Evidence That Mating by the *Saccharomyces cerevisiae* *gpa1*^{Val50} Mutant Occurs Through the Default Mating Pathway and a Suggestion of a Role for Ubiquitin-mediated Proteolysis

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The yeast G α subunit, Gpa1p, plays a negative role in the pheromone response pathway. The *gpa1*^{Val50} mutant was previously shown to have a growth defect, consistent with the GTPase defect predicted for this mutation, and greatly reduced mating. Various explanations for the mating defect have been proposed. One approach to analyze the *gpa1*^{Val50} mating defect involved epistasis analysis. The low mating of the *gpa1*^{Val50} mutant was independent of the pheromone receptor; therefore, it results from intracellular activation of the pathway, consistent with a GTPase defect. This result suggests that *gpa1*^{Val50} mating occurs through the default rather than the chemotropic pathway involved in pheromone response. We therefore tested the effect of a *spa2* mutation on *gpa1*^{Val50} mating, because Spa2p has been implicated in the default pathway. The *spa2* mutation greatly reduced the mating of the *gpa1*^{Val50} mutant, suggesting that *gpa1*^{Val50} mating occurs predominantly through the default pathway. In a second approach to investigate the *gpa1*^{Val50} phenotypes, suppressors of the *gpa1*^{Val50} mating defect were isolated. Two suppressor genes corresponded to *SON1/UFD5* and *SEN3*, which are implicated in ubiquitin-mediated proteolysis. On the basis of these results, we suggest that a positive component of the default mating pathway is subject to ubiquitin-mediated degradation.

INTRODUCTION

In the budding yeast *Saccharomyces cerevisiae*, haploid cells of *a* and α mating type secrete the peptide pheromones *a*-factor and α -factor, respectively. Binding of the pheromones to membrane receptors expressed by cells of the opposite mating type initiates activation of the pheromone response pathway. Response to pheromone results in cell cycle arrest in the G₁ phase, morphological changes, and transcriptional induction of genes involved in various aspects of pheromone response and mating (reviewed in Sprague and Thorner, 1992; Kurjan, 1993). Response to pheromone is essential for the mating of *a* and α cells to produce the *a*/ α diploid. The *a*-factor and

α -factor receptors, Ste3p and Ste2p, respectively, act through a heterotrimeric G protein composed of Gpa1p (α), Ste4p (β), and Ste18p (γ).

In G protein-mediated signal transduction pathways, the G protein exists as a heterotrimer with GDP bound to the α subunit in the absence of the signal (reviewed in Neer, 1995). Activation of the receptor results in guanine nucleotide exchange and dissociation of α -GTP from $\beta\gamma$. In most pathways, α -GTP activates the downstream effector, but in some cases $\beta\gamma$ can activate an effector. An intrinsic GTPase activity of the α subunit acts to return the system to the basal state.

In the yeast pheromone response pathway, the α subunit is a negative component that prevents activation of the pathway; loss-of-function *gpa1* mutations

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result in growth arrest due to constitutive activation of the pathway (Dietzel and Kurjan, 1987a; Miyajima *et al.*, 1987; Jahng *et al.*, 1988). $\beta\gamma$ is the positive component that activates the downstream pathway; loss-of-function *ste4* or *ste18* mutations result in defects in pheromone response and mating (Whiteway *et al.*, 1989). The downstream pathway includes a mitogen-activated protein kinase cascade (reviewed in Levin and Errede, 1995) and the transcriptional activator Ste12p, which binds to the promoter of pheromone-inducible genes and activates their expression.

The Gly-50 residue of *GPA1* was an interesting target for mutagenesis because it is analogous to the Gly-12 residue of Ras; mutations of Ras Gly-12 are present in many tumors, and mutation of Ras Gly-12 to any amino acid except Pro resulted in a GTPase defect and a transforming phenotype (Barbacid, 1987). The analogous Gs protein α subunit (*Gas*) mutation, *Gas*^{Val49}, resulted in a GTPase defect both in vitro and in vivo (Graziano and Gilman, 1989; Masters *et al.*, 1989). Consistent with this GTPase defect, *Gas*^{Val49} showed increased stimulation of adenylate cyclase in vitro; however, in vivo the mutation resulted in a slight decrease in adenylate cyclase activity due to an inability to dissociate from $\beta\gamma$.

On the basis of the Ras and *Gas* results, mutations of the *GPA1* Gly-50 residue would be predicted to lead to a GTPase defect, resulting in constitutive activation of the pheromone response pathway. *gpa1*^{Val50} and *gpa1*^{Asp50} have been shown to result in a complex set of phenotypes (Miyajima *et al.*, 1989; Stone and Reed, 1990; Kurjan *et al.*, 1991) that are not straightforward to explain based on a GTPase defect. In addition, some of the phenotypes differed depending on whether the *gpa1* mutation was examined as a genomic replacement or on a plasmid. Our previous analysis of the *gpa1*^{Val50} mutation as a gene replacement indicated that it resulted in a growth defect and changes in cell morphology (Kurjan *et al.*, 1991); *gpa1*^{Val50} cell morphology was intermediate to wild-type cells exposed to pheromone and *gpa1* null mutant cells. The *gpa1*^{Val50} growth defect was less severe than the defect of *gpa1* null mutants; *gpa1* null mutants were unable to form colonies (Dietzel and Kurjan, 1987a; Miyajima *et al.*, 1987), whereas the *gpa1*^{Val50} mutant formed small colonies (Kurjan *et al.*, 1991). These phenotypes are consistent with a shift in the equilibrium toward the GTP-bound form of Gpa1p, as expected for a GTPase defect. The *gpa1*^{Val50} mutant also showed greatly reduced mating, which was not predicted for a mutant with a GTPase defect, and an unusual pheromone response phenotype described below. One hypothesis based on the latter phenotypes was that the *gpa1*^{Val50} mutant was supersensitive to pheromone, resulting in reduced mating, and that Gpa1p-GTP activates desensitization to pheromone (Miyajima *et al.*, 1989; Stone and Reed, 1990). To further examine the *gpa1*^{Val50} mat-

ing defect, we performed epistasis tests with *gpa1*^{Val50} in combination with other mutations and isolated suppressors that resulted in increased mating of the *gpa1*^{Val50} mutant.

MATERIALS AND METHODS

Strains and Gene Disruptions

The yeast strains used are listed in Table 1. *Escherichia coli* strain TG1 was used for plasmid analysis and the isolation of single-stranded

Table 1. Yeast strains

Strain	Genotype ^a
W303-1A	a
W303-1B	α
W303	a/ α
K276	a/ α <i>GPA1/gpa1</i> ^{Val50}
BXK1	<i>STE2/ste2::LEU2</i> in K276
BXK2	<i>STE4/ste4::ADE2</i> in K276
BXK10	a <i>gpa1</i> ^{Val50} <i>HIS3</i>
BXK3	a <i>gvm1-1 gpa1</i> ^{Val50}
BXK4	a <i>gvm2-1 gpa1</i> ^{Val50}
BXK5	a <i>gvm3-1 gpa1</i> ^{Val50}
BXK6	a <i>gvm1-1</i>
BXK7	a <i>gvm2-1</i>
BXK8	a <i>gvm3-1</i>
BXK11	α <i>gvm1-1</i>
BXK12	α <i>gvm2-1</i>
BXK13	α <i>gvm3-1</i>
BXK15	a/a <i>gpa1</i> ^{Val50} / <i>gpa1</i> ^{Val50} <i>gvm1-1/gvm1-1</i>
BXK16	a <i>gpa1</i> ^{Val50} <i>gvm1::LEU2</i>
BXK20	a <i>gvm2-1 gvm1::HIS3</i> [YCpGVM1-URA3]
BXK21	a/ α <i>GVM1/gvm1::LEU2</i>
BXK22	a/ α <i>GVM2/gvm2::ADE2</i>
BXK23	a <i>gvm1::LEU2</i>
RAK62	a <i>ste20::URA3</i>
RAK59	a <i>ste21::URA3</i>
TCK21	a <i>far1::ADE2</i>
RAK29	a <i>fus3::LEU2</i>
SDK1	a Δ <i>gpa1::URA3</i> [pTRP1-MAT α]
BXK24	a <i>gpa1</i> ^{Lys355}
BXK25	a <i>gpa1</i> ^{Lys364}
BXK26	a <i>ste20::URA3 gvm1::HIS3</i>
BXK27	a <i>ste21::URA3 gvm1::HIS3</i>
BXK28	a <i>far1::ADE2 gvm1::HIS3</i>
BXK29	a <i>fus3::LEU2 gvm1::HIS3</i>
BXK30	a <i>gpa1</i> ^{Lys355} <i>gvm1::LEU2</i>
BXK31	a <i>gpa1</i> ^{Lys364} <i>gvm1::LEU2</i>
BXK32	a <i>spa2::TRP1</i>
RAK39	a <i>sst2::ADE2</i>
Tn44-1A	a <i>sst2::LEU2</i>
Tn44-1B	α <i>sst2::LEU2</i>
BXK33	a <i>spa2::TRP1 gpa1</i> ^{Val50}
BXK34	a <i>sst2::ADE2 gpa1</i> ^{Val50}
BXK35	a <i>gpa1</i> ^{Lys355} <i>ste2::LEU2</i>
BXK36	a <i>gpa1</i> ^{Lys364} <i>ste2::LEU2</i>
N435-1A	a <i>his7 lys7 met6 arg1 gal4 MAL2 SUC</i>
N435-2A	α <i>his7 lys7 met6 arg1 gal4 MAL2 SUC</i>

^aWith the exception of the mating testers (N435-1A and N435-2A), all strains are isogenic to W303-1A and contain the markers *ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15* (diploids are homozygous for these markers) in addition to the other markers indicated.

