Partial Constitutive Activation of Pheromone Responses by a Palmitoylation-Site Mutant of a G Protein α Subunit in Yeast[†]

Jianping Song and Henrik G. Dohlman*

Department of Pharmacology and Molecular Cardiobiology Program, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06536-0812

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ABSTRACT: G protein α subunits are often myristoylated and/or palmitoylated near their amino terminus. The G protein α subunit in the yeast *Saccharomyces cerevisiae* (*GPA1* gene product, Gpa1p) is known to be myristoylated, and this modification is essential for G protein activity *in vivo*. Here we examined whether Gpa1p is palmitoylated and determined the functional consequences of this modification. [^{3}H]-Palmitic acid was incorporated into Gpa1p in cells expressing myc-tagged Gpa1p or Gpa1p-Gst. The label was released upon hydroxylamine treatment. Substitution of the conserved Cys 3 for Ser blocked incorporation of the label (Gpa1p^{C3S}). Palmitoylation was also blocked by a mutation that prevents myristoylation (Gly2Ala), whereas the palmitoylation-site mutation had no effect on myristoylation of Gpa1p. Gpa1p^{C3S} complemented the *gpa1*Δ mutation *in vivo* and formed a complex with $G_{\beta\gamma}$ that was able to undergo nucleotide exchange *in vitro*. However, basal and pheromone-induced *FUS1*−*lacZ* transcription were 2−5-fold higher in the C3S mutant. Pheromone-induced growth arrest was also enhanced by the mutation, but recovery from arrest was not affected. Like wild-type Gpa1p, the C3S mutant was predominantly membrane-associated. Upon Triton X-114 partitioning or high pH treatment, no difference in the membrane-binding properties of the wild-type Gpa1p and the C3S mutant was detected. By sucrose density gradient centrifugation of membranes, however, most of the mutant protein was mislocalized to a non-plasma membrane compartment, whereas $G_{\beta\gamma}$ localization was unaltered. Taken together, our data suggest that Gpa1p is palmitoylated via a thioester bond at Cys 3 and that palmitoylation plays a role in modulating Gpa1p signaling and membrane localization.

Heterotrimeric G proteins are involved in transducing extracellular signals by coupling to cell surface receptors and intracellular effector molecules. The pheromone-dependent mating pathway in the yeast *Saccharomyces cerevisiae* is mediated through G protein-coupled receptors. In preparation for mating, each of the two haploid cells types (*MAT***a** and *MAT*α) secretes a specific pheromone that binds to a seven-transmembrane-segment receptor on the opposite cell type. The receptor then activates a heterotrimeric G protein, resulting in nucleotide exchange on the G protein α subunit and dissociation of G_α from $G_{\beta\gamma}$. In yeast, the α subunit of the heterotrimeric G protein is encoded by the *GPA1* gene (Dietzel & Kurjan, 1987; Miyajima et al., 1987), and *βγ* subunits are encoded by *STE4* and *STE18* genes (Whiteway et al., 1989), respectively. In mammals, both G_α and $G_{\beta\gamma}$ can activate effectors (Birnbaumer, 1992; Clapham & Neer, 1993). In yeast, it is the $G_{\beta\gamma}$ complex that activates the downstream signaling pathway, resulting in transcriptional activation and growth arrest at the G1 phase in the cell cycle. Thus Gpa1p is thought to negatively regulate signaling by controlling the levels of free $G_{\beta\gamma}$ in the cell (Schultz et al., 1995; Sprague & Thorner, 1992).

The α subunit of all G proteins undergoes myristoylation and/or palmitoylation near the amino terminus. These modifications are often necessary for membrane association and in some cases are essential for normal signaling function (Casey, 1995; Wedegaertner et al., 1995). Myristoylation of the G protein α subunits involves the covalent addition of a 14-carbon saturated fatty acid through an amide bond to the amino-terminal Gly 2. This modification is usually, but not always, irreversible (Manenti et al., 1994). $G_{o\alpha}$, $G_{i\alpha}$, $G_{z\alpha}$, and Gpa1p are known to be N-terminally myristoylated (Hallak et al., 1994; Jones et al., 1990; Mumby et al., 1990; Stone et al., 1991). Myristoylation of $G_{i\alpha}$ promotes membrane association, binding to $G_{\beta\gamma}$, and direct inhibition of adenylyl cyclase *in vitro* (Kokame et al., 1992; Linder et al., 1991; Taussig et al., 1993; Wilson & Bourne, 1995). In yeast, mutations in the N-myristoyltransferase (*NMT1*) gene (Duronio et al., 1989; Johnson et al., 1993; Stone et al., 1991) or mutations that replace the myristoylated Gly residue of Gpa1p (Stone et al., 1991) result in constitutive activation of the pheromone response pathway. Myristoylation of Gpa1p is not required for membrane association but is needed for specific localization to the plasma membrane (Song et al., 1996).

Palmitoylation of G protein α subunits involves the addition of a 16-carbon saturated fatty acid through a thioester bond to internal Cys residues. Nearly all G protein α subunits, including $G_{s\alpha}$, $G_{i\alpha}$, $G_{q\alpha}$, $G_{12\alpha}$, $G_{13\alpha}$, $G_{o\alpha}$, and $G_{z\alpha}$, are palmitoylated (Linder et al., 1993; Parenti et al., 1993; Veit et al., 1994). Palmitoylation occurs at residue Cys 3 of $G_{s\alpha}$, $G_{i\alpha}$, $G_{o\alpha}$, $G_{z\alpha}$ (Degtyarev et al., 1993a,b, 1994; Mumby et al., 1994; Parenti et al., 1993), and Cys 9 and 10 of $G_{q\alpha}$ (Wedegaertner et al., 1993). Unlike myristoylation, palmitoylation is readily reversible and thus can be regulated in a dynamic manner. Indeed, Wedegaertner et al. showed that receptor activation stimulates depalmitoylation of $G_{s\alpha}$

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* Corresponding author.

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and translocation from the membrane to cytosol (Wedegaertner & Bourne, 1994). This observation suggests that activation-dependent depalmitoylation regulates G protein signaling by controlling its membrane localization. However, others have shown that palmitoylation is not required for membrane attachment of $G_{s\alpha}$ (Degtyarev et al., 1993a; Mumby et al., 1994) or $G_{q\alpha}$ (Edgerton et al., 1994; McCallum et al., 1995), in apparent contradiction to the results of Wedegaertner et al. (1993). Paradoxically, palmitoylation does contribute to membrane association of proteins that are also myristoylated, such as $G_{o\alpha}$ (Mumby et al., 1994), $G_{i\alpha}$ (Degtyarev et al., 1994), and $G_{z\alpha}$ (Wilson & Bourne, 1995), but palmitoylation of these proteins has not been shown to be regulated in the manner of G_s.

The functional consequences of palmitoylation on effector activation and receptor coupling have been variable and unexpected. Mutations that prevent palmitoylation have no effect on effector coupling to $G_{z\alpha}$ (Wilson & Bourne, 1995), modestly inhibit coupling to $G_{s\alpha}$ (Wedegaertner et al., 1993), and disrupt coupling to $G_{q\alpha}$ (Edgerton et al., 1994; Wedegaertner et al., 1993). Palmitoylation of $G_{s\alpha}$ is required for coupling with α2-adrenergic receptors (Wedegaertner et al., 1993) and for coupling of $G_{q\alpha}$ to neurokinin (Edgerton et al., 1994) and α2-adrenergic receptors (Wedegaertner et al., 1993). Interestingly, enzymatic depalmitoylation of $G_{q\alpha}$ *in vitro* has no effect on coupling to M1 muscarinic cholinergic receptors or PLC-β1, while mutations that prevent palmitoylation substantially impair these interactions (Hepler et al., 1996).

Gpa1p contains a Cys at the third amino acid, which corresponds to the palmitoylation site in $G_{s\alpha}$, $G_{i\alpha}$, $G_{o\alpha}$, and $G_{z\alpha}$. However, to date it has not been determined if Gpa1p is palmitoylated and, if so, what the functional consequences of this modification are *in vivo*. In this report, we show that Gpa1p is palmitoylated through a thioester bond at Cys 3. Mutation of Cys 3 to Ser in Gpa1p blocks palmitoylation. This nonpalmitoylated Gpa1p complements the *gpa1*Δ mutant *in vivo*, binds to $G_{\beta\gamma}$ *in vitro*, and is predominantly membrane-attached. However, the $Gpa1p^{C3S}$ mutant exhibits reduced plasma membrane localization, increased basal signaling, and increased pheromone sensitivity. These results suggest that palmitoylation of Gpa1p plays a role in Gpa1p localization and as a consequence affects signal sensitivity.

EXPERIMENTAL PROCEDURES

Strains, Media, and Transformation. Standard methods for the growth and maintenance of yeast and bacteria were used throughout (Ausubel et al., 1987). The *Escherichia coli* strain DH 5α was used for the maintenance of plasmids. Yeast cells were grown in complete medium (YPD) (1% Bacto-yeast extract, 2% Bacto-peptone, 2% Dextrose) or in synthetic medium supplemented with amino acids, adenine, and 2% glucose (SCD); uracil or/and leucine were omitted to maintain selection for plasmids. *Saccharomyces cerevisiae* strains used in this study were YGS5 (YPH499 *gpa1*::hisG *ste11*^{ts}) (Song et al., 1996) and BJ2168 (*MAT***a** *leu*2 *trp*1 *ura*3-52 *prb*1-1122 *prc*1-407 *pep*4-3) (Jones, 1991). YGS5 cells are viable at 34 °C because the mating pathway is blocked at *ste11*. At 24 °C, however, cells will not grow because of the *gpa1*Δ mutation which results in constitutive signaling and G1 arrest.

