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Inhibition of G-Protein Signaling by Dominant Gain-of-Function Mutations in Sst2p, a Pheromone Desensitization Factor in *Saccharomyces cerevisiae*

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Genetic analysis of cell-cell signaling in *Saccharomyces cerevisiae* has led to the identification of a novel factor, known as Sst2p, that promotes recovery after pheromone-induced growth arrest (R. K. Chan and C. A. Otte, Mol. Cell. Biol. 2:11–20, 1982). Loss-of-function mutations lead to increased pheromone sensitivity, but this phenotype is partially suppressed by overexpression of the G protein α subunit gene (*GPA1*). Suppression is allele specific, however, suggesting that there is direct interaction between the two gene products. To test this model directly, we isolated and characterized several dominant gain-of-function mutants of *SST2*. These mutations block the normal pheromone response, including a loss of pheromone-stimulated gene transcription, cell cycle growth arrest, and G protein myristoylation. Although the *SST2* mutations confer a pheromone-resistant phenotype, they do not prevent downstream activation by overexpression of Gβ (*STE4*), a constitutively active Gβ mutation (*$STE4^{Hpl}$*), or a disruption of *GPA1*. None of the *SST2* alleles affects the expression or stability of Gα. These results point to the G protein α subunit as being the direct target of Sst2p action and underscore the importance of this novel desensitization factor in G-protein-mediated signaling.

The yeast *Saccharomyces cerevisiae* is a unicellular eukaryote that can grow vegetatively either as a diploid or as one of two haploid cell types, known as the mating types **a** and α. Haploid cells secrete specific peptide pheromones (**a** and α mating factors) that act on cells of the opposite type to promote cell fusion, leading to the formation of an **a**/α diploid (28, 46).

Pheromone signaling is mediated through receptors of the seven-transmembrane-segment class (other examples include the β-adrenergic receptor and rhodopsin) and is coupled to downstream events via a guanine nucleotide-binding regulatory protein (G protein) (28). Agonist binding to these receptors promotes exchange of GDP for GTP on the G-protein α subunit and dissociation of Gα from the G protein β and γ subunits (4, 6, 11). In yeast cells, it is the β/γ moiety that activates downstream signaling events leading to mating, which include alterations in gene transcription, morphological and cytoskeletal changes, and growth arrest in the G_1 phase of the cell cycle. Indeed, overexpression of the β subunit or loss of the α subunit leads to mating even in the absence of pheromone or receptor (13, 17, 25, 36, 39, 53).

A property of signal-response systems in general, and of G-protein-coupled receptors in particular, is that prolonged stimulation often results in a loss of responsiveness over time (desensitization) (23). The molecular basis for this phenomenon has been extensively characterized for vertebrate G-protein-coupled receptors (2, 32) and has also been described for *S. cerevisiae* (3, 8–10, 12, 16, 19, 27, 33, 37, 40, 41, 47). Despite the profound physiological and morphological changes that yeast cells undergo in preparation for mating, cells that fail to mate become desensitized to the continued presence of pheromone and eventually resume growth as normal haploids (recovery).

Genetic analysis of the yeast mating response pathway has led to the identification of several mutations that affect pheromone sensitivity and desensitization (3, 8–10, 12, 16, 27, 29, 35, 37, 40, 41, 47, 49). One such mutation in the *SST2* gene (supersensitivity to pheromone), *sst2-1*, has no effect on normal cell growth but prevents recovery from pheromone-induced growth arrest (8, 9, 16). Moreover, *SST2* is expressed only in pheromone-responsive (haploid) cells, and expression is strongly induced by pheromone treatment (16, 18a). These observations suggest that *SST2* encodes a key regulatory component of the pheromone desensitization pathway. Recently, Madura and Varshavsky have reported that *SST2* promotes degradation of Gpa1p via the N-end rule pathway, resulting in pheromone-independent growth arrest (34).

Despite its critical role in pheromone desensitization, the point at which *SST2* regulates the signal transduction cascade is not known. Here, we describe the isolation and characterization of a novel class of *SST2* mutations that inhibit receptor-mediated signaling. A detailed genetic analysis of these and other alleles of *SST2* reveals that Sst2p controls pheromone signaling through direct interactions with the G protein, Gpa1p.

MATERIALS AND METHODS

Strains, media, and transformation. The *S. cerevisiae* strains used in this study were RK512-5B (*MAT**a** ura3-52 his3-Δ1 ade2-1^{oc} sst2-1*), BC138 (*MAT**a** leu2-3,112 ura3-52 ade2 sst2-4^{oc}*), BC159 (*MAT**a** leu2-3,112 ura3-52 his3-Δ1 ade2-1^{oc}*), BC180 (*MAT**a** leu2-3,112 ura3-52 his3-Δ1 ade2-1^{oc} sst2-Δ2*) (15, 18a), YPH499 (*MAT**a** ura3-52 lys2-801^{am} ade2-101^{oc} trp1-Δ63 his3-Δ200 leu2-Δ1*) (44), YDM400 (YPH499 *sst2-Δ2*) (from J. Thorner), YDK499 (YPH499 *bar1::hisG*) (from J. Thorner), YDK499-βgal (YDK499 *FUS1-lacZ*) (transformed with the integrating plasmid pSB286, containing the bacterial *lacZ* gene under the control of the pheromone-inducible *FUS1* promoter and *URA3* as a selectable marker; from J. Trueheart) (50), YGS5 (YPHY99 *gpa1::hisG ste11^{ts}*) (this work), DJ706-2-4 (*MAT**a** cry1 ade2-1 his4-580 lys2 trp1 try1 SUP4-3 leu2 ura3 ste5-3 $STE4^{Hpl}$*) (from D. Jenness) (3), and DC17 (*MATα his1*). *Escherichia coli* DH5α was used

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for the maintenance of plasmids. Strain MH1066 (Δ*lacX74 hsr rpsL pyrF*::Tn*5 leuB600 trpC9830 galE galK*; from J. Boeke) was used to determine *LEU2*-complementing activity (see below). Standard methods for the growth and maintenance of yeast cells (21) and bacteria (1) were used. Yeast strains were grown in rich medium (YPD) or synthetic medium supplemented with amino acids, uracil, adenine, and 2% glucose (SCD) or 2% galactose plus 0.2% sucrose (SCG); nutrients were omitted to maintain selection for plasmids as necessary (e.g., SCD-Leu is SCD lacking leucine).

