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The Yeast G Protein α Subunit Gpa1 Transmits a Signal through an RNA Binding Effector Protein Scp160

Short Article

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Summary

In yeast *Saccharomyces cerevisiae* the G protein βγ subunits (Ste4/Ste18) have long been known to transmit the signal required for mating. Here we demonstrate that GTPase-deficient mutants of Gα (Gpa1) directly activate the mating response pathway. We also show that signaling by activated Gpa1 requires direct coupling to an RNA binding protein Scp160. These findings suggest an additional role for Gpa1 and reveal Scp160 as a component of the mating response pathway in yeast.

Introduction

All cells have the capacity to respond to chemical and sensory stimuli in their environment. In many cases, signal detection occurs through cell surface receptors coupled to G proteins. In humans, receptors of this type can detect hormones, neurotransmitters, odors, taste, and light. In the yeast *Saccharomyces cerevisiae*, G protein-coupled receptors bind peptide ligands that promote haploid cell fusion, or mating. In this instance, haploid **a** and α cell types respond to pheromones secreted by cells of the opposite type. Receptor stimulation leads to activation of a G protein composed of an α subunit (Gpa1) and a tightly associated βγ dimer (Ste4/Ste18). Upon stimulation, Gα releases GDP, binds GTP, and dissociates from the βγ subunits, which in turn activate downstream signaling proteins. Events that precede mating include new gene transcription, morphological and cytoskeletal changes, and cell cycle arrest in G1 (Dohlman, 2002).

Gβγ propagates the mating signal through activation of at least three effector proteins: the Cdc42 GDP-GTP exchange factor Cdc24 (Zheng et al., 1994), a protein kinase Ste20 (Leeuw et al., 1998), and a kinase scaffolding protein Ste5 (Feng et al., 1998; Inouye et al., 1997; Whiteway et al., 1995). Activation of Cdc24 and Cdc42 is required for the morphological changes that accompany pheromone stimulation. Binding of Gβγ to Cdc24 occurs indirectly through Far1, a scaffold protein that is also required for pheromone-induced cell division arrest in G1. Activation of Ste20 initiates a signaling cascade that includes three other protein kinases associated with Ste5. The first is Ste11, which phosphorylates and activates a dual-specificity kinase Ste7, which phosphorylates and activates two mitogen-activated protein kinases (MAPKs) Fus3 and Kss1. Among the known MAPK substrates is the transcription factor Ste12, which is responsible for induction of most pheromone-inducible genes (reviewed in Dohlman and Thorner, 2001).

Gβγ has long been regarded as the sole signal-transmitting component of the G protein in yeast. Deletion of genes encoding either Gβ or Gγ results in a pheromone-insensitive sterile phenotype, while overexpression leads to constitutive signaling and growth arrest. Deletion of the Gα gene *GPA1* leads to constitutive signaling and growth arrest, apparently due to uncontrolled signaling by free Gβγ. Conversely, overexpression of *GPA1* leads to diminished signaling due to sequestration of free Gβγ. These findings demonstrated that the βγ moiety is sufficient to transmit the pheromone signal (reviewed in Dohlman and Thorner, 2001).

Studies in other systems have revealed examples of cooperative (or in some cases antagonistic) regulation of signaling pathways by both Gα and Gβγ (Jordan et al., 2000). Here, we propose a positive signaling function for the Gα subunit in yeast. These findings are significant because Gpa1 has not previously been shown to transmit a signal to any known effector. Moreover, the effector in this case, Scp160, has not previously been recognized to act in the pheromone response pathway, and more generally, RNA binding proteins have not previously been identified as targets of G protein activation.

Results

GTPase-Deficient Gpa1 Mutants Activate the Mating Pathway

Our goal here was to identify an effector and signaling pathway activated by Gpa1. Any such effector will, by definition, bind and respond only to the GTP-bound form of the protein. Thus, our initial approach was to determine the functional consequences of a Gpa1 mutant that cannot hydrolyze GTP, since this would result in permanent activation of any effector. Such a mutant will not directly affect signaling by Gβγ, since Gβγ binds only to the inactive GDP-bound form of Gα (Sprang, 1997). To this end, we expressed in wild-type cells two

