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Genome-Scale Analysis Reveals Sst2 as the Principal Regulator of Mating Pheromone Signaling in the Yeast *Saccharomyces cerevisiae*†

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A common property of G protein-coupled receptors is that they become less responsive with prolonged stimulation. Regulators of G protein signaling (RGS proteins) are well known to accelerate G protein GTPase activity and do so by stabilizing the transition state conformation of the G protein α subunit. In the yeast *Saccharomyces cerevisiae* there are four RGS-homologous proteins (Sst2, Rgs2, Rax1, and Mdm1) and two Gα proteins (Gpa1 and Gpa2). We show that Sst2 is the only RGS protein that binds selectively to the transition state conformation of Gpa1. The other RGS proteins also bind Gpa1 and modulate pheromone signaling, but to a lesser extent and in a manner clearly distinct from Sst2. To identify other candidate pathway regulators, we compared pheromone responses in 4,349 gene deletion mutants representing nearly all nonessential genes in yeast. A number of mutants produced an increase (*sst2*, *bar1*, *asc1*, and *ygl024w*) or decrease (*cla4*) in pheromone sensitivity or resulted in pheromone-independent signaling (*sst2*, *pbs2*, *gas1*, and *ygl024w*). These findings suggest that Sst2 is the principal regulator of Gpa1-mediated signaling in vivo but that other proteins also contribute in distinct ways to pathway regulation.

G protein-coupled receptors respond to a vast array of chemical and sensory signals, including hormones, neurotransmitters, odors, and light. Approximately one-third of all drugs act by binding directly to receptors of this class (64). Upon agonist stimulation of the receptor, a cognate G protein α subunit will exchange GDP for GTP and undergo dissociation from the G protein βγ subunit dimer. The dissociated subunits bind to effector enzymes, which in turn activate protein kinases, trigger new gene transcription, and ultimately produce programmed changes in cell homeostasis or differentiation (90). Regulators of G protein signaling (RGS proteins) function as GTPase-accelerating proteins (GAPs) and, in this manner, promote rapid inactivation or desensitization of the signal (89).

Whereas mammalian genome analysis has revealed at least 16 Gα- and ~40 RGS-encoding genes (89, 106), a similar analysis in the yeast *Saccharomyces cerevisiae* reveals only two Gα subunits but at least four RGS protein homologues. Gpa1 mediates cellular responses to mating pheromones. These pheromones, called **a**-factor and α-factor, are produced by haploid **a** and α cells and bind to G protein-coupled receptors on cells of the opposite mating type. Upon activation of pheromone receptors, Gpa1 binds to GTP and dissociates from the Gβγ dimer Ste4/Ste18, and the dissociated subunits activate a multitude of downstream effectors leading to cell fusion (mating) to form an **a**/α diploid (36, 50). Prominent among the known effectors are components of a MAP (mitogen-activated protein) kinase cascade comprised of Ste20, Ste11, Ste7, and Fus3. A parallel signaling pathway responds to glucose stimulation, leading to activation of a distinct receptor (Gpr1) (66, 73, 76, 99, 124), a distinct G protein α subunit (Gpa2), and an atypical G protein βγ complex comprised of Gpb1 or Gpb2 and Gpg1 (4, 54).

Among the RGS proteins in yeast, Sst2 is by far the best characterized. The gene was originally identified through a screen for negative regulators of the pheromone response (15, 16). Subsequent analyses revealed that Sst2 interacts genetically (33, 37) and physically (39) with Gpa1 and can accelerate Gpa1 GTPase activity (2, 133). A second yeast RGS protein, Rgs2, was identified as a multicopy suppressor of Gpa2-dependent loss of heat shock resistance in stationary-phase cells and was also shown to accelerate Gpa2 GTP binding and hydrolysis (123).

Two additional RGS protein homologues in yeast have not been implicated previously in G protein signaling events (Fig. 1). *RAX1* (*r*evert to *ax*ial) was identified as a suppressor of *axl1*/*ste22* mutations (46). Axl1 is a haploid-specific endoprotease required for maturation of **a**-factor mating pheromone and for the normal axial budding pattern of haploid cells (23, 26, 75, 91). Conversely, diploid *rax1*/*rax1* mutants exhibit a random or axial-like budding pattern normally found only in haploid cells (46, 91). These findings suggest a role for Rax1 in the establishment and maintenance of cell polarity. A fourth RGS protein is Mdm1. This is the least conserved member of the RGS family in yeast, but the one most similar to human RGS-PX1 (also known as SNX13) (137). Both Mdm1 and RGS-PX1 have a Phox (PX) domain (137), which binds to SH3 domains and phosphoinositides and contributes to membrane localization in vivo (20, 58, 134). Overexpression of human

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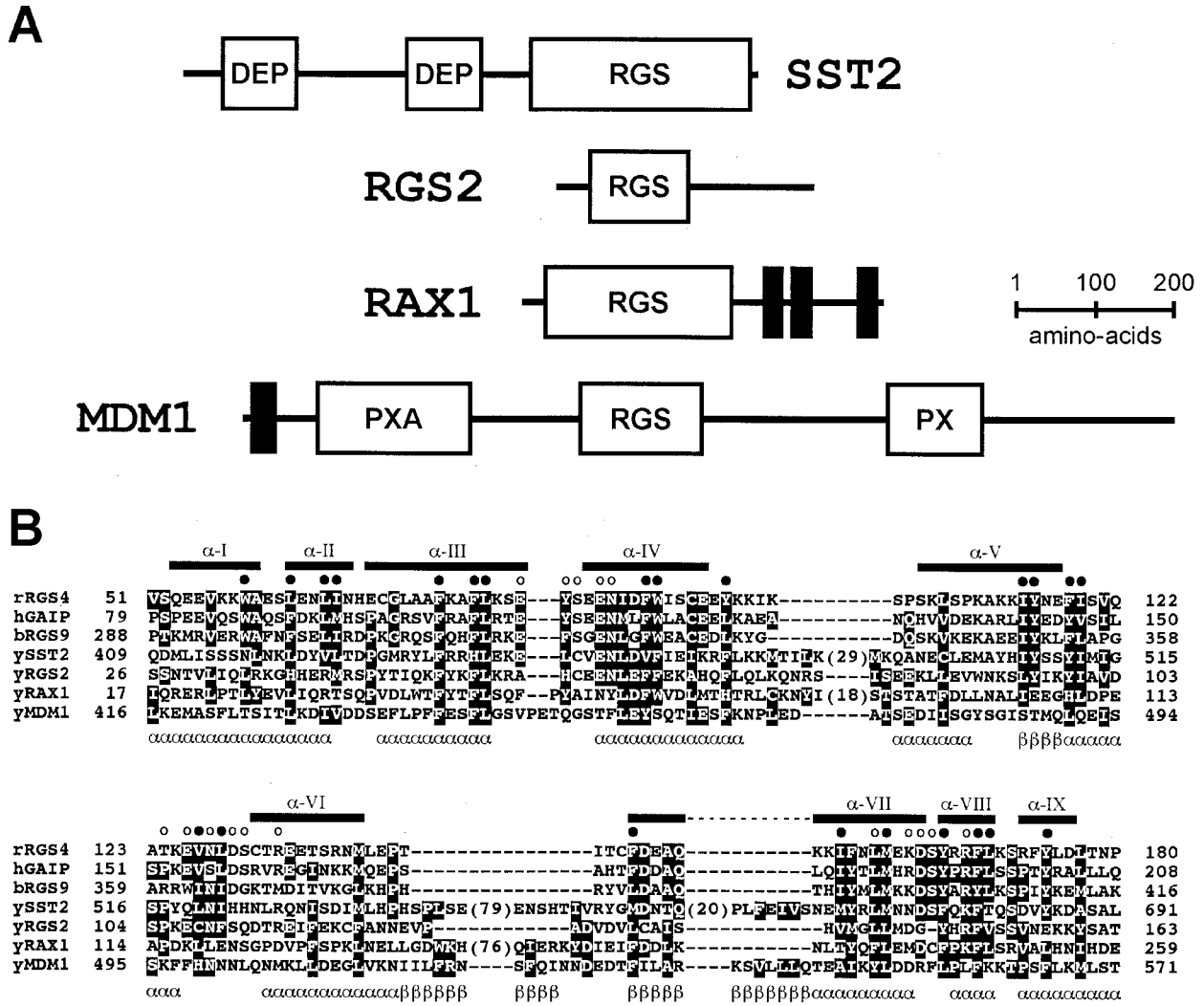

FIG. 1. Architecture of the four RGS proteins in yeast. (A) Schematic of the multiple domains of Sst2, Rgs2, Rax1, and Mdm1. DEP, Dishevelled/EGL-10/pleckstrin homology domain; RGS, regulator of G-protein signaling domain; PXA, PX-associated domain; PX, $p40^{phox}$ and $p47^{phox}$ homology domain. Putative transmembrane regions are denoted by solid vertical bars. (B) Multiple sequence alignment of the RGS box regions of rat (r) RGS4, human (h) GAIP/RGS19, bovine (b) RGS9, and the four yeast (y) RGS box proteins. Boxed residues are conserved amino acids identified using Clustal-X (120). Numbers in parentheses within Sst2 and Rax1 protein sequences denote the number of residues not shown in each insert region. The nine α-helices observed within the nuclear magnetic resonance solution structure of human GAIP are numbered in roman numerals and marked with horizontal bars above the sequences (29). Closed circles (●) denote conserved residues forming the RGS box hydrophobic core. Open circles (○) highlight conserved residues making direct contacts with Gα in the RGS4/Gαi1 crystal structure (119). Predicted α-helical (α) and β-strand (β) secondary structure within the Mdm1 RGS-box, based on the PSI-Pred algorithm (81), is denoted underneath the Mdm1 sequence. Primary sequences in the alignment are derived from rat RGS4 (SwissProt accession no. P49799), human GAIP/RGS19 (SwissProt accession no. P49795), bovine RGS9 (SwissProt accession no. O46469), yeast Sst2 (SwissProt accession no. P11972), yeast Rgs2 (GenBank accession no. NP_014750), yeast Rax1 (GenBank accession no. NP_014945), and yeast Mdm1 (GenBank accession no. NP_013603).

RGS-PX1 inhibits transport of epidermal growth factor receptors from endosomes to lysosomes, thereby enhancing the growth factor receptor signal (137). Yeast Mdm1 is expressed predominantly in late G_1 to early S phase of the cell cycle and appears to be required for proper nuclear and mitochondrial inheritance in cells grown at elevated temperatures (45, 80).

While the GAP function of RGS proteins is well established, not all RGS proteins exhibit this activity. Two prominent examples are Axin and the G protein-coupled receptor kinase GRK2 (13, 101, 113). Even when GAP activity has been documented, the physiological function of most RGS protein family members remains poorly understood. Moreover, there is growing evidence in mammalian cells that RGS proteins regulate specific Gα subunits in vivo, even when such specificity is absent in vitro. For example, RGS4 and Gα-interacting protein (GAIP) behave similarly towards $G_i\alpha$ and $G_o\alpha$ in vitro (7) yet have dissimilar effects in cultured neuronal cells (35). Likewise, in yeast it is not known if more than one RGS protein specifically regulates Gpa1 (or Gpa2) signaling. The promiscuity of RGS-Gα coupling observed in vitro highlights the need to analyze the specificity of RGS-Gα interactions in vivo, as well as in vitro.

