6-1-1990

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Effects of expression of mammalian G alpha and hybrid mammalian-yeast G alpha proteins on the yeast pheromone response signal transduction pathway

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Effects of Expression of Mammalian Gα and Hybrid Mammalian-Yeast Gα Proteins on the Yeast Pheromone Response Signal Transduction Pathway

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Received 7 December 1989/Accepted 27 February 1990

Scg1, the product of the Saccharomyces cerevisiae SCG1 (also called GPA1) gene, is homologous to the α subunits of G proteins involved in signal transduction in mammalian cells. Scg1 negatively controls the pheromone response pathway in haploid cells. Either pheromonal activation or an scg1 null mutation relieves the negative control and leads to an arrest of cell growth in the G1 phase of the cell cycle. Expression of rat Gas was previously shown to complement the growth defect of scg1 null mutants while not allowing mating. We have extended this analysis to examine the effects of the short form of Gas (which lacks 15 amino acids present in the long form), Gα2, Gβγ, and Scg1-mammalian Gα hybrids. In addition, we have found that constructs able to complement scg1 are also able to inhibit the response to pheromone and mating when expressed in a wild-type SCG1 strain. Overexpression of Scg1 has a similar inhibitory effect. These results are consistent with a model proposed for the action of Scg1 as the α component of a heterotrimeric G protein in which the βγ component (Ste4/Ste18) activates the pheromone response after dissociation from Scg1. They suggest that the Gα constructs able to complement scg1 can interact with βγ to prevent activation of the pathway but are unable to interact with pheromone receptors to activate the pathway.

The G protein family transduces hormonal and sensory signals received by a major class of transmembrane receptors in eucaryotic cells. These receptors respond to an extracellular signal by directly activating a G protein on the cytoplasmic face of the membrane (for a review, see references 12, 26, and 29). Mammalian G proteins are heterotrimers of α (39 to 52 kilodaltons), β (35 to 36 kilodaltons), and γ (8 to 10 kilodaltons) subunits. The α subunits have highly conserved regions that are similar to the guanine nucleotide-binding pockets of the structurally defined GTP-binding proteins, EF-Tu and c-H ras (9, 31). In the unstimulated state, the nucleotide-binding domain of α is occupied by GDP, and the G protein exists as a stable, membrane-bound heterotrimer. Interaction of an agonist with the appropriate receptor induces a receptor-G protein interaction that results in replacement of GDP by GTP and dissociation of α(GTP) from βγ. In the phototransduction and β-adrenergic systems, α(GTP) subunits directly activate effectors; however, recent evidence suggests that βγ can stimulate phospholipase A2 activity in cardiac cells (4, 17).

In the yeast Saccharomyces cerevisiae, haploid cells of a and α mating types are prepared for mating by mutual interactions mediated by peptide pheromones (for a review, see reference 8). α cells secrete a-α factor, a tridecapeptide that binds to the α-α factor receptor present on a cells, and a cells secrete a-α factor, a modified dodcapeptide that binds to the α-α factor receptor present on a cells. Binding of either pheromone to its receptor results in an arrest of the cell cycle in G1 and other responses that are necessary for mating. Sequencing of the structural genes for the a- and α-factor receptors (STE3 and STE2, respectively; 5, 13, 25) suggests that both have the seven transmembrane-spanning domain topology characteristics of mammalian G protein-coupled receptors.

Genes involved in pheromone response and mating that encode α, β, and γ homologs have been identified. The α homolog (Scg1) is encoded by SCG1 (10) (also called GPA1; 22, 23), and the β and γ subunits (Ste4, Ste18) are encoded by STE4 and STE18, respectively (32). Null mutations in these genes result in phenotypes in haploids but not diploids. The ste4 and ste18 null mutants are sterile (i.e., are defective in pheromone response and mating), indicating that wild-type Ste4 and Ste18 play positive roles in activation of the pheromone response pathway. The scg1 null mutants show cell cycle arrest and morphological changes characteristic of pheromone response; this phenotype represents constitutive activation of the pheromone response pathway, indicating that wild-type Scg1 plays a negative or inhibitory role (10, 14, 22, 24).

Genetic results indicate that β and γ act downstream of α in the pathway (1, 24, 32). A model consistent with these results suggests that, in the resting state, the G protein exists as a heterotrimer, α(GDP)βγ (10, 14, 32). After activation of the pathway and guanine nucleotide exchange, α(GTP)βγ dissociate, and βγ stimulates pheromone response by activation of a downstream effector. The negative role of α results from inhibition of βγ activity.

