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Functional Coupling of a Mammalian Somatostatin Receptor to the Yeast Pheromone Response Pathway

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A detailed analysis of structural and functional aspects of G-protein-coupled receptors, as well as discovery of novel pharmacophores that exert their effects on members of this class of receptors, will be facilitated by development of a yeast-based bioassay. To that end, yeast strains that functionally express the rat somatostatin receptor subtype 2 (SSTR2) were constructed. High-affinity binding sites for somatostatin ([^{125}I-Tyr-11]S-14) comparable to those in native tissues were detected in yeast membrane extracts at levels equivalent to the α-mating pheromone receptor (Ste2p). Somatostatin-dependent growth of strains modified by deletion of genes encoding components of the pheromone response pathway was detected through induction of a pheromone-responsive *HIS3* reporter gene, enabling cells to grow on medium lacking histidine. Dose-dependent growth responses to S-14 and related SSTR2 subtype-selective agonists that were proportional to the affinity of the ligands for SSTR2 were observed. The growth response required SSTR2, G_α proteins, and an intact signal transduction pathway. The sensitivity of the bioassay was affected by intracellular levels of the G_α protein. A mutation in the *SST2* gene, which confers supersensitivity to pheromone, was found to significantly enhance the growth response to S-14. In *sst2*Δ cells, SSTR2 functionally interacted with both a chimeric yeast/mammalian G_α protein and the yeast G_α protein, Gpa1p, to promote growth. These yeast strains should serve as a useful in vivo reconstitution system for examination of molecular interactions of the G-protein-coupled receptors and G proteins.

The cyclic tetradecapeptide somatostatin is a potent inhibitor of secretion of several hormones, including the growth hormone from the pituitary, glucagon and insulin from the pancreas, and gastrin from the gut. Somatostatin also acts as a neurotransmitter and has been shown to have broad modulatory effects in central nervous system and peripheral tissues (3). The effects of somatostatin are transduced through binding of the hormone to high-affinity, plasma membrane-localized somatostatin receptors (SSTRs) (41). The SSTRs, encoded in five distinct subtypes which account in part for tissue-specific differences in responses to somatostatin (4, 26, 28, 33, 37, 56, 59), compose a subset of the seven-transmembrane domain, G-protein-coupled receptor superfamily.

G-protein-coupled receptors transduce the information inherent in ligand binding to the cell interior through interaction with guanine nucleotide-binding proteins (G proteins) (10). G proteins are heterotrimeric proteins composed of α, β, and γ subunits. The α-subunit bears the guanine nucleotide binding and catalytic sites and interacts with both receptors and effectors, defining the activity of the G-protein complex. Receptor contact sites residing in the carboxy-terminal domain within the G_α subunit are sufficient to confer receptor interaction specificity (10).

SSTRs have been shown to modulate the activity of several different classes of effector molecules including activation of potassium channels, serine/threonine and tyrosine phosphatases, and Na^+/H^+ antiporters and inhibition of calcium channels and adenylyl cyclases (17, 20, 27, 29, 44, 50, 51, 55, 58). Several lines of evidence implicate $G_{\alpha i}$ in signaling by the SSTRs. With the exception of modulation of the Na^+/H^+ antiporter (20), the effector-modulating activities of the SSTRs are sensitive to pertussis toxin-catalyzed ADP ribosylation, which inactivates $G_{\alpha i}$ and $G_{\alpha o}$ (27, 45, 57). Antibodies directed at the carboxy-terminal domains of $G_{\alpha i1\text{-}3}$ block formation of high-affinity binding sites in soluble brain SSTRs, in agreement with inhibition of the formation of an SSTR-$G_{\alpha i}$ complex (35). Recently, the SSTR subtype 2 was shown to interact with and copurify with $G_{\alpha i2}$ and $G_{\alpha i3}$ (16, 30).

Detailed analyses of somatostatin receptor-ligand and receptor–G-protein interactions, as well as the discovery of novel SSTR subtype-selective ligands, would be facilitated by functional expression of these proteins in yeast cells (14). *Saccharomyces cerevisiae* utilizes G-protein-coupled receptors and heterotrimeric G proteins to regulate the mating process (47). Haploid yeast cells detect the presence of peptide mating pheromones secreted by potential mating partners through binding to G-protein-coupled pheromone receptors encoded by the *STE2* and *STE3* genes. Activated pheromone receptors catalyze dissociation of a heterotrimeric G protein, encoded by the *GPA1* (α), *STE4* (β), and *STE18* (γ) genes. Gpa1p-mediated negative regulation is thereby relieved, allowing the complex of Ste4p and Ste18p to activate a signal transduction pathway composed of elements of a mitogen-activated protein kinase (MAP kinase) cascade (18). The pathway is composed of MAP kinase homologs encoded by the *FUS3* and *KSS1* genes and upstream regulatory protein kinases encoded by the *STE7* (MAP kinase kinase or MEK) and *STE11* (MEK kinase) genes. The result of activation of this pathway is cell cycle arrest and transcriptional induction of pheromone-responsive genes. Mutations in elements of the MAP kinase pathway abolish these responses.

Cell cycle arrest in response to pheromone is mediated by the product of the *FAR1* gene (8). *FAR1* encodes a negative

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regulator of G_1 cyclins and is thought to serve as the primary interface between the pheromone response pathway and cell cycle regulatory machinery (8, 39, 53). Deletion of the *FAR1* gene allows for continued cell growth and transcriptional induction of pheromone-responsive genes in the presence of an activated mating signal transduction pathway.

The yeast cell response to chronic stimulation of the pheromone response pathway is induction of specific desensitization or adaptation mechanisms. The primary adaptation mechanism is mediated by the product of the *SST2* gene (6, 7). Yeast cells lacking functional Sst2p exhibit hypersensitivity to the presence of pheromone and inability to recover from pheromone-induced cell cycle arrest.

