Dual Lipid Modification Motifs in G_{α} and G_{γ} Subunits Are Required for Full Activity of the Pheromone Response Pathway in *Saccharomyces cerevisiae*

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To establish the biological function of thioacylation (palmitoylation), we have studied the heterotrimeric guanine nucleotide-binding protein (G protein) subunits of the pheromone response pathway of *Saccharomyces cerevisiae*. The yeast G protein γ subunit (Ste18p) is unusual among G_{γ} subunits because it is farnesylated at cysteine 107 and has the potential to be thioacylated at cysteine 106. Substitution of either cysteine results in a strong signaling defect. In this study, we found that Ste18p is thioacylated at cysteine 106, which depended on prenylation of cysteine 107. Ste18p was targeted to the plasma membrane even in the absence of prenylation or thioacylation. However, G protein activation released prenylation- or thioacylation-defective Ste18p into the cytoplasm. Hence, lipid modifications of the G_{γ} subunit are dispensable for G protein activation by receptor, but they are required to maintain the plasma membrane association of $G_{\beta\gamma}$ after receptor-stimulated release from G_{α} . The G protein α subunit (Gpa1p) is tandemly modified at its N terminus with amide- and thioester-linked fatty acids. Here we show that Gpa1p was thioacylated in vivo with a mixture of radioactive myristate and palmitate. Mutation of the thioacylation site in Gpa1p resulted in yeast cells that displayed partial activation of the pathway in the absence of pheromone. Thus, dual lipidation motifs on Gpa1p and Ste18p are required for a fully functional pheromone response pathway.

INTRODUCTION

Lipid modifications anchor heterotrimeric guanine nucleotide-binding proteins (G proteins) to the inner leaflet of the plasma membrane. G protein α subunits are fatty acylated with amide-linked myristate, thioester-linked palmitate, or both. G protein γ subunits are prenylated with either farnesyl or geranylgeranyl moieties through stable thioether linkages. Prenylation of γ subunits and myristoylation of α subunits are essential for the function of G proteins. These modifications promote plasma membrane association and facilitate high-affinity protein-protein interactions (reviewed in Wedegaertner *et al.*, 1995). The functional consequences of thioacylation are less well understood. Thioacy-

lation does not appear to be a major determinant of membrane avidity, at least in the presence of $G_{\beta\gamma}$ subunits, but may play a role in targeting G_{α} specifically to the plasma membrane (Dunphy *et al.*, 1996; Morales *et al.*, 1998; Fishburn *et al.*, 1999; Huang *et al.*, 1999). In vitro studies have demonstrated the importance of thioester-linked lipid in mediating protein-protein interactions of G_{α} subunits. Thioacylation increases the affinity of $G_{s\alpha}$ for $G_{\beta\gamma}$ approximately fivefold (Iiri *et al.*, 1996) and negatively regulates the interaction between regulators of G protein signaling and G_{α} subunits (Tu *et al.*, 1997). Thus, thioacylation may impact protein-protein interactions, as well as the subcellular distribution of modified proteins.

We have investigated thioacylation of G proteins in the genetically tractable organism *Saccharomyces cerevisiae*. The pheromone response pathway in this species of yeast is regulated by a heterotrimeric G protein encoded by *GPA1* (α subunit), *STE4* (β subunit), and *STE18* (γ subunit) (reviewed in Sprague and Thorner, 1992; Leberer *et al.*, 1997). In this pathway, $\beta\gamma$ propagates the signal from the receptor via the

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Abbreviations used: bp, base pair; GFP, green fluorescent protein; G proteins, heterotrimeric guanine nucleotide-binding proteins; HA, hemagglutinin; kb, kilobase; WT, wild type.

Table 1. Yeast strains used in this study

Strain	Genotype	Source or Reference
W303a	<i>MATa ade2-1 ura3-1 his3-11,15 leu2-3, 112 trp1-1 can1-100</i>	
W303a/ α	<i>MATa/α, ade2-1/ade2-1, ura3-1/ura3-1, his3-11,15/his3-11,15, leu2-3,112/leu2-3,112, trp1-1/trp1-1, can1-100/can1-100</i>	
SWY 518	W303aADE2	Bucci and Wente, 1997
SWY 595	W303a/ α ADE2/ADE2	Bucci and Wente, 1997
RK511-6B-1	<i>MATa ade2 ura3-52 his3-Δ1 his6 leu2-3,112 trp1-1 sst1-3 ste18Δ::URA3</i>	Grishin <i>et al.</i> , 1994b
YML20	W303a/ α <i>GPA1/gpa1Δ::URA3</i>	This work
CMY23	W303a <i>gpa1Δ::URA3-TRP1-GPA1 sst1Δ</i>	This work
CMY24	W303a <i>gpa1Δ::URA3-TRP1-gpa1-C3A sst1Δ</i>	This work
CMY26	CMY23 <i>ste2Δ</i>	This work
CMY25	CMY24 <i>ste2Δ</i>	This work
DY150	<i>MATa ade2-1 ura3-52, his3-11 leu2-3, 112 trp1-1 can 1-100</i>	AMRAD Biotech

activation of a MAP kinase cascade. This initiates cellular responses associated with mating, including growth arrest in the G_1 phase of the cell cycle. In cells lacking functional Gpa1p, $\beta\gamma$ subunits activate the mating pathway constitutively, resulting in irreversible growth arrest. The *GPA1* gene product acts as a negative regulator of the pathway, sequestering $\beta\gamma$ subunits and preventing activation of downstream effectors in the absence of a mating partner.

The lipid modifications identified on mammalian G protein subunits are conserved in yeast G proteins. The *GPA1* gene product (Gpa1p) is myristoylated, and this modification is essential for viability of haploid yeast (Stone *et al.*, 1991). A cysteine residue is found adjacent to the N-myristoylated glycine in Gpa1p, and palmitoylation of Gpa1p at this site has been reported (Song and Dohlman, 1996). However, the radioactive fatty acids incorporated into Gpa1p were not identified. Cells expressing thioacylation-defective Gpa1p are supersensitive to pheromone and may display partial constitutive activity of the pathway (Song and Dohlman, 1996).

The G_γ subunit Ste18p is prenylated, most likely with a farnesyl moiety, on a C-terminal cysteine (Finegold *et al.*, 1990). Loss of the G_γ prenylation site results in sterility. Replacement of the C-terminal lipidation motif in Ste18p with a transmembrane domain results in a functional protein, arguing that the function of the C-terminal domain is membrane localization of $G_{\beta\gamma}$ (Pryciak and Huntress, 1998). Recent work by Pryciak and Huntress (1998) strongly suggests that an important function of $G_{\beta\gamma}$ is to recruit Ste5p, a scaffolding protein that binds to the kinases in the cascade, to the plasma membrane. Green fluorescent protein (GFP)-Ste5p is recruited to the plasma membrane upon pheromone stimulation in a $G_{\beta\gamma}$ -dependent manner. In addition, increased expression of Ste4p results in GFP-Ste5p recruitment in the absence of pheromone treatment. Interestingly, constitutive targeting of Ste5p to the plasma membrane bypasses the requirement for pheromone or $G_{\beta\gamma}$ for pathway activation, suggesting that the primary function of $G_{\beta\gamma}$ in signal transduction is the recruitment of Ste5p to the plasma membrane (Pryciak and Huntress, 1998).

