Regulation of Membrane and Subunit Interactions by N-Myristoylation of a G Protein $\alpha$ Subunit in Yeast*

(Received for publication, May 7, 1996)

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Initiation of the mating process in yeast Saccharomyces cerevisiae requires the action of secreted pheromones and G protein-coupled receptors. As in other eukaryotes, the yeast G protein $\alpha$ subunit undergoes N-myristoylation (GPA1 gene product, Gpa1p). This modification appears to be essential for function, since a myristoylation site mutation exhibits the null phenotype in vivo (gpa1G2A). Here we examine how myristoylation affects Gpa1p activity in vitro. We show that the G2A mutant of Gpa1p, when fused with glutathione-S-transferase, can still form a complex with the G protein $\beta\gamma$ subunits. The complex is stabilized by GDP and is dissociated upon treatment with guanosine 5'-O-(thiotriphosphate). In addition, there is no apparent difference in the relative binding affinity of G$_\alpha_{\text{G2A}}$ for mutant and wild-type Gpa1p. Using sucrose density gradient fractionation of cell membranes, Gpa1p associates normally with the plasma membrane whereas Gpa1pG2A is mislocalized to a microsomal membrane fraction. A portion of G$_\alpha_{\text{G2A}}$ is also mislocalized in these cells, as it is in a gpa1 strain. In contrast, wild-type Gpa1p reaches the plasma membrane in cells that do not express G$_\alpha_{\text{G2A}}$, or cell surface receptors. These findings indicate that mislocalization of Gpa1pG2A is not caused by a redistribution of G$_\alpha_{\text{G2A}}$, nor is it the result of any difference in G$_\alpha_{\text{G2A}}$ binding affinity. These data suggest that myristoylation is required for specific targeting of Gpa1p to the plasma membrane, where it is needed to interact with the receptor and to regulate the release of G$_\alpha_{\text{G2A}}$.

The actions of many extracellular stimuli (hormones, neurotransmitters, odorants, light) are elicited by signaling cascades that involve a membrane-bound receptor, a regulatory G protein composed of three subunits ($\alpha$, $\beta$, $\gamma$), and an effector that propagates the signal. Upon receptor activation, the G protein $\alpha$ subunit releases GDP, binds GTP, and dissociates from the G$_\alpha_{\text{G2A}}$ subunit complex. The G protein remains in the active dissociated state until GTP is hydrolyzed. In yeast, most components of this signaling cascade were discovered genetically, through gene mutations that block pheromone signaling and mating. Disruption of genes encoding G$_\alpha$ (STE4) or G$_\gamma$ (STE18) result in a sterile phenotype. Loss of G$_\alpha$ (GPA1, also known as SCG1) leads to constitutive signaling and mating even in the absence of pheromone. Thus it appears that G$_\alpha_{\beta\gamma}$ activates the downstream signaling pathway, and the primary role of Gpa1p is to regulate the levels of free G$_\alpha_{\text{G2A}}$ in the cell (1–3).

In order to respond to extracellular signals, G proteins and receptors must associate with the plasma membrane of the cell. Since G proteins are not integral membrane proteins, however, it is not clear how they become attached to the lipid bilayer or how they are targeted specifically to the plasma membrane. One mechanism for controlling protein localization is post-translational modification (4). Indeed, it has been shown that all G protein $\alpha$ subunits undergo some form of fatty acylation. G$_\alpha_{\text{G2A}}$, G$_\alpha_{\text{G2A}}$, G$_\alpha_{\text{G2A}}$, and G$_\alpha_{\text{G2A}}$ are palmitoylated (5–10); G$_\alpha_{\text{G2A}}$, G$_\alpha_{\text{G2A}}$, and Gpa1p are N-terminal myristoylated (10–18); G$_\alpha_{\text{G2A}}$ is heterogeneously acylated (14, 19, 20).

In yeast, mutations in the N-myristoyltransferase (NMT1) gene (11, 21, 22), or mutations that replace the myristoylated Gly residue of Gpa1p (11), result in constitutive activation of the pheromone response pathway. Thus nonmyristoylated Gpa1p appears unable to bind G$_\alpha_{\text{G2A}}$ in vivo, even though it remains associated with the cell membrane (11). The ability of myristoylated or nonmyristoylated Gpa1p to bind G$_\alpha_{\text{G2A}}$ has never been documented, however. Moreover, while nonmyristoylated Gpa1p sediments with the membrane fraction, the type of membrane was never defined (11). Thus it is not known if myristoylation is necessary for high affinity binding to G$_\alpha_{\text{G2A}}$, or for specific binding to the plasma membrane, or both. For example, Gpa1p could be recruited to the plasma membrane via myristoylation-dependent coupling to G$_\alpha_{\beta\gamma}$. Conversely, Gpa1p might require myristoylation to reach the plasma membrane, and consequently is able to interact with other plasma membrane proteins such as G$_\alpha_{\text{G2A}}$, or the receptor.

