The Gβγ Complex of the Yeast Pheromone Response Pathway

SUBCELLULAR FRACTIONATION AND PROTEIN-PROTEIN INTERACTIONS*

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Genetic evidence suggests that the yeast STE4 and STE18 genes encode Gβ and Gγ subunits, respectively, that the Gβγ complex plays a positive role in the pheromone response pathway, and that its activity is subject to negative regulation by the Ga subunit (product of the GPA1 gene) and to positive regulation by cell-surface pheromone receptors. However, as yet there is no direct biochemical evidence for a Gβγ protein complex associated with the plasma membrane. We found that the products of the STE4 and STE18 genes are stably associated with plasma membrane as well as with internal membranes and that 30% of the protein pool is not tightly associated with either membrane fraction. A slower-migrating, presumably phosphorylated, form of Ste4p is enriched in the non-membrane fraction. The Ste4p and Ste18p proteins that had been extracted from plasma membranes with detergent were found to cosediment as an 8 S particle under low salt conditions and as a 6 S particle in the presence of 0.25 M NaCl; the Ste18p in these fractions was precipitated with anti-Ste4p antiserum. Under the conditions of our assay, Gpa1p was not associated with either particle. The levels of Ste4p and Ste18p accumulation in mutant cells provided additional evidence for a Gβγ complex. Ste18p failed to accumulate in ste4 mutant cells, and Ste4p showed reduced levels of accumulation and an increased rate of turnover in ste18 mutant cells. The gpa1 mutant blocked stable association of Ste4p with the plasma membrane, and the ste18 mutant blocked stable association of Ste4p with both plasma membranes and internal membranes. The membrane distribution of Ste4p was unaffected by the ste2 mutation or by down-regulation of the cell-surface receptors. These results indicate that at least 40% of Ste4p and Ste18p are part of a Gβγ complex at the plasma membrane and that stable association of this complex with the plasma membrane requires the presence of Ga.

The pheromone response pathway of Saccharomyces cerevisiae provides a microbial model for studying function of heterotrimeric G proteins. α-Factor and a-factor pheromones are peptides synthesized by haploid cells of α and a mating type, respectively. Each pheromone binds specific receptors located on cells of the opposite mating type; receptor activation causes cell division arrest at G1 and expression of genes controlling the conjugation of the two mating types. Both α-factor receptors (encoded by the STE2 gene) and a-factor receptors (encoded by STE3) are members of the rhodopsin/β-adrenergic receptor family in that they contain seven transmembrane segments, and they require homologs of mammalian Ga, Gβ, and Gγ protein subunits (encoded by GPA1, STE4, and STE18, respectively) for signal transduction. The receptor appears to form a direct physical association with the G-protein subunits since α-factor binds ste4 mutant cells more weakly (1) and since it dissociates more rapidly from membranes assayed in the presence of GTP analogs or when the membranes are prepared from mutants with defects in GPA1, STE4, or STE18 (2).

Genetic evidence indicates that Gβγ, rather than Ga, activates subsequent events in the response pathway, that is loss of function mutations in STE4 and STE18 block the signal (3–5) whereas loss of function mutations in GPA1 activate signaling (6–8). Moreover, gain of function mutations affecting STE4 (designated STE4Hpa (9, 10) and overexpression of wild-type Ste4p (11–13) cause constitutive activation of the pathway. Gpa1p and Ste18p are modified covalently with myristoyl and farnesyl moieties, respectively (14, 15).

Genetic studies also suggest that Gβ associates with the Ga and Gγ homologs in vivo as well as with other proteins thought to play roles in the pheromone response pathway. However, biochemical characterization of these potential interactions has not been reported. Interactions between Gpa1p and Ste4p were inferred from two-hybrid genetic analysis (10, 16). Moreover, overexpression of Gpa1p compensates for overexpression of Ste4p (11–13); STE4Hpa mutations are suppressed by mutations in GPA1 (10), and one allele is suppressed by overexpression of the wild-type Gpa1p (17). Two-hybrid genetic analysis also predicts that Ste4p binds to Ste18p (16). Mutations in the STE18 gene have been identified that either suppress or enhance partial defects in STE4 (16, 18). Some ste4 alleles are suppressed by overexpression of Ste18p (16, 19), while certain ste18 alleles are suppressed by overexpression of Ste4p (20).

Genetic tests also suggest that Ste4p interacts with the products of the STE5 (21, 22), STE20 (22–25), CDC24 (26), SYG1 (27), and AKR1 (28, 29) genes; the interaction with Ste5p has been confirmed by demonstrating that epitope-tagged Ste4p coimmunoprecipitated with Ste5p (21). Thus, these proteins may function as either effectors or regulators of the Gβγ complex.