The remarkable similarities in the functional properties of the components of the pheromone response pathway with mammalian systems suggest that the inherent advantages of *S. cerevisiae* as an experimental organism may be put to productive use for analysis of G-protein-coupled receptors and G proteins. In this report, we describe a novel expression technology in which yeast cells containing the rat SSTR subtype 2 were rendered dependent on agonist-stimulated activation of the receptor and downstream elements of the pheromone response pathway for growth on selective medium. The technology was exploited to examine aspects of ligand-receptor and receptor–G-protein interactions.

MATERIALS AND METHODS

Plasmid constructions. Molecular biological manipulations were performed by standard methods (43). Plasmids bearing deletion alleles of *GPA1* and *FAR1* were assembled in vitro. The *gpa1ΔhisG* allele was constructed by subcloning *Eco*RI-*Hin*dIII and *Sph*I-*Sna*BI fragments of *GPA1* into corresponding sites in YIp5 (42), producing plasmid pLP12. The *hisG-URA3-hisG* fragment from pNKY51 (1) was placed in the *Bam*HI site between the *GPA1* fragments, producing pLP16. The *far1ΔLYS2* allele was constructed by amplifying two fragments of the *FAR1* gene (8) from yeast genomic DNA (YPH501) with synthetic oligonucleotides that introduce an *Eco*RI site at position 295 and include the natural *Hin*dIII site at position 1201 in the 5′ fragment and an *Hin*dIII site at position 2017 and a *Sal*I site at position 2821 in the 3′ fragment. The fragments were cloned in a three-part ligation reaction into the *Eco*RI-*Sal*I fragment of pBSK (Stratagene). The resulting plasmid (pLP59) was digested with *Hin*dIII-*Nhe*I, and a *Hin*dIII-*Xba*I fragment of *LYS2* was inserted, producing pLP80.

The multistep construction of the SSTR2 expression plasmid, pJH2 (Fig. 1A), was initiated by inserting an *Sph*I-*Nar*I fragment of the 3′ untranslated region from the *STE7* gene (52) and an *Eco*RI-*Bam*HI fragment of the *GAL1/10* promoter (60) into appropriate sites in YEp352 (19), creating pEK1. A DNA fragment encoding the open reading frame and transcriptional termination sequences of the *GAL4* gene (23) was amplified by PCR from YPH500 genomic DNA with oligonucleotides containing 5′ *Eco*RI (5′ ACTGAATTCAAAGAT GAAGCTACTGTCTTCTATCGAA) and 3′ *Hin*dIII (5′ CGTTATATGAGCG AAGCTTCTGAATAAG) sites and subcloned into pGEM3 (Promega). An *Eco*RI-*Nae*I fragment (*Nae*I from pGEM3) was then inserted between the *Eco*RI and *Pvu*II sites in pEK1, creating pMP3. The cDNA encoding rat SSTR2 (50) was modified by PCR using oligonucleotides (5′ AAAGATCTAAAAAAT GGAGATGAGCTCTGAG and 5′ TCTAGAAGATCTTCAGATACTGGTTT GGAGGT) that added a *Bgl*II site in the DNA sequences encoding the amino terminus of SSTR2 and a *Bgl*II site immediately after the translational stop site. SSTR2 coding sequences were inserted as a *Bgl*II fragment into the *Bam*HI site of pMP3, creating pJH2.

The chimeric G_α-protein expression plasmid pLP82 (Fig. 1B) was constructed by first replacing the *Xho*I-*Eco*RI promoter fragment in pPGKH-*SCG1*-$G_{\alpha s}$ (24) with a modified *SCG1/GPA1* promoter fragment (12, 22, 34, 36) amplified from yeast genomic DNA by using oligonucleotides that introduce 5′ *Xho*I and 3′ *Eco*RI sites at positions −200 and −42, producing plasmid pLP61. When placed upstream of the *SCG1*-$G_{\alpha s}$ fragment, the combined sequence reconstitutes the functional *GPA1* promoter found in the complementing plasmid, pC3 (12). The *Bam*HI fragment encoding the $G_{\alpha s}$ domain was replaced with a comparable fragment encoding $G_{\alpha i2}$, producing plasmid pLP71. The *Xho*I-*Sal*I fragment of pLP71 encoding the chimeric G_α protein (designated Gpa1-$G_{\alpha i2}$) expressed under the control of the *GPA1* promoter was transferred to the *Sal*I site in pRS414 (*ARSH4 CEN6 TRP1*; Stratagene), producing pLP82. Plasmid pLP83 was constructed by replacing the *Eco*RI fragment in pLP71 with the *Eco*RI fragment encoding *SCG1/GPA1* from pPGKH-*SCG1* (24), producing plasmid pLP75. The *Xho*I-*Sal*I fragment encoding *GPA1/SCG1* was transferred to the *Sal*I site in pRS414, producing plasmid pLP83. Plasmid pEK36 was constructed by transferring the *Xho*I-*Sal*I fragment from pPGKH-*SCG1*-$G_{\alpha i2}$ to the *Sal*I site of pRS414. Plasmid pEK38 was constructed by transferring the *Xho*I-*Sal*I fragment from pPGKH-*SCG1*-$G_{\alpha i2}$ to the *Sal*I site of pRS424 (*ARSH4 TRP1*; Stratagene).