Previous studies have revealed a possible role for G$_\alpha_{\text{G2A}}$ in targeting G$_\alpha$ to the plasma membrane. Sternweis (23) has shown that binding of purified G$_\alpha_{\text{G2A}}$ and G$_\alpha_{\text{G2A}}$ to lipid vesicles requires co-reconstitution with G$_\alpha_{\beta\gamma}$, suggesting that G$_\alpha_{\beta\gamma}$ can serve as a membrane anchor for the $\alpha$ subunit in mammalian cells. However, other proteins must also be involved since expression of G$_\alpha_{\text{G2A}}$ in excess of G$_\alpha_{\text{G2A}}$ does not prevent membrane association (24), and disruption of the G protein $\beta$ and $\gamma$ genes in yeast (STE4, STE18) does not lead to solubilization of Gpa1p (25). Again, the type of membrane was not defined in these experiments, so they do not reveal whether G$_\alpha_{\text{G2A}}$ can direct G$_\alpha_{\text{G2A}}$ to a specific cell membrane compartment.

Past studies have also revealed several functional changes associated with the myristoylation of G proteins. For example, myristoylation of G$_\alpha_{\text{G2A}}$ promotes binding to G$_\alpha_{\beta\gamma}$, direct inhibition of adenyl cyclase in vitro, as well as binding to cell membranes (16, 17, 26–29). Myristoylated peptides corresponding to the N-terminal domain of G$_\alpha_{\text{G2A}}$ were found to inhibit binding to G$_\alpha_{\text{G2A}}$. In these experiments, a random myristoylated peptide

*This work was supported in part by funding from the Yale-Lederle Cooperative Agreement, the Patrick and Catherine Weldon Donahue Medical Research Foundation, and the American Heart Association (to H. G. D.), as well as from National Institutes of Health Grant GM 34719 (to Duane Jenness). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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was also an effective inhibitor, suggesting that the lipid moiety can play an important role in $G_{\gamma}$ recognition (14, 15). Less direct experiments (e.g. N-terminal deletions or protein) have yielded similar conclusions (reviewed in Ref. 30).

More recently, Chabre and colleagues have proposed an alternative model for how $G_{\gamma}$ myristoylation contributes to subunit and membrane interactions. They showed that tight association of purified subunits requires the presence of lipids or detergent, suggesting that acylation contributes indirectly to subunit interaction by restricting their mobility to the two-dimensional plane of the membrane or micelle (31). These experiments were possible because of the unique ability of $G_{\gamma}$ to be solubilized even in the absence of detergent. We reasoned that the $G$ protein in yeast would also be useful in this regard, because of the unusual ability of Gpa1p to remain membrane associated even when myristoylation is blocked (11). Thus a direct role for myristoylation in subunit binding can be examined without the confounding effects of membrane dissociation. Moreover, strains that bear disruption mutations of $G$ protein subunits are available, so a role for $G_{\gamma}$ expression in $G_{\mu}$ trafficking can be tested directly.

In this report, we investigate the role of myristoylation in $G$ protein subunit and membrane interactions. These experiments reveal that $G_{\gamma}$ binds to myristoylated and nonmyristoylated Gpa1p with similar affinity, but that myristoylation is required for Gpa1p to reach the plasma membrane where $G_{\gamma}$ is normally located. These findings reveal a role for myristoylation in $G$ protein subcellular localization and highlight the importance of proper membrane localization for normal $G$ protein function.

**EXPERIMENTAL PROCEDURES**

Strains, Media, and Transformation—Standard methods for the growth and maintenance of yeast and bacteria were used throughout (32). The Escherichia coli strain DH5α was used for the maintenance of plasmids. Yeast cells were grown in synthetic medium supplemented with amino acids, adenine, and 2% glucose (SCD) or 2% galactose plus adenine, and 2% glucose (SDG). Enzymes and buffers were obtained from New England Biolabs. PCR reagents were from Perkin-Elmer. Sequencing reagents were purchased from U. S. Biochemical Corp. Expression plasmids used in this study were pRS316 (ampR, CEN/ARS, URA3), or pRS316-ADH (pRS316 with ADH1 promoter and terminator from pAD4M, described below) (33), or pRS316-GAL.

A double epitope tagged version of GPA1 in pBluescript (Stratagene) (pBSSH-E-GPA1-myc) was constructed by ligation of two annealed synthetic oligonucleotides (5'-GCT TAT AGA CAA AAA TTA ATT TCG GAT ATT TAA GAA CAA AAA TTA ATT TCG TAT ATT TAA GAG TTT TTA TTT AAG ATG AGG TTT CGG TAT AAG CGT ATG TCG A-3') in tandem into the unique HindIII site of GPA1. This oligonucleotide encodes an epitope, Ala-Glu-Glu-Glu-Ile-Ser-Glu-Glu-Asp-Leu-Lys, recognized by the c-myc monoclonal antibody 9E10 (39).

Replacement of the N-terminal Gly2 codon was achieved by PCR using mismatched primers (32) with pBSH/E-GPA1 or pBSSH/E-GPA1-myc as templates. The forward primers contain a BamHI site and encode Met-Gly-Cys-Thr (5'-GGG GAT CCC ATG GGG TGT ACA-3'), or Met-Ala-Cys-Thr (5'-GGG GAT CCC ATG GGG TGT ACA-3')

The reverse primer (5'-ATG AGA CCT ATG GGG TGT ACA-3') is complementary to nucleotides 398-414 of the GPA1 open reading frame. The PCR products were digested with BamHI and HindIII. pRS316-GPA1 and pRS316-GPA1-G2A (5'-CGA CCT ATG GGG TGT ACA-3') were also effective inhibitors, suggesting that the lipid moiety contributes to subunit interaction by restricting their mobility to the two-dimensional plane of the membrane or micelle. These experiments were possible because of the unique ability of $G_{\gamma}$ to be solubilized even in the absence of detergent. We reasoned that the $G$ protein in yeast would also be useful in this regard, because of the unusual ability of Gpa1p to remain membrane associated even when myristoylation is blocked (11). Thus a direct role for myristoylation in subunit binding can be examined without the confounding effects of membrane dissociation. Moreover, strains that bear disruption mutations of $G$ protein subunits are available, so a role for $G_{\gamma}$ expression in $G_{\mu}$ trafficking can be tested directly.