Although heterotrimeric G proteins are present among plant, animal, and fungal kingdoms, our current understanding of G protein structure and subcellular localization is based largely on studies of mammalian proteins (reviewed in Refs. 30–32). Mammals are known to contain over 20 different isoforms of Ga representing four different classes; there are 5 and 11 known

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isofoms of Gβ and Gγ, respectively. X-ray crystallographic structures have been solved for two G protein heterotrimers (33, 34), as well as for the transducin α-subunit bound to GDP and to GTP analogs (35) and for the transducin βγ dimer (36). G proteins are bound to the plasma membrane where they mediate a variety of hormonal responses; they also associate with internal membrane compartments where they play a role in membrane trafficking. Stable association of G proteins with membranes involves cooperative interactions among the three subunits as well as lipid modifications of Ga and Gγ (see Ref. 37). Tight binding of Ga to the plasma membrane is thought to occur only when it contains myristoyl and palmitoyl modifications and when it is complexed with Gβγ (37). It is unknown whether Gβγ requires Ga for membrane association.

The object of the present study was to initiate biochemical characterization of Gβγ from an organism that is amenable to genetic analysis. We sought to determine whether the products of the STE4 and STE18 genes form a Gβγ protein complex that is associated with plasma membranes. We found that approximately 40% of both proteins was bound tightly to plasma membranes, whereas the remainder was associated with internal membranes or was not tightly bound to membranes. Stable association of the Gβγ complex with plasma membranes required Ga but did not require the receptor. After detergent extraction of plasma membranes, Ste4p and Ste18p were found to cosediment and to coinmunoprecipitate. The size of the resulting protein complex depended on salt concentration, suggesting that Ste4p and Ste18p interact with other proteins in a salt-dependent manner.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Strains used in this study are described in Table I. All strains except JH59 and JH60 are congenic to strain 381G (4). The designation 381G indicates that the genotype is the same as strain 381G except for the markers shown.

| Strain† | Genotype‡ |
|---------|-----------|
| 381G    | MATa cya1 ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3‡ |
| DJ602-15-1 | 381G MATa ade3 leu2 TYR1† ura3 |
| DJ1006-17-2 | 381G MATa ste18::LEU2 ade3 leu2 ura3 |
| DJ1004-11-1 | 381G MATa leu2 ura3 ade3 ste4::LEU2 |
| DJ147-1-2 | 381G MATa leu2 ura3 TYR1† |
| DJ240-4-1 | 381G MATa ste2-10::LEU2 ura2 ura3 TYR1† |
| DJ803-11-1 | 381G MATa ste5-3 leu2 ura3 bar1-1 can1 cyh2 TYR1† ADE2† |
| DJ803-2-1b | 381G MATa ste5-3 gpa1::LEU2 leu2 ura3 bar1-1 can1 cyh2 TYR1† |
| DJ211-5-3 | 381G MATa bar1-1 leu2 ura3 |
| JH59    | MATa/MATa ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1/trp1 ura3-1/ura3-1 can1-100/can1-100 containing plasmids pPgk-Scg, pEL37 and pBH21 |
| JH60    | 381G MATa ade3 leu2 TYR1† ura3 containing plasmid M91p1 |

† All strains except JH59 and JH60 are congenic to strain 381G (4). The designation 381G indicates that the genotype is the same as strain 381G except for the markers shown.

‡ The temperature-sensitive allele ste5-3 (4) leads to sterility and a-factor resistance at 34 °C. Mutation gpa1::LEU2 is the sce1::LacZ6[LEU2] allele described by Dietz and Kurjan (6). Mutation bar1-1 (67) inhibits a-factor degradation in a cell cultures. Plasmid pPgk-Scg (17) directs high level expression of Gpa1p; plasmid pEL37 results in high level expression of Gpa1p and Ste4p; plasmids pBH21 and M91p1 lead to high level expression of Ste15p.

The abbreviations used are: ATCase, aspartate transcarbamoylase (ATCase) was a gift from Y. R. Yang and H. K. Schachman; anti-Gda1p (provided by C. Hirschberg), specific for the yeast Golgi guanosine diphosphatase; anti-Gpa1p (provided by H. Dohlman), specific for the C terminus of Gpa1p (43). Mouse monoclonal anti-Pma1p (provided by J. Aris), C56 (44), is specific for the yeast plasma membrane ATPase (45). Mouse monoclonal anti-Vpk1p (obtained from Molecular Probes, Eugene, OR) is specific for the 100-kDa subunit of the yeast vacuolar ATPase. Peroxidase-labeled goat anti-mouse IgG was from Life Technologies, Inc.; peroxidase-labeled goat anti-rabbit IgG was from Kirkegaard and Perry.

Anti-Strept oligonucleotide was prepared by immunizing rabbits with full-length Strept expressed from plasmid pBH19 in E. coli strain BL21(DE3) (46). Plasmid pBH19 was constructed by cloning the 1.3-kilobase NcoI/SalI fragment (containing the STE4 gene) from plasmid pL19 (11) into plasmid pET-2c (46). Antibodies were affinity purified by using a resin that had been coupled to bacterially expressed Strept. Anti-Strept was purified by injecting rabbits with the peptide, TSVQNSRPLQQPQE, conjugated with glutaraldehyde to keyhole limpet hemocyanin.