Strain constructions. Yeast strains were constructed and growth media and culture conditions were formulated by standard procedures (42). DNA-mediated transformation of *S. cerevisiae* was carried out by the lithium acetate method (49). A diploid yeast strain, LY43, heterozygous at the *GPA1* locus was created by integrative transformation of YPH501 (Table 1) with the *Eco*RI-*Sal*I *gpa1ΔhisG-URA3-hisG* fragment from pLP16. A derivative exhibiting spontaneous loss of the *URA3* marker identified by growth on 5-fluoro-orotic acid-containing medium (42) was transformed with pPGKH-*SCG1*-$G_{\alpha i2}$ (24) and induced to sporulate. A congenic descendent, LY150, of a *MAT***a** *gpa1ΔhisG* segregant containing pPGKH-*SCG1*-$G_{\alpha i2}$ was chosen for further analysis. The *FAR1* gene in LY150 was deleted by using the *far1ΔLYS2* construct in pLP80 and then cultured on nonselective medium to allow loss of pPGKH-*SCG1*-$G_{\alpha i2}$, producing LY172. The *Eco*RI fragment encoding the *FUS1-HIS3* reporter gene (pSL1497) (48) was used to transform LY172 to histidine prototrophy, producing LY228. The *SST2* gene (13) was deleted from the LY228 genome by using the *sst2ΔADE2 Sal*I fragment in plasmid p*sst2ΔADE2* (26a), yielding LY252. Strains based on LY228 and LY252 were constructed by transformation with the designated SSTR2 and G_α expression plasmids described above. The *STE2* gene was deleted in strain LY754 by using the *ste2ΔLEU2 Bam*HI fragment in pAB506 (5), and the *STE7* gene was deleted in strain LY784 by using the *ste7ΔLEU2 Sac*I fragment in pNC113 (9).

Bioassay. Yeast strains were grown overnight in 2 ml of synthetic complete (SC) liquid medium containing glucose (2%) and lacking uracil and tryptophan, washed to remove residual glucose, cultured for 4 to 8 h in 5 ml of SC medium containing lactate (3%) and lacking uracil and tryptophan, collected by centrifugation, and grown overnight in 5 ml of SC medium containing galactose (2%) and lacking uracil and tryptophan. Molten (50°C) SC agar medium containing galactose (2%) and lacking uracil, tryptophan, and histidine (35 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) was inoculated with the overnight culture (2×10^4 cells per ml) and poured into square petri plates (9 by 9 cm). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 μl of sterile water containing the indicated amounts of the designated compounds. Compounds applied to the surface of the plate diffused radially from the site of application and bound to SSTR2 expressed on the surface of cells embedded in the agar, resulting in induction of *FUS1-HIS3* expression. The responding cells formed a dense zone readily detectable over the limited growth of cells observed in response to basal *FUS1-HIS3* expression. Plates were incubated at 30°C for 3 days. Somatostatin (S-14, S-28), tumor-inhibiting peptide, Met-enkephalin, and growth hormone-releasing factor were from Bachem. The α-mating pheromone, oxymetazoline, isoproterenol, and carbachol were purchased from Sigma. MK678 (40) and octreotide (32) were prepared synthetically.

Radiolabeled agonist saturation binding assays. Crude yeast membrane extracts from late-log-phase cultures were prepared by glass bead lysis and centrifugation at $40,000 \times g$ by using a published procedure (2). The protein content of crude membrane fractions was measured by using the Bio-Rad protein assay kit according to the manufacturer's instructions. Radio-ligand binding assays were conducted as described by Strnad et al. (50) by using [^{125}I-Tyr-11]S-14 labeled by the chloramine T method and purified by high-pressure liquid chromatography (16). Nonspecific binding was defined as that observed in the presence of 1 μM S-14. The effect of a GTP analog on radio-ligand binding to SSTR2 in crude membrane fractions prepared from SSTR2-transfected CHO cells (50) and LY268 was assayed in the presence or absence of the nonhydrolyzable GTP analog GppNHp (100 μM; Sigma). Data are expressed as percent specific binding remaining in membranes after treatment with GppNHp.

RESULTS

Somatostatin induces growth of yeast cells that express SSTR2. A selective and sensitive bioassay of functional expression of SSTR2 would be facilitated by yeast strains that respond to somatostatin by exhibiting a growth response rather than cell cycle arrest. Therefore, several modifications were introduced into a typical laboratory strain as follows. (i) The *FAR1* gene was deleted. Deletion of the *FAR1* gene allows for continued cell growth and transcriptional induction of pheromone-responsive genes in the presence of an activated mating signal transduction pathway. (ii) The *FUS1* gene was replaced with a reporter gene construct made by fusing transcriptional control elements of the pheromone-responsive *FUS1* gene to *HIS3* protein coding sequences (48), thereby placing expression of His3p under the control of the pheromone response pathway. Thus, receptor activation by an agonist leads to activation of the pheromone response pathway, induction of the