In Ste18p, a cysteine residue is found immediately upstream of the prenylated cysteine that is a potential site for thioacylation. Mutation of this site also causes a severe defect in the activity of Ste18p (Whiteway and Thomas, 1994). While our manuscript was in revision, Hirschman and Jen-

ness (1999) reported that Ste18p is thioacylated. Their analysis of the localization of prenylation- and thioacylation-defective mutants of Ste18p led them to conclude that lipid modifications on Ste18p are required for plasma membrane localization of $G_{\beta\gamma}$ (Hirschman and Jenness, 1999). However, these findings are inconsistent with previous data showing that nonprenylated Ste18p functionally couples to the plasma membrane-bound receptor Ste2p in *in vitro* assays (Grishin *et al.*, 1994a). This suggests that nonlipidated Ste18p is localized at the plasma membrane at least transiently and suggests that the signaling defect caused by lack of thioacylation is associated with defective interactions with downstream components of the pathway or maintenance of $G_{\beta\gamma}$ on the plasma membrane.

In this study, we present a biochemical analysis of the thioacylation of Ste18p and Gpa1p and characterize the phenotypic consequences of loss of thioacylation. Our analysis of the localization of GFP-tagged Ste18p and its lipidated C-terminal domain fused to GFP points to the importance of protein-protein interactions, as well as lipid modifications, in targeting proteins to the plasma membrane.

MATERIALS AND METHODS

Yeast Strains, Media, and Microbiological Techniques

The *S. cerevisiae* strains used in this study are described in Table 1. Yeast cultures were grown in rich medium (YPD) or synthetic minimal medium (Sherman, 1991), supplemented with amino acids to satisfy auxotrophic requirements but maintain plasmids. Glucose was added to a final concentration of 2% (wt/vol) unless otherwise stated. Yeast transformations were performed by the alkali cation method (Ito *et al.*, 1983). Genetic manipulation of yeast cells was as described (Sherman and Hicks, 1991).

Construction of Plasmids Expressing GFP Fused to Full-Length Ste18p and the C-Terminal Nine Amino Acids of Ste18p (GFP-CTSte18)

To create the GFP-Ste18p full-length fusions, we PCR amplified the coding sequence of GFP from pBJ646 (GFP-S65T) (Waddle *et al.*, 1996) using oligonucleotides that destroy the GFP stop codon and add the influenza virus hemagglutinin (HA) epitope (Wilson *et al.*, 1984) to the 3' end of GFP. The PCR product and pVTU-HA-Ste18 (Finegold *et al.*, 1990) were digested with *Pst*I and *Nhe*I and ligated together. This resulted in a GFP-Ste18p fusion with the HA tag as a

Table 2. Sequences from Ste18p fused to the C terminus of GFP

Construct	Amino acid sequence ^a
Wild type	-S-N-S-V-C-C-T-L-M-COOH
C107S	-S-N-S-V-C-S-T-L-M-COOH
C106S	-S-N-S-V-S-C-T-L-M-COOH

^a Amino acids are indicated by their single letter code. Mutated amino acids are underlined.

linker region. Point mutations C106S and C107S were generated by PCR. Complementation of *ste18Δ* was confirmed by transforming RK511-6B-1 with Trp-marked versions of pVTUGFP-HA-Ste18 (wild type [WT], C106S, and C107S).

To construct fusions of GFP with the last nine amino acids of Ste18p in frame, we used a synthetic linker strategy. GFP was PCR amplified from pBJ646 (Waddle *et al.*, 1996), adding *Xba*I and *Xho*I sites to the ends. pVT102U (Vernet *et al.*, 1987) was digested with *Xho*I and *Xba*I and ligated with the GFP fragment, creating pML1. Synthetic complementary oligonucleotides encoding wild-type or mutant Ste18p sequences (see Table 2) were annealed and ligated with pML1 digested with *Xho*I and *Not*I. The resulting plasmids were pML2 (WT), pML3 (C107S equivalent), and pML4 (C106S equivalent).

Construction of Plasmids to Generate Recombinant Baculovirus

A single recombinant baculovirus expressing Ste4p from the polyhedrin promoter and Ste18p from the P10 promoter was generated using the plasmid p2Bac (Invitrogen, San Diego, CA). The *STE4* sequence was derived from pBM258-Ste4His8 (Rodriguez and Blumer, unpublished observations) that encodes a polyhistidine tag at the C terminus of Ste4p. An *Eco*RI fragment containing the *STE4* sequence was ligated into p2Bac at the *Eco*RI site, resulting in p2BacSte4.

To subclone wild-type or mutated *STE18* into p2BacSte4, we used pVT-HA-Ste18 (Finegold *et al.*, 1990) as a template for mutagenic PCR. The PCR products were digested with *Eco*RV and *Bam*HI and subcloned into pBluescript SK⁺ (Stratagene, La Jolla, CA). *Sac*I/*Apa*I fragments from pBS-Ste18 (wild type, C106S, or C107S) were ligated into p2BacSte4 digested with *Stu*I and *Apa*I.

The 1.9-kilobase (kb) *Eco*RI fragment of *GPA1* containing the promoter and the entire coding region was isolated from YCplac22 (Stone and Reed, 1990) and subcloned into a pBluescript SK⁺ vector that was modified to delete the *Hind*III to *Kpn*I sites in the multiple cloning site, resulting in plasmid pBS-Gpa1. A *Nco*I site was created in *GPA1* at the initiator methionine using inverse PCR (Weiner *et al.*, 1994). A baculovirus plasmid encoding an HA epitope-tagged form of Gpa1p was created with NGpa1 as a template for PCR that adds sequence encoding the HA epitope at the internal *Hind*III site. The PCR product was digested with *Nco*I and *Hind*III and ligated into NGpa1, replacing the corresponding *Nco*I-*Hind*III fragment. The resulting plasmid NGpa1-HA was digested with *Eco*RI, end filled with Klenow, and cut with *Nco*I. The *GPA1* fragment was ligated into p2Bac that had been digested with *Hind*III, end filled, and cut with *Nco*I, generating p2BacGpa1. Recombinant baculoviruses were generated as described (Iniguez-Lluhi *et al.*, 1992).

Radiolabeling of GFP-Ste18p Fusions Expressed in Yeast

GFP vectors (pVTU-GFP-HA-Ste18p WT, C106S, or C107S) were transformed into yeast strain SWY518 along with pAG3STE4 (Grishin *et al.*, 1994a). Early logarithmic cells (A_{600} , 0.3–0.5) were

treated with a final concentration of 25 μ M cerulenin (Sigma, St. Louis, MO) and 2% (final concentration) galactose for 105 min. [9,10-³H]palmitic acid (56.5 Ci/mmol; DuPont NEN, Wilmington, DE) was added in ethanol (final concentration, 200 μ Ci/ml), and the cells were labeled for 15 min. Nonradioactive samples for immunoblotting were processed in parallel in the presence of vehicle. The labeling of cells expressing GFP-Ste18 peptide fusions was performed similarly, except that galactose was omitted during the cerulenin treatment.