Plasmid construction and mutagenesis. Standard methods for the manipulation of DNA were used throughout (1). The plasmids used in this study were pG1501, pG1501Δ, pRS315, pRS316, pAD4M, pRS315-GAL, pRS316-GAL, pRS315-SST2, pRS315-GAL-SST2, pRS316-GAL-STE4, pAD4M-SST2, and pAD4M-GPA1. pG1501 is a centromere-based shuttle vector containing *URA3* as a selectable marker, the *GAL1* promoter, *GPA1*, and the transcription termination region from *TRP5* (35). pG1501Δ is identical to pG1501 but lacks the *GPA1* gene (this work). pRS315 is a *CEN* vector derived from pBluescript (Stratagene) bearing the *LEU2* marker (44). pRS315-SST2 contains the 4.5-kb *Hin*dIII-*Hin*dIII fragment of the *SST2* gene in pRS315 (16). pRS315-GAL and pRS316-GAL contain the *Eco*RI-*Bam*HI fragment of the *GAL1/10* promoter at the corresponding sites within pRS315 and pRS316, respectively. pRS315-GAL-SST2 contains *GAL1/10* followed by the 3.6-kb *SST2 Bam*HI-*Hin*dIII fragment (mutant *Bam*HI site at bp −33 relative to the initiator AUG) and a duplication of the *Hin*dIII-*Eco*RI cloning region from pBluescript. pRS316-GAL-STE4 was constructed by ligation of the *Eco*RI-*Sal*I fragment from pL19 (containing *GAL1/10* and *STE4*; from M. Whiteway [53]) into pRS316. pAD4M is a 2μm vector containing *LEU2* and the *ADH1* promoter and terminator (from P. McCabe, Cetus Corp.). pAD4M-GPA1 was constructed by ligation of a *Sma*I-*Sac*I product of *GPA1* in pBluescript (containing the *Xba*I-*Xba*I fragment from pG1501 [35]) into the *Sma*I and *Sac*I sites of pAD4M. pAD4M-SST2 was constructed by digestion of *SST2* in pBluescript (*Hin*dIII-*Hin*dIII fragment) with *Sal*I (mutant site at bp −55 relative to the initiator ATG) and *Sac*I and ligation into the corresponding sites of pAD4M. The *SST2* mutations were obtained by treating pRS315-GAL-SST2 with hydroxylamine and transforming *E. coli* MH1066 (to 96% survival; determined by replica plating to M9 medium lacking leucine, as described previously) (43). Mutagenized plasmid DNA was prepared by scraping cells grown on ampicillin-containing LB plates, purified (Qiagen), and used to transform YDK499 by the method of Schiestl and Gietz (42).

Growth inhibition by α-factor. To identify mutations in *SST2* that confer resistance to α-factor, strain YDK499 was transformed with the library of mutagenized pRS315-GAL-SST2 plasmids and plated on SCG-Leu plus 2% agar (three 100-mm petri dishes; 16,000 colonies total). After 3 days, the colonies were replica stamped to SCG-Leu plates (~30 ml) containing 300 μg of synthetic α-factor (~6 μM concentration). Then 48 colonies were restreaked on SCG-Leu plates containing 100 μg (~2 μM) of α-factor and, after 3 days, were replica stamped to SCG-Leu and SCD-Leu plates containing 100 μg of α-factor. Of the 48 α-factor-resistant colonies, 5 appeared to be dependent on galactose induction. To verify that α-factor resistance was conferred by the plasmid, episomal DNA was prepared from all five colonies, amplified in *E. coli*, and used to retransform YDK499 (24). Of the five plasmids tested, three were sufficiently α-factor resistant to warrant further study. All cultures exposed to α-factor were maintained at 24°C. However, none of the mutants were temperature dependent (tested at 24, 30, and 34°C), and all subsequent experiments were performed at 30°C.

The growth inhibition assay (halo assay) was described previously (21, 45). Briefly, cells were transformed with either pG1501, pG1501Δ, pRS315, pRS315-GAL, pRS315-SST2, or pRS315-GAL-SST2 (including mutant alleles). An overnight culture of each transformant (in SCD-Leu or SCD-Ura) was diluted (1:40) into 2 ml of SCD-Leu, SCG-Leu, or SCG-Ura, followed by the addition of an equal volume of 1% (wt/vol) dissolved agar (50°C), and spread onto a culture dish of the same medium. Sterile filter disks were spotted with synthetic α-factor (0.5 or 1.5 μg for YDK499; 5 or 15 μg for YPH499, and YDM400; 0.3, 1, or 3 μg for all other strains) and placed on the nascent lawn. The resulting halo of growth-arrested cells surrounding the source of α-factor was photographed after 3 days at 30°C.

Growth in the presence of pRS316-GAL or pRS316-GAL-STE4 (coexpressed with pRS315-GAL-SST2 plasmids in YDK499) was determined by spotting 10 μl of an overnight culture (in SCD-Ura-Leu) onto SCD-Ura-Leu or SCG-Ura-Leu plates and streaking out the nascent colonies. In some cases, a 600 nM concentration of α-factor was added to the plates, as indicated. The growth of strains DJ706-2-4 and YGS5 carrying pRS315-GAL or pRS315-GAL containing wild-type or mutant *SST2* was determined in the same manner except that these temperature-sensitive strains were grown initially at the permissive temperature and then spotted to SCG-Leu plates at either the restrictive or permissive temperature, as indicated. The resulting colony growth was documented after 3 days.

DNA sequencing. Base substitutions were determined by oligonucleotide-primed double-stranded DNA sequencing (US Biochemicals Sequenase version 2.0, used according to the manufacturer's instructions). All of the sequences match that of the previously published wild-type *SST2* sequence (16), except for the following codon substitutions: CCG→CTG, encoding a Pro-for-Leu substitution at position 20 (*SST*2-1, *SST*2-2, and *SST*2-3), and GGA→AGA, encoding a Gly-for-Arg substitution at position 22 (*SST*2-2 and *SST*2-3). Thus, *SST*2-2 and *SST*2-3 are identical within their coding regions. In addition, the wild type and all three mutants differed from the published sequence at codons 615 and 616, where ACC GAT (Thr-Asp) was replaced by ACG CAT (Thr-His).

Immunoblot analysis. Strains YDK499, YPH499, and YDM400 were transformed with plasmid pRS315-GAL or pAD4M containing either no insert, wild-type *SST2*, or mutant *SST2* alleles. Transformed cells were grown in SCD-Leu or SCG-Leu medium, as indicated. In some cases, cells were treated with 5 μM synthetic α-factor for 1 h. Cell extracts from strains RK512-5B, BC138, BC159, and BC180 were prepared in the same manner except that cells were not transformed and were maintained in rich medium (YPD). To monitor the loss of Gpa1p over time, cells were treated with cycloheximide (10 μg/ml in 0.1% ethanol, final concentrations) for 1 to 4 h. In the course of membrane fractionation and immunoprecipitation studies, we have found Gpa1p to be difficult to solubilize and susceptible to degradation following cell lysis (18a). Therefore, an advantage of this method is that treated cells are lysed directly in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 100°C (see below), resulting in complete solubilization and protease inactivation, allowing total cellular Gpa1p to be determined by immunoblotting. Cell growth was stopped at mid-logarithmic phase by the addition of NaN_3 (10 mM final) and chilling, and the cells were harvested by centrifugation (4°C). Cells were resuspended in 5 mM NaN_3, and equivalent numbers of cells were transferred to 1.5-ml Eppendorf tubes and centrifuged briefly. The resulting cell pellets were resuspended in a fourfold excess volume of SDS-PAGE lysis buffer (0.05 M Tris-HCl [pH 6.8], 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.0015% bromophenol blue), boiled for 10 min, and subjected to Vortex homogenization with glass beads (0.50 mm) for 4 min. The resulting extracts were then resolved by SDS-PAGE with a Bio-Rad MiniProtean apparatus according to the manufacturer's instructions, electrophoretically transferred to nitrocellulose, and probed with polyclonal anti-Sst2p or anti-Gpa1p antiserum (18, 18a) and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; Bio-Rad) (1). The second antibody was detected by using Kodak XAR5 film and the Amersham enhanced chemiluminescence detection reagent, with the transfer, hybridization, and wash conditions recommended by the manufacturer.