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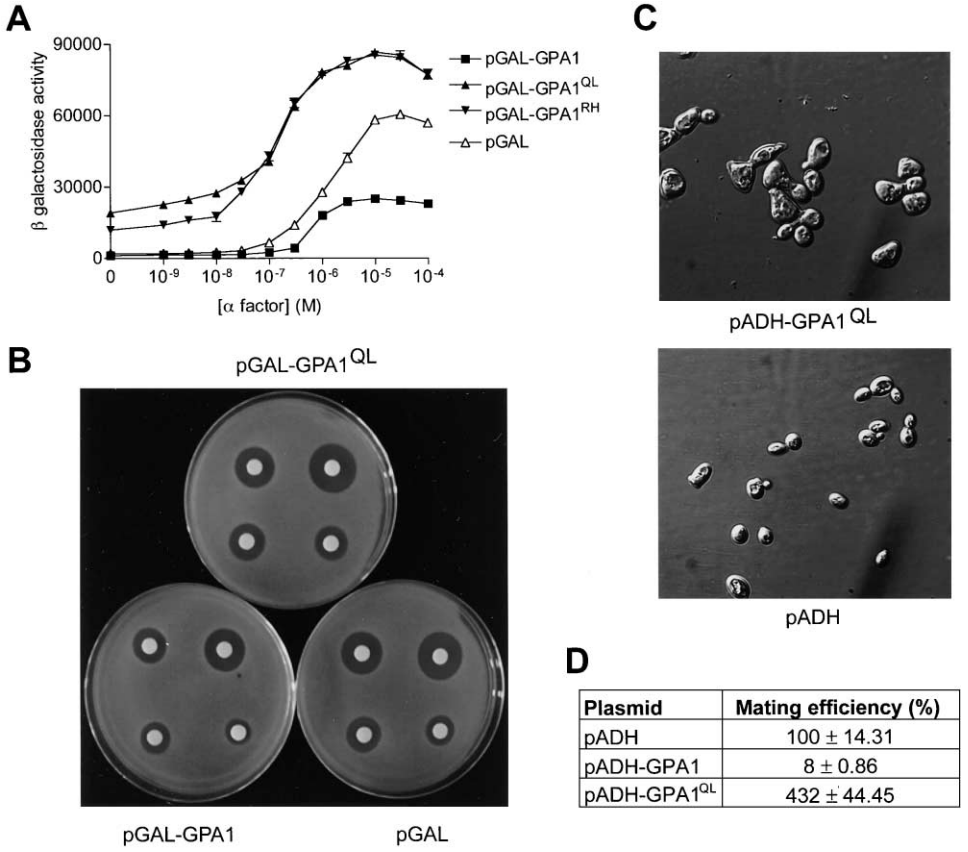

Figure 1. A Positive Signaling Role for Gpa1

(A) Wild-type cells were transformed with plasmid pG1501 (pGAL) containing no insert, *GPA1*, *GPA1*^{Q323L}, or *GPA1*^{R297H}, then grown in the presence of galactose for 6 hr to induce Gpa1 expression, and treated with the indicated concentration of α factor pheromone for 90 min. β-galactosidase activity was determined spectrofluorometrically using a pheromone-responsive *FUS1* promoter-lacZ reporter (plasmid pRS423-FUS1-lacZ).

(B) The growth inhibition plate assay was performed on the same cells, using 5, 15, 45, or 60 μg α factor for 48 hr.

(C) Wild-type cells were transformed with plasmid pAD4M containing no insert, *GPA1*^{Q323L}, or *GPA1* (not shown) and grown to mid-log phase.

(D) Mating efficiency was determined for the same cells. All data are representative of at least three independent experiments performed in triplicate (β-galactosidase assay, mating assay) or duplicate (halo assay). Error bars, ±SEM.

Gpa1 mutations that block (Q323L) or slow (R297H) GTP hydrolysis (Apanovitch et al., 1998) and measured pheromone-dependent gene transcription using a reporter-transcription assay (*FUS1* promoter, lacZ reporter). Because activation of the pheromone pathway can also trigger growth arrest, expression of the Gpa1 mutants was initially done using a galactose-inducible promoter that was activated shortly before performing the assay.

As shown in Figure 1A, both Gpa1 mutants produced a marked increase in signaling over the entire range of pheromone concentrations tested. Compared with the empty vector control, basal signaling was increased by 10-fold (Gpa1^{R297H}) or 15-fold (Gpa1^{Q323L}), and the maximum response was increased by ~40%. There was no difference in EC_{50}. In contrast, overexpression of wild-type Gpa1 diminished the maximum response by more than half. This inhibitory effect was anticipated, since the wild-type protein in the GDP-bound state will help to sequester free Gβγ. As an additional control we tested the Gpa1^{E364K} mutation, which was previously shown to inhibit pheromone-dependent growth arrest (Stratton et al., 1996). In this case we observed an inhibition of pheromone-dependent transcription nearly identical to that seen with overexpression of wild-type Gpa1 (data not shown). We also found that overexpression of Gpa1^{Q323L} leads to increased phosphorylation of Ste4 (data not shown), an event that requires activation of the MAP kinases Fus3 and/or Kss1 (Li et al., 1998). These results indicate that the GTP-bound form of Gpa1 can activate the mating response pathway leading to new gene transcription and MAP kinase activation.