Here we present a comprehensive analysis of G protein signal regulation in yeast. Through systematic overexpression and disruption of all four RGS genes, we establish the ability of each to regulate the well-characterized Gpa1 signaling pathway. We show that all four RGS proteins regulate signaling to some extent, but by distinct mechanisms, and with Sst2 as by far the most important contributor. To determine whether additional factors regulate Gpa1 signaling, we present results of pheromone dose-response profiles for nearly all viable gene deletion mutants in yeast. This analysis reveals several additional components that regulate the pheromone signaling pathway, either by restricting basal activity or by pheromone-induced activation of the G protein.

MATERIALS AND METHODS

Strains. Standard methods for the growth, maintenance, and transformation of yeast and bacteria, the preparation of growth medium, and the manipulation of DNA were used throughout (3). The yeast *Saccharomyces cerevisiae* strains used in this study were DC17 (*MAT*α *his1*) (from J. Thorner, University of California—Berkeley), BJ2168 (*MAT***a** *ura3-52 leu2-*Δ*1 trp1-*Δ*63 prb1-1,122 prc1-407 pep4-3*) (from E. Jones, Carnegie Mellon University), YPH499 (*MAT***a** *ura3-52 lys2-801*^{amber} *ade2-101*^{ochre} *trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1*), BY4741 (*MAT***a** *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ), and commercially available gene deletion strains in BY4741 (Research Genetics, Huntsville, AL).

Multiple RGS deletion strains were made in the BY4741 background and designated BY4741-sst2/rgs2 (*sst2*Δ::G418^R *rgs2*Δ::hisG), BY4741-sst2/rax1 (*sst2*Δ::G418^R *rax1*Δ::hisG), BY4741-sst2/mdm1 (*sst2*Δ::G418^R *mdm1*Δ::hisG), BY4741-rgs2/rax1 (*rgs2*Δ::G418^R *rax1*Δ::hisG), BY4741-rgs2/mdm1 (*rgs2*Δ::G418^R *mdm1*Δ::hisG), BY4741-rax1/mdm1 (*rax1*Δ::G418^R *mdm1*Δ::hisG), and BY4741-sst2/rgs2/rax1/mdm1 (*sst2*Δ::G418^R *rgs2*Δ::hisG *rax1*Δ::hisG *mdm1*Δ::hisG). Multiple gene disruptions were constructed by replacing a portion of the indicated gene with the hisG-*URA3*-hisG cassette from pUC19-hisG-URA3, containing the hisG-*URA3*-hisG BamHI-BglII digestion product from pNK51 (1) ligated into the BamHI site of pUC19. To this end, *RGS2* was PCR amplified using primers 5′-CTGTC ATTTG CCTTA CTGTT-3′ and 5′-TGAGC CACAG ACATT GGGTC-3′, cloned into pYES2.1/V5 TOPO (Invitrogen), digested with SacI, blunt-ended with T4 polymerase, and digested with EcoRI. The deleted portion of the gene was replaced with hisG-*URA3*-hisG digested in the same manner. *RAX1* was PCR amplified using primers 5′-CGAAT GGGAG TTATT TGGCC-3′ and 5′-CGGTA GGAAG GATCA GTCAT-3′, digested with XhoI (blunt ended) and SacI, and ligated with hisG-*URA3*-hisG digested with SalI (blunt ended) and AscII. *MDM1* was PCR amplified using primers 5′-CCACT ATCTG TTGCA ATACA-3′ and 5′-GCTGC GCCAA TTAAT TGTCC-3′, digested with HindIII (blunt ended) and BamHI, and ligated to hisG-*URA3*-hisG digested with SalI (blunt ended) and BamHI. Cells transformed with the hisG-*URA3*-hisG mutants were grown in 5-fluoroorotic acid to select against *URA3*. The integrity of the mutants was confirmed by PCR amplification of the disrupted gene.

Constitutively active mutants (group VI) were introduced into YPH499 and YPH499-based sterile mutants YDK101 (*ste2*::*HIS3*, from J. Thorner, University of California), YTG4 (*ste4*::*hisG*) (47), JTY2556 (*ste7*::*ADE2*, from J. Thorner), and YDK12/JDY3 (*ste12*::*LEU2*) (28). Primers designed to amplify the G418^R selection gene module from genomic DNA derived from the deletion strains of interest were either purchased (GenePairs PCR primers; Research Genetics) (for *SST2* and *YGL024W*) or custom designed for *PBS2* (5′-GCAAA GGTCT AGATT TCTTG C-3′, 5′-GGTAA TTCTA GACTG TTTTC C-3′) and *GAS1* (5′-GAATC TTCCG AGCTC ACAAC C-3′, 5′-CCTAA CGTGA GCTCG TACAC G-3′). The amplification products were gel purified, transformed, and plated on yeast extract-peptone-dextrose medium containing 300 μg/ml G418. Proper integration of the gene deletion cassette was confirmed by PCR.

Plasmids. Previously described expression plasmids used in this study were pRS316-ADH (*CEN*, amp^R, *URA3*, *ADH1* promoter/terminator) (112), pAB27 (2μm, amp^R, *URA3*, *leu2-d*, *GAP1* promoter and terminator; from Anthony Brake, University of California—San Francisco), pAD4M (2μm, amp^R, *LEU2*, *ADH1* promoter/terminator; from Peter McCabe, Onyx Pharmaceutical), pAD4M-GPA1^{Q323L} (39), pAD4M-GPA1-GST, pAD4M-GST (112), and *HOG1* in pRS426 (from M. Gustin, Rice University) (11). pAD4M-GPA2-GST was constructed as described previously for pAD4M-GPA1-GST (112), using PCR primers 5′-GCG**GT CGAC**A TGGGT CTCTG CGCAT CTTCA-3′ and 5′-GCG**GA GCTC**T TGTAA CACTC CAGAG TCTTT-3′. The boldface print indicates added SalI or SacI restriction sites used to digest the product prior to ligation to the corresponding sites in pAD4M.

Overexpression of RGS proteins was achieved by subcloning each gene into the blunt-ended BglII site of pAB27. For construction of pAB27-RGS2 the *RGS2* gene was PCR amplified using primers 5′-CGC**GG ATCC**G CCAGA TGGCG AGTGT ACCAA GTCTA-3′ and 5′-CGC**GG ATCC**C TATCT TTGTT GATGA CTGTT-3′. For pAB27-RAX1 the gene was PCR amplified using primers 5′-CGC**GG ATCC**T CATCA TGAAG GAAGA GCTCA GCAA A-3′ and 5′-CGC**GG ATCCGAATTC**T CATAC ACGAC GGCCG GGAAC-3′. For pAB27-MDM1 the gene was PCR amplified using primers 5′-GACGC **GTCGA C**GCCT TCTGA TATGA TATCG T-3′ and 5′-GACGC **GTCGA C**TAGA TTGTT CGGTA CTTAG T-3′. The boldface print indicates added BamHI, EcoRI, or SalI sites, which were used to digest the product prior to blunt-ending and ligation to pAB27. Construction of a similar plasmid used to overexpress *SST2* (pAB23-SST2) was described previously (39).

Constitutive expression of RGS proteins was achieved by subcloning PCR amplification products into pRS316-ADH. For construction of pRS316-ADH-RGS2, *RGS2* was amplified using primers 5′-CCG**GT CGAC**G CCAGA TGGCG AGTGT ACCAA GTCA-3′ and 5′-CA**GGA TCC**CT ATCTT TGTTG ATGAC TGTT-3′. The boldface print indicates added SalI or BamHI sites, used to subclone the PCR product into the corresponding sites in pRS316-ADH. pRS316-ADH-RAX1 was constructed by PCR amplification of *RAX1* using the same primers as for pAB27-RAX1 and subcloned as a BamHI-EcoRI fragment. pRS316-ADH-MDM1 was constructed by PCR amplification of *MDM1* using the same primers as for pAB27-MDM1 and subcloned as a SalI fragment. Construction of pRS316-ADH-SST2 was described previously (61).

Constitutive expression of epitope-tagged RGS proteins was achieved by subcloning PCR amplification products containing the primer-encoded Myc epitope into pRS316-ADH. pRS316-ADH-RGS2-Myc was constructed by PCR amplification of *RGS2* using primers 5′-CCG**GT CGAC**G CCAGA TGGCG AGTGT ACCAA GTCA-3′ and 5′-CGC**GG ATCC**T TACCT CTTCC TGAGG AGGTC CTCTT CGCTG ATTAA TTTCT GCTCC TCGAG TCTTT GTTGA TGACT GTTTT GTCCT TTCAA-3′ and subcloned as a SalI-BamHI fragment into pRS316-ADH. pRS316-ADH-MDM1-Myc was constructed by PCR amplification of *MDM1* using primers 5′-ACGC**G TCGAC** CTTCT GATAT GATAT CGTAT G-3′ and 5′-GC**GAG CTC**TT ACCTC TTCCT GAGGA GGTCC TCTTC GCTGA TTAAT TTCTG CTCCT CGAGG TCATT ACATA TTATG TCCAA TAAAA TTGC-3′ and subcloned as a SalI-SacI fragment. pRS316-ADH-RAX1-Myc was constructed by amplification of *RAX1* using primers 5′-C GC**GG ATCC**T CATCA TGAAG GAAGA GCTCA GCAAA-3′ and 5′-C CG**GA ATTC**T TACCT CTTCC TGAGG AGGTC CTCTT CGCTG ATTAA TTTCT GCTCC TCGAG TACAC GACGG CCGGG AACAC AGCTG AAA A-3′ and subcloned as a BamHI-EcoRI fragment. pRS316-ADH-SST2-Myc was constructed by subcloning *SST2*-Myc as a BamHI digestion product from pBS-SST2-Myc (39) into pRS316-ADH. Added BamHI, SalI, EcoRI, or SacI sites are in boldface print, and the Myc epitope is underlined.

A slightly different strategy was used to clone non-RGS regulators. GenePairs PCR primers (Research Genetics) were used to PCR amplify group I, group II, and group III genes. Custom primers were used to amplify *YAL047c* (5′-GATCG AATTC ACCAC CATGG TACGT CGATG GATTC GTAG-3′ and 5′-G ATCC CCGGG AATTG CCATG TTAGG GATTG TTGAT TGAT-3′) and *YDR462w* (5′-GATCG AATTC ACCAC CATGC TGGCA CAAAC ATTCA AAAA-3′ and 5′-GATCC CCGGG AATTG CCATG TCATT TTCGG AAGTT ATAAT GCCAT AACG-3′). The resulting PCR products were subcloned directly into pYES2.1/V5 TOPO (Invitrogen). The integrity of *BAR1*, *SST2*, *YGL024W*, *ASC1*, *UBC4*, and *CLA4* genes was confirmed by DNA sequencing.