Transcription analysis. Strain YDK499-βgal was transformed with plasmid pRS315-GAL containing no insert, wild-type *SST2*, or the mutant alleles of *SST2*. Cells were grown to mid-log phase in SCG-Leu medium and treated with 1 to 300 nM α-factor for 90 min. Cells (1.4 ml) were harvested and resuspended in 1 ml of Z buffer (0.1 M $NaPO_4$ [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol) with 0.5% (vol/vol) chloroform and 0.005% (wt/vol) SDS. After 15 min at 30°C, 0.2 ml of the *o*-nitrophenyl-β-D-galactoside substrate in Z buffer was added (0.225 mM final concentration). After 90 min, the reaction was stopped with the addition of 0.5 ml Na_2CO_3 (0.4 M final concentration). β-Galactosidase activity units are expressed as the ratio of *o*-nitrophenol product to cell density determined colorimetrically (optical density at 420 nm [OD_{420}]/OD_{600}), as described by Guarente (20).

Analysis of mating competence. Quantitative mating assays were performed as described previously (45). Briefly, *MAT***a** and *MAT*α cells were grown to mid-log phase in SCG-Leu, mixed at a ratio of 10:1 with the DC17 tester strain in excess, and collected onto sterile membranes by vacuum filtration. The filtered cells were placed on a YPG plate overnight, resuspended in water, and plated to various dilutions on synthetic medium containing 2% galactose and 0.2% sucrose but lacking amino acids and uracil to select for diploids (SG medium). The relative mating efficiencies of strains bearing the *SST2* mutations in pRS315-GAL were calculated by counting diploids and are expressed relative to that of wild-type (pRS315-GAL-SST2-transformed) cells.

RESULTS

Overexpression of *GPA1* restores recovery in *sst2* mutants. To characterize the role of *SST2* in pheromone desensitization, we used the growth inhibition assay (halo assay) to compare the effects of *GPA1* overexpression on a variety of known *sst2* mutants (21, 26). In this procedure, cells are plated on solid medium and exposed to α-mating factor spotted on sterile filter disks. Differences in the zone of inhibition (halo size and turbidity) are indicative of a particular strain's ability to arrest, desensitize, and resume growth within the time period of the assay (typically 2 to 5 days).

Four strains were tested: one in which a complete disruption of *SST2* had been generated in vitro (*sst2*-Δ*2*), two carrying alleles previously isolated in a genetic screen for supersensitivity (*sst2-1* and *sst2-4*), and a wild-type strain (9, 15). As shown in Fig. 1A, wild-type cells exhibit small turbid halos that rapidly fill in, the *sst2-1* (missense) and *sst2*-Δ*2* (disruption) mutants yield large clear halos of similar size that never fill in, and the *sst2-4* (nonsense) mutant exhibits clear halos of intermediate size. Immunoblot analysis reveals that *sst2-1* cells pro-

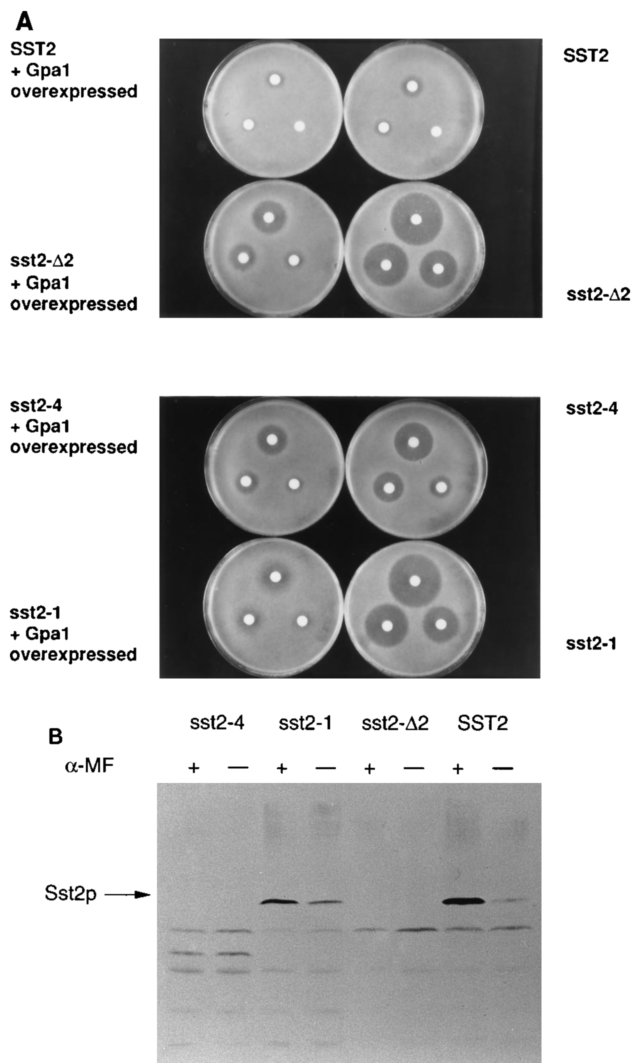

FIG. 1. Allele-dependent suppression of *sst2* mutations by *GPA1*. (A) Strains carrying various alleles of *SST2* were transformed with a high-copy vector containing *GPA1* under control of the *GAL1/10* promoter (pG1501; Gpa1 overexpressed) or the parent vector alone (right). The strains either have a complete disruption of *SST2* generated in vitro (*sst2-Δ2*) or express either the wild-type *SST2* gene (SST2) or one of two missense alleles previously isolated in a genetic screen for supersensitivity (*sst2-1* or *sst2-4*). Individual transformants were plated as a nascent lawn on galactose selective plates, exposed to α-factor, and photographed after 3 days at 30°C. The order of sensitivity is altered by *GPA1* overexpression in an *sst2* allele-specific manner, as indicated by relative halo size: *sst2-Δ2* = *sst2-1* > *sst2-4* >> *SST2* (right) versus *sst2-Δ2* + Gpa1 overexpressed > *sst2-4* + Gpa1 overexpressed > *sst2-1* + Gpa1 overexpressed >> *SST2* + Gpa1 overexpressed (left). (B) Immunoblotting was used to detect the wild-type (*SST2*) and any expressed mutant (*sst2-1*, *sst2-4*, and *sst2-Δ2*) gene products, as detailed under Materials and Methods. Cells were grown in YPD medium to the mid-logarithmic phase, collected, homogenized, and resolved by SDS-PAGE and electrophoretic transfer to nitrocellulose. The anti-Sst2p antiserum detects a single major band of ~82 kDa, corresponding to the *SST2* gene product (Sst2p), and is induced by pheromone (α-mating factor, α-MF) addition (5 μM, 1 h). These data indicate that the *sst2-1* mutant product is expressed as a full-length product.

duce a specific product of the same molecular weight as Sst2p, indicating that the mutant gene is expressed and may retain some function (Fig. 1B).

When *GPA1* is overexpressed in these strains, smaller and more turbid halos are observed in every case, indicating an enhanced ability to desensitize (Fig. 1A, left). One interpretation of this effect is that increased Gpa1p expression leads to sequestration of β/γ and a reduced overall response to pheromone, independent of any effects of *SST2* (17, 28).