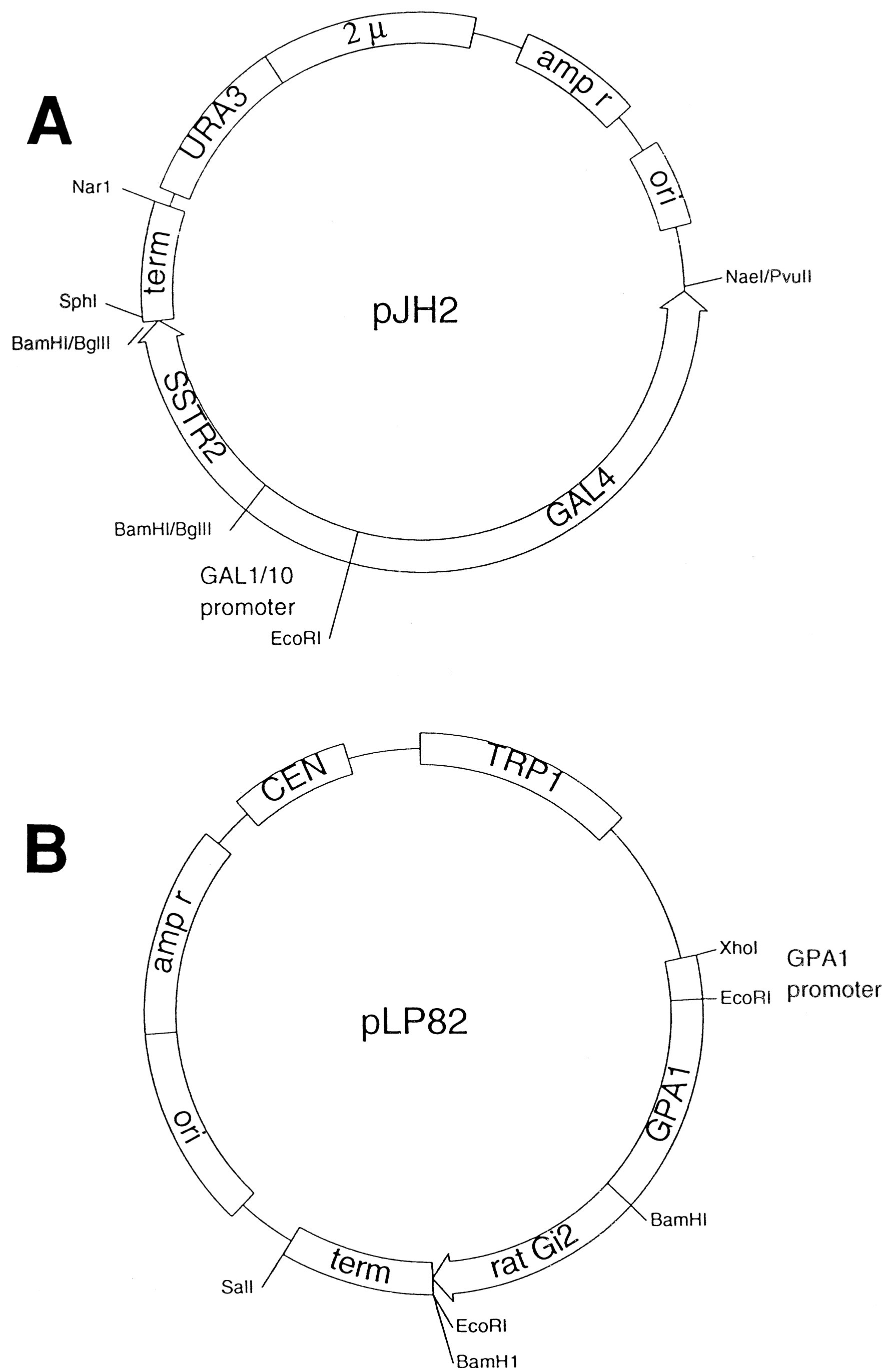

FIG. 1. Plasmids used in this study. The SSTR2 expression plasmid, pJH2 (A), and the G_{α}-protein expression plasmid, pLP82 (B), were constructed as described in Materials and Methods.

FUS1 promoter, and increased *HIS3* protein expression, permitting growth of auxotrophic (*his3*) yeast strains on medium lacking histidine. (iii) The *GPA1* gene was deleted and replaced with plasmid-borne G_{α} genes.

Yeast cells described above were transformed with the SSTR2 expression plasmid pJH2 (Fig. 1A) and the chimeric G_{α} protein expression plasmid, pLP82 (Fig. 1B), and tested for response to somatostatin. The expressed chimeric G_{α} protein (Gpa1-$G_{\alpha i2}$) was composed of an amino-terminal $G_{\beta\gamma}$ interaction domain from *GPA1* and a carboxy-terminal receptor interaction domain from rat $G_{\alpha i2}$ (24). This construct was chosen to promote efficient interaction between SSTR2 and G_{α} protein. A bioassay was performed with this strain (LY288) as described in Materials and Methods. Somatostatin (S-14) induced a dose-dependent increase in the growth response of yeast cells (Fig. 2A). No detectable S-14-dependent induction of growth was observed in cells (LY228) that lack pJH2 (data not shown).

Mutation of the *SST2* gene promotes increased sensitivity to the ligand. Mutations in the *SST2* gene are known to cause

TABLE 1. Yeast strains used in this study

Strain	Markers[a]
YPH501[b]	*MAT***a**/α *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*
LY43	YPH501 *gpa1ΔhisG/GPA1* (pPGKH-*SCG1*-$G_{\alpha i2}$)
LY150	*MAT***a** *gpa1ΔhisG* (pPGKH-*SCG1*-$G_{\alpha i2}$)
LY172	LY150 *far1ΔLYS2* (loss of pPGKH-*SCG1*-$G_{\alpha i2}$)
LY228	LY172 *FUS1-HIS3*
LY288	LY228 (pJH2, pLP82)
LY252	LY228 *sst2ΔADE2*
LY262	LY252 (pJH2, pEK36)
LY266	LY252 (pJH2, pLP83)
LY268	LY252 (pJH2, pLP82)
LY284	LY252 (pJH2, pEK38)
LY754	LY266 *ste2ΔLEU2*
LY784	LY266 *ste7ΔLEU2*

[a] All strains are congenic with YPH501; only markers different from background are given.
[b] Obtained from Stratagene.

supersensitivity to the mating pheromone (6, 7), suggesting that sensitivity to S-14 might be improved by inactivating the *SST2* gene. The effect of an *SST2* deletion mutation on the sensitivity of the growth response to S-14 was examined by comparing strains LY288 (*SST2*) and LY268 (*sst2Δ*) (Fig. 2A and B). LY268 cells exhibited a detectable growth response around a 10-fold smaller amount of S-14 (60 pmol) than did LY288 cells (600 pmol of S-14). Additionally, the zones of growth displayed by *sst2Δ* mutant cells were substantially denser than those exhibited by *SST2* cells, an apparent result of the increased growth rate of responding cells within the halo of growth. These results suggest that Sst2p was capable of promoting adaptation of the mating response even in the sole presence of Gpa1-$G_{\alpha i2}$.