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G Protein Myristoylation and Localization

GST were grown to mid-logarithmic phase in selective medium. Approximately 1.3 x 10^8 cells were harvested by centrifugation at 1000 x g for 10 min. The cells were washed twice with 10 ml of YPD plus 1% ethanol and resuspended at 2.5 x 10^7 cells/ml in YPD plus 1% ethanol. A 2.0 ml aliquot 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 μC/ml [3H]myristic acid (Du Pont NEN, ~39 Ci/mmol) and grown for 1.5 h. Growth was stopped by the addition of NaN3 to 10 mM. All subsequent manipulations were carried out at 0–4 °C. Cells were harvested by centrifugation and washed once with 10 mM NaCl and once with 100 mM triethanolamine, pH 7.4, 8.0, 10 mM MgCl2 and either 100 μM GTP and is essential for the conformational change leading to G

The lysate was centrifuged twice at 500 g for 3 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. 100 μl of glutathione-Sepharose 4B resin (Pharmacia Biotech Inc.) (20% slurry) was added to the soluble material and mixed at 4 °C for 2 h. The resin was washed three times with 20 ml sodium Pi, pH 7.3, 350 mM NaCl. The bound proteins were eluted by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 10 min. The purified GST were precipitated by mixing with a monoclonal antibody 9E10 for 1 h, followed by washing and subsequent manipulations were carried out at 0–4 °C. Approximately 2 x 10^6 cells were harvested by centrifugation and washed once with 10 mM NaCl and once with 100 mM triethanolamine, pH 7.4, 8.0, 10 mM MgCl2 and either 100 μM GTP and is essential for the conformational change leading to G

All subsequent manipulations were performed at 0–4 °C. 7.5 x 10^4 cells were harvested by centrifugation at 1000 x g for 10 min and washed in lysis buffer A. Cell pellets were resuspended in 100 μl of buffer A containing 50 mM MgCl2, 100 μM GDP, 10 mM NaN3, and 30 μM AlCl3 to promote Gαi dissociation and subjected to glass bead vortex homogenization for 1 min. The resulting supernatant was treated with 1% Triton X-100 (final concentration) at 4 °C for 1 h. 100 μl of glutathione-Sepharose 4B resin (Pharmacia) (20% slurry) was added to the lysate and mixed at 4 °C for 2 h. After removing the supernatant, the resin was used for Gβγ binding in solution and determination of relative Gβγ binding affinity.

For isolation of Gβγ, J TY2117 cells expressing Gβγ, prepared as described below. After 2 h, the resin was centrifuged and resuspended in buffer A containing 50 mM MgCl2 and either 100 μM GDP or 100 μM GTPyS. The resin was washed three times with 20 ml sodium Pi, pH 7.3, 350 mM NaCl. The bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting with antibodies against GST, Ste6p or Ste28p, as described above.

For determination of relative Gβγ binding affinity, the resin was mixed with lyses from J TY2117 cells expressing Gβγ, and centrifuged at 10,000 x g for 10 min and resuspended in SCG-uracil medium at 0.4 A590nm/ml. Cells were grown for 30 min in buffer A containing 1% Triton X-100 (final concentration) at 4 °C for 1 h as described above.

Cell Disruption and Membrane Fractionation—YGS5 cells expressing pRS316-ADH, pRS316-ADH-GPA1-myrc, or pRS316-ADH-GPA1-G2Amyc were grown and metabolically labeled with [3H]myristic acid as described above. All subsequent manipulations were carried out at 0–4 °C. Approximately 2 x 10^6 cells were harvested by centrifugation and washed once with 10 mM NaCl and once with 100 mM triethanolamine, pH 7.4, 8.0, 10 mM MgCl2 and either 100 μM GTP and is essential for the conformational change leading to G

Gα binding affinity, the resin was mixed with lyses from J TY2117 cells expressing Gα, and centrifuged at 10,000 x g for 10 min and resuspended in SCG-uracil medium at 0.4 A590nm/ml. Cells were grown for 30 min in buffer A containing 1% Triton X-100 (final concentration) at 4 °C for 1 h as described above.

Sucrose Gradient Fractionation—Methods for cell membrane fractionations have been described in detail elsewhere (48). Briefly, cells were grown in selective media to mid-logarithmic phase, centrifuged, and resuspended in YPD at 0.5 A590nm/ml. After one doubling, growth was stopped by addition of NaCl to 10 mM. Approximately 3 x 10^6 cells were harvested by centrifugation at 500 x g for 10 min and washed twice with 0.2 M sucrose, 0.1 M potassium Pi, pH 7.5.