However, the data in Fig. 1A also reveal that suppression is allele dependent with regard to *SST2*. Whereas the *sst2-1* and *sst2-Δ2* mutants normally respond equally to pheromone, the *sst2-1* mutant becomes much less sensitive when *GPA1* is overexpressed. This difference is difficult to explain simply in terms of the β/γ sequestration model described above. Rather, we favor an alternative model in which Sst2p and Gpa1p interact to promote desensitization. The *sst2-1* gene product may be partially unfolded or mislocalized under normal circumstances but can form a stable or functional complex when Gpa1p is overexpressed.

Isolation of dominant inhibitory *SST2* mutations. Allele-specific suppression of *sst2* by *GPA1* suggests that the products of these genes interact. Thus, we devised a genetic strategy to test this model directly. We reasoned that if loss-of-function mutations in *SST2* amplify pheromone signaling, gain-of-function mutations that block signaling in a dominant manner could be obtained. By activating the signal at various points throughout the pathway, the dominant inhibitory mutations could then be used to establish where Sst2p operates. If Sst2p indeed acts directly on the G protein, gain-of-function mutations should block signaling through the receptor but not activating mutations in the G protein. An added advantage is that sequencing and phenotypic characterization of dominant gene mutations can provide useful information about structure-function relationships that is not available from simple gene knockout or overexpression experiments.

The following strategy was used to identify *SST2* mutations that inhibit pheromone signaling. Strain YDK499 was transformed with a hydroxylamine-mutagenized plasmid containing *SST2* under the control of the *GAL1* promoter (pRS315-GAL-SST2), as described in Materials and Methods. After transformation and several days of growth on galactose-containing medium, the colonies were replica stamped to plates containing very high (normally lethal) concentrations of α-factor (~6 μM). To verify that any surviving α-factor-resistant colonies were dependent on *SST2* expression, they were restreaked on α-factor-containing plates and replica stamped to dextrose- or galactose-containing medium (to repress or induce gene expression, respectively) in the presence of α-factor. To verify that growth was conferred by the plasmid, episomal DNA was prepared from all surviving (galactose-dependent) colonies, amplified in *E. coli*, and used to retransform the original YDK499 strain. Plasmids pRS315-GAL (lacking the *SST2* gene) and pRS315-GAL-SST2 (wild-type *SST2* gene) were used as negative controls. After these manipulations, three clones were sufficiently α-factor resistant to warrant further study and were designated *SST*2-1, *SST*2-2, and *SST*2-3.

Sequencing of *SST2* mutations. To determine the structural basis for the change in α-factor sensitivity, the entire *SST2* coding region of the wild type and clones *SST*2-1, *SST*2-2, and *SST*2-3 was sequenced as described in Materials and Methods. All three mutants differ from the wild type at codon 20, where CCG (Pro) was replaced with CTG (Leu). In addition, *SST*2-2 and *SST*2-3 carry a substitution at codon 22, where GGA (Gly) has been changed to AGA (Arg). Both missense mutations are likely due to hydroxylamine treatment, which hydroxylates cytidine and leads to mispairing with adenine (43).

***SST2* mutations prevent α-factor-dependent growth arrest.** In order to characterize the role of *SST2* in desensitization, pheromone sensitivity was evaluated by the halo assay. Whereas wild-type cells normally exhibit small turbid halos that rapidly fill in (consistent with their ability to desensitize,

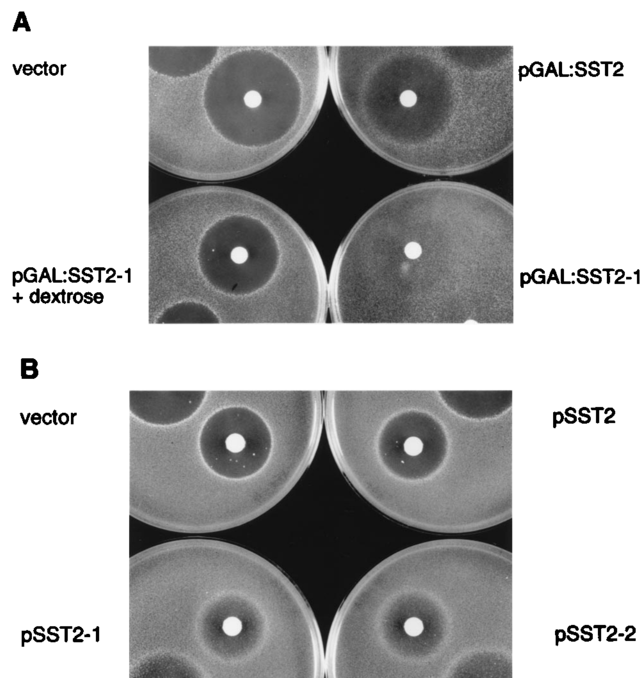

FIG. 2. *SST2* suppression of α-factor-induced growth arrest. The halo assay was used to evaluate the ability of wild-type and pheromone-unresponsive *SST2* mutants to respond and desensitize to α-factor. (A) As detailed under Materials and Methods, plasmid pRS315-GAL (vector) or pRS315-GAL containing *SST2* (pGAL:SST2) or *SST*2-1 (pGAL:SST2-1) was introduced into strain YDK499. Transformed cells were plated as a nascent lawn on selective plates containing galactose or dextrose, as indicated. Lawns were exposed to α-factor and photographed after 3 days at 30°C. Pheromone responsiveness was reduced by *SST2* and blocked by SST2-1 overexpression, as indicated by relative halo size and turbidity. (B) To determine if overexpression contributes to the loss of pheromone signaling, mutations were expressed from a single-copy plasmid with the wild-type *SST2* promoter. Compared with the wild type, *SST*2-1 and *SST*2-2 yield slightly smaller and considerably more turbid halos (pSST2 versus pSST2-1 and pSST2-2), indicating that the *SST2* mutations must be overexpressed to fully block signaling.

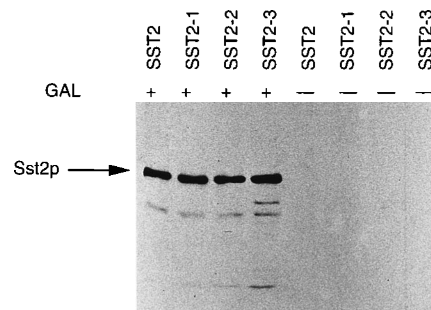

FIG. 3. Immunodetection of Sst2p. Immunoblotting was used to detect the wild-type (*SST2*) and mutant products, as detailed under Materials and Methods. Cells were grown in dextrose (lanes —) or galactose (lanes +) to mid-log phase, collected, homogenized, and resolved by SDS-PAGE and electrophoretic transfer to nitrocellulose. The anti-Sst2p antiserum detects a single major band of ~82 kDa, corresponding to the *SST2* gene product (Sst2p), and is dependent on galactose induction. These data indicate that the wild-type and *SST2* mutant products are expressed at comparable levels following galactose induction.

Fig. 1A), YDK499 exhibits large clear halos that do not fill in (because of the absence of *BAR1*, the α-factor-specific protease) (8–10, 33). To avoid the confounding effects of *BAR1* expression, strain YDK499 was used for most of the experiments described below.