SSTR2 productively interacts with the yeast G_α protein, Gpa1p. Structural aspects of SSTR2-G_α protein interactions were tested by examining the growth response of *sst2Δ* cells containing either Gpa1p or Gpa1-$G_{\alpha i2}$. Surprisingly, yeast cells containing Gpa1p (LY266) were observed to grow in response to S-14, suggesting that SSTR2 engages in a functional interaction with the wild-type yeast G_α protein (Fig. 3B). The growth response of LY266 cells was comparable to that of cells (LY268) containing Gpa1-$G_{\alpha i2}$ (Fig. 3A), implying that coupling between SSTR2 and Gpa1p was effective in stimulating downstream signaling in spite of the absence of a mammalian receptor–G-protein interaction domain. Growth of *SST2* cells containing Gpa1p in response to S-14 was barely detectable, consistent with effective *SST2*-mediated adaptation to the chronic presence of an agonist resulting in reduced growth (data not shown). Exchange of the carboxy-terminal domain of Gpa1p for that of $G_{\alpha i2}$ had a more profound effect on the α-mating pheromone-induced growth response. The α-mating pheromone induced the growth of cells containing Gpa1-$G_{\alpha i2}$, suggesting coupling of the chimeric G_α protein to Ste2p (Fig. 3A, center). However, the zone of growth elicited by the α-mating pheromone was substantially smaller than that in cells containing Gpa1p, and growth proceeded to a lesser extent (Fig. 3B, center).

Elevated levels of G_α protein attenuate the SSTR2-dependent growth response. To examine the effects of G_α-protein expression levels on signaling, G_α protein genes were placed under the control of either the *GPA1* promoter or the more powerful *PGK1* promoter in multicopy or low-copy, centromere (CEN)-bearing plasmids. Yeast cells (LY268) containing an expression plasmid that conferred expression of Gpa1-$G_{\alpha i2}$ at levels comparable to Gpa1p exhibited the strongest growth response to S-14 (Fig. 3A). The growth responses of yeast cells bearing plasmids that confer higher levels of Gpa1-$G_{\alpha i2}$ expression were attenuated (Fig. 3C and D), consistent with the observation that overexpression of G_α subunits inhibits signaling, presumably by sequestering the free $G_{\beta\gamma}$ subunit complex (24).

SSTR2 transmits its growth-promoting signal through activation of the pheromone response pathway. SSTR2-dependent expression of His3p likely proceeds via activation of elements of the pheromone response pathway. To test this hypothesis, the *STE7* gene was inactivated by deletion, and the growth response of cells lacking Ste7p was examined. LY784 (*ste7ΔLEU2*) cells failed to exhibit a growth response to the applied α-mating pheromone and S-14 but were rescued by exogenous histidine (Fig. 4A). The histidine-dependent growth rate of LY784 was reduced compared with that of LY266 (Fig. 4C), and agonist-independent background growth of LY784 was not detectable, consistent with a reduction of basal FUS1-HIS3 expression in cells lacking Ste7p. These results demonstrate that an intact pheromone response pathway is necessary for both SSTR2- and Ste2p-mediated signaling.

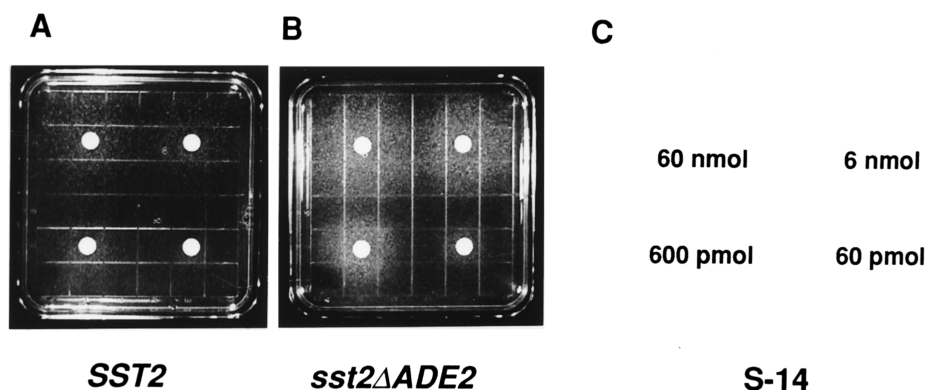

FIG. 2. Effect of mutation in the *SST2* gene on the growth response to S-14. Congenic derivatives of LY252 bearing the SSTR2 expression plasmid pJH2 and the G_α-protein expression plasmid pLP82 (Table 1) were cultured and assayed for S-14-dependent growth as described in Materials and Methods. (A) LY288 (*SST2*); (B) LY268 (*sst2ΔADE2*); (C) amounts of S-14 applied.

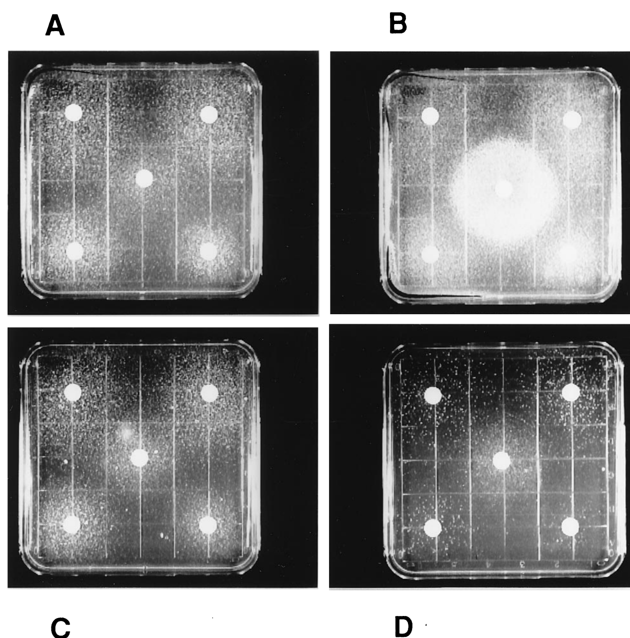

FIG. 3. Effects of G_α-protein expression on the growth response. Congenic derivatives of LY252 bearing the SSTR2 expression plasmid pJH2 and G_α-protein expression plasmids (Table 1) were cultured and assayed for S-14 or α-mating pheromone-dependent growth as described in Materials and Methods. S-14 was applied in amounts indicated in Fig. 2C. The yeast α-mating pheromone (60 nmol) was applied to the center disk of each plate. (A) LY268 (pLP82: pRS414 [CEN] *GPA1* promoter-*GPA1*-$G_{\alpha i2}$); (B) LY266 (pLP83: pRS414 [CEN]-*GPA1*); (C) LY262 (pEK36: pRS414 [CEN] PGK1 promoter-*GPA1*-$G_{\alpha i2}$); (D) LY284 (pEK38: pRS424 [multicopy] PGK1 promoter-*GPA1*-$G_{\alpha i2}$).