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Spheroplasts were prepared by resuspending cells in 10 ml of SK, 1 mg of zymolyase 100T (Kirin Brewery), 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, washed once with SK, and once with lysing buffer C (0.8% sucrose, 20 mM triethanolamine, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol, 2 μM AEBSF, 10 μg/ml each of leupeptin, pepstatin, benzamidine, and aprotinin). Cell pellets were resuspended in 1.0 ml of lysing 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. 606 mg of sucrose were 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 of 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 170,000 × g. Samples were then centrifuged in a Beckman L80 ultracentrifuge. 16 samples were then centrifuged in a Beckman SW50Ti swinging bucket rotor for 16 h at 170,000 × g in a Beckman L80 ultracentrifuge. 16 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 through 13 or 14 (fractions 14, 15, and 16 do not contain Gpa1p, Ste4p, or Pma1p) were resolved by 8% SDS-PAGE and immuno-blotting 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 Saccharomyces cerevisiae; from C. Slayman, Yale University) (49).

RESULTS

Expression of Gpa1p—All experiments were performed in strains YPH501 (MATα/a), YPH499 (MATα), JTY2117 (a protease deficient MATα/a strain), B2168 (a protease deficient MATα strain) or derivatives of YPH499 lacking Gα (ste8Δ), the α-factor receptor (ste11Δ), or Gαα (gpa1Δ). The absence of Gα1 would normally lead to constitutive signaling and growth arrest; however, the gpa1Δ strain is viable at 34 °C due to a temperature sensitive mutation (ste11) that blocks the signal downstream of the G protein.

In general, Gpa1p was expressed under the control of a heterologous gene promoter from ADH1. This promoter was used to normalize expression of Gα1, which is significantly reduced in ste11 and ste8 mutants (35) and is absent in a/a diploid cells (36, 50). As shown in Fig. 1A, expression of Gα1 under control of the ADH1 promoter only modestly reduces pheromone responsiveness, as demonstrated by the growth inhibition (halo) assay (see “Experimental Procedures”).

Epitope Tagging and Expression of Gpa1p-GST—For immunoprecipitation experiments, an epitope-tagged version of Gpa1p was prepared using a well defined antigenic domain of the myc oncogene product. The monoclonal antibody 9E10 binds to this epitope with high affinity and specificity, and does not recognize any endogenous yeast proteins (39, 51). The epitope tag does not interfere with Gpa1p function in vivo. As shown in Fig. 1A, Gpa1p-myc can restore growth to the gpa1Δ strain, and confers a normal pheromone response. Moreover, tagged and untagged versions of Gpa1p are expressed at equal levels in the cell and fractionate to the same membrane compartment following sucrose density gradient fractionation of membranes (see below, Figs. 7 and 8). Thus, epitope-tagging provides a convenient method for the sensitive and selective detection of functional Gpa1p.

For Gαβφ, binding experiments, we prepared a full-length version of Gpa1p fused to glutathione S-transferase (Gpa1p-GST). We placed the GST domain at the C terminus of Gpa1p so as not to interfere with myristoylation and Gαβφ binding (functions requiring an intact N terminus) (30). Indeed, the Gpa1p-GST fusion was able to complement the growth defect of the gpa1Δ mutant, indicating that it can bind Gαβφ in vivo (Fig. 1B). However, the fusion protein did not produce a halo when expressed in this mutant strain (data not shown). This is presumably because an intact C-terminal domain of Gαβφ is required for binding to the receptor (30). As expected, the gpa1Δ mutation is not complemented by either Gpa1p-G2A or the Gpa1pG2A-GST fusion (Fig. 1B) (11).

Gpa1p and Gpa1pG2A Bind Reversibly to Gαβφ—As discussed above, it is not known why Gpa1pG2A cannot function in vivo. One possibility is that myristoylation is required for proper folding or binding to Gαβφ. To test this hypothesis, we examined whether the Gαβφ subunits can bind to Gpa1pG2A in vitro. Because Gpa1p is not available in purified form we devised a new strategy to detect subunit interactions using the Gpa1p-GST fusion. GST was chosen because it is a well characterized soluble protein for which antibodies and a glutathione-based affinity resin are available. Thus Gpa1p-GST (and any bound Gαβφ) can be easily purified using this resin. Moreover, Gαβφ binding can be easily tracked using Gαβφ antibodies. Functional interaction of Gαβφ with Gpa1p can be demonstrated by showing specific binding in the presence of GDP, but is reversed by GTPγS which promotes subunit dissociation.

To confirm that the fusion protein is still myristoylated, we purified Gpa1p-GST as well as Gpa1pG2A-GST and GST alone from cells metabolically labeled with [3H]myristic acid. As
shown in Fig. 2A, only the wild-type fusion protein was labeled. Labeling was abolished by the G2A mutation, and was absent from GST. Moreover, labeling was resistant to hydroxylamine treatment, indicating that the incorporated label had not been converted to palmitic acid (myristic acid forms an amide linkage that is hydroxylamine resistant, whereas palmitic acid forms a thioester linkage that is hydroxylamine labile) (44). Essentially identical results were obtained when we immunoprecipitated the epitope-tagged (fully functional) version of Gpa1p (Fig. 2B) (11). Thus, while immunoprecipitation provides a convenient way to detect lipid modification of functional Gpa1p, the Gpa1p-GST fusion is similarly modified and can also be used to measure \( G_{bg} \) binding in vitro.

To determine if Gpa1p G2A retains the ability to bind \( G_{bg} \), we purified Gpa1p-GST and examined the effects of guanine nucleotides on \( G_{bg} \) subunit interaction. Detergent solubilized lysates from equal numbers of cells expressing Gpa1p-GST, Gpa1pG2A-GST, or GST alone were prepared (plasmid pAD4M) in B) 2168 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, then centrifuged and resuspended in buffer containing either GDP or GTP\(_S\). 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.