Another way to measure pheromone sensitivity is the growth inhibition plate assay (halo assay). In this method, a nascent lawn of cells is exposed to different amounts of pheromone spotted onto paper disks. The resulting zone of growth inhibition provides an indication of the cellular response to pheromone after several days. Thus, we compared growth arrest in cells that overexpress Gpa1^{Q323L} or Ste4 versus the empty vector control. As shown in Figure 1B, Gpa1^{Q323L} produced nearly normal-sized halos, indicating that activation of Gpa1 has no effect on the growth arrest response (Apanovitch et al., 1998). In contrast, overexpression of Ste4 completely inhibited growth such that no cells were visible even after prolonged incubation (data not shown).

Cells treated with high concentrations of pheromone

will eventually arrest growth and assume an enlarged and elongated "shmoo" morphology. Thus, we compared growth arrest in cells that overexpress Gpa1^{Q323L} or the empty vector control. As shown in Figure 1C, overexpression of Gpa1^{Q323L} produced an enlarged and elongated cell morphology; however, the cells continued to bud and divide. This is consistent with the results presented above, indicating that Gpa1 can initiate a signal leading to transcription but it does not affect the growth arrest response. A number of other signaling mutants (e.g., *fus3*, *far1*) have likewise been shown to simultaneously shmoo and divide in the presence of pheromone.

The ultimate purpose of pheromone stimulation is to promote mating. Thus, we compared the mating efficiency of cells that overexpress the Gpa1^{Q323L} mutant with those that overexpress wild-type Gpa1 and cells that express normal levels of Gpa1. As shown in Figure 1D, Gpa1^{Q323L} improved the mating frequency by more than 4-fold, while overexpression of wild-type Gpa1 diminished mating efficiency more than 10-fold, as compared with the control strain. These data mirror the results of the reporter-transcription, Ste4 phosphorylation, and cell morphology assays presented above, and support the model that GTP-bound Gpa1 can activate the mating-response pathway in parallel with Gβγ.

Genetic Analysis of Gpa1 Signaling

The data above suggest that Gα and Gβγ can each promote activation of the mating response pathway. Activation by each moiety could occur through separate effectors; one effector responds to Gβγ and promotes morphological changes, transcriptional induction, and growth arrest, while a second effector responds to Gα and promotes morphological changes and transcription only. An alternative possibility is that the Gpa1 effector acts in opposition to Ste4/Ste18 to inhibit the growth arrest response. These models are considered in more detail below.

To determine the point at which the two pathways converge, we examined Gpa1 signaling in a series of gene deletion mutants affecting known components of the pathway; these include the cell surface receptor (*ste2*Δ) and G protein βγ subunits (*ste4*Δ), as well as two downstream kinases (*ste11*Δ, *ste7*Δ). As shown in Figure 2A, Gpa1 transcriptional activation was abolished in the *ste4*Δ, *ste11*Δ, and *ste7*Δ mutants. Transcriptional induction was still observed in the *ste2*Δ strain; however, the activity was diminished compared with that of the wild-type strain, presumably because unliganded receptor can promote a low level of GDP-GTP exchange on Gα (Siekhaus and Drubin, 2003). As expected, pheromone treatment had no additional stimulatory effect on signaling in any of these sterile mutants. Taken together, these results suggest that the Gpa1 effector acts downstream of the receptor, but at or upstream of Gβγ.

Since Gpa1 can stimulate transcription but fails to promote growth arrest, we considered whether it might act specifically through the MAPK Kss1. Deletion of *KSS1* results in diminished transcription yet does not alter the growth arrest response. Deletion of the other MAPK in the pathway, *FUS3*, diminishes both activities (Cherkasova et al., 1999; Cook et al., 1997; Madhani et al., 1997). Moreover, Kss1 and/or Fus3 appear to phosphorylate a number of proteins that bind to Gpa1 (Ste3, Sst2, Ste4) and Ste4 (Ste5, Far1), consistent with a close association of Gpa1 and the MAPKs (Dohlman and Thorner, 2001; Metodiev et al., 2002). Thus, we compared signaling by Gpa1^{Q323L} in strains lacking either *FUS3* or *KSS1*. As shown in Figure 2B, pheromone-induced transcription was diminished to a similar extent in both the *fus3*Δ and *kss1*Δ gene deletion mutants. Gpa1^{Q323L}-initiated signaling was also similar in both mutants, indicating that Gα signaling can occur through either kinase and not Kss1 specifically.

