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Schizosaccharomyces pombe Adenylate Cyclase Suppressor Mutations Suggest a Role for cAMP Phosphodiesterase Regulation in Feedback Control of Glucose/cAMP Signaling

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ABSTRACT

Mutations affecting the *Schizosaccharomyces pombe* cAMP phosphodiesterase (PDE) gene *cgs2*$^+$ were identified in a screen for suppressors of mutant alleles of the adenylate cyclase gene (*git2*$^+$/*cyr1*$^+$), which encode catalytically active forms of the enzyme that cannot be stimulated by extracellular glucose signaling. These mutations suppress both the *git2*$^-$ mutant alleles used in the suppressor selection and mutations in *git1*$^+$, *git3*$^+$, *git5*$^+$, *git7*$^+$, *git10*$^+$, and *git11*$^+$, which are all required for adenylate cyclase activation. Notably, these *cgs2* mutant alleles fail to suppress mutations in *gpa2*$^+$, which encodes the Gα subunit of a heterotrimeric G protein required for adenylate cyclase activation, although the previously identified *cgs2-2* allele does suppress loss of *gpa2*$^+$. Further analysis of the *cgs2-s1* allele reveals a synthetic interaction with the *gpa2*^{R176H}-activated allele, with respect to derepression of *fbp1-lacZ* transcription in glucose-starved cells. In addition, direct measurements of cAMP levels show that *cgs2-s1* cells maintain normal basal cAMP levels, but are severely defective in feedback regulation upon glucose detection. These results suggest that PDE activity in *S. pombe* may be coordinately regulated with adenylate cyclase activity as part of the feedback regulation mechanism to limit the cAMP response to glucose detection.

NUTRIENT sensing is a critical process for microorganisms as they must regulate their growth and metabolism in response to changes in the growth environment. Signal transduction pathways that are activated by either the presence or the absence of specific nutrients, the best studied of which is glucose, have been identified. Both yeast and fungi, including a wide variety of pathogenic fungi, have been shown to regulate metabolic pathways, sexual development, and growth morphology in response to nutrient signaling (Lengeler *et al.* 2000).

In the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, glucose detection leads to a transient cAMP signal, which activates the cAMP-dependent protein kinase PKA. The increase in cAMP is due to the activation of adenylate cyclase, which converts ATP to cAMP, and not to the inactivation of cAMP phosphodiesterase (PDE), which converts cAMP to AMP (Nikawa *et al.* 1987; Byrne and Hoffman 1993). Genetic and molecular analyses to characterize the mechanism of glucose detection and adenylate cyclase activation have uncovered some related components between these two cAMP signaling pathways, as well as several features that are unique to each pathway (Hoffman 2005). For example, both yeasts express seven-transmembrane G-protein-coupled receptors (GPCRs; *S. cerevisiae* Gpr1 and *S. pombe* Git3), which are required to activate similar Gpa2 Gα subunits (Nocero *et al.* 1994; Xue *et al.* 1998; Yun *et al.* 1998; Kraakman *et al.* 1999; Lorenz *et al.* 2000; Welton and Hoffman 2000). However, *S. pombe* Gpa2, which has recently been shown to directly bind an N-terminal domain of adenylate cyclase (Ivey and Hoffman 2005), functions together with the Git5-Git11 Gβγ dimer (Landry *et al.* 2000; Landry and Hoffman 2001), while the only authentic *S. cerevisiae* Gβγ dimer, Ste4-Ste18, does not act in conjunction with Gpa2 (Liu *et al.* 1993). In addition, Ras proteins play an important role in adenylate cyclase activation in *S. cerevisiae*, but have no role in the *S. pombe* cAMP pathway (Toda *et al.* 1985; Fukui *et al.* 1986; Mbonyi *et al.* 1988; Hoffman and Winston 1991).