When overexpressed in YDK499, wild-type *SST2* yields slightly smaller and more turbid halos, consistent with its role in mediating desensitization (Fig. 2A, pGAL:SST2 versus vector). Transformation with *SST*2-1 yields extremely turbid halos, indicating a greatly enhanced ability to desensitize (pGAL: SST2-1). This effect is absolutely dependent on galactose induction, since it is blocked by growth in dextrose (pGAL: SST2-1 + dextrose). Identical results were obtained for *SST*2-2 and *SST*2-3 (data not shown). Loss of α-factor sensitivity was also observed with all three mutant alleles expressed in a *BAR1* (YPH499) strain as well as in an *sst2*-Δ*2* (YDM400) mutant, as expected for a true dominant allele (data not shown).

The *SST2* mutations were isolated based on their ability to block pheromone-induced growth arrest when overexpressed under control of the galactose-inducible *GAL1* promoter. Accordingly, we tested whether "normal" levels of expression would also confer α-factor resistance. As shown in Fig. 2B, we performed halo assays on each of the mutants expressed from a single-copy plasmid containing the wild-type *SST2* promoter. Compared with the wild type, *SST*2-1 and *SST*2-2 yield smaller and considerably more turbid halos (Fig. 2B, pSST2 versus pSST2-1 and pSST2-2). Identical results were obtained for *SST*2-3 (data not shown). These data indicate that the Pro-20 to Leu mutation in Sst2p results in a greatly attenuated pheromone response but that overexpression is required to fully block signaling.

Equal expression of wild-type and mutant alleles of *SST2*. As shown in Fig. 2, overexpression of wild-type *SST2* can promote recovery from pheromone-induced growth arrest, as manifested by more turbid halos after prolonged exposure to α-factor. To rule out the possibility that the pheromone-resistant phenotype displayed by the *SST2* mutants results from gross overexpression (e.g., due to a mutation that stabilizes mRNA or protein), expression of the *SST2* products was evaluated by immunoblotting.

Transformed yeast cultures were grown in either dextrose or galactose, harvested, and prepared for SDS-PAGE and immunoblotting as detailed under Materials and Methods. As shown in Fig. 3, anti-Sst2p antibodies detect a prominent protein species of ~82 kDa. Moreover, all three mutant products are expressed at levels comparable to wild-type Sst2p, and expression is dependent on galactose induction (Fig. 3). Untransformed cells treated with α-factor also express an 82-kDa *SST2* product which is absent in the *sst2*-Δ*2* mutant strain (Fig. 1).

***SST2* mutations attenuate pheromone-dependent gene induction.** There are several caveats to using the halo assay described above for measuring the response to α-factor. For example, the effects on growth are measured over several days and may not reflect short-term changes in pheromone responsiveness. Moreover, differences in "turbidity" are difficult to quantitate. One short-term assay that is amenable to quantitation is pheromone-dependent gene transcription. This is conveniently done with a reporter gene (β-galactosidase) under the control of a pheromone-responsive gene promoter (from *FUS1*) (45, 50).

As shown in Fig. 4, we used the β-galactosidase assay to test the ability of the *SST2* alleles to attenuate pheromone-dependent gene transcription. Strain YDK499-βgal was transformed with either vector alone, wild-type *SST2*, or mutant *SST2* plasmids, as detailed under Materials and Methods. Cells were treated for 90 min with α-factor at concentrations ranging from 1 to 300 nM. Compared with cells transformed with the vector alone, overexpression of wild-type *SST2* leads to a modest reduction in β-galactosidase induction, with a <2-fold change in the 50% effective concentration (EC_{50}) for the ligand.

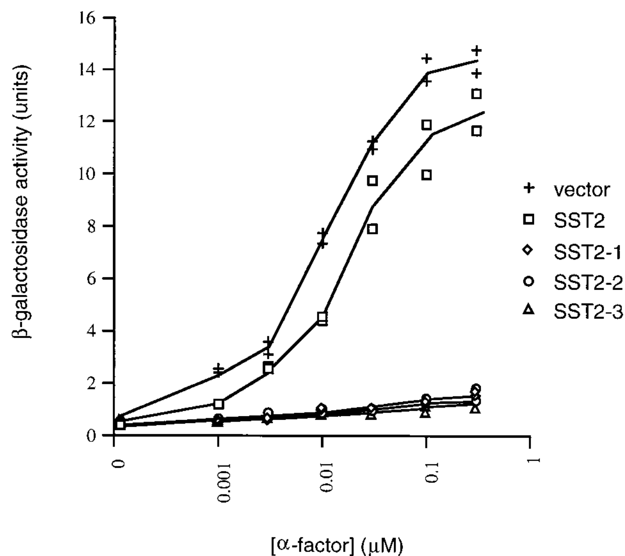

FIG. 4. β-Galactosidase induction in cells overexpressing wild-type and mutant alleles of *SST2*. Strain YDK499 (*bar1*Δ) was transformed with the integrating plasmid pSB286, containing the bacterial *lacZ* gene under the control of the pheromone-inducible *FUS1* promoter, followed by transformation with pRS315-GAL alone (vector) or containing wild-type *SST2* or the *SST2* mutant *SST*2-1, *SST*2-2, or *SST*2-3. Cells were grown to mid-logarithmic phase in galactose-containing medium and treated with 1 to 300 nM α-factor for 90 min, as indicated. Cells were harvested and tested for induction of β-galactosidase activity, as detailed under Materials and Methods. β-Galactosidase units are expressed as the ratio of *o*-nitrophenol product to cell density (determined colorimetrically; OD_{420}/OD_{600}). Individual data points (duplicates) were plotted, and an interpolating line was drawn for each series. The experiment is representative of results obtained from two independent transformants. α-Factor sensitivity is reduced by the overexpression of *SST2* and is abolished by the *SST2* mutations.

Moreover, overexpression of mutant *SST2* leads to a virtually complete loss of β-galactosidase induction (Fig. 4), which is evident even at very high α-factor concentrations (5 μM) (data not shown). This reduction in the biological efficacy but not the potency of the agonist is typical of what is observed after desensitization of mammalian G-protein-coupled receptors (2, 23, 32).

SST2* mutations do not prevent mating.** A number of recessive mutations that block pheromone signaling have been described previously. Many of these result in a pheromone-unresponsive phenotype and an inability to mate (sterile phenotype). These include genes for the mating factor receptors (*STE2* and *STE3*) (7, 22, 38) and the G protein β and γ (*STE4* and *STE18*) subunits (52). To determine whether cells carrying the dominant *SST2* mutations are similarly impaired in their ability to mate, a quantitative mating assay was performed (Table 1). Transformed *MATa** cells (YDK499) were mixed with a 10-fold excess of a *MAT*α tester strain, DC17, and plated on SG medium to select diploids, as described under Materials and Methods. The relative mating efficiencies of cells bearing the mutant *SST2* alleles (or vector alone) were calculated relative to those of cells transformed with wild-type *SST2*. Since *bar1* mutants mate with a reduced efficiency relative to wild-type cells, we also tested an isogenic *BAR1* strain, with similar results (YPH499, Table 1).

Despite the profound loss of pheromone responsiveness observed in other assays (Fig. 2 and 4), the mating efficiencies of cells bearing the *SST2* mutations were comparable to that of the wild type, within 30% in every case (Table 1). Although these results seem paradoxical, they were not entirely unexpected. An *sst2-1* mutant strain was reported to be ~100-fold more sensitive than the wild type by the halo assay, but 10- to 30-fold less likely to mate (9). Thus, the dominant inhibitory alleles of *SST2* appear to have a phenotype opposite that of *sst2*, exhibiting a greatly reduced sensitivity to pheromone but an unimpaired ability to mate.