Plasmid Construction. Standard methods for the manipulation of DNA were used throughout (Ausubel et al., 1987). Enzymes and buffers were obtained from New England Biolabs. PCR reagents were from Perkin-Elmer Cetus. Sequencing reagents were purchased from U.S. Biochemical Corp. Expression plasmids used in this study were pRS316 (amp^r, *CEN/ARS*, *URA3*) and pRS316-ADH (pRS316 with *ADH1* promoter and terminator from pAD4M, described below) (Sikorski & Hieter, 1989).

Replacement of the N-terminal Cys 3 codon was achieved by PCR using mismatched primers (Ausubel et al., 1987) with *GPA1* or an epitope-tagged version, *GPA1-myc*, as templates (Song et al., 1996). The forward primer contains a *Bam*HI site and encodes Met-Gly-Ser-Thr (5′ GGG GAT CCC ATG GGG TCT ACA 3′). The reverse primer (5′ ATC AGA ACC ACC GGC AA 3′) is complementary to nucleotides 398−414 of the *GPA1* open reading frame. pRS316-$GPA1^{C3S}$, pRS316-ADH-$GPA1^{C3S}$, and pAD4M-$GPA1^{C3S}$-GST were constructed as described before for $GPA1^{G2A}$ (Song et al., 1996).

Metabolic Labeling and Purification of Gpa1p-Gst. BJ2168 cells expressing pAD4M-GST, pAD4M-GPA1-GST, pAD4M-$GPA1^{C3S}$-GST, or pAD4M-$GPA1^{G2A}$-GST were grown to midlogarithmic phase in selective medium. Approximately 1.3×10^8 cells were harvested by centrifugation at 1000*g* for 10 min. The cells were washed twice with 10 mL of YPD plus 1% ethanol and resuspended at 2.5×10^7 cells/mL in YPD plus 1% ethanol. After 10 min of growth at 30 °C, cells were treated with cerulenin (2 μg/mL) to inhibit endogenous fatty acid synthesis 15 min before the addition of 30 μCi/mL [^{3}H]myristic acid (DuPont−New England Nuclear, ∼39 Ci/mmol) or 300 μCi/mL [^{3}H]palmitic acid (DuPont−New England Nuclear, ∼16 Ci/mmol) and grown for 1.5 h. Growth was stopped by the addition of NaN_3 to 10 mM. All subsequent manipulations were carried out at 0−4 °C. Cells were harvested by centrifugation and washed once with 10 mM NaN_3 and once with lysis buffer A (40 mM triethanolamine, pH 7.2, 2 mM EDTA, 2 mM dithiothreitol, 0.15 M NaCl, 2 μM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride], and 10 μg/mL each leupeptin, pepstatin, benzamidine, and aprotinin). Cell pellets were resuspended in 300 μL of lysis buffer A and subjected to glass bead vortex homogenization for 4 min. The lysate was centrifuged twice at 500*g* for 10 min, and the resulting supernatant was treated with 1% Triton X-100 (final concentration) at 4 °C for 1 h to solubilize membrane proteins. One hundred microliters of glutathione−Sepharose 4B resin (Pharmacia) (20% slurry) was added to the soluble material and mixed at 4 °C for 2 h. The resin was washed three times with 20 mM NaP_i, pH 7.3, and 350 mM NaCl. The bound proteins were eluted by boiling in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample buffer (62.5 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 1% β-mercaptoethanol, and 0.0005% bromophenol blue) for 10 min. For palmitic acid labeling, dithiothreitol or β-mercaptoethanol was omitted in the lysis buffer and SDS−PAGE sample buffer to prevent reduction of the thioester bond. Samples were subjected to SDS−PAGE with a linear gradient of 7−15% polyacrylamide and fixed in H_2O/2-propanol/acetic acid (65:25:10 v/v/v) for 30 min. To cleave possible thioester-linked fatty acids, identical gels were soaked either with 1 M hydroxylamine, pH 7.0, at room temperature for 18 h or with 1 M Tris·HCl, pH 7.0, as a control. The gels were then fixed again and treated with Amplify (Amersham) for 30 min, vacuum-dried, and exposed to X-ray film (Kodak) (Masterson & Magee, 1992).

Metabolic Labeling and Immunoprecipitation. YGS5 cells expressing pRS316-ADH, pRS316-ADH-GPA1-myc, pRS316-ADH-GPA1^{C3S}-myc, or pRS316-ADH-GPA1^{G2A}-myc were grown to midlogarithmic phase at 34 °C in SCD −ura. Cells were metabolically labeled with [^{3}H]myristic acid or [^{3}H]-palmitic acid as described above. Cells were harvested by centrifugation and washed once with 10 mM NaN_3 and once with RIPA buffer (150 mM NaCl, 50 mM Tris·HCl, pH 7.4, 5 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 2 μM AEBSF, and 10 μg/mL each leupeptin, pepstatin, benzamidine, and aprotinin). Cells were then resuspended in 150 μL of RIPA buffer. Acid-washed glass beads were added and the suspension was vortexed at high speed for 4 min. The lysates were centrifuged twice at 500*g* for 10 min. The supernatant was precleared by mixing with 10% protein A−Sepharose CL-4B (Pharmacia) for 30 min and then immunoprecipitated by mixing with a monoclonal antibody, 9E10, for 1 h, followed by the addition of 10% protein A−Sepharose CL-4B for 30 min. The protein A resin was washed four times with RIPA buffer, and the bound proteins were eluted by boiling in SDS−PAGE sample buffer for 10 min. Samples were subjected to SDS−8% PAGE, fixed, and soaked with 1 M hydroxylamine or with 1 M Tris·HCl as described above.

β-Galactosidase Assay. YGS5 cells bearing pRS316-GPA1, or pRS316-GPA1^{C3S}, or pRS316-ADH-GPA1, or pRS316-ADH-GPA1^{C3S} were transformed with plasmid pBJ 207 (Ma et al., 1987) which contains the *lacZ* gene under the control of *FUS1* promoter (Sprague, 1991; Trueheart et al., 1987). Cells were grown at 24 °C to midlogarithmic phase in SCD −ura −leu medium and treated with α-factor for 1.5 h. Cells were harvested and assayed for β-galactosidase activity as described before (Ausubel et al., 1987).

Growth Inhibition Assay. The growth inhibition assay (halo assay) was performed as described previously (Julius et al., 1983). YGS5 cells containing plasmids pRS316-GPA1, pRS316-GPA1^{C3S}, pRS316-ADH-GPA1, or pRS316-ADH-GPA1^{C3S} were grown at 34 °C. Briefly, 100 μL from an overnight culture grown at 34 °C was diluted with 2 mL of SCD −ura, followed by the addition of an equal volume of 1% (w/v) dissolved agar (55 °C), and the mixture was poured onto a culture dish of the same medium. Sterile filter discs were spotted with 15 μg of synthetic α-factor and placed on the nascent lawn. The resulting halo of the G1-arrested cells closest to the source of α-factor was documented after 36 h at 24 °C.

Copurification of Gpa1p-Gst with $G_{\beta\gamma}$. BJ2168 cells expressing pAD4M-GST, pAD4M-GPA1-GST, or pAD4M-GPA1^{C3S}-GST were grown to midlogarithmic phase in SCD −leu medium. Growth was stopped by the addition of NaN_3 to 10 mM. All subsequent manipulations were performed at 0−4 °C. Approximately 5×10^8 cells were harvested by centrifugation at 1000*g* for 10 min and washed in lysis buffer A. Cell pellets were resuspended in 350 μL of lysis buffer A, 3 mM $MgCl_2$, and 10 μM GDP and subjected to glass bead vortex homogenization for 4 min. The lysate was centrifuged twice at 500*g* for 10 min, and the resulting supernatant was treated with 1% Triton X-100 (final concentration) at 4 °C for 1 h to solubilize membrane proteins. One hundred microliters of glutathione−Sepharose 4B resin (Pharmacia) (20% slurry) was added to the lysate and mixed at 4 °C for 2 h. The resin was resuspended in either buffer A containing 3 mM $MgCl_2$, 10 μM GDP, and 1% Triton X-100 (GDP buffer) or 50 mM $MgCl_2$, 20 μM GTPγS, and 1% Triton X-100 (GTPγS buffer) and mixed at 4 °C for 2 h. The resin was washed three times with 20 mM NaP_i, pH 7.3, and 350 mM NaCl. The bound proteins were eluted by boiling in 100 μL of SDS−PAGE sample buffer for 10 min. The purified proteins were resolved by SDS−12.5% PAGE, transferred to nitrocellulose, and probed with antibodies against Gst (from J. Steitz, Yale University) or Ste4p (D. D. Jenness, 1996, personal communication). Antibody detection was achieved using horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and the ECL chemiluminescence system (Amersham), according to the manufacturer's instructions.