To determine which, if any, group III mutants were truly sterile, we PCR amplified the wild-type genes of all open reading frames not previously reported to be sterile and expressed each clone in the corresponding deletion strain. All remained unresponsive to α-factor, indicating they were likely false positives (data not shown). These mutants were likely to have switched mating type or to contain contaminating diploid cells, as reported previously for some strains in the collection, and would therefore be unresponsive to α-factor. Note that the *sir* mutants are likely to cause a loss of silencing at the *HML*α locus in **a** cells, resulting in expression of **a**- and α-type genes, creating a pseudodiploid state that is unresponsive to α-factor (79).

Assay of RGS binding to Gα proteins. Binding assays were performed as described previously for monitoring the interaction of Gpa1 and Ste4/Ste18, with minor modifications (112). Briefly, BJ2168 cells were transformed with the pRS316-ADH plasmids containing Myc epitope-tagged RGS proteins and either pAD4M-GST, pAD4M-GPA1-GST, or pAD4M-GPA2-GST. Cells were grown

to an A_{600} of ~1.0 in selective medium and transferred to ice, and growth was stopped by addition of NaN_3 to 10 mM. All subsequent manipulations were carried out at 0 °C to 4°C.

Thirty A_{600} units of cells (per treatment) was harvested by centrifugation at 1,000 × *g* for 10 min, washed once with 10 mM NaN_3, and washed once with lysis buffer (40 mM triethanolamine [pH 7.2], 2 mM EDTA, 150 mM NaCl, 2 mM dithiothreitol, 1 mM benzamidine, protease inhibitor cocktail [Sigma no. P8215], 3 mM $MgCl_2$, 10 μM GDP) (condition 1) or the same buffer plus 60 μM $AlCl_3$ and 20 mM NaF (condition 2). Cells were subjected to glass bead vortex homogenization for 8 × 1 min, with 1 min on ice in between. The lysate was centrifuged twice at 1,000 × *g* for 10 min, and the resulting supernatant was supplemented with 1.5% Triton X-100 (final concentration) for 1 h to solubilize membrane proteins. The insoluble material was removed by centrifugation at 1,000 × *g* for 10 min. The "applied" samples contain 30 μl of the solubilized supernatant mixed with an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 10 min. The glutathione *S*-transferase-purified ("GST-purified") sample was obtained by mixing a 40% slurry of 100 μl glutathione-Sepharose 4B resin in lysis buffer with the remainder of the solubilized lysate for 12 h. The resin was then washed five times with phosphate-buffered saline containing 1% Triton X-100 supplemented with 300 mM NaCl. Bound proteins were eluted by boiling in 30 μl of 2× SDS-PAGE sample buffer for 10 min. Samples were resolved by SDS-10% PAGE, transferred to nitrocellulose, and probed with 9E10 anti-Myc mouse monoclonal antibodies (ascites) at 1:1,000 to detect the RGS proteins (44) or anti-GST rabbit polyclonal antibodies at 1:1,500 to confirm equal expression and recovery of the GST proteins (from J. Steitz, Yale University). Immunoreactive species were visualized by enhanced chemiluminescence detection (Pierce) of horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (Bio-Rad).

For immunoblot detection of whole-cell lysates, cell growth was stopped by the addition of 10 mM NaN_3 and transfer to an ice bath. Cells were washed and resuspended directly in SDS-PAGE sample buffer, boiled for 10 min, subjected to glass bead homogenization for 2 min, and clarified by microcentrifugation for 2 min at maximum speed. Following SDS-PAGE and transfer to nitrocellulose, the membrane was probed with antibodies to phospho-p44/p42 MAP kinase at 1:500 (Cell Signaling Technology no. 9101L), phospho-Tyr (4G10; Upstate Cell Signaling Solutions no. 05–321), phospho-p38 MAP kinase at 1:500 (Cell Signaling Technologies no. 9211L), Gpa1 at 1:1,000 (38), Ste4 at 1:2,000 (from D. Jenness, University of Massachusetts), Sst2 at 1:2,000 (39), and Pgk1 at 1:75,000 (from J. Thorner) as indicated. Specificity of antibody detection was established using diploid cells (which do not express the G protein or RGS protein) or the corresponding gene deletion mutants.

Growth, transcription, and mating bioassays. Analysis of RGS gene deletion and overexpression using the reporter-transcription assay was carried out as described previously (60). The methods used to screen the yeast deletion strain array and to analyze data have been published previously (18). Briefly, gene deletion mutants in the parental strain BY4741 were arrayed in 96-well plates provided by Research Genetics and transformed with the pheromone response reporter plasmid pRS423-FUS1-lacZ (60). β-Galactosidase activity assays were performed at mid-log-phase growth (corresponding to an A_{600} of 0.420 to 0.9) (60). β-Galactosidase activity values were normalized to an A_{600} of 0.8 and used to calculate the change in activity (*n*-fold) over that of the wild type, determined for the parent wild-type strain present on each plate. Dose-response curves (variable slope) were plotted and the 50% effective concentration (or concentration necessary to achieve 50% of the maximum response) (EC_{50}) was calculated for each data set using the GraphPad Prism software package (GraphPad Software, San Diego, CA). Selected mutants were arrayed, retransformed, and reanalyzed at a wider range of α-factor concentrations as described above (60).

The quantitative mating assay was described previously (114). Briefly, 1×10^7 to 1.5×10^7 DC17 "tester" cells were mixed with 2×10^6 to 5×10^6 exponentially growing **a** cells containing an RGS gene deletion or overexpression plasmid (pAB27). The starting concentration of input α cells was measured by counting the number of viable colonies formed after serial dilution of the cultures. The mixtures were collected using a 0.45-μm 25-mm nitrocellulose filter (HAWP02500; Millipore Corp.) and SWINNEX filter cartridge (SX0002500; Millipore Corp.) and were placed on solid yeast extract-peptone-dextrose medium for 12 h. The filters were resuspended in sterile distilled water, serially diluted, and plated onto synthetic medium lacking uracil and all amino acids to select for diploids. Mating frequency for deletion mutants was expressed as the ratio of diploid colonies obtained for each mutant strain divided by the number of colonies obtained for the isogenic wild-type control strain (BY4741). Mating frequency with RGS gene overexpression was compared to that for the same wild-type strain containing the parent vector.

RESULTS

Multiple RGS proteins in yeast. In the yeast *Saccharomyces cerevisiae*, Sst2 and Rgs2 have been identified previously as RGS proteins. Sst2 regulates the pheromone signaling pathway through mating factor receptors and the G protein α subunit Gpa1 (36). Rgs2 regulates dextrose signaling through a distinct receptor and Gα protein Gpa2 (123). We searched the yeast genome sequence database and identified two additional RGS homologues, Rax1 and Mdm1 (Fig. 1). Neither Rax1 nor Mdm1 has been characterized previously with respect to G protein signaling. Thus, we employed methods established for Sst2 to determine whether any of the other RGS proteins modulate Gpa1 signaling in vivo.

Initially, we tested the ability of each RGS protein to bind to Gpa1. Most RGS proteins act as GTPase-accelerating proteins and exert this function by stabilizing the transition state conformation of the G protein α subunit. RGS proteins that function as GAPs bind with low affinity to Gα in the GTP- or GDP-bound state but with high affinity to Gα in the presence of GDP-AlF_4^- (6, 119, 126), which functions as a transition state mimic (111, 117). Thus, preferential binding of an RGS protein to Gα-GDP-AlF_4^- correlates with GAP activity, as documented previously for Sst2 and Gpa1 as well as for Rgs2 and Gpa2 (2, 123).

In order to determine which RGS proteins interact with Gpa1 in a guanine nucleotide-dependent manner, we fused full-length Sst2 (positive control), Rgs2, Rax1, and Mdm1 to the Myc epitope tag and fused Gpa1 to GST. The resulting RGS-Myc and Gpa1-GST fusions were coexpressed in yeast, and the GST fusion proteins were purified by glutathione-Sepharose affinity chromatography. GST alone was purified as a negative control, to detect nonspecific binding. The purified samples (Fig. 2, lanes labeled "GST-Purified"), as well as the starting material applied to each column (Fig. 2, lanes labeled "Applied"), were resolved by SDS-PAGE. Each RGS protein was detected by immunoblotting using the anti-Myc antibodies, and equal loading of each lane was confirmed using anti-GST antibodies. For unknown reasons, the majority of cellular Rax1 migrated well above the predicted molecular weight of 50,160 (Fig. 2A, lane 4). A similar (though less prominent) higher-molecular-weight form of Sst2 was also detected (Fig. 2A, lane 1) and has been demonstrated previously to result from Sst2 ubiquitination (53).

As shown in Fig. 2B, all four RGS proteins bound detectably to Gpa1-GST. Whereas Sst2 binding was enhanced by the addition of GDP-AlF_4^- (2), the other RGS proteins exhibited equal binding in the presence of either GDP or GDP-AlF_4^-. Binding in the presence of GTP or GTP analogs was not tested because it is limited by the slow dissociation of GDP, whereas AlF_4^- binds to the GDP-occupied form of the G protein without the need for exchange (132). In no case was binding to GST alone detected. Thus, it appears that all four RGS proteins can bind to Gpa1, but Sst2 alone binds preferentially to the transition state conformation of this G protein.

As an additional control, we tested the ability of each RGS protein to bind Gpa2. Again, all four RGS proteins bound detectably to Gpa2-GST, but not to GST alone. In this case, Rgs2 binding was enhanced by the addition of GDP-AlF_4^- (123), while the other proteins exhibited equal binding in the