To confirm the specificity of the effect of S-14 on its cognate receptor, the *STE2* gene was deleted and yeast cells bearing this mutation (LY754) were tested for response to the α-mating pheromone and S-14 (Fig. 4B). The lack of Ste2p completely eliminated signaling in response to the α-mating pheromone as expected, but left the S-14 growth response intact, indicating that the growth observed was attributable to S-14 interaction with SSTR2 and not with Ste2p. Control cells (LY266) exhibited robust growth responses to both ligands and to histidine as well (Fig. 4C).

Somatostatin binding to SSTR2 expressed in yeast membranes. Most G-protein-coupled receptors exhibit both high and low agonist-dependent affinity states. High-affinity agonist binding is dependent on functional association of the receptor with a heterotrimeric G protein. If the receptor does not associate with, or is uncoupled from, the G protein, agonist binding will be of low affinity and undetectable in radiolabeled agonist saturation binding assays. In crude yeast membrane fractions, the agonist [^{125}I-Tyr-11]S-14 bound to SSTR2 with high affinity and in a saturable manner (Fig. 5), demonstrating that (i) a functional ligand-binding conformation of SSTR2 was expressed and (ii) the receptor functionally associated with Gpa1-$G_{\alpha i2}$, resulting in a high-affinity agonist binding state. SSTR2 expressed in yeast membranes displayed an affinity for [^{125}I-Tyr-11]S-14 (K_d, 600 pM) comparable to the high-affinity binding state of SSTR2 expressed in mammalian cells (K_d, 133 pM [50]). The total number of [^{125}I-Tyr-11]S-14 binding sites observed (B_{max}, 190 fmol/mg) was consistent with values obtained for the yeast α-mating pheromone receptor (B_{max}, 200 fmol/mg [54]). No specific or saturable [^{125}I-Tyr-11]S-14 binding was detected in membranes prepared from a strain (LY296) lacking SSTR2 (Fig. 5). The properties of [^{125}I-Tyr-11]S-14 binding to SSTR2 in LY288 membranes (B_{max}, 203 fmol/mg; K_d, 940 pM) were equivalent to those of LY268 membranes, indicating that the lack of Sst2p had no detectable effect on the binding properties of the receptor and, by implication, no effect on receptor interaction with Gpa1-$G_{\alpha i2}$.

For many G-protein-coupled receptors, high-affinity agonist binding is sensitive to GTP and its analogs. GTP analogs induce dissociation of the receptor–G-protein complex, resulting in a low-affinity agonist binding state. Addition of GppNHp (100 μM), a nonhydrolyzable GTP analog, to an agonist binding assay decreased specific binding of [^{125}I-Tyr-11]S-14 to SSTR2 by more than 90% in crude membrane fractions from LY268 and SSTR2-transfected CHO cells (50). These results represent a further indication of functional coupling between SSTR2 and Gpa1-$G_{\alpha i2}$.

SSTR2 retains agonist selectivity when expressed in yeast cells. Somatostatin (S-14) and its peptide analogs, MK678 and octreotide, induced a dose-dependent growth response of LY268 cells (Fig. 6). The assay was selective: the diameter of the growth zones was proportional to the reported affinity of the ligands for the SSTR2 (MK678 = S-14 > octreotide), reflecting the ability of the bioassay to discriminate between

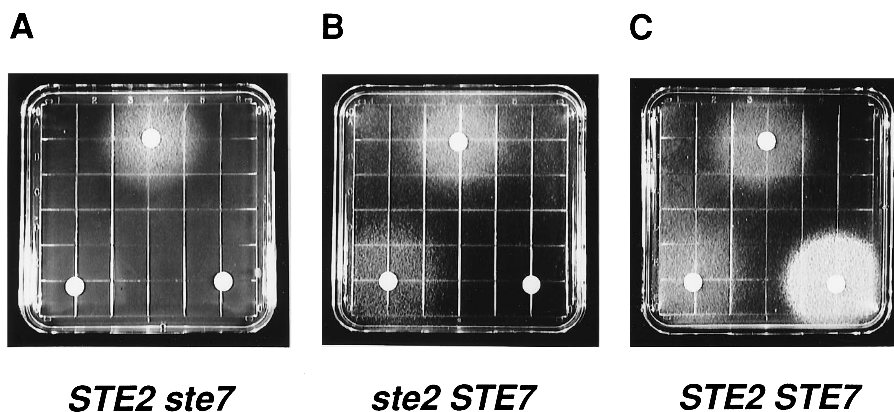

FIG. 4. Effects of mutations in the mating-pheromone signal transduction pathway. Congenic derivatives of LY252 bearing the SSTR2 expression plasmid pJH2 and the G_α-protein expression plasmid pLP83 (pLP83: pRS414 [CEN]-GPA1) (Table 1) were cultured and assayed for S-14-dependent growth as described in Materials and Methods. Top, 10 nmol of L-histidine; lower left, 6 nmol of S-14; lower right, 6 nmol of the α-mating pheromone. (A) LY784 (*STE2 ste7*); (B) LY754 (*ste2 STE7*); (C) LY266 (*STE2 STE7*). LY784 was incubated for 5 days at 30°C. LY754 and LY266 were incubated for 3 days at 30°C.