**FIG. 2.** Gpa1p-GST is myristoylated. A, to determine if the Gpa1p-GST fusion is myristoylated, B) 2168 cells expressing Gpa1p-GST, Gpa1pG2A-GST, or GST alone (plasmid pAD4M) were metabolically labeled with \(^{3}H\)myristic acid. All three 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 (top) or 1 M Tris-HCl (bottom), and analyzed by fluorography. B, to demonstrate that epitope-tagged Gpa1p is myristoylated and that myristoylation is abolished by the G2A mutation, YGS5 cells bearing the vector alone, GPA1-myc, or gpa1G2A-myc (pRS316-ADH) were metabolically labeled with \(^{3}H\)myristic acid. Proteins were immunoprecipitated from solubilized cell lysates with the myc monoclonal antibody 9E10. The immunoprecipitates were subjected to SDS-PAGE and treated with hydroxylamine (top) or Tris-HCl (bottom) as described above.

**FIG. 3.** Ste4p copurifies with Gpa1p and Gpa1pG2A. To determine if nonmyristoylated Gpa1p is able to bind to \( G_{bg} \) in vitro, Gpa1p-GST, Gpa1pG2A-GST, or GST alone were expressed (pAD4M) in B) 2168 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, then centrifuged and resuspended in buffer containing either GDP or GTP\(_S\). 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.
found that G\(_\beta\) (presumably as the G\(_{\beta\gamma}\) complex) co-purified with Gpa1p-GST and Gpa1pG2A-GST, and not at all with GST (Fig. 3, bottom). For both the wild-type and mutant fusion proteins, addition of GTP\(_\gamma\)S led to 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 Gpa1pG2A mutant, and that either complex is stabilized in the presence of GDP and dissociated in the presence of GTP\(_\gamma\)S.

To determine if Gpa1pG2A-GST can bind to G\(_{\beta\gamma}\) in solution, we prepared Gpa1p-GST and G\(_{\beta\gamma}\) separately and tested their ability to interact in a guanine nucleotide dependent manner. To obtain Gpa1p without G\(_{\beta\gamma}\), we purified Gpa1p-GST and Gpa1pG2A-GST in the presence of GDP and AlF\(_4^\text{-}\), AlF\(_4^\text{-}\) was used because it mimics the \(\gamma\)-phosphate of GTP when bound to GDP, and promotes complete subunit dissociation when added to the lysis buffer (53, 54). To obtain G\(_{\beta\gamma}\), without Gpa1p, we expressed G\(_{\beta\gamma}\) in a MAT\(a\) strain, where G protein subunits are normally not expressed (36, 50). The expression of G\(_{\beta\gamma}\) was confirmed by immunoblotting with Ste4p and Ste18p antibodies (Fig. 4). The fusion proteins Gpa1p-GST, Gpa1pG2A-GST, or GST alone were immobilized on the glutathione resin, washed extensively, and mixed with lysates from diploid cells expressing G\(_{\beta\gamma}\). After removing the supernatant, the resin was treated with buffer containing GDP or GTP\(_\gamma\)S. The bound proteins remaining were analyzed by immunoblotting with GST, Ste4p, and Ste18p antisera. As shown in Fig. 5, both Ste4p and Ste18p bound well to Gpa1p-GST and Gpa1pG2A-GST, but not to GST alone in the presence of GDP. For both the wild-type and mutant fusion proteins, treatment with GTP\(_\gamma\)S led to dissociation of Ste4p and Ste18p. In both binding experiments (Figs. 3 and 5), however, GTP\(_\gamma\)S-dependent dissociation of G\(_{\beta\gamma}\) from the mutant was more pronounced than from the wild-type Gpa1p fusion. This could be due to a number of differences in G protein function, as discussed below. Since our primary concern was whether there was a myristoylation-dependent change in subunit affinity, we examined this parameter directly.

To determine if there is any difference in the relative G\(_{\beta\gamma}\) binding affinity for mutant and wild-type Gpa1p, equal amounts of Gpa1p-GST and Gpa1pG2A-GST were purified in the presence of GDP and AlF\(_4^\text{-}\). The purified fusion proteins did not contain endogenous G\(_{\beta\gamma}\), as shown by immunoblotting with Ste4p (Fig. 6) and Ste18p antisera (data not shown). The resin was subsequently mixed with increasing amounts of lysates from cells expressing G\(_{\beta\gamma}\) in the presence of GDP. Lysates from cells expressing G\(_{\beta\gamma}\) were mixed with lysates from cells that do not express G\(_{\beta\gamma}\), to correct for any difference in the concentration of other proteins. The binding of G\(_{\beta\gamma}\) was analyzed by SDS-PAGE and immunoblotting with Ste4p antiserum. Each lane contained equal amounts of fusion protein. As shown in Fig. 6, both the wild-type and G2A mutant fusions bind to G\(_{\beta\gamma}\) in a saturable and concentration-dependent manner, and with no significant difference in affinity.