DNA using helper phage R408. The *ste2::LEU2* disruption was made by cleaving plasmid pAB506 (provided by J. Konopka, SUNY at Stony Brook) with *Bam*HI and transforming the *a/α GPA1/gpa1^{Val50}* strain (K276; Kurjan *et al.*, 1991). The *ste4::ADE2* disruption was made in K276 by using *pste4::ADE2* (provided by R. Akada, Yamaguchi University) cleaved with *Bam*HI and *Sal*I. The *a/α GPA1/gpa1^{Val50} STE4/ste4::ADE2* and *a/α GPA1/gpa1^{Val50} STE2/ste2::LEU2* diploids were sporulated and dissected to obtain the *gpa1^{Val50} ste4::ADE2* and *gpa1^{Val50} ste2::LEU2* double mutants. A *spa2::TRP1* fragment was obtained by cleavage of p211 (provided by M. Snyder, Yale University) with *Kpn*I and *Stu*I, and an *sst2::ADE2* fragment was obtained by cleavage of *psst2::ADE2* (provided by R. Akada) with *Sal*I. The *spa2::TRP1* and *sst2::ADE2* disruptions were made in the wild-type strain W303-1A and in an *gpa1^{Val50}* [pGPA1-URA3] strain; the GPA1-containing plasmid was lost from the *gpa1^{Val50} spa2::TRP1* and *gpa1^{Val50} sst2::ADE2* strains by streaking on 5-fluoroorotic acid (5-FOA) medium (Boeke *et al.*, 1984).

Mating and Pheromone Response Assays

Qualitative mating assays were done by replica plating masters to minimal plates that had been spread with a thick lawn of mating tester cells in YEPD and incubating at 30°C for 2 d (Sprague, 1991). Quantitative mating assays were done by mixing 3×10^6 experimental cells with 1.5×10^7 tester cells from logarithmic phase cultures. The mixtures were filtered onto 25 mm in diameter nitrocellulose filters (Millipore, Bedford, MA) and incubated on YEPD plates at 30°C for 4 h. The cells were resuspended and diluted in water before plating. Mating efficiencies were calculated by dividing the number of diploids (minimal medium) by the total number of diploids and nontester haploids (–Met –Arg double-dropout medium).

Pheromone spotting assays were done by spreading approximately 1×10^6 cells onto plates, spotting 4 μ l of 1 mg/ml α -factor (Sigma, St. Louis, MO) on the lawn, and incubating the plates at 30°C for 24 to 48 h. The pheromone-inducible *FUS1-lacZ* reporter construct on pTCFL1 (*TRP1*; provided by T. Chen, University of Vermont) or pSB234 (*URA3*; Trueheart *et al.*, 1987) was used to assay pheromone response. Strains were grown in medium selective for plasmids to an OD₆₀₀ of 0.5–1.0 and harvested. For pheromone induction, cells were grown in YEPD medium for 90 min and then were induced with 2.4 μ M α -factor for 1 h before harvesting. Cells were prepared and permeabilized, *o*-nitrophenyl β -D-galactopyranoside was used as substrate, and β -galactosidase units were calculated as described (Miller, 1972).

Mating Suppressor Isolation

K276 (*a/α GPA1/gpa1^{Val50}*) was transformed with the 1.8-kb *HIS3 Bam*HI fragment from pUC18::*HIS3* to construct a *HIS3/his3–11,15* diploid, which was sporulated and dissected to obtain *GPA1 HIS3* and *gpa1^{Val50} HIS3* strains; *HIS3* prototrophy allowed for selection of diploids in isogenic crosses of the mutants isolated to strains containing YEp51B (*LEU2*).

The *a gpa1^{Val50} HIS3* (BXK10) cells were grown in YEPD medium overnight, harvested, washed with double-distilled H₂O, and approximately 600 cells/plate were spread on YEPD plates. The plates were exposed to UV light (model TM-36 transilluminator, 302-nm wavelength; UVP, San Gabriel, CA) for 20 s and incubated at 30°C for 2 to 3 d, which gave a 50% survival rate. After picking to master plates, the mutagenized colonies were screened by the qualitative mating assay, and colonies showing increased mating were streaked for single colonies and retested. Out of approximately 20,000 colonies tested, three mutants that showed reproducible increases in mating were obtained.

Cloning and Analysis of GVM1

An *a/α gpa1^{Val50}/gpa1^{Val50} gvm1–1/gvm1–1* diploid (BXK15) was used for cloning of GVM1 by complementation to avoid the isola-

tion of sterile suppressors of the *gpa1^{Val50}* growth defect. BXK15 was obtained by mating type switching of the isogenic *a/α* diploid using pGAL-HO (Herskowitz and Jensen, 1994). A YCp50 library (provided by M. Rose; Rose *et al.*, 1987) was transformed into BXK15, transformants were picked to –Ura master plates, which were replica plated to mating testers. Of approximately 30,000 transformants tested, one transformant showed reduced mating. After growth on nonselective medium, the Ura[–] colonies showed the original mating phenotype, indicating that the phenotype was plasmid dependent. DNA was isolated from this transformant, and the plasmid DNA (p70) was recovered from *E. coli* and transformed into various yeast strains to test for complementation of the *gvm1–1* mutation.

To test linkage of the p70 insert to GVM1, an internal 1.6-kb *Bam*HI fragment from the insert was subcloned into the integrating vector pRS306 to produce YIp70. YIp70 was cleaved with *Pml*II, which cuts at a unique site within the insert, and transformed into the *α gpa1^{Val50} GVM1* strain. The integrant was crossed with the *gpa1^{Val50} gvm1–1* mutant and the diploid was subjected to tetrad analysis.

To delineate the functional region of the 15.5-kb p70 insert, the 6.5-kb *Eco*RI–*Bam*HI, 10.0-kb *Eco*RI–*Bam*HI, and 4.0-kb *Hind*III–*Bam*HI fragments were subcloned into YCp50 and transformed into the *gpa1^{Val50} gvm1–1* strain to test complementation. This analysis indicated that an internal *Bam*HI site was within the GVM1 functional region. Therefore, single-stranded DNA was made from pRS306 plasmids containing the 1.6-kb *Bam*HI fragment in both orientations and both ends of the fragment were sequenced by dideoxynucleotide sequencing using Sequenase (United States Biochemical, Cleveland, OH) and the M13 –40 primer. A BLAST search (Altschul and Lipman, 1990) indicated that one end of the fragment was in the *SON1* open reading frame (GenBank accession no. L00921; Nelson *et al.*, 1993). The oligonucleotides 5'-TTCGGC-GAAGCTTGTTGA (322 bp upstream of the *SON1* initiation codon) and 5'-GGTCTTAAGCTTATCAGA (321 bp downstream of the *SON1* termination codon) were used to amplify the *SON1* gene by polymerase chain reaction (PCR) from the wild-type and *gvm1–1* strains. The 2.2-kb *Hind*III fragments (the restriction sites are underlined in the oligonucleotide sequences) were subcloned into pRS316, and the resulting plasmids were transformed into the *gpa1^{Val50} gvm1–1* strain to test complementation.

To construct GVM1 disruption plasmids, the wild-type 2.2-kb *Hind*III fragment obtained by PCR was subcloned into pCR-Script Amp SK (+) (Stratagene, La Jolla, CA) to produce pBX2.2H. The 3.1-kb *LEU2 Bgl*III and 1.8-kb *HIS3 Bam*HI fragments were subcloned into the *Bgl*III site of pBX2.2H to produce *gvm1* disruption plasmids. These plasmids were cleaved at flanking restriction sites and used to transform the wild-type diploid (W303) and other strains described below. In addition, the 2.2-kb *Hind*III fragment was subcloned into YEp352 to create YEp352-GVM1.

