Mutation of *RGA1*, which encodes a putative GTPase-activating protein for the polarity-establishment protein Cdc42p, activates the pheromone-response pathway in the yeast *Saccharomyces cerevisiae*

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We have selected yeast mutants that exhibit a constitutively active pheromone-response pathway in the absence of the β subunit of the trimeric G protein. Genetic analysis of one such mutant revealed that it contained recessive mutations in two distinct genes, both of which contributed to the constitutive phenotype. One mutation identifies the *RGA1* locus (Rho GTPase activating protein), which encodes a protein with homology to GAP domains and to LIM domains. Deletion of *RGA1* is sufficient to activate the pathway in strains lacking the G β subunit. Moreover, in wild-type strains, deletion of *RGA1* increases signaling in the pheromone pathway, whereas over-expression of *RGA1* dampens signaling, demonstrating that Rga1p functions as a negative regulator of the pheromone response pathway. The second mutation present in the original mutant proved to be an allele of a known gene, *PBS2*, which encodes a putative protein kinase that functions in the high osmolarity stress pathway. The *pbs2* mutation enhanced the *rga1* mutant phenotype, but by itself did not activate the pheromone pathway. Genetic and two-hybrid analyses indicate that an important target of Rga1p is Cdc42p, a p21 GTPase required for polarity establishment and bud emergence. This finding coupled with recent experiments with mammalian and yeast cells indicating that Cdc42p can interact with and activate Ste20p, a protein kinase that operates in the pheromone pathway, leads us to suggest that Rga1p controls the activity of Cdc42p, which in turn controls the magnitude of signaling in the pheromone pathway via Ste20p.

[Key Words: *RGA1*; GAPs; *Saccharomyces cerevisiae*; pheromone-response pathway; Cdc42p]

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Mating between the haploid cell types of *Saccharomyces cerevisiae* is controlled by the reciprocal exchange of extracellular peptide pheromones. Binding of pheromone to specific cell-surface receptors activates an intracellular pathway that leads to transcriptional induction of genes required for the mating process and to arrest of the mitotic cell cycle in the G₁ phase (for review, see Marsh et al. 1991; Sprague and Thorner 1992; Kurjan 1993). The current view of the pathway, based both on genetic

epistasis analysis and on biochemical reconstitution of a segment of the pathway, is summarized below.

Pheromone binds to cell type-specific receptors at the cell surface—a-factor binds to Ste3p on α cells and α -factor binds to Ste2p on a cells. These receptors belong to the seven transmembrane family and transduce their signal through a trimeric G protein common to both cell types. Binding of ligand to receptor causes the G α subunit, Gpa1p, to dissociate from the G $\beta\gamma$ dimer, Ste4p–Ste18p, which then activates a downstream component of the pathway. The identity of this G $\beta\gamma$ target molecule(s) remains unknown. Subsequent signal transmission, however, depends on the protein kinase Ste20p (Leberer et al. 1992; Ramer and Davis 1993) and on a

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three-tiered MAP kinase module composed of Ste11p, Ste7p, and the partially redundant MAP kinases Fus3p and Kss1p (Cairns et al. 1992; Gartner et al. 1992; Stevenson et al. 1992; Errede et al. 1993; Zhou et al. 1993; Neiman and Herskowitz 1994). Another pathway component, Ste5p, is required for efficient signal transmission, in part at least because it appears to organize the MAP kinase module into a complex (Choi et al. 1994; Kranz et al. 1994; Marcus et al. 1994; Printen and Sprague 1994). The targets of the MAP kinases include Ste12p, a transcription factor that mediates induction of pheromone-responsive genes (Dolan and Fields 1989; Errede and Ammerer 1989), and Far1p, a protein that mediates cell-cycle arrest (Chang and Herskowitz 1990; Peter et al. 1993; Tyers and Futcher 1993). Ste12p appears to be a substrate for both Fus3p and Kss1p (Elion et al. 1993), whereas Far1p is a substrate only for Fus3p (Elion et al. 1993; Peter et al. 1993; Tyers and Futcher 1993). Thus, these studies imply that the pheromone response pathway components function in the order: receptor → G protein → Ste20p → Ste11p → Ste7p → Fus3p, Kss1p → Ste12p and Far1p. At odds with this strictly linear arrangement, however, are recent findings that suggest that Ste20p activity is modulated by several inputs, or has several output targets. In particular, Ste20p is partially redundant with Cla4p, a related protein kinase; the double mutant cannot maintain septin rings at the bud neck and cannot undergo cytokinesis (Cvrcková et al. 1995).

Most of the pathway components described above were identified by an identical genetic strategy—the isolation of mutants (or strains carrying genes on multicopy plasmids) that were nonresponsive to pheromone. Because the connection between the G protein and the MAP kinase module is poorly understood and because components or regulators encoded by essential or redundant genes might have been missed by application of that strategy, we elected to isolate constitutive mutants in which pathway signaling occurs autonomously of a receptor–ligand interaction (Stevenson et al. 1992; J. Shultz, A. Rogat, and G.F. Sprague, Jr., unpubl.). Here we present the analysis of one such mutant, which identifies the *RGA1* (Rho GTPase activating protein) gene. The *RGA1* product has homology to GTPase activating proteins (GAPs) for p21 GTPases of the Rho subfamily. We present two-hybrid and genetic data that Cdc42p, a protein known to be required for polarity establishment and bud emergence, is an important target of Rga1p. Loss of Rga1p function activates the pheromone response pathway in *ste4Δ* (Gβ) mutants, but not in *ste11Δ* mutants, implying that Rga1p serves as a negative regulator of the pathway at a step between the G protein and the MAP kinase module. These findings, together with the recent findings that a mammalian homolog of Cdc42p activates a homolog of Ste20p (Manser et al. 1994), that yeast Cdc42p and Ste20p interact (Simon et al. 1995; Zhao et al. 1995), and that Cdc42p is required for pheromone response (Simon et al. 1995; Zhao et al. 1995), imply that the activity of Cdc42p is an important modulator of signaling in the pheromone response pathway.

Results

rga1-1 activates the pheromone signal transduction pathway

To isolate mutants in which signal transmission through the pheromone-response pathway is activated or enhanced independent of a receptor–ligand interaction, we used a *ste4Δ his3* mutant strain carrying a chromosomal *FUS1::HIS3* construct (Stevenson et al. 1992). In this strain, the basal pathway signal normally seen in wild-type cells is absent. As a result, the *FUS1* promoter is inactive (Hagen et al. 1991; also see Table 1) and the cells have a His[−] phenotype. We selected mutants that could grow on medium lacking histidine and containing 3 mM amino triazole (AT), a competitive inhibitor of the His3p enzyme (Stevenson et al. 1992). This condition requires that pathway activity be restored to basal level or higher. The His⁺ phenotype of mutant 27 was recessive (Stevenson et al. 1992), implying that it identified a negative regulator of the pheromone pathway. This mutant was chosen for further study.

Genetic analysis of mutant 27 revealed that it contained two recessive, independently segregating mutations (see Materials and methods). One mutation, subsequently designated *rga1-1* (see below), activated the *FUS1* promoter as evidenced by the His⁺ phenotype of a *ste4Δ his3 FUS1::HIS3* strain. The effect of the *rga1-1* mutation was quantitated by monitoring the expression of a *FUS1-lacZ* reporter gene. As shown in Table 1, *rga1-1* restored pathway signaling to the basal level seen in wild-type cells (compare strains BSY179, 187, and 191). The second mutation (later identified as an allele of *PBS2* and dubbed *pbs2-99*) did not activate *FUS1::HIS3* on its own, but enhanced the phenotype conferred by *rga1-1* (Table 1). Indeed, together the *rga1-1* and *pbs2-99* mutations restored some mating competence to *ste4Δ* strains, but neither mutation alone did (Table 1 and data not shown). The second mutation (*pbs2-99*) conferred an additional phenotype, sensitivity to osmotic stress, providing a clue to its identity.

RGA1 encodes a large protein with homology to LIM domains and to Rho GAP domains

As a first step in the molecular characterization of the two genes identified by mutant 27, it was transformed with a YE24-based yeast genomic library (Carlson and Botstein 1982), and colonies were isolated that exhibited reduced expression of *FUS1::HIS3* (see Materials and methods). Five clones were identified. Two of the five clones, which contained overlapping DNA inserts as determined by restriction-enzyme mapping, also complemented the osmotic-sensitivity phenotype of the original strain. We reasoned that these clones harbored the *PBS2* gene on the basis of the following observations: The cloned DNA complemented and was linked genetically to the mutation that conferred sensitivity to osmotic stress; the cloned DNA mapped to chromosome X, where others have mapped *PBS2* (Boguslawski and Polazzi 1987); and the restriction-enzyme map and par-

Table 1. *FUS1-lacZ* expression and mating capacity of *rga1 ste* strains

Strain	Genotype ^a	<i>FUS1-lacZ</i> expression ^b	Mating efficiency ^c
BSY179	<i>RGA1 PBS2</i>	5.3	0.9
BSY183	<i>rga1-1 PBS2</i>	11.5	0.9
BSY185	<i>rga1-1 pbs2-99</i>	20.2	1.0
BSY187	<i>ste4Δ::LEU2 RGA1 PBS2</i>	0.3	$<3 \times 10^{-8}$
BSY191	<i>ste4Δ::LEU2 rga1-1 PBS2</i>	5.4	$<3 \times 10^{-8}$
BSY193	<i>ste4Δ::LEU2 rga1-1 pbs2-99</i>	17.0	5.5×10^{-5}
BSY231	<i>ste20Δ::URA3 RGA1 PBS2</i>	0.4	5.3×10^{-2}
BSY233	<i>ste20Δ::URA3 rga1-1 PBS2</i>	2.1	3.6×10^{-2}
BSY235	<i>ste20Δ::URA3 rga1-1 pbs2-99</i>	6.1	0.1
BSY219	<i>ste5Δ::URA3 RGA1 PBS2</i>	0.3	$<5 \times 10^{-8}$
BSY221	<i>ste5Δ::URA3 rga1-1 PBS2</i>	7.6	$<4 \times 10^{-8}$
BSY223	<i>ste5Δ::URA3 rga1-1 pbs2-99</i>	20.5	7.0×10^{-5}
BSY225	<i>ste11Δ::URA3 RGA1 PBS2</i>	0.3	$<3 \times 10^{-8}$
BSY227	<i>ste11Δ::URA3 rga1-1 PBS2</i>	0.3	$<4 \times 10^{-8}$
BSY229	<i>ste11Δ::URA3 rga1-1 pbs2-99</i>	0.3	$<3 \times 10^{-8}$

^aAll strains also have the markers of SY2002.