The mechanism of fungal adenylate cyclase activation remains largely unknown. In budding yeast, the GTP-binding Ras proteins have been shown to bind to a sequence far from the catalytic domain, while, in mammals, G proteins bind directly to adenylate cyclase catalytic domains. In *S. pombe*, most of the genes known to encode components of the glucose/cAMP pathway have been identified in a selection for mutants that fail to glucose repress transcription of the *fbp1*$^+$ gene, encoding the gluconeogenic enzyme fructose-1,6-bisphosphatase (Hoffman and Winston 1990). This collection of mutants includes 31 strains carrying mutations in the *git2*$^+$/*cyr1*$^+$ adenylate cyclase gene, some of which compose

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two intragenic complementation groups (Hoffman and Winston 1990, 1991). *In vitro* assays of adenylate cyclase activity showed wild-type catalytic activity in *git2-7* and *git2-210* strains from one group and reduced catalytic activity in a *git2-61* strain from the second group (Hoffman and Winston 1991). Presumably, the first group is defective in a step involved in *in vivo* activation, such as binding an activator, while the second group is defective in catalytic activity.

In this study, we carried out a suppressor selection on two activation-defective adenylate cyclase mutant strains in an effort to identify gain-of-function mutations in a gene presumed to encode an adenylate cyclase activator. From 120 independently isolated mutants from two parental strains, we identified two dominant mutations that failed to suppress an adenylate cyclase deletion. Furthermore, a synthetic interaction with a gain-of-function *gpa2*^{R176H} allele suggested that these mutations affected a gene that acted in concert with Gpa2 to activate adenylate cyclase. Screening of a genomic library, constructed with insert DNA from a strain carrying one of these dominant mutations, failed to identify the suppressor gene. Surprisingly, a genetic mapping approach revealed that these suppressors are alleles of the *cgs2*$^+$/*pde1*$^+$ cAMP phosphodiesterase gene (DeVoti *et al.* 1991; Mochizuki and Yamamoto 1992). Similar to the alleles identified in this study, the *cgs2-2* allele, which we show to possess a frameshift mutation, also behaves as a dominant suppressor of mutations in *git* genes, although the *cgs2-2* allele is able to suppress a *gpa2* disruption. Therefore, these appear to be dominant-negative mutations with dominance possibly due to protein poisoning or haplo-insufficiency. Measurement of cAMP levels in a *cgs2-s1* strain shows that these cells possess wild-type basal cAMP levels, but exhibit a dramatically increased glucose-stimulated response indicative of a loss of feedback regulation. These data are consistent with a model in which the wild-type Cgs2 cAMP phosphodiesterase becomes activated almost immediately after adenylate cyclase activation to limit the cAMP response to glucose in fission yeast.

MATERIALS AND METHODS

Yeast strains and growth media: Yeast strains used are listed in Table 1. The *fbp1::ura4*$^+$ and *ura4::fbp1-lacZ* reporter constructs have been previously described (Hoffman and Winston 1990). Only relevant genotypes are given in the text, tables, and figure legends.

Standard rich media yeast extract agar (YEA) and YEL (Gutz *et al.* 1974) were supplemented with 2% casamino acids. *S. pombe* minimal media (PM; Watanabe *et al.* 1988) were supplemented with required nutrients at 75 mg/liter, except for leucine, which was added to 150 mg/liter. Glucose was generally present at a concentration of 3%, unless otherwise specified. Sensitivity to 5-fluoroorotic acid (5-FOA) was determined on SC solid media containing 8% glucose as previously described (Hoffman and Winston 1991). Strains were grown at 30°.

Recombinant DNA methodology: Standard recombinant DNA techniques, including DNA restriction digests, ligations, and *Escherichia coli* transformations, were done according to Ausubel *et al.* (1998).