Cloning and Analysis of GVM2

The lethality of the *gvm1::HIS3 gvm2–1* double mutant allowed the use of a plasmid-shuffle screen (Mann *et al.*, 1987) to clone GVM2. p70 (YCpGVM1-URA3) was transformed into the *gvm2–1* mutant, and a *gvm1::HIS3* disruption was constructed to produce the *gvm1::HIS3 gvm2–1* [pGVM1-URA3] strain (BXK20), which was 5-FOA-sensitive (Boeke *et al.*, 1984) due to the inability to lose pGVM1-URA3. BXK20 was transformed with a pRS318 (*LEU2*) genomic library (provided by R. Akada), transformant colonies were replica plated onto 5-FOA plates lacking leucine, and 5-FOA-resistant colonies were picked and tested for plasmid dependency.

Of 120 5-FOA-resistant colonies tested, 2 were unable to lose the *LEU2* plasmid. Plasmids (p39 and p101) were recovered in *E. coli* and transformed back into BXK20 to confirm the phenotypes. p101 contained the GVM1 gene, whereas p39 contained a nonoverlapping insert that could complement the *gpa1^{Val50} gvm2–1* phenotype. To test linkage of the p39 insert to GVM2, an internal 3.5-kb *Hind*III

fragment was subcloned into the YIp vector pRS306, which was cleaved at the unique *HpaI* site within the insert and integrated into the α *gpa1^{Val50}* mutant. A stable integrant was crossed to the *gpa1^{Val50} gvm2-1* strain and used for tetrad analysis.

To delineate the functional region of the 5.0-kb p39 insert, 1.0-kb *BamHI*–*HindIII*, 3.5-kb *HindIII*, and 1.8-kb *Sall*–*BamHI* fragments were subcloned into pRS316 and retransformed into the *gpa1^{Val50} gvm2-1* strain to test complementation. Because one of two internal *HindIII* sites was implicated in function, single-stranded DNA was made from pRS306 plasmids containing the 3.5-kb *HindIII* fragment in both orientations and sequenced. A BLAST search (Altschul and Lipman, 1990) indicated that one end of the fragment was within the open reading frame of the *SEN3* gene (GenBank accession no. L06321; DeMarini *et al.*, 1995). The oligonucleotides 5'-GCCATTATGAATTCTAGGG (199 bp upstream of the *SEN3* initiation codon) and 5'-TTAGAATTCTGCGTAAAG (142 bp downstream of the *SEN3* termination codon) were used to amplify the *SEN3* gene by PCR from the wild-type and *gvm2-1* strains. The resulting 3.1-kb *EcoRI* fragments (the restriction sites are underlined in the oligonucleotide sequences) were subcloned into pRS314 and pRS316. The pRS314 constructs were transformed into *gvm1::HIS3 gvm2-1* [pGVM1-URA3] (BXK20), and the pRS316 constructs were transformed into *gpa1^{Val50} gvm2-1* to test complementation.

To construct a GVM2 disruption plasmid, a 3.2-kb *PstI*–*Sall* GVM2 fragment was subcloned into pCR-Script Amp SK (+) vector to create pBX3.2PS. The 2.3-kb *ADE2 BglII* fragment was inserted into the *BglII* site of pBX3.2PS to produce a *gvm2::ADE2* disruption plasmid, which was cleaved with *PstI* and *Sall* and used to transform W303. In addition, the 5.0-kb *PstI*–*XbaI* GVM2 fragment was subcloned into YEp352 to create YEp352-GVM2.

Construction of *gpa1^{Lys355}* and *gpa1^{Lys364}* Mutants

Site-directed mutagenesis was used to create the *gpa1^{Lys355}* and *gpa1^{Lys364}* mutations (Stone and Reed, 1990). Single-stranded DNA from pRS306-GPA1, which contains the 1.9-kb *GPA1 EcoRI* fragment, was mutagenized with the oligonucleotides 5'-CAGATGTTGTTTAAGGATGAAAG (*gpa1^{Lys355}*) and 5'-GAACAGAATGCATAAATCAATAATGC (*gpa1^{Lys364}*; mutated nucleotides are underlined) using the Sculptor in vitro mutagenesis kit (Amersham, Arlington Heights, IL). The presence of the mutation was confirmed by sequencing. The mutant 1.9-kb *EcoRI* fragments were subcloned into the YCp plasmid pRS316. To construct gene replacements, the mutant 1.9-kb *EcoRI* fragments were used to replace the wild-type fragment in pSK-GPA1/US, which contains the 1.9-kb *GPA1* fragment and an additional upstream 1.5-kb fragment (DeSimone and Kurjan, unpublished results). The 3.4-kb *XhoI*–*PstI* fragments from pSK-*gpa1^{Lys355}*/US and pSK-*gpa1^{Lys364}*/US constructs were cotransformed with pRS425 (*LEU2*) into the Δ *gpa1::URA3* [pTRP-MAT α] strain (SDK1; DeSimone and Kurjan, unpublished results), and Leu⁺ transformants were replica-plated onto 5-FOA plates to select for Ura⁻ colonies. For each mutant, several Ura⁻ colonies were tested for the ability to lose the pTRP-MAT α plasmid and the resulting *gpa1^{Lys355}* and *gpa1^{Lys364}* replacement mutants were tested for various phenotypes.

β -Galactosidase Assays for Ubiquitin-mediated Proteolysis Defects

The wild-type, *gvm1-1*, *gvm2-1*, and *gvm3-1* strains were transformed with pLR20 (Deg1- β gal; URA3-CEN; Chen *et al.*, 1993) and with pUB-Leu- β gal and pUB-Pro β gal (URA3-2 μ ; Bachmair *et al.*, 1986); these plasmids were provided by M. Hochstrasser (University of Chicago). Cell patches were tested for β -galactosidase activity by using the colony lift assay (Breedon and Nasmyth, 1985).

RESULTS

The Low Mating of the *gpa1^{Val50}* Mutant Is Dependent on *Ste4p* but Not *Ste2p*

The growth defect resulting from a *gpa1* null mutation can be suppressed by mutations in downstream components of the pheromone response pathway, including *STE4* (Nakayama *et al.*, 1988; Blinder *et al.*, 1989). We constructed *gpa1^{Val50} ste4::ADE2* double mutants to determine whether the *ste4* mutation could suppress the *gpa1^{Val50}* phenotypes that were proposed to result from constitutive activation of the pheromone response pathway (Kurjan *et al.*, 1991). The *ste4::ADE2* mutation suppressed the *gpa1^{Val50}* growth (Figure 1A) and cell morphology defects. Assays of the pheromone inducible reporter gene *FUS1-lacZ* had indicated that the *gpa1^{Val50}* mutation results in high basal activation of the reporter construct and only slight induction by pheromone (Figure 1B; Kurjan *et al.*, 1991). The *ste4::ADE2* mutation eliminated both the basal and induced reporter gene expression; these results indicate that the growth defect and high basal β -galactosidase expression result from constitutive activation of the pathway. The *gpa1^{Val50} ste4::ADE2* mutant was defective for cell cycle arrest in response to pheromone (our unpublished results). In addition, mating by the *gpa1^{Val50} ste4::ADE2* mutant was undetectable (Figure 1C and Table 2). Therefore, the *ste4::ADE2* mutation was epistatic to the *gpa1^{Val50}* mutation with respect to all phenotypes.

Intracellular activation of the pheromone response pathway by galactose-induced expression of *STE4* or *STE12* allows low levels of mating that are independent of the pheromone receptor (Dolan and Fields, 1990; Cole *et al.*, 1990; Nomoto *et al.*, 1990; Whiteway *et al.*, 1990). A GTPase defect due to the *gpa1^{Val50}* mutation should also lead to intracellular activation of the pathway. To determine whether the low mating of the *gpa1^{Val50}* mutant was independent of the receptor, we tested the effect of a null mutation in the α -factor receptor gene (*ste2::LEU2*) on the *gpa1^{Val50}* phenotype. The *ste2::LEU2* mutation did not suppress the *gpa1^{Val50}* growth defect (Figure 1A), as expected for a mutation in an upstream component. The *gpa1^{Val50} ste2::LEU2* mutant retained the high basal activation of *FUS1-lacZ*, consistent with constitutive intracellular activation of the pathway. However, the *ste2::LEU2* mutation prevented response to pheromone, as indicated by the absence of the mild pheromonal induction of the reporter gene observed for the *gpa1^{Val50} STE2* single mutant (Figure 1B), and a defect in growth arrest of the *gpa1^{Val50} ste2::LEU2* double mutant using the pheromone spotting assay (our unpublished results), in which α -factor spotted on a lawn of wild-type cells results in a zone of growth arrest. Mating by the *gpa1^{Val50} ste2::LEU2* double mutant was reduced less than threefold in comparison to the *gpa1^{Val50} STE2*

Figure 1. Epistasis tests of *gpa1*^{Val50} combined with *ste2::LEU2* and *ste4::ADE2* disruptions. (A) Colony growth. The strains indicated were streaked for single colonies and grown for 2 d at 30°C. (B) Reporter gene assays. Cells containing the *FUS1-lacZ* reporter gene on pSB234 were assayed for β -galactosidase activity. β -Galactosidase units were the average of at least three determinations. (C) Mating assays. *GPA1* (top) and *gpa1*^{Val50} (bottom) strains wild-type for *STE2* and *STE4* (left), with the *ste2::LEU2* disruption (middle) or with the *ste4::ADE2* disruption (right), were tested by qualitative mating assays with the tester strain N435-2A. WT, wild-type strain (W303-1A).

single mutant (Figure 1C and Table 2), indicating that this mating is predominantly independent of exposure to pheromone.