It was previously shown that expression of rat Gas (the α subunit of the G protein that activates adenylate cyclase) under the control of the yeast CUP1 promoter complemented the growth and morphological defects of an scg1 null mutant (10). The scg1[Gas] cells were sterile. In this paper, we have extended the analysis of the complementation of scg1 by mammalian Gα genes to Gα2 (one of three related Gα proteins involved in inhibition of adenylate cyclase) and
TABLE 1. Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotypic characteristics</th>
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<td>DJ211-3-4</td>
<td>barl-1 derivative of 147-1-2</td>
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* Markers shown in brackets are homozygous.

Gao (a G protein of unknown function abundant in brain tissue). We have also constructed and tested hybrid Scgl and mammalian Gα genes. We show that expression of many of these constructs efficiently complements the scgl growth defect but does not allow mating. In addition, their expression exerts a dominant inhibitory effect on the response to α-factor and on mating in both a and α cells. Overexpression of SCG1 has the same inhibitory effect.

MATERIALS AND METHODS

Yeast strains, transformations, and expression vectors. The yeast strains used are described in Table 1. Transformants of these strains with the plasmid constructs described below were selected using uracil or tryptophan prototrophy and maintained on SC-Ura medium (synthetic complete medium lacking uracil; 3) or SC-Trp medium. All media contained 2% glucose except media designed for induction of pGAL constructs, which contained 2% sucrose and 1% galactose. YEP-Gal medium, used for tetrad analysis, is yeast extract-peptone (3) containing 2% galactose. All plasmid constructs were propagated in Escherichia coli AKE28 (20) under ampicillin selection.

Expression vectors, pPGK (YEpdT-PGK; Fig. 1). The segment comprising AmpR, Ori, and URA3 was derived from YIp5. The 2-μm replication origin (a 1,424-base-pair (bp) HaeIII-to-HpaI fragment from YEp24) was inserted at the EcoRI site of YIp5, as in p13A1 (3). The PGK expression cassette was inserted between the Clal and NruI sites (converted by linker insertion to Xhol and SalI, respectively) and consisted of two fragments derived from pMA91 (20). pMA91 contained a 2.9-kilobase HindIII fragment of yeast DNA containing the PGK gene and flanking sequences and a deletion which joined nucleotide −2 to the BgII site 30 codons from the C terminus of the gene. The 672-bp fragment comprising UASPGK was derived by inserting an Xhol linker at the DraI site at −670 and an EcoRI linker at the BglII site of pMA91 at −2. The 300-bp translation and transcription termination fragment was derived by Bal 31 recombination at the BgII site followed by insertion of a BamHI linker one codon upstream of the termination codon (TAA) in the PGK reading frame. Terminators occurred in all three reading frames within 22 bp of the BamHI site, immediately followed by a Clal site (GGATCCGGGA AATTTGTTGATTCATTTA). A SalI linker was inserted at the HindIII site 300 bp from this BamHI site, downstream of the PGK transcription terminator. Filling in of the two PGK fragments cut at the their EcoRI and BamHI sites, followed by religation with a HindIII linker, regenerated both sites (GAATCCCAAGTCTTGGGATCC), producing YEpdT-PGK with unique EcoRI, HindIII, and BamHI cloning sites for expression (Fig. 1).

pGAL (YCpDT-GAL; Fig. 1). The 672-bp XhoI-EcoRI fragment comprising UASPGK in pPGK was replaced with the 685-bp BamHI-EcoRI UASPGK fragment from pBM272 (provided by Mark Johnston) after filling in both XhoI and BamHI sites. The XhoI site was reorganized. The product was YEpdT-GAL. The 985-bp XhoI-Sall fragment of YEpdT-GAL comprising the expression cassette was reinserted into pBM272 between the EcoRI and SalI sites after filling in both the EcoRI and XhoI sites, producing YCpDTGAL (Fig. 1). This vector contained the ARS1, CEN4, and URA3 yeast fragments (indicated in Fig. 1) derived from pBM272.

pCUP (pYSK-136; Fig. 1). The pCUP vector (11) comprised the AmpR and Ori fragments of pBR322, the 2-kilobase EcoRI origin fragment of 2 μm, a 900-bp TRP1 fragment, and an expression cassette consisting of a 430-bp UASGAL fragment and a 310-bp CYC1 terminator fragment separated by unique NcoI and Asp718I expression sites.

cDNAs and expression of full-length constructs. Ge cDNAs. Rat Gα cDNAs were provided by Randall R. Reed (15) as EcoRI fragments in a phage or by Gary Johnson as sub-
clones in pGEM2. They included Gai2, Gao, and both Gas long (Gus) and short [Gas(sh)].