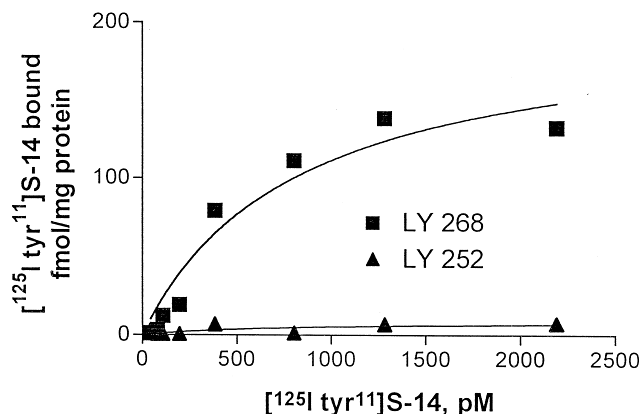

FIG. 5. Ligand binding to the SSTR2 expressed in yeast membranes. Overnight liquid cultures of LY268 and LY252 (Table 1) in SC medium containing glucose (2%) and lacking uracil and tryptophan were transferred to SC medium containing lactate (2%) and lacking uracil and tryptophan and subsequently to SC medium containing galactose (2%) and lacking uracil and tryptophan. Crude membrane fractions were prepared and agonist saturation binding assays were conducted as described in Materials and Methods. Saturation binding was performed with 25 to 2,200 pM [^{125}I-Tyr-11]S-14 (20 μg of protein per tube). Squares, LY268; triangles, LY252. Nonspecific binding for each point ranged from 20 to 60% of cpm bound in the presence of 1 μM unlabeled S-14. The data are representative for experiments performed in duplicate.

ligands of different potencies (32). Growth was also induced in response to the 28-amino-acid form of somatostatin (S-28) and the somatostatin analog, tumor-inhibiting peptide, but not by a variety of agonists selective for other G-protein-coupled receptors including Met-enkephalin, oxymetazoline, growth hormone-releasing factor, isoproterenol, and carbachol (data not shown).

DISCUSSION

In this report, we describe a yeast expression system that permits detection of somatostatin agonists by ligand- and receptor-dependent activation of the pheromone response pathway. Functional expression of SSTR2 was necessary for the development of a useful bioassay. Expression of the rat SSTR2 was placed under the control of the *GAL1* promoter in a plasmid vector (pJH2; Fig. 1A) that confers inducible overexpression of the *GAL4* gene product under the control of the *GAL10* promoter. Overexpression of Gal4p has been shown to increase expression of proteins from galactose-inducible promoters (46). In this way, functional somatostatin agonist-binding sites were expressed in yeast cells with properties comparable to those in native tissues. Previous reports of mammalian G-protein-coupled receptors (β_2-adrenergic and muscarinic acetylcholine receptors) expressed in yeast cells showed detectable antagonist binding but did not demonstrate high-affinity agonist binding in membrane fractions. In two of these reports of receptor expression (21, 38), mammalian G_α was not coexpressed, suggesting that the lack of high-affinity binding was due to the inability of the heterologous receptors to form productive interactions with yeast G_α protein. In a study reported by King et al., the human β_2-adrenergic receptor was coexpressed in yeast cells along with mammalian $G_{\alpha s}$ (25). However, radio-ligand binding assays were performed in the presence of GTP. GTP and its analogs shift high-affinity agonist binding to a low affinity state, making it difficult to detect high-affinity agonist binding sites. Further, expression of the β_2-adrenergic receptor required amino-terminal sequences from the Ste2p. In the present report, SSTR2 was expressed efficiently without modification of its coding sequences.

The results of the agonist bioassay indicate that SSTR2 forms high-affinity ligand binding sites in vivo by association with a chimeric G_α protein, Gpa1-$G_{\alpha i2}$. The chimeric construct, formed from DNA sequences encoding an amino-terminal $G_{\beta\gamma}$ interaction domain from *GPA1* and a carboxy-terminal receptor interaction domain from rat $G_{\alpha i2}$, was chosen to promote efficient interaction between SSTR2 and G_α protein. In addition, it had been reported that mammalian G_α proteins and chimeric G_α proteins composed of domains from yeast and various mammalian G_α proteins rescued the growth arrest phenotype of a *gpa1* mutant (24), indicating a functional interaction with $G_{\beta\gamma}$. Strains bearing these constructs were mating deficient, presumably because of inefficient interaction between the pheromone receptor, Ste2p, and the receptor interaction domain from the heterologous G_α proteins (24). The growth responses exhibited by cells bearing Gpa1-$G_{\alpha i2}$ demonstrate that the chimeric G_α protein is capable of assembling into a configuration that can form productive protein-protein interactions with both SSTR2 and yeast $G_{\beta\gamma}$.

SSTR2 was also capable of forming sufficiently productive interactions with Gpa1p to produce a growth response comparable to that observed in concert with Gpa1-$G_{\alpha i2}$. Interestingly, the highest level of amino acid similarity between Gpa1p and related mammalian G_α subunit proteins exists with $G_{\alpha i2}$. An alignment of the presumptive receptor contact domains of $G_{\alpha i2}$ (amino acids 135 to 355) and Gpa1p (amino acids 252 to 472) revealed a 52% identity, which increases to 63% when conservative substitutions are included. The critical role of carboxy-terminal sequences in receptor–G_α-protein coupling has been demonstrated in studies in which replacement of the 4, 5, or 9 amino acids proximal to the carboxy terminus of $G_{\alpha q}$

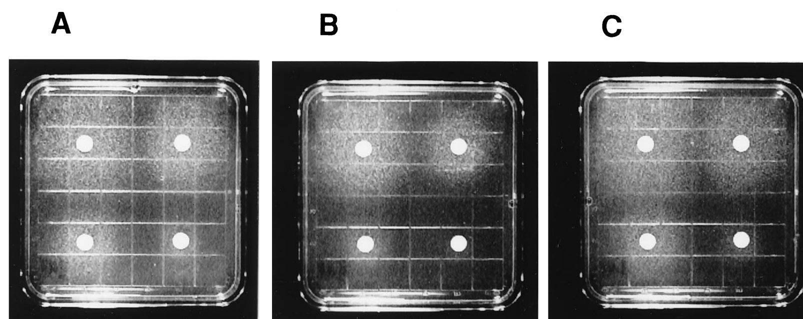

FIG. 6. Growth of *S. cerevisiae* in response to somatostatin receptor agonists. LY268 cells (Table 1) were cultured and assayed for S-14-dependent growth as described in Materials and Methods. Compounds were applied in amounts indicated in Fig. 2C. (A) MK678; (B) octreotide/sandostatin; (C) somatostatin (S-14).

