence similarity to GPCRs, yet also function as GEFS.
The existence of these proteins was likely overlooked for so long because screens for activators of Gα subunits have focused on identifying proteins with structural, not functional similarity to GPCRs. However, in recent years, investigators have returned to identifying activators using functional criteria, and the result has been the discovery of at least 3 unrelated families of non-receptor Gα activators: Ric-8 and its homologs, the AGS family, and most recently the yeast protein Arr4 (26, 88, 151). All three discoveries were made with the help of the genetic tools available in model organisms such as *S. cerevisiae* and *C. elegans*. Ric-8 and AGS proteins have been reviewed extensively elsewhere (15, 65); here we will focus on the dominant-negative screening method used to identify the yeast protein Arr4.

**Dominant negative mutant screen**

Dominant negative mutants are loss-of-function alleles that when over-expressed also block the activity of the wild-type protein. Dominant negative activity is often achieved if the mutant product is still capable of interacting with the same binding partners, but has lost functional properties. The classic example is a transcription factor that contains a DNA binding domain, but lacks a transactivation domain. Dominant negative mutants can be potent tools for studying protein function.

Recently, two different mutations in the yeast Gα subunit Gpa1 were claimed to cause constitutive activity. Paradoxically, over-expression of the two mutants caused opposite phenotypes. Expression of Gpa1Q323L caused signal activation (62). Expression of Gpa1N388D caused signal inhibition (147). Gpa1Q323L had already been characterized biochemically, and shown to bind but not hydrolyze GTP (3). To resolve this debate, we
characterized the Gpa1^{N388D} mutant, and found that it functioned as a dominant negative (171). Gpa1^{N388D} could still bind to and hydrolyze GTP, and still bound to Gβγ. The dominant negative activity arose because this mutant formed a stable complex with the ligand-bound GPCR Ste2. The critical finding was that Ste2 bound better to the Gpa1^{N388D} mutant than to the wild-type version of Gpa1, but this was only true of the ligand-bound receptor (Figure 5.8). Stated differently, the dominant negative mutant did not simply exhibit enhanced binding to receptors, it exhibited enhanced binding to GEFs.

It is not clear why the “ND” mutant binds GEFs, but the simplest explanation is that Gpa1^{N388D} is nucleotide-empty or somehow mimics the empty state. Indeed, Gpa1^{N388D} has at least two hallmarks of empty Gα subunits: specific interaction with activated receptors in vivo and protein instability in vitro (53). In our previous study, adding the “ND” mutation to either Gpa1 or Gα_{i1} resulted in decreased protein yield, decreased activity, and increased sensitivity to time and temperature in vitro; and activating the receptor stabilized the protein in vivo (171). One reason “ND” might favor the empty state could be decreased affinity of the G protein for guanine nucleotide. Asn-388 lies within the conserved NKXD motif, required for guanine recognition (141). Structural evidence shows that both the Asn and the Asp in this motif form hydrogen bonds with the guanine ring, and other investigators have found that mutational substitution of within this region is sufficient to switch nucleotide specificity from guanine to xanthosine (141, 172).
Figure 5.8

A

\[
p\text{GAL}^H\text{-GPA1}^{N388D} \quad p\text{GAL}^H\text{-GPA1}
\]

B

<table>
<thead>
<tr>
<th></th>
<th>Vec</th>
<th>GPA1</th>
<th>GPA1^{N388D}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ste2:</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ste2-FLAG:</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>α-MF:</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Applied FLAG Ab

IP FLAG Ab

Applied Gpa1 Ab

IP Gpa1 Ab
Figure 5.8 Stable interaction between Gpa1<sup>N388D</sup> and the ligand-bound receptor Ste2. (A) ste7Δ gpa1Δ mutant strain was transformed with plasmids containing STE7 under the control of the GAL1/10 promoter and either wild-type GPA1 or GPA1<sup>N388D</sup> under the control of the GAL<sup>H</sup> promoter. Cells were plated onto galactose-containing medium and exposed to α-factor pheromone (counter-clockwise from top, 75, 25, 8, 0 µg). The resulting zone of pheromone-dependent cell growth was recorded after 2 days. (B) To determine if Gpa1<sup>N388D</sup> forms a stable complex with the receptor, diploid cells (strain YPH501) were transformed with plasmids containing either tagged or untagged STE2, STE4/18 (Gβγ), and either wild-type or mutant GPA1 and then treated with 2.5 µM α-factor (α-MF) as indicated. Detergent-solubilized lysates were immunoprecipitated with anti-FLAG antibodies. Samples of total cell lysates (Applied) and purified protein (IP) were resolved by 7.5% SDS-PAGE and subjected to immunoblotting with antibodies that detect Ste2 (FLAG Ab) and Gpa1. Data shown are representative of three independent experiments. Arrows indicate the proteins specifically detected by the indicated antibodies.
Identification of the non-receptor GEF Arr4

If our hypothesis is correct, that Gpa1\textsuperscript{N388D} had dominant negative activity because it mimics the empty state, this mutant will selectively interact with any protein that stabilizes the empty state. Thus we reasoned that the selective interaction between a GEF (ligand bound Ste2) and Gpa1\textsuperscript{N388D} could be a useful tool to match receptors to their cognate G\textgreek{a} subunits and also to screen for novel GEFs. This is particularly valuable in light of the fact that GEFs for G\textgreek{a} subunits lack any physical, structural, or sequence similarity to allow for their identification \textit{in silico}.