**pCUP expression constructs.** The Gas cDNA clone was cleaved at the AfII site 27 bp past the translation termination codon. After being filled in with DNA polymerase (Klenow fragment) and cut with NcoI at the initiation codon (boldface) (CCATGGG), the 1,218-bp fragment of Gas was cloned into pCUP between the NcoI and filled-in Asp718I sites. The Gas(sh) construct was made by exchanging the internal BamHI fragment of Gas in pCUP-αs for the equivalent fragment of Gas(sh). Similar procedures were used to insert minimal-length fragments of Gai2 and Gao cDNAs lacking rat 5’ sequences into the same expression sites.

**pGAL and pPGK expression constructs (Fig. 2).** To insert rat Gas into pPGK, the pGem clone was cleaved at the unique NcoI site at the translation start point. Insertion of a 10-bp HindIII linker after filling in produced a HindIII site at -9, leaving only 1 bp of rat 5’ sequence before the initiation codon (CCAACTG). The 1,374-bp HindIII fragment starting at this point and ending 191 bp downstream of the translation terminator for Gas was inserted into the HindIII sites of pPGK and pGAL for expression.

Rat Gai2 cDNA was provided as a 1,750-bp EcoRI fragment in pGEM2. pPGK-αi was produced by partial BglII digestion of the pGEM2 clone followed by treatment with the Klenow fragment of DNA polymerase and insertion of a 10-bp EcoRI linker, producing an EcoRI site at -22 and retaining 14 bp of rat 5’ sequence (CCCAATCCGCGCGGCGGAGAGAGAGAGAG). The 1,619-bp EcoRI fragment from this site to the EcoRI site 525 bp downstream of the translation terminator for Gai2 was inserted into the EcoRI sites of pPGK and pGAL for expression (Fig. 2).

The yeast SCG1 gene was cloned into M13 mp10 as a 1,931-bp EcoRI fragment. Mutagenesis with the oligonucleotide 5'-TTAGCATCACATCAAGAATTCAGAG-3' (underlined nucleotides indicate positions resulting in nucleotide changes) was employed to insert an EcoRI site at -42 (Fig. 2). The new 1,762-bp EcoRI fragment was cloned into M13 mp19 and mutated with 5'-GAAGTGGATCCATTGTT TCG-3' to create a silent BamHI site at +989, producing Scg-1(b). The 1,762-bp EcoRI fragment, with and without this mutation, was cloned into pGAL for expression, producing pGAL-Scg and pGAL-Scg(b) (Fig. 2). pPGK-Scg was constructed similarly.

**Gα fusion constructs (Fig. 2).** Sgc-αi. The 1,031-bp N-terminal fragment of SCG1 from the EcoRI site at -42 to the mutant BamHI site at 989 and the 1,018-bp C-terminal fragment of Gai2 from the BamHI site at 634 to the downstream HindIII site of the pGEM2 multicloning site were inserted simultaneously into pGAL and also into pPGK, both cut with EcoRI and HindIII. The sizes of the protein

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**FIG. 2.** Ga proteins and fusions: open reading frames with the lengths (in amino acid residues) of various regions indicated. Symbols: □, SCG1; □, mammalian Ga cDNAs. The HindIII site in SCG1 is approximately at the start of the unique 110-residue loop in Sgc1 which has no counterpart in the mammalian Ga proteins (10, 23). A BamHI site was introduced into SCG1 at a position homologous to a BamHI site in Gas, Gai2, and Gao (see Discussion), and the hybrid genes shown were constructed. The sizes (in base pairs) of the 5’ and 3’ sequences included when these constructs were inserted in the pGAL and pPGK expression vectors (Fig. 1) are also indicated. The pCUP constructs utilized an NcoI site at the Ga ATG initiation codon; therefore, no rat 5’ sequences were present upstream of the initiation codon in these constructs (data not shown).
fragments in the hybrids resulting from this and other fusions are indicated in Fig. 2.

Scg-αs. The same SCG1 fragment and the 666-bp C-terminal fragment of Gas from the BamHI site at 707 to the downstream HindIII site were inserted into the expression vectors as described above.