Cell Disruption and Membrane Fractionation. YGS5 cells expressing pRS316-ADH-GPA1 or pRS316-ADH-GPA1^{C3S} were grown to midlogarithmic phase in SCD −ura. Approximately 5×10^8 cells were harvested and washed in lysis buffer B (same as lysis buffer A but lacking NaCl). All subsequent manipulations were carried out at 0−4 °C. Cell pellets were resuspended in 200 μL of lysis buffer B and subjected to glass bead vortex homogenization for 4 min. The lysate was centrifuged twice at 500*g* for 10 min, and the resulting supernatant was centrifuged at 140000*g* for 30 min. A portion of the supernatant (S fraction) was diluted with an equal volume of 2× SDS−PAGE sample buffer and boiled for 10 min. The pellet (P fraction) was resuspended in lysis buffer B at the original sample volume, diluted with an equal volume of 2× SDS−PAGE sample buffer, and boiled for 10 min. The extracts were resolved by SDS−8% PAGE and transferred to nitrocellulose (Harlow & Lane, 1988). Blots were probed with antiserum against Gpa1p (Dohlman et al., 1993) and horseradish peroxidase-conjugated goat anti-rabbit IgG, as described above.

Sucrose Gradient Fractionation. Methods for cell membrane fractionation have been described in detail elsewhere (Bowser & Novick, 1991). Briefly, YGS5 cells expressing pRS316-ADH-GPA1 or pRS316-ADH-GPA1^{C3S} were grown to midlogarithmic phase in SCD −ura. Cells were centrifuged and resuspended in YPD at 0.5 OD_{600nm}/mL. After one doubling, growth was stopped by addition of NaN_3 to 10 mM. Approximately 3×10^9 cells were harvested by centrifugation at 500*g* for 10 min and washed twice with SK buffer (1.2 M sorbitol and 0.1 M KP_i, pH 7.5). Spheroplasts were prepared by resuspending cells in 10 mL of SK, 1 mg of zymolyase 100T (Kirin Brewery), and 28.8 mM β-mercaptoethanol for 45 min at 30 °C. All subsequent manipulations were performed at 0−4 °C. Spheroplasts were centrifuged at 500*g* for 10 min and washed once with SK and once with lysis buffer C (0.8 M sucrose, 20 mM triethanolamine, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol, 2 μM AEBSF, and 10 μg/mL each leupeptin, pepstatin, benzamidine, and aprotinin). Cell pellets were resuspended in 1.0 mL of lysis buffer C and disrupted with 25 strokes of a motor-driven Potter-Elvehjem homogenizer. The lysate was cleared of unbroken cells and debris by centrifuging twice at 500*g* for 10 min. Sucrose (606 mg) was added to 650 μL of the supernatant and dissolved (final sucrose concentration 70% w/v). The sample was transferred to a Beckman thin-walled polypropylene tube and overlaid with 1 mL sucrose solutions of 60%, 50%, 40%, and 30% (w/v), respectively. The samples were then centrifuged in a Beckman SW50Ti swinging-bucket rotor for 16 h at 170000*g* in a Beckman L80 ultracentrifuge. Sixteen samples of 300 μL each were collected from the bottom of the gradient, diluted 1:1 with 2× SDS−PAGE sample buffer, and boiled

for 10 min. Fractions 1−13 (fractions 14−16 do not contain Gpa1p, Ste4p, or Pma1p) were resolved by SDS−8% PAGE and immunoblotting as described above. Blots were probed with antibodies to Gpa1p and Ste4p (see above) as well as Pma1p (raised against the plasma membrane H^+ ATPase from *Neurospora crassa* and specifically recognizes Pma1p in *S. cerevisiae*, from C. Slayman, Yale University) (Hager et al., 1986)

High pH Treatment. YGS5 cells expressing pRS316-GPA1 or pRS316-GPA1^{C3S} were grown to midlogarithmic phase in SCD −ura. Approximately 5×10^8 cells were harvested by centrifugation at 500*g* for 10 min. All subsequent manipulations were carried out at 0−4 °C. Cell pellets were resuspended in cold 10 mM NaN_3, washed with 200 μL of lysis buffer B, and subjected to glass bead vortex homogenization for 4 min. The lysate was centrifuged twice at 500*g* for 10 min, and the resulting supernatant was centrifuged at 140000*g* for 30 min. Pellets were resuspended in either buffer B or buffer B containing 0.1 mM $NaHCO_3$, pH 11, incubated on ice for 15 min, and centrifuged at 140000*g* for 30 min. A portion of the supernatant was diluted with an equal volume of 2× SDS−PAGE sample buffer and boiled for 10 min. The pellet was resuspended in lysis buffer B at the original sample volume, diluted with an equal volume of 2× SDS−PAGE sample buffer, and boiled for 10 min. The extracts were resolved by SDS−8% PAGE and analyzed on immunoblots with antiserum against Gpa1p as described above.

Triton X-114 Phase Partitioning. Triton X-114 phase partitioning was carried out as described before (Bordier, 1981). Cells lysates were prepared as described above and mixed with Triton X-114 to a final concentration of 1% for 1 h at 4 °C. After removal of insoluble materials by centrifugation, a 200-μL portion of the lysate was layered over a 60-μL sucrose cushion (6% sucrose, 0.06% Triton X-114, 40 mM triethanolamine, pH 7.2, 0.15 M NaCl) and incubated at 30 °C for 5 min. The clouding solution was centrifuged at 13000*g* for 5 min at room temperature. The upper aqueous phase (A) and the detergent phase (D) were collected and adjust with Triton X-114 and buffer B to equalize composition and volume of the two fractions. Samples were diluted with an equal volume of 2× SDS−PAGE sample buffer and boiled for 10 min. The extracts were resolved by SDS−8% PAGE and analyzed on immunoblots with antiserum against Gpa1p as described above.

RESULTS

Gpa1p Is Palmitoylated through a Thioester Bond. To determine if Gpa1p is palmitoylated, we performed a metabolic labeling experiment using a myc epitope-tagged version of Gpa1p. This epitope tag does not interfere with Gpa1p function, expression, or membrane localization (Song et al., 1996). Cells lacking *GPA1* (strain YGS5) were transformed with vector (pRS316-ADH) or vector containing the myc-tagged GPA1 (pRS316-ADH-GPA1-myc) and metabolically labeled with [^{3}H]palmitic acid. Gpa1p was immunoprecipitated with the myc (9E10) monoclonal antibody, and analyzed by SDS−PAGE and fluorography. As shown in Figure 1, [^{3}H]palmitic acid was incorporated into wild-type Gpa1p but not into cells that do not express Gpa1p. Upon treatment of the gel with hydroxylamine, which will cleave thioester linkages but not amide bonds (Masterson & Magee, 1992), the incorporation of label was abolished. These data suggest that Gpa1p is palmitoylated through a hydroxylamine-labile thioester bond.

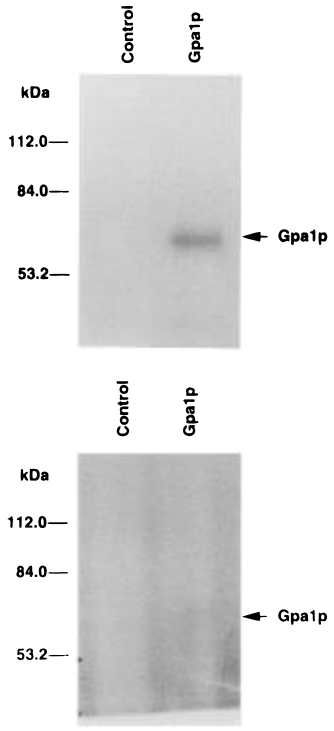

FIGURE 1: Gpa1p is palmitoylated. To determine whether Gpa1p is palmitoylated, YGS5 cells (*gpa1*Δ) bearing the vector alone (control) or *GPA1*-myc (Gpa1p) were metabolically labeled with [^{3}H]palmitic acid. Proteins were immunoprecipitated from solubilized cell lysates with the myc monoclonal antibody 9E10 and subjected to SDS−PAGE. Gels were treated with either 1 M hydroxylamine to cleave thiol ester-bonded fatty acids (bottom) or 1 M Tris·HCl (top) and analyzed by fluorography.

Mutation of C3S Prevents Palmitoylation. The metabolic labeling experiments suggest that Gpa1p undergoes palmitoylation. Gpa1p contains a conserved Cys residue at position 3 that is palmitoylated in $G_{i\alpha}$, $G_{o\alpha}$, $G_{z\alpha}$, and $G_{s\alpha}$. To test if Cys 3 is the palmitoylation site for Gpa1p, we examined the incorporation of [^{3}H]palmitic acid in cells expressing a Gpa1p mutant in which Cys 3 was substituted for Ser. As shown in Figure 2A, [^{3}H]palmitic acid was incorporated into the wild-type Gpa1p as observed before, and incorporation was abolished in the Gpa1p^{C3S} mutant, suggesting that Cys 3 is the palmitoylation site. The Gpa1p^{C3S} mutant still incorporates [^{3}H]myristic acid, though to a lesser extent than wild-type Gpa1p (Figure 2B). However, the reduced labeling of the mutant appears to be due to reduced stability and lower recovery of the protein following cell lysis (see below, Figure 6A).