If Gα and Gβγ activate a common signaling pathway, they should induce the same set of gene transcripts. To test this, we transformed wild-type cells with *GPA1*^{Q323L} or an empty vector and grew these in the absence or presence of α factor for 1 hr. For these experiments we used a low dose of pheromone sufficient to trigger new gene transcription but not sufficient to invoke cell division arrest or induction of cell cycle-regulated genes (since Gpa1^{Q323L} does not trigger arrest). Biotin-labeled cRNA was prepared from each strain and hybridized to an Affymetrix GeneChip representing ~7000 genes and open reading frames. Under these conditions pheromone induced 20 transcripts, 17 of which were also induced by Gpa1^{Q323L} (Figure 2C). These data provide further evidence that Gpa1 and Ste4/Ste18 activate the same gene promoter elements, most likely through a common transcription factor Ste12.

The data presented in Figure 2A indicate that Gpa1 signals through Ste4/Ste18. If Gα and Gβγ are redundant, signaling by both components is likely to be nonadditive. If they have distinct functions, signaling might appear additive or even synergistic. To distinguish between these possibilities, we compared the transcription response in cells that overexpress Gpa1^{Q323L}, Ste4, or both. As shown in Figure 2D, overexpression of either protein produced a marked increase in signaling over the entire range of pheromone concentrations tested. Overexpression of both Ste4 and Gpa1^{Q323L} further enhanced signaling across the entire dose-response profile. In the absence of added pheromone or when the effects of pheromone were subtracted, activation by Gpa1^{Q323L} and Ste4 was additive. We conclude from these data that GTP-bound Gpa1 can contribute to signaling even when a cell is already activated by Gβγ. Stated differently, activation by both Gα-GTP and Gβγ is required for full activation of the pathway.

The experiments described above were performed in cells that contain endogenous wild-type Gpa1. We also examined the transcription response of Gpa1^{Q323L} in a *gpa1*Δ mutant. Deletion of *GPA1* will ordinarily result in constitutive growth arrest (through uncontrolled release of Gβγ) but can be maintained by placing a downstream effector kinase under control of an inducible promoter (*GAL1* promoter, *STE7* gene). As shown in Figure 2E, overexpression of Gpa1^{Q323L} produced an increase in *FUS1*-lacZ activity, similar to that observed in wild-type cells treated with pheromone (Figure 1A) or cells that lack Gpa1 altogether. Gpa1^{Q323L} was also unable to suppress the constitutive (Gβγ-mediated) growth arrest phenotype of the *gpa1*Δ strain (Apanovitch et al., 1998). These results show that mutationally activated Gpa1 can function in the absence or presence of endogenous wild-type Gpa1 and cannot sequester Gβγ in vivo.