^bβ-Galactosidase activity was determined as described in Materials and methods. The reported values are the averages of at least three determinations.

^cAbsolute mating efficiencies were determined as described in Materials and methods.

tial DNA sequence of the cloned DNAs matched that published for *PBS2* (data not shown; Boguslawski and Polazzi 1987; see Materials and methods). *PBS2* encodes a protein kinase thought to occupy the middle tier of a MAP-kinase module that functions in the high osmolarity stress (HOG) pathway.

The remaining three clones contained overlapping DNA inserts that complemented the His⁺ phenotype of the original strain (conferred by the *rga1-1* mutation) but not the osmotic sensitivity. The smallest of the cloned inserts (pRGA1.3) is shown in Figure 1. To ensure that this cloned DNA represented the *RGA1* locus, and not

an unlinked high-copy suppressor, we performed genetic linkage analysis (see Materials and methods). The cloned DNA showed linkage to the *rga1-1* mutation indicating that it indeed represented the *RGA1* locus. Hybridization of a radiolabeled 0.3-kb *Bam*HI fragment (see Fig. 1) to a blot of intact chromosomes and to blots of an ordered array of lambda clones (Riles et al. 1993) revealed that *RGA1* is located on the right arm of chromosome XV (data not shown).

The *RGA1* gene was localized within pRGA1.3 by testing the ability of fragments of the clone to complement mutant 27 (Fig. 1). This analysis indicated that the minimal complementing sequence was a 3.6-kb *Xho*I–*Hind*III fragment (Fig. 1). The DNA sequence of the 4.1-kb *Clai*I–*Hind*III fragment, shown in Figure 2, contains one long open reading frame capable of encoding a protein of 1007 amino acids. Rgalp has a predicted molecular weight of 112,802 daltons, and is likely to be an intracellular protein, because it contains no obvious signal sequence for secretion nor any putative transmembrane segments. The complement of nucleotides 3306 through 3328 of this sequence (double underline in Fig. 2) encode the last six residues and the translation stop of the Ade2 protein, indicating that *RGA1* and *ADE2* are convergently transcribed, and confirming the assignment of *RGA1* to the right arm of chromosome XV.

A homology search of the current GenBank data base by use of the BLAST algorithm (Altschul et al. 1990) identified two nonoverlapping segments of Rgalp that show significant similarity to previously identified proteins. Residues 13 through 66 in the amino-terminal region of Rgalp match the consensus of a LIM domain, and a second sequence (residues 70–122) is an imperfect match, with a conserved histidine replaced by a cysteine

Figure 1. Restriction enzyme map of the *RGA1* locus and location of the *rga1*-complementing activity. The 8.9-kb genomic insert carried by the pRGA1.3 plasmid is shown as a gray rectangle. The open region corresponds to the *RGA1* open reading frame, and the hatched and solid boxes within the open reading frame represent the LIM domains and the GAP homology region, respectively. Restriction enzyme sites shown are: (B) *Bam*HI, (Bs) *Bst*XI, (H) *Hind*III, (K) *Kpn*I, (P) *Pst*I, (X) *Xho*I. The ability of deletion derivatives of the insert to complement the His⁺ phenotype in strain SY1731 (*rga1-1 pbs2-99*) is shown to the right.