To test whether myristoylation is needed for palmitoylation of Gpa1p, we also examined the incorporation of [^{3}H]palmitic acid in a nonmyristoylated mutant, Gpa1p^{G2A}. As shown in Figure 2A,B, the G2A mutation prevents both myristoylation and palmitoylation, suggesting that myristoylation is a prerequisite for G_α palmitoylation in yeast.

To corroborate the immunoprecipitation results, we purified wild-type and mutant forms of Gpa1p which had been fused in-frame with the glutathione-S-transferase (*GST*) gene. An additional advantage of this method is that the wild type and mutant fusion proteins appear to be equally stable following cell lysis (see below, Figure 4). The wild-type fusion protein is myristoylated and palmitoylated, as determined by metabolic labeling of cells with [^{3}H]palmitic acid (Figure 2C) or [^{3}H]myristic acid (Figure 2D), as observed for the myc-tagged protein. Moreover, the Gpa1p^{G2A}-Gst mutant fails to incorporate both myristate and palmitate,

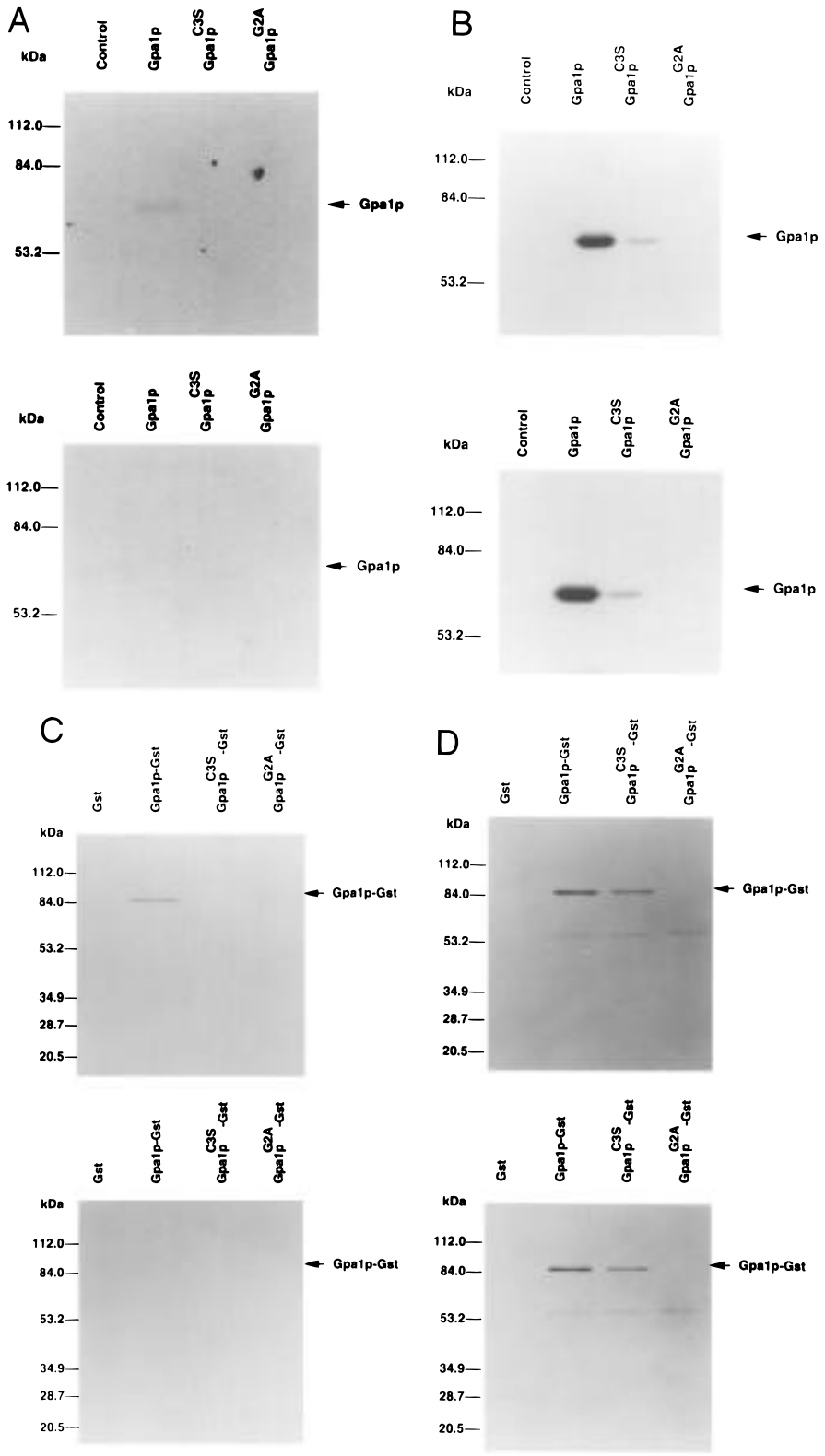

FIGURE 2: Gpa1p is palmitoylated at Cys 3 and myristoylation precedes palmitoylation. (A) To demonstrate that palmitoylation is abolished by the C3S mutation and that myristoylation is a prerequisite for palmitoylation, YGS5 cells bearing the vector alone (control), *GPA1*-myc (Gpa1p), *gpa1*^{C3S}-myc (Gpa1p^{C3S}), or *gpa1*^{G2A}-myc (Gpa1p^{G2A}) were metabolically labeled with [^{3}H]palmitic acid. Proteins were immunoprecipitated from solubilized cell lysates with the myc monoclonal antibody 9E10, subjected to SDS−PAGE, and treated with hydroxylamine (bottom) or Tris·HCl (top) as described in Figure 1. (B) To determine if palmitoylation has any effect on myristoylation, cells were metabolically labeled with [^{3}H]myristic acid, immunoprecipitated, and analyzed as described above. To determine if the Gpa1p-Gst fusion is myristoylated and palmitoylated, BJ2168 cells expressing Gpa1p-Gst, Gpa1p^{G2A}-Gst, Gpa1p^{C3S}-Gst, or Gst alone were metabolically labeled with (C) [^{3}H]palmitic acid or (D) [^{3}H]myristic acid. Proteins were purified from solubilized whole-cell lysates using a glutathione−Sepharose 4B resin and resolved by SDS−PAGE. Gels were treated with either 1 M hydroxylamine to cleave thiol ester-bonded fatty acids (bottom) or 1 M Tris·HCl (top) and analyzed by fluorography.

while Gpa1p^{C3S}-Gst incorporates myristate but not palmitate, again as observed for the myc-tagged protein. Thus the fusion proteins and the epitope-tagged proteins are similar in their ability to undergo myristoylation and palmitoylation.

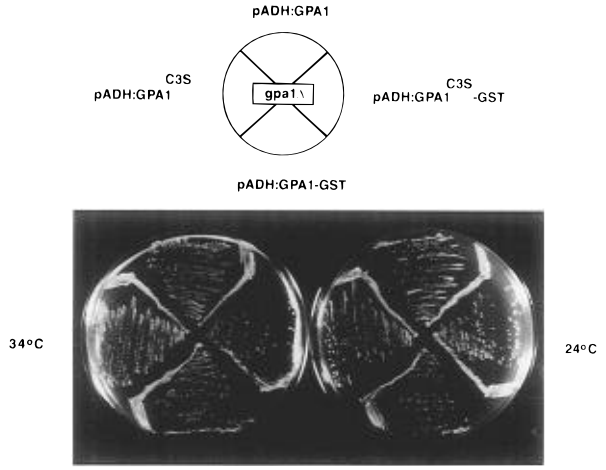

FIGURE 3: Gpa1p^{C3S} and Gpa1p^{C3S}-Gst complement *gpa1*Δ. To determine if Gpa1p^{C3S} and Gpa1p^{C3S}-Gst can bind $G_{\beta\gamma}$ *in vivo*, YGS5 cells containing *GPA1-GST*, *GPA1*, *gpa1*^{C3S}*-GST*, or *gpa1*^{C3S} (in plasmid pRS316-ADH) were streaked out at 34 °C, or at 24 °C to allow functional expression of the temperature-sensitive *ste11* gene product. Growth at 24 °C indicates complementation of the *gpa1*Δ mutation.

Gpa1p^{C3S} Binds to $G_{\beta\gamma}$ in a GDP-Dependent Manner. Since the primary role of Gpa1p is to regulate signaling through sequestering $G_{\beta\gamma}$ in the cell, disruption of Gpa1p interaction with $G_{\beta\gamma}$ will lead to constitutive activation of the signaling pathway including growth arrest. We first tested if the nonpalmitoylated mutant of Gpa1p can still bind to $G_{\beta\gamma}$ *in vivo*. Plasmids containing *GPA1-GST*, *gpa1*^{C3S}*-GST*, *GPA1*, or *gpa1*^{C3S} were introduced into the *gpa1*Δ strain, YGS5. The absence of *GPA1* would normally lead to constitutive signaling and growth arrest; however, YGS5 cells are viable at 34 °C due to a temperature-sensitive mutation of *ste11* that blocks the signal downstream of the G protein. At 24 °C cells can grow only when a functional Gpa1p is expressed. As expected, cells expressing wild-type Gpa1p are viable at 24 °C. The Gpa1p^{C3S} mutant cells are also viable at 24 °C (Figure 3), indicating that the nonpalmitoylated Gpa1p is able to complement the *gpa1*Δ mutation. The corresponding Gst fusion proteins of wild-type and mutant Gpa1p are also able to complement the *gpa1*Δ mutation (Figure 3). In contrast, cells expressing Gpa1p^{G2A} or no Gpa1p fail to grow at 24 °C (Song et al., 1996; data not shown). These data suggest that palmitoylation is not essential for $G_{\beta\gamma}$ binding and that the Gst fusion protein behaves the same as the nonfusion protein in its ability to bind $G_{\beta\gamma}$ *in vivo*.