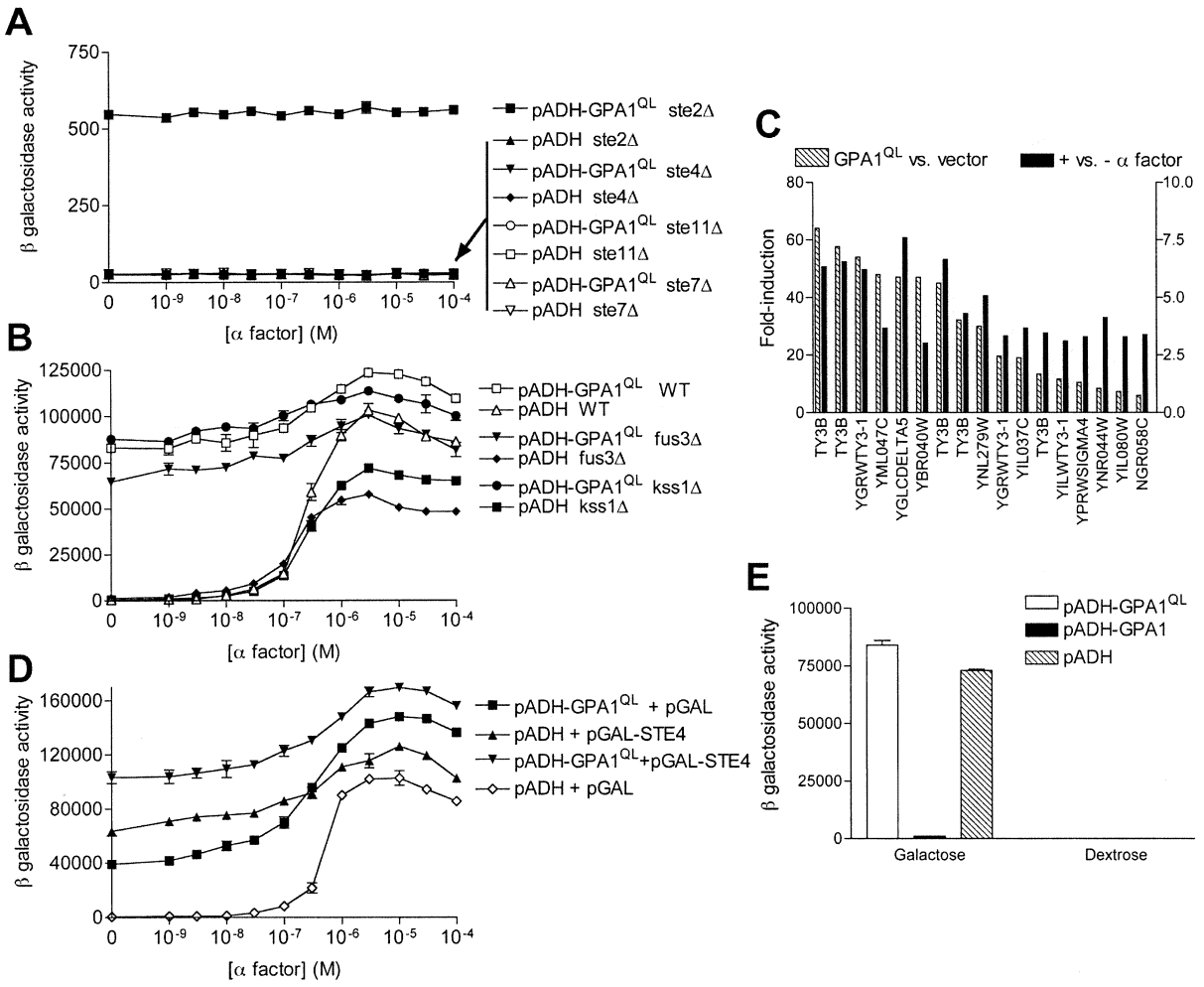

Figure 2. Gpa1 and Ste4/Ste18 Converge on a Common Signaling Pathway

(A and B) Plasmid pAD4M containing *GPA1*^{Q323L} (pADH-GPA1^{QL}) or no insert (pADH) was transformed into wild-type and mutant strains, as indicated. The reporter transcription assay was performed as described in the legend to Figure 1, except that the cells were grown in the presence of dextrose.

(C) Wild-type cells were transformed with plasmid pAD4M containing no insert (vector) or *GPA1*^{Q323L}. Biotin-labeled cRNA was hybridized to an Affymetrix GeneChip. Data are from values generated by averaging three replicate chips per strain. Comparisons are Gpa1^{Q323L} versus vector (hatched bars, left axis) or vector plus pheromone versus no pheromone (black bars, right axis). Shown are genes induced by >3-fold after 2.5 nM α factor (20 total) that are also induced by Gpa1^{Q323L}.

(D) Reporter transcription activity was measured in wild-type cells transformed with plasmid pAD4M containing no insert or *GPA1*^{Q323L}, and plasmid pRS316-GAL containing *STE4* (pGAL-STE4) or no insert (pGAL).

(E) Reporter transcription activity was measured in a *gpa1Δ ste7Δ* mutant strain transformed with plasmid pAD4M containing no insert, *GPA1*, or *GPA1*^{Q323L}, and plasmid pYES containing *STE7* and the *GAL1* promoter. Cells were grown in dextrose and then shifted to galactose to induce *STE7* (as indicated) for 6 hr.

Identification of a Gpa1 Effector by Genome-Wide Two-Hybrid Screening

One way to ascertain the Gpa1 effector is through the identification of associated proteins. To this end, we carried out a two-hybrid screen against an array of nearly all yeast open reading frames and identified Scp160 as a candidate binding partner (Uetz et al., 2000). To determine whether Scp160 is the Gpa1 effector, we examined whether the *scp160Δ* mutant blocks signaling by Gpa1^{Q323L}. As shown in Figure 3, Gpa1^{Q323L} elevates both the pheromone-dependent and -independent signals, while wild-type Gpa1 diminishes signaling. When the same experiment was performed in *scp160Δ* cells, however, the Gpa1^{Q323L} signal was completely abolished. In contrast, the ability of wild-type Gpa1 to diminish pheromone signaling (through sequestration of Ste4/Ste18) was preserved (Figure 3). Taken together, these data indicate that Scp160 could be an effector for Gpa1.