Immunoblotting Methods and Quantitation—Protein samples were diluted 1:3 with sample buffer (1 g of urea dissolved in 1 ml of 17.5 mM Tris-HCl, pH 6.8, 1.75% sodium dodecyl sulfate, 1% β-mercaptoethanol, bromphenol blue) and heated to 37 °C for 10 min. Proteins were resolved by using SDS-PAGE (47) and transferred (48) to an Immobilon membrane (Millipore Corp., Bedford, MA). Membranes were blocked with 20 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20 containing 5% nonfat dried milk; they were probed with primary antisera and secondary antibodies diluted in blocking buffer. Secondary antibodies were either goat anti-rabbit or goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Conjugates were visualized by using chemiluminescence reagents (Renassiance kit, DuPont NEN; or SuperSignal kit, Pierce). Autoradiographic results were quantified by using a densitometer.
Preparation of Cleared Lysates—Unless otherwise indicated, a 150-ml culture was grown overnight at 30°C in YM1 medium (39) to 10^7 cells/ml. Cells were collected by centrifugation, washed twice with ice-cold membrane buffer (10 mM Tris acetate, pH 7.6, 1 mM magnesium acetate, 0.1 mM EDTA, 8% glycerol) containing 1× protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A), and resuspended in 0.5 ml of the same buffer. Glass beads were added, and cells were lysed by mechanical disruption. Unbroken cells were removed from the lysate by centrifugation for 5 min at 330 × g.

Renografin Density Gradients—Membranes from the cleared lysates were resuspended in Renografin gradients as described (45).

Plasma Membrane Purification—Strain JH59 was cultured to approximately 5 × 10^7 cells/ml in minimal galactose medium. 12.5 g (wet weight) of cells were mixed with glass beads and analyzing the crude extracts directly, we found that the technique separates plasma membranes and the G protein subunits by using density gradient centrifugation to fractionate the crude cellular membranes. A gradient of the G protein homolog, Gpa1p, was released efficiently with this technique, but not with 1.0 M NaCl. However, with this technique, it is not possible to distinguish whether the Ste4p and Ste18p in the particulate fraction are associated with membranes or with other non-membranous particles.

We tested whether plasma membranes contain tightly bound G protein subunits by using density gradient centrifugation to fractionate the crude cellular membranes. A gradient of Renografin was layered over a cleared cell lysate that had been adjusted to 38% Renografin (ionic strength, roughly equivalent to 0.5 M NaCl), and the membranes were then allowed to float in a centrifugal field until they reached their buoyant density. We had previously shown that this technique separates plasma membranes from non-membrane proteins and from the more buoyant internal membrane species, i.e., endoplasmic reticulum, Golgi complex, and vacuole (45). The advantages of this fractionation technique are 2-fold. First, by lysing cells rapidly with glass beads and analyzing the crude extracts directly, we minimized potential problems with protein degradation and redistribution of proteins among the cellular membranes. Second, the high ionic strength of Renografin apparently strips the loosely associated proteins from the membranes, maximizing the difference in density between plasma membranes and the internal membranes and thus limiting our analysis to the G

**Table II**

| Wash conditions | Percentage of protein in the supernatant fraction |
|-----------------|--------------------------------------------------|
| Ste4p           | Ste18p                                          |
| No additions    | 5%                                              |
| 0.25 m NaCl     | 6%                                              |
| 0.5 m NaCl      | 15%                                             |
| 1.0 m NaCl      | 22%                                             |
| Detergent     | 24%                                             |
| Detergent + 0.25 m NaCl | 80%  |
| 4 m urea      | 15%                                             |
| 100 mM Na_2CO_3, pH 11 | 73%  |

a Detergent was a mixture of 2 mg/ml dodecyl-β-D-maltoside and 0.4 mg/ml cholesterol hemisuccinate (68).

b Although a Ste18p band was evident on the immunoblot, the amount of protein was below the linear range of the assay.
The distribution of protein markers for the cytosol (Pgk1p, phosphatase (Gda1p) cofractionated with Ost1p and Vph1p (not shown). The fractions from the Renografin gradient depicted in Fig. 1 (strain DJ602-15-1) and probed with anti-Ste18p antiserum. B, the ste18 mutant strain (DJ1006-17-2) was analyzed as in A. C, Ste18p over-producing strain (JH60) was analyzed as in A except that the cells were cultured in selective medium (−Ura + CAA).