To determine if palmitoylation of Gpa1p contributes to $G_{\beta\gamma}$ binding or release *in vitro*, we copurified $G_{\beta\gamma}$ with Gpa1p-Gst and examined the effects of guanine nucleotides on subunit interaction. In this method the N-terminus is unaltered and will undergo myristoylation and palmitoylation (Figure 2C,D), so the functional role of these modifications can be tested directly. Detergent-solubilized lysates from equal numbers of cells expressing Gpa1p-Gst, Gpa1p^{C3S}-Gst, or Gst alone were prepared in the presence of GDP and mixed with the glutathione resin. The washed resin was then treated with GTPγS to promote subunit dissociation or with GDP to preserve the complex. Equal levels of Gpa1p and Gpa1p^{C3S} fusions remained bound to the resin after these washes, as indicated by blotting with anti-Gst antiserum (Figure 4, top). By probing the same extracts with Ste4p antiserum, we found that G_{β} (representing the $G_{\beta\gamma}$ complex) (Clark et al., 1993; Song et al., 1996) copurified with Gpa1p-Gst and Gpa1p^{C3S}-Gst but not with Gst (Figure 4, bottom). For both the wild-type and mutant fusion proteins, addition of GTPγS led to the complete dissociation of $G_{\beta\gamma}$. These data reveal that $G_{\beta\gamma}$ can form a complex with wild-type Gpa1p as well as with the Gpa1p^{C3S} mutant, both *in vivo* and *in vitro*, and that either complex is stabilized in the presence of GDP and dissociated in the presence of GTPγS.

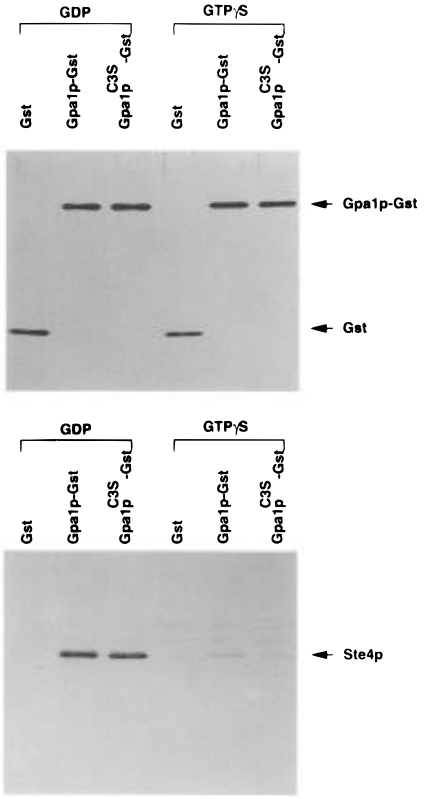

FIGURE 4: Ste4p copurifies with Gpa1p and Gpa1p^{C3S}. To determine if nonpalmitoylated Gpa1p is able to bind to $G_{\beta\gamma}$ *in vitro*, Gpa1p-Gst, Gpa1p^{C3S}-Gst, or Gst alone were expressed (plasmid pAD4M) in BJ2168 and purified using the glutathione affinity resin. Equal numbers of cells were disrupted in buffer containing GDP. The lysates were solubilized with Triton X-100, incubated with the glutathione−Sepharose resin, and then centrifuged and resuspended in buffer containing either GDP or GTPγS. The resin was subsequently washed in high-salt buffer, and the bound protein was eluted and analyzed by immunoblotting with antibodies against Gst (top) or Ste4p (bottom), as indicated.

Palmitoylation Modulates G Protein Signaling in Vivo. The data presented above indicate that nonpalmitoylated Gpa1p can complement the *gpa1*Δ mutation and thus binds $G_{\beta\gamma}$ *in vivo*. In yeast the G protein effector has not been positively identified. Yeast $G_{\beta\gamma}$ has been shown to interact with Ste5p (Whiteway et al., 1995) and Cdc24p (Zhao et al., 1995). These interactions have not been shown to be stimulus-dependent, however, and their role in propagating the pheromone signal is not yet clear. Alternatively, there are a number of simple bioassays available to assess overall G protein activity *in vivo*.

Since overexpression of Gpa1 could affect pheromone sensitivity, the following experiments were carried out using a single-copy plasmid bearing *GPA1* and the homologous *GPA1* promoter. "Normal" expression was confirmed by quantitative immunoblotting of wild-type cell lysates containing this plasmid vs the plasmid without an insert (data not shown). First, we examined whether palmitoylation affects pheromone-dependent gene transcription using a pheromone-responsive promoter and a reporter gene con-

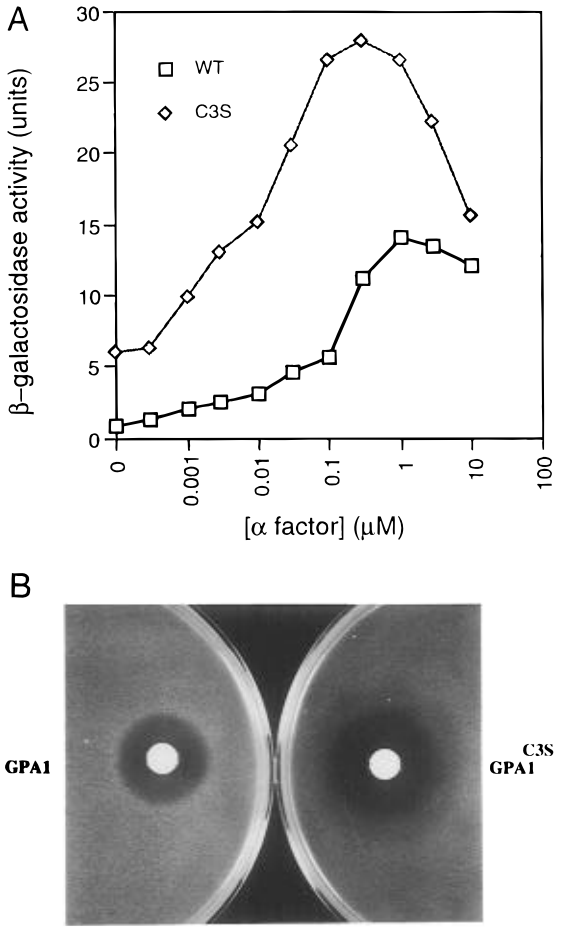

FIGURE 5: Loss of palmitoylation leads to an elevated pheromone response. (A) To determine if palmitoylation has any effect on pheromone-dependent gene transcription, YGS5 cells bearing *GPA1* or *gpa1*^{C3S} (plasmid pRS316) were transformed with plasmids containing the *lacZ* gene under the control of the *FUS1* promoter. Cells were grown at 24 °C, treated with various concentrations of α-factor for 90 min, and assayed for β-galactosidase activity. (B) To determine if palmitoylation has any effect on pheromone-dependent growth arrest, YGS5 cells expressing *GPA1* or *gpa1*^{C3S} were embedded in agar and exposed to 15 μg of α-factor at 24 °C for 36 h. Units, $(A_{420nm} - 1.75A_{550nm})/A_{600nm}$.

struct, *FUS1-lacZ*. *FUS1* encodes a protein that is required for cell fusion during mating and contains a pheromone-responsive element in its promoter region. Its mRNA level and protein level are induced up to 100-fold by pheromone. Thus *FUS1*−*lacZ* has been used as an indicator of pheromone-dependent gene transcription (Sprague, 1991; Trueheart et al., 1987). YGS5 cells expressing either wild-type Gpa1p or the Gpa1p^{C3S} mutant protein were transformed with a plasmid containing *FUS1*−*lacZ*, grown at 24 °C, and treated with varying concentrations of α-factor for 1.5 h, after which the β-galactosidase activity was measured. In the absence of pheromone, the basal β-galactosidase activity was about 5-fold higher in the C3S mutant than in the wild type, indicating that there is a partial activation of the signaling pathway in the nonpalmitoylated mutant. Upon pheromone stimulation, the C3S mutant showed a dose-dependent induction of β-galactosidase activity, as did the wild type. However, the mutant exhibited a 2-fold increase in the maximum level of pheromone-induced gene transcription and a >10-fold decrease in the EC_{50} compared to the wild type (Figure 5A).