After radiolabeling, yeast were collected by centrifugation, washed in sterile water, and suspended in lysis buffer (50 mM Tris, pH 8, 10% glycerol, 0.1 M NaCl, 11 mM EDTA, 1 mM EGTA, and protease inhibitors [2.1 μ g/ml aprotinin, 1 mM benzamidine hydrochloride, 1 μ g/ml chymostatin, 8.2 μ g/ml leupeptin, 3.2 μ g/ml lima bean trypsin inhibitor, 1 mM Pefabloc SC (Boehringer Mannheim), and 1 μ g/ml pepstatin A]). SDS was added to a final concentration of 2%, and the cells were lysed by vortexing with 0.5-mm glass beads (Biospec Products, Bartlesville, OK), eight times for 1 min each. The lysates were heated for 5 min at 65°C and centrifuged at 100,000 $\times$ g for 30 min at 4°C. To reduce the concentration of SDS to 0.2%, we diluted the supernatants fivefold in water and then twofold in immunoprecipitation buffer (50 mM Tris, pH 8, 100 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, 0.5 mM EGTA, and protease inhibitors [listed above]).

GFP-CTSte18 fusion proteins were immunoprecipitated using GFP polyclonal antibody (Clontech, Cambridge, United Kingdom) and washed Pansorbin cells (Calbiochem, San Diego, CA). GFP-HA-Ste18p proteins (full-length Ste18p fusions) were immunoprecipitated using an affinity-purified antibody against the HA epitope (12CA5; Boehringer Mannheim, Indianapolis, IN) and Protein G Sepharose (Pharmacia, Piscataway, NJ). Immunoprecipitates were washed three times in immunoprecipitation buffer. Protein was eluted from the beads in SDS sample buffer by heating at 100°C for 1 min and was resolved by SDS-PAGE. The gels were stained with Coomassie blue (Sigma, St. Louis, MO), destained, treated with Amplify (Amersham, Arlington Heights, IL) for 30 min, dried, and exposed to film (Kodak X-Omat; Eastman Kodak, Rochester, NY) for the indicated time at –75°C.

Radiolabeling of Ste18p in Insect Cells

Sf9 cells were coinfecting with Gpa1p and Ste4p/Ste18p (WT, C106S, or C107S) viruses and radiolabeled with [³⁵S]methionine or [³H]palmitate as described (Linder *et al.*, 1995). Cell lysates were prepared (Linder *et al.*, 1995), and Ste18p proteins were immunoprecipitated using monoclonal antibody 12CA5 (Wilson *et al.*, 1984) and processed for fluorography.

Fluorescence Microscopy

SWY518 cells transformed with the appropriate plasmids were grown to early log phase (A_{600} , 0.1–0.3) in synthetic media to select for GFP-CTSte18 or GFP-HA-Ste18 plasmids. After concentration by centrifugation, the cells were suspended in 1/10th volume of synthetic medium and viewed using an Olympus epifluorescence microscope (Olympus, Mellville, NY) equipped with UG-1, BP490, and BP545 dichroic filters (Chroma Technology, Battleboro, VT) and a cooled charged-coupled device camera (Dage, Michigan City, IN). Confocal microscopy was performed on GFP full-length Ste18p fusions. Live cells were imaged using an Axioplan microscope (Zeiss, Thornwood, NJ) coupled to an MRC-1000 Laser Scanning Microscope (Bio-Rad, Richmond, CA). For pheromone treatment of cells, early log cultures were collected by centrifugation, washed once with 10 volumes of prewarmed media, and suspended in the same amount of prewarmed media. Pheromone (α -factor; Sigma) was added to a final concentration of 5 μ M, and the cells were incubated at 30°C for 2 h for maximal response. The images represent single planes obtained from the middle of the cell using a 63 $\times$

objective. Images were processed using Adobe Photoshop 5.0 (Adobe, Mountain View, CA).

Construction of Plasmids for Radiolabeling *Gpa1p* in Yeast

Gpa1p was expressed from a copper-inducible promoter using plasmid pKM1362-2 (Madura and Varshavsky, 1994). This plasmid encodes *GPA1* fused in frame to the HA epitope at the 3' end of the *GPA1*-coding sequence. pKM1362-2 was renamed pCMGpa1-HA. N-terminal mutations (G2A, C3A, and G2A/C3A) in *Gpa1p* were created using PCR mutagenesis.

Radiolabeling and Immunoprecipitation of *Gpa1p*

Yeast cells (DY150) carrying pVT-HA-Ste18 (Finegold *et al.*, 1990), pAG3STE4 (Grishin *et al.*, 1994a), and wild-type or mutated pCMGpa1-HA were grown to midlogarithmic phase (A_{600} , 0.5–0.7) in sucrose (2% wt/vol) synthetic medium at 30°C. *Gpa1p* and *Ste4p* were induced by adding CuSO_4 (final concentration, 100 μM) and galactose (final concentration, 2% wt/vol), respectively. *Ste18p* is constitutively expressed from the *ADH1* promoter. Four hours after induction, cerulenin was added to a final concentration of 25 μM . Immediately after cerulenin addition, [9,10- ^3H]myristic acid (11.2 Ci/mmol; DuPont NEN) was added to a final concentration of 100 $\mu\text{Ci/ml}$, and the cells were incubated for an additional 2 h. For palmitate labeling, [^3H]palmitate was added (final concentration, 200 $\mu\text{Ci/ml}$) 105 min after cerulenin, and the cells were incubated for an additional 15 min.

Cell lysates were prepared as described above, and *Gpa1p* proteins were immunoprecipitated using the 12CA5 antibody. Immunoprecipitates were processed for fluorography. Radioactive fatty acids incorporated into *Gpa1p* were hydrolyzed and analyzed by TLC as described (Linder *et al.*, 1995).

Deletion of *GPA1* and Integration of the *gpa1-C3A* Allele into the Yeast Genome

To delete the chromosomal copy of *GPA1*, we created pBS-*gpa1/URA3* with the *URA3* gene inserted in the *GPA1* sequence (*NcoI* to *SphI*) from plasmid NGpa1, replacing the coding sequence for amino acids 1–400. The plasmid was digested with *EcoRI* and transformed into yeast strain W303a/ α . Deletion of *GPA1* was confirmed by tetrad analysis and Southern blotting.