-793 Clai
 ATCGATAAAATTCGGGAGGGGACACTTTGGGACCTGCTGAGCATGCATGTTGGCACCCAAAAATATTTGTG
 -720 GCCATGACAATATTGGATTCTGCTTTAAATCTCAGGAAGTATTTTCAACGCCATCTAGAAGTGACCTTTGAACCTCTTTGAAGAATATCCATTTTCTCGTATATTCGTGACT
 -600 AATCGCGCTCCAGGCGACTACTGATCTTTGCCATCTCAGTGGGCTAGTATTTAAAGCAAAATTCAGTAATGGAATCCCAAAATACAGAAACTTCTCGTAAATCCATATTTGTGCACAGT
 -480 TTAAGCGTACGAACAGATGATAATGTAGAGCTAGTCTTGGTTCTGTATGTGTTACACAGTGAAGGTGTAAGTGCCCTTATTTTCCGCGCTTTATGCCGTATTAGGAAAAAGTG
 -360 AAAAAAAGGAGGCTCTCGAGTTTAGATTGATATAATTAACATGTGCTTTTCTATTGACCTTTGATGAGATTGTATCAGCAGCTGCTTAATTCATTGAACGTTTTCACAG
 -240 GGTAGGACTGCTCAACAGACTAAATTTCTTTGCCAAATAGGGTGTGTTGAGTTTGTAGTCAATCTTGTATCAATTCACAGTTCCTTAAATGAGAAAAAATGATCGGTATCA
 -120 CTAAATTTTAAAGAACTGAAAGCACCACGGTTTGTACATTTATCTCTATTATAGCTTTTGTTCAGCAAGAGATAGCTGATTCAGGTCTAGTCTGAGAGAGAGCGGCATATTTAA
 1 ATGGCATCAACTGCTCCCAATGAACAATTTCCATCTGCGTACGATGCAAGAATTTATTACACGGGCGATGCATATGAGTTGGGTTGTGATAGATGGCACACACATTTGTTCCGCTGT
 M A S T A P N E Q F P S C V R C K E F I T T G H A Y E L G C D R W H T H C F A C 40
 121 TACAATGTGAGAACCAATTAAGCTGCAATCTGATTTTATGCTCTTGAACAGGTCCTTTGATCTGCTTTGATGTTTCCGATCTTGTAAAAATTCGGGTAAAAAGATTGATGATTG
 Y K C E K P L S C E S D F L V L G T G A L I C F D C S D S C K N C G K I D D L 80
 241 GCCATAATCTCTCTCTCAATAGGCTTATTTGTCAGATTGTTTAAATGCTGTAATGTGGTGAATAATTTGCTGACCTACGGTACCGGAAAAACCAAGAGGTTTATTTGTTTA
 A I I L S S S N E A Y C S D C F K C C K C G E N I A D L R Y A K T K R G L F C L 120
 361 AGCTGCACGAAGGCTATTAGCCAAAGAAAAATCTACGAAGAGAGAAAGGCGACTCAAAAGAATTTACCAAGTTTACCCACACCTGTGATTGATAATGGCATCTGATGAGGTC
 S C H E K P L S C E S D F L V L G T G A L I C F D C S D S C K N C G K I D D L 160
 481 TCAAGCTTCTGAGTCTCCGAGAAAAAATTTAGCAGACTGCTAGTAACTGAGATTCTTCAAGGTTCTGAACCTTCAAGGACATAGAACCAATTCGAGTGTATTTGTTCCG
 S A S A V L P E K T F S R P A S L V N E I P S G S E F S K D I E T N S S D I V P 200
 601 CATTTTATCTAGTGGTATTAACGATAGGATGACAACTCTGGAAGTTCAAAATTCGGTTCAAAATGTCCATAGATGTTATAGGACCGAAGAAAAATAGCAGGAGCATTTAAATGATGAT
 H F I T G Y N D S D D N S G S S K F G S N V S I D V I G P E E N S T E H V N D D 240
 721 GTTAAGAGGAGCAGAACGCTCTCAGCAATATGCTACTCAATGTGCTACGGATCCAGCCCTAAGTTGTAAAGAACCTCTAGCATTGAGGAATTTGTTAAATAAACACCGCTG
 V K E E A E A P S A N M S L N V A T D P T L S C K E P P S H S R N L L N K F Q E Q A 280
 841 AGAAATCTCTGGTCAATCTCGCAAAATCTCAAGCTCTATAGCAGGGAATAATTTGTAACGATAGTTTGAAGAGAGGATCAAAATGATCTCCAAATTAACAGTTCACGAAT
 R N S S G Q Y L A K S P S S Y R Q G I I V N D S L E E S D Q I D P P N N S S R N 320
 961 GCAAGTGAATTTGTTGACCTCGGTATTTGATAGTCCAGTTCTGTTAATGAATAATCCGAGGTTCAAAATACGGATATTTTCAACACCGTGAATATCCCAATTTGATCTCTGCTA
 A S E L L T S V L H S P V S V N M K N P K G S N T D I F N T G E I S Q M D P S L 360
 1081 TCAAGGAAGTATGAATAATTTGCGAAGAACCAACGCACTTCAAGGCGAGTTTGAAGTTGTCAGGAAGACAGAGGTTTCTGACCTAGCAGGTTTCAACAGAGCAAGCA
 S R K V L N N I V E E T N A L Q R P V V E V V K E D R S V P D L A G V Q E Q A 400
 1201 GAGAAATATCTTATTAACAATAGTGGAAAGCAGAAAAATTTCAAGATCAATTTGTCAGGAAGTCCAAAGATTGTGATTAATCTAAATCCAGGCGCAGGCAACAGGATAGT
 E K Y S Y S N N S G K G R K I S R S L S R R S K D L M I N L K S R A T G K Q D S 440
 1321 AACGTAAATCTTACCTGCTCTTAAAGTGACATCTAGACCTCACAAGGTTAATGAGGATATGATTCACATCTGCTGACACACCAATTTCAACAGCATTCTTTAGATATT
 N V K L S P A S K V T S R R S Q E L M R D N D S H T G L D T P N S N S T S L D I 480
 1441 TTGCTGAATATCAAAAGGCTAAACTATAAAGATTTCGCGATATGCGACTCTTAGGTTCACTAGTGGAAAGAACCCGCCCTTGAAGAACAGAAATACAGATTTTAAATCTCCG
 L V N G K S L N Y K R F T D N G T L R V T S G K E P A L E B Q K N H S F T P L 520
 1561 TCACCAATGATCATTTTGTACAACTCCAGCTACACCAAGTAAATGTATCATGTATCGAACTCCGCTTTAGATAGCTCATTGACATTTGATAGCGAAATGGGCTTACATACAGTAAC
 S P I D H L L Q S P A T P S N V S M Y R T P P L D S S L T F D R R N G S S Y S N 560
 1681 CAAATATAGCATACCTAGTTGCGAAGAACCTCAAGACCCCAATTTGAGAAATAGCGAAATTTTGAAGAACAGAAACACTTTACGAAATAGCGAATCTAGAAACGATCCCTCG
 Q N Y S I P S W Q K T P K T Q L E N S D N F E E Q K E T L Y E N S E S R N D P S 600
 1801 CTTGATAAGAAATTTGTCACGAGGACTATCTGAACAATTTAAAAATAAATTTGAAGAACTTGAATCTCAAGGGAAGAAATTAAGAAAGAGATCACTGAAATGAAGTCTATGAA
 L D K E I V T A E H Y L K Q L K I N L K E L E S Q R E E L M K E I T E M K S M F 640
 1921 GAGGCTCTAGCGGACATATTAATCTTAAATAGCGAGAAATTAACATATATCTGACTCCAATGAATATCAATAATCCACGATGATAAATGAATCAGCTTAGGTGAGTGGCA
 E A L R R H I E S Y N S E K N K L Y L D S N E L S N N P P M I N E I S L G E S P 680
 2041 CCGGTGAACATGTGCACTGCAAGTTCTGTAGCGGCAAGTTCTGTAAACCAAAATTTTGAAGTTTCTTCACTGCAAGGCCAGAGGAGCAATCTATACAGGTGTGAGTACA
 P V K H V A T A S S V A R S S V K P K F W K F F S S A K P Q T E Q S I Q G V T 720
 2161 AATAACAAATTCATCTGTTAAAGTGCCCAAGTTTCTGCTCTCTCTCTCTGTTAGTAAATTCGGGCGTTTGAATAATTCACCACTGTGCTACAAATCTATGAATTTAGT
 N N T N S I V K S A P V L L S A P S S G S N S G R L E I S P P V L Q N P N E F S 760
 2281 GAGCTTAGATTAGTACCTTACGAGAACGATGCAAAATAGGCAAGAAATGAAGATGGAAGAAATATTTGGATGGAAGCAATTTGATGGTTCAAGTCTGTTCTAGGTGCAATTTATGAA
 D V R L V P I E N D A N H G Q S K D G E E Y L D G S N L Y G S S L V A R C N Y E 800
 2401 AACAATGAATACCGATGATATCTGCTGATAGATTTTATGAATCAGACGAAGAAATATGAGATCGGAGGCAATTTATAGAAATCAGGTTCCAGCTAGTATAGAGAAATA
 N N E I P M I L S V C I D F I E S D E E N M R S E G I Y R K S G S Q L V I E E I 840
 2521 GAAAGCAATTTCTGATGGAAGTACAAACAAATACGAAAGCCAAATATTTAAACGGAACAGATCTCAACCGCTTACTGCTGTGTTGAAGGATACCTTAAGAAAGCTCCCAAC
 E K Q F S A W K V Q N T E T P N I L T E Q D L N A V T G V L K R Y L R K L P N 880
 2641 CCTATCTTACCTTCAAAATACGAGCGGTTGATGAGGTTAGTTAAATCTAGAAAAATGAGAAACTTGCATTTGTTGAGGAAAGTTGCTCTAGAAAGCAAAATTCGGACACT
 P I F T F Q I Y E P L M R L V K S R K M M E N L P F V G G K L S L E A K N S D T 920
 2761 TATATGTCAGGCAAGGCGCTTTAAAAACATATTTGAAGACCTTCAAGGGAACATTATAGGTTGTTAAGAGTACTGAGTGAACATATAGAAAGGTTACACGCTACAGTCTGGAAT
 Y M S S G A L K N I L E D L P R E H Y R V L R V L S E H I E K V T R Y S H W N 960
 2881 CGAATGACGCTTTAATAATTTGGCTTTAGTTTGTCTCCAGGTTTGTATGTTTCACTGAGAAAGGATATTTATGATGAAAGAAAGAACTATATTTGCGCATTTATATTTGGG
 R M T L Y N L A L V F A P G L I R D F S G E K D I I D M K E R N Y I V A F I F G 1000
 3001 AACTACAAAGATATCTGACGTAGCGCTATCTCGGTGATGATGAGCGGCTTATATGAAGTGTATGAAACGTTATTTTAAATGACAGCTTAAGCAGGTAATTTATCTCTGCTT
 N Y K D I L T 1007
 3121 CTGTTATCTGATATGATATGATATAAATAGTATCTTATGATGAAATCTTAAAAAGGACACCTGTAAGCGTTGATTTCTATGATGAGTCCACATTTGATGATATCATACAA
 3241 AGCCTAAAAATAGGTATATCATTTTATATTATTTGCTGTGCAAGTATATCAATAAATCTATATATTTACTTGTGTTTCTAGATAGCTT
 HindIII

Figure 2. Sequence of the *RG1* gene. The DNA sequence of the 4.1-kb *Clai*–*HindIII* fragment is shown, numbered to the left, with the predicted amino-acid sequence of Rgalp below, numbered to the right. The *SpeI* and *Eco47III* restriction enzyme sites used to construct *rga1Δ::LEU2* are underlined. The complement of the DNA sequence coding for the last 6 residues of Ade2p is double underlined.

Rgalp (1)	13	CVRCKEFTTGHAYELG.CDRWHTCFACYKCEKPLSCSDFLVLGTGALICFDC	66
Rgalp (2)	70	CKNGGKIDDLAILSSNEAYSCDFKCKCKGE..NIADLRVAKTKRGLFCLSC	122
cCRP (1)	10	CGVCQKAVYFAEEVQCE.GSSPHKSCFLCMVCKKNL..DSTTVAVHGDIEYCKSC	61
cCRP (2)	118	CPRCQAVYAAEKVIGA.GKSMHKSFCRAKCGKSL..ESTTLADKDGIEYCKGC	169
Lin-11 (1)	135	CAACAQPIIDRVVFTVL.GKCMHQSCLRCDCRAPM..SMTFCFS.RDGLILCKTD	185
Lin-11 (2)	306	CAGCDGKLEKEDLVRRARDKVFHRCFCQSVQRLDLDGQQLVIMEGNRFVQSD	360
LIM CONSENSUS		C..C.....H..C..C.....C.(C,H,D)	

Figure 3. Rgalp contains two sequences with homology to LIM domains. The LIM domains of Lin-11 (Freyd et al. 1990) and cCRP (Crawford et al. 1994) are shown together with the homologous sequences from Rgalp. 1 and 2 refer to the position of the domains with respect to the amino terminus of the protein. The consensus sequence of the residues involved in zinc coordination are shown in boldface letters. Note that the imperfect Rgalp (2) sequence contains a cysteine in place of the conserved histidine.

(Fig. 3). LIM domains bind zinc ions (Michelsen et al. 1993; Archer et al. 1994) and are thought to mediate protein-protein interactions. They were first characterized in the homeo domain-containing proteins Lin-11, Isl-1, and Mec-3 (Way and Chalfie 1988; Freyd et al. 1990; Karlsson et al. 1990), but have since been found in proteins that lack homeo domains, such as the chicken cysteine-rich protein (cCRP; Crawford et al. 1994).