Scp160 Binds to the Active Conformation of Purified Gpa1

Any G protein effector will bind Gα in the active GTP-bound state, but not the inactive GDP-bound state. Thus, we examined whether Scp160 can interact with Gpa1 in a guanine-nucleotide-dependent manner. To this end, we coexpressed full-length Scp160 fused to

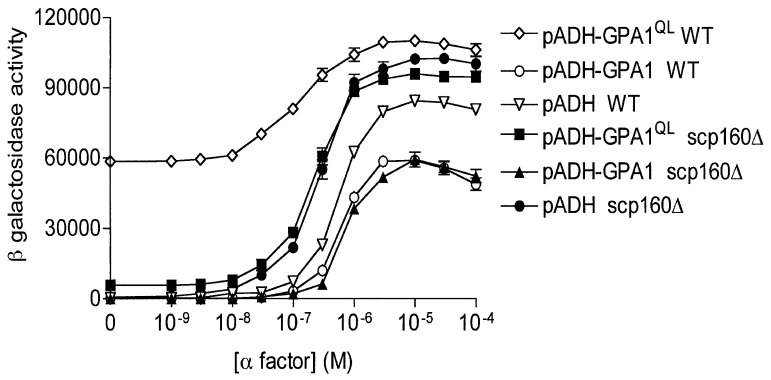

Figure 3. Gpa1 Signaling Is Blocked by *scp160Δ*

Plasmid pAD4M containing *GPA1*, *GPA1*^{Q323L}, or no insert (pADH) was transformed into wild-type (WT) and *scp160Δ* cells. The reporter transcription assay was performed as described in Figure 1.

the Myc epitope tag and Gpa1 fused to glutathione S-transferase (GST). Gpa1-GST was purified by glutathione-Sepharose affinity chromatography. The purified samples (Figure 4, Bound), as well as the starting material applied to each column (Applied), were resolved by SDS-PAGE and immunoblotting. Scp160 was tracked by immunodetection using the anti-Myc antibodies (Myc Ab). Gβγ binding was monitored using anti-Ste4 antibodies, as a positive control for Gpa1 function (Ste4 Ab). Equal loading of each lane was confirmed by immunodetection of identical samples using anti-GST antibodies (GST Ab).

As shown in Figure 4, Scp160 bound specifically to Gpa1-GST but not to GST alone. Significantly, Scp160 binding was diminished by addition of GDP and was enhanced by addition of GDP-AlF_4^-, a transition state mimic that closely resembles the active (GTP) conformation of Gα (Sprang, 1997). Gβγ (Ste4) binding followed the opposite pattern, with strong binding in the presence of GDP but not GDP-AlF_4^-. Another GTP mimic, GTPγS was less effective at promoting Scp160 binding or at inhibiting Ste4 binding. This is likely due to slow spontaneous release of GDP, which is required for binding of GTPγS but which is not a prerequisite for binding of AlF_4^-. Thus, Scp160 can bind specifically to the activated form of Gpa1 in the manner of known Gα effectors.

Discussion

Over the past fifteen years the paradigm of Gβγ-initiated signaling in yeast has held firm. Here, we propose a signaling function for the Gα subunit Gpa1. Moreover, we identify Scp160 as a binding partner and an essential component of the Gpa1 signaling pathway. No RNA binding protein has been identified previously as a G protein effector.

Other investigators have suggested previously that Gpa1 transmits a signal independently of Gβγ (Metodiev et al., 2002; Stratton et al., 1996; Zhou et al., 1999). The basis for this model comes from the ability of two dominant mutants, Gpa1^{E364K} and Gpa1^{N388D}, to promote recovery from G1 arrest following prolonged pheromone stimulation. Both mutants were presumed (but not demonstrated) to slow GTPase activity, which if true would lock the protein in a constitutively active state (Stratton et al., 1996; Zhou et al., 1999). However, Gpa1^{E364K} has not been characterized biochemically, and a published attempt to purify Gpa1^{N388D} yielded an inactive product devoid of measurable guanine nucleotide or Gβγ binding activity (Cismowski et al., 2001). In contrast, we have previously purified Gpa1^{Q323L} and documented its ability to bind guanine nucleotides and its inability to hydrolyze GTP (Apanovitch et al., 1998). Here we demonstrate that Gpa1^{Q323L} stimulates the transcription-induction response in the manner of pheromone or free βγ, and in a manner opposite to that of the Gpa1^{E364K} and Gpa1^{N388D} mutations. Using the same assay, we found that Gpa1^{E364K} inhibits transcription even when combined with the Q323L mutation (Gpa1$^{E364K/Q323L}$, data not shown). We speculate that Gpa1^{E364K} and Gpa1^{N388D} inhibit growth arrest because they form an inactive complex with the receptor and/or Gβγ and thereby prevent normal activation of the endogenous wild-type Gpa1. This model was proposed to explain a similar phenotype in Gpa1^{G50V}, Gpa1^{R327S}, and Gpa1$^{R327S/Q323L}$ mutants (Apanovitch et al., 1998; Kallal and Fishel, 2000).