Plasmid NGpa1C3A was generated by PCR mutagenesis. To create the plasmids for integration of wild type or *gpa1-C3A*, we added genomic sequences flanking the 1.9-kb *EcoRI* fragment to facilitate integration. To add sequence downstream of the *GPA1* locus, including a *BglII* site to direct integration, we PCR amplified a 500–base pair (bp) segment of genomic DNA from yeast strain W303a. The resulting PCR fragment was subcloned into NGpa1 or NGpa1C3A using *NsiI* and *XbaI*. A *KpnI*–*SacI* fragment containing the 2.4-kb fragment *GPA1* or *gpa1-C3A* was ligated into pRS304 (Sikorski and Hieter, 1989), creating pCM1 (wild type) and pCM2 (C3A). Additional genomic sequence 5' of *GPA1* was PCR amplified and ligated into pCM1 or pCM2 digested with *KpnI* and *NcoI*. The resulting plasmids (pCM4 and pCM5) contained an additional 960-bp noncoding sequence upstream of the *EcoRI* fragment of *GPA1*. pCM4 and pCM5 were digested with *BglII* and transformed into YML20. Trp^+ transformants were sporulated, and tetrad analysis was performed. Ura^+ Trp^+ colonies were analyzed further by Southern analysis to confirm proper integration downstream of *gpa1::URA3*. The *SST1* gene was disrupted using plasmid pJGst1) (Reneke *et al.*, 1988) digested with *SalI* and *EcoRI* and transformed into yeast strains to create CMY23 and CMY24. Successful disruptions (as assayed by halo assays) were streaked on media containing 5'-fluoro-orotic acid, so that the *STE2* disruption could be performed.

The *STE2* disruptions were created in CMY23 and CMY24 using the integrating plasmid Ypste2), which contains the 5'- and 3'-untranslated regions of *STE2* but no coding sequence (Stefan *et al.*, 1998). The plasmid was digested with *Clal* and transformed into yeast. Ura^+ cells were streaked on media containing 5'-fluoro-orotic acid. Cells deleted for *STE2* were identified by patch-mating tests.

Phenotypic Analysis of Mutants

Pheromone-induced gene expression was assayed using a plasmid (pRS424-*FUS1-lacZ* [Johnson *et al.*, 1994]) that contains the pheromone-inducible reporter *FUS1-lacZ*. Cells in early logarithmic phase (A_{600} , 0.35–0.5) were treated with various concentrations of α -factor for 2 h. β -Galactosidase activity in permeabilized cells was determined (McCaffrey *et al.*, 1987) and expressed in Miller units: $[\text{OD}_{405}/(\text{OD}_{600} \times \text{volume of cells [in milliliters]} \times \text{time [in minutes]})] \times 1000$.

Gpa1p protein levels were determined by immunoblot of 100 μg of yeast total cell lysate from CMY23 and CMY24. Endogenous *Gpa1p* was detected with 9126, a polyclonal antibody against *Gpa1p* (generously provided by D. Stone, University of Illinois, Chicago, IL). The blots were probed with [^{125}I]-labeled secondary antibody and exposed to a phosphorimaging screen for quantitation using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Yeast *G γ* Subunits Are Thioacylated

The yeast G protein γ subunit is a candidate for thioacylation at cysteine 106, the amino acid adjacent to the prenylated cysteine (Finegold *et al.*, 1990; Whiteway and Thomas, 1994). Therefore, we sought to determine whether *Ste18p* is thioacylated and how this modification impacts *Ste18p* function. Attempts to isolate epitope-tagged *Ste18p* expressed in yeast were unsuccessful because of low levels of protein expression (Grishin *et al.*, 1994a). However, we were able to detect expression of GFP fused to full-length *Ste18p* using a linker of 18 amino acids, including an HA epitope, between GFP and the N terminus of *Ste18p*. Expression of this fusion protein complemented a *ste18 Δ* mutation, whereas a *Ste18p*-GFP fusion lacking the linker was not functional (our unpublished observations).

To determine whether the GFP-*Ste18p* fusion is a substrate for thioacylation in yeast, cells expressing GFP-*Ste18p* fusions (WT, C106S, or C107S) were labeled with [^3H]palmitate. Wild-type *Ste18p* incorporated radioactivity (Figure 1A, lane 1) that was sensitive to neutral hydroxylamine (our unpublished observations), consistent with the incorporation of radioactivity into the protein through a thioester linkage. Mutation of the thioacylation site, cysteine 106 to serine, abolished the incorporation of radioactive palmitate (Figure 1A, lane 2), suggesting that cysteine 106 is the thioacylation site. These results confirm those published recently by Hirschman and Jenness (1999). As in Ras proteins that are both prenylated and thioacylated (Hancock *et al.*, 1989; Fujiyama *et al.*, 1991), the incorporation of palmitate into *Ste18p* was dependent on an intact prenylation site (Figure 1A, lane 3). Mutation of the prenylation site cysteine 107 abolished incorporation of radioactivity into *Ste18p*. Thus, *Ste18p* thioacylation appears to require cysteine 106 and previous prenylation at cysteine 107.

Because of the low signals of [^3H]palmitate-labeled *Ste18p* expressed in yeast cells, we used a heterologous expression system to confirm our results. The baculovirus expression

Figure 1. $[^3\text{H}]$ palmitate incorporation into wild-type and mutated Ste18p proteins. (A) Top, yeast cells expressing GFP-HA-Ste18p (wild type, C106S, or C107S) were incubated with $[^3\text{H}]$ palmitate. After cell lysis, GFP-HA-Ste18p was immunoprecipitated with 12CA5 antibody and analyzed by SDS-PAGE and fluorography. The arrow indicates GFP-HA-Ste18p. The top band (*) is nonspecific binding of free $[^3\text{H}]$ palmitate to heavy-chain IgG that becomes evident in long exposures. The film was exposed for 2 mo. Bottom, an immunoblot performed on yeast cell lysates from mock-labeled cells expressing GFP-HA-Ste18p (wild type, C106S, or C107S) is shown. Equal amounts of protein were loaded in each lane. The arrow indicates the GFP-HA-Ste18p protein. The higher molecular weight band is a nonspecific band from yeast cytosol. (B) Sf9 cells were coinfectd for 26 h with viruses encoding Gpa1p and Ste4p/Ste18p (wild type, C106S, or C107S). Cells were labeled with $[^{35}\text{S}]$ methionine or $[^3\text{H}]$ palmitate. Ste18p was immunoprecipitated and analyzed by SDS-PAGE and fluorography. The film was exposed for 2 d. Palm, palmitate; Met, methionine.

system has been used successfully to study lipid modifications of heterotrimeric G proteins (Iniguez-Lluhi *et al.*, 1992; Linder *et al.*, 1993). Therefore, we expressed epitope-tagged Ste18p in insect cells using recombinant baculovirus to determine whether it is a substrate for thioacylation. To facilitate stable expression of Ste18p and its association with membranes, we expressed the yeast heterotrimer by coinfecting cells with viruses encoding Gpa1p, Ste18p, and Ste4p. Confirming the results in yeast, wild-type Ste18p incorporated radioactivity when cells were labeled with $[^3\text{H}]$ palmitate (Figure 1B, lane 1). Mutation of the putative thioacylation site (C106) or prenylation site (C107) to serine in Ste18p abolished incorporation of radioactive palmitate (Figure 1B, lanes 2 and 3). Base hydrolysates from radiolabeled Ste18p were analyzed by high-pressure liquid chromatography. The radiolabel released from wild-type Ste18p coeluted with a palmitate standard (our unpublished observations). The data from yeast and this heterologous system

support the hypothesis (Whiteway and Thomas, 1994) that Ste18p is posttranslationally modified with a thioester-linked lipid on cysteine 106.