The carboxy-terminal 200 residues of Rgalp show high similarity to sequences common to GTPase-activating proteins (GAPs), particularly to GAPs of the Rho subclass, which includes Rho, Rac, and Cdc42-like proteins (Fig. 4). GAPs enhance the low intrinsic GTPase activity of p21 GTP-binding proteins and consequently promote their inactivation. Several other GAPs have been identified in *S. cerevisiae*. Bud2p, Bem2p, and Bem3p, have functions required for bud morphogenesis or bud-site positioning (Park et al. 1993; Zheng et al. 1993, 1994; Peterson et al. 1994). Ira1p and Ira2p function as GAPs for Ras1p and Ras2p (Tanaka et al. 1990). An additional GAP, Lrg1p, has an unknown function, but is expressed at highest levels in sporulating cells (Müller et al. 1994).

Surprisingly, the data base search found an exact match between residues 570–639 of Rgalp and a par-

tially sequenced gene, *THE1* (Ramer et al. 1992). Galactose-mediated overexpression of an amino-terminal truncation of *THE1* leads to cell death in all cell types of *S. cerevisiae*. Assuming that *RGA1* is identical to *THE1*, the cause of the lethality is likely to be overexpression of the GAP region of Rgalp. We tested this directly by constructing an allele of *RGA1* lacking the amino-terminal 537 amino acids, under control of the *GAL1* promoter. Wild-type cells containing this construct grew well on glucose-containing medium but did not grow on medium containing galactose as the sole carbon source (data not shown), confirming that overexpression of the GAP domain of *RGA1* leads to growth arrest. C. Chan (pers. comm.) has also identified *RGA1* in an independent genetic screen as a suppressor of *bem2* mutations.

RGA1 is a nonessential gene

The phenotype(s) of a null mutation of *RGA1* were examined by constructing a *MATa/MATα* diploid strain heterozygous for a deletion that removes all of the *RGA1* open reading frame (*rga1Δ::LEU2*, see Materials and methods). Sporulation of this strain (BSY237) gave rise to four viable spores per tetrad, indicating that *RGA1* is not an essential gene. We assayed *FUS1-lacZ* expression and mating efficiency in strains that contained *rga1Δ::LEU2*, alone or in combination with a *pbs2Δ::URA3* allele, in a wild-type or *ste4Δ* background. As shown in Table 2, deletion of *RGA1* increased the expression of *FUS1* in wild-type and *ste4Δ* strains. In the absence of a functional G protein (*ste4Δ* strains), only the *rga1Δ::LEU2 pbs2Δ::URA3* double mutant showed some mating ability. These results are similar to those obtained with *rga1-1* (Table 1), suggesting that *rga1-1* is a loss-of-function allele, consistent with its recessiveness.

Many genes that affect the functioning of the pheromone signal-transduction pathway are selectively expressed in the haploid cell types or are inducible by pheromone (Sprague and Thorner 1992). To investigate the expression of *RGA1*, the centrally located 0.3-kb *Bam*HI

Block 1			
Rgalp	805	PMILSVICIDFIESDEENMRSEGIYRKSSQSLVIEIE.....KQFSANKVQONTETPNILTEQ	862
Bem2p	1981	PTIVVKLLIEIEIRG..LDEVLGRIIPG...SIGSIN.....ALKNAFDEEGATDNTSFTLEDD	2033
Bem3p	927	PSVVYRCLEYL.YKNRGIQEEGIFRLSGSSSTVIKTLQERFDKEYDVLCLRYNESIEAKDDEASPS	990
Lrg1p	755	PIIDELISSLSQMD..MSVDEGIFRKNGNIRRLRELT.....A.....TDSNTPAEPDFDSKE	805
Bcr	1068	PVIVROCVETERRG..MEVVGIVYVSG...VATDIQ.....ALKAAPFDVNN.KDVSVMMSE.	1118
Chimaerin	122	PMVVDICIRIEESRG..LNSEGLYRVSG...FSDLIE.....DVKMAFDDEKAD...ISVN	171
p50rhoGAP	260	PVIVRETVAYLQAHG..LTTEGIFRRSANTQVVRVQ.....Q.....KY..NMGLPVDPDQY	308
CONSENSUS		P.I.....G.YR..G	
Block 2			
Rgalp	863	..DLNAVTVGLRKRLKLPNPIFTFQIYEPLMRVLVSRKMMENLPVFGKLSLEAKNSDITYMS	924
Bem2p	2034	RWFEVNAIAGCFKMYLRELPLDLSFHAMVNDF.....TD.LAIKYKAHAMVNE.....EY	2082
Bem3p	991	LYIGVNTVSGLLKLYLRLPHLLFGDEQFLSKRVVDENHNNPVQISLGFKELTESG.....	1047
Lrg1p	806	NAIQLSA...LLKKFIREQPPLSTDLIELMIKASKIDLEDEKQL.....	849
Bcr	1119	..MDVNAITAGLKLIFRELPEPLFTDEFYVNF.....AEGTAL...SDPVAKE.....SC	1163
Chimaerin	172	MYEDINIITGALKLYFRDLPIPLITYDAYFKF.....IESAKIMDPDEQLET.....	218
p50rhoGAP	309	NELHLPAV...ILKTEFLRELPEPLTFDLYPHVVGFLNID.ESQVPA.....	352
CONSENSUS		..LK.YLR.LP...F.....Y..F..	
Block 3			
Rgalp	925	KGALKNILEDPLPREHYVRLVLSHEHIEKVT.....RYSHNNRMTLYNLALVFAPGLIRDFSGEKD	984
Bem2p	2083	KRMNNEILQKLPTCYQYQLTKRIVFHLNKVH.....QHVVNKNMDASNLAIVFSMSFINQEDLANS	2142
Bem3p	1048	..LVPHANLSLMYALPELLVRIN.....ENSKFNKNLRLKICVFSPTLNIPIKMLQP	1098
Lrg1p	850	..ILLIYSLPLTYNRNLLEALLSLFHWTSFSSYIENEMSGKMDIHLNFTVITPNTILYLRHKEIS	911
Bcr	1164	..MLNLLSLPEANLITFLFLDLHLKRVV.....EKEAVNKMNSLHNLATVFGPTLLRPSSEKESK	1220
Chimaerin	219	..LHEALKLLPFAHCETRLYLAHLKRVV.....LHEKENLMAENLGIIVFGPTLLRPSPELDAM	275
p50rhoGAP	353	..TLQVLQTLPEENYQVLRFLTAFLVQISAHG.....DQNKMTNLAIVFGPNLLWAKDAIT	409
CONSENSUS		LP.....L..L..L..V.....N..M..NL..V.....	

Figure 4. Rgalp has homology to Rho-GAP proteins. An alignment of the carboxy-terminal region of Rgalp with the Rho-GAP domains of six other proteins is presented, derived by use of the pileup program of the UWGCG package (Devereux et al. 1984). A consensus sequence, with invariant residues in bold, is shown for the highly conserved subdomains (Blocks 1–3; Zheng et al. 1993). The proteins are: *S. cerevisiae* Bem2p (Zheng et al. 1994); *S. cerevisiae* Bem3p (Peterson et al. 1994); *S. cerevisiae* Lrg1p (Müller et al. 1994); human breakpoint cluster region (Bcr) protein (Heisterkamp et al. 1985); human n-chimaerin (Hall et al. 1990); and p50rhoGAP (Barford et al. 1993; Lancaster et al. 1994).

Table 2. Pheromone-response pathway activity in *rga1Δ::LEU2* and *GAL-RGA1* strains

A. Strain	Genotype ^a	<i>FUS1-lacZ</i> expression ^b	Mating efficiency ^c
SY2003	<i>RGA1 PBS2</i>	4.6	0.9
BSY240	<i>rga1Δ::LEU2 PBS2</i>	9.3	0.9
BSY242	<i>rga1Δ::LEU2 pbs2Δ::URA3</i>	25.9	0.9
BSY171	<i>ste4Δ::URA3 RGA1 PBS2</i>	0.3	$<4 \times 10^{-8}$
BSY244	<i>ste4Δ::URA3 rga1Δ::LEU2 PBS2</i>	2.5	$<3 \times 10^{-8}$
BSY246	<i>ste4Δ::URA3 rga1Δ::LEU2 pbs2Δ::URA3</i>	31.2	7×10^{-5}

B. Strain	Genotype ^a	<i>FUS1-lacZ</i> expression ^b	
		uninduced	pheromone treated
SY2002	<i>RGA1</i>	2.4	53.4
SY2002 <i>pGAL-RGA1</i>	<i>GAL-RGA1</i>	1.4	19.3
SY3116	<i>rga1-1</i>	4.9	57.7

^aAll strains are isogenic to SY2002 except as indicated.^bβ-Galactosidase activity was determined as described in Materials and methods, except for strains in *B*, which were grown in YEP-GAL medium. The reported values are the averages of at least three determinations.^cAbsolute mating efficiencies were determined as described in Materials and methods.

fragment was radiolabeled and used for Northern analysis. The *RGA1* probe hybridized with a 3.3-kb RNA that was expressed in all cell types and was not inducible by pheromone (data not shown).