One surprise here was the identification of an RNA binding protein as the effector for Gpa1 (Li et al., 2003). *SCP160* was cloned fortuitously by screening an expression library with antiserum raised against RNase H (Wintersberger et al., 1995). *SCP160* deletion mutants exhibit diminished viability, an enlarged and abnormal cell morphology, as well as elevated protein, RNA, and DNA content following sporulation. These elevated DNA levels led to speculation that Scp160 is needed for proper nuclear segregation, and the gene was named accordingly (*S*accharomyces protein *c*ontrolling *p*loidy, *160* kDa). Those phenotypes were not complemented by an episomal plasmid-expressing *SCP160* (Wintersberger et al., 1995). In contrast, the signaling phenotypes we observed here are rescued by *SCP160* (data not shown).

We have shown here that Gpa1^{Q323L} amplifies the pheromone signal, and this activity requires Scp160. Coexpression of Gβγ further amplifies the signal, but with some distinct properties. Whereas Gpa1^{Q323L} signaling requires expression of *SCP160,* Gβγ does not. Whereas Gβγ promotes growth arrest, Gpa1^{Q323L} does not. Perhaps the Gpa1^{Q323L} signal falls below a threshold needed to induce cell cycle arrest. Alternatively, Scp160 might regulate a component of the cell cycle machinery that diminishes the growth arrest response.

G proteins have traditionally been regarded as regulators of enzymes that produce some form of chemical second messenger. Recent studies have revealed new types of G protein effectors that are not necessarily