Previous workers (49) have resolved electrophoretically distinct forms of Ste4p that differ by their state of phosphorylation. We found that about 10% of Ste4p migrates more slowly during SDS electrophoretic analysis, although we have no direct evidence that this species is in fact phosphorylated. In our Renografin density gradient analysis (Fig. 1A), we found that nearly all of this slower-migrating form was associated with the non-membrane fractions (fractions 13–15). Moreover, it was also largely excluded from the particulate fraction in non-membrane fractions (fractions 13 and 14) mutant strain (Table II); thus, it is likely that the particulate fraction contains Ste4p that is not associated with membranes. Consistent with this interpretation, two-thirds of the Ste4p present in each fraction was evaluated by using densitometric analysis of the immunoblot in A. The dashed line indicates the densities (g/ml) of the fractions. C, shown is the distribution of protein markers for the cytosol (Pgk1p, endoplasmic reticulum (Ost1p, vacuole (Vph1p, plasma membrane (Pma1p, α; and Ste2p, β). The Golgi-specific guanosine diphosphatase (Gda1p) cofractionated with Ost1p and Vph1p (not shown). The Ste4p was contained in three different regions of the gradient (data not shown). The fractions from the Renografin gradient depicted in Fig. 1A were assayed for the presence of Ste18p (Fig. 2A). In order to distinguish Ste18p from other cross-reacting proteins, we included control strains that either contained a deletion of the STE18 gene (Fig. 2B) or contained an over-producing plasmid (Fig. 2C). Like Ste4p, a portion of Ste18p was found to be tightly associated with plasma membranes (fractions 7–10) in addition to its association with internal membranes (fractions 2–6) and to the non-membrane fractions (fractions 13 and 14). We also examined Renografin gradients for Gpa1p; it was present in both internal membrane and plasma membrane fractions (not shown). We could not determine whether Gpa1p was present in the particulate fraction (85%) at comparable ionic strength (Table II); thus, it is likely that the particulate fraction contains Ste4p that is not associated with membranes. Consistent with this interpretation, two-thirds of the Ste4p present in each fraction was evaluated by using densitometric analysis of the immunoblot in A. The dashed line indicates the densities (g/ml) of the fractions. C, shown is the distribution of protein markers for the cytosol (Pgk1p, endoplasmic reticulum (Ost1p, vacuole (Vph1p, plasma membrane (Pma1p, α; and Ste2p, β). The Golgi-specific guanosine diphosphatase (Gda1p) cofractionated with Ost1p and Vph1p (not shown). The Ste4p was contained in three different regions of the gradient (data not shown). The fractions from the Renografin gradient depicted in Fig. 1A were assayed for the presence of Ste18p (Fig. 2A). In order to distinguish Ste18p from other cross-reacting proteins, we included control strains that either contained a deletion of the STE18 gene (Fig. 2B) or contained an over-producing plasmid (Fig. 2C). Like Ste4p, a portion of Ste18p was found to be tightly associated with plasma membranes (fractions 7–10) in addition to its association with internal membranes (fractions 2–6) and to the non-membrane fractions (fractions 13 and 14). We also examined Renografin gradients for Gpa1p; it was present in both internal membrane and plasma membrane fractions (not shown). We could not determine whether Gpa1p was
in the non-membrane fraction due to the presence of cross-reacting proteins.

**Solubilized Plasma Membranes Contain a Complex of Ste4p and Ste18p**—To obtain biochemical evidence for a Ste4p-Ste18p complex, we extracted these proteins from purified plasma membranes with detergents and then examined their sedimentation properties under two different solvent conditions. To maximize detection of the proteins in this experiment, we used a diploid strain containing plasmids that express strong promoters. Renografin gradient analysis of Ste4p and Ste18p from this strain showed a similar profile as observed for the wild-type haploid strain (see Figs. 1 and 2). Plasma membranes were purified by using Renografin gradient centrifugation and then solubilized in buffer containing deoxy-β-d-maltoside and cholesterol hemisuccinate in the presence and in the absence of 250 mM NaCl. The detergent-extracted proteins were resolved on 8–30% glycerol gradients containing 250 mM NaCl (A) or lacking NaCl (B). Immunoblotting methods were used to assay the fractions for Ste4p (C), Ste18p (D), and Gpa1p (E). The pellet fractions (not shown) contained 13% of the total Ste4p, 45% of the total Ste18p, and 88% of the total Gpa1p in A, and 62% of the total Ste4p, 86% of the total Ste18p, and 75% of the total Gpa1p in B. Sedimentation markers were bovine serum albumin (4.3 S, small-dashed line), mouse gamma globulin (7 S, dotted line), and E. coli ATCase (11.7 S, large-dashed line).

**FIG. 3.** Sedimentation profile of Ste4p, Ste18p, and Gpa1p in glycerol gradients. Proteins were extracted from purified plasma membranes with detergent and resolved on 8–30% glycerol gradients containing 250 mM NaCl (A) or lacking NaCl (B). Immuno blotting methods were used to assay the fractions for Ste4p (C), Ste18p (D), and Gpa1p (E). The pellet fractions (not shown) contained 13% of the total Ste4p, 45% of the total Ste18p, and 88% of the total Gpa1p in A, and 62% of the total Ste4p, 86% of the total Ste18p, and 75% of the total Gpa1p in B. Sedimentation markers were bovine serum albumin (4.3 S, small-dashed line), mouse gamma globulin (7 S, dotted line), and E. coli ATCase (11.7 S, large-dashed line).

...despite the large difference in their molecular masses (51 and 15 kDa, respectively (5)). When compared with marker proteins, the apparent sedimentation coefficient was 6 S at 250 mM NaCl (Fig. 3A) and 8 S under low salt conditions (Fig. 3B). The complexes depicted in Fig. 3 did not appear to be a consequence of protein overproduction or expression in diploid cells since identical sedimentation coefficients under both low and high salt conditions were obtained for the Ste4p that had been extracted from the plasma membranes of wild-type haploid cells (not shown); however, in these experiments, the concentration Ste18p was below the limit of detection. It is presently unclear whether the larger complex (Fig. 3B) results from dimerization of Gβγ, from increased association of detergent, or from the binding of additional protein factor(s). Although the Gpa1p peak overlapped with Ste4p and Ste18p in 250 mM NaCl (Fig. 3A), the sedimentation coefficient of Gpa1p was not influenced by salt (Fig. 3B). Hence, under the conditions of our assay, we could detect no association of the Gα subunit homolog, Gpa1p, with the Gβγ complex.