Isolation and genetic analysis of *git2-7* and *git2-216* suppressor mutations: 5-FOA-resistant (5-FOA^R) derivatives from strains CHP7 (*git2-7*) and CHP216 (*git2-216*) were isolated by plating 10^5 cells onto YEA plates, subjecting them to 175 J of UV irradiation, and replica plating to 5-FOA medium after a 24-hr grow out period. Colonies that formed within 3–6 days were single colony purified on YEA and retested for 5-FOA^R growth. Sixty 5-FOA^R derivatives from each strain were isolated and characterized. Strains unable to grow on PM-ura medium were discarded as these most likely carry mutations in either *ura4*$^+$ (within the *fbp1-ura4*$^+$ reporter) or *ura5*$^+$ and are not strains with reduced *fbp1-ura4*$^+$ transcription. (PM-ura medium is less restrictive than SC-ura for growth of strains carrying a functional *fbp1-ura4*$^+$ reporter.) Random spore analysis was carried out on progeny from CHP7 derivatives crossed with CHP386 (*git2*Δ::*his7*$^+$) or from CHP216 derivatives crossed with CHP398 (*git2*Δ::*his7*$^+$). Intragenic suppressor mutations were identified by the fact that all His$^-$ progeny were 5-FOA^R, while all His$^+$ progeny were 5-FOA sensitive (5-FOA^S). Suppressor mutations able to suppress *git2*Δ::*his7*$^+$ were identified by the fact that approximately half of the His$^+$ (*git2*Δ) progeny were 5-FOA^R. Allele-specific suppressors were identified by the fact that approximately half of the His$^-$ progeny, containing the *git2* point mutations, were 5-FOA^S, while all of the His$^+$ progeny were 5-FOA^S. The allele-specific nature of the suppressor mutations in strains KGP5 and KGP6 was confirmed by tetrad dissection. Dominance-recessiveness testing was carried out by mating the suppressor strains with a *git2*Δ strain and determining whether the resulting diploid strain was 5-FOA^S (indicating a recessive suppressor) or 5-FOA^R (indicating a dominant suppressor).

Construction and screening of an *S. pombe* genomic DNA library: Genomic DNA from strain KGP5 (*git2-7 sog1-1*) was isolated and used as insert DNA for the construction of a genomic library in the *his7*$^+$ cloning vector pEA500 (Apolinario *et al.* 1993), using a partial fill-in cloning strategy as described by Ohi *et al.* (1996). The insert DNA was generated by a *Sau*3AI partial digestion and partial Klenow fill-in, while the plasmid was linearized at the *Xho*I site in the polylinker followed by a partial Klenow fill-in reaction. The library consists of ~250,000 independent clones, of which 70% contain inserts with an average size of 3.9 kb. CHP7 (*git2-7*; 50,000 colonies screened) or FWP110 (*git1-1*; 40,000 colonies screened) transformants were screened for 5-FOA^R growth, indicative of glucose repression of *fbp1-ura4*$^+$ expression. Candidate transformants were subjected to a plasmid-loss experiment to determine whether or not the 5-FOA^R growth was plasmid conferred. Plasmids shown to confer 5-FOA^R growth were rescued into *E. coli* (Hoffman and Winston 1987) and subjected to a DNA sequence analysis using vector-specific sequencing primers to determine the two ends of the insert DNA.

Mapping of *sog1-1* to the *cgs2*$^+$ locus: Chromosomal mapping of *sog1-1* to chromosome 3 was carried out by benomyl-induced haploidization of an *h*$^-$/*mat2-102* diploid strain as previously described (Alfa *et al.* 1993). The *sog1-1* allele was further mapped by tetrad dissection, scoring for its ability to suppress either a *git3-14* or a *git3*Δ allele. Initial crosses involved strains carrying mutant alleles of chromosome 3 genes, including *ura4*$^+$, *wee1*$^+$, *cdc21*$^+$, *ade6*$^+$, *git3*$^+$, *cdc11*$^+$, and *ade5*$^+$. Additional markers were constructed by cloning fragments (~1 kb) of chromosome 3 into the *LEU2*$^+$-marked TOPO cloning vector pNMT41 (Invitrogen, Carlsbad, CA), linearizing the plasmid within the insert DNA, and integrating the plasmid into the chromosome by homologous recombination (Bähler *et al.* 1998) to place the *LEU2*$^+$ marker at specific sites within the chromosome.