Gpa1pG2A Does Not Bind to Plasma Membranes—The data presented above reveal that Gpa1pG2A retains the ability to bind G\(_{\beta\gamma}\). Thus, we considered an alternative possibility that the loss of function exhibited by this mutant is due to mislocalization within the cell. It was shown previously that both Gpa1p and Gpa1pG2A bind to cell membranes (11). However, those studies did not examine if either protein was specifically associated with the plasma membrane (11). Thus, signaling could be disrupted because Gpa1pG2A does not reach the plasma membrane where G\(_{\beta\gamma}\) is normally located. To test this hypothesis, we used differential and sucrose density gradient centrifugation methods to determine if the subcellular distribution of Gpa1pG2A is altered in any way.

Cells expressing Gpa1p were homogenized, centrifuged at low speed to remove unbroken cells and nuclei, and then separated into soluble (S) and membrane (pelleted, P) fractions by centrifugation at 140,000 \(\times\) g. As shown in Fig. 7A, wild-type Gpa1p and Gpa1pG2A both fractionate exclusively with the 140,000 \(\times\) g membrane fraction. Moreover, the antibodies recognize a single product for Gpa1pG2A (migrating at 56 kDa) but two products for wild-type Gpa1p, corresponding to the myristoylated (54 kDa) and nonmyristoylated (56 kDa) forms of the
Previous studies have also shown that myristoylation of \( G_\alpha \) protein (11, 35). Identical results were obtained using myc-tagged Gpa1p (data not shown). These results confirm that myristoylated and nonmyristoylated pools of wild-type Gpa1p, as well as nonmyristoylated Gpa1p mutant, are all predominantly associated with cell membranes (11).

To determine if nonmyristoylated 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) (49). Immunoblots for cells expressing myc-tagged Gpa1p are shown, but similar results were obtained using untagged Gpa1p (see Fig. 7E). As presented in Fig. 7B, wild-type Gpa1p associates predominately with the plasma membrane fractions (containing Pma1p), while Gpa1pG2A accumulates in earlier fractions that contain Golgi and other microsomal membrane proteins (Fig. 7C).3

To determine if mislocalization of Gpa1pG2A results in a concomitant redistribution of the \( G_\beta\gamma \) complex, we probed the same fractions with polyclonal antibodies to Ste4p. As shown in Fig. 7B, Ste4p is normally associated with the plasma membrane, but is partially mislocalized in cells expressing Gpa1pG2A (Fig. 7C). This redistribution of Ste4p could be the result of binding to the mislocalized population of Gpa1pG2A. Alternatively, functional Gpa1p may be required for proper targeting of \( G_\beta\gamma \) to the plasma membrane. To clarify the relationship between the localization of Gpa1p and Ste4p, we also examined the distribution of Ste4p in a gpa1Δ strain. As shown in Fig. 7D, Ste4p localization is similar to that seen in cells expressing Gpa1pG2A. These results suggest that Gpa1pG2A is largely excluded from the plasma membrane and that any loss of functional Gpa1p expression leads to partial mislocalization of the \( G_\beta\gamma \) subunits.

We also attempted to compare the distribution of the 54-kDa myristoylated and 56-kDa nonmyristoylated forms of the wild-type protein, since this would have provided the best possible internal control for the influence of myristoylation on localization. However, the lower abundance of the nonmyristoylated species in these preparations (Fig. 7A), the high concentrations of sucrose in the gradient samples (which can alter protein mobility) and the long centrifugation times (which can lead to sample degradation) were all confounding factors that made it impossible to reliably quantify myristoylated versus nonmyristoylated wild-type Gpa1p by this method.

The data in Fig. 7A indicate that the overall expression levels of wild-type Gpa1p are higher than the mutant. This difference is not surprising since a greater fraction of wild-type Gpa1p is myristoylated than nonmyristoylated in these cells. Nonetheless, to rule out the possibility that the lower expression of Gpa1pG2A might be responsible for the difference in membrane localization, we examined the distribution of Gpa1pG2A overexpressed from a multi-copy plasmid (pAD4M). As shown in Fig. 7F, expression of both the mutant and wild-type protein is much higher than that with the single copy plasmid (using the same ADH1 promoter), yet they still remain completely associated with the 140,000 \( \times \) g membrane fraction. We then used the sucrose gradient method to determine if overexpression of the protein alters its subcellular localization. As shown in Fig. 7, G and H, the distribution of overexpressed mutant and wild-type Gpa1p is indistinguishable from that seen with the single copy plasmid. However, overexpression of the mutant appears to partially restore normal \( G_\beta\gamma \) localization. This is unexpected, particularly since the distribution of Gpa1pG2A is unaltered in this case. Nevertheless, these data indicate that even large differences in Gpa1p expression have no apparent effects on its subcellular distribution.

**DISCUSSION**

Lipid modification of proteins can have profound effects on their function and subcellular localization (4). Although only a small number of cellular proteins are \( N \)-myristoylated, these include almost all \( G \) protein \( \alpha \) subunits (10–18). In \( S.\ cerevisiae \), it has been documented that Gpa1p is myristoylated and that a myristoylation site mutant exhibits the null phenotype (11). However, it is not clear why this modification is essential for \( G \) protein function.

Past studies have revealed a likely role for myristoylation in \( G \) protein subunit interaction. Jones et al. (17) have shown that nonmyristoylated \( G_{12\alpha} \) requires higher concentrations of \( G_{12\beta\gamma} \) to support ADP-ribosylation by pertussis toxin (which only recognizes the heterotrimer), suggesting that subunit binding affinity is reduced by the lack of myristoylation. Linder et al. (26) observed an even more dramatic reduction in toxin labeling for nonmyristoylated \( G_{12\alpha} \). They also showed that nonmyristoylated \( G_{12\alpha} \) fails to bind a \( G_{12\beta\gamma} \) affinity matrix and is insensitive to \( G_{12\beta\gamma} \)-dependent inhibition of GTP\( \gamma \)S binding.