TABLE 1. Mating efficiencies[a]

Plasmid	Relative mating efficiency (%)	
	YDK499	YPH499
Vector	88	102
SST2 (wild type)	100	100
SST2-1	79	81
SST2-2	74	ND[b]
SST2-3	75	69

[a] Mating efficiencies for *SST2* mutants in the vector pRS315-GAL, expressed in strain YPH499 or YDK499 (*bar1*). Values represent the number of diploids formed as a percentage of the wild-type number (pRS315-GAL-SST2). Values are averages from two independent experiments, in which >250 colonies were counted.

[b] ND, not determined.

***SST2* mutations do not prevent growth arrest following direct G-protein activation.** Any mutation that compensates for some other mutation, resulting in a normal phenotype in the double mutant, is known as a suppressor allele. Suppressor alleles can be extremely useful in ordering components in a signaling pathway. Accordingly, we examined whether the *SST2* mutations could suppress signaling by the G protein as well as the receptor. If Sst2p interacts directly with Gpa1p, our gain-of-function mutations should not inhibit signaling in the absence of protein (*gpa1*Δ mutant) or if signal regulation is subverted by overexpression or constitutively active mutants of β/γ.

As shown in Fig. 5A, strain YDK499 was cotransformed with plasmids containing *STE4* and either wild-type or mutant *SST2*, each under the control of the *GAL* promoter. Cells were first grown in dextrose-containing liquid cultures and then spotted and streaked onto galactose-containing medium (in some cases supplemented with α-factor). As anticipated, galactose induction of *STE4* expression (Fig. 5A, galactose, octants 1 to 4) and treatment with high concentrations of pheromone (galactose + α-factor) led to permanent growth arrest. However, whereas the *SST2* mutations restored growth in α-factor-containing medium (Fig. 5A, galactose + α-factor, octants 5 to 7), they were not able to restore growth in Gβ-overexpressing cells, whether or not α-factor was present (Fig. 5A, galactose and galactose + α-factor, octants 2 to 4). The *SST2* mutations also conferred α-factor resistance in cells carrying a second plasmid with the *GAL* promoter but lacking *STE4* (octants 5 to 7 versus octant 8, pGAL:vector), ruling out any confounding effects of competition for promoter binding.

Suppression of receptor-mediated but not β/γ-mediated growth arrest indicates that *SST2* most likely regulates signaling at the level of the receptor or G protein. The experiments shown in Fig. 5B and C were designed to rule out another possibility, that overexpressed Ste4p competes with Sst2p for binding to a downstream effector molecule. Accordingly, we expressed wild-type *SST2* or *SST*2-1 or *SST*2-2 in a strain (DJ706-2-4) carrying a single genomic copy of $STE4^{Hpl}$. $STE4^{Hpl}$ encodes a dominant constitutively active Gβ mutation that leads to pheromone-independent signaling and growth arrest. Strain DJ706-2-4 also carries a downstream temperature-sensitive mutation in *STE5* (*ste5-3*), which blocks signaling by $STE4^{Hpl}$ when maintained at 34°C (the permissive temperature) but not at 24°C (restrictive temperature). As shown in