TABLE 1

Strain list

Strain	Genotype
CHP7	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-7*
CHP14	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3-14*
CHP216	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-216*
CHP384	*h*$^+$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ cgs2-2*
CHP386	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-2::his7*$^+$
CHP398	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-2::his7*$^+$
CHP732	*h*$^-$ *ade6-M216 leu1-32 ura4::fbp1-lacZ cgs2-s1 gpa2*^{R176H}
CHP733	*h*$^-$ *ade6-M216 leu1-32 ura4::fbp1-lacZ cgs2-s1*
CHP769	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *cgs2-s1 git3Δ::kan*
CHP774	*h*$^+$ *ade6-M216 his7-366 leu1-32 ura4-D18 fbp1::ura4*$^+$ *git1-1 cgs2-s1*
CHP863	*h*$^-$ *ade6-M216 his3-D1 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *gpa2::his3*$^+$
CHP934	*h*$^-$ *his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3Δ::kan*
CHP939	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ git3Δ::kan*
FWP17	*mat2-102 lys1-131 ura4-294*
FWP72	*h*$^-$ *leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$
FWP101	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$
FWP110	*h*$^+$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git1-1*
FWP111	*h*$^+$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git1-1*
FWP112	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$
FWP114	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-7*
FWP188	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-1::LEU2*$^+$
FWP190	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-1::LEU2*$^+$
KGP5	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-7 cgs2-s1*
KGP6	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-216 cgs2-s4*
KGP7	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-1::LEU2*$^+$ *cgs2-s1*
KGP8	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-1::LEU2*$^+$ *cgs2-s4*
KGP9	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2*$^+$ *cgs2-s4*
KGP10	*h*$^+$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2*$^+$ *cgs2-s1*
KGP11	*h*$^+$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2-7 gpa2-249*
LWP20	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3-14 c1676-890::LEU2*$^+$
LWP28	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3-14 cgs2-s1 c1676-890::LEU2*$^+$
LWP30	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3-14 cdc11-123*
LWP31	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3-14 cgs2-s1 snf1Δ::kan*
LWP38	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3-14 cgs2-s1 c1676-804::LEU2*$^+$
LWP39	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3-14 cgs2-s1 c1676-751::LEU2*$^+$
LWP40	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3-14 cgs2*$^+$ *int::LEU2*$^+$
LWP41	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3-14 cgs2-s1 cgs2*$^+$ *int::LEU2*$^+$
LWP96	*h*$^+$ *ade6-M216 his3-D1 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *cgs2-2*
LWP98	*h*$^+$ *ade6-M216 his3-D1 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *cgs2-2 gpa2::his3*$^+$
LWP99	*h*$^+$ *ade6-M216 his3-D1 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$
LWP110	*h*$^-$ *leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2*$^+$-TAP::*kan*
LWP127	*mat2-102 ade6-M210 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git2*$^+$-13myc::*kan*
LWP156	*mat2-102 his3-D1 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3Δ::kan*
LWP159	*h*$^-$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *cgs2-2 git3Δ::kan*
LWP161	*h*$^+$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ cgs2-2 git3Δ::kan*
LWP167	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ cgs2-s1 git3Δ::kan*
LWP181	*h*$^+$ *ade6-M216 leu1-32 ura4::fbp1-lacZ cgs2*$^+$ *int::LEU2*$^+$ *gpa2::ura4*$^+$
LWP191	*h*$^+$ *ade6-M216 leu1-32 ura4::fbp1-lacZ cgs2-s1 gpa2::ura4*$^+$
LWP238	*h*$^-$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ cgs2-s4*
RWP1	*h*$^-$ *ade6-M216 leu1-32 ura4::fbp1-lacZ gpa2*^{R176H}
RWP9	*h*$^-$ *leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^+$ *git3Δ::kan*
SP578	*h*^{90} *ade6-M216 leu1-32 cgs2-2*