To verify that the Ste4p and Ste18p on the glycerol gradient (Fig. 3A) were contained in the same complex, we tested for the ability of anti-Ste4p antiserum to precipitate Ste18p. As shown in Fig. 4, Ste18p was precipitated specifically with either anti-Ste4p (compare lanes 1 and 2) or with anti-Ste18p antiserum (compare lanes 3 and 4). Ste4p was not analyzed as it comigrated with immunoglobulin heavy chains on SDS-PAGE. Sera used in the immunoprecipitation methods. Ste4p was not analyzed as it comigrated with immunoglobulin heavy chains on SDS-PAGE. Sera used in the immunoprecipitation methods. Ste4p was not analyzed as it comigrated with immunoglobulin heavy chains on SDS-PAGE.

**FIG. 4.** Coprecipitation of Ste4p and Ste18p. Pooled fractions 10, 11, and 12 from the glycerol gradient depicted in Fig. 3A were analyzed by immunoprecipitation. Ste18p in supernatant fractions (S, upper panel) and pellet fractions (P, lower panel) was detected by using immunoblotting methods. Ste4p was not analyzed as it comigrates with immunoglobulin heavy chains on SDS-PAGE. Sera used in the immunoprecipitation reactions were anti-Ste4p (lane 1), preimmune (lane 2), anti-Ste18 (lane 3), and preimmune (lane 4). One control reaction contained no serum (lane 5), and the other contained neither serum nor protein A-agarose beads (lane 6).

...and to a reduced rate of synthesis, we examined the rate at which Ste4p disappeared from cultures that had been blocked for protein synthesis. Cycloheximide was added to growing cultures of STE× and stel8 strains, and samples were assayed for the amount of Ste4p that remained in the culture over a 3-h time course (Fig. 5). The kinetics of Ste4p decay in the stel8 mutant were consistent with a first-order reaction and a half-life of 90 min; whereas in the wild-type control, the level of Ste4p (Fig. 5) and Ste18p (not shown) remained undiminished for the duration of the time course. The rate of decay in the stel8 mutant is expected to produce a 2-fold reduction in the steady-state level of Ste4p in growing cultures since Ste4p is also diluted as a result of cell division (90 min doubling time). Ste18p was barely detectable in the ste4 mutant, consistent with rapid decay (Table III). These results suggest that Ste4p and Ste18p form a stable protein complex in vivo.

**Mutations in Ste18p and Gpa1p Affect Localization of Ste4p**

...activity of anti-Ste4p antiserum to precipitate Ste18p. As shown in Fig. 4, Ste18p was precipitated specifically with either anti-Ste4p (compare lanes 1 and 2) or with anti-Ste18p antiserum (compare lanes 3 and 4). Ste4p was not analyzed as it comigrated with immunoglobulin heavy chains on SDS-PAGE. Sera used in the immunoprecipitation methods. Ste4p was not analyzed as it comigrated with immunoglobulin heavy chains on SDS-PAGE. Sera used in the immunoprecipitation methods. Ste4p was not analyzed as it comigrated with immunoglobulin heavy chains on SDS-PAGE. Sera used in the immunoprecipitation methods. Ste4p was not analyzed as it comigrated with immunoglobulin heavy chains on SDS-PAGE.

...acylase crude extract from the strain depicted in Fig. 3A and B. The collected fractions were analyzed by immunoblotting methods. Ste4p and Ste18p were precipitated specifically with anti-Ste4p (lanes 1 and 2) or with anti-Ste18 antiserum (lanes 3 and 4). Ste4p was not analyzed as it comigrated with immunoglobulin heavy chains on SDS-PAGE. Sera used in the immunoprecipitation methods. Ste4p was not analyzed as it comigrated with immunoglobulin heavy chains on SDS-PAGE.
TABLE III

| Strain         | Mutation       | Amount of protein (\% wild type) |
|----------------|----------------|---------------------------------|
|                |                | ste4p | ste18p |
| DJ1006-17-2    | ste18::LEU2    | 50    | 5   |
| DJ1004-11-1    | ste4::LEU2     | 120   | 110  |
| DJ803-2-1      | gpa1::LEU2     | 100   | 115  |
| DJ240-4-1      | ste2-10::LEU2  | 120   | 110  |

* Strain DJ002-15-1 served as the wild-type control for strains DJ1006-17-2 and DJ1004-11-1. Strain DJ803-11-1 was the control for DJ240-4-1. ** Background value from the ste18 mutant was subtracted.

###FIG. 5. Turnover of Ste4p in the ste18 mutant. Cultures of the wild-type strain (ΔJ002-15-1, ○) and the ste18 mutant (ΔJ1006-17-2, △) received cycloheximide at time 0. Samples were withdrawn at the times indicated, and Ste4p remaining in the cleared lysates was determined.