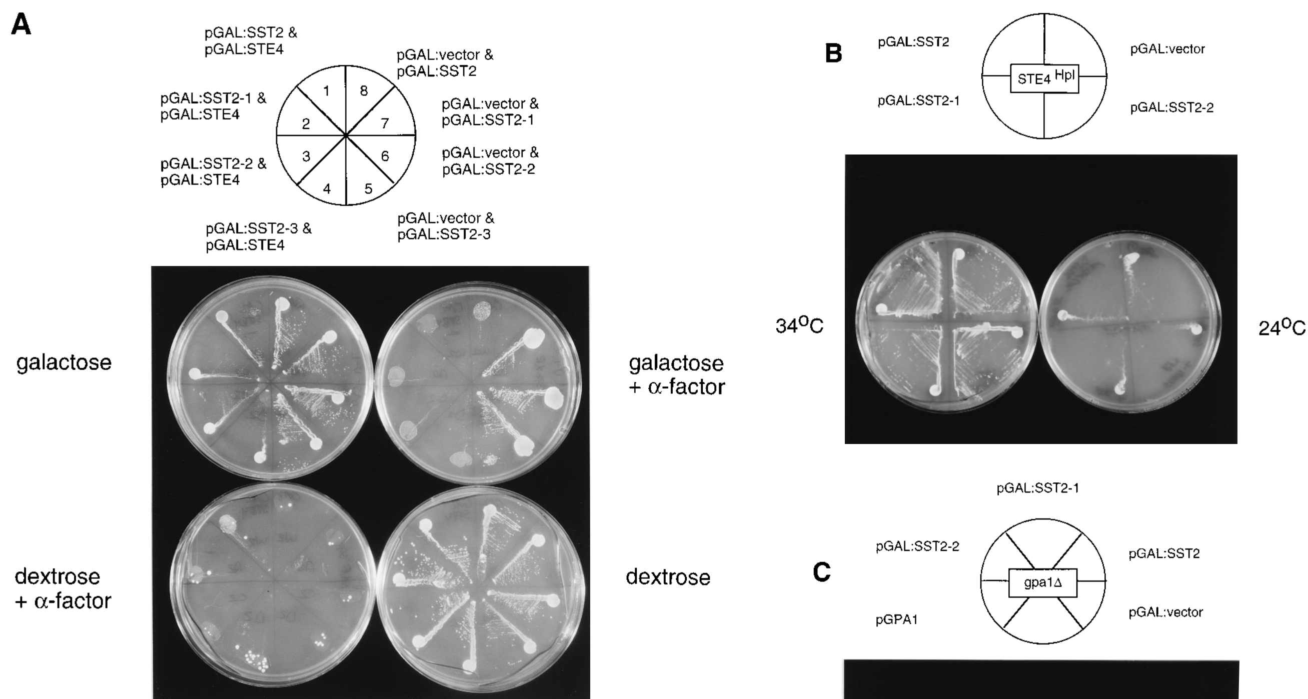

FIG. 5. *SST2* suppression of pheromone-induced but not G-protein-induced growth arrest. Epistasis analysis was used to determine if the *SST2* mutants suppress signaling by the G protein as well as by the receptor. (A) Strain YDK499 was cotransformed with plasmid pRS316-GAL (octants 5 to 8, pGAL:vector) or pRS316-GAL-STE4 (octants 1 to 4, pGAL:STE4, overexpressing Gβ) and with pRS315-GAL bearing wild-type or mutant *SST2*. Transformed cells were grown in dextrose-containing liquid cultures and then spotted and streaked onto galactose or dextrose-containing plates, in some cases supplemented with α-factor. Whereas *SST2* mutants restored growth in α-factor-containing medium (galactose + α-factor, octants 5 to 7 versus octant 8), they were not able to restore growth to *STE4*-overexpressing cells (galactose, octants 2 to 4) or *STE4*-overexpressing cells treated with α-factor (galactose + α-factor, octants 2 to 4). No growth was observed for cells treated with α-factor under noninducing conditions (dextrose + α-factor, octants 1 to 8). (B) To rule out the possibility that overexpression of Ste4p interferes with Sst2p function, strain DJ706-2-4 ($STE4^{Hpl}$ *ste5-3*) was transformed with pRS315-GAL bearing wild-type *SST2* (pGAL:SST2) mutant *SST2* (pGAL:SST2-1 or pGAL:SST2-2), or no insert (pGAL:vector). $STE4^{Hpl}$ encodes a dominant constitutively active Gβ mutant that causes pheromone-independent signaling and growth arrest at the restrictive temperature (24°C; *ste5-3*). Transformed cells were grown in galactose at the permissive temperature and then streaked onto galactose-containing plates at either 34 or 24°C. The *SST2* mutants did not restore growth to the $STE4^{Hpl}$-arrested cells. (C) To test whether *SST2* mutants could suppress constitutive signaling resulting from a disruption of *GPA1*, strain YGS5 was transformed with pRS315-GAL bearing wild-type (pGAL:SST2) or mutant *SST2* (pGAL:SST2-1 or pGAL:SST2-2) or no insert (pGAL:vector). A *GPA1* plasmid was used as a positive control. YGS5 carries a downstream temperature-sensitive mutation in *STE11* that blocks signaling at 34°C (permissive temperature). Transformed cells were grown in galactose at the permissive temperature and then streaked onto galactose-containing plates at either 34 or 24°C. The *SST2* mutants did not restore growth to the *gpa1*Δ-arrested cells.

Fig. 5B, cells were first grown in galactose at 34°C and then streaked onto galactose-containing plates at either at 34 or 24°C. As anticipated, cells maintained at 34°C grew in the presence or absence of the *SST2*-containing plasmids. However, neither *SST*2-1 nor *SST*2-2 was able to restore growth when the cells were shifted to 24°C.

The experiments in Fig. 5C were designed to test whether the *SST2* mutations could suppress another type of constitutive signaling mutation, disruption of *GPA1* (strain YGS5). The absence of Gα in YGS5 leads to unregulated expression of free β/γ and persistent signaling in the absence of pheromone. Strain YGS5 carries a downstream temperature-sensitive mutation in *STE11* which blocks growth arrest when maintained at 34°C (the permissive temperature) but not at 24°C (restrictive temperature). As shown in Fig. 5C, cells carrying *GPA1* (positive control), wild-type *SST2*, *SST*2-1, *SST*2-2, or vector alone (negative control) were grown in galactose at 34°C and then spotted and streaked onto galactose-containing plates at either 34 or 24°C. Whereas all of the transformed cells grew at 34°C, only *GPA1* conferred growth at 24°C. Thus, whereas the *SST2* mutations block α-factor-dependent growth arrest, they are ineffective at blocking G-protein-mediated signaling.

***SST2* regulation of Gpa1p expression.** The data presented in Fig. 5 demonstrate that *STE4* and *GPA1* are epistatic to *SST2*, suggesting that Sst2p acts on the receptor or G protein. A recent report from Madura and Varshavsky (34) reveals a possible mode of action for Sst2p. These investigators have shown that overexpression of the ubiquitin-conjugating enzymes in yeast cells leads to increased turnover of Gpa1p and inhibition of cell growth and that this phenotype is suppressed by an *sst2*Δ mutation. The authors proposed (but did not show) that Sst2p acts to destabilize Gpa1p. A paradox of this model is that *SST2* mutants are more sensitive to α-factor, but elevating Gpa1p expression reduces α-factor sensitivity (e.g., Fig. 1). Moreover, Gpa1p turnover was monitored by using overexpressed epitope-tagged protein, and only in the absence of pheromone. Since the epitope tags were placed at the functionally important amino and carboxy termini of the protein, Gpa1p should not be functional (14). Since *SST2* transcription

is highly dependent on pheromone induction, very little protein would be expressed (Fig. 1) (16).

The experiments in Fig. 6 were designed to test whether *SST2* can influence the expression of endogenous functional Gpa1p and whether expression is affected by pheromone stimulation. Initially, we examined Gpa1p in strains YPH499, YDM400 (*sst2-Δ2*), and YDM400 overexpressing wild-type *SST2* or SST2-1, grown in the presence or absence of 5 μM α-factor. As shown in Fig. 6A, the Gpa1p antibodies recognize two species of Gpa1p, representing the myristoylated (~54 kDa) and unmyristoylated (~56 kDa) forms of the protein (48). Only the myristoylated species is capable of binding to β/γ, since a mutation that blocks this modification ($gpa1^{Gly2Ala}$) cannot complement the loss of *GPA1* (48). Moreover, it was previously shown that α-factor stimulation leads to an increase in Gpa1p myristoylation (18). In agreement with those findings, we detect an increase in the myristoylated 54-kDa species in cells treated with pheromone, and the extent of pheromone-stimulated myristoylation correlates well with pheromone-dependent growth arrest and transcription (i.e., *sst2*Δ > *SST2* > SST2-1). However, there is little or no effect of *SST2* on overall Gpa1p expression. Overexpression of *SST2* and *SST*2-1 was confirmed by immunoblotting the same extracts with *SST2* antibodies (data not shown).

To determine if *SST2* affects Gpa1p stability, we examined cells grown in the presence or absence of 5 μM α-factor for 1 h and then treated with cycloheximide to block new protein synthesis. Immunoblot analysis revealed that cells bearing an *SST2* disruption or wild-type *SST2* do not differ significantly in the rate of loss of myristoylated (i.e., functional) Gpa1p after cycloheximide addition (Fig. 6B and C). Indeed, the loss of unmyristoylated Gpa1p appears to be somewhat reduced in wild-type cells compared with cells bearing *sst2-Δ2*. As before, differences in the levels of myristoylated and unmyristoylated protein correlate with α-factor sensitivity. To determine if *SST*2-1 influences Gpa1p turnover, expression of genomic Gpa1p was monitored following α-factor stimulation and cycloheximide administration. Immunoblots prepared from cells overexpressing wild-type *SST2* or *SST*2-1 reveal little difference in Gpa1p degradation (data not shown). We conclude that while turnover of functional, myristoylated Gpa1p is indeed rapid, overall expression or stability is not significantly affected by *SST2.*