Gap repair cloning of the *cgs2* allele from wild-type and mutant strains: The *cgs2* alleles from various strains were cloned by gap repair to avoid PCR-based amplification artifacts. Two sets of PCR primers were used to amplify regions flanking the *cgs2* gene and were joined into a single product in a two-step PCR reaction (Pearson *et al.* 1998). Primers cgs2gap1 (5′-TTATTTGGAAATGGAGAGTCACG-3′) and cgs2gap2 (5′-ACGGGATCCAGGAGCCTTTGCTGCAGCGTG-3′) amplify a 330-bp fragment ~800 bp downstream of the *cgs2* STOP codon, while primers cgs2gap3 (5′-CCTGGATCCCGTTAGCAGG CATGGAGTGCA-3′) and cgs2gap4 (5′-AAACATCAAGAAAT GGAGACTCG-3′) amplify a 260-bp fragment ~1.7 kb upstream

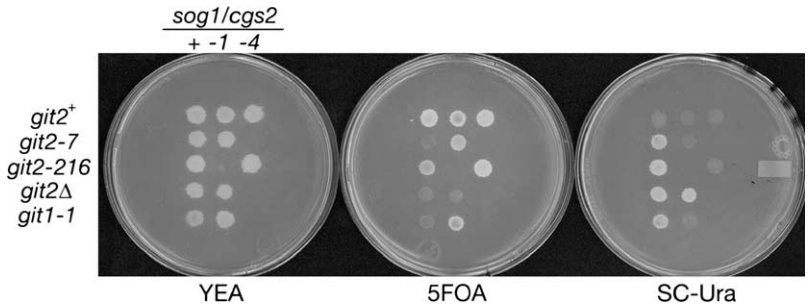


FIGURE 1.—*sog1-1* and *sog1-4* mutations suppress spontaneous mutations, but not deletions, of the *git2*$^+$/*cyr1*$^+$ adenylate cyclase gene. Strains were spotted onto YEA-rich medium and grown overnight before replica plating to YEA, 5-FOA, and SC-Ura media. Plates were photographed after 2 days. Glucose repression of the *fbp1-ura4*$^+$ reporter confers 5-FOA^R growth, while a defect in glucose repression allows strains to grow on SC-Ura as previously shown (HOFFMAN and WINSTON 1990). Note that the *git2-216* allele allows some growth on 5-FOA medium, although this amount of growth did not preclude our ability to carry out a screen for suppressor mutations that allows single-colony formation on 5-FOA. Suppression of the *git1-1* mutation by *sog1-1* is also shown.

of the *cgs2* START codon. Primers cgs2gap2 and cgs2gap3 contain complementary sequences (underlined in the sequences shown above) that include a unique *Bam*HI site, allowing the two PCR products to be combined into a single product by a second PCR reaction. This second-round PCR product was cloned into the pNMT41 TOPO cloning vector to create a plasmid that can be linearized with *Bam*HI to facilitate gap repair cloning of *cgs2* alleles from the yeast chromosome. The *cgs2* allele was cloned from strains FWP112 (*cgs2*$^+$), KGP10 (*sog1-1/cgs2-s1*), KGP9 (*sog1-4/cgs2-s4*), and SP578 (*cgs2-2*) and the plasmids were rescued into *E. coli* by the "smash and grab" method (HOFFMAN and WINSTON 1987). The sequence of each *cgs2* allele was determined on both strands using custom oligonucleotides together with the CEQ DTCS-Quick Start kit (Beckman Coulter) and the CEQ 8000 genetic analysis system.