Although *RGA1* is not an essential gene, loss of Rgalp activity causes a distinct change in cell morphology. Cells carrying the *rga1-1* or *rga1Δ::LEU2* allele appear elongated and display at most a few bud scars, usually at both poles of the cell (Fig. 5). Wild-type haploid cells typically display a chain of bud scars centered at one cell pole (Fig. 5; Chant and Herskowitz 1991; Chant and Pringle 1995). Pedigree analysis confirmed that *rga1-1* mutants produce buds in a bipolar manner, whereas wild-type cells generated buds in the expected axial pattern. Although a bipolar bud-site selection pattern is typical of *a/α* cells, *rga1-1* mutants are otherwise phenotypically *a* or *α*; they mate with cells of the opposite mating type, and the resulting diploids produce viable spores.

Overexpression of *RGA1* reduces both basal pathway activity and response to pheromone

We next tested the effect of overexpression of *RGA1* on signaling in the pheromone-response pathway. A single-copy plasmid containing *RGA1* under the control of the *GAL1* promoter was introduced into a wild-type strain, and pheromone pathway activity was monitored by use of the *FUS1-lacZ* reporter gene. When grown in the presence of 0.4 M galactose, cells containing the *GAL1-RGA1* gene produced about 50% less β-galactosidase activity than cells lacking the *GAL1-RGA1* gene (Table 2). Similarly, in the presence of pheromone, the *GAL1-RGA1* cells produced 65% less β-galactosidase activity than cells with wild-type levels of Rgalp. These results are consistent with the hypothesis that Rgalp negatively regulates the pheromone-response pathway: Increased Rgalp reduces signaling through the pathway, whereas loss of Rgalp increases signaling.

Rga1p functions at or before *Ste11p* in the pheromone pathway

To determine at which point *rga1-1* activates the pheromone-response pathway, we performed double-mutant analysis with known components of the pathway. Strains carrying deletions of *STE5*, *STE11*, *STE7*, *STE12*, or *STE20* were crossed to a strain carrying both *rga1-1* and *pbs2-99*, and segregants with *ste* deletions and

Figure 5. Bud-scar staining and cellular morphologies of *RGA1* and *rga1-1* strains. BSY187 and BSY191 were grown to exponential phase and stained with Calcofluor to illuminate bud scars as described in Materials and methods. *RGA1* cells viewed with fluorescence (A) or Nomarski optics (B); *rga1-1* cells viewed by fluorescence (C) or Nomarski optics (D).

rga1-1 were assayed for *FUS1-lacZ* expression. As expected, otherwise wild-type strains containing *ste* deletions expressed *FUS1* at very low levels and showed no detectable mating activity. [We note that in contrast to published reports (Leberer et al. 1992; Ramer and Davis 1993), deletion of *STE20* in our genetic background severely reduced the basal level of *FUS1* expression but decreased the mating efficiency only about 10-fold below that of the *STE*⁺ strain (Table 1). The reason for these differences is not known.] Activation of *FUS1-lacZ* expression by *rga1-1* was apparent in *ste5Δ* strains, and to a lesser extent in *ste20Δ* strains. In contrast, *rga1-1* had no effect in *ste11Δ* strains (Table 1) or in *ste7Δ* or *ste12Δ* backgrounds (data not shown). The *pbs2-99* allele enhanced the activity of *rga1-1* by two- to threefold in *ste5Δ* and *ste20Δ* strains, and allowed *ste5Δ rga1-1* cells to mate, albeit at a low level (Table 1). These results suggest that *rga1-1* exerts its effect before or at the same level as *STE11* in the pathway.

Rga1p exhibits genetic and two-hybrid interactions with *Cdc42p*

The sequence of *RGA1* suggests that it encodes a GAP for a member of the p21 GTPase superfamily, most likely a Rho protein. We used the two-hybrid technique (Fields and Song 1989; Gyuris et al. 1993) to investigate the possibility that Rga1p interacts with known Rho/Ras proteins, specifically Rho1p, Rho2p, Rho3p, Rho4p, Rsr1p (Bud1p), or Cdc42p. Interaction was detected between a Rga1 activation-domain (AD) fusion protein and LexA-Cdc42p (Table 3A). The interaction with Cdc42p was substantially enhanced by introducing a C188S sub-

stitution, which prevents prenylation, and a G12V or Q61L substitution, which should trap Cdc42p in the GTP-bound form. Conversely, a D118A substitution, which should lock Cdc42p out of the GTP-bound form, dramatically reduced interaction with Rga1p. Bem3p, a protein with demonstrable GAP activity on Cdc42p in vitro (Zheng et al. 1994), also interacted with Cdc42p, although in this case, significant interaction was observed only with Cdc42p containing the Q61L and C188S substitutions. Because the LexA-Cdc42p fusions showed some ability to activate the *lexAop-lacZ* reporter on their own, we re-examined the interactions using LexA-Rga1p and -Bem3p fusion proteins in conjunction with Cdc42p-AD fusions. Interaction was again detected and was again stronger when Cdc42p contained the C188S and G12V or Q61L substitutions (Table 3B); indeed, for Bem3p, interaction was detected only for Cdc42p^{Q61L,C188S}. Rga1p-AD also interacted weakly with LexA-Rsr1p and -Rho1p fusions (Table 3A), although in the former case, the G12V substitution apparently weakened the interactions. The Rho4p fusion interacted strongly with a RhoGDI (data not shown), indicating that the failure of this protein to interact with Rga1p and Bem3p is not simply because the *RHO4* construct was faulty.

In a separate experiment, the ability of Rga1p to interact with components of the pheromone-response pathway was tested. No interactions were detected between Rga1p and Ste20p, Ste5p, Ste11p, Ste7p, or Fus3p (data not shown).

If Rga1p interacts with and serves as a GAP for Cdc42p, as the data above suggest, we reasoned that one should observe genetic interactions between *RGA1* and

Table 3. Two-hybrid interactions between *Rga1p* and Rho/Ras proteins

A.	pJG4-5- <i>RGA1</i>	pJG4-5- <i>BEM3</i>	pJG4-5
pEG202- <i>CDC42</i>	16	3	2
pEG202- <i>CDC42</i> ^{C188S}	191	12	40
pEG202- <i>CDC42</i> ^{G12V,C188S}	4777	108	323
pEG202- <i>CDC42</i> ^{Q61L,C188S}	3956	1798	311
pEG202- <i>CDC42</i> ^{D118A,C188S}	12	37	6
pEG202- <i>RHO1</i>	84	20	7
pEG202- <i>RHO1</i> ^{C206S}	14	17	30
pEG202- <i>RHO2</i>	6	14	20
pEG202- <i>RHO2</i> ^{C188S,C189S}	10	26	26
pEG202- <i>RHO3</i>	4	27	6
pEG202- <i>RHO3</i> ^{C228S}	1	18	10
pEG202- <i>RHO4</i>	8	15	7
pEG202- <i>RHO4</i> ^{C288S}	26	20	69
pEG202- <i>RSR1</i>	189	13	5
pEG202- <i>RSR1</i> ^{G12V}	121	N.D.	4
pEG202- <i>RAS2</i>	9	15	4
B.	pEG202- <i>RGA1</i>	pEG202- <i>BEM3</i>	pEG202
pJG4-5- <i>CDC42</i>	2	3	20
pJG4-5- <i>CDC42</i> ^{C188S}	26	1	18
pJG4-5- <i>CDC42</i> ^{G12V,C188S}	561	4	26
pJG4-5- <i>CDC42</i> ^{Q61L,C188S}	2016	268	18
pJG4-5	5	5	17

CDC42 and between *RGA1* and genes encoding known regulators of Cdc42p. We first examined the effect of overexpression of *RGA1* on a *cdc42^{ts}* mutant, reasoning that an increase in Cdc42p GAP activity should reduce the amount of active Cdc42p and therefore lower the maximum temperature at which the mutant can grow. Indeed, expression of *RGA1* or *BEM3* from multicopy plasmids lowered the restrictive temperature of the *cdc42^{ts}* mutant (Fig. 6A). Even at room temperature, the presence of high-copy *RGA1* or *BEM3* caused a significant increase in the proportion of large round, unbudded cells, the terminal phenotype exhibited by *cdc42-1* mutants (data not shown). We also examined the phenotype of a double-mutant strain containing mutations in *RGA1* and *CDC24*, which encodes an essential GDP release factor (GRF) that stimulates the exchange of GTP for GDP on Cdc42p (Zheng et al. 1994). Deletion of *RGA1* raised the restrictive temperature of *cdc24^{ts}* mutants (Fig. 6B) and restored wild-type cell morphology at 30°C (Fig. 7, top row). In addition, the multi-copy suppression of *cdc24^{ts}* by *CDC42* (Bender and Pringle 1989) was abolished if either *RGA1* or *BEM3* was also present on a high-copy plasmid (Pringle et al. 1995). These findings support the hypothesis that Rga1p and Bem3p have a function opposite to that of Cdc24p; that is, they serve as GAPs, or negative regulators, of Cdc42p.

If both Rga1p and Bem3p serve as GAPs for Cdc42p, an

rga1 bem3 double mutant should have a more extreme phenotype than either single mutant. Indeed, in contrast to the single mutants, the *rga1Δ bem3Δ* double mutant had an aberrant, elongated cell morphology (Fig. 7, bottom row). The *rga1 bem3* mutant, however, was viable, even at elevated temperatures (Fig. 6C), suggesting that there may also be additional GAPs that regulate Cdc42p.