The Low Mating of the *gpa1*^{Val50} Mutant Is Dependent on *Spa2p*

Recent results have indicated that mating occurs primarily by using a chemotropic pathway by which cells respond to the spatial pheromone signal provided by potential mating partners (Jackson and Hartwell, 1990a,b; Dorer *et al.*, 1995). At high levels of pheromone, however, this spatial signal is saturated and cells instead mate by a much less efficient default pathway. Mutations that lead to constitutive intracellular activation of the pheromone response pathway,

such as the *gpa1*^{Val50} mutation, might mimic saturation by pheromone, and mating might, therefore, occur through the default pathway.

To test the hypothesis that the low mating of the *gpa1*^{Val50} mutant occurs through the default pathway, we compared the effect of mutations that predominantly affect the default pathway (*spa2*), that only affect the chemotropic pathway (*far1*), or that shift mating from the chemotropic to the default pathway (*sst2*) on *gpa1*^{Val50} mating (Dorer *et al.*, 1995; Valtz *et al.*, 1995). Consistent with previous results (Dietzel and Kurjan, 1987b), the *sst2::ADE2* mutation did not affect the low mating resulting from the *gpa1*^{Val50} mutation (Figure 2B and Table 2); mating by the *gpa1*^{Val50} *sst2::ADE2* double mutant was only reduced 1.7-fold

Table 2. Quantitative mating assays for epistasis tests

GPA1 allele	Mating frequency ^a				
	WT	<i>ste2::LEU2</i>	<i>ste4::ADE2</i>	<i>spa2::TRP1</i>	<i>sst2::ADE2</i>
<i>GPA1</i>	1.0	$<1.4 \times 10^{-6}$	$<5.6 \times 10^{-6}$	0.37	9.4×10^{-2}
<i>gpa1</i> ^{Val50}	1.4×10^{-4}	4.9×10^{-5}	$<4.9 \times 10^{-6}$	3.1×10^{-6}	8.0×10^{-5}
<i>gpa1</i> ^{Lys355}	3.7×10^{-5}	$<1.6 \times 10^{-6}$	NT	NT	NT
<i>gpa1</i> ^{Lys364}	5.0×10^{-6}	$<1.5 \times 10^{-6}$	NT	NT	NT

^aMating frequencies are calculated relative to the wild type strain W303-1A. WT, *STE2 STE4 SPA2 SST2*; NT, not tested.

Figure 2. Epistasis tests of *gpa1^{Val50}* combined with *sst2::ADE2* and *spa2::TRP1* disruptions. The strains indicated were tested for the growth of colonies (A) and mating (B) as described in Figure 1. WT, wild-type strain (W303-1A).

in comparison to the *gpa1^{Val50}* *SST2* mutant. The *far1::ADE2* mutation also did not affect *gpa1^{Val50}* mating (our unpublished results). In contrast, the *gpa1^{Val50}* *spa2::TRP1* double mutant resulted in a 45-fold decrease in mating in comparison to the *gpa1^{Val50}* *SPA2* mutant (Figure 2B and Table 2). Neither the *sst2::ADE2* nor the *spa2::TRP1* mutation had any effect on the *gpa1^{Val50}* growth (Figure 2A) or pheromone spotting phenotypes.

The *gpa1^{Lys355}* and *gpa1^{Lys364}* Phenotypes Differ from the *gpa1^{Val50}* Phenotypes

In the pheromone spotting assay for growth arrest, the response of the *gpa1^{Val50}* mutant was unusual (Miyajima *et al.*, 1989; Kurjan *et al.*, 1991). Wild-type cells show a small region of growth arrest that fills in as cells recover from cell cycle arrest (Figure 3A). The *gpa1^{Val50}* mutant shows a large zone of growth arrest similar in size to the supersensitive mutant *sst2*, which is defective in recovery from pheromone arrest (Dietzel and Kurjan, 1987b); however, the *gpa1^{Val50}* zone is turbid in contrast to the clear zone seen with the *sst2* mutant. The large turbid pheromone spotting phenotype observed with the *gpa1^{Val50}* mutant (Miyajima *et al.*, 1989; Kurjan *et al.*, 1991) was also observed with *gpa1^{Asp50}*, *gpa1^{Lys355}*, and *gpa1^{Lys364}* mutants when assayed on a centromere plasmid (Stone and Reed,

1990), although the latter mutants did not show the *gpa1^{Val50}* growth defect and mating was not tested.

Because the interpretation of the *gpa1^{Val50}* pheromone spotting phenotype has been uncertain, we made the *gpa1^{Lys355}* and *gpa1^{Lys364}* mutants by site-directed mutagenesis and constructed gene replacements to allow a comparison to the *gpa1^{Val50}* phenotype. The *gpa1^{Lys355}* and *gpa1^{Lys364}* mutants showed several differences from the *gpa1^{Val50}* mutant. The *gpa1^{Lys355}* and *gpa1^{Lys364}* did not show growth defects. The *gpa1^{Lys355}* and *gpa1^{Lys364}* mating defects were more severe than the *gpa1^{Val50}* defect (Table 2), and unlike the *gpa1^{Val50}* *ste2::LEU2* double mutant, which retained the low mating phenotype of the *gpa1^{Val50}* mutant, the *gpa1^{Lys355}* *ste2::LEU2* and *gpa1^{Lys364}* *ste2::LEU2* double mutants were sterile. In reporter gene assays, both the *gpa1^{Lys355}* and *gpa1^{Lys364}* mutants showed basal expression levels similar to the wild-type strain, rather than the high basal level of the *gpa1^{Val50}* mutant (Figure 3B). Thus, the lack of a growth defect of these mutants, the wild-type reporter gene expression in the absence of α -factor, and the elimination of mating by the *ste2::LEU2* mutation indicate that the *gpa1^{Lys355}* and *gpa1^{Lys364}* mutations did not result in constitutive activation of the pheromone response pathway.

The phenotypes of the *gpa1^{Lys355}* and *gpa1^{Lys364}* mutants are instead consistent with defects in signal

Figure 3. *gpa1^{Lys355}* and *gpa1^{Lys364}* phenotypes. (A) Pheromone spotting assays. Thin lawns of cells were spread on plates, 4 μ g of α -factor was spotted, and the plates were incubated at 30°C for 2 d. (B) Reporter gene assays were done as described in Figure 1. WT, wild-type strain (W303-1A).

transmission. In the reporter gene assays, the *gpa1^{Lys355}* and *gpa1^{Lys364}* mutants showed 10-fold and 3-fold induction by α -factor in contrast to 30-fold induction in the wild-type strain (Figure 3B). The *gpa1^{Lys364}* mutant, which showed the greatest mating defect (Table 2), did not show detectable growth inhibition in the pheromone spotting assay (Figure 3A), in contrast to the turbid zone of growth inhibition observed for this mutation as assayed on a centromere plasmid (Stone and Reed, 1990). The *gpa1^{Lys355}* mutant did show a turbid zone in this assay, although it differed from the *gpa1^{Val50}* phenotype; the zone of inhibition was somewhat similar to the unexplained ring phenotype observed with some *ste4* and *ste18* mutants (Whiteway *et al.*, 1989), in which a central region of poor growth inhibition is surrounded by a ring of greater growth inhibition. A final difference of

the *gpa1^{Lys355}* and *gpa1^{Lys364}* mutants from the *gpa1^{Val50}* mutant was that YCp plasmids containing either the *gpa1^{Lys355}* or the *gpa1^{Lys364}* mutant gene were able to complement the growth defect of the *gpa1::LEU2* null mutant, whereas we were unable to obtain *gpa1::LEU2* strains containing YCp-*gpa1^{Val50}*.