We then tested whether palmitoylation of Gpa1p has any effect on pheromone-dependent growth inhibition using the halo bioassay. Differences in the zone of growth inhibition (halo size) are indicative of pheromone sensitivity, while differences in recovery from growth arrest (halo turbidity) provide an indication of that strain's ability to undergo long-term desensitization. YGS5 cells transformed with plasmids containing *GPA1* or *gpa1*^{C3S} were grown at 34 °C and spread onto solid medium. Sterile filter discs spotted with synthetic α-factor were placed on the nascent lawn and incubated for 36 h at 24 °C. As shown in Figure 5B, cells expressing the nonpalmitoylated mutant of Gpa1p grow at 24 °C and exhibit an increase in halo size (compared to wild type) but no change in turbidity. These results confirm that the C3S mutant complements the *gpa1*Δ mutation and is considerably more sensitive to pheromone than the wild type. Taken together, our data suggest that palmitoylation of Gpa1p is not essential for $G_{\beta\gamma}$ binding or signaling *in vivo* but does plays a role in modulating the initial pheromone response, leading to gene transcription and growth arrest.

Palmitoylation Is Not Required for Membrane Association. The data presented above reveal that Gpa1p^{C3S} can bind $G_{\beta\gamma}$ *in vitro*. We then considered whether the increased pheromone sensitivity exhibited by this mutant *in vivo* is due to reduced membrane attachment or specific plasma membrane localization. Recently, we have used membrane fractionation and sucrose density gradient centrifugation methods to show that Gpa1p binds to Golgi and plasma membranes (Dohlman et al., 1996). The same methods were also used to show that myristoylation of Gpa1p is not required for membrane attachment but is needed for specific association with the plasma membrane (Song et al., 1996).

First, we used high-speed centrifugation to determine whether palmitoylation is required for the membrane attachment of Gpa1p (Figure 6A). The resulting pellet (P) and cytosolic (S) fractions were analyzed by SDS−PAGE and immunoblotting with the Gpa1p antibody. There are two populations of Gpa1p in wild-type cells; one migrates at 56 kDa representing nonmyristoylated Gpa1p and another migrates at 54 kDa representing myristoylated Gpa1p (Dohlman et al., 1993; Stone et al., 1991). Both forms of Gpa1p were exclusively in the pellet fraction. The same pattern was observed for the C3S mutant, suggesting that palmitoylation is not required for membrane attachment. However, the amount of mutant protein recovered in the pellet after lysis and centrifugation was lower, despite the fact that the starting expression levels of Gpa1p and Gpa1p^{C3S} were the same in these cells (T). Moreover, the ratio of the myristoylated (54 kDa) and nonmyristoylated (56 kDa) species is similar in the wild type and the C3S mutant, even though the overall recovery of the mutant is significantly lower (Figure 6A). This suggests that the mutant protein is less stable in the cell-free system, perhaps because it is more accessible to protease degradation, but its ability to undergo myristoylation is unaltered.

Then we tested if protein−protein interactions contribute to the membrane association of Gpa1p^{C3S}. Gα proteins are peripheral membrane proteins that attach to the lipid bilayer through electrostatic or hydrogen-bond interactions. High-pH treatment will deprotonate charged residues of proteins and will therefore often disrupt protein−protein association. Thus it can be used as a crude measurement of this type of interaction. The membrane-bound Gpa1p was treated with either neutral-pH or high-pH buffer. After centrifugation, the resulting pellet (P) and cytosolic (S) fractions were analyzed by SDS−PAGE and immunoblotting

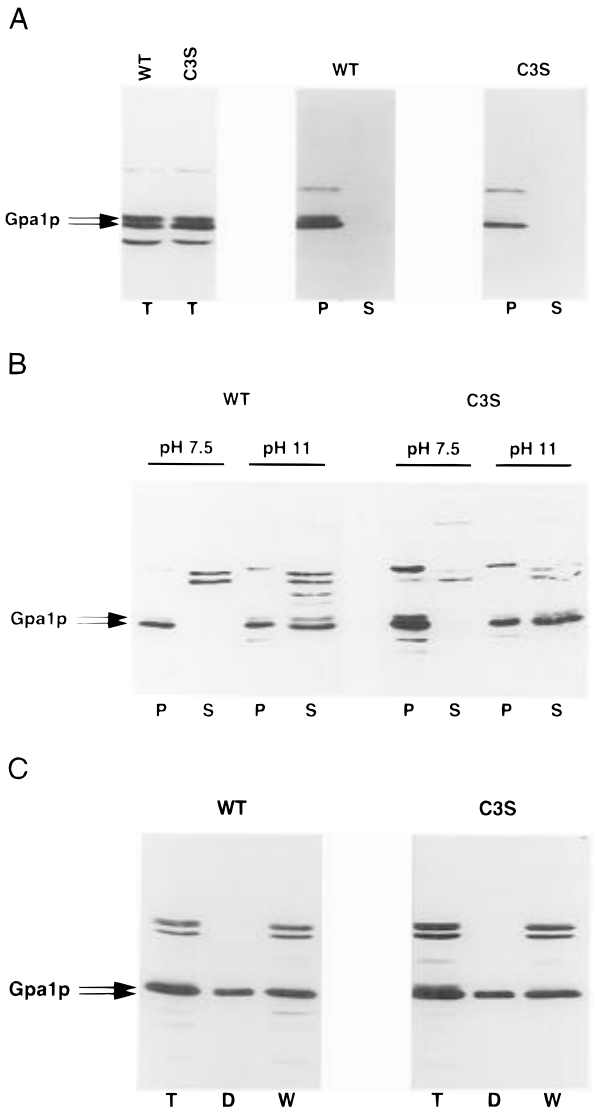

FIGURE 6: Palmitoylation does not affect membrane attachment. (A) YGS5 cells bearing *GPA1* or *gpa1*^{C3S} (plasmid pRS316-ADH) were lysed in SDS−PAGE sample buffer. Total expression of Gpa1p (T) was analyzed by immunoblotting with antiserum against Gpa1p. The arrows indicate the position of nonmyristoylated (upper band, 56 kDa) and myristoylated (lower band, 54 kDa) forms of Gpa1p. Additional immunoreactive bands are nonspecifically recognized by the Gpa1p antibody, as they are also present in a *gpa1*Δ mutant (Dohlman et al., 1993). YGS5 cells containing *GPA1* or *gpa1*^{C3S} were lysed, and the precleared homogenates were centrifuged at 140000*g* for 30 min. The cytosolic (S, supernatant) and membrane (P, pellet) fractions were analyzed by immunoblotting. To compare recovery of wild-type and mutant Gpa1p, an equal amount of protein was loaded in the WT and C3S lanes, resolved in the same gel, and exposed to film for an equivalent amount of time. (B) YGS5 containing *GPA1* or *gpa1*^{C3S} (plasmid pRS316) were lysed, and the precleared homogenates were centrifuged at 140000*g* for 30 min. The membrane fractions were incubated with buffer alone (pH 7.5) as a control or with buffer containing 0.1 mM $NaHCO_3$ (pH 11) to solubilize peripheral membrane proteins. The pellet fraction (P) and soluble fraction (S) were separated, resolved by SDS−8% PAGE, and analyzed by immunoblotting with antiserum against Gpa1p as described above. (C) YGS5 cells containing *GPA1* or *gpa1*^{C3S} were lysed and subjected to Triton X-114 phase partition as described under Experimental Procedures. Samples from the detergent phase (D), the aqueous phase (W), and the untreated sample (T) were subjected to SDS−8% PAGE and analyzed by immunoblotting with antiserum against Gpa1p.

with the Gpa1p antibody. As shown in Figure 6B, wild-type Gpa1p, and the C3S mutant, remained completely membrane-associated at neutral pH. An equivalent portion of wild-type and mutant was solubilized from the membrane under high-pH conditions, indicating that the membrane association of at least a portion of cellular Gpa1p is due to protein−protein interaction and that palmitoylation is not essential for this interaction.

We also tested if the membrane association of Gpa1p^{C3S} is due to hydrophobic interactions. Triton X-114 is a detergent that exists stably in a single phase at 4 °C but partitions into detergent-rich and detergent-depleted (aqueous) phases at 30 °C. This behavior is extremely useful for separating integral and peripheral membrane proteins, and has also been used to characterize protein hydrophobicity (Bordier, 1981; Justice et al., 1995). The capacity of wild type and the C3S mutant to partition between the detergent phase (D) and aqueous phase (W) was examined. As shown in Figure 6C, wild-type Gpa1p and the C3S mutant distributed equally in both detergent and aqueous phases, indicating that the membrane association of at least a portion of Gpa1p is due to hydrophobic membrane interactions and that palmitoylation is not essential for this interaction.

Palmitoylation Contributes to Plasma Membrane Localization. Finally, we tested if nonpalmitoylated Gpa1p still associates specifically with the plasma membrane. Precleared cell lysates were subjected to high-speed centrifugation through a 70−30% sucrose flotation gradient. Fractions were collected from the bottom of the gradient, prepared for immunoblotting, and probed with antisera against Gpa1p and Pma1p (an integral plasma membrane protein marker) (Hager et al., 1986). Immunoblots for cells expressing myc-tagged Gpa1p are shown, but similar results were obtained using untagged Gpa1p (Song et al., 1996; data not shown). As presented in Figure 7, wild-type Gpa1p associates predominantly with the plasma membrane fractions (containing Pma1p) and to a lesser extent in early fractions which contain Golgi and other microsomal membranes (Dohlman et al., 1996). Gpa1p^{C3S} is also at the plasma membrane, but a significant portion accumulates in the microsomal fractions. Probing the same fractions with polyclonal antibodies to Ste4p, we find that $G_{\beta\gamma}$ is mostly associated with the plasma membrane in cells expressing either Gpa1p or Gpa1p^{C3S} (Figure 7). These results indicate that palmitoylation promotes the plasma membrane association of Gpa1p. These data also provide a possible explanation for the increased level of signaling by the nonpalmitoylated mutant; specifically, reduced Gpa1p^{C3S} at the plasma membrane leaves a pool of unsequestered $G_{\beta\gamma}$ at the plasma membrane, which leads to an increase in basal signaling and a more persistent response after pheromone stimulation.