Scg-αo. Rat Gas cDNA, provided as a 1,485-bp EcoRI fragment in pGEM2, was cloned directly into the EcoRI site of pPGK, producing pPGK-αo. This construct had 116 bp of 5' and 298 bp of 3'-flanking cDNA sequence (Fig. 2). The fragment of Gas from the BamHI site at 635 to the BamHI site just downstream of the EcoRI site in pPGK-αo (Fig. 1) was isolated and used to replace the BamHI fragment from 989 to the same vector site in pPGK-Scg(B), producing pPGK-Scg-αo (Fig. 2).

α1-Scg. The 656-bp N-terminal fragment of Gas from the EcoRI site at -22 to the BamHI site at 634 and the 783-bp C-terminal fragment of SCG1 from the mutant BamHI site at 989 to the downstream HindIII site were inserted into expression vectors simultaneously, as described above.

αs-Scg. The 710-bp N-terminal fragment of Gas from the HindIII site at -9 to the second BamHI site was obtained by partial BamHI hydrolysis and cloned into the pPGK and pGAL vectors cleaved with HindIII and BamHI. The 752-bp C-terminal SCG1 BamHI fragment from 989 to the downstream BamHI site was subsequently inserted in the correct orientation to create the αs-Scg fusions.

Assays for effects on the pheromone response pathway. Complementation of the growth defect due to scgl null mutations was tested by transformation of strain D109 or DI11 (Table 1) and sporulation and dissection of the diploids. Diploids containing pGAL constructs were dissected on YEP-Gal medium, diploids containing pCUP constructs were dissected on SC-Trp + Cu (100 μM) medium, and diploids containing the constitutive pPGK constructs were dissected on SC-Ura medium. Tetrad s were monitored for growth of more than two spores per tetrad, and viable segregants were picked and tested for the marker present at scgl and for mating.

Response to α-factor was tested by monitoring growth inhibition. Transformants of strain DJ211-3-4 (Table 1) were plated as lawns in soft agar overlays on SC-Ura medium (pH 4.7) with appropriate carbon sources. Disks containing different quantities of α-factor (10 μl of 0 \times 10^{-6}, 2 \times 10^{-6}, 5 \times 10^{-6}, 10 \times 10^{-6} M) were placed on the lawns. After 24 and 48 h at 30°C, zones of growth inhibition surrounding the disks were inspected for diameter and density of growth compared with the rest of the lawn. Clear, sharp-edged zones whose diameters were proportional to the log of α-factor concentration were produced on controls containing the vectors without inserts. Constructs that inhibited pheromone response allowed some growth within the α-factor zones; estimates of growth density were made visually. Repetition with multiple independent transformants gave reproducible results.

Inhibition of mating was tested by quantitative mating assays. Transformants of strains DJ147-1-1 and DJ147-1-2 (Table 1) were grown to mid-exponential phase on SC-Ura medium (pH 4.7) containing appropriate carbon sources, and mating efficiency was assayed by a membrane filter procedure (1).

RESULTS

Expression of heterologous and hybrid Ga proteins in yeast. In mammalian cells, major forms of Gas are expressed that differ by the presence or absence of 15 amino acids due to differential splicing (12). The form previously shown to complement the growth and morphological defects of scgl null mutants was Gas under the control of the copper-inducible CUP1 promoter (10). We have extended this analysis to Gas(sh), Gao2, and Gao (Fig. 2).

To examine the functional aspects of Ga protein domains, hybrids were made between SCG1 and rat Gas cDNAs (diagrammed in Fig. 2) as described in Materials and Methods. Construction of these hybrids involved introducing a silent mutation in the Ile-coding codon 331 of SCG1 that introduces a BamHI site to produce SCG1(B). BamHI sites were present in Gas, Gao, and Gao2 at the analogous position and were used to construct in-frame fusions between SCG1 and the three rat Ga cDNA genes.

The heterologous and hybrid Ga proteins were expressed in yeast by using three different vectors, pCUP, pGAL, and pPGK (Fig. 1). To allow efficient expression, the heterologous or hybrid gene in each of the plasmids was flanked by a yeast upstream regulatory region and a yeast terminator. pGAL is a YCP (stable, low-copy-number) vector with the galactose-inducible GAL1/10 promoter and PGK terminator. pPGK is a YEp (high-copy-number) vector with the constitutive PGK promoter and the PGK terminator. pCUP is a YEp vector with the copper-inducible CUP1 promoter and the CYC1 terminator.