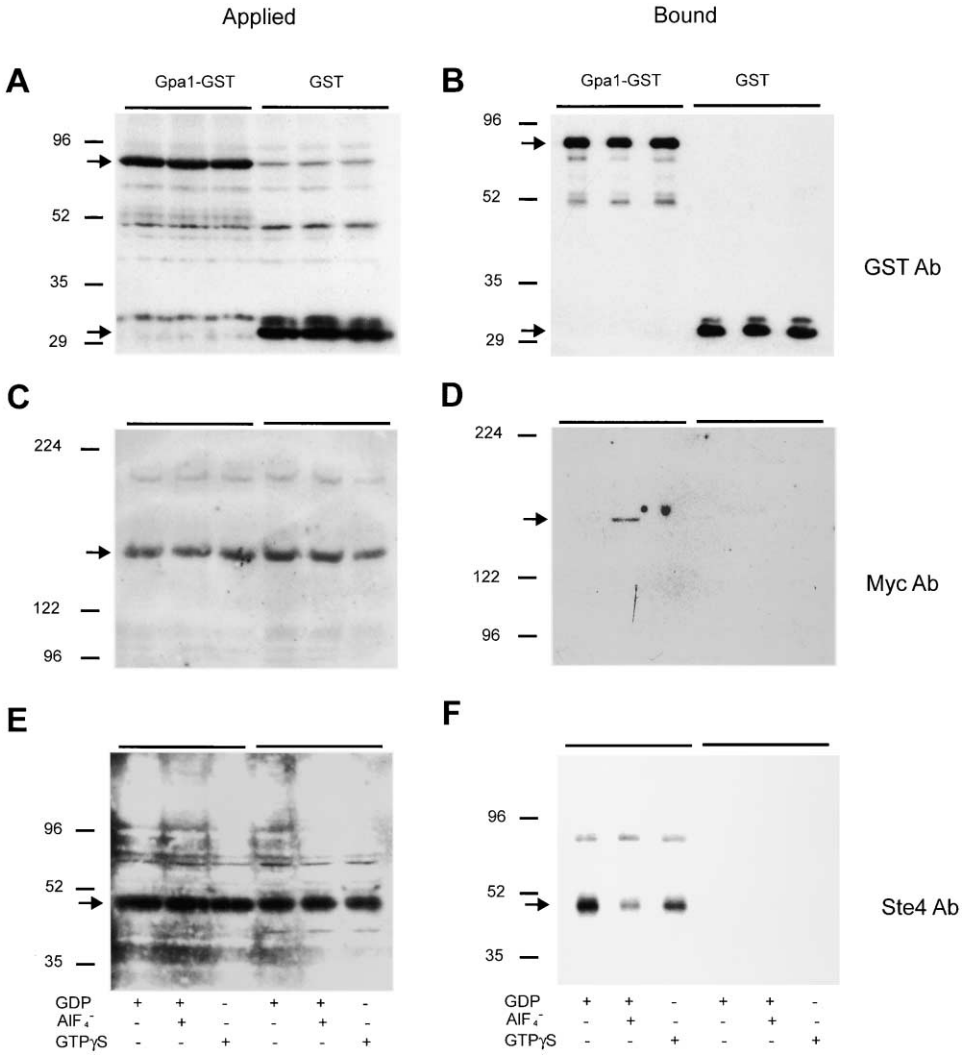

Figure 4. Scp160 Binds to the Active State of Gpa1

Cells expressing Scp160-Myc (in plasmid pYES) and either Gpa1-GST or GST alone (plasmid pAD4M) were grown to mid-log phase and then lysed in the presence of GDP, GDP+AlF_4^-, or GTPγS as indicated. Detergent-solubilized lysates were immobilized on glutathione-Sepharose, washed, and eluted with SDS-PAGE sample buffer. Retained protein (Bound) was detected by immunoblotting with antibodies against (B) GST, (D) Myc, and (F) Ste4. Samples of the soluble cell lysate (Applied) were similarly probed with antibodies against (A) GST, (C) Myc, and (E) Ste4 to confirm equivalent levels of protein expression. Arrows indicate the protein specifically recognized by the antibody.

enzymes but which indirectly regulate such enzymes. The $G_z\alpha$ effector Eya2 functions as a transcription coactivator (Fan et al., 2000). $G_z\alpha$ and $G_i\alpha$ have been reported to activate Rap1GAP, a GTPase accelerating protein for the monomeric G protein Rap1 (Meng et al., 1999; Mochizuki et al., 1999). $G_{12}\alpha$ and $G_{13}\alpha$ bind cadherin and promote dissociation of the transcriptional activator β-catenin (Meigs et al., 2001). $G_{12}\alpha$ and $G_{13}\alpha$ activate p115RhoGEF, which is a guanine nucleotide exchange factor for RhoA (Hart et al., 1998; Kozasa et al., 1998). Our findings suggest that Scp160 is an RNA binding effector that is activated by the G protein α subunit. These findings propose that the signaling network is larger and more complex than previously recognized, and raise the intriguing prospect of RNA as a form of "second messenger."

Experimental Procedures

Strains and Plasmids

Standard methods for the growth, maintenance, and transformation of yeast were used throughout (Ausubel et al., 1987). The yeast *S. cerevisiae* strains used in this study were BJ2168 (*MATa ura3*-52 *leu2*-Δ1 *trp1*-Δ63 *prb1*-1122 *prc1*-407 *pep4*-3) (from E. Jones, Carnegie-Mellon University), BY4741 (*MATa leu2*Δ *met15*Δ *ura3*Δ *his3*Δ), and BY4741-derived mutants lacking YBR116c, *SCP160*, *STE2*, *STE4*, *STE11*, *STE7*, *FUS3*, and *KSS1* (Research Genetics).

pYES-SCP160, pYES-SCP160-MYC, and pYES-STE7 were constructed by subcloning PCR-amplification products into pYES2.1 TOPO TA (Invitrogen Corporation). Other plasmids used here have been described previously (Dohlman et al., 1995, 1996; Song et al., 1996).

Functional Assays

The functional assays described previously include microarray analysis (Burchett et al., 2002) and mating efficiency (Dohlman et al.,

1995), as well as halo and reporter-transcription assays (Hoffman et al., 2002). RNA for microarray was isolated from BY4741 cells transformed with either pAD4M-GPA1^{Q323L} or pAD4M and treated at mid-log phase with 0 or 2.5 nM α factor for 1 hr. Binding was performed as described earlier for Gpa1 binding to Gβγ, with minor modifications (Song et al., 1996). Samples were resolved using 6% (for Scp160-Myc) or 10% SDS-PAGE, and detected with antibodies to GST (from J. Steitz, Yale University), Myc, or Ste4 (from D. Jenness, University of Massachusetts), and the ECL chemiluminescence system (Amersham Pharmacia Biotech).

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