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**Yeast Gβγ Complex**

Steady-state levels of Ste4p and Ste18p in mutants

Cleared lysates of wild-type and mutant strains were assayed for Ste4p and Ste18p by using immunoblotting methods. Linearity was evaluated by analyzing dilutions of the wild type control.

| Strain         | Mutation       | Amount of protein (\% wild type) |
|----------------|----------------|---------------------------------|
|                |                | ste4p | ste18p |
| DJ1006-17-2    | ste18::LEU2    | 50    | 5   |
| DJ1004-11-1    | ste4::LEU2     | 120   | 110  |
| DJ803-2-1      | gpa1::LEU2     | 100   | 115  |
| DJ240-4-1      | ste2-10::LEU2  | 120   | 110  |

* Strain DJ002-15-1 served as the wild-type control for strains DJ1006-17-2 and DJ1004-11-1. Strain DJ803-11-1 was the control for DJ240-4-1. ** Background value from the ste18 mutant was subtracted.

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**to the Plasma Membrane**—As a second criterion for the presence of specific protein complexes in vivo, we examined whether Gβ requires the receptor or the other G protein subunits for its tight association with plasma membranes. Lysates from ste2, ste18, and gpa1 mutant cells were subjected to Renografin gradient analysis, and the fractions were assayed for Ste4p. In the ste18 mutant, greater than 90% of Ste4p was found in non-membrane fractions (Fig. 6A), indicating that the Gγ homolog is required for stable association of Gβ with both the plasma membrane and the internal membranes. In the gpa1 mutant, Ste4p was present only in internal membrane and non-membrane fractions (Fig. 6B), suggesting that Gα is required for stable accumulation of Gβ at the plasma membrane. In contrast to ste18 and gpa1, the ste2 mutation did not significantly affect distribution of Ste4p (Fig. 6C), indicating that the receptor is not required for Gβ to accumulate at either membrane location.

**Effect of Ste18p on the Aggregation State of Ste4p**—As an additional test for interaction between Ste4p and Ste18p, we examined the sedimentation properties of Ste4p in the ste18 mutant. When lysates of the ste18 mutant were analyzed as described in Table II, 84% of the Ste4p protein was found in the particulate fraction, and 65% of this particulate fraction was solubilized by extracting it with a combination of detergent and 0.25 M NaCl. When the solubilized extract was analyzed by using glycerol gradient centrifugation (as described in Fig. 3A), we found a value of 4 S for the sedimentation coefficient of Ste4p from the ste18 mutant (consistent with a 54-kDa Ste4p monomer), whereas we obtained a value of 8 S for the STE18+ control strain under these conditions. Thus, Ste18p is necessary for assembly of Ste4p into a more rapidly sedimenting complex. This complex is likely to contain additional protein components since Ste18p (15 kDa) alone is too small to have an appreciable influence on sedimentation of Ste4p. The observation that the sedimentation coefficient for the wild-type control is larger than the value obtained for purified plasma membranes from diploid cells (Fig. 3A) or from haploid cells (not shown) implies that protein factors associated with Gβγ are removed during plasma membrane purification.

**DISCUSSION**

Consistent with the proposed function of Ste4p and Ste18p in the pheromone response pathway, we have found that these Gβ and Gγ homologs form a complex associated with the plasma membrane. First, approximately 40% of Ste4p and Ste18p fractionated with the plasma membrane ATPase in Renografin buoyant density gradients, and association of Ste4p with plasma membranes was dependent upon functional Ste18p. Second, the Ste4p and Ste18p that had been extracted from plasma membranes were found to co-sediment under two different solvent conditions, and the Ste18p in these fractions was precipitated in the presence of anti-Ste4p antisera. Finally, Ste4p and Ste18p were interdependent for their stability in vivo. Levels of Ste18p in ste4 mutant cells were barely detectable; in ste18 mutant cells, Ste4p was reduced in amount and exhibited a more rapid turnover rate. These results represent the first direct evidence for a Gβγ complex at the plasma membrane. Other biochemical evidence supporting a direct interaction between Ste4p and Ste18p was provided by Song et al. (2) who found that Ste4p and Ste18p coprecipitate with Gpa1p in a guanine nucleotide-dependent manner. Other indirect evidence was provided in a G protein-coupling assay, that is, the ability of guanine nucleotides to influence α-factor binding is disrupted in both ste18 and STE4/STE18 mutants (2).