Previous studies have also shown that myristoylation of \( G_\alpha \)
Fig. 7. Membrane fractionation of Gpa1p and Gpa1pG2A. Membrane fractionation and immunoblot analysis were used to determine the subcellular localization of Gpa1p and Gpa1pG2A. A, YGS5 cells expressing GPA1 or gpa1G2A were lysed, and the precleared homogenates were fractionated into membrane (P) and soluble (S) fractions.

D

E

F

G

H

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FIG. 7. Membrane fractionation of Gpa1p and Gpa1pG2A. Membrane fractionation and immunoblot analysis were used to determine the subcellular localization of Gpa1p and Gpa1pG2A. A, YGS5 cells expressing GPA1 or gpa1G2A were lysed, and the precleared homogenates were fractionated into membrane (P) and soluble (S) fractions.
promotes membrane association. Blocking myristoylation leads to dissociation of G\textsubscript{aG} and G\textsubscript{G}, but not G\textsubscript{a}. The lack of a myristoyl group in G\textsubscript{aG} leads to a reduction in subunit binding affinity and consequently to dissociation of G\textsubscript{a} from the membrane. Alternatively, the myristoyl group could bind directly to the lipid bilayer. In this case, the lack of a myristoyl group would lead to reduced membrane association and only indirectly to a loss of subunit binding. That most G protein subtypes require myristoylation for membrane attachment has made it difficult to distinguish between such direct and indirect effects on subunit binding. Transducin is an exception in this regard, since it can be solubilized from membranes without the use of detergents. Indeed, Chabre and colleagues (31) have recently shown that transducin subunits form a tight complex in the presence of lipids or detergent, but are readily dissociated in aqueous solution. They proposed that lipid modifications of both G\textsubscript{a} and G\textsubscript{G} contribute indirectly to protein-protein interactions, by restricting their relative mobility to the two-dimensional plane of a lipid bilayer or detergent micelle. They also suggested that direct lipid-lipid interactions might play a role in subunit association, a model that is supported by the recent finding that the modified residues of G\textsubscript{a} and G\textsubscript{G} are in close proximity in the crystal structure of the heterotrimer (53, 54). However, none of these reports attempted to quantify the relative hydrophobicity or G\textsubscript{\textalpha} versus nonmyristoylated G\textsubscript{\textalpha}.

G Protein Myristoylation and Localization

The G protein in yeast is another attractive system for investigating how myristoylation affects subunit interaction, since Gpa1p remains associated with the lipid bilayer even in the absence of myristoylation. Thus a direct role for myristoylation in subunit binding can be examined without the confounding effects of membrane dissociation. In the experiments described above, we examined the ability of myristoylated and nonmyristoylated Gpa1p to bind G\textsubscript{aG} and to localize specifically to the plasma membrane.

Initially we asked if the Gpa1p\textsuperscript{G2A} mutant was still able to bind G\textsubscript{aG}. Previous studies have used the "2-hybrid" protein association assay to monitor G protein subunit interactions in yeast (52). This method was not appropriate in our case since it requires that the mutants do not form a DNA binding domain or nuclear localization sequence be fused to the N terminus of Gpa1p, an arrangement that would block myristoylation. In addition, this approach cannot be used to detect guanine-nucleotide dependent effects on subunit binding and dissociation. Thus we developed an alternative method in which we purified Gpa1p fused to GST, and examined its ability to bind G\textsubscript{aG}. The N terminus is unaltered, so the functional role of N-myristoylation can be tested explicitly. To carry out these experiments, we first showed that the fusion and non-fusion variants behave similarly. Wild-type Gpa1p and Gpa1p-GST are myristoylated and can bind G\textsubscript{aG} in vivo, whereas a G2A mutation blocks myristoylation and fails to complement gpa1\textalpha. Both wild-type and mutant fusions can bind to Ste4p/Ste18p in vitro, and binding is reversed by treatment with GTP\textgamma\textsubscript{S}. Thus Gpa1p-GST is myristoylated, can bind guanine nucleotides, and can undergo the conformational change needed for subunit dissociation (Figs. 3 and 5). By probing the same samples with Ste4p and Ste18p antibodies, we confirmed that these proteins form a complex that associates with G\textsubscript{aG}, and this complex is stable under conditions that prevent subunit dissociation (Figs. 3 and 5).

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The binding of Ste4p/Ste18p to wild-type and mutant Gpa1p-GST appears very similar. However, one consistent difference is that G\textsubscript{aG} dissociates more readily from the mutant when treated with GTP\textgamma\textsubscript{S}. The mechanism underlying this difference is obscure, and could result from any one of a number of changes in G protein structure or function. Some possibilities (as yet untested) include changes in magnesium or guanine nucleotide binding affinity, alterations in protein stability or modification, differences in the rate of GTP hydrolysis or exchange, or competition with other G protein-binding proteins. Our primary concern was to rule out a difference in subunit binding affinity. Purified G\textsubscript{aG} subunits are not available from yeast however, so a direct binding assay could not be performed. Thus we used a method that relies only on the available G\textsubscript{\textalpha} antibodies. Specifically, we equilibrated mutant and wild-type Gpa1p with G\textsubscript{aG}, G\textsubscript{\textalpha}, or vector alone (D) were lysed, and the precleared homogenates were resolved by 30–70% sucrose density gradient centrifugation. 16 Fractions were collected from the bottom of gradient and analyzed by SDS-PAGE and immuno blotting as described in Fig. 7.
wild-type Gpa1p-GST with a range of G_{pr} concentrations (generated by mixing lysates from G_{pr}-expressing and -nonexpressing cells), and performed immunoblots to detect the bound protein. In this case, we could not detect any difference in binding of G_{pr} to the wild-type and mutant proteins (Fig. 6).