Isolation and Characterization of Suppressors of the *gpa1^{Val50}* Mating Defect

As an alternative approach to characterize the *gpa1^{Val50}* mating defect, we undertook a screen for suppressors of *gpa1^{Val50}* that showed increased mating. Screening of approximately 20,000 UV mutagenized *gpa1^{Val50}* colonies identified three mutants that showed significantly higher mating than the original *gpa1^{Val50}* mutant (Figure 4A and Table 3). The

Figure 4. Phenotypes of *gpa1^{Val50} gvm* double mutants. Mating (A), colony growth (B), pheromone spotting (C), and reporter gene assays (D) were done as in Figure 1. The growth and pheromone spotting phenotypes of the *gpa1^{Val50} gvm2-1* and *gpa1^{Val50} gvm3-1* mutants were similar to the *gpa1^{Val50} gvm1-1* phenotype. WT, wild-type strain (W303-1A).

gpa1^{Val50} gvm1-1, *gpa1^{Val50} gvm2-1*, and *gpa1^{Val50} gvm3-1* mutants (*gvm* = *gpa1^{Val50}* mating defect suppressors) retained the growth defect (Figure 4B) and aberrant cellular morphology of the *gpa1^{Val50}* single mutant (Kurjan *et al.*, 1991). In *FUS1-lacZ* reporter gene assays, the *gpa1^{Val50} gvm1-1* and *gpa1^{Val50} gvm3-1* mutants showed phenotypes similar to the *gpa1^{Val50}* single mutant (Figure 4D). Although both the basal and induced levels of β -galactosidase were approximately 7.5-fold lower for the *gpa1^{Val50} gvm2-1* mutant than for the *gpa1^{Val50}* single mutant, the basal activity

was significantly elevated in comparison to the wild-type strain. The *gpa1^{Val50} gvm* mutants retained the large turbid zone of growth arrest observed with the *gpa1^{Val50}* GVM mutant (Figure 4C).

To determine whether suppression of the *gpa1^{Val50}* mating defect resulted from single *gvm* mutations, the *gpa1^{Val50} gvm* mutants were crossed with the *gpa1^{Val50}* GVM strain and followed by tetrad analysis. Approximately 20 tetrads were analyzed for each of the *gvm* mutants, all of which showed 2:2 segregation patterns for the original *gpa1^{Val50}* mating phenotype and the increased *gpa1^{Val50} gvm* mating phenotype, indicating that a single mutation was sufficient for suppression by all three *gvm* mutants.

To test linkage of the *gvm* mutations to *GPA1*, the *gpa1^{Val50} gvm* mutants were crossed to the wild-type strain (W303-1B) and subjected to tetrad analysis. For the *GPA1/gpa1^{Val50} GVM2/gvm2-1* and *GPA1/gpa1^{Val50} GVM3/gvm3-1* diploids, the tetrads showed the same pattern of growth as the singly heterozygous *GPA1/gpa1^{Val50}* diploid; tetrads consisted of two spores that formed colonies of wild-type size and two spores that formed small colonies. These results sug-

Table 3. Quantitative mating assays of *gvm* mutants

GPA1 allele	Mating of strains with various <i>gvm</i> alleles ^a			
	WT	<i>gvm1-1</i>	<i>gvm2-1</i>	<i>gvm3-1</i>
GPA1	1.0	0.82	1.0	1.3
<i>gpa1^{Val50}</i>	1.4×10^{-4}	1.8×10^{-2}	5.8×10^{-3}	1.0×10^{-2}

^aMating frequencies are calculated relative to the wild-type strain W303-1A. WT, GVM1 GVM2 GVM3.

gested that the *gvm2-1* and *gvm3-1* mutations did not affect growth at 30°C. Of the small *gpa1*^{Val50} colonies, approximately half (46% for the *gvm2-1* cross and 48% for the *gvm3-1* cross) showed the low mating of the parental *gpa1*^{Val50} mutant and half showed increased mating similar to the original *gpa1*^{Val50} *gvm* double mutant, indicating that the *gvm2-1* and *gvm3-1* mutations segregated independently of *GPA1* and had similar effects in *a* and α cells.

Analysis of the *GPA1/gpa1*^{Val50} *GVM1/gvm1-1* tetrads was more complicated. Of 32 tetrads analyzed, 5 tetrads gave rise to two small colonies and two wild-type size colonies (parental ditype), 6 gave four small colonies (nonparental ditype), and 21 gave three small colonies and one wild-type colony (tetratype). Microscopic examination of cells from the small colonies indicated that two spores from each tetrad showed the aberrant morphology of *gpa1*^{Val50} *GVM* or *gpa1*^{Val50} *gvm1-1* mutants, whereas the cells from the other small colonies showed wild-type morphology. Of the small colonies with morphologically aberrant cells, approximately half (52%) showed mating levels similar to the *gpa1*^{Val50} *GVM* mutant and half showed mating similar to the *gpa1*^{Val50} *gvm1-1* double mutant. These results indicated that the *gvm1-1* mutation segregated independently of *GPA1* and resulted in increased mating of the *gpa1*^{Val50} mutant in both *a* and α cells. In addition, the *gvm1-1* mutation itself resulted in slow growth of spore colonies at 30°C.

The phenotypes of single *GPA1 gvm* mutants obtained from nonparental ditype (NPD) tetrads were analyzed. For the *gvm1-1* mutation, the NPD tetrads could be clearly distinguished, because all four spores formed small colonies. The *gpa1*^{Val50} *GVM1* and *GPA1 gvm1-1* spores could be distinguished by two phenotypes: 1) the *gpa1*^{Val50} *GVM1* spores showed the aberrant cell morphology associated with the *gpa1*^{Val50} mutation (Kurjan *et al.*, 1991), whereas *GPA1 gvm1-1* cells showed normal morphology; and 2) the *gpa1*^{Val50} *GVM1* spores formed small colonies after outgrowth, whereas the *GPA1 gvm1-1* spores formed colonies of wild-type size after outgrowth. The *gvm1-1* growth defect was therefore mild, although in transformation experiments, *gvm1-1* transformants grew slowly at 23°C. For the *gvm2-1* and *gvm3-1* mutations, tetrads in which the two *gpa1*^{Val50} spores showed the original mating phenotype were assumed to correspond to NPD tetrads. Growth of the *gvm2-1* and *gvm3-1* mutants was similar to the wild-type strains at all temperatures. No significant differences from the wild-type strains and the *gvm1-1*, *gvm2-1*, and *gvm3-1* mutants were observed in assays of mating (Table 3), pheromone spotting (unpublished results), or *FUS1-LacZ* assays (Figure 5B).

To test dominance, *a/a gpa1*^{Val50}/*gpa1*^{Val50} *GVM/gvm* diploids were transformed with a pGAL-HO plasmid (Herskowitz and Jensen, 1994) and grown in galactose

Figure 5. Phenotypes of *gvm1* and *gvm2* disruptions. (A) Tetrad analysis. *a/a GVM1/gvm1::LEU2* and *GVM2/gvm2::ADE2* diploids were dissected and incubated on YEPD plates at 30°C for 2–3 d. (B) Reporter gene assays. The wild-type strain (W303-1A) and the *gvm1::LEU2* disruption mutant were compared with the original *gvm1-1* mutant. The *gvm2-1* and *gvm3-1* phenotypes were similar to the *gvm1-1* phenotype in the reporter gene assays.

medium to obtain *a/a* and α/α diploids by mating type interconversion. All of the resulting strains showed similar mating levels as *a/a* and α/α *gpa1*^{Val50}/*gpa1*^{Val50} *GVM/GVM* diploids, indicating that *gvm1-1*, *gvm2-1*, and *gvm3-1* were recessive mutations.

To determine whether the *gvm* mutations were allelic, crosses were made between the various *gpa1*^{Val50} *gvm* mutants and the diploids were characterized by tetrad analysis. Tetrad analysis indicated that *gvm3-1* segregated independently of *gvm1-1* (P:N:T = 2:3:13) and of *gvm2-1* (P:N:T = 5:4:14). We were unsuccessful in obtaining diploids in crosses between *gpa1*^{Val50} *gvm1-1* and *gpa1*^{Val50} *gvm2-1* mutants. Results obtained after cloning of the *GVM1* gene, described be-

low, indicated that *gvm1-1* and *gvm2-1* were not allelic; therefore, this screen identified three genes.

Cloning of GVM1

Because the *gvm* mutations were recessive, it was possible to screen a library for transformants that showed the low mating of the original *gpa1^{Val50}* mutant. A potential complication of this screen was that the poor growth of the *gpa1^{Val50}* mutant led to a high frequency of spontaneous suppressors of the growth defect, most of which were sterile (our unpublished results). To avoid the isolation of recessive sterile suppressors, the YCp50 library (Rose *et al.*, 1987) was screened in an *a/a gpa1^{Val50}/gpa1^{Val50} gvm1-1/gvm1-1* diploid (BXK15). Plasmid p70, which resulted in a plasmid-dependent, low mating phenotype, was recovered and transformed into the BXK15 diploid and the *gpa1^{Val50} gvm1-1* haploid. p70 complemented the increased mating phenotype in both strains, although the difference between the p70-containing and the vector-containing strains was more clear in the haploid than in the diploid strain. Transformation of *gvm1-1* cells with p70 gave rise to colonies that appeared at the same time as the wild-type strain at 23°C, in contrast to the slow growth of the *gvm1-1* colonies transformed with the vector, indicating that the plasmid also complemented the mild *gvm1-1* growth defect. p70 did not complement the increased mating phenotype of the *gpa1^{Val50} gvm2-1* or *gpa1^{Val50} gvm3-1* mutants.