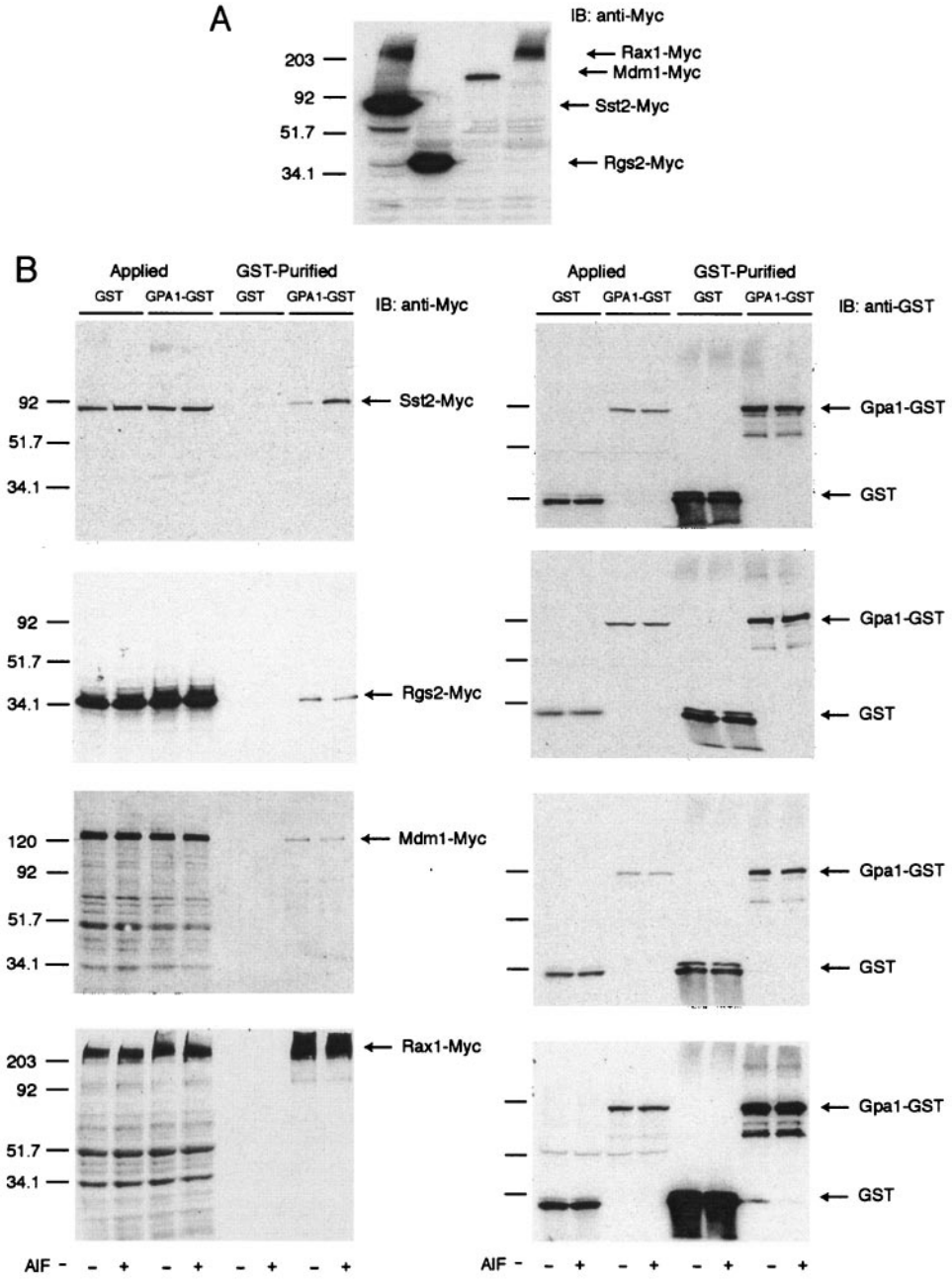

FIG. 2. Binding of RGS proteins to Gpa1. Plasmid pRS316-ADH, containing Myc epitope-tagged *SST2*, *RGS2*, *RAX1*, or *MDM1*, was cotransformed with plasmid pAD4M, containing GPA1-GST or GST, into strain BJ2168. (A) Whole-cell lysates were resolved by 10% SDS-PAGE and detected by immunoblotting (IB) with anti-Myc antibodies. Note that the Rax1 sample ran anomalously whether or not the sample was boiled. (B) Cells were grown to mid-log phase and then lysed in the presence of GDP (−) or GDP+AlF_4^- (+) as indicated. Detergent-solubilized and clarified extracts were immobilized on glutathione-Sepharose, washed, and eluted with SDS-PAGE sample buffer. Total cell lysate ("Applied") and retained protein ("GST-Purified") were subjected to immunoblotting and probed with anti-Myc antibodies (left panels) or anti-GST antibodies (right panels) (to ensure equal loading), as indicated. The Applied sample represents ~8% of the total cell lysate in every case. The arrows indicate bands specifically recognized by each antibody. Numbers at left of gels indicate molecular weights (in thousands). AlF, AlF_4^-.

presence of GDP or GDP-AlF_4^- (data not shown). Taken together, our data confirm that Sst2 binds the transition state conformation of Gpa1 and Rgs2 binds the transition state conformation of Gpa2. In addition, we show these are the only RGS-Gα pairs in yeast that distinguish between the transition state and the inactive state of either G protein. Other RGS proteins bind detectably to Gpa1 and Gpa2, but not in the manner of the canonical GAPs.

Multiple RGS proteins regulate Gpa1. Having established that all four RGS proteins bind to Gpa1, we then examined whether any RGS proteins other than Sst2 influence pheromone signaling. Initially we measured mating efficiency in cells

TABLE 1. Effects of RGS protein expression on mating efficiency[a]

Strain	Plasmid	Mating % (SEM %)
WT		100 (4.4)
*sst2*Δ		55 (7)
*rgs2*Δ		120 (1.1)
*rax1*Δ		130 (1.5)
*mdm1*Δ		120 (9)
WT	pAB	100 (6)
WT	pAB-SST2	86 (10)
WT	pAB-RGS2	90 (5)
WT	pAB-RAX1	110 (8)
WT	pAB-MDM1	90 (7)

[a] Mating, mating efficiency was determined as the mating frequency of the mutant divided by the mating frequency of the WT control; control was defined as 100%. SEM is expressed as a percentage. Data are representative of assays performed three times in triplicate.

lacking individual RGS genes. Each mutant (as the **a** mating type) was mixed with an excess of α cells and then plated on medium to select for the growth of **a**/α diploids. Mating efficiency was calculated as the percentage of **a** cells that formed a viable diploid. The *sst2*Δ-mutant strain mated with about half the frequency of wild-type cells (Table 1), as reported previously (15). In contrast, deletion of *RGS2*, *RAX1*, or *MDM1* each had a moderate enhancing effect on mating efficiency.

A second measure of pheromone response is the growth inhibition plate assay (halo assay). In this method a nascent lawn of cells is exposed to a point source of α-factor, and the resulting zone of growth inhibition, or halo, provides a measure of pheromone sensitivity over several days. The *SST2*-deficient strain is considerably more sensitive to pheromones, producing much larger zones of growth inhibition surrounding the source of α-factor, as reported previously (15, 16). In contrast, and in accord with the mating assay, the *RGS2*-, *RAX1*-, and *MDM1*-deficient strains all produced halos very similar to those of the wild-type strain (data not shown).

A third measure of pheromone signaling is the reporter transcription assay, which provides quantitative information about pheromone sensitivity over a relatively short time. For this we used the pheromone-inducible *FUS1* promoter and *lacZ* (β-galactosidase) reporter gene. As shown in Fig. 3A and in Fig. 4, the *mdm1*Δ and *rgs2*Δ mutants produced a modest increase in maximum responsiveness (efficacy), while the *rax1*Δ mutant showed a significant reduction in this activity. The *rax1*Δ strain was also unique in that it exhibited an increase in the EC_{50} for pheromone (i.e., decreased potency). All of these effects were in striking contrast to the *SST2*-deletion mutant, which exhibited a >10-fold decrease in the EC_{50} as well as an increase in basal activity (activity in the absence of added pheromone). These data reveal a minor role for Mdm1, Rgs2, and Rax1 in pheromone-dependent gene regulation. Moreover, the phenotypes exhibited by the three mutants are distinct from that of the *sst2*Δ strain, suggesting that these other RGS proteins function in a manner different from Sst2.

The data presented above indicate that all four RGS proteins regulate Gpa1 signaling activity. However, unlike Sst2, the other RGS proteins (i) do not distinguish between the transition state and inactive conformations of the G protein, (ii) increase rather than decrease mating efficiency, (iii) do not

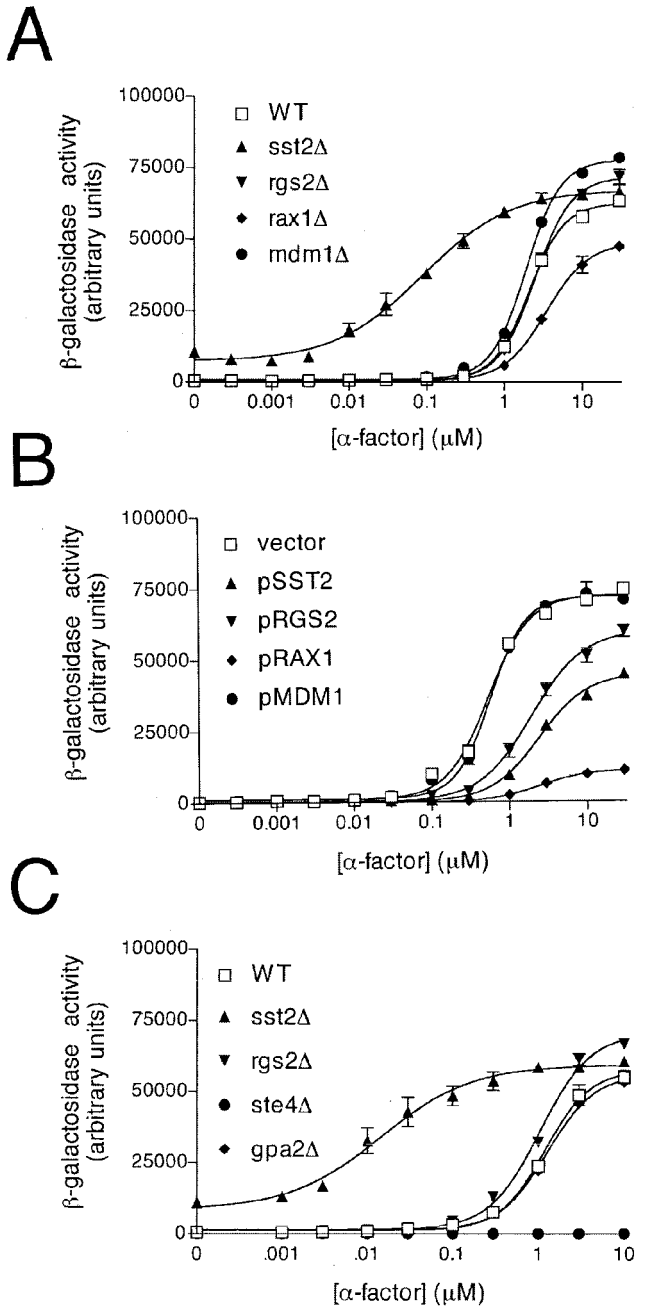

FIG. 3. Pheromone response after deletion or overexpression of RGS proteins. (A) Wild-type (WT) cells and the indicated mutant cells were transformed with a plasmid containing the pheromone-inducible *FUS1* promoter and *lacZ* reporter gene (pRS423-FUS1-lacZ), grown to mid-log phase, and treated with the indicated concentration of α-factor for 90 min. β-Galactosidase activity was measured spectrofluorometrically and expressed as arbitrary fluorescence units. (B) Wild-type cells that overexpress the indicated RGS protein (in plasmids pAB27 or pAB23), cotransformed with the *FUS1-lacZ* reporter, were assayed for β-galactosidase activity as described above. (C) Transcription reporter assay control experiments. Wild-type cells and the indicated gene deletion mutants were assayed for β-galactosidase activity as described above. Data shown are typical of three independent experiments performed in triplicate. Error bars indicate standard errors of the means (SEM).