The differences in plasmid copy number and promoter strength should have resulted in different expression levels for each of the vectors. Relative steady-state levels of transcripts, as determined by Northern (RNA) blot analysis with appropriate probes for the heterologous Gas proteins, were estimated to be 1:50:250 for pGAL, pPGK, and pCUP constructs, respectively (data not shown). The induced pGAL-Scg transcript in strain DJ211-3-4 was present at about twice the level of the endogenous 1,700-bp transcript derived from the wild-type gene (data not shown).

A polyclonal antiserum raised against a fusion of Gao to the N-terminal 81 residues of the influenza virus NS1 protein (34) was used to detect total cellular Gao proteins by Western blot (immunoblot) (data not shown). Protein was undetectable in cells of strain DJ211-3-4 expressing pGAl-αs. Proteins of the expected sizes were detected in cells expressing pCUP-αs, pPGK-αs, pPGK-αs-Scg, and pPGK-Scg-αs. All were detected at similar levels in spite of the higher transcript levels expressed by pCUP-αs. For some constructs (e.g., pCUP-αs and pPGK-αs), therefore, protein and RNA levels did not correlate well. Protein levels in cells expressing pCUP-αs(sh) were 5- to 10-fold higher than for pCUP-αs.

Complementation of scgl null mutations. In haploid yeast, scgl null mutations result in a growth arrest due to constitutive activation of the pheromone response pathway (10, 14, 24). The scgl spores that arise from dissection of an SCG1/scgl diploid form microcolonies (see Fig. 3; tetrad s with vector alone). It was previously shown that expression of pCUP-αs in scgl spores resulted in spore colonies that were smaller than those of the wild type (10; see Fig. 3) but that growth was similar to that of the wild type after the colonies were picked to fresh plates. The reason for the initial growth lag is unclear; however, the size of scgl spore colonies expressing the various Ga constructs provided a measure of complementation of the scgl growth defect. The ability of mammalian Ga genes and hybrids between the SCG1 and mammalian genes to complement scgl was tested by transformation of SCG1/scgl diploids with the various constructs and examination of the growth and mating of
spores obtained by sporulation of the diploids and tetrad analysis. Representative tetrads are shown in Fig. 3.

All of the $\alpha$S constructs complemented the $scgl$ growth defect, but none allowed mating (Fig. 3 and Table 2). Complementation was poorest with pGAL-$\alpha$s, intermediate with pCUP-$\alpha$s, and best with pPGK-$\alpha$s. Expression of Ga(s)(sh) under the control of the CUP1 promoter [pCUP-$\alpha$s(sh)] produced results indistinguishable from those produced with the long form (pCUP-$\alpha$s; Fig. 3).

Ga2i2 expressed from pCUP or pPGK but not from pGAL weakly complemented the $scgl$ growth defect (Fig. 3 and Table 2). Again, pPGK-Gai2 gave the strongest effect. The weakly complemented cells were sterile. No complementation of $scgl$ was seen in cells expressing Gao from either pCUP or pPGK.

A silent BamHI site was introduced into $SCGI$ to allow construction of the hybrid genes. Growth and mating were normal in both $scgl$ and $SCGI$ cells expressing either pGAL-Scg or pGAL-Scg(h) (Fig. 3), consistent with the presumptively silent mutation. The Scg-$\alpha$s, Scg-$\alpha$i, and Scg-$\alpha$o hybrids efficiently complemented the $scgl$ growth defect on either pGAL or pPGK vectors (Fig. 3 and Table 2), and the resulting cells were sterile. Again, the pPGK constructs were more effective than the pGAL constructs. The remaining hybrids, $\alpha$s-Scg and $\alpha$i-Scg, expressed from either pGAL or pPGK, were unable to complement $scgl$.

Inhibition of pheromone response and mating by expression of Ga proteins. The sterility of $scgl$ cells expressing heterologous or hybrid Ga proteins suggested that these proteins were able to interact with a downstream component of the pheromone response pathway to keep the pathway inactivated but were unable to interact with the pheromone receptors to elicit activation of the pathway in response to pheromone (see Discussion). If the heterologous and hybrid