Subcellular Localization of Ste18p Is Dependent on Lipid Modifications and Protein-Protein Interactions

Mutation of the prenylation site or thioacylation site in Ste18p results in cells that are sterile or severely compromised in their ability to mate, respectively (Whiteway and Thomas, 1994). While this work was in progress, Hirschman and Jenness (1999) proposed that the signaling defect caused by the loss of thioacylation or prenylation of Ste18p was caused by mislocalization from the plasma membrane. However, a previous study showed that a heterotrimer of nonprenylated Ste18p, Ste4p, and Gpa1p coupled to the pheromone receptor Ste2p in membrane preparations, consistent with nonprenylated γ being localized at the plasma membrane (Grishin *et al.*, 1994a). To investigate further the relationship between localization and the signaling capacity of Ste18p, we compared the localization of wild-type GFP-tagged Ste18p with lipidation-defective mutants in living cells. In agreement with previous studies (Hirschman *et al.*, 1997; Pryciak and Huntress, 1998), the wild-type protein was localized at the plasma membrane in the basal state and remained there after pheromone treatment (Figure 2). Wild-type Ste18p had a punctate appearance in its staining of the plasma membrane, but the nature of these structures, which are clearly at the cell surface, is unknown. GFP-Ste18p with mutations in either the thioacylation site or prenylation site also localized to the plasma membrane effectively under basal conditions (Figure 2), in agreement with the demonstration that prenylation is dispensable for receptor-G protein coupling in yeast plasma membrane fractions *in vitro* (Grishin *et al.*, 1994a). However, upon pheromone treatment, which disassociates the heterotrimer, prenylation- or thioacylation-defective GFP-Ste18p was released into the cytoplasm (Figure 2). These data suggest that in the absence of lipid modifications, binding to G_{α} is sufficient to target $G_{\beta\gamma}$ to the plasma membrane in yeast. However, lipid modifications of Ste18p are required for the maintenance of plasma membrane association when $G_{\beta\gamma}$ dissociates from G_{α} .

To provide additional support for the hypothesis that interactions with Gpa1p promote membrane targeting of the thioacylation-defective mutant of Ste18p, we expressed this mutant in diploid cells in the absence or presence of Gpa1p. Gpa1p, Ste4p, and Ste18p are not expressed in diploid cells, eliminating the possibility that targeting of the GFP-tagged construct might be caused by association with endogenous G protein subunits. When GFP-Ste18 was coexpressed with Ste4p in diploid cells, wild-type Ste18p localized to the plasma membrane, whereas the C106S mutant was cytoplasmic (Figure 3). However, when Gpa1p was expressed with Ste4p and GFP-Ste18C106S in diploid cells, mutant Ste18p was now localized at the plasma membrane (Figure 3). These data are consistent with the hypothesis that thioacylation-defective Ste18p can be targeted to membranes via interactions with Gpa1p.

Taken together, our data suggest that lipid modifications of Ste18p are dispensable for the initial targeting of $G_{\beta\gamma}$ to

Figure 2. Lipid modifications are not required for targeting but are essential for the maintenance of Ste18p at the plasma membrane after release from Gpa1p. Yeast cells expressing GFP-HA-Ste18p (wild type, C106S, or C107S) were visualized by confocal microscopy (–). Cells were treated with α -factor (+) as described in MATERIALS AND METHODS and visualized. Note that cells expressing GFP-HA-Ste18p wild type exhibit the characteristic morphological change known as “shmooing,” which is associated with activation of the signaling cascade, whereas the mutants C106S and C107S do not.

the plasma membrane when G_{α} is expressed but are required for maintenance of the plasma membrane association when $\beta\gamma$ dissociates from G_{α} . Thus, the signaling defect that is associated with mutation of the prenylation or thioacylation sites in Ste18p is downstream of interactions with receptor and Gpa1p, consistent with the data of Grishin *et al.* (1994a). The presence of lipidation-defective mutants of Ste18p in the cytoplasm after receptor activation prevents the recruitment of Ste5p to the plasma membrane and disables their interactions with other plasma membrane-bound effectors. This results in defective signaling.

A Prenylation and Thioacylation Motif Is Sufficient for Plasma Membrane Targeting

To determine the role of thioacylation in the plasma membrane association of G_{γ} , independent of its interaction with Gpa1p, we fused the last nine amino acids of Ste18p to the C terminus of GFP (Table 2). The construct containing wild-type sequence includes the dual lipidation motif. Similar to

Figure 3. Thioacylation-defective Ste18p is localized at the plasma membrane in diploid cells only when Gpa1p is also present. Live yeast cells expressing GFP-HA-Ste18p (wild type or C106S) and β (Ste4p), in the absence ($\beta\gamma$ only) or presence (trimer) of Gpa1p, were visualized by confocal microscopy.

full-length Ste18p in yeast and Sf9 cells, these C-terminal peptides from Ste18p fused to GFP were substrates for thioacylation in yeast. When GFP-CTSte18p (WT) was expressed in yeast cells radiolabeled with [3 H]palmitate, radioactivity was incorporated into the immunoprecipitated protein (Figure 4A, lane 1) that was hydroxylamine sensitive (our unpublished observations). Thioacylation of these constructs appeared to be dependent on the cysteine equivalent to C106 in wild-type Ste18p and previous prenylation at cysteine 107 (in WT Ste18p), because no radioactivity was incorporated into these mutant proteins (Figure 4A, lanes 2 and 3). Therefore, these constructs contain the lipid modifications found on full-length wild-type Ste18p.

When viewed by fluorescence microscopy, GFP-CTSte18 was localized to the plasma membrane (Figure 4B), confirming that the last nine amino acids of Ste18p (Table 2) are sufficient to target a heterologous protein to the plasma membrane (Srinivasa *et al.*, 1998). Mutation of the cysteine corresponding to C106 in Ste18p, the thioacylation site, or the residue corresponding to the farnesylation site (equivalent to C107) resulted in a loss of plasma membrane localization, suggesting that both lipid modifications are required for plasma membrane association in the absence of protein-protein interactions with Gpa1p (Figure 4B). Interestingly, GFP-CTSte18 exhibited uniform staining of the plasma membrane. The punctate staining seen with full-length Ste18p (Figures 2 and 3) was not apparent, suggesting that features of Ste18p other than the lipidated sequence or GFP contribute to this localization pattern.