Possible cross talk between the high osmolarity–stress pathway and the pheromone-response pathway

The ability of a *pbs2* mutation to potentiate pheromone-pathway signaling in an *rga1* mutant background was surprising because previous reports suggested that inactivation of *PBS2* had no effect on the mating pathway (Boguslawski 1992). To determine whether the ability of *pbs2* to potentiate pheromone-pathway activity was specific for *rga1*, we performed two experiments. First, we tested the ability of *pbs2-99* to enhance *FUS1-lacZ* expression in *ste4Δ* strains containing weakly constitutive alleles of *STE7* (Yashar et al. 1995). *FUS1-lacZ* expression was fourfold higher in the *pbs2-99* strain than in the *PBS2* strain (data not shown). Second, we stimulated the osmotic stress pathway by the addition of 1 M sorbitol to the growth medium. This condition increased expression of *FUS1-lacZ* by 10-fold in *ste4Δ pbs2Δ* cells but not in *ste4Δ PBS2* cells (Table 4; data not shown). Thus,

Figure 6. Genetic interactions among *RGA1*, *BEM3*, *CDC42*, and *CDC24*. Cells were grown on SD-Leu or YEPD medium at 23, 30, 35, or 37°C for 24–36 hr. (A) Strain DJTD2-16D (*cdc42-1*) with plasmids YEp13, YEp13-*RGA1*, and YEp13-*BEM3*. (B) Segregants from diploid SY3043, with relevant genotypes *cdc24^{ts}*, *rga1Δ::URA3*, and *cdc24^{ts} rga1Δ::URA3*. (C) Segregants from diploid SY3032 with relevant genotypes *rga1Δ::URA3*, *bem3Δ::LEU2*, and *rga1Δ::URA3 bem3Δ::LEU2*.

Figure 7. Cell morphologies of *rga1Δ cdc24^{ts}* and *rga1Δ bem3Δ* strains. Heterozygous diploids SY3043 (*cdc24^{ts}/CDC24 rga1Δ::URA3/RGA1*) and SY3032 (*bem3Δ::LEU2/BEM3 rga1Δ::URA3/RGA1*) were sporulated and dissected. Cells from a representative tetrad type tetrad from each diploid are shown. For each diploid, at least five segregants of each genotype were examined and found to exhibit the phenotype typified by the photographs. Cells were grown at 30°C in YEPD medium and prepared and photographed as described in Materials and methods.

under certain conditions, transfer of signal is possible between the osmotic-stress pathway and the pheromone-transduction pathway.

Discussion

We have identified a new regulator of the yeast pheromone-response pathway by identifying mutations that activate the pathway in *ste4Δ* strains, which lack the β subunit of the G protein. Wild-type cells that have not been stimulated with pheromone exhibit a basal signal in the pheromone pathway, presumably because such cells contain some free G $\beta\gamma$ subunit; *ste4Δ* mutants lack this basal signal. Recessive mutations in *RGA1*, which encodes the newly identified regulator, create a signal in *ste4Δ* mutants and increase the basal signal in wild-type cells. Conversely, overexpression of *RGA1* reduces the basal signal in wild-type cells and dampens response to pheromone. Thus, we conclude that *RGA1* encodes a negative regulator of the pheromone signal-transduction pathway. The Rga1p protein sequence shows striking sequence similarity to GTPase-activating proteins (GAPs) that promote GTP hydrolysis by p21 GTPases of the Rho subfamily. It seems likely, therefore, that Rga1p functions to modulate the pheromone-response pathway by

regulating a Rho/Rac GTPase, which itself functions as a positive regulator of the pathway.

Genetic and two-hybrid experiments indicate that Cdc42p, a Rho/Rac GTPase required for polarity establishment during budding, is an important target of Rga1p, and we suggest that Cdc42p is also a positive regulator of the pheromone-response pathway. Support for this suggestion comes from biochemical studies with mammalian cells and genetic and biochemical studies with yeast. Manser et al. (1994) showed that the GTP-bound form of mammalian Cdc42p could bind to a Ste20p homolog, p65^{PAK}, stimulating it to autophosphorylate and thereby become an active protein kinase capable of phosphorylating other substrates. Consistent with these observations, Simon et al. (1995) and Zhao et al. (1995) found that yeast Cdc42p and Ste20p interact both in the two-hybrid system and in vitro. Moreover, these authors found that *cdc42* and *cdc24* temperature-sensitive mutants failed to respond to pheromone at the restrictive temperature as measured by induction of a *FUS1-lacZ* reporter and were released from cell-cycle arrest when cells arrested by pheromone at permissive temperature were shifted to restrictive temperatures. Taken together, these data suggest that Rga1p acts as a GAP for Cdc42p, which in turn affects signaling in the pheromone-response pathway through the Ste20p protein kinase.

Two observations complicate the relatively simple picture presented above. First, deletion of *STE20* reduces, but does not abolish, pathway activation caused by mutation of *RGA1*. Perhaps GTP-bound Cdc42p can activate other protein kinases that can substitute, at least partially, for Ste20p. For example, Cdc42p also interacts with Cla4p, a protein that is homologous to Ste20p (Cvrcková et al. 1995). These two protein kinases are partially redundant in function during vegetative growth: the double mutant is inviable, whereas either single mutant is viable (Cvrcková et al. 1995). Perhaps there is also some redundancy during pheromone signaling. Another possibility is that GTP-bound Cdc42p may directly activate Ste11p. The second complication is that mutations that should trap Cdc42p in the GTP-bound state [e.g.,

Table 4. *FUS1-lacZ* expression in *pbs2Δ* strains subjected to osmotic stress

Strain	<i>FUS1-lacZ</i> activity ^a	
	– sorbitol	+ sorbitol ^b
<i>ste4Δ pbs2Δ</i>	0.23	2.2
<i>ste5Δ pbs2Δ</i>	0.21	2.3
<i>ste20Δ pbs2Δ</i>	0.4	0.9
<i>ste11Δ pbs2Δ</i>	0.2	0.2

^aValues are averages of four determinations (two each in *MATa* and *MATα* strains).

^bCells were grown in 0.4 M sorbitol for 1 hr at 30°C.

Cdc42p^{G12V}) do not by themselves cause constitutive activation of the pheromone pathway, although they do make cells more sensitive to pheromone (Simon et al. 1995). Perhaps Cdc42p must hydrolyze the bound GTP to achieve stimulation of the pheromone pathway. Perhaps Rga1p is itself a target of one of the early components of the pheromone pathway and, unless it has received a signal from that component, prevents Cdc42p from activating Ste20p. Or, perhaps an as-yet-unidentified GTPase is the relevant target of Rga1p with respect to the pheromone pathway signal generated in *rga1* mutants.

The postulated role of Cdc42p in pheromone signal transmission raises the possibility that it may be an integral part of a switch that controls cellular morphology: Vegetative cells form buds at discrete and predictable sites, whereas pheromone-stimulated cells cease budding and form mating projections oriented toward the pheromone source. Cdc42p is thought to participate in bud-site selection and bud initiation by interaction with other proteins in a morphogenetic hierarchy that includes proteins (such as Rsr1p) dedicated to bud-site selection, as well as other proteins (such as Cdc24p) that are essential for polarity establishment (Bender and Pringle 1989; Park et al. 1993; Zheng et al. 1994, 1995; Pringle et al. 1995). The constellation of proteins with which Cdc24p and Cdc42p interact may change upon pheromone stimulation. For example, they may interact, directly or indirectly, with the membrane-bound G β γ dimer. Such new interactions may recruit Cdc24p and Cdc42p away from the predetermined site for bud emergence and to a site appropriate for projection formation. Similarly, the new interactions may facilitate interaction with, and stimulation of, Ste20p. A dual role for p21 GTPases is not unprecedented. In the fission yeast *Schizosaccharomyces pombe*, Ras1p is argued to participate both in mating and in morphogenesis (Chang et al. 1994).

The possibility that Cdc42p has dual (or multiple) roles, or at least is subject to two or more regulatory inputs, is also suggested by the existence of two GAPs for this protein. Outside the GAP domains, Bem3p and Rga1p show no sequence similarity. For example, Rga1p contains two LIM domains, which are thought to be zinc-binding domains that mediate protein-protein interaction, whereas Bem3p lacks LIM domains. Thus, Rga1p and Bem3p likely interact with different proteins and may, therefore, provide a way for distinct signals to influence the activity of Cdc42p. Moreover, it is possible that there are additional GAPs for Cdc42p. A cell lacking either Cdc42p or Cdc24p activity is inviable, whereas a cell lacking both Rga1p and Bem3p is viable, although noticeably misshapen. Cdc42p may have sufficient inherent, unstimulated GTPase activity to maintain viability (Zheng et al. 1994). On the other hand, perhaps an additional protein(s) with GAP activity towards Cdc42p remains to be discovered. Understanding the regulatory inputs that influence Cdc42p activity, and the spectrum of physiological processes that Cdc42p in turn governs, are important challenges for the future.