Under high salt conditions, the protein complex containing Ste4p and Ste18p sedimented more rapidly than predicted for a simple complex of these two proteins, and this discrepancy was greater under low salt conditions. An increase in the sedimentation rate can be explained by the binding of additional molecules of protein and/or detergent, whereas deviations in shape or hydration would both tend to decrease the rate. It is therefore likely that more protein or detergent binds under low salt conditions than under high salt conditions. Although we have not determined the relative contributions of protein and
detergent to the sedimentation behavior, there are a number of proteins predicted to interact with Ste4p. Candidates include Gpa1p, the pheromone receptor, Ste5p (21, 22), Ste20p (22–25), Cdc24p (26), Akr1p (28, 29), and Syg1p (27). Ste5p and the receptor were not expressed in the diploid cells analyzed in Fig. 3. Although Gpa1p was expressed in these cells, its sedimentation rate was not influenced by salt; hence, under the conditions of our assay, Gpa1p does not appear to bind the Gβγ complex. Ste20p, Cdc24p, Akr1p, and Syg1p are expressed in both haploid and diploid cells; hence, they are potentially components of the protein complex that contains Ste4p and Ste18p (Fig. 3). Ste20p encodes a protein kinase (52); analyses of double mutant strains suggest that Ste20p acts in the pheromone response pathway at a step that is executed after Ste4p (23, 24) and before Ste5p (25). Cdc24p is required for budding and for generating cell polarity (53); recent evidence also suggests that it is involved in pheromone signaling (26, 54). The ankyrin repeat-containing protein Akr1p is required for normal bud and projection formation and appears to have a negative effect on signaling (28, 29). Interestingly, in the two-hybrid system, Akr1p shows an interaction with free Gβγ but not with the Gaβγ heterotrimer (28). Consistent with this requirement for binding, we do not detect Gpa1p in the complexes containing Ste4p and Ste18p. Finally, the truncated form of a putative transmembrane protein, designated Syg1p, has been shown to interact with Ste4p by using the two-hybrid system, and it has also been shown to suppress the lethality of a gpa1 deletion (27). Syg1p is proposed to be a transmembrane signaling component that can respond to or transduce signals through Gβγ.

In mammalians, Gβγ subunits apparently have a broad range of functions as indicated by the variety of proteins with which they interact. In addition to interacting with Ga, Gβγ subunits appear to bind receptors (55) and several effector proteins including phospholipase Cβ and certain isozymes of adenylyl cyclase (reviewed in Ref. 56). In other examples, Gβγ complexes are known to promote attenuation of signaling by binding the β-adrenergic receptor kinase (57) and by binding phosducin (58). Pumiglia et al. (59) used the two-hybrid system to show that the protein kinase, Raf-1, binds the Gβγ subunit, thereby implicating Gβγ in regulation of the mitogen-activated protein kinase pathway. The small GTPase ARF, a component required for transport of proteins among Golgi compartments, has also been shown to bind Gβγ (60), supporting the notion that heterotrimeric G proteins are involved in the control of vesicular protein traffic.

As judged from our Renografin gradient analysis, a substantial portion of Gβγ was not tightly associated with plasma membranes. Approximately 30% of Ste4p and Ste18p was associated with internal membrane fractions (containing membranes of the endoplasmic reticulum, Golgi, and vacuole), while the remaining 30% was not tightly bound to membranes. The biological significance of this fractionation pattern is as yet unclear. Association with different cellular compartments may reflect independent pools of Ste4p and Ste18p or it may reflect the presence of intermediates in the assembly, turnover, or activation of Gβγ. Some, or all, of the Ste4p that was confined to the non-membrane fractions on Renografin gradients may be associated with membranes in vivo. Roughly 25% of Ste4p and Ste18p was released from the particulate fraction in the pres-

**Fig. 6.** Membrane fractionation and localization of Ste4p in ste18, gpa1, and ste2 mutants. Cultures were subjected to Renografin gradient analysis as depicted in Fig. 1. A, ste18 mutant (DJ1006-17-2, □) and wild-type control (DJ602-15-1, ■). Both strains contained the ste5-3 mutation and were cultured at 34 °C; consequently, the gpa1 mutation did not result in constitutive arrest of the cell division. C, ste2 mutant cells (DJ240-4-1, □) and wild-type control (DJ147-1-2, ■). Amount of Ste4 protein (solid lines) is the percentage of Ste4p present in each fraction; for the ste18 mutant (A), this value was normalized to reflect the reduced level of Ste4p described in Table III. The plasma membrane marker, Pma1p, is indicated by the dashed line for each mutant strain and by the dotted line for each wild-type control.
Yeast Gβγ Complex

Fig. 7. Ste4p remains at the plasma membrane after endocytosis of the receptor. Identical exponential cultures (strain DJ211-5-3) that had been treated with cycloheximide (10 μg/ml) for 5 min received 10^{-6} M α-factor (B) or remained untreated (A) for an additional 20 min. Membranes were resolved on Renografin gradients as in Fig. 1. Percentage of Ste4p (●) and Ste2p (○) in each fraction was determined by using immunoblotting methods.

ence of 1.0 M NaCl (Table II); thus, the Gβγ that was weakly associated with membranes may be released in the presence of 38% Renografin (ionic strength roughly equivalent to 0.5 M NaCl). This material appears, at least in part, to represent a chemically distinct form of Gβγ since a Ste4p species that migrated more slowly on SDS-polyacrylamide gels was primarily limited to the non-membrane fractions of the Renografin gradient (Fig. 1) and since it was extracted from the particulate fraction with 1.0 M NaCl (not shown). Interestingly, the γ subunit of transducin (Gγ) is modified with the C_{15} farnesyl group (61), as is Ste18p, and transducin does not require detergent for extraction from the membrane (62). In contrast, the γ subunit of Gα, Gγ, and Gε contains the C_{20} geranylergeranyl lipid, and these G proteins require detergent for extraction (63, 64).