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FIG. 3. Complementation of $scgl$ growth defect by Ga constructs. Strain D111 ($SCGI/scgl$) containing the indicated constructs was sporulated and dissected onto medium that provided the appropriate selection and induction of the various plasmid constructs (see Materials and Methods). With vector alone, tetrads gave rise to two wild-type colonies and two barely visible colonies. The larger colony size for more than two spores per tetrad indicates complementation of the $scgl$ growth defect. The relative complementation by these constructs is summarized in Table 2.
produced clear, sharp-edged zones of growth inhibition on lawns of the $SCGI$ bar1 strain (DJ211-3-4) carrying the vectors without insert (Fig. 4). Cells containing constructs that complemented the $scgl$ growth defect also showed zones of growth inhibition of similar sizes, but this inhibition was partial; i.e., a variable extent of growth was seen within the $\alpha$-factor zone. It has been suggested that growth within the zone of inhibition reflects more rapid recovery from growth inhibition, and a role has been proposed for $Scg1(GTP)$ in this adaptation phenotype (6, 7, 21). Analysis of growth kinetics in $scgl$ strains expressing the heterologous and hybrid $\alpha$ constructs (C. Mang and D. J. Tipper, unpublished data) suggested that the primary effect was on initiation of the response rather than on adaptation. We therefore interpret our assay as measuring inhibition of pheromone response. The relative levels of growth within the $\alpha$-factor zones were used to estimate the degree of inhibition (Table 2).

Expression of the same constructs also resulted in an inhibition of mating as determined by quantitative mating assays (Table 2). Inhibition was more effective when a particular construct was expressed in cells of both mating types rather than in only one of the mating pair; e.g., 94% inhibition of mating was seen when both a and $\alpha$ cells contained pPGK-$\alpha$; whereas inhibition was about 85% when cells of either mating type contained pPGK-$\alpha$ and the strain of the opposite mating type contained the pPGK vector. The results summarized in Table 2 represent mating frequencies with strains of both mating types expressing identical constructs.

An excellent correlation between the ability to complement the $scgl$ growth defect and the ability to inhibit pheromone response and mating in the $SCGI$ strain was seen (Table 2). Inhibition by $Ga$ was considerably greater than that by $Gai2$ expressed from the same construct, and $Gao$ did not cause detectable inhibition. Inhibition was greater for a particular $\alpha$ protein expressed from pPGK or pCUP than when expressed from pGAL. The Scg-$\alpha$ and Scg-$\alpha$ constructs had the strongest effects in all three assays of any construct tested.

Overexpression of $SCGI$ inhibited mating and response to $\alpha$-factor. Growth rates were normal in both $scgl$ and $SCGI$ cells expressing pPGK-Scg. However, while mating was normal in both $scgl$ and $SCGI$ cells expressing pGAL-Scg, and expression of pGAL-Scg did not affect the $\alpha$-factor

<table>
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<th>Plasmid</th>
<th>Growth ratea</th>
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* Colony size was estimated for $scgl$ segregants carrying the indicated plasmids (Fig. 3). Symbols (colony size): +++, indistinguishable from $Scg1^{*}$; +, somewhat smaller; +, small; (+), very small; -, identical to $scgl$ null spores.

* Assessed from the relative densities of growth within zones of inhibition surrounding $\alpha$-factor disks on test plates (Fig. 4). Symbols: +++, lack of a detectable inhibition zone; +++, density 60 to 80% of lawn; ++, density 30 to 60% of lawn; +, density 10 to 30% of lawn; (+), several isolated colonies inside zone of inhibition; -, normal response.

* Assayed as described in Materials and Methods. Mating efficiency was normalized to matings of pairs of cells containing the PGK vector. ND, Not determined.

Ga proteins are able to interact with the downstream component in the presence of Scg1, they should inhibit activation of the response pathway in the presence of pheromone, resulting in dominant inhibition of mating. We therefore tested $\alpha$-factor response and mating in wild-type $SCGI$ strains expressing the various constructs.

Pheromone response was assayed by a plate assay for the inhibition of growth by $\alpha$-factor. Disks containing $\alpha$-factor

FIG. 4. Plate assays of $\alpha$-factor sensitivity. Cells of strain DJ211-3-4 (MATa $SCGI$ bar1), transformed with the indicated plasmids, were spread as a soft agar overlay onto SC-Ura or SC-Trp (100 $\mu$M Cu++) buffered to pH 4.7 and exposed to disks containing increasing quantities of $\alpha$-factor (see Materials and Methods). Zones are shown after 48 h at 30°C.
response in the wild-type a strain (DJ211-3-4) (Fig. 4), expression of pPGK-Scg efficiently inhibited α-factor re-
response in this strain. The test plates failed to show even faint zones of inhibition surrounding α-factor disks (Fig. 4). Mating efficiency was also reduced by 86% when pPGK-Scg was expressed in both a and α cells (Table 2).