β-Galactosidase assays: Strains were cultured overnight under repressing conditions (8% glucose) in YEL medium before subculturing into YEL medium under repressing or derepressing conditions (0.1% glucose plus 3% glycerol). Cultures were grown for 24 hr to a final cell density of ~1 × 10^7 cells/ml. Protein lysates were prepared on ice and assayed for β-galactosidase activity as previously described (NOCERO *et al.* 1994).

cAMP assays: Intracellular cAMP levels were measured by radioimmune assay in glucose-starved cells (basal level) and in the same cultures 1 and 10 min after exposure to 100 mM glucose as previously described (BYRNE and HOFFMAN 1993).

RESULTS

Isolation of allele-specific adenylate cyclase suppressor mutations: In an effort to identify direct activators of *S. pombe* adenylate cyclase, the following suppressor screen was carried out. Strains CHP7 (*git2-7*) and CHP216 (*git2-216*) carry mutations in the adenylate cyclase gene that display intragenic complementation with the *git2-61* mutation, which confers a partial defect in catalytic activity (HOFFMAN and WINSTON 1991). As such, the *git2-7* and *git2-216* mutations may affect the binding of an activator. These mutations confer a 5-FOA^S growth defect (Figure 1; the 5-FOA^S phenotype conferred by the *git2-216* mutation is leaky, although sufficient to allow selection of suppressor mutations) due to derepression of transcription of an *fbp1-ura4*$^+$ reporter gene, which normally is glucose repressed. Sixty independently isolated 5-FOA^R derivatives from each of strains CHP7 and CHP216 were isolated and put through genetic tests to identify candidate gain-of-function mutations in an adenylate cyclase activator gene (see MATERIALS AND METHODS). One suppressor mutation from each screen displayed the desired genetic characteristics of being unlinked to the original *git2* mutation, being dominant to wild type for suppression of the *git2* point mutations, and being unable to suppress a *git2* deletion (*git2*Δ; Figure 1). These mutations, designated *sog* for *s*uppressor *o*f *g*it, are single nuclear mutations, segregating 2:2 in 18 tetrads each. Finally, these mutations can suppress either the *git2-7* or the *git2-216* mutation and are tightly linked to each other, suggesting that they are allelic, as a cross between strains KGP5 (*git2-7 sog1-1*) and KGP6 (*git2-216 sog1-4*) yielded only 5-FOA^R progeny in 38 tetrads.

Genetic interactions between *sog1-1* and "upstream" *git* genes: To determine if *sog1-1* is a gain-of-function allele of any of the known *git* genes that are required for adenylate cyclase activation, we crossed strain KGP10 (*git2*$^+$ *sog1-1*) with strains carrying a mutation in *git1*$^+$, *git3*$^+$, *git5*$^+$, *git7*$^+$, *gpa2*$^+$, *git10*$^+$, or *git11*$^+$. With the exception of the cross with a *gpa2*$^-$ mutant strain, most tetrads from each cross contained three 5-FOA^R to one 5-FOA^S progeny, indicating that the *sog1-1* allele is not linked to the *git* mutation in the other parental strain and that the *sog1-1* allele suppresses the *git* mutant allele in the double-mutant progeny (see Figure 1 for suppression of *git1-1* by *sog1-1*). On the other hand, every tetrad from the *gpa2*$^-$ mutant cross contained two 5-FOA^R progeny and two 5-FOA^S progeny, indicating that *sog1-1* either is tightly linked to the *gpa2* locus or fails to suppress a *gpa2*$^-$ mutation. To distinguish between these two possibilities, progeny from a cross between KGP11 (*git2-7 gpa2-249*) and KGP5 (*git2-7 sog1-1*) were analyzed. Tetrads with fewer than two 5-FOA^R progeny were readily obtained (data not shown), indicating that *git2-7 gpa2-249 sog1-1* 5-FOA^S progeny were being generated. Thus, *sog1-1* fails to suppress a mutation in the *gpa2*$^+$ gene that encodes the Gα of the heterotrimeric G protein of the glucose/cAMP pathway (NOCERO *et al.* 1994), but does suppress mutations in the other *git* genes required for adenylate cyclase activation. In addition, strains carrying