DISCUSSION

In this report, we investigated how palmitoylation of Gpa1p affects pheromone signal transduction in yeast. This is the first demonstration of palmitoylation of Gpa1p. More importantly, this is the first attempt to investigate how palmitoylation affects G protein function in a homologous, *in vivo* expression system. Previous studies of G protein palmitoylation have relied on mutant alleles expressed transiently in cultured cells or on purified proteins reconstituted into lipid vesicles. These approaches are certainly useful and valid in most cases, but they have some obvious limitations. First, overexpression may alter the stoichiometry of G_α to other cellular components, which can have

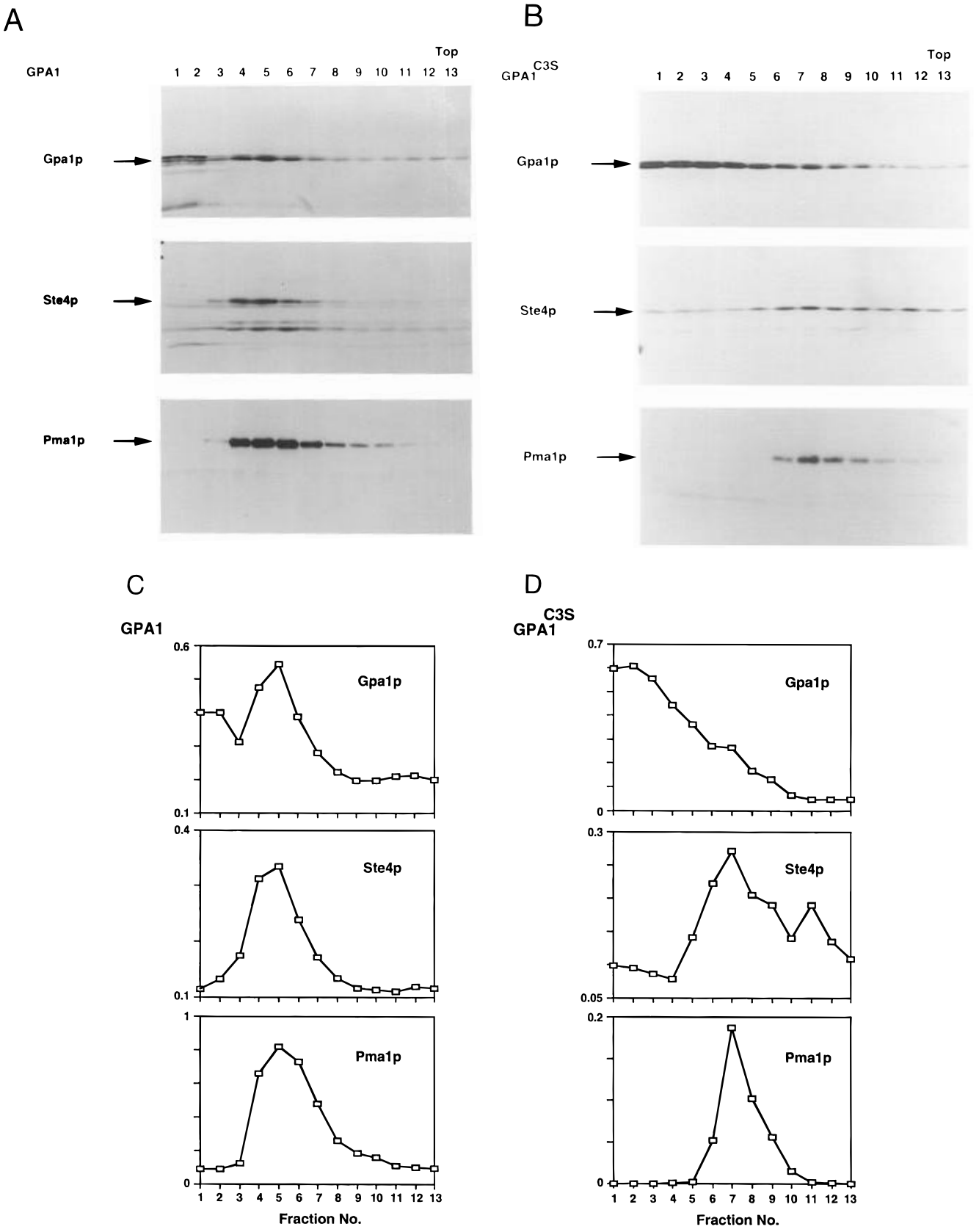

FIGURE 7: Palmitoylation promotes plasma membrane localization. Cells containing *GPA1* (A) or *gpa1*^{C3S} (B) (plasmid pRS316-ADH) were lysed, and the precleared homogenates were resolved by 30−70% sucrose density gradient centrifugation. Sixteen fractions were collected from the bottom of gradient and analyzed by SDS−PAGE and immunoblotting using antibodies against Gpa1p, Ste4p (G_β), or Pma1p (H^+ ATPase, plasma membrane marker). Fractions 14−16 are not shown and did not contain Gpa1p, Ste4p, or Pma1p. Immunoblots from cells expressing myc-tagged Gpa1p are shown here, but similar results were obtained with untagged protein (data not shown). Panels C and D show densitometric scanning analysis of the specific immunoreactive bands in panels A and B, respectively.

unanticipated effects on its localization or signaling properties. Second, heterologous expression or protein reconstitution systems may lack the normal signaling proteins that regulate G_α function. Third, reconstituted proteins may lack other (perhaps uncharacterized) posttranslational modifications. Thus we sought to determine if G protein activity is

altered by palmitoylation using "normal" levels of G_α expression in a completely homologous system. Our finding that the nonpalmitoylated mutant of Gpa1p confers partial constitutive activation of the signaling pathway indicates that this modification profoundly alters Gpa1p function *in vivo*. Our demonstration that nonpalmitoylated Gpa1p is partially mislocalized provides a likely explanation for this unique phenotype.

In yeast the covalent modification of proteins by two saturated fatty acids, myristate (C14:0, myristoylation) and palmitate (C16:0, palmitoylation), have been described (Towler et al., 1988). Myristate is attached cotranslationally via an amide bond to the N-terminal Gly residue following cleavage of the initiator Met. Palmitoylation occurs post-translationally via a thioester linkage at internal Cys residues. Gpa1p contains a consensus sequence Met-Gly-Cys-X-X-X-Ser/Thr at the N-terminus, which is found in many proteins that undergo both myristoylation and palmitoylation. Examples include mammalian G protein α subunits and members of the Src family of protein tyrosine kinases (Resh, 1994). Gpa1p is known to be myristoylated, and this modification is essential for its activity *in vivo* (Stone et al., 1991). The incorporation of [^{3}H]palmitate indicates that Gpa1p also undergoes palmitoylation (Figure 1). Although palmitic acid can be converted to myristic acid (Christgau et al., 1992), this is unlikely to have occurred since the labeling is hydroxylamine-sensitive and is blocked by a Cys-to-Ser mutation (Figure 2A). These characteristics are consistent with a palmitoyl-thioester modification. In contrast, incorporation of [^{3}H]myristate is neither hydroxylamine-sensitive nor blocked by the Cys mutation (Figure 2B).

While the C3S mutation in Gpa1p blocked palmitoylation, it did not prevent myristoylation (Figure 2). Thus, palmitoylation is not needed for myristoylation of Gpa1p, consistent with previous studies in mammalian systems (Degtyarev et al., 1994; Grassie et al., 1994; Mumby et al., 1994). [^{3}H]Myristate labeling of Gpa1p^{C3S} was lower than that of wild type (Figure 2B), however, most likely due to reduced recovery of the mutant following cell lysis (Figure 6A). Indeed, a much smaller difference was observed for the Gst fusions (Figure 2D), which appear to be less susceptible to degradation (Figure 4). In contrast, the G2A mutation in Gpa1p blocked both myristoylation and palmitoylation. This observation suggests that myristoylation is a prerequisite for palmitoylation, consistent with studies of $G_{o\alpha}$, $G_{i1\alpha}$, and $G_{z\alpha}$ (Galbiati et al., 1994; Hallak et al., 1994; Mumby et al., 1994). However, members of the $G_{s\alpha}$ and $G_{q\alpha}$ family are palmitoylated but are not normally myristoylated (Linder et al., 1993). In addition, Wilson and Bourne showed that $G_{z\alpha}^{G2A}$ still incorporates palmitic acid, though to a much lesser extent than the wild-type protein (Wilson & Bourne, 1995). Another study found that a nonmyristoylated mutant of $G_{i\alpha}$ could incorporate palmitate when cooverexpressed with $G_{\beta\gamma}$, presumably by virtue of its ability to form a complex with G_α and restore membrane binding. The authors concluded from this observation that palmitoylation requires membrane localization but not myristoylation per se (Degtyarev et al., 1994). Thus one explanation for our inability to detect incorporation of [^{3}H]palmitate to the Gpa1p^{G2A} mutant is that it is not at the plasma membrane, where palmitoyl acyltransferase is thought to reside (Dunphy et al., 1996). Indeed we have previously shown that Gpa1p^{G2A} is membrane-associated but is not localized to the plasma membrane (Song et al., 1996). We cannot rule out other possibilities, however, since palmitoyl transferases have not been fully characterized. For example, it is also possible that the G2A mutant is more readily depalmitoylated during purification, since cell extracts are known to contain palmitoyl thioesterase activity (Wedegaertner & Bourne, 1994). Alternatively, the absence of palmitoylation in the Gpa1p^{G2A} mutant could simply be due to changes in sequence context or protein secondary structure.