Materials and methods

Strains, plasmids, and microbiological techniques

The yeast strains used in this work are listed in Table 5. The pheromone responsive *FUS1::HIS3* construct and plasmids used to introduce deletions at the chromosomal *STE* loci have been described previously (Stevenson et al. 1992). *STE20* was deleted by use of pEL45 (*ste20 Δ ::URA3*; Leberer et al. 1992). For the deletion of *PBS2*, the *SacI* fragment of pJB40, containing *pbs2 Δ ::URA3* (Brewster et al. 1993), was subcloned into the nonreplicating vector pRS306 (Sikorski and Hieter 1989), and the resultant plasmid was digested with *SacI* before transformation. A deletion allele of *RGA1* (*rga1 Δ ::LEU2*) was created by subcloning of the 5.8-kb *HindIII* fragment into pIC19H (Marsh et al. 1984) and replacement of the largest *SpeI*-*Eco47III* fragment with an *NheI*-*SmaI* fragment containing the *LEU2* gene from YDpL (Berben et al. 1991). This deletion removes all of the *RGA1* ORF. An *rga1::URA3* allele was constructed by replacing the larger *XhoI*-*BamHI* fragment with the *URA3* gene carried on a *SalI*-*SmaI* fragment from YDpU (Berben et al. 1991). This allele deletes the amino-terminal third of the *RGA1* open reading frame and gives rise to a phenotype indistinguishable from *rga1::LEU2*. Constructs used for segregation analysis were produced by subcloning of fragments from the cloned genomic DNAs into modified versions of the integrating vector pRS306 (Sikorski and Hieter 1989); a 5.8-kb *HindIII* fragment was subcloned from pRGA1.3 to give pRGA1.INT, and a 6.1-kb *PstI* fragment was subcloned from pPBS2.16 to give pPBS2.INT. Chromosomal deletions of genes were produced by one-step gene replacement (Rothstein 1991) and were verified by Southern analysis (Southern 1975). The pGAL-*RGA1* expression plasmid was constructed by introducing *SacI* restriction sites 5' of the presumed initiation codon and 3' of the stop codon through PCR amplification. The resulting fragment was ligated into pSL1597 (constructed by C. Boone, Simon Fraser University, Burnaby, British Columbia, Canada), a pRS316-based plasmid (Sikorski and Hieter 1989) containing a 0.8-kb fragment of the *GAL1* promoter. The multicopy plasmid YEp13-*RGA1* was constructed by ligation of the 5.8-kb *HindIII* fragment including *RGA1* into YEp13 (Broach et al. 1979). YEp13-BEM3 was a gift from A. Bender (Indiana University, Bloomington).

Yeast and bacterial strains were propagated by use of standard methods. YEPD and SD media were prepared as described (Rose et al. 1990). Yeast transformations were performed by use of a modification of the Li⁺ ion method (Gietz et al. 1992). Bacterial transformations, DNA preparations, and plasmid constructions were performed by standard methods (Sambrook et al. 1989).

Two-hybrid assays

Two different two-hybrid systems were used in the analysis of Rga1p. In the study of interactions with Ste proteins, a *lexA*-*RGA1* gene fusion was constructed by the polymerase chain reaction (PCR) addition of *EcoRI* restriction sites immediately 3' of the *RGA1* initiation codon (creating a deletion of the first three amino acids and an addition of two amino acids, EF) and 3' of the stop codon. The resulting fragment was ligated into plasmid pMA424 (Ma and Ptashne 1987). The Gal4 activation domain fusions containing Ste4p, Ste5p, Ste11p, Ste7p, and Fus3p were previously described (Printen and Sprague 1994). Interactions were assayed in strain CTY10-5D (gift of S. Fields).

In the Cdc42p experiments, the two-hybrid system of Gyuris et al. (1993) was used. To construct fusions of various GTPase proteins to the LexA DNA-binding domain (DBD) in pEG202, *S. cerevisiae* CDC42 (Johnson and Pringle 1990), CDC42^{C188S} (Ziman et al. 1991), *RHO1* and *RHO2* (Madaule et al. 1987), *RHO3*

Table 5. Yeast strains

Strain	Genotype	Source
227	<i>MATa lys1 cry1</i>	I. Herskowitz (University of California, San Francisco)
YEF24H	<i>MATα cdc24-H^{ts} ura3 trp1 leu2 his4 ade2</i>	J. Pringle
DJTD2-16D	<i>MATα cdc42-1^{ts} ura3 trp1 leu2 his4 gal2</i>	Johnson et al. (1987)
ABY646	<i>MATa bem3::LEU2 ura3 leu2</i>	A. Bender (Indiana University, Bloomington)
CTY10-5D	<i>MATa ade2 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3::lacZop-lacZ</i>	S. Fields (University of Washington, Seattle)
EGY48	<i>MATα his3 trp1 ura3-52 lexA-LEU2</i>	Gyuris et al. (1993)
SY1731 (27)	<i>MATα ste4Δ::LEU2 rga1-1 pbs2-99 FUS1::HIS3 leu2 trp1 ura3 his3Δ200::ura3 pep4Δ::ura3 can1</i>	Stevenson et al. (1992)
SY2002	<i>MATa FUS1::HIS3 his3 mfa2-Δ1::FUS1-lacZ ade1 leu2 trp1 ura3</i>	lab strain
SY2003	<i>MATα FUS1::HIS3 his3 mfa2-Δ1::FUS1-lacZ ade1 leu2 trp1 ura3</i>	lab strain
SY3032	<i>MATa/MATα diploid from SY3064 × ABY646</i>	this study
SY3043	<i>MATa/MATα diploid from BSY109 and YEF24H</i>	this study
SY3064	<i>SY2003 except rga1::URA3</i>	this study
SY3116	<i>SY2002 except rga1-1</i>	this study
BSY109	<i>SY2002 except RGA1::URA3::RGA1</i>	this study
BSY110	<i>SY2002 except PBS2::URA3::PBS2</i>	this study
BSY106	<i>MATα ste4Δ::LEU2 rga1-1 pbs2-99 FUS1::HIS3 his3Δ200 ade1 leu2 trp1 ura3</i>	this study
BSY146	<i>MATα ste4Δ::LEU2 rga1-1 pbs2-99 FUS1::HIS3 his3 mfa2-Δ1::FUS1-lacZ ade1 leu2 trp1 ura3</i>	this study
BSY179 ^a	<i>MATα FUS1::HIS3 his3 mfa2-Δ1::FUS1-lacZ ade1 leu2 trp1 ura3</i>	this study
BSY183	<i>BSY179 except rga1-1</i>	this study
BSY185	<i>BSY179 except rga1-1 pbs2-99</i>	this study
BSY187	<i>BSY179 except ste4Δ::LEU2</i>	this study
BSY191	<i>BSY179 except ste4Δ::LEU2 rga1-1</i>	this study
BSY193	<i>BSY179 except ste4Δ::LEU2 rga1-1 pbs2-99</i>	this study
BSY194	<i>MATa ste4Δ::LEU2 rga1-1 pbs2-99 FUS1::HIS3 his3 mfa2-Δ1::FUS1-lacZ ade1 leu2 trp1 ura3</i>	this study
BSY171	<i>SY2003 except ste4Δ::URA3</i>	this study
BSY173	<i>SY2003 except ste5Δ::URA3</i>	this study
BSY160	<i>SY2003 except ste11Δ::URA3</i>	this study
BSY217	<i>SY2003 except ste20Δ::URA3</i>	this study
BSY215	<i>SY2003 except ste4Δ::URA3 pbs2Δ::URA3</i>	this study
BSY244	<i>SY2003 except ste4Δ::URA3 rga1Δ::LEU2</i>	this study
BSY246	<i>SY2003 except ste4Δ::URA3 rga1Δ::LEU2 pbs2Δ::URA3</i>	this study
BSY240	<i>SY2003 except rga1Δ::LEU2</i>	this study
BSY242	<i>SY2003 except rga1Δ::LEU2 pbs2Δ::URA3</i>	this study
BSY219 ^b	<i>MATα ste5Δ::URA3 FUS1::HIS3 his3 mfa2-Δ1::FUS1-lacZ ade1 leu2 trp1 ura3</i>	this study
BSY221	<i>BSY219 except rga1-1</i>	this study
BSY223	<i>BSY219 except rga1-1 pbs2-99</i>	this study
BSY225 ^c	<i>MATα ste11Δ::URA3 FUS1::HIS3 his3 mfa2-Δ1::FUS1-lacZ ade1 leu2 trp1 ura3</i>	this study
BSY227	<i>BSY225 except rga1-1</i>	this study
BSY229	<i>BSY225 except rga1-1 pbs2-99</i>	this study
BSY231 ^d	<i>MATα ste20Δ::URA3 FUS1::HIS3 his3 mfa2-Δ1::FUS1-lacZ ade1 leu2 trp1 ura3</i>	this study
BSY233	<i>BSY231 except rga1-1</i>	this study
BSY235	<i>BSY231 except rga1-1 pbs2-99</i>	this study

^aBSY179–BSY194 are isogenic segregants from a BSY146 × SY2002 cross.^bBSY219–BSY223 are isogenic segregants from a BSY194 × BSY173 cross.^cBSY225–BSY193 are isogenic segregants from a BSY194 × BSY215 cross.^dBSY231–BSY235 are isogenic segregants from a BSY194 × BSY217 cross.

and *RHO4* (Matsui and Toh-e 1992; YKRO55W, accession no. Z28280, MIPS), *RSR1* (Bender and Pringle 1989), *RSR1*^{G12V} (Ruggieri et al. 1992), and *RAS2* (Powers et al. 1984) full-length coding sequences were amplified by PCR with Vent polymerase (New England Biolabs). Plasmids containing the genes were used as templates, and appropriate restriction sites were introduced with the primers. The mutant genes *CDC42*^{G12V,C188S}, *CDC42*^{Q61L,C188S}, *CDC42*^{D118A,C188S}, *RHO1*^{C206S}, *RHO2*^{C188S,C189S}, *RHO3*^{C228S}, and *RHO4*^{C288S} were amplified by use of plasmids containing *CDC42*^{G12V}, *CDC42*^{Q61L}, *CDC42*^{D118A} (Ziman et al. 1991), *RHO1*, *RHO2*, *RHO3*, and *RHO4* as templates and reverse primers that specifically introduced the various C-S mutations (bold letters) as well as a *XhoI* cloning site (italic letters): *CDC42*^{C188S}, 5'-ATATACTACTCGAGCTACAAAATTGTA-GATTTTACTTTTCTTG-3'; *RHO1*^{C206S}, 5'-GAGATCG-AGCTCGAGCTATAACAAGACAGACTTCTTCTTCTTCT-3'; *RHO2*^{C188S,C189S}, 5'-CAGTGTAGCTCGAGTTATAAAA-TTATGGAAGAGTTAGCCCTGG-3'; *RHO3*^{C228S}, 5'-TATATATACTCGAGTTACATAATGGTAGAGCTGGATC-CACTG-3'; *RHO4*^{C288S}, 5'-CTTCATCACTCGAGTTACAT-TATAATAGACTTGTTTTTCTTAA-3'.