Cole and Reed (49) found that phosphorylated forms of Ste4p migrate more slowly on SDS-polyacrylamide gels and that the abundance of these species increases upon exposure to α-factor. Furthermore, these modifications are likely to regulate the Ste4p activity since ste4 mutants that block phosphorylation become hypersensitive to pheromones. In our experiments, when a cell cultures were treated with α-factor and the membranes resolved on Renografin gradients (not shown), Ste4p was converted to slower-migrating species in plasma membrane, internal membrane, and non-membrane fractions. Assuming that these species represent phosphorylated forms, then the phosphorylated Ste4p that was tightly associated with membranes may represent a transient intermediate that subsequently is either dephosphorylated, degraded, or reduced in its affinity for membranes. Two models are consistent with the presence of phosphorylated forms of Ste4p in both membrane fractions. Protein kinases that become activated upon α-factor binding may modify Ste4p molecules both at the plasma membrane and at internal membrane sites; alternatively, Ste4p may be modified at one site and then transported to the other site.

While loss of the receptor did not affect localization of Ste4p, loss of Gpa1p prevented stable association of Ste4p with the plasma membrane. Gpa1p did not influence association of Ste4p with internal membranes. These results suggest that Gα exerts a physical effect on Gβγ in vivo; Gpa1p may help to anchor Gβγ to the plasma membrane. Lipid modifications of Gα and Gγ are thought to mediate (at least in part) the membrane attachment of G proteins (see Ref. 37). It has been proposed (37) that the Gα subunit facilitates binding of Gβγ to membranes by supplementing the low binding energy provided by the Gγ isoprenyl group. Ste18p appears to be farnesylated since its sequence contains a consensus site for farnesylation and since mutants affecting that site or the farnesyl transferase enzyme affect signal transduction (15, 20, 65, 66). Gpa1p is both myristoylated (14) and palmitoylated.5

In a recent related paper focusing on N-myristoylation of Gpa1p, Song et al. (51) report on a number of observations that pertain to subcellular localization of Gβγ. Some of their results differ from ours and provide possible insights for properties of Gβγ and limitations of the two approaches. Using sucrose gradient fractionation, Song et al. (51) could detect Ste4p only in fractions containing plasma membranes. Lack of Ste4p in the non-membrane fractions is likely to reflect the lower salt conditions used in their assay. In Table II, we found that a portion of Ste4p was associated with the particulate fraction only under low salt conditions. Perhaps, this species is associated weakly with the plasma membrane and suggests the presence of at least two populations of Gβγ species at this location. The inability of Song et al. (51) to detect Ste4p in other membrane fractions may have resulted from strain differences, from a failure to resolve these membrane species from the plasma membrane, or from degradation of Ste4p during spheroplast formation. In gpa1 mutants, Song et al. (51) found that only a portion of the Ste4p was resolved from plasma membrane-containing fractions, whereas in the present study, we found that essentially all of the tightly associated Ste4p was resolved from the plasma membrane. These results may reflect either the presence of a Ste4p species that is weakly associated with plasma membranes or insufficient resolution of the sucrose gradient technique. When we examined the sedimentation behavior of detergent-solubilized Gβγ on glycerol gradients, we found no evidence for association with Gpa1. In contrast, Song et al. (51) found that at least a portion of Ste4p and Ste18p from whole cell extracts coprecipitates with the Gpa1p that had been

5 C. Manahan and M. Linder, personal communication; J. Song and H. Dehlmam, personal communication.
tagged with glutathione S-transferase. The apparent absence of such a complex in our study may reflect the differences in the assay conditions or the fact that we limited our analysis to purified plasma membranes.

Although a number of proteins have been proposed to interact with Ste4p, the mechanism by which Gβγ activates subsequent events in the pheromone response pathway remains unknown. The activity of Gβγ is thought to be stimulated upon its dissociation from Go, that is when occupied receptors stimulate guanine nucleotide exchange or when Go is inactivated by a mutation in the GPA1 gene. When cells were treated with a saturating concentration of α-factor for 20 min, we found that cellular distribution of Ste4p was not altered discernibly. As this is sufficient time to provide maximal response to α-factor, it appears unlikely that activation of the response pathway requires movement of Gβγ from the plasma membrane to another cellular compartment or that removal of Gβγ from the plasma membrane regulates its activity. However, we cannot rule out the possibility that the exit of a small quantity of Gβγ in response to α-factor is sufficient for maximal activation. In contrast, gpa1 mutant cells failed to accumulate Gβγ that was tightly bound to the plasma membrane, thus cells lack Gpa1p are not in the same physiological state as wild-type cells treated with pheromone. Ga may retain some contacts with Gβγ during α-factor stimulation; Ga may facilitate the assembly of Gβγ into a structure that remains tightly bound to the plasma membrane when activated by α-factor, or chronic stimulation of Gβγ in the gpa1 mutant may disrupt its stable contacts with the plasma membrane. Even though Gβγ is no longer tightly associated with the plasma membrane in the gpa1 mutant, the requisite events for signal transduction may be mediated by Gβγ molecules that are only weakly associated with the plasma membrane.

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