Using this knowledge, we designed a screen in yeast for novel GEFs for Gpa1. We began by compiling a list all proteins that have previously been found to interact with Gpa1. This list was collected from a combination of published data (yeast 2-hybrid, genome-wide interaction screens, etc.) and unpublished data from our own lab. Our screen involved 2 steps: first determining which proteins interact with Gpa1 in a nucleotide-dependent manner, and second, identifying which proteins bound selectively to the dominant negative mutant Gpa1\textsuperscript{N388D}. GDP-selective binding is the only characteristic maintained across all GEFs, and as described above, selective binding to the Gpa1\textsuperscript{N388D} mutant is a characteristic we believe to be unique to GEFs. We identified 7 proteins that interact with the GDP-bound form of Gpa1, but not the GTP-bound form (see \textit{Chapter IV}). Of those 7 proteins, we identified 2—Arr4 and Reg1—that bind preferentially to Gpa1\textsuperscript{N388D}. We subsequently found \textit{in vitro} that Arr4 binds to the G protein in a manner similar to receptors and functions as a GEF for Gpa1, and \textit{in vivo} that Arr4 functions in cooperation with receptors to promote activation of the mating signal through Gpa1 (88).
Figure 5.9

A

Basal $\beta$-galactosidase Activity

WT Δreg1 WT+REG1 rescue

B

wild-type reg1Δ

Gpa1-GFP

DIC
Figure 5.9  Reg1 regulates Gpa1 signaling and localization. BY4741 or BY4741-derived cells lacking *REG1* were transformed with pAD4M, pAD4M-REG1 or pRS316-REG1. Cells were also transformed with the reporter plasmid pRS423-FUS1-lacZ. Data are basal β-galactosidase activity; results are the mean ± SEM for 3 individual experiments each performed in triplicate. (B) Wild-type and *reg1Δ* cells were co-transformed with integrating plasmids pRS406-GPA1-GFP. Cells were visualized using a fluorescent microscope.
Considerations when using the “ND” mutant

The major limitation in using the dominant negative G\(\alpha\) is the instability of this protein. In our previous works, we have combated the poor expression associated with Gpa1\(^N388D\) using a couple different means. In our recent published work, rather than monitoring absolute binding, we focused on binding relative to total expression, as a crude measure of affinity (see Chapter IV). In other studies we used diploid yeast cells, which do not express most of the mating components including G\(\alpha\), \(\beta\gamma\), and receptor; we found that over-expression of the receptor stabilized the expression of the Gpa1\(^N388D\) mutant (171). Although diploidizing cells is not likely to be of widespread usefulness, receptor-mediated stability provides a universal mechanism for stabilizing this mutant for study and another potential screening phenotype. Future studies aimed at identifying GEFs could focus on identifying proteins that when over-expressed enhance ND mutant stability. Furthermore, identifying receptor-derived peptides that enhance ND mutant stability could provide insight into the elusive mechanism by which GPCRs function as GEFs. In addition, identifying secondary mutations that stabilize the Gpa1\(^N388D\) mutant but do not block its activity will be of immense value.

Reg1—Validation of the “ND Screen”

Our finding that Arr4 is a GEF for validates the Gpa1\(^N388D\) screening principle. Rigorous validation, however, will require that both proteins identified in the screen are GEFs. Reg1 is the regulatory subunit of a protein phosphatase, known to regulate the expression of glucose repressible genes in yeast (158). Our preliminary attempts to purify Reg1 from bacteria for characterization \textit{in vitro} have failed; Reg1 is a 112 kDa protein with
no known domain structure. Purification from other sources, such as insect cell expression systems or direct purification from yeast, has not been attempted. In the absence of *in vitro* biochemistry, it is difficult to make any conclusions about Reg1 function, but some *in vivo* evidence does exist suggesting that Reg1 regulates Gpa1 function. In addition to the GEF-like binding profile exhibited by Reg1, when mating pathway activation is monitored at the level of gene transcription, Reg1 associated phenotypes are consistent with it functioning as a GEF. Over-expression of *REG1* results in increased pathway activation downstream of Gpa1, whereas deletion of *REG1* results in a decrease in basal pathway activity (Figure 5.9 A). Similar results were found for the GEF Arr4 as well as the GPCR Ste2 (88). Furthermore, in another screen performed by our group, *REG1* deletion was found to block G\(\alpha\) signaling in yeast (136). This screen utilized a constitutively active mutant, Gpa1\(^{Q323L}\), which should have bypassed the need for GEFs, however, other non-receptor GEFs such as Ric-8a, have been shown to regulate protein activity and protein localization (100). A similar case could be made for Reg1. In fact, preliminary data suggests that this might be the case; we found that Gpa1-GFP mislocalizes in cells that have REG1 deleted (Figure 5.9 B).