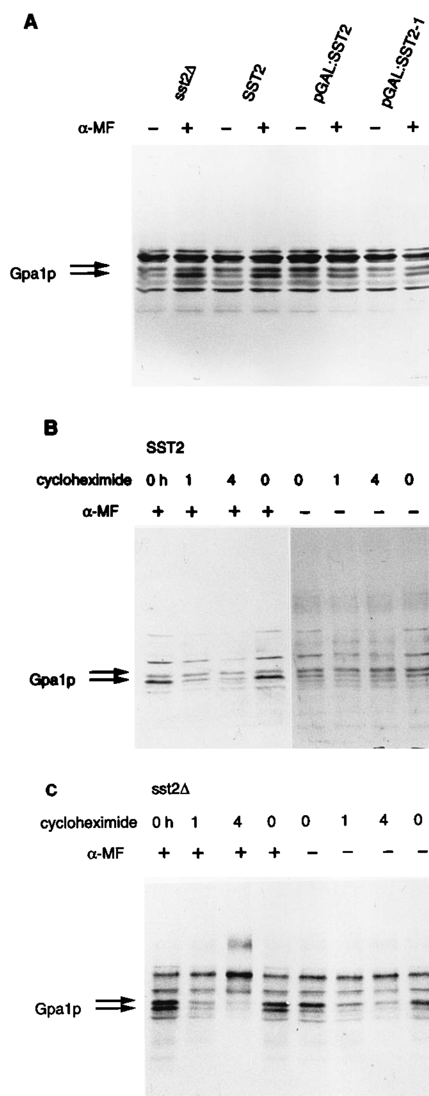

FIG. 6. *SST2* regulation of Gpa1p expression. To determine if *SST2* alters expression or turnover of Gpa1p, we examined Gpa1p in cells lacking *SST2*, expressing *SST2* at high or low copy, or overexpressing *SST*2-1, in the presence and absence of 5 μM α-factor. (A) Immunoblot analysis was used to monitor Gpa1p in cells that carry the *sst2-Δ2* mutation (strain YDM400 containing pRS315-GAL, sst2Δ) or wild-type *SST2* (YPH499/pRS315-GAL, SST2) or overexpress *SST2* (YDM400/pRS315-GAL-SST2, pGAL:SST2) or *SST*2-1 (YDM 400/pRS315-GAL-SST2-1, pGAL:SST2-1). These data indicate that overall Gpa1p expression is not significantly altered by *SST2*. The shift from 56 to 54 kDa after α-factor administration reflects increased myristoylation of Gpa1p (18). (B and C) To determine how *SST2* influences Gpa1p stability, wild-type (B) and mutant (C) cells (strains YPH499 and YDM400, respectively) were exposed to α-factor (α-MF) for 1 h or not treated, and cycloheximide was used to block further protein synthesis. Aliquots were taken at 0, 1, and 4 h after cycloheximide addition and analyzed by immunoblotting. These data indicate that *SST2* does not promote Gpa1p degradation and has a slight stabilizing effect on the unmyristoylated (54-kDa) form of the protein.

DISCUSSION

Desensitization is an important phenomenon in biology and has been extensively characterized for a number of vertebrate receptors, such as the β-adrenergic receptor and rhodopsin. Receptor desensitization is thought to represent the molecular basis for attenuation to light, odors, chemical stimulants, and narcotics and is also a factor that limits the efficacy of therapeutic drugs (31).

The mechanisms of receptor desensitization are complex and include rapid alterations, such as receptor phosphorylation, uncoupling from the G protein, and protein sequestration within minutes of agonist activation (2, 19, 23, 31, 32). Like vertebrate cells, yeast cells become desensitized to the continued presence of pheromone and will eventually recover from pheromone-induced G_1 growth arrest (3, 8–10, 12, 16, 19, 27, 33, 37, 40, 41, 47). Accordingly, genetic methods have been used to identify several factors required for efficient recovery after pheromone stimulation in yeast cells. One of these, Sst2p, appears to be particularly important for this process. Loss of Sst2p can have dramatic (100- to 300-fold) effects on pheromone sensitivity. However, neither the specific role of Sst2p in signaling nor its target(s) of action is known. In order to better characterize the role of this important component of the transmembrane signaling pathway, we sought to identify *SST2* mutations that antagonize pheromone action in vivo. Our objective was to find dominant alleles that would behave like steriles, allowing cells to grow in what would normally be lethally high concentrations of α-factor. These mutants would be useful for genetic experiments, such as those presented in

Fig. 5, to determine where *SST2* functions in the signaling pathway. They will also be useful in biochemical experiments, which are in progress, to examine how Sst2p affects G protein function. For example, a constitutively active variant of Sst2p might bind to its target with a higher affinity, allowing copurification or coimmunoprecipitation.

Sequencing of mutations with well-characterized phenotypes can also provide information about the importance of structural domains in protein function. All of the *SST2* mutations described here prevent pheromone-induced growth arrest (Fig. 2 and 5) and transcription (Fig. 4). These changes are not due to any difference in mutant and wild-type *SST2* expression (Fig. 3). Since *SST*2-1, *SST*2-2, and *SST*2-3 behave similarly, the shared Pro-to-Leu substitution at position 20 must be responsible for the change in phenotype. Indeed, the loss of a Pro residue is likely to have profound effects on the secondary structure of the protein product, since it is the only naturally occurring amino acid in which the nitrogen atom forms a rigid ring structure that cannot rotate.

It is worth emphasizing that the strategy used here should be useful for characterizing the epistasis relationships of additional desensitization mutations as they become known. Already, a wide variety of mutations have been found to affect signaling. For example, *MAT***a** cells secrete a protease, the *BAR1* product, that cleaves and inactivates α-factor (10, 33), resulting in a prolonged response to α-factor stimulation (8, 9). Domains of cell surface receptors may also play a role in desensitization. Removal of the C-terminal cytoplasmic domain of the α-factor receptor has no effect on ligand-binding activity but prevents pheromone-stimulated phosphorylation and internalization (27, 41), resulting in up to 100-fold-greater sensitivity to added α-factor. Recently, point mutations in the third cytoplasmic loop of the **a**-factor receptor that cause ~20-fold-greater sensitivity to pheromone and result in partial constitutive activation of the signaling pathway have been described (5). Finally, a deletion mutation in Gβ ($ste4^{\Delta 310\text{-}346}$) prevents pheromone-stimulated phosphorylation of the protein and results in an approximately sixfold increase in pheromone sensitivity (12). For all of these mutations, however, the effects on pheromone sensitivity are enhanced by the loss of *SST2*, suggesting distinct modes of signal regulation.

A number of alleles that reduce pheromone sensitivity have also been described. *GPA1* and *KSS1* (MAP kinase homolog) were originally cloned as high-copy suppressors of the pheromone-supersensitive *sst2-1* mutation (15, 17). Blumer and colleagues have described recessive mutations in the cytoplasmic domains of the α-factor receptor that interfere with receptor-G protein coupling, resulting in a higher EC_{50} for the pheromone and enhanced recovery from pheromone-induced growth arrest (51). The ability of these mutants to effect recovery was dependent on the functional expression of *SST2*, suggesting that the receptor operates upstream of Sst2p. Leberer et al. have characterized dominant negative alleles of *STE4* that block pheromone-induced growth arrest yet have little effect on gene transcription or mating (30). These mutations were isolated in a *bar1*Δ *sst2*Δ strain, however, and it was not determined how these phenotypes are affected by the absence of *SST2.*

Finally, a number of *GPA1* mutations have been found to promote desensitization (29, 35, 49). One of these, $GPA1^{Gly50Val}$, was effective in *sst2-1* (35) but not in *sst2*Δ (29) cells. These findings support a model in which Gα directly activates a downstream desensitization pathway (3, 35). Since overexpression of Gpa1p is also allele specific with regard to *SST2*, and since dominant *SST2* mutations suppress receptor-mediated but not β/γ-mediated signaling, we postulate that Sst2p is the "desensitization effector" controlled by Gα. Additional support for this model comes from recent experiments demonstrating that Gpa1p and Sst2p are both localized at the cytoplasmic surface of the Golgi and plasma membranes (18a). Thus, having established that Sst2p and Gpa1p cooperate to promote desensitization in vivo, we are developing methods to characterize this interaction in vitro. A critical question for the future is to determine how Sst2p affects G-protein subunit interactions and GTPase activity.

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