Gpa1p Is Dually Myristoylated

Song and Dohlman (1996) have reported previously that Gpa1p is palmitoylated at cysteine 3 in a manner that is de-

Figure 4. GFP-CTSte18 localization at the plasma membrane requires cysteine residues that are sites for lipid modification. (A) Top, SWY518 cells expressing GFP-CTSte18 (WT, C107S equivalent, or C106S equivalent) were incubated with $[^3\text{H}]$ palmitate. After cell lysis, GFP-CTSte18 was immunoprecipitated and analyzed by SDS-PAGE and fluorography. The film was exposed for 2 wk. Bottom, yeast lysates from cells expressing GFP-CTSte18 fusions were transferred to nitrocellulose and immunoblotted for GFP. Because GFP-CTSte18-C107S is expressed at lower levels than other proteins, twice as much lysate was immunoprecipitated to get equal amounts of protein. (B) Yeast cells expressing GFP-CTSte18 fusions (wild type, C107S equivalent, or C106S equivalent) or GFP alone (GFP) were visualized by Nomarski and GFP fluorescence (Fluor.).

pendent on previous N-myristoylation of the protein. We sought to confirm these results and to analyze the fatty acids incorporated into Gpa1p through thioester linkages. Yeast cells expressing high levels of Gpa1p, Ste4p, and Ste18p were incubated with $[^3\text{H}]$ palmitate and $[^3\text{H}]$ myristate. Wild-type Gpa1p incorporated radioactivity when cells were labeled with $[^3\text{H}]$ palmitate (Figure 5A, lane 1), consistent with thioacylation of the protein. As expected (Stone *et al.*, 1991), $[^3\text{H}]$ myristate was incorporated into Gpa1p (Figure 5A, lane 1). To determine whether the cysteine residue at position 3 is required for thioacylation of Gpa1p, a mutant protein with an alanine substitu-

Figure 5. $[^3\text{H}]$ palmitate and $[^3\text{H}]$ myristate incorporation into wild-type and mutated Gpa1p proteins in yeast. (A) DY150 cells expressing HA-tagged Gpa1p (wild type, C3A, G2A, or G2A/C3A), Ste4p, and Ste18p were incubated with $[^3\text{H}]$ palmitate or $[^3\text{H}]$ myristate. Gpa1p was immunoprecipitated and analyzed by SDS-PAGE and fluorography. The film was exposed for 3 d. (B) Gpa1p (wild type or C3A) was immunoprecipitated from yeast cells labeled with $[^3\text{H}]$ palmitate and resolved by SDS-PAGE. The Gpa1p bands were excised and processed (Linder *et al.*, 1995). The base hydrolysates were analyzed by reversed-phase TLC. Radioactive standards were run in parallel, and their positions are indicated by arrows. The TLC plates were treated for fluorography and exposed to film for 5 wk. Myr, myristate; C14:0, myristate; C16:0, palmitate; C18:0, stearate.

tion at this site (C3A Gpa1p) was expressed in yeast. C3A Gpa1p incorporated very little radioactivity when cells were incubated with $[^3\text{H}]$ palmitate (Figure 5A, lane 2).¹ Incorporation

¹ The residual signal in C3A Gpa1p labeled with $[^3\text{H}]$ palmitate was not sensitive to hydroxylamine (our unpublished observations), suggesting that the radioactivity was incorporated into C3A Gpa1p as amide-linked myristate. This interpretation is supported by the finding that mutation of the N-myristoylation site (G2A) completely abolished incorporation of either radioactive fatty acid into Gpa1p (see Figure 5, lane 3). Metabolic interconversion of radioactive palmitate into myristate and the subsequent incorporation into protein through an amide linkage have been well documented (Linder *et al.*, 1993; Wilson and Bourne, 1995).

ration of radioactive myristate into C3A Gpa1p was unaffected by the cysteine-to-alanine substitution (Figure 5A, lane 2); thus, N-myristoylation at glycine 2 occurred normally. However, thioacylation of Gpa1p at cysteine 3 was dependent on glycine 2, suggesting that N-myristoylation is a prerequisite for thioacylation. Our results confirm those published previously (Song and Dohlman, 1996).

Studies of a number of mammalian and viral proteins have revealed that fatty acids other than palmitate are incorporated into proteins through thioester linkages when cells are incubated with radiolabeled palmitate (Fujimoto *et al.*, 1993; Nadler *et al.*, 1994; Revery *et al.*, 1996). In a murine T cell line (LSTRA) fed [^3H] palmitate, p56lck incorporates predominately [^3H]palmitate, whereas the transferrin receptors incorporate predominately [^3H]stearate (Nadler *et al.*, 1994). Importantly, heterogeneous fatty acylation of proteins has also been revealed by mass spectrometric analysis of endogenous fatty acids released from rhodopsin (O'Brien *et al.*, 1987) and the Band 3 protein from human erythrocytes (Okubo *et al.*, 1991). To determine the type of fatty acid attached to Gpa1p in yeast incubated with [^3H]palmitate, we treated Gpa1p with base to release thioester-linked fatty acids and analyzed the hydrolysates by TLC (Figure 5B, left). Surprisingly, the radioactive fatty acid released from wild-type Gpa1p labeled with [^3H]palmitate comigrated with the myristate standard (Figure 5B). Smaller amounts of a species that comigrated with the palmitate standard were also detected. These data suggest that yeast take up [^3H]palmitate and convert a substantial fraction to [^3H]myristate in <15 min. Even in the absence of cerulenin, a specific inhibitor of fatty acid synthetase (Omura, 1976), myristate was incorporated preferentially in a base-sensitive manner into Gpa1p (our unpublished observations). As expected, no radioactive fatty acids were released from C3A Gpa1p after alkaline hydrolysis (Figure 5B, right). These data demonstrate that thioacylation of Gpa1p is heterogeneous; both myristate and palmitate can be incorporated into Gpa1p in a manner that is dependent on cysteine 3. When Gpa1p was expressed in insect cells using recombinant baculovirus, palmitate was the only fatty acid recovered after base hydrolysis (our unpublished observations). Thus, heterogeneous thioacylation of Gpa1p is not dictated by the protein but must reflect the acyl-CoA pools, the substrate specificity of thioacyltransferases (Nadler *et al.*, 1994), and other factors. The interpretation of these studies is limited by how well the incorporation of radioactive fatty acids reflects the endogenous fatty acids associated with the protein. Definitive identification of the posttranslational modifications of Gpa1p using mass spectroscopy will be an important step in clarifying the extent of heterogeneous fatty acylation.

The C3A Gpa1p Mutant Can Partially Rescue the *gpa1* Null

Song and Dohlman (1996) characterized the phenotype of thioacylation-defective Gpa1p using plasmid-based expression. They reported partial constitutive signaling through the pheromone response pathway in yeast cells expressing thioacylation-defective Gpa1p compared with wild-type cells. It is possible that this apparent constitutive activity is caused by autocrine secretion of α -factor or spontaneous loss of the Gpa1p C3S plasmid. To characterize further the phe-

Table 3. Constitutive expression of *FUS1-lacZ* in strains lacking the pheromone receptor and expressing wild-type Gpa1p or C3A Gpa1p from a chromosomal allele

	Trial 1	Trial 2
WT Gpa1p	1.7 $\pm$ 1.4	0.5 $\pm$ 0.7
C3A Gpa1p	51.4 $\pm$ 2.2	53.9 $\pm$ 15.0
No. isolates	4	6

notype of thioacylation-defective Gpa1p, we chose to replace the wild-type allele of *GPA1* in the chromosome with thioacylation-defective *gpa1*. Integration of the mutant allele into the chromosome eliminates variability in plasmid copy number that affects protein expression levels. The pheromone response pathway is exquisitely sensitive to changes in Gpa1p expression (Cole *et al.*, 1990; Kang *et al.*, 1990). Studies of other *GPA1* mutations have revealed different phenotypes when expressed on a centromere-based plasmid instead of the chromosomal locus (Miyajima *et al.*, 1989; Kurjan *et al.*, 1991). We found that haploid cells expressing the mutant allele as the sole copy of *GPA1* were viable. The *gpa1*-C3A cells grew normally and at the same rate as wild-type cells at 30, 37, and 25°C (our unpublished observations). Protein levels of the Gpa1p mutant and wild type were indistinguishable in the two strains as measured by quantitative immunoblotting experiments (our unpublished observations).