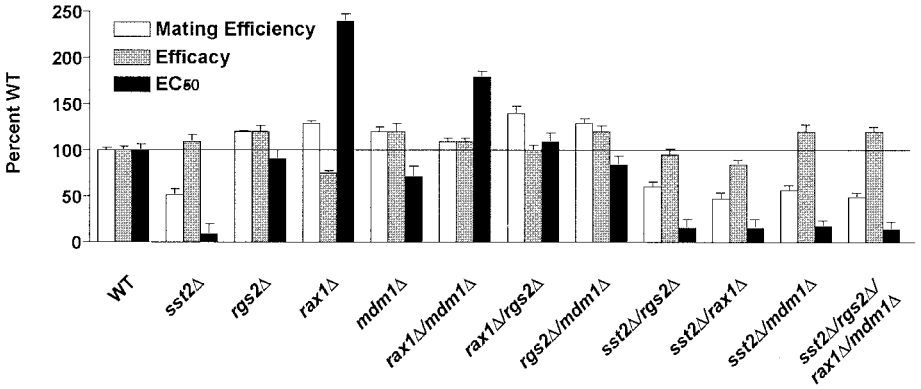

FIG. 4. Pheromone response after deletion of RGS proteins alone or in combination. WT cells and the indicated mutant cells were assayed for mating efficiency and also transformed with the *FUS1-lacZ* reporter and assayed for β-galactosidase activity as described in the legend to Fig. 3. Mating efficiency as well as pheromone efficacy and potency was calculated, as described in Materials and Methods. Data shown are expressed as a percentage of WT strain activity and are typical of three independent experiments performed in triplicate. Error bars indicate SEM. The horizontal line indicates 100% of wild type.

alter the growth arrest response, (iv) do not alter basal transcriptional activity, and (v) exhibit unique changes in pheromone potency and/or efficacy. If the mechanisms of action are indeed different, we would predict that deletion of multiple RGS proteins should produce nonoverlapping or additive effects on signaling. To test this model we constructed all possible combinations of double mutants, as well as a quadruple mutant lacking all four RGS-encoding genes, and then measured pheromone responses for each. Any multigene deletion mutant lacking *SST2* exhibited a larger halo, decreased EC_{50}, and an elevated basal response, as seen with *sst2*Δ alone (Fig. 4 and data not shown). Thus, in every case where *SST2* was deleted, the robust mutant phenotype prevailed over that of any other deletion, and significantly quantifiable differences were not observed. For the other multigene deletions, however, approximately additive effects were observed in the mating efficiency and reporter transcription assays (Fig. 4). These data further support our model of distinct modes of regulation by each of the four RGS proteins, but with Sst2 as the principal regulator of the pathway.

We then examined whether high-level expression of any RGS protein affects pheromone signaling. Overexpression of a gene product typically results in a phenotype opposite that of the gene disruption but can sometimes reveal additional functions not evident from simple knockout mutations. We first examined whether RGS overexpression affects mating efficiency, and we observed no appreciable differences for any of the genes, including *SST2* (Table 1). We then examined the effects of RGS overexpression, using the growth inhibition halo assay. Overexpression of *SST2* led to a reduced arrest response as previously reported (37), while overexpression of the other RGS genes had no effect (data not shown). Finally, we measured pheromone response using the reporter-transcription assay. In this case, overexpression of Sst2, Rgs2, or Rax1 diminished pheromone potency and, most dramatically, pheromone efficacy, while overexpression of Mdm1 had no effect (Fig. 3B). The effects of Rax1 were especially strong, equaling or exceeding even those of Sst2 (it is worth noting that of the three "atypical" RGS proteins tested, Rax1 appeared to bind with the highest affinity to Gpa1 [Fig. 2]). Taking these results together, it appears that deletion of *SST2* or *RGS2* can enhance the maximum transcription response slightly, while overexpression of these proteins diminishes the response. Deletion of *MDM1* promotes signaling slightly, while overexpression has no effect. Either deletion or overexpression of *RAX1* diminishes the pheromone signal.

The results described above reveal that all four RGS proteins can—in distinct ways—alter Gpa1-mediated signaling events. These effects are best detected using the reporter transcription assay, which is more quantitative, more sensitive, and, presumably, more selective than the longer-term growth arrest or mating efficiency assays. To confirm that the transcription reporter assay is indeed specific for the Gpa1 pathway (and not that of Gpa2), we performed several control experiments using activated or inactive mutants of each G protein and monitored signaling using the reporter transcription assay. First, we examined the transcription response in a *gpa1*Δ mutant. Deletion of *GPA1* ordinarily results in constitutive growth arrest (through uncontrolled release of Gβγ) but can be maintained by placing a downstream effector kinase under the control of an inducible promoter (83, 88). The *gpa1*Δ mutation resulted in an increase in *FUS1-lacZ* activity similar to that observed in wild-type cells treated with pheromone (50) (data not shown). Conversely, *ste4*Δ-mutant cells, which lack the Gβ subunit associated with Gpa1, were completely unresponsive to pheromone (129) (Fig. 3C). Finally, cells lacking the only other Gα subunit in yeast (*GPA2*) responded normally, in a manner indistinguishable from wild-type cells and in striking contrast to the *RGS2*-deficient cells (Fig. 3C). These results strongly suggest that the changes in *FUS1-lacZ* expression reflect the activation state of Gpa1 and not Gpa2.

Additional regulators of G protein signaling. Having established that Sst2 is the principal RGS regulator of Gpa1, we sought to determine whether other proteins regulate the same signaling pathway, perhaps in a manner distinct from Sst2. Indeed, in mammalian cells, G protein signaling is regulated by a number of non-RGS proteins. For example, phospholipase C-β1 is a downstream effector of G_q but also serves to regulate pathway activation by accelerating $G_q\alpha$ GTPase activity (9). Likewise, GRK2 (*G* protein-coupled *r*eceptor *k*inase 2) can

TABLE 2. Group I and II mutants alter pheromone potency[a]

Group	Strain	EC_{50} (fold vs WT)	Efficacy (fold vs WT)	Halo size	Plasmid rescue?
I	yil015w (*bar1*Δ)	7.9	0.93	++	Yes
I	ylr452c (*sst2*Δ)	12.0	1.1	++	Yes
I	ygl024w (*ygl024w*Δ)	3.3	1.3	+	Yes
I	ymr116c (*asc1*Δ)	2.5	1.6	+	Yes
I	ybr082c (*ubc4*Δ)	1.9	1.3	+	ND
II	ynl298w (*cla4*Δ)	0.52	1.0	=	Yes

[a] Group I (lower EC_{50}) and group II (higher EC_{50}) mutants retested over a wide range of α-factor concentrations to quantitate EC_{50} and maximum response ("Efficacy"). EC_{50} and efficacy are expressed as the ratio of mutant and WT activities, where the activity of the fully stimulated wild type equals 1. Halo size is expressed as larger (+) or smaller (−) than the wild type. ND, not done.

TABLE 3. Group III mutants are pheromone-unresponsive[a]

Gene (strain)	ID?	Role
STE2 (YFL026W)	YES	α-factor receptor
STE3 (YKL178C)	NA	**a**-factor receptor
STE4 (YOR212W)	YES	G protein β subunit
STE5 (YDR103W)	YES	Kinase scaffold
STE6 (YKL209C)	NT	**a**-factor export
STE7 (YDL159W)	YES	MAP kinase kinase
STE8/*SIR3* (YLR442C)	YES	Transcription silencing
STE9/*SIR4* (YDR227W)	YES	Transcription silencing
STE11 (YLR362W)	YES	MAP kinase kinase kinase
STE12 (YHR084W)	NT	Transcription factor
STE13 (YOR219C)	NA	α-factor processing
STE14 (YDR410C)	ND	**a**-factor processing
STE16/*RAM1* (YDL090C)	NA	**a**-factor processing
STE18 (YJR086W)	NT	G protein γ subunit
STE20 (YHL007C)	ND	MAP kinase kinase kinase
STE21/*MSN5* (YDR335W)	NO	Nuclear import/export
STE22/*AXL1* (YPR122W)	NA	**a**-factor processing
STE23 (YLR389C)	NA	**a**-factor processing
STE24 (YJR117W)	YES	**a**-factor processing
STE50 (YCL032W)	YES	Ste11 regulator
SIR2 (YDL042C)	YES	Transcription silencing

[a] List of genes known to be required for pheromone signaling were identified ("ID") by α-factor screening ("YES") or unidentified because they are required for **a**-factor responsiveness or for pheromone processing and are not applicable (NA) or unidentified because they were absent from the collection and not tested (NT) or strains containing them failed to grow and so they were not done (ND). Deletion of *STE21* results in partial sterility (http://www.yeastgenome.org).

downregulate G protein signaling by phosphorylation and inactivation of the receptor as well as binding to Gα in competition with effectors (13, 32). Considering the precedent of multiple alternative modes of G protein signal modulation, we anticipated that additional proteins might also modulate G protein signaling in yeast.

To this end, we measured the pheromone response in each of 4,847 available yeast gene deletion mutants, representing nearly all nonessential genes in the genome. To conduct this analysis on several thousand strains, we adapted the reporter-transcription assay described above to a 96-well microplate format and employed a robotic liquid handling system (18). Mutant strains in microplates were transformed with the *FUS1-lacZ* reporter and then exposed to five different concentrations of α-factor pheromone or left untreated, in triplicate. From the resulting β-galactosidase activity, we tabulated for each mutant the EC_{50} and the maximum response to pheromone, as well as the basal activity. Any mutant whose results differed from the wild type by more than twofold and at least 2 standard deviation units in either the EC_{50} or the maximum response, or by more than fivefold in basal activity, was rearrayed and retested. (See the supplemental material for a complete list of the 90 mutants that showed a reproducibly altered signaling phenotype after two rounds of testing in the 96-well format.) A subset of these mutants were validated further as described below and ultimately classified into six functional groups. Group I consists of five validated strains that exhibited a decrease in the EC_{50} for α-factor. These include known pathway components *sst2*Δ, *bar1*Δ (also known as *sst1*Δ), and *ubc4*Δ, as well as two novel mutants with a similar phenotype, *ygl024w*Δ and *asc1*Δ. Group II consists of one validated mutant, *cla4*Δ, that exhibited an increase in the EC_{50} (Table 2). Group III mutants exhibited no pheromone response whatsoever. These include mutants shown previously to block or diminish the α-factor response (*ste2*Δ, *ste4*Δ, *ste5*Δ, *ste7*Δ, *ste8*Δ/*sir3*Δ, *ste9*Δ/*sir4*Δ, *ste11*Δ, *ste50*Δ, and *sir2*Δ) (Table 3). Mutants with several additional known-sterile genes were not present in the strain collection (*ste12*Δ, *ste18*Δ) or failed to reach an appropriate cell density and were not tested (*ste20*Δ). Group IV consists of 38 mutants that exhibited a >50% decrease in maximum responsiveness, compared with the wild-type strain. Group V contains six mutants with a more than twofold increase in maximum responsiveness. Group IV and V mutants were shown to have reproducible phenotypes, but they were not validated or characterized further (see Table S1 in the supplemental material). Finally, group VI contains four mutants with a more than fivefold elevation in basal activity; of these, *sst2*Δ and *gas1*Δ were previously shown to exhibit pheromone-independent signaling (25, 105) (Table 4). The identification of known sterile, supersensitive, and consti-

TABLE 4. Group VI mutants are constitutively active[a]

Gene	ORFΔ	α-MF	ORF^+ STE^+	orfΔ STE^+	orfΔ ste2Δ	orfΔ ste4Δ	orfΔ ste7Δ	orfΔ ste12Δ
ylr452c	*sst2*Δ	−	1.0	12.3				
		+	143	189				
ygl024w		−	1.0	11.4	10.6	1.7	1.4	0.8
		+	161	188	8.2	1.6	1.2	0.76
yjl128c	*pbs2*Δ	−	1.0	6.7	26.2	0.3	0.3	0.3
		+	269	280	23.3	0.51	0.35	0.57
ymr307w	*gas1*Δ	−	1.0	14.1	26.8	0.15	0.18	0.14
		+	116	146	29.2	0.19	0.20	0.23

[a] Group VI gene ("ORF") or gene deletion mutants ("ORFΔ") in mating-competent ("STE^+") or the indicated sterile mutant strain background. Values represent reporter transcription activities expressed as ratios, where activity of the unstimulated WT equals 1.0. Complete data for *sst2*Δ have been published elsewhere (105).

tutively active mutants confirmed the integrity of the strain collection and the validity of the screening method used.