**DISCUSSION**

Scgl, the product of the yeast SCGI gene, is a yeast Ga homolog involved in pheromone response and mating (10). It was previously shown that the expression of the long form of rat Gαs in yeast was able to complement the growth and morphological defects resulting from null mutations in scg1 (10). In this paper, we have extended this analysis to examine the effects of Gas5(sh) (which lacks 15 amino acids present in the long form; 12), Ga12, Gαo, and Scg1 and mammalian Gaαs hybrids. In experiments with the heterologous construct able to complement the growth defect in an scg1 mutant are also able to inhibit the response to α-factor and mating when expressed in a wild-type SCGI strain. Overexpression of Scg1 has a similar inhibitory effect.

These results are consistent with a simple model (Fig. 5A) for the role of the yeast Gα(Scg1), Gβ (Ste4), and Gγ (Ste18) subunits in the activation of the pheromone response pathway. Genetic results (1, 24, 32) indicate that 13y functions downstream of α to activate the pathway, presumably by activating a downstream effector which is currently unidentified. In the absence of pheromone, α is presumed to bind GDP tightly and interact with βγ to inhibit the pathway. In the presence of pheromone, the pheromone receptor interaction relieves this negative control by promoting GDP and GTP exchange on α, resulting in dissociation of a from βγ; free βγ then activates the pathway. In an scg1 null mutant, free βγ is present and constitutively activates the pathway (Fig. 5B), leading to G1 arrest and morphological alterations.

The ability of a mammalian Gα protein to complement the scg1 growth defect suggests that the heterologous protein is able to interact with yeast βγ as an analog of Scg1(GDP), thus preventing activation of the pathway (Fig. 5C). The resulting cells are sterile and unable to respond to pheromone, suggesting that the heterologous protein cannot interact functionally with the pheromone receptors. The ability of heterologous proteins to complement the scg1 growth defect correlates well with their ability to inhibit pheromone response and mating when expressed in wild-type SCGI cells. This effect is also consistent with the model, because the heterologous or hybrid Gα protein competes with Scg1 for binding to βγ (Fig. 5D). Upon pheromone exposure, only that portion of βγ bound to Scg1 is freed and any excess heterologous Gα protein competes with Scg1 for binding to βγ, resulting in reduced activation of the pathway and reduced mating. The degree of inhibition should reflect the level of expression of the heterologous Gα protein, as was observed. Greater inhibition of mating was observed when both mating partners expressed the heterologous protein compared with the inhibition observed when expression occurred in only one of the two partners, because both mating partners must respond for mating to occur.

Comparison of Gαs with other rat G proteins by our functional assays showed relative levels of effectiveness of Gαs = Gas5(sh) > Ga12 > Gαo. The lower activity of Ga12 compared with that of Gαs is somewhat surprising, given that sequence conservation between Scg1 and Ga12 (10, 23) is significantly higher than for Gαs. Intriguingly, in vitro studies of Gα proteins (2, 12, 16, 27, 28) indicate relative affinities of mammalian βγ of Gαs > Ga12 > Gαo, which correlates with relative activities in our assays. Direct assays of G protein subunit interactions will be necessary to determine the relevance of this correlation.

The construction of Scg1 and mammalian Gaαs hybrids was undertaken to analyze the role of various domains of Ga subunits (18) in the yeast signal transduction pathway. Fusions between SCGI and rat Gaα cDNAs were made at a site within a highly conserved sequence of 32 amino acids predicted by analogy to ras (9) and EF-Tu (18) to comprise a critical component of the nucleotide-binding pocket of Ga proteins. The fusions, therefore, should be likely to retain normal structure in this region, and this region is unlikely to be involved in differential Gα functions. A Gαi-Gαs hybrid with the analogous fusion position has been shown to function in differential Gα functions. A Gαi-Gαs hybrid with the analogous fusion position has been shown to function in mammalian systems (19). The Scg1-αs, Scg1-α2 and Scg1-αo hybrids (Fig. 2) inhibited pheromone response and mating in an SCGI strain and allowed growth but not mating of the scg1 null mutant. These results indicate sufficient conservation of structure between the yeast and mammalian Ga proteins to allow the function of appropriate domains in the hybrids and suggest that, like intact Gαs and Gai, these hybrid proteins can interact with βγ but not with the pheromone receptors (Fig. 5). The sterility of scg1 strains and inhibition of mating in SCGI strains expressing the hybrids are consistent with an essential role for the C terminus of Scg1 in receptor interactions, as demonstrated in mammalian systems (18, 19, 30). High-level expression of the N-terminal 330 amino acids of Scg1 by itself was without effect in our assays (data not shown), and a 22-amino-acid C-terminal truncation of Scg1 results in an scg1 null phenotype (S. Hirsch, C. Dietzel, and J. Kurjan, unpublished data). Thus, the C-terminal region of Scg1 is essential to prevent activation of the pheromone response pathway by Scg1 in the absence of pheromone but can be replaced by heterologous Gα sequences.