In conclusion, we’ve characterized the dominant negative mutant Gpa1\(^{N388D}\), and used this mutant to identify the GEF Arr4. Future studies in yeast will likely include characterization of Reg1, and also an expanded genome-level screen for Gpa1\(^{N388D}\) interacting partners. However, studies in yeast are only the beginning. This residue is conserved in all G proteins, and it is likely that similar mutations in other G\(\alpha\) subunits will also function as dominant negatives. Whether as a tool for identifying new GEFs, or for revealing the mechanism of receptor-mediated activation, this dominant negative could provide a unique resource for studying G\(\alpha\) activation. Studies of this type would benefit
from a greater understanding of this mutant. For example, it is not clear why this mutation results in a dominant negative phenotype, and it will likely remain unresolved until structural data is collected for this mutant. Structural data will also resolve whether or not it is possible to separate the dominant negative activity from the instability of the ND mutant protein. Identifying GEFs for Gα subunits has long been a difficult endeavor; the development of new tools, such as dominant negative Gα mutants, can only help in answering these, and many other fundamental questions.

ACKNOWLEDGEMENTS

I would like to thank Dr. Janeen Vanhooke and Dr. Laurie Betts for generously supplying their expertise to the early protein purification and crystallization trial efforts.

EXPERIMENTAL PROCEDURES

Strains and plasmids—Standard methods for growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout. Yeast Saccharomyces cerevisiae strains used in this study were BY4741 (MATα leu2Δ met15Δ his3Δ ura3Δ), BY4741-derived mutants constructed using the KanMX G418-resistance marker (Yeast Deletion Clones, Invitrogen; originally purchased from Research Genetics), BY4741-derived strains containing a C-terminal tandem affinity purification (TAP) tag (Yeast TAP-Fusion Library, Open Biosystems), and DC17 (MATα his1). The arr4Δ strain from Research Genetics did not produce a phenotype that could be rescued by addition of a plasmid-borne copy of ARR4, so the strain was remade by PCR amplification of the original deletion cassette and transformation of the parent strain.
Yeast shuttle plasmids used were pRS305 (amp\textsuperscript{R}, \textit{LEU2}) and pAD4M (2\mu m, amp\textsuperscript{R}, \textit{LEU2}, \textit{ADH1} promoter and terminator). Expression plasmids described previously were pAD4M-GPA1 (139), pAD4M-GPA1\textsuperscript{Q323L} (62), pRS423-FUS1-lacZ (74), pAD4M-GPA1\textsuperscript{N388D} (171), pAD4M-ARR4 (88), pRS406-GPA1-GFP (164). pRS406-GFP-Arr4 was created by inserting the \textit{ARR4} ORF into pUG36 (N-terminal GFP vector) using the restriction sites XmaI (FWD) and SalI (REV). The XmaI-SalI fragment was then subcloned into pRS406. FLAG- and MYC-ARR4 were created using the pRS316FLAG- and pRS316MYC-plasmids created previously; ARR4 was subcloned into these vectors using pRS305-ARR4 as an insert (174).

\textit{E. coli} expression of 6xHIS fusion proteins—6xHIS-Gpa1 expression plasmid was described previously (4). Plasmids for bacterial expression of 6xHIS-Arr4 fusion protein were generated by ligation-independent cloning as described previously (143). \textit{ARR4} was PCR-amplified from genomic DNA (Forward primer: 5’—TAC TTC CAA TCC AAT CGC ATG GAT TTA ACC GTG GAA CC—3’; reverse primer: 5’—TTA TCC ACT TCC AAT GCG CTA CTA TTC CTT ATC TTC TAA CTC—3’), annealed to the gapped 6XHIS vector pMCSG7 or a version of pMCSG7 modified to contain a GST tag (from Jason Snyder and John Sondek, Univ. North Carolina), and transformed into BL21 (DE3) \textit{E. coli}. Overnight cultures from single colonies grown at 37\degree C in Luria Broth (LB) supplemented with 100 \mu g/ml carbenicillin were diluted 1:100 into fresh media and grown to A\textsubscript{600 nm} \sim 0.7. 6xHIS-Gpa1 and 6xHIS-Gpa2 expression was induced by addition of 0.1 mM isopropyl \textbeta-D-1-thiogalactopyranoside and incubation at room temperature for 5 h with shaking. 6xHIS-Arr4 was induced by addition of 0.1 mM isopropyl \textbeta-D-1-thiogalactopyranoside and incubation at 18\degree C for 14 h. Cells were harvested by centrifugation, resuspended in Buffer A (20 mM Tris...
pH 8.0, 200 mM NaCl, 5% glycerol, 1 mM DTT), and homogenized using an Emulsiflex-C5 Homogenizer (Avestin Inc.). Lysates were clarified by centrifugation at 12,000 x g for 30 min and the resulting supernatant was mixed with Ni-NTA resin (Amersham Pharmacia Biotech AB) equilibrated with Buffer A, and incubated for 2 h at 4°C with gentle rotation. Resin was collected by centrifugation at 500 x g for 5 min and washed 3 times in Buffer A, and eluted in 250mM imidazole. Elution was concentrated using Vivaspin concentrators (Vivascience AG).