To determine whether p70 contained the GVM1 gene, a fragment from the insert was subcloned into an integrating *URA3* plasmid and integrated into an *α gpa1^{Val50} GVM1* strain. This strain was crossed to an *α gpa1^{Val50} gvm1-1* strain and the diploid was subjected to tetrad analysis. In 23 tetrads, the two *Ura⁺* spores showed the low *gpa1^{Val50}* mating phenotype and the two *Ura⁻* spores showed the higher *gpa1^{Val50} gvm1-1* mating phenotype, indicating that integration had occurred within 2.2 centimorgans of the GVM1 locus, consistent with p70 containing the GVM1 gene.

Cloning of GVM2

Attempts to clone GVM2 and GVM3 by the approach described above were unsuccessful. The lethal phenotype of the *gvm1::HIS3 gvm2-1* double mutant, described below, allowed the use of a plasmid shuffle screen (Mann *et al.*, 1987) to clone GVM2. A pRS318 (CEN-LEU2) genomic library was screened in the *gvm1::HIS3 gvm2-1* [YCpGVM1-*URA3*] strain (BXK20). Two 5-FOA-resistant colonies were unable to lose the *LEU2* plasmid, indicating that the ability to grow in the absence of YCpGVM1-*URA3* was plasmid dependent. Plasmids p39 and p101 were recovered from the two transformants and transformed into the *gvm1::HIS3 gvm2-1* [YCpGVM1-*URA3*] strain to confirm the phenotype.

Plasmids isolated in this screen could contain either GVM1 or GVM2. Restriction mapping indicated that the p101 insert overlapped with the YCpGVM1 (p70) insert (Figure 6A), and p101 was able to complement the increased mating phenotype of the *gpa1^{Val50} gvm1-1* mutant but not the *gpa1^{Val50} gvm2-1* mutant. Restriction mapping indicated that p39 did not contain GVM1 (Figure 6B), and p39 was able to complement the increased mating phenotype of the *gpa1^{Val50} gvm2-1* mutant but not the *gpa1^{Val50} gvm1-1* or *gpa1^{Val50} gvm3-1* mutants.

To determine whether p39 contained the GVM2 gene, a fragment from the insert was subcloned into an integrating vector and tested for allelism to *gvm2-1*, as described for GVM1 above. In 30 tetrads tested, the two *Ura⁺* spores showed the low *gpa1^{Val50}* mating phenotype and the two *Ura⁻* spores showed the higher *gpa1^{Val50} gvm2-1* mating phenotype, indicating that integration had occurred within 1.7 centimorgans of GVM2, consistent with p39 containing the GVM2 gene.

Delineation of GVM1 and GVM2 Functional Regions

Restriction mapping indicated that the YCpGVM1 (p70) insert was approximately 15.5 kb. Subcloning and complementation assays of various fragments indicated that the region around the internal 0.9-kb *EcoRI-BamHI* fragment was within the GVM1 functional region (Figure 6A). Partial sequence from the region of the *BamHI* site was obtained and used for a BLAST search (Altschul and Lipman, 1990). The search indicated that this sequence was within the *SON1* gene, which was identified as an extragenic suppressor of a temperature-sensitive *sec63* mutation and could encode a protein of 531 amino acids (Nelson *et al.*, 1993). The *SON1* gene, including the open reading frame and flanking sequences, was amplified from both wild-type and *gvm1-1* strains by PCR and subcloned into a YCp vector. The PCR product from the wild-type strain could complement the *gpa1^{Val50} gvm1-1* mating phenotype, whereas the PCR product from *gvm1-1* could not complement the phenotype, indicating that the *gvm1-1* mutation was in *SON1*.

Restriction mapping indicated that the YCpGVM2 (p39) insert was 5.0 kb. Subcloning and complementation assays of various fragments indicated that one of two *HindIII* sites was within the functional gene (Figure 6B). Sequence extending from both ends of the *HindIII* fragment was obtained and used for a BLAST search (Altschul and Lipman, 1990). The search indicated that the sequence from the left end of the *HindIII* fragment was within the *SEN3* gene, which was identified as being involved in tRNA splicing and could encode a protein of 945 amino acids (DeMarini *et al.*, 1995). The *SEN3* gene, including the open reading frame and flanking sequences, was amplified from the wild-type and *gvm2-1* strains by using PCR and sub-

Figure 6. Restriction maps and functional analysis of GVM1 and GVM2 inserts. The GVM1 (A) and GVM2 (B) inserts are indicated. Various insert fragments were subcloned into YCp plasmids and tested for complementation; for the GVM1 constructs, complementation of the *gpa1^{Val50} gvm1-1* double mutant was tested, and for the GVM2 constructs, complementation of several strains was tested, as described in the text. Regions amplified by PCR are indicated with the region of the open reading frame shaded; the 5' to 3' direction is indicated by arrows. The 2.2-kb GVM1 PCR product includes 322 bp upstream and 321 bp downstream of the open reading frame, respectively, and the 3.2-kb GVM2 PCR product includes 199 bp upstream and 142 bp downstream of the open reading frame, respectively. The *Bgl*III sites at which insertions were made to construct disruption mutations are indicated by open-headed arrows; insertions of *LEU2* and *HIS3* were made in GVM1, and an insertion of *ADE2* was made in GVM2. E, *Eco*RI; B, *Bam*HI; H, *Hind*III; Bg, *Bgl*III; S, *Sal*I; 'P, *Pst*I site from the vector multiple cloning site (MCS); X', *Xba*I site from the MCS.

cloned into YCp vectors. The PCR product from the wild-type strain was able to complement the *gpa1^{Val50} gvm2-1* increased mating phenotype and the *gvm1::HIS3 gvm2-1* growth defect, whereas the PCR product from the *gvm2-1* mutant was unable to complement either *gvm2-1* phenotype. Therefore, the *gvm2-1* mutation was in *SEN3*.

***gvm1* and *gvm2* Null Phenotypes**

To examine the *gvm1* and *gvm2* null phenotypes, gene disruptions were constructed in the wild-type *a/α* diploid (W303) and characterized by tetrad analysis. The *gvm1::LEU2* spores showed the same small colony phenotype as the *gvm1-1* mutant (Figure 5A). Mating (Table 4) and pheromone response as assayed by pheromone spotting (our unpublished results) were wild-type. However, the *gvm1::LEU2* mutant showed 3.5-fold higher induction of *FUS1-LacZ* induction than the wild-type strain (Figure 5B). A *gpa1^{Val50} gvm1::LEU2* double mutant showed the same increased mating phenotype as the original *gpa1^{Val50} gvm1-1* mutant (Tables 3 and 4). An *a/α gvm1::HIS3/gvm1::HIS3* diploid was defective in sporulation.

To determine whether the *gvm1::LEU2* growth defect was due to activation of the pheromone response pathway, disruptions of components of the pathway were tested for the ability to suppress the growth

Table 4. Effect of *gvm1* disruptions on mating

Strain	Mating of wild-type versus <i>gvm1</i> strains ^a		
	GVM1	<i>gvm1</i> ^b	Ratio <i>gvm1</i> /GVM1
GPA1	1.0	0.80	0.8
<i>gpa1^{Val50}</i>	1.4×10^{-4}	2.4×10^{-2}	171.4
<i>gpa1^{Lys355}</i>	3.7×10^{-5}	1.0×10^{-3}	27.0
<i>gpa1^{Lys364}</i>	5.0×10^{-6}	2.4×10^{-5}	4.8
<i>far1::ADE2</i>	8.3×10^{-6}	2.4×10^{-5}	2.9
<i>fus3::LEU2</i>	1.7×10^{-4}	2.0×10^{-2}	11.8
<i>ste20::URA3</i>	4.5×10^{-6}	4.7×10^{-4}	104.4
<i>ste21::URA3</i>	1.8×10^{-2}	4.0×10^{-2}	2.2

^aMating frequencies are calculated relative to the wild-type strain W303-1A.

^b*gvm1* mutant was *gvm1::LEU2* for the wild-type and *gpa1* mutants and *gvm1::HIS3* for the remainder of the mutants.

Table 5. β -Galactosidase activity of ubiquitin system substrates in *gvm* mutants

Construct	β -Galactosidase activity in yeast strains ^a			
	WT	<i>gvm1-1</i>	<i>gvm2-1</i>	<i>gvm3-1</i>
Deg1-LacZ	–	++	+	+
Ub-Leu-LacZ	+	+++	++++	++
Ub-Pro-LacZ	+	+++	++++	++

^a Wild-type and *gvm* strains containing the indicated constructs on plasmids were tested for β -galactosidase activity by the colony lift assay. The Deg1-LacZ construct on plasmid pLR20 was tested in α strains grown on glucose medium, and the other two constructs were tested in α strains grown on galactose medium, because the Ub-X-LacZ constructs were expressed under the *GAL* promoter. –, white; +, blue with the number of plus signs indicating the intensity.

defect. Neither the *ste4::HIS3* nor the *ste12::ADE2* mutation suppressed the *gvm1::LEU2* slow growth of transformants at 23°C.