The efficient activity of the Scg1-α2 and Scg1-αo hybrids, in contrast to that of the intact Gαi2 and Gαo proteins, suggests that the domain critical for interaction with βγ lies within the N-terminal 330 amino acids of Scg1. These results, therefore, are consistent with data from mammalian systems that have indirectly implicated the amino termini of Ga proteins in βγ interactions (18). The relative activities of the fusion proteins (Scg1-αi = Scg1-αo = Scg1-αs), unlike those of the intact proteins, do reflect sequence similarity to Scg1. The effectiveness of these fusions may depend, therefore, on the extent to which these heterologous C-terminal sequences interact appropriately with the N-terminal domain.

Because our results suggest that Gαs can bind to yeast βγ, we speculated that the α-Scg1 hybrid (Fig. 2) might be able to interact with both βγ and the pheromone receptors and thus allow pheromone response and mating. Instead, expression of this hybrid protein at a level similar to the levels of the functional constructs failed to produce a detectable phenotype. No definitive conclusion can be made from these negative results, although the lack of function of this hybrid protein suggests that its structure may be abnormal.

We also found that very high level expression of Scg1 resulted in the inhibition of pheromone response and mating (Table 2). One explanation for this result is that free α may play a role in adaptation (7, 21) and that increased levels of α could desensitize the pathway, leading to the inhibition of pheromone response and mating. Alternatively, this result may reflect the importance of the balance between levels of the G protein subunits for normal activation of the pathway.
FIG. 5. Model for pheromone response pathway and effect of expression of heterologous or hybrid Ga proteins. (A) In the absence of pheromone (•), the pheromone response pathway (E) is inactive (in) because Ga(GDP) (Scg1) binds Gβγ (Ste4/Stel8). Interaction of pheromone with its receptor induces a GDP-GTP exchange on Ga, resulting in the release of Gβγ, which positively activates (act) the effector pathway, leading to cell cycle arrest and other aspects of the pheromone response. (B) In an scgl mutant, Gβγ is free, leading to constitutive activation of the pathway and cell cycle arrest. (C) A heterologous or hybrid Ga protein (Gaα) complements the scgl growth defect in proportion to its ability to bind Gβγ. Because it cannot interact with the receptor, the pathway cannot be activated, so the cells are sterile. (D) When both Scg1 and a Gα are present, they compete for binding to Gβγ. Gα remains bound to Gβγ after exposure to pheromone, and any excess Gα will compete with effector in binding Gβγ released by Scg1(GTP), reducing activation of the pathway and mating. The degree of inhibition should be proportional to the level of Gα and its affinity for Gβγ. (E) In a strain greatly overproducing Scg1, conversion of Scg1(GDP)βγ to dissociated Scg1(GTP) and βγ by activated receptor is unaffected, but excess Scg1(GDP) is present and binds to free βγ. The presumptive intrinsic GTPase activity of Scg1 may result in the maintenance of Scg1(GDP) levels sufficient to keep the pathway inactivated.

It has recently been shown (6, 33) that increasing the level of Ste4 (β) constitutively activates the pathway, while a simultaneous increase in the level of Scg1 (α) prevents this constitutive activation. Our data suggest that greatly increasing α alone may result in the persistence of sufficient α(GDP) to complex the available βγ, even in the presence of a fully activated receptor (Fig. 5E). Increased levels of either receptor or βγ, therefore, might alleviate the inhibitory effect of increased α on the pheromone response.

ACKNOWLEDGMENTS

We thank J. Kevin O’Donnell for the preparation of the antiserum to the Gas fusion protein, Randall Reed and Gary Johnson for the generous gifts of cDNA clones, Duane Jenness for yeast strains, and Ted Wright for expert technical assistance. We thank Lorraine Marsh, Jeanné Hirsch, and Didi Robins for comments on the manuscript and Elliott Ross for pointing out the correlation for the Gα subunits between their activity in yeast and affinity for mammalian βγ.
This work was supported by grants GM20755 and DK32520 (a pilot grant) to D.J.T. and by Public Health Service grant GM 40585 to J.K. from the National Institutes of Health.

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