with corresponding sequences from $G_{\alpha i2}$ switches the receptor interaction of the chimeric G_{α} protein to one that involves exclusively $G_{\alpha i}$-interacting receptors (11). An alignment of the last 21 amino acids of the two G_{α} subunits examined in this study (amino acids 334 to 355 of $G_{\alpha i2}$ and amino acids 451 to 472 of Gpa1p) shows 80% similarity when conservative substitutions are considered in the analysis. This degree of homology appears to be sufficient to explain the coupling of SSTR2 to Gpa1p. In contrast, the α-mating factor-induced growth response of cells bearing Gpa1-$G_{\alpha i2}$ was substantially reduced compared with cells bearing Gpa1p, suggesting significant differences in the association of Ste2p with Gpa1-$G_{\alpha i2}$. Nevertheless, a detectable growth signal was elicited, illustrating the substantial functional homology between G-protein-coupled receptors and G_{α} proteins that have limited primary sequence similarity. These results reflect the emerging view that considerable flexibility exists in the interactions of the G-protein-coupled receptors and G_{α} proteins. This flexibility may serve to integrate signals from several sources to a single signal transduction pathway and/or to promote activation of multiple signal transduction pathways by a single receptor.

The sensitivity of the bioassay was greatly improved by deleting the *SST2* gene. *SST2* encodes a protein thought to be involved in adaptation and recovery in response to pheromone treatment (6, 7). Lack of functional Sst2p greatly increases sensitivity to the mating pheromone and prolongs recovery from pheromone-induced cell cycle arrest (6, 7). Genetic evidence implies a direct interaction between Sst2p and Gpa1p. Dominant gain-of-function mutations in *SST2* block mating signal transduction (14). Overexpression of Gpa1p suppresses the hypersensitivity of *sst2-1* mutants (13), and the signaling block of the gain-of-function mutations act in an *SST2* allele-specific fashion (14). In this report, we demonstrate that Gpa1-$G_{\alpha i2}$ is compatible with *SST2*-mediated adaptation. Thus, either structural aspects of G_{α} proteins that permit their action in the *SST2*-mediated adaptation pathway are present in the carboxy-terminal domain of the protein and are conserved between species (see above) or domains involved in this process are found in the amino terminus. Deletion of *SST2* dramatically enhances signaling of both S-14, through the SSTR2, and the α-mating pheromone, through Ste2p. However, preliminary data suggest that the effect of deleting *SST2* is greater in strains containing Gpa1p than in Gpa1-$G_{\alpha i2}$-containing strains. In *SST2* cells, S-14-induced growth of cells containing Gpa1p was barely detectable, while Gpa1-$G_{\alpha i2}$-containing cells exhibited a significant growth response. In *sst2Δ* cells, SSTR2-mediated signaling through Gpa1p was comparable to that seen with Gpa1-$G_{\alpha i2}$, responding to as little as 60 pmol of S14. These observations support the idea that Sst2p acts at the level of the G_{α} protein to promote adaptation and return to the mitotic cell cycle and are consistent with the prediction that Sst2p would interact most efficiently with the native G_{α} subunit. Accordingly, exchange of carboxy-terminal domains in the chimeric G_{α} protein leads to a protein structure upon which Sst2p acts less efficiently, resulting in a reduced extent of adaptation and an enhanced growth response in the bioassay.

One potential function for a presumptive Gpa1p-Sst2p complex may be to target Gpa1p for degradation by the N-end rule pathway (31). Madura and Varshavsky reported that overexpression of the targeting proteins Ubr1p and Ubc2p destabilized Gpa1p in the presence of Sst2p while Gpa1p was stabilized in cells lacking functional Sst2p (31). Paradoxically, our results and those of others demonstrate that increased Gpa1p levels decrease pheromone sensitivity, and mutations in *SST2* increase pheromone sensitivity (6, 7). However, the overexpressed form of Gpa1p examined by Madura and Varshavsky was likely to be nonfunctional, as it was modified at the functionally critical amino and carboxy termini (10). In contrast, expression of functional myristoylated Gpa1p is unaffected by the lack of Sst2p (14).

Heterologous expression of G-protein-coupled receptors and G_{α} proteins in yeast cells provides several useful advantages over expression in mammalian cells. Ligand binding data for mammalian cells are often confounded by the presence of multiple receptor types. The mating pheromone receptors are the only known G-protein-coupled receptors in haploid yeast cells. Thus, the effects of ligands at receptors other than the receptor of interest is limited to *STE2* or *STE3*, and these may be eliminated by homologous recombination using appropriate deletion constructs. In addition, yeast cells express a single type of G_{α} protein, *GPA1*, that participates in the pheromone response pathway. Detailed examination of G_{α}-protein subtypes may be carried out without regard to confusion caused by the cellular G_{α}-protein composition.

Implementation of the sensitive and specific yeast expression system described in this report will facilitate description of structural and functional aspects of receptor-ligand and receptor–G-protein interactions. Powerful genetic selection schemes, made possible by modification of elements of the pheromone response pathway, may be employed to identify aspects of the receptor that have effects on agonist selectivity, ligand stereo selectivity, and determinants of agonist or antagonist binding. The role of proteins (e.g., Sst2p, arrestins, and receptor kinases) that modify the response of receptors and G proteins to the ligand may be worked out in detail with assistance of this powerful genetic system. Finally, the system is adaptable to other combinations of G-protein-coupled receptors and G proteins and, therefore, should be useful in identifying ligands for orphan G-protein-coupled receptors and for discovering novel therapeutically useful ligands for receptors of medical or agricultural importance.

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