TABLE 2

Multicopy suppressors identified from *sog1-1* library

Gene cloned	No. of clones	
	Suppressor of *git1-1*	Suppressor of *git2-7*
pka1$^+$	9	1
esc1$^+$	0	4
pyp1$^+$	0	1
mcs4$^+$	0	3
sck2$^+$	0	2
spc1$^+$/*sty1*$^+$	0	1

the *sog1-1* allele together with the activated *gpa2*^{R176H} allele display a synthetic defect in *fbp1-lacZ* derepression (see below), suggesting that Sog1 and Gpa2 may act in concert to activate adenylate cyclase.

Efforts to clone *sog1-1* from a genomic library identify only multicopy suppressors: As the *sog1-1* mutation is dominant (*i.e.*, a *git2-7*/*git2*Δ *sog1-1*/*sog1*$^+$ diploid is 5-FOA^R), we constructed a genomic library from strain KGP5 (*git2-7 sog1-1*) and screened it for plasmids able to confer 5-FOA^R growth to either CHP7 (*git2-7*) or CHP110 (*git1-1*) transformants (see MATERIALS AND METHODS). Surprisingly, all nine candidate plasmids that suppress the *git1-1* mutation carry the *pka1*$^+$ gene, which encodes the catalytic subunit of the cAMP-dependent protein kinase (Table 2). The plasmids obtained from the *git2-7* transformants carried a more varied collection of genes, including *pka1*$^+$, *sck2*$^+$, *pyp1*$^+$, *mcs4*$^+$, *spc1*$^+$, and *esc1*$^+$. However, it seemed unlikely that these clones carried *sog1-1*, as they also confer 5-FOA^R growth to strains carrying deletions of either *git2*$^+$ or *pka1*$^+$ (see Figure 2 for suppression by *esc1*$^+$). In addition, homologous integration of the plasmids, followed by a linkage analysis, demonstrated that none of these genes are linked to *sog1*$^+$; therefore these genes represent multicopy suppressors.

***sog1-1* maps to the *cgs2*$^+$ locus:** Due to the inability to clone *sog1-1* by a library screen, we adopted a genetic mapping strategy to identify the *sog1*$^+$ gene. We first mapped *sog1-1* to chromosome 3 using a diploid haploidization strategy (see MATERIALS AND METHODS), which takes advantage of the *mat2-102* mating-type allele to construct stable diploids by mating a *mat2-102* strain with an *h*$^-$ strain. Benomyl treatment of diploid cells formed by mating strains FWP17 and KGP5, which carry markers to identify all three chromosomes from each parental strain, resulted in haploid derivatives without undergoing meiotic recombination so that markers on each chromosome remain genetically linked. Of the haploid strains that carried chromosome 2 from the KGP5 parent (with the *git2-7* and *fbp1-ura4*$^+$ alleles), only the strains carrying chromosome 3 from the *sog1-1* parental strain were 5-FOA^R, due to the suppression of the *git2-7* mutation by *sog1-1* (data not shown). Thus, the *sog1*$^+$ gene is present on chromosome 3.

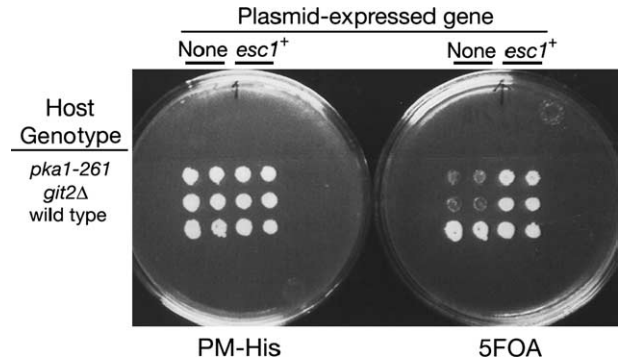