*Steric-exclusion chromatography*—Purified protein was subject to steric-exclusion chromatography using an Akta FPLC system and a Sephacryl 26/60 S200 column (GE Healthcare). Protein was equilibrated in 20 mM Tris pH 8.0, 100 mM NaCl, 5% glycerol, 1 mM DTT, 2 mM MgCl₂, 20 µM GDP (when added), and 150 nM CuSO₄ (when added). Protein was separated at 0.5 ml/min, and collected in 7 ml fractions. UV absorbance of each fraction (A₂₈₀nm) was recorded and data analyzed using the UNICORN program associated with the FPLC system. A 20 µl sample from each fraction was separated by SDS-PAGE and either probed with penta-HIS antibody or stained with Coomassie Brilliant Blue R250 (Bio-Rad).

*MAPK phosphorylation assays*—Yeast strains were grown with shaking at 30°C in selective media to A₆ₐ₀nm ~0.8 and treated with either 3 µM α-factor pheromone or 500 mM KCl. Samples were removed at timed intervals, collected by centrifugation, and stored at -70°C. To prepare extracts, cell pellets were thawed on ice and resuspended in 250 µl of ice cold TCA buffer (10 mM Tris pH 8.0, 10% trichloroacetic acid, 25 mM NH₄OAc, 1 mM EDTA). Cells were disrupted by vortexing with 100 µl of glass beads in 5 x 1 min bursts with chilling on ice in between. Lysates were transferred to new tubes and centrifuged for 10 min at
16,000 x g at 4°C. Pellets were resuspended in 0.1 M Tris pH 11.0, 3% SDS, and boiled for 5 min, then centrifuged at 16,000 x g. The resulting supernatant was separated and protein concentration was determined using the DC protein assay (Bio-Rad Laboratories). 20 µg of protein in 2x SDS-PAGE sample buffer was used per time point. MAPK phosphorylation was determined by 12% SDS-PAGE and immunoblotting with p44/42 MAPK antibody or p38 MAPK antibody at 1:500 (Cell Signaling Technology). Densitometry of developed blots was determined using ImageJ. The MAPK signaling defect in arr4 cells was rescued by a plasmid-borne copy of ARR4 (data not shown).

Transcription reporter assay—Cells were transformed with pRS423-FUS1-lacZ, and β-galactosidase activity was determined as described previously (74). Briefly, cells were grown to A600 nm ~ 0.8, and treated with α-factor mating pheromone for 90 min. 1 mM fluoro-di-D-galactopyranoside (FDG) diluted in 25 mM PIPES pH 7.2 was added, and cells were incubated at 37°C in the dark for 45 min. Reaction was quenched by addition of Na2CO3 to a final concentration of 200 µM and fluorescence at 485-530 nm was measured using a VersaMax optical plate reader. The transcription-reporter defect in arr4 cells was rescued by a plasmid-borne copy of ARR4 (data not shown).

Microscopy analysis. Cells expressing single-copy, integrated GFP-tagged gene fusions were visualized by differential interference contrast (DIC) and fluorescence microscopy using a Nikon eclipse E600EN, photographed with a Hamamatsu digital camera, and analyzed with MetaMorph Version 5.0 software.

FLAG-IP. Cells expressing FLAG-tagged ARR4 were grown to A600nm ~ 0.8, treated with α-factor mating pheromone to a final concentration of 3 µM, and grown for an additional 90 minutes. Cells were harvested by centrifugation, lysed using a bead beater, and
purified using M2 anti-FLAG resin, according to the manufacturer’s protocol (Sigma).
Protein was eluted off of M2 FLAG resin using 3x-FLAG peptide.
REFERENCES


mechanism that is dependent on pheromone and independent of Gβγ sequestration. Mol Cell Biol 16:6325-37.