After promoting receptor-dependent signaling, the major role of Gpa1p is to act as a negative regulator of the pathway by binding to $\beta\gamma$ and attenuating further signaling. Therefore, we tested the effect of mutating the thioacylation site on this process. In agreement with the results of Song and Dohlman (1996), the mutant cells did show alterations in their response to pheromone (our unpublished observations). The halo assay represents a test of the cells' ability to respond and adapt to pheromone. The strain expressing C3A Gpa1p gave rise to larger halos, demonstrating an increased response, two- to fivefold greater than that of wild-type cells. A shorter-term assay of signaling through the pheromone response pathway is the measurement of pheromone-induced increases in transcriptional activity of the *FUS1* gene using a β -galactosidase reporter construct. The strain expressing C3A Gpa1p was $\sim$ 10-fold more sensitive to pheromone (EC_{50} , $\sim$ 3 nM) than was wild-type Gpa1p (EC_{50} , $\sim$ 30 nM) (our unpublished observations).

We also observed that the strain expressing C3A Gpa1p exhibited β -galactosidase activity in the absence of pheromone (our unpublished observations), suggesting partial constitutive signaling through the pathway. To eliminate the possibility that this phenotype is caused by autocrine secretion of α -factor, we deleted the *STE2* gene encoding the α -factor receptor. Even in the absence of receptor, *FUS1-lacZ* expression was 20- to 50-fold higher in cells expressing C3A Gpa1p compared with that in wild type (Table 3). Constitutive signaling through the pathway indicates that a partial loss of function is associated with mutation of the thioacylation site in Gpa1p.

DISCUSSION

Role of Ste18p Thioacylation In Vivo

In this study, we report that the G protein γ subunit of *S. cerevisiae* is modified with a thioester-linked fatty acid at the cysteine residue immediately N-terminal to the farnesylated cysteine. One proposed function for thioacylation of Ste18p is constitutive association with the plasma membrane (Hirschman and Jenness, 1999). This hypothesis is consistent with the data of Hirschman and Jenness and the localization of our Ste18p C-terminal constructs (Figure 4). However, the role of thioacylation of Ste18p in vivo appears to be more complex. When Ste18p fused to GFP was expressed and visualized in haploid yeast cells, it localized to the plasma membrane, even when the sites of lipidation were mutated (Figure 2). This localization is consistent with previous work showing that prenylation-defective Ste18p (C107Y) is able to couple functionally to the receptor Ste2p, suggesting that this mutant is localized at the plasma membrane (Grishin *et al.*, 1994a). However, mutations in the prenylation site or the thioacylation site result in the severe loss of function in Ste18p (Whiteway and Thomas, 1994). Therefore, the signaling defect in these lipidation mutants must be associated with events downstream of receptor coupling.

Plasma membrane localization of $G_{\beta\gamma}$ is required for productive interactions not only with the receptor but also with effectors (Leberer *et al.*, 1997; Pryciak and Huntress, 1998). Our data suggest a model to explain the signaling phenotypes associated with the loss of prenylation or thioacylation (Figure 6). We propose that wild-type Ste18p/Ste4p is properly localized to the plasma membrane and, upon disassociation from Gpa1p, remains at the plasma membrane through Ste18p's lipid modifications. This sustained membrane association permits recruitment of Ste5p to the plasma membrane, which underlies activation of the pathway (Pryciak and Huntress, 1998), and interaction with other plasma membrane-associated effectors (Figure 6A). $G_{\beta\gamma}$ complexes containing Ste18p mutants lacking prenylation or thioacylation are targeted initially to the plasma membrane via their association with Gpa1p (see below). However, when dissociated from Gpa1p after pheromone treatment, the mutants are released into the cytoplasm (Figure 6B). The resulting spatial separation of $\beta\gamma$ from the plasma membrane prevents signaling to effectors.

Our data suggest that the lipids play a role in the membrane association of Ste18p, but only after subunit dissociation. This is in contrast to the recent work reporting constitutive loss of plasma membrane association by nonthioacylated Ste18pC106S (Hirschman and Jenness, 1999). The methods used in the two studies are different and may underlie the discrepancy in the data. Hirschman and Jenness (1999) followed Ste18p/Ste4p localization using high-ionic strength Renografin gradients of yeast cell lysates. This method might have resulted in the release of lipidation-defective Ste18p from membranes after cell lysis. Our results determine localization of these mutants in vivo using GFP-tagged Ste18p. We presume that the localization of GFP-tagged Ste18p is representative of native Ste18p because the wild-type fusion protein rescues mating in a *ste18 Δ* strain. Importantly, the evidence that pheromone treatment results in the release of the lipidation mutants into the cytosol suggests that the mutants are

Figure 6. Ste18p requires lipid modifications for signal propagation. (A) Yeast cells expressing wild-type Ste18p signal because $G_{\beta\gamma}$ is at the plasma membrane and remains there after release from Gpa1p. This permits $G_{\beta\gamma}$ to recruit and interact with plasma membrane-bound effectors. (B) The lipidation mutants (C106S or C107S) are targeted to the plasma membrane via their association with Gpa1p, which is dually acylated. However, upon receipt of a signal, Gpa1p binds GTP and releases $G_{\beta\gamma}$. Because the lipidation mutants have low affinity for the plasma membrane, $G_{\beta\gamma}$ is released into the cytosol. This spatial separation of $G_{\beta\gamma}$ from its membrane-bound effectors prevents it from signaling, and therefore, the cells are sterile.

binding Gpa1p and receptor, and this coupling is responsive to pheromone treatment (Figure 2). The dynamic localization of lipidation-defective GFP-Ste18p argues against its association with the plasma membrane via aggregation or any other nonspecific mechanism.

Plasma Membrane-targeting via Lipid Modifications and Protein-Protein Interactions

Many proteins involved in signal transduction pathways are associated with the plasma membrane via tandem lipid modifications, either N-myristoylation and thioacylation or farnesylation and thioacylation. We and others have demonstrated that short peptide sequences are sufficient to direct the addition of both lipids and confer plasma membrane targeting (Schroeder *et al.*, 1996, 1997; Wolven *et al.*, 1997) (this study). Biophysical measurements of the association of lipid-modified peptides with artificial bilayers provide a rationale for the requirement of two lipid modifications in targeting proteins to membranes (Peitzsch and McLaughlin,

1993; Silviu and l'Heureux, 1994; Shahinian and Silviu, 1995). A C15 farnesyl isoprenoid or an N-myristoyl group does not provide sufficient hydrophobicity to anchor a peptide stably to membranes. However, the addition of a second lipid modification dramatically slows the rate of interbilayer transfer such that the peptide is essentially permanently anchored. Shahinian and Silviu (1995) suggested the kinetic bilayer-trapping model as a mechanism for targeting proteins to a specific membrane. The model proposes that a farnesylated or N-myristoylated protein diffuses through the cytosol transiently associating with all membranes until it encounters the site where the second lipid is added. The dually modified protein becomes a resident of that membrane because of its extremely slow dissociation rate. Proteins that are thioacylated after N-myristoylation or prenylation could be "trapped" at the plasma membrane via the action of a plasma membrane-specific protein acyltransferase. Indeed, protein acyltransferase activity for $G_{i\alpha 1}$ is enriched in plasma membranes isolated from rat liver (Dunphy *et al.*, 1996). Furthermore, the trafficking of newly synthesized $G_{z\alpha}$ suggests that thioacylation is coincident with the protein's arrival at the plasma membrane (Fishburn *et al.*, 1999).

Characterization of thioacylation-defective Gpa1p and Ste18p provides support for the membrane-trapping model (Song and Dohlman, 1996; Hirschman and Jenness, 1999) (this study). In both proteins, thioacylation is dependent on a previous modification with myristate or a prenyl group. Similar to Ste18p, thioacylation-defective Gpa1p exhibits a partial loss of function phenotype that can be attributed to reduced plasma membrane localization (Song and Dohlman, 1996). A strain expressing thioacylation-defective Gpa1p exhibits partial constitutive activation of the pheromone response pathway (Table 3) (Song and Dohlman, 1996). This suggests a reduced interaction with $G_{\beta\gamma}$ subunits, allowing the pathway to be activated in the absence of pheromone. The reduced interaction is most likely explained by the finding that relative to wild-type Gpa1p, thioacylation-defective Gpa1p is reduced in plasma membrane fractions and increased in microsomal membrane fractions (Song and Dohlman, 1996). Reduced affinity for $G_{\beta\gamma}$ subunits is also potentially a determinant of the mutant phenotype. Although thioacylation-defective Gpa1p binds to $G_{\beta\gamma}$ complexes (Song and Dohlman, 1996), the affinity of these subunit interactions has not been determined quantitatively. In mammalian systems, thioacylation of $G_{s\alpha}$ increases its affinity for $G_{\beta\gamma}$ subunits approximately fivefold (Irie *et al.*, 1991). We found that modest increases in the expression of Gpa1p eliminated constitutive activity of the pathway (our unpublished observations). Increased levels of the mutant protein may compensate for a defect attributable to mislocalization or reduced subunit affinity. Thus, thioacylation of Gpa1p may contribute to both membrane association and subunit interactions.

The importance of protein-protein interactions in targeting signaling proteins to the plasma membrane is suggested by our analysis of lipidation-defective Ste18p. Binding of Ste18p/Ste4p to Gpa1p independent of Ste18p lipid modifications can be inferred from the ability of lipidation-defective mutants to support G protein coupling to receptor (Grishin *et al.*, 1994a). This raises the possibility that Gpa1p targets lipidation-defective $G_{\beta\gamma}$ to the plasma membrane.

Two lines of evidence support this hypothesis. First, treatment of yeast with pheromone, which would disassociate G_{α} from $G_{\beta\gamma}$, resulted in the maintenance of the wild-type Ste18p association with the plasma membrane (Figure 2). However, in yeast cells in which Ste18p was mutated in the sites for thioacylation or prenylation, $G_{\beta\gamma}$ was released from the plasma membrane into the cytoplasm upon receptor-stimulated release from Gpa1p (Figure 2). Second, expression of $G_{\beta\gamma}$ (C106S) in diploids resulted in a cytoplasmic localization of Ste18p (Figure 3). Diploid yeast cells do not express Ste18p, Ste4p, Gpa1p, or Ste2p, the receptor (Sprague and Thorner, 1992). However, association of $G_{\beta\gamma}$ (C106S) with the plasma membrane was recovered by coexpression with Gpa1p in diploids (Figure 3), suggesting that association of $G_{\beta\gamma}$ with Gpa1p can reconstitute the targeting of the thioacylation mutant to the plasma membrane.

For mammalian G_{α} subunits, association with $G_{\beta\gamma}$ is important for plasma membrane targeting. Reduction of free $G_{\beta\gamma}$ levels in cells results in the mistargeting of newly synthesized $G_{z\alpha}$ (Fishburn *et al.*, 1999). Furthermore, thioacylation of myristoylation-defective G_{α} in mammalian cells is restored by increased expression of $G_{\beta\gamma}$ subunits (Degtyarev *et al.*, 1994; Morales *et al.*, 1998). Interestingly, Gpa1p appears to be different from mammalian G protein α subunits that are modified with amide- and thioester-linked fatty acids. Although Gpa1p can escort nonlipidated Ste18p to the plasma membrane, dually lipidated Ste18p cannot rescue the loss of myristoylation or thioacylation of Gpa1p. Loss of myristoylation results in mislocalization of Gpa1p to intracellular membranes (Song *et al.*, 1996), and these yeast cells arrest growth because of activation of the pathway by free $G_{\beta\gamma}$. Cells expressing nonmyristoylated Gpa1p cannot be targeted to the plasma membrane and rescue a *gpa1Δ* mutation by overexpression of Ste4p/Ste18p (our unpublished observations). Myristoylation-defective Gpa1p expressed with $G_{\beta\gamma}$ in yeast or insect cells did not incorporate [3 H]palmitate (our unpublished observations). This failure to become palmitoylated even in the presence of $G_{\beta\gamma}$ subunits may reflect myristoylation-dependent trafficking of the newly synthesized Gpa1p to the plasma membrane in yeast.

A recent study of mammalian N-Ras, which is farnesylated and thioacylated, has shown that the protein transits through the secretory pathway to be targeted to the plasma membrane (Choy *et al.*, 1999). In yeast and mammalian cells, the enzymes that prenylate proteins are found in the cytoplasm, but those that mediate proteolysis and carboxylmethylation are localized in the endoplasmic reticulum (reviewed in Magee and Marshall, 1999). Where thioacylation of Ras occurs has not been established, but it has been suggested that thioacylation also occurs early in the secretory pathway (Choy *et al.*, 1999). The membrane-trapping model does not exclude thioacylation from occurring on intracellular membranes. After the protein is anchored to membranes by farnesylation and thioacylation, it could move to the plasma membrane by vesicle-mediated transport. Thioacylation and plasma membrane association of yeast Ras2p are facilitated by expression of an integral membrane protein, Erf2p, that is localized in the endoplasmic reticulum (Bartels *et al.*, 1999). The mechanism by which this protein facilitates Ras thioacylation and plasma membrane association is unknown. There is no evidence to suggest that

Erf2p is a protein acyltransferase. Proteolysis and carboxyl-methylation of Ste18p almost certainly occur in the endoplasmic reticulum, but Ste18p's trafficking pathway to the plasma membrane and its subcellular site of thioacylation remain to be determined.

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