These results appear to be at odds with previous studies showing reduced binding of G_{pr} to nonmyristoylated G_{ax} in mammals. However, another way that myristoylation could contribute to G protein subunit interaction is to promote co-localization within the cell. Loss of subunit binding in mammals could stem from a reduction in membrane or detergent micelle partitioning, rather than a direct loss of subunit binding affinity. In yeast, previous studies have shown that myristoylation of Gpa1p is not needed for membrane attachment, so the possibility remained that Gpa1pG2A is not at the plasma membrane and is therefore unable to bind G_{pr}. Thus we examined the subcellular distribution of wild-type and mutant Gpa1p, using a sucrose density gradient centrifugation method. We found that the distribution of the two proteins differs markedly, with wild type mostly at the plasma membrane and Gpa1pG2A absent from these fractions. These experiments suggest that myristoylation can indeed play a role in subunit localization in vivo (Fig. 7). This method was also used to examine the distribution of wild-type Gpa1p in cells that lack specific components of the signal transduction apparatus. In these experiments, Gpa1p is properly localized to the plasma membrane even in cells that do not express G_{pr} (Fig. 8). Thus, even if myristoylation contributes to the affinity of subunit interaction in vivo, this cannot explain the mislocalization of Gpa1pG2A since Gpa1p is still able to reach the plasma membrane even in the absence of G_{pr} expression.

It is interesting that Ste4p is mislocalized in cells expressing Gpa1pG2A, as well as in cells that lack GPA1 altogether. The mislocalization is not complete, however, since substantial amounts of the protein still associate with the plasma membrane fractions that are devoid of Gpa1pG2A. This is in marked contrast to our finding that Gpa1p localization is unaltered in cells that lack G_{pr}. Thus it appears that functional Gpa1p expression is required for proper localization of G_{pr}, but G_{pr} is not required for proper localization of Gpa1p. It is also interesting that the mislocalization of G_{pr} appears less severe in cells that overexpress Gpa1pG2A. Although the mechanism for this difference is not clear, it suggests that the mutant form of the protein retains some ability to direct G_{pr} to the proper membrane compartment. Perhaps a small (undetectable) amount of the mutant reaches the plasma membrane and helps to recruit G_{pr} as well. Alternatively, the mutant could compete with G_{pr} for a common binding target in the microsomal membrane compartment, and this competition allows G_{pr} to disengage and reach the plasma membrane. In any case, a substantial pool of G_{pr} is detached from Gpa1pG2A.

Finally, we also considered a third possibility, that reduced expression of Gpa1pG2A could be responsible for the difference in localization or function. The expression levels of both the mutant and nonmyristoylated wild-type forms of Gpa1p are similar (Fig. 7A), so this difference appears to reflect the in vivo situation. Nevertheless, differences in expression cannot explain the change in localization since increasing the expression of Gpa1pG2A does not change its subcellular distribution and still fails to complement the gpa1 mutation (Fig. 7).

In summary, we have characterized the role of myristoylation in G protein subunit interaction and subcellular localization. The central findings of this study are that myristoylation of Gpa1p is required for its plasma membrane localization and is not required for its interaction with Ste4p/Ste18p. These conclusions are based on the observations that the Gpa1pG2A mutant fails to reach the plasma membrane, but is still able to interact with G_{pr} subunits in vitro. Moreover, plasma membrane localization of Gpa1p does not require expression of G_{pr}.

Collectively, these data suggest that the loss of function exhibited by Gpa1pG2A is most likely due to a difference in G_{pr} and Gpa1p localization. The resulting pool of unsequestered G_{pr} is then free to activate the downstream effector, leading to constitutive signaling in vivo.

These results may have significance with regard to the regulation of G protein signaling. Once thought to be co-translational and irreversible, there is emerging evidence that myristoylation can occur post-translationally and is reversed in a stimulus-dependent manner (58–60). Several groups have also described proteins (including Gpa1p) that undergo myristoylation in response to hormone stimulation (35, 61–64). The functional significance of hormone or pheromone-regulated myristoylation is not clear. However, one possibility, suggested by the results described here, is that myristoylation regulates the recruitment or retention of Gpa1p to the plasma membrane where it is available to transmit a signal from the receptor to G_{pr}. A challenge for the future will be to determine how pheromone-regulated changes in myristoylation affect G protein signaling efficiency, and what additional proteins or modifications are required to recruit myristoylated Gpa1p to the plasma membrane.

Acknowledgments—The authors are grateful to Ken Blumer, J effrey Gordon, Duane Jenness, Carolyn Slayman, J eremy Thormer, and Malcolm Whiteway for providing research materials and valuable advice; to John Sondok, Don Apanovitch, Kathy Gillen, and Paul DiBello for critical review of the manuscript, and to Gregory Taylor for expert secretarial assistance.

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