Attempts to disrupt *SON1/GVM1* in the *gvm2-1* mutant gave rise to very few transformants, suggesting that the double mutation was detrimental. Therefore, to determine the phenotype of the *gvm1::HIS3 gvm2-1* double mutant, an α/α *GVM1/gvm1::HIS3 GVM2/gvm2-1* diploid was constructed and characterized by tetrad analysis. Of 12 tetrads dissected, 3 had two viable spores (both His[–]), 1 had four viable spores (two His⁺ and two His[–]), and 8 had three viable spores (one His⁺ and two His[–]). This segregation pattern indicated that *gvm1* and *gvm2* were unlinked and that the *gvm1::HIS3 gvm2-1* double mutant was inviable. Microscopic examination indicated that the *gvm1::HIS3 gvm2-1* spores went through several rounds of cell division, indicating that the defect was in growth rather than spore germination. The lethality of the *gvm1::HIS3 gvm2-1* double mutant allowed the use of the plasmid shuffle screen described above to clone *SEN3/GVM2*.

Tetrads from the α/α *GVM2/gvm2::ADE2* diploid segregated two viable Ade[–] spores and two inviable spores (Figure 5A), indicating that *SEN3/GVM2* is an essential gene and that the original *gvm2-1* mutation was a partial loss-of-function mutation. Microscopic examination indicated that the *gvm2::ADE2* spores went through several rounds of cell division, similar to the *gvm1::HIS3 gvm2-1* mutant.

Overexpression of *SON1/GVM1* and *SEN3/GVM2*

The effect of *SON1/GVM1* and *SEN3/GVM2* when present on multicopy plasmids was tested in a wild-type α strain (W303-1A). The wild-type strains containing YEp352-GVM1 or YEp352-GVM2 showed mild growth defects. Pheromone spotting assays with the

Figure 7. *GVM1* and *GVM2* overexpression assays. (A) Pheromone spotting assays. The assays were done as in Figure 3, except that the cells were grown on medium selective for the plasmids and the assays were done on selective plates. (B) Reporter gene assays. The assays were done as in Figure 1, except that the *FUS1-lacZ* reporter gene was on pTCFL1.

strains containing YEp352-GVM1 or YEp352-GVM2 showed turbid zones of growth inhibition in comparison to the strain containing the control plasmid (Figure 7A). In assays of the pheromone inducible reporter gene, YEp352-GVM1 and YEp352-GVM2 did not affect basal expression of the reporter construct but resulted in only two- to threefold induction of expression in the presence of pheromone in contrast to approximately 40-fold induction in the strain with the control plasmid (Figure 7B).

The increased induction of the pheromone-inducible reporter gene in the *gvm1::LEU2* mutant and the decreased induction in strains overexpressing *GVM1* or *GVM2* suggested that *GVM1* and *GVM2* negatively regulate the pheromone response pathway. An α *gpa1::LEU2* [pTRP1-MAT α] strain can grow due to

expression of both MAT α and MAT α but cannot lose pTRP1-MAT α due to the haploid-specific *gpa1* growth defect (Hirsch *et al.*, 1991). The a *gpa1::LEU2* [pTRP1-MAT α] strains containing YEp352, YEp352-GVM1, or YEp352-GVM2 were all unable to lose pTRP1-MAT α , indicating that the inhibitory effect of overexpression of GVM1 or GVM2 was not sufficient to suppress the *gpa1::LEU2* growth defect.

Overexpression of GVM1 or GVM2 was also tested for the ability to suppress the other *gvm* mutations as assayed by the higher mating of *gpa1^{Val50} gvm* mutants. YEp352-GVM1 and YEp352-GVM2 complemented the increased mating phenotype in the *gpa1^{Val50} gvm1-1* and *gpa1^{Val50} gvm2-1* double mutants, respectively, but neither plasmid showed an effect in the other double mutant or in the *gpa1^{Val50} gvm3-1* mutant.

The Effect of *gvm1* Disruptions on Mating Is Not Specific to the *gpa1^{Val50}* Mutation

To test for allele specificity, the effect of the *gvm1::LEU2* mutation on mating by the *gpa1^{Lys355}* and *gpa1^{Lys364}* mutants was tested. The *gvm1::LEU2* disruption resulted in increased mating in both mutants, although the effect was milder than observed for the *gpa1^{Val50}* mutant (Table 4).

We also tested whether disruption of GVM1 could suppress the mating defects resulting from mutations in other components of the pheromone response pathway. The *gvm1::LEU2* disruption did not suppress the mating defect of mutations that completely eliminated mating, including several partially dominant GPA1 sterile mutations (*GPA1^{Asn55}*, *GPA1^{Ala322}*, and *GPA1^{Och468}*; Kurjan *et al.*, 1991; Hirsch *et al.*, 1991; Xu, unpublished results), and *ste4::ADE2* or *ste12::ADE2* disruption mutations (our unpublished results). However, the *gvm1::HIS3* disruption was able to increase the mating of mutants that showed partial mating defects (Table 4; Chang and Herskowitz, 1990; Elion *et al.*, 1991; Leberer *et al.*, 1992; Ramer and Davis, 1993; Akada *et al.*, 1996). The degree of suppression of the mating defect varied greatly; strong suppression was seen for *ste20::URA3*, an intermediate effect was seen for *fus3::LEU2*, and very mild suppression was seen for *far1::ADE2* and *ste21::URA3*. The degree of suppression was not correlated with the severity of the mating defect associated with the various mutations or with the level of action of the component in the pheromone response pathway.

The *gvm* Mutants Are Defective in Ubiquitin-mediated Proteolysis

The SON1/GVM1 and SEN3/GVM2 genes have been implicated in ubiquitin-mediated proteolysis (DeMarini *et al.*, 1995; Johnson *et al.*, 1995; Yokota *et al.*, 1996). The Deg1- β gal, Pro- β gal, and Leu- β gal constructs are

subject to ubiquitin-mediated proteolysis through different pathways. The N-terminal ubiquitin portion of the Ub-Leu- β gal fusion is removed by a ubiquitin-C-terminal hydrolase, leaving the destabilizing N-terminal Leu residue, leading to degradation by the N-end rule pathway (Bachmair *et al.*, 1986; Johnson *et al.*, 1995). The ubiquitin-Pro- β gal fusion is not a substrate for ubiquitin-C-terminal hydrolase, but the intact fusion is degraded by the ubiquitin fusion degradation (UFD) pathway. The Deg1- β gal fusion, which contains a region of Mata2p that targets it for proteolysis, is degraded by the Deg pathway (Chen *et al.*, 1993). These three constructs were used to test for defects in the *gvm* mutants. The wild-type strain showed low levels of β -galactosidase activity with all three constructs (Table 5). The *gvm1-1* mutant showed large increases in β -galactosidase activity with all three constructs. The *gvm2-1* mutants showed large increases with the Leu- β gal and Pro- β gal constructs and a more mild increase with the Deg1- β gal construct. The *gvm3-1* mutant showed mild increases in β -galactosidase activity with all three constructs.

DISCUSSION

The *gpa1^{Val50}* mutation was previously shown to lead to a complex set of phenotypes (Miyajima *et al.*, 1989; Kurjan *et al.*, 1991). On the basis of analogous mutations in Ras and G α s, the *gpa1^{Val50}* mutation should lead to a GTPase defect (Barbacid, 1987; Graziano and Gilman, 1989; Masters *et al.*, 1989), which would be predicted to result in constitutive activation of the pheromone response pathway. Some of the *gpa1^{Val50}* phenotypes, the growth defect, cell morphology changes, and high basal expression of a pheromone-inducible reporter gene were consistent with a GTPase defect (Kurjan *et al.*, 1991); however, the *gpa1^{Val50}* mating defect did not seem to be consistent with such a defect.

The *gvm* Mutants Identify Components Involved in Ubiquitin-mediated Proteolysis

One approach to analyze the *gpa1^{Val50}* mating defect further involved a screen for suppressors that resulted in increased mating of the *gpa1^{Val50}* mutant. This screen identified recessive mutations in three genes (GVM1, GVM2, and GVM3). GPA1 *gvm* mutants did not show detectable effects on pheromone response or mating. The *gvm1-1* mutation had mild effects on the growth of spore and transformant colonies, whereas no detectable growth phenotype was detected for the *gvm2-1* and *gvm3-1* mutants. *gvm1* disruption mutants showed the same mild growth defect as the *gvm1-1* mutant; however, the *gvm2::ADE2* disruption mutation was lethal, indicating that the original *gvm2-1* mutation was a partial loss-of-function mutation. The *gvm1::HIS3 gvm2-1* double mutant was invi-

able; this synthetic lethal phenotype suggested that Gvm1p and Gvm2p may be involved in the same process. Unlike the original *gvm1-1* mutant, the *gvm1::LEU2* disruption resulted in a fourfold increase in pheromone induction of the *FUS1-LacZ* reporter gene (Figure 5B). In addition, overexpression of either *GVM1* or *GVM2* inhibited induction of *FUS1-LacZ* by pheromone (Figure 7B). The effects on pheromone induction indicated that Gvm1p and Gvm2p inhibit pheromone response.