The PCR products were cloned either at the *EcoRI*-*XhoI* sites (all but *RSR1*) or at the *BamHI*-*XhoI* sites (*RSR1*) of pEG202. Accordingly, all constructs, except for that involving *RSR1*, contain two additional amino acids (EF) between the last amino acid of the LexA DBD and the first amino acid (M) of the fused protein. LexA-Rsr1p contains five additional amino acids (EFGPI) between the LexA DBD and the first amino acid (R, corresponding to the second amino acid of Rsr1p) of the fused Rsr1p. All constructs contain the original stop codons of the fused genes.

To construct fusions of Bem3p (Bender and Pringle 1991; Zheng et al. 1994) and Rga1p to the LexA DBD in pEG202, both full-length coding sequences were amplified by PCR as described above, by use of plasmids containing either *BEM3* or *RGA1* as template. The PCR products were cloned either at the *EcoRI*-*NcoI* sites (*BEM3*) or at the *NcoI*-*XhoI* sites (*RGA1*) of pEG202. Accordingly, the Bem3p construct contains two additional amino acids (EF) and the Rga1p construct nine additional amino acids (EFGPIRRPW) between the last amino acid of the LexA DBD and the first amino acid (M) of the fused protein. Both constructs contain the original stop codons of the fused genes.

For construction of fusions of the various proteins to the activation domain (AD) in pJG4-5, the desired sequences were amplified by PCR as described above and cloned into pJG4-5 by use of the *EcoRI* and *XhoI* sites (*CDC42*, *CDC42*^{C188S}, *CDC42*^{G12V,C188S}, and *CDC42*^{Q61L,C188S}), the *EcoRI* and *NcoI* sites (*BEM3*), or the *NcoI* and *XhoI* sites (*RGA1*) of a pJG4-5 that had been modified to include an *NcoI* site in its polylinker.

For assessment of β -galactosidase activities, strain EGY48 containing the *lexAop-lacZ* reporter plasmid pSH18-34 (Gyuris et al. 1993) was cotransformed either with pEG202 or with a pEG202-derived plasmid expressing a LexA DBD fusion protein and with pJG4-5 or a pJG4-5-derived plasmid expressing an AD-fusion protein. β -Galactosidase activities were measured in three to six different isolates of each strain after growth for 16 hr at 30°C in minimal medium containing 2% galactose, 1% raffinose, and 0.1 mg/ml L-leucine. The average β -galactosidase activities (in Miller units) are reported.

Genetic analysis of mutant 27

Mutant 27 (SY1731; Stevenson et al. 1992) was isolated on the basis of its ability to activate expression of *FUS1::HIS3* and to mate in the absence of a functional G protein. Preliminary char-

acterization of this strain revealed that, unlike the parent strain, it grew poorly on media of high osmolarity (1 M sorbitol). To determine if these phenotypes were linked, the mutant was crossed four times to SY2002. SY2002 carries *FUS1::HIS3* at *his3* and a *FUS1-lacZ* construct at *mfa2 Δ* (Boone et al. 1993). Analysis of the progeny of these crosses indicated that two unlinked mutations were responsible for the phenotypes of the original mutant. One of the mutations (*rga1-1*) activated expression of *FUS1* in a *ste4 Δ* background, but did not by itself restore mating ability. The second mutation (*pbs2-99*) conferred an osmotic sensitivity phenotype but did not activate expression of *FUS1*. The presence of both *rga1-1* and *pbs2-99*, however, gave higher expression of *FUS1* (than *rga1-1* alone) and allowed mating in a *ste4 Δ* background (see Table 1).

Cloning and sequencing of RGA1

Mutant 27 (SY1731) was transformed to uracil prototrophy with a high-copy, yeast genomic library (Carlson and Botstein 1982), and colonies were replica-plated to solid medium lacking histidine to screen for plasmid-mediated reduction of expression of *FUS1::HIS3*. A total of five distinct plasmids that conferred the same phenotype upon retransformation into SY1731 were analyzed further by restriction enzyme analysis. Three plasmids (designated pRGA1.3, pRGA1.4, and pRGA1.17) contained overlapping inserts representing about 20 kb of genomic DNA. This group of plasmids complemented the *FUS1* expression phenotype, but not the osmotic-sensitivity phenotype, of the original strain. The second group, comprising two plasmids with overlapping inserts (designated pPBS2.1 and pPBS2.16) complemented both phenotypes. In a separate experiment, several plasmids with inserts overlapping those of pPBS2.1 and pPBS2.16 were cloned by screening for growth of mutant 27 on media containing 1 M sorbitol after transformation with a CEN-based genomic library.

Linkage analysis was performed to verify that the cloned genes represented wild-type versions of the mutant alleles and not high-copy suppressors. SY2002 (*MATa FUS1::HIS3*) was transformed with either pRGA1.INT linearized with *XhoI* or pPBS2.INT linearized with *XbaI*. Strains with correct integration of pRGA1.INT (BSY109) or pPBS2.INT (BSY110) were mated to BSY106 (*MATa ste4 Δ ::LEU2 rga1-1 pbs2-99 ura3 FUS1::HIS3*). The diploids were sporulated and segregants assayed for the presence of the cloned gene (*Ura*⁺), expression of *FUS1::HIS3* in *ste4 Δ* cells (*His*⁺ or *His*⁻), and growth on high osmolarity medium (1 M sorbitol). In 19 tetrads analyzed from the BSY109 $\times$ BSY106 cross all except one of the 38 *ste4 Δ* segregants were *Ura*⁺ *His*⁻ (15) or *Ura*⁺ *His*⁺ (22), and about half of all *Ura*⁺ (20/37) and half of all *Ura*⁻ (18/39) segregants were able to grow well on 1 M sorbitol. These data indicate that the cloned pRGA1 DNA is linked to the *rga1-1* locus. In the BSY110 $\times$ BSY106 cross, all segregants were either *Ura*⁺ and grew well on 1 M sorbitol (34) or *Ura*⁻ and sensitive to 1 M sorbitol (34) (i.e., parental ditype), indicating that the cloned pPBS2 was indeed linked to the mutation (*pbs2-99*) which conferred the osmotic-sensitivity phenotype.

The *RGA1* gene was located within pRGA1.3 by subcloning of restriction enzyme fragments of the genomic insert into pRS316 (Sikorski and Hieter 1989) and testing for complementation after transformation into SY1731. For generation of nested deletions for DNA sequencing by use of exonuclease III, the complementing 5.8-kb *HindIII* fragment was cloned in both directions in pRS316 and digested with *SacI* (exonuclease III-resistant ends) and *EcoRI* (exonuclease III-sensitive ends) prior to exonuclease III treatment. Useful deletions were converted to single-stranded templates by use of M13 KO7 helper phage

(Vieira and Messing 1987) and their sequences were determined by use of the dideoxy chain-termination method (Sanger et al. 1977). The 4.1-kb *Clai-HindIII* fragment was completely sequenced on both strands. The *RGA1* DNA sequence has been deposited in the EMBL, GenBank and DDJB nucleotide sequence databases under accession number X90950.

β -Galactosidase and mating assays

Cells containing a *FUS1-lacZ* construct integrated at *mfa2 Δ* were diluted from a fresh overnight culture in YEPD medium and grown for about 6 hr, to mid-log phase at 30°C. Cells were prepared and assayed as described previously (Jarvis et al. 1988). Quantitative mating assays using 227 as a tester strain were performed by a filter mating assay as described (Clark and Sprague 1989).

Microscopy

Cells were grown in YEPD medium to a density of $4-8 \times 10^6$ cells/ml, sonicated for 5 sec, then concentrated by centrifugation. Cell pellets were either resuspended in water or in 1 μ g/ml Calcofluor (Sigma) for 20 min, then washed with water and visualized on a Zeiss Axioplan photomicroscope with a 100 $\times$ objective.

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Mutation of RGA1, which encodes a putative GTPase-activating protein for the polarity-establishment protein Cdc42p, activates the pheromone-response pathway in the yeast *Saccharomyces cerevisiae*.

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