The proportion of all strains tested was approximately 89%. This value was calculated as the number of mutants for which full dose-response profiles were obtained (4,349) divided by the total number of mutant strains available (4,847). Of the strains not analyzed, 54 failed to transform and the remainder grew poorly and failed to reach a suitable cell density even after two or more attempts. The identity of strains not tested is provided in Table S2 in the supplemental material.

We focused our initial efforts on mutants that alter the EC_{50} for pheromone, since this is the parameter most affected by deletion of the prototypical G protein regulator *SST2*. To obtain more accurate EC_{50} values, we retested the best-performing strains from group I (eight mutants originally) and group II (three mutants originally) over a wider range of α-factor concentrations (0.001 to 100 μM). Examples of these higher-resolution dose-response curves are presented in Fig. 5. In these experiments, an increase in agonist potency was evident for group I mutants, in order of magnitude as follows: *sst2*Δ, *bar1*Δ, *ygl024w*Δ, *asc1*Δ, and *ubc4*Δ (Table 2). Note that, upon retesting, the activity of the *ubc4*Δ strain fell just below the twofold threshold. The group II mutant *cla4*Δ exhibited an ~50% reduction in agonist potency (Table 2). Additional mutants from groups I and II produced a reproducible phenotype but could not be validated using a genetic complementation test (see below); these putative false positives are listed in Table S1 in the supplemental material but are not listed in Table 2 and were dropped from further consideration.

To further validate the mutants we performed a growth arrest assay on the 90 strains identified in our initial screen (see Table S1 in the supplemental material). As expected for group I mutants, substantially larger-than-normal halos were observed for, in order of magnitude, *sst2*Δ, *bar1*Δ, *asc1*Δ, and *ubc4*Δ. The group II mutant *cla4*Δ responded much like the wild-type strain (Table 2).

We then investigated whether the phenotype of each mutant from group I and group II could be reversed by transformation with a plasmid-borne copy of the wild-type gene. This control was necessary to ensure that each deletion was properly constructed and catalogued and that the observed phenotype was due to the original mutation and not due to a suppressor mutation or another secondary mutation. Each gene (including the native promoter and transcription termination sequence) was PCR amplified and cloned into a yeast expression vector. These expression plasmids were then transformed together with the *FUS1-lacZ* reporter plasmid into the corresponding deletion strain and tested using the growth arrest and reporter transcription assays. Plasmid-borne expression of the wild-type gene conferred wild-type growth arrest and transcription induction responses to the *asc1*Δ, *ygl024w*Δ, and *cla4*Δ mutants as well as the control mutants *bar1*Δ and *sst2*Δ (Table 2). Figure 6 shows rescue data for a representative group I mutant (*asc1*Δ) and the sole group II mutant *cla4*Δ. Rescue was not observed for *yjl123c*Δ, *eap1*Δ, *ylr068w*Δ, or *trf5*Δ (see Table S1 in the supplemental material). Considering that each plasmid-borne gene was fully sequenced and each gene deletion was authenticated by PCR, it appears that a second genomic mutation was responsible for the phenotype observed in these strains. For *trf5*Δ, the signaling defect appears to result from alterations in *CLA4*. Within the yeast genome, *TRF5* and *CLA4* are juxtaposed, suggesting that deletion of *TRF5* interferes with the expression of *CLA4*. For *yjl123c*Δ, the additional mutation appears to be in *SST2*, since in this case full rescue

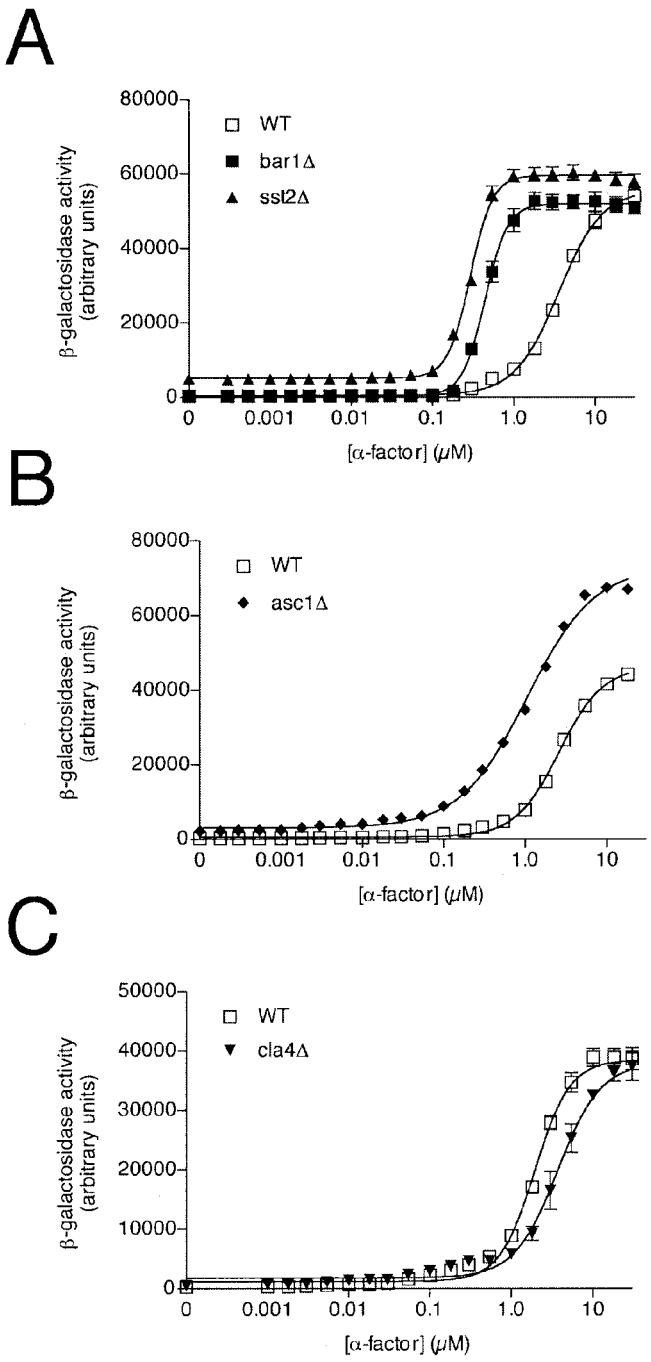

FIG. 5. Pheromone response after deletion of group I or group II genes. WT cells and the indicated mutant cells were transformed with the *FUS1-lacZ* reporter plasmid and treated with the indicated concentration of α-factor for 90 min. (A) Wild-type strain and group I control mutants *sst2*Δ and *bar1*Δ. (B) Wild-type strain and representative group I mutant *asc1*Δ. (C) Wild-type strain and the group II mutant *cla4*Δ. Data shown are typical of three independent experiments performed in triplicate. Error bars indicate SEM.

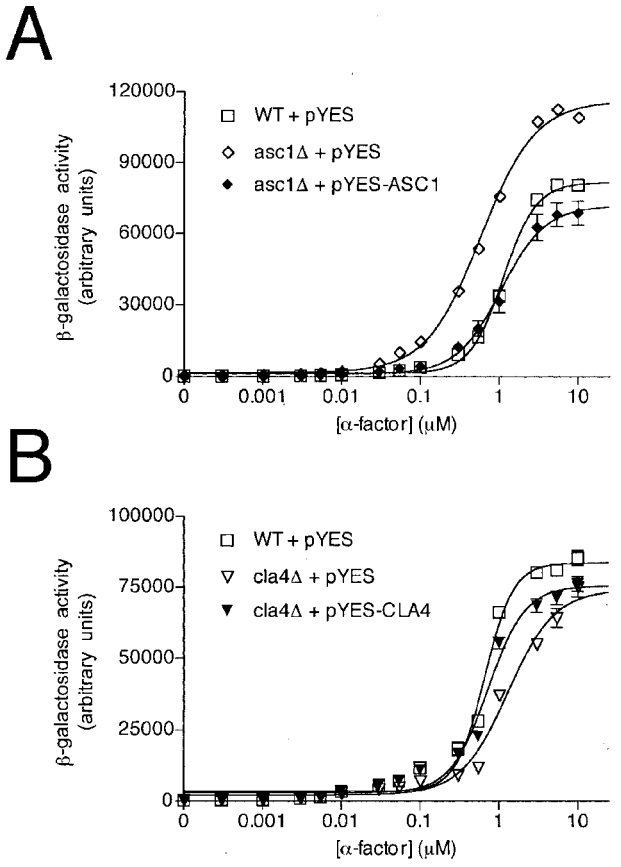

FIG. 6. Plasmid rescue of group I or group II mutants. WT cells and the indicated mutant cells were transformed with the *FUS1-lacZ* reporter plasmid and either an empty vector (pYES) or the same vector containing the absent gene, as indicated. (A) Wild-type strain and the group I mutant *asc1*Δ. (B) Wild-type strain and the group II mutant *cla4*Δ. Data for the remaining mutants are summarized in Table 2.

was observed with transformation of the *SST2* expression plasmid (data not shown).

We then examined the function of the group VI mutants *sst2*Δ, *pbs2*Δ, *gas1*Δ, and *ygl024w*Δ, all of which exhibited pheromone-independent signaling activity. Since *sst2*Δ and *ygl024w*Δ exhibited an elevated basal signal as well as increased pheromone sensitivity, these two mutants met the criteria of both group I and group VI mutants. We first attempted to establish where in the pathway this activation occurs. For example, high basal transcriptional activity could result from hyperactivation of the receptor, G protein, effector kinase, or transcription factor. A similar analysis indicated previously that deletion of *SST2* promotes a moderate level of constitutive activity and that this activity was dependent on components downstream of the receptor (105). To determine whether the novel mutants functioned similarly, the activities of *pbs2*Δ, *gas1*Δ, and *ygl024w*Δ strains were examined in cells lacking either the pheromone receptor (*ste2*Δ), G protein β subunit (*ste4*Δ), effector MAP kinase kinase (*ste7*Δ), or the transcription factor (*ste12*Δ). Elevated basal activity was detected for all three mutants in an otherwise mating-competent (*STE*$^+$) strain, as well as in the absence of the receptor gene *STE2* (Table 4). However, basal activity was not elevated in cells lacking *STE4*, *STE7*, or *STE12*. As expected, all of the *ste*$^-$ mutants also lost the ability to respond to α-factor pheromone. We conclude from this analysis that Ygl024w, Pbs2, and Gas1 regulate the pheromone pathway downstream of the receptor and most likely at the level of the G protein.

We then considered possible mechanisms by which the group VI mutants might increase basal activity, all of which have some precedent in the literature. Initially, we considered whether any mutant altered the expression of Sst2, Gpa1, or Ste4. Deletion of *SST2* was shown previously to result in pheromone-independent activation of the mating pathway, as described above (105). An even more dramatic response occurs upon deletion of *GPA1* or overexpression of *STE4* (24, 34, 83, 92, 130). Thus, a reduction of Sst2 or a change in the relative abundance of Gpa1 versus Ste4 could account for the constitutive signaling phenotype of the group VI mutants. To test these possibilities, lysates from wild-type and constitutively active mutant cells (group VI), as well as from all validated group I and II mutants, were resolved by gel electrophoresis and immunoblotting using antibodies against Gpa1, Ste4, and Sst2. Equal loading of each lane was confirmed using anti-Pgk1 antibodies. In most of the mutants, all three proteins were expressed at normal levels, making it unlikely that the constitutive signaling phenotype resulted from altered expression of the RGS or G protein (Fig. 7), but there were two exceptions. First, in the *gas1*Δ strain the apparent expression levels of Gpa1, Ste4, and Sst2 were reduced to similar degrees (Pgk1 expression was only slightly diminished). Perhaps this is a result of the upregulation of the cell integrity pathway with the deletion of *GAS1* as a competing signaling pathway (97). Second, in the *ygl024w*Δ strain, we observed a substantial decrease in Sst2 abundance, with no alteration in Gpa1, Ste4, or Pgk1. Indeed, *ygl024w*Δ resembles *sst2*Δ in having elevated basal signaling as well as a leftward shift in the pheromone dose-response profile. These data, together with the genetic epistasis data presented above, suggest that deletion of the *YGL024W* open reading frame regulates the pheromone pathway by promoting full expression of Sst2. However, since Ygl024w does not resemble any other known protein, the mechanism of action is not obvious. Unfortunately, our repeated attempts to epitope tag the protein were unsuccessful, making it difficult to characterize potential interactions with the G protein or Sst2.

We then considered whether any of the group I, II, or VI mutants could alter signaling by MAP kinases other than Fus3. To this end, we examined the phosphorylation state of Kss1 (which promotes invasive growth and, under some circumstances, can substitute for Fus3), Hog1 (which functions in the high-osmolarity glycerol pathway), and Slt2 (which functions in the cell integrity pathway). It has been shown previously that deletion of Hog1 or its upstream activator, the MAP kinase kinase Pbs2, blocks the high-osmolarity response but also allows spurious activation of the mating pathway (52, 94, 118). Similarly, deletion of *GAS1* was previously shown to result in activation of the cell integrity pathway, including hyperphosphorylation of the MAP kinase Slt2 (14, 31, 67, 96), as well as spurious activation of the mating response pathway (25). Thus, whole-cell extracts were probed by immunoblotting using phospho-p42/p44 antibodies, which recognize the dually phos-

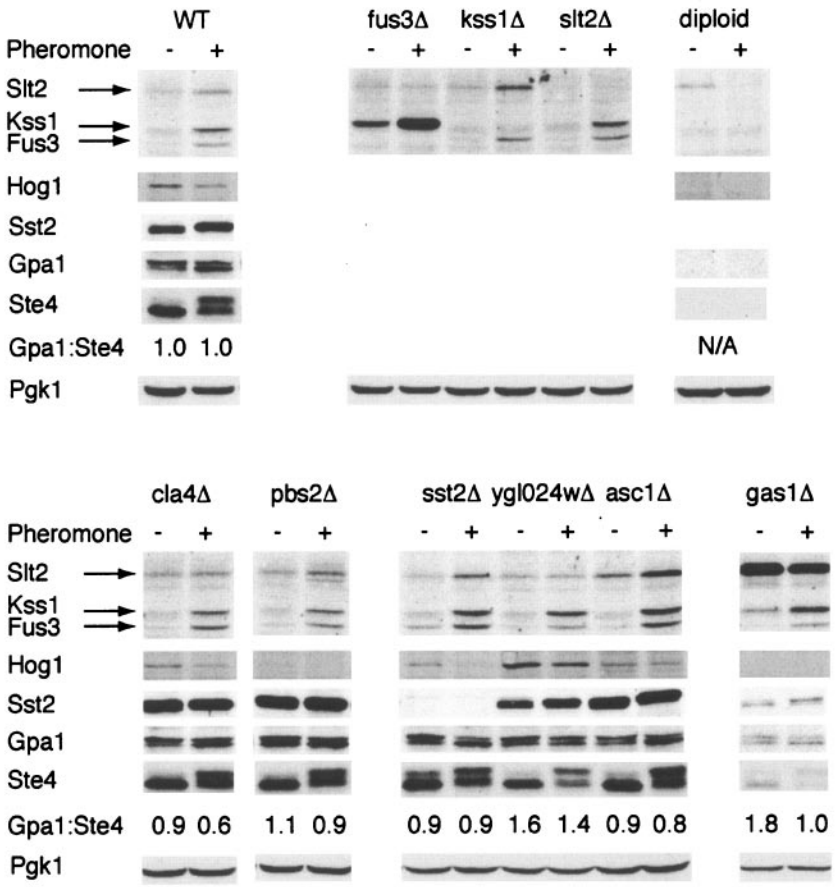

FIG. 7. Expression of G protein components and activated MAP kinases. For immunoblot detection of phosphorylated MAP kinases, WT cells and the indicated mutant cells were grown to mid-log phase, treated for 1 h with (+) or without (−) 2.5 μM α-factor pheromone as indicated, lysed, and resolved by SDS-PAGE and immunoblotting. Membranes were probed with antibodies to phospho-p44/p42 (to detect phosphorylated Fus3, Kss1, and Slt2), phospho-Tyr (to detect phosphorylated Hog1), Sst2, Gpa1, Ste4, or Pgk1 (loading control), as indicated. Specificity of antibody detection was established using the corresponding gene deletion mutant and/or diploid cells (which do not normally express Sst2, Gpa1, Ste4, or Fus3) (data not shown). Gpa1:Ste4, ratio of Gpa1 and Ste4 expression, as calculated by scanning densitometry; N/A, not analyzed.

phorylated and activated forms of Fus3, Kss1, and Slt2, as well as with antibodies against phospho-p38, which detect the phosphorylated form of Hog1. As shown in Fig. 7, pheromone treatment of wild-type cells resulted in increased phosphorylation of Fus3, Kss1, and Slt2 but decreased phosphorylation of Hog1. In the *sst2*Δ strain it appears the MAP kinases Fus3, Kss1, and Slt2 were activated to a greater degree than in the wild-type strain; however, it was interesting to note that in the absence of pheromone, Fus3 alone appeared to be upregulated. In the *gas1*Δ strain there was a striking increase in basal phosphorylation of Slt2 (Fig. 7); this phenomenon has been noted before, and suggests that cell wall defects activate the cell integrity MAP kinase signaling pathway (14, 67) and perhaps indirectly activate the pheromone response pathway. In addition, the *GAS1* null mutant showed activation of Kss1 in the absence of pheromone, suggesting that the constitutive upregulation of the yeast pheromone pathway could be mediated through Kss1. However, *fus3*Δ strains demonstrate even more robust activation of Kss1, both with and without the addition of pheromone, mitigating this possibility and supporting the model that pheromone pathway activation is mediated through, or occurs in concert with, Slt2 activation. Slt2 phosphorylation was also elevated slightly in the *asc1*Δ strain, and Hog1 phosphorylation was enhanced in the *ygl024w*Δ strain. Thus, it is possible that *asc1*Δ and *ygl024w*Δ mutants promote spurious activation of the mating pathway via dysregulation of Slt2 or Hog1.

DISCUSSION

Previous genetic studies in yeast have revealed a large number of components required for signal propagation. Most were identified through the isolation of mating-deficient, or sterile (*ste*), gene mutations. Examples include genes that encode the α-factor pheromone receptor (*STE2*), G protein βγ subunits (*STE4* and *STE18*), downstream protein kinases (*STE20*, *STE11*, and *STE7*), kinase-binding proteins (*STE5* and *STE50*), and a transcription factor (*STE12*) (40).

Yeast genetic analysis has also led to the discovery of several components required for signal modulation, most notably *BAR1* (*SST1*) and *SST2*. The *BAR1* gene encodes a secreted pepsin-like protease that cleaves and inactivates α-factor (78).

The *SST2* gene product is the founding member of the RGS protein family and is known to accelerate Gpa1 GTPase activity (2). Cells deficient in either *BAR1* or *SST2* exhibit sustained activation of the Gpa1 signaling pathway and an inability to recover from prolonged pheromone stimulation (15, 16).

A second RGS protein in yeast, Rgs2, was identified as a multicopy suppressor of Gpa2-dependent loss of heat shock resistance in stationary-phase cells and was shown also to accelerate GTP binding and hydrolysis by Gpa2 (123). The role of the remaining two RGS protein family members, Rax1 and Mdm1, has not been characterized previously with respect to G protein signaling.

Acceleration of GTPase activity is likely the predominant mechanism by which RGS proteins regulate Gα signaling in vivo. However, there is growing evidence that RGS proteins can regulate signaling even in the absence of GAP activity (62, 89). Regardless of the mechanism, it is important to know whether a given RGS protein regulates a specific Gα subtype and, if so, under what circumstances and, ultimately, by what mechanism. A related question is whether other proteins also contribute to G protein-mediated signaling. Even in the genetically tractable yeast system, few such regulatory factors have been identified or characterized.

Our objective here was to identify new pheromone response regulators through systematic analysis of all RGS proteins and nearly all other nonessential genes in yeast. Our approach was to construct cells that lack the four RGS-encoding genes, individually or in combination, and compare their abilities to regulate the well-characterized Gpa1 signaling pathway. We found that Sst2 alone binds preferentially to the transition state conformation of Gpa1, while the other RGS proteins bind equally to the transition state and inactive-state conformations. Sst2 functions primarily to reduce basal signaling and to reduce pheromone potency, while Rgs2 and Rax1 regulate pheromone efficacy.

Based on our detailed understanding of pathway regulation by Sst2, we devised a strategy to also assess regulation by other nonessential, non-RGS genes in yeast. We found that *asc1*Δ and *ygl024w*Δ mutants reduce the EC_{50} for pheromone stimulation, in the manner of the known regulatory mutants *sst2*Δ and *bar1*Δ. Conversely, we found that deletion of *CLA4* results in an unusual increase in the EC_{50} for pheromone. Additional mutants exhibit some degree of pheromone-independent signaling; these include those with deletions of *PBS2* and *YGL024W* as well as the previously documented *GAS1* and *SST2* (25, 105). These findings suggest that although Sst2 is the principal regulator of Gpa1 signaling, many other proteins also modulate pathway activity.

Our analysis benefited from powerful genomic tools, many of which are unique to the yeast system. With the sequencing of the yeast genome, all RGS domain-containing proteins could be readily identified through sequence similarity analysis. With the deletion of most open reading frames, new regulatory factors could be screened in a systematic manner (10, 49, 131). Since each mutant has been arrayed and annotated, new pathway components could be identified immediately and with few false negatives. By using a highly specific reporter, only pathway components would be identified and with few false positives. By using a full range of pheromone concentrations, even small differences in signal responsiveness could be detected and quantified.

A systematic approach has also been used to study the function of all RGS proteins in *Caenorhabditis elegans* (41, 51, 56). It has long been known that the RGS protein EGL-10 inhibits signaling by GOA-1 ($G_o\alpha$) (65). Activation of GOA-1 leads to diminished egg-laying and locomotor functions, and these activities are amplified in *egl-10*-deficient mutants. To identify other RGS regulators of GOA-1, Koelle and colleagues overexpressed each of the 13 RGS-homologous proteins in *C. elegans* and examined their effects on $G_o\alpha$-dependent signaling. By this approach they found two, the products of *rgs-1* and *rgs-2*, that mimic the effects of *egl-10* overexpression. The ability of RGS-1 and RGS-2 to regulate GOA-1 was confirmed by isolating loss-of-function mutants for the RGS genes, as well as by demonstrating that they too are needed for proper egg-laying behavior; however, the effects of *rgs-1* and *rgs-2* mutants differ from those for *egl-10*, in that they are only manifest following a period of starvation and refeeding (41, 56). These findings were interpreted to mean that EGL-10 controls "baseline" signaling while RGS-1 and RGS-2 modulate signaling under specific physiological circumstances. We observed a similar pattern of activity in yeast; whereas Sst2 controls basal signaling as well as signaling at low doses of pheromone, the other RGS proteins modulate signaling exclusively at high doses of pheromone.

An unresolved question is how RGS proteins that fail to bind preferentially to the transition state form of Gpa1 can nevertheless regulate Gpa1 signaling. One possibility is that these proteins have a function similar to GRK2. GRK2 was originally identified as a kinase that phosphorylates and desensitizes G protein-coupled receptors but was later shown to also have an N-terminal RGS homology domain that binds specifically to $G_q\alpha$ (13, 101, 104). In contrast to most other RGS proteins, however, GRK2 binds with high affinity to the active conformation as well as the transition state conformation of the $G_q\alpha$ protein and has a negligible effect on GTP hydrolysis (13, 101). GRK2 appears to function primarily by blocking $G_q\alpha$ coupling to its effector enzyme, phospholipase Cβ. The structural basis for this activity was first deduced from the available crystal structure of the RGS4-$G_i\alpha$ complex. Whereas $G_i\alpha$ binds to the so-called "A-site" of the RGS core domain (and in so doing stabilizes the transition state and reduces the activation energy for GTP hydrolysis) (115, 119), mutagenesis studies revealed that $G_q\alpha$ binds instead to a distinct site within the RGS core domain of GRK2 designated the "C-site" (116). Binding in this case would not be expected to influence the conformation of the G protein or to alter GTP hydrolysis but could easily account for the ability of GRK2 to diminish G protein-mediated signaling by effector antagonism.

Competition for Gα binding to effectors has likewise been reported for one of the more typical RGS family members, RGS2 (13, 55). Moreover, there is at least one report of a mammalian RGS protein that can directly bind and inhibit an effector enzyme, adenylyl cyclase (108). RGS proteins have also been reported to bind to receptors (8, 109), possibly in competition with Gα (8), as well as to Gβγ, possibly in competition with Gα or effectors (42, 103, 119, 125). Regardless of mechanism, inhibition of G protein subunit association will indirectly prevent receptor activation, since receptors recog-

nize only the assembled G protein heterotrimer (127). Currently we are attempting to determine which, if any, of these mechanisms account for the ability of Rgs2, Rax1, and Mdm1 to modulate the Gpa1 signal.

Our analysis also revealed a number of non-RGS domain-containing regulators of the Gpa1 signaling pathway. Of these, Bar1, Ubc4, and Gas1 have been described before. Bar1 is a secreted pepsin-like protease that cleaves and inactivates α-factor pheromone (78). Like *SST2*, deletion of *BAR1* dramatically amplifies the α-factor pheromone response. *UBC4* encodes an E2 ubiquitin-conjugating enzyme that promotes pheromone-stimulated ubiquitination and down-regulation of the α-factor receptor Ste2 (57). *GAS1* encodes a glycosylphosphatidylinositol-anchored β-1,3-glucanosyltransferase localized to the external face of the plasma membrane (85, 86, 93). A *gas1* mutant exhibits anomalous cell wall composition, including reduced β-glucan and increased chitin and mannan content, suggesting a role in proper cell wall assembly (12, 68, 97, 122). In agreement with this idea, *gas1* mutants exhibit chronic hyperactivation of Slt2, which is the MAP kinase that regulates the cell integrity pathway (14, 31, 67, 96).

We also identified genes that had not been previously associated with the yeast pheromone signaling pathway. *YGL024W* is a novel gene with no known function or obvious homologues. It encodes a 111-amino-acid protein with two putative transmembrane domains. However, there is uncertainty whether *YGL024W* is an authentic gene, and it has been designated as "dubious" by the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). Neither the gene nor its deduced translation product is conserved in any other species. Further, the *YGL024W* open reading frame overlaps the promoter and 5′-coding regions of the *PGD1* gene present on the complementary Crick strand. *PGD1* encodes a protein reported to mediate RNA polymerase II activity (87). Thus, it is possible that loss of *YGL024W* disrupts mRNA synthesis and, in this manner, modulates expression of some protein involved in pheromone signaling. On the other hand, deletion of the *PGD1* gene did not result in a signaling phenotype in our high-throughput screen. Moreover, we observed full rescue of the *ygl024w*Δ-mutant phenotype following plasmid-borne expression of the *YGL024W* gene, as demonstrated by both the reporter transcription and halo assays (data not shown). There are several possibilities that might explain these results. First, there may be a library annotation error or a secondary suppressor mutation within the *pgd1* mutant strain. Indeed, secondary mutations were detected in multiple strains, as evidenced by the numerous false-positive sterile mutants identified. Another possibility is that the N-terminal and C-terminal domains of Pgd1 affect pheromone signaling by independent and functionally antagonistic mechanisms. According to this model, the Pgd1 N-terminal domain is a negative regulator and the C-terminal domain is a positive regulator of the pathway. Deletion of *YGL024W* removes the N-terminal portion of the Pgd1 protein and enhances signaling activity, while deletion of the entire *PGD1* gene removes both the N-terminal and C-terminal portions, and signaling is unaffected. Despite numerous attempts, we have been unable to epitope tag Ygl024w and therefore unable to demonstrate expression or characterize the protein in situ. Thus, we have been unable to establish how *YGL024W* affects signaling, and considerable further investigation will be needed to fully resolve all of these complex issues.

Another novel regulator gene, *ASC1*, encodes a protein with seven WD40 repeats (17), a domain structure found in all G protein β subunits (110). Asc1 is functionally and structurally similar (54% sequence identity) to mammalian RACK1 (*r*eceptor of *a*ctivated *C k*inase 1) (17, 59), a protein that is best known to serve as an adapter for protein kinase C (102). RACK1 has also been shown to interact with a variety of other proteins, including dynamin-1, Src, and β-integrins, all of which have been suggested to bind directly or indirectly to G proteins (102, 121). More recently, reports from the laboratory of Hamm and colleagues have reported that RACK1 binds directly with Gβγ subunits, either alone or in complex with Gα (21, 22, 30). We have obtained evidence that Asc1 functions similarly, associating preferentially with the inactive form of yeast G protein heterotrimers (C. Zeller and H. G. Dohlman, unpublished data). While the functional significance of these interactions is not known, it raises the intriguing possibilities that Gpa1 can regulate protein kinase C or some related protein kinase and that these interactions are in turn regulated by Asc1.

One model currently being considered is that Asc1 regulates Cla4. Cla4 and Ste20 are both members of the p21-activated protein kinase family implicated in cell signaling and in the establishment of cell polarity (27, 43, 70, 98, 128). Cla4 and Ste20 are at least partially functionally redundant, since neither is essential for viability but a double deletion is inviable (27, 82). Both kinases are activated by the GTP-bound form of Cdc42 (63, 95, 107, 136), and Ste20 is activated as well by binding to the Gβγ subunits Ste4/Ste18 (72, 136). Ste20 in turn phosphorylates Ste11 and ultimately promotes activation of the MAP kinases Fus3 and Kss1 (27, 43, 70–72, 84, 95, 98, 107, 136).

Another mechanism by which Asc1 could modulate signaling is by altering protein translation, possibly in conjunction with the candidate effector protein Scp160 (50). Asc1 has been identified as an integral component of the 40S ribosomal subunit (74) and is required for efficient association of Scp160 with ribosomes (5, 48). Although the precise function of Asc1 and Scp160 is not established, one possibility is that these proteins coordinately regulate translation efficiency or ribosome biosynthesis (5, 17, 69).

Finally, we found that deletion of *YGL024W* and *PBS2* results in elevated basal pathway activation. A similar constitutively active phenotype was reported previously for *sst2* and *gas1* mutants (25, 105). In all four cases, the constitutively active phenotype was evident in the absence of the receptor gene *STE2* but not in the absence of the G protein β subunit *STE4* or other downstream signaling components (Table 4) (105). Based on the genetic epistasis data, we postulate that all of these proteins act at the level of the G protein. These proteins might prevent coupling of the receptor to its G protein, thereby leading to diminished G protein activation. Alternatively, they could indirectly diminish G protein activation or otherwise promote G protein subunit inactivation. Our data suggest that Ygl024w (or Pgd1) acts indirectly, by promoting full expression of Sst2 (Fig. 7). Yet another possibility is that these novel regulators bind to an effector kinase such as Ste20 or Cla4, thereby leading to diminished signaling. Currently we

are attempting to establish which, if any, of these mechanisms account for the ability of *YGL024W*, *GAS1*, and *ASC1* to modulate the pheromone signal.

The yeast system is now well recognized as a powerful resource for molecular pharmacology research. The discovery of Sst2 in yeast, and of RGS proteins generally, has established a new paradigm in signaling (19, 40, 89). The results presented here reveal several additional genes that also modulate agonist sensitivity in vivo. Most of the newly identified components have human homologues, which are thus likely to have functions similar to their yeast counterparts. The identification of new pathway modulators in yeast, whether affecting basal activity or agonist sensitivity, could eventually reveal potential new drug targets in humans. Drugs that modulate hormone or neurotransmitter signaling may be useful in situations where the receptor exhibits diminished activity or is chronically desensitized (89). A number of drugs that indirectly modulate G protein responsiveness have already been developed. For instance, inhibitors of serotonin reuptake have proven very useful in the treatment of depression. Competitive inhibitors of cGMP phosphodiesterase, an enzyme that degrades second messengers, have been developed for treatment of erectile dysfunction. Finally, if mutations or polymorphisms exist in agonist sensitivity genes such as those described here, that information could be used to reveal genetic predispositions to neurological or cardiovascular disorders or to dictate treatments for the most drug-sensitive individuals (100).

In conclusion, we have used a genome-scale approach to identify several new components of the pheromone signaling pathway. The identification of these agonist sensitivity genes provides a far more complete view of G protein signaling in yeast. Significantly, we found no gene as important to pathway regulation as *SST2*. Nevertheless we believe a similar analysis of agonist sensitivity in mammals would likewise reveal additional pathway components and may serve to validate potential drug targets within these fundamentally important signaling pathways. This goal may soon be realistic, given recent advances in homologous gene replacement and RNA interference technologies (77, 135).

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