The effect of the *gvm1* disruptions on mating was not specific to the *gpa1^{Val50}* mutant. Although it could not suppress the mating defects of complete steriles, it allowed increased mating when combined with several mutations that result in partial mating defects, including other mutations in *GPA1* and mutations in downstream components (Table 4).

Cloning of *GVM1* and *GVM2* indicated that they were identical to previously identified genes, called *SON1* and *SEN3*, respectively (Nelson *et al.*, 1993; DeMarini *et al.*, 1995). Recent results have indicated that both *SON1/GVM1* (also called *UFD5*) and *SEN3/GVM2* are involved in ubiquitin-mediated proteolysis (DeMarini *et al.*, 1995; Johnson *et al.*, 1995; Yokota *et al.*, 1996). Assays with reporter constructs subject to ubiquitin-mediated proteolysis indicate that *GVM3* is also likely to play a role in ubiquitin-mediated proteolysis (Table 5).

The *SEN3/GVM2* gene was originally identified by an effect on the *SEN1* gene, which is necessary for the endonuclease activity of the tRNA splicing machinery (Winey and Culbertson, 1988); the *sen3-1* mutant allowed overexpression of Sen1p (DeMarini *et al.*, 1995). Sen3p is a component of the regulatory complex of the 26S proteasome (Peters *et al.*, 1993; Hochstrasser, 1995; Yokota *et al.*, 1996). The regulatory complex consists of at least 16 subunits; some show sequence similarity to ATPases and others are novel. *GVM2/SEN3* is one of the novel components of the regulatory complex (DeMartino *et al.*, 1994; Yokota *et al.*, 1996).

SON1/GVM1 was originally identified by the isolation of suppressors of a temperature-sensitive *sec63* mutant (Nelson *et al.*, 1993). *SEC63* is involved in translocation of secreted proteins into the endoplasmic reticulum and in localization of proteins to the nucleus. The *UFD5* gene, which was identified by a defect in the UFD pathway, was also shown to be identical to *SON1* (Johnson *et al.*, 1995). Son1p/Ufd5p was shown to function at a postubiquitination step of both the UFD and N-end rule pathways of ubiquitin-mediated proteolysis, and Son1p has been proposed to be a component of the 26S proteasomal regulatory complex (Yokota *et al.*, 1996).

The effects of mutations in *SON1/UFD5/GVM1* and *SEN3/GVM2* on tRNA splicing, nuclear protein import, and pheromone response and mating are likely to be an indirect effect of stabilization of proteins

involved in each of these processes. The stimulatory effects of the *gvm1* and *gvm2* mutations on mating and the inhibitory effect of overexpression of the genes on pheromone response suggest that Gvm1p and Gvm2p act negatively on this process.

The *gvm* mutations result in significant increases in mating in the *gpa1^{Val50}* strain but no increase, and possibly a small decrease, in mating in the *GPA1* strain (Table 3). Because our results indicate that *gpa1^{Val50}* mating occurs primarily through the default pathway, as discussed below, these results may indicate that a positive component(s) of the default mating pathway is a substrate of ubiquitin-mediated proteolysis.

The Low Mating of the *gpa1^{Val50}* Mutant Occurs Primarily Through the Default Mating Pathway

The *gpa1^{Val50}* growth defect, cell morphology changes, and high basal expression of a pheromone-inducible reporter gene were consistent with constitutive activation of the pheromone response pathway (Kurjan *et al.*, 1991). Consistent with this interpretation, epistasis analysis indicated that disruption of a downstream component (the G β subunit Ste4p) eliminated these *gpa1^{Val50}* phenotypes (Figure 1 and Table 2); the *gpa1^{Val50} ste4::ADE2* double mutant showed normal growth and morphology and was defective in response to pheromone. In addition, the *ste4::ADE2* mutation eliminated the low level of *gpa1^{Val50}* mating. Disruption of an upstream component (the α -factor receptor Ste2p) had no effect on the *gpa1^{Val50}* growth and morphology defects or the high basal reporter gene activity, as predicted for constitutive activation of the pathway at the level of the G α subunit. Interestingly, the *ste2::LEU2* mutation did not eliminate the low mating of the *gpa1^{Val50}* mutant. The latter result indicates that the low level of mating of the *gpa1^{Val50}* mutant is independent of exposure to pheromone and, therefore, that it also results from intracellular activation of the pathway.

In previous studies of the *gpa1^{Val50}* mutant (Miyajima *et al.*, 1989), the low mating was proposed to be due to the supersensitivity to pheromone indicated by the large zone of growth arrest in the pheromone spotting assay. In addition, it was proposed that high levels of Gpa1p-GTP due to the expected GTPase defect promoted desensitization to pheromone, consistent with the turbid zone of growth arrest and some other results (Miyajima *et al.*, 1989; Stone and Reed, 1990). Both the *gpa1^{Val50} ste2::LEU2* and *gpa1^{Val50} ste4::ADE2* mutants were defective in response to pheromone and, therefore, do not provide information concerning this pheromone response phenotype. The *gvm* mutations increased the mating of the *gpa1^{Val50}* mutant but did not affect either the growth or pheromone spotting phenotypes. Finally, the *spa2::TRP1* and *sst2::ADE2* mutations did not affect the growth or pheromone spotting phenotypes of the

gpa1^{Val50} mutant, and the *spa2::TRP1* but not the *sst2::ADE2* mutation affected *gpa1*^{Val50} mating. These results indicate that the *gpa1*^{Val50} pheromone spotting phenotype is independent of effects on mating, and its explanation remains enigmatic. It remains possible that this phenotype and some effects of other *GPA1* mutations do reflect a role for Gpa1p-GTP in the promotion of desensitization (Stone and Reed, 1990; Stratton *et al.*, 1996).

The mating frequency of the *gpa1*^{Val50} mutant and other cases in which the pathway is activated intracellularly, e.g., in *ste2* mutants expressing *STE4* or *STE12* under galactose control (Cole *et al.*, 1990; Dolan and Fields, 1990; Nomoto *et al.*, 1990; Whiteway *et al.*, 1990), is much lower than the mating of a wild-type strain. Wild-type strains respond to pheromone primarily through the chemotropic pathway in which cells respond to a spatial signal provided by pheromone and preferentially choose a mating partner that produces the highest level of pheromone (Jackson and Hartwell, 1990a). When cells are exposed to very high levels of pheromone, the chemotropic pathway is saturated and cells instead respond by the default pathway (Dorer *et al.*, 1995). Mating by the default pathway is much less efficient than mating by the chemotropic pathway. In cells in which the pheromone response pathway is activated intracellularly, such as the *gpa1*^{Val50} mutant, the cells cannot be responding to a spatial extracellular signal and, therefore, mating must occur through the default pathway.

To test the hypothesis that *gpa1*^{Val50} mating occurs by the default pathway, we examined the effect on *gpa1*^{Val50} mating of mutations that primarily affect one or the other pathway. *far1* mutations result in chemotropic mating defects (Dorer *et al.*, 1995; Valtz *et al.*, 1995), and a *far1::ADE2* mutation did not affect *gpa1*^{Val50} mating (our unpublished results). *sst2* mutations shift mating from the chemotropic to the default mating pathway, and the *sst2::ADE2* mutation did not affect the growth, pheromone spotting, or mating phenotypes of the *gpa1*^{Val50} mutant (Figure 2 and Table 2; Dietzel and Kurjan, 1987b). In contrast, a *spa2::TRP1* mutation, which has been shown to result in a default mating defect (Dorer *et al.*, 1995), greatly reduced *gpa1*^{Val50} mating. Unlike the *gpa1*^{Val50} *ste4::ADE2* double mutant, however, the *gpa1*^{Val50} *spa2::TRP1* double mutant retained the growth defect and pheromone spotting phenotype of the *gpa1*^{Val50} mutant. The reduction of *gpa1*^{Val50} mating by the *spa2* default pathway mutation but not by the *far1* and *sst2* chemotropic pathway mutations indicates that the low mating of the *gpa1*^{Val50} mutant occurs primarily through the default mating pathway. Thus, our current picture is that the *gpa1*^{Val50} mutation shifts the equilibrium of the G protein toward the Gpa1p-GTP form, due to the proposed GTPase defect. The resulting constitutive intracellular activation of the pathway mimics response to

very high pheromone concentrations, in which cells are unable to discriminate pheromone concentrations, resulting in a shift from the chemotropic to the default mating pathway.

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