In yeast, activation of the G protein signaling pathway results in transcriptional activation of certain genes and growth arrest at the G1 phase of the cell cycle. Thus overall G protein activity can be easily monitored *in vivo*. Replacement of the palmitoylation-site Cys still allows complementation of the *gpa1*Δ mutation (Figure 3) but partially impairs Gpa1p function *in vivo* (Figure 5). The C3S mutant responded to pheromone, yet it exhibited a 2-fold increase in the maximum level of pheromone-induced transcriptional activation, as well as an increase in the sensitivity of pheromone-induced growth arrest. Moreover, the mutant exhibited a 5-fold elevation in basal transcription, comparable to that seen in wild-type cells treated with ∼100 nM pheromone. This increase in basal signaling might be expected to lead to growth arrest. However, pheromone-induced growth arrest is usually transient, particularly at such low doses, presumably because the receptor or G protein undergoes desensitization (Dohlman et al., 1996).

What is the mechanism underlying the partial constitutive activation of the signaling pathway and the increased pheromone sensitivity? Previous studies have suggested a possible functional role of palmitoylation in coupling of G proteins to receptors and effectors in mammals. While palmitoylation is required for coupling of $G_{q\alpha}$ to neurokinin NK2 (Edgerton et al., 1994) and α2-adrenergic receptors (Wedegaertner et al., 1993) and to its effector, PLC-β1 (Edgerton et al., 1994; Wedegaertner et al., 1993), it is not required for signaling by $G_{z\alpha}$ (Wilson & Bourne, 1995). It is unlikely that palmitoylation will affect receptor coupling to Gpa1p since the C3S mutant is still able to respond to pheromone. In yeast, Gpa1p appears not to interact with downstream effectors since disruption of *GPA1* results in constitutive activation of the signaling pathway. Rather, the primary role of Gpa1p is to regulate the levels of free $G_{\beta\gamma}$ in the cell. Thus, the functional consequences of the C3S mutation suggest that palmitoylation may alter the interaction of Gpa1p with $G_{\beta\gamma}$ *in vivo*.

As a preliminary test of this model, we determined that the wild-type and nonpalmitoylated mutant forms of Gpa1p are similarly able to bind $G_{\beta\gamma}$ and that complex formation in either case could be reversed with the addition of GTPγS (Figure 4). This was not unexpected, since we had shown previously that the Gpa1p^{G2A} mutant, which is neither myristoylated nor palmitoylated, also binds normally to $G_{\beta\gamma}$ (Song et al., 1996). These experiments suggest that subunit binding affinity is unaltered; however, methods to demonstrate this directly are not yet available in yeast.

An alternative possibility is that the hydrophobic palmitoyl group could alter Gpa1p binding to cell membranes in general or to the plasma membrane in particular. For example, a reduction of Gpa1p at the plasma membrane could lead to a pool of unsequestered $G_{\beta\gamma}$ free to activate the pathway. Indeed, previous studies in mammalian cells have revealed a possible role of G protein acylation in membrane binding. While palmitoylation contributes to

membrane association of $G_{o\alpha}$ (Mumby et al., 1994), $G_{i\alpha}$ (Degtyarev et al., 1994), and $G_{z\alpha}$ (Wilson & Bourne, 1995), it is not required for membrane attachment of $G_{s\alpha}$ (Mumby et al., 1994) or $G_{q\alpha}$ (Edgerton et al., 1994; McCallum et al., 1995). However, another group showed that palmitoylation is required for membrane binding of $G_{s\alpha}$ and $G_{q\alpha}$ (Wedegaertner et al., 1993). The reason for the discrepancy is not clear and could be due to cell line differences or variations in protein expression (e.g., receptors, $G_{\beta\gamma}$ subtypes). These differences could also explain the discordant results regarding the effects of G2A mutants on palmitoylation, as outlined above. Thus an important advantage of the yeast system is that G_α palmitoylation can be readily studied in a homologous expression system, without overexpression or epitope tagging.

Loss of palmitoylation does not alter association of Gpa1p to the membrane fraction (Figure 6A). This is not surprising since $Gpa1p^{G2A}$, which is neither myristoylated nor palmitoylated, also remains bound to the membrane (Song et al., 1996; Stone et al., 1991). Presumably some feature of the primary sequence of Gpa1p, rather than myristoylation or palmitoylation, is responsible for its membrane association. The membrane association of Gpa1p is at least partially due to protein−protein interactions, since wild-type Gpa1p is partially solubilized from the membrane upon high-pH treatment. However, palmitoylation is not essential for these interactions since the nonpalmitoylated mutant (the C3S mutation) behaves the same as wild type. Similarly, membrane association is at least partially due to hydrophobic interactions, since wild-type Gpa1p partitions in both the detergent-rich and aqueous phases in Triton X-114 solution. Again, palmitoylation is not essential for these interactions since the C3S mutant behaves the same as wild type. Presumably myristoylation is sufficient for both the protein−protein and hydrophobic−hydrophobic modes of interaction. Thus, by at least three criteria, palmitoylation does not contribute to the membrane binding properties of Gpa1p.

Using a sucrose density gradient centrifugation method to purify plasma membranes, we have previously shown that myristoylation is required for plasma membrane localization (Dohlman et al., 1996; Song et al., 1996). A much smaller representation of Gpa1p in the plasma membrane fraction was also observed for the nonpalmitoylated mutant, as compared to wild type, and this was accompanied by an increase of Gpa1 in the microsomal membrane fraction (Figure 7). Thus, while palmitoylation is not essential for Gpa1p attachment to the lipid bilayer, it does promote the recruitment or retention of Gpa1p to the plasma membrane, where it is needed to transmit the signal from the receptor to $G_{\beta\gamma}$. This partial mislocalization of nonpalmitoylated Gpa1p may contribute to the observed increase in basal signaling, since it would leave a pool of unsequestered $G_{\beta\gamma}$ at the plasma membrane free to activate the effector. Indeed, even a small change in the stoichiometry of G_α to $G_{\beta\gamma}$ is likely to affect signaling, since it has been shown previously that expression of one additional copy of *STE4* (G_β) is sufficient to activate the signaling pathway (Cole & Reed, 1991).

Finally, palmitoylation has been proposed to play a role in the desensitization of receptors (Moffett et al., 1993) and G proteins. Palmitoylation of $G_{s\alpha}$ is accelerated upon G protein activation (Degtyarev et al., 1993b; Wedegaertner & Bourne, 1994). Hormone stimulation or a mutation that blocks GTPase activity results in a rapid loss of [^{3}H]palmitate from $G_{s\alpha}$, as well as increased [^{3}H]palmitate incorporation to previously unlabeled $G_{s\alpha}$ (Wedegaertner & Bourne, 1994). In addition, activation leads to $G_{s\alpha}$ translocation from the membrane to the cytosol (Levis & Bourne, 1992; Negishi et al., 1992; Ransnas et al., 1989), but only the membrane-bound pool of G_α is palmitoylated (Wedegaertner & Bourne, 1994). These data provide a mechanism for dynamic regulation of protein localization by palmitoylation. However, stimulus-dependent depalmitoylation of dually acylated G proteins (i.e., those that are also myristoylated) has not been demonstrated. Moreover, preliminary experiments have failed to reveal any stimulus-dependent change of Gpa1p palmitoylation or binding to the plasma membrane (data not shown). In contrast, myristoylation of Gpa1p is regulated by pheromone treatment (Dohlman et al., 1993), but the functional significance of this change is not clear.

In summary, the central findings of this study are that Gpa1p is palmitoylated and that palmitoylation is required for normal pheromone responsiveness *in vivo*. These conclusions are based on the following observations. [^{3}H]-Palmitic acid was incorporated into Gpa1p. The C3S mutation in Gpa1p prevented incorporation of the label. Cells expressing the nonpalmitoylated Gpa1p were viable but exhibited an increase in both basal and pheromone-stimulated responses. Finally, the nonpalmitoylated mutant exhibited no change in membrane binding but a reduced plasma membrane association. Collectively, these data suggest that the elevated basal signal and the increased pheromone sensitivity exhibited by $Gpa1p^{C3S}$ are probably due to reduced plasma membrane association of Gpa1p, leaving a small pool of unsequestered $G_{\beta\gamma}$ free to activate the downstream effector, thus leading to increased basal signaling *in vivo*. These experiments highlight a previously unsuspected role of palmitoylation in membrane binding *specificity* and suggest that any dynamic changes in G protein subcellular distribution could have profound effects on transmembrane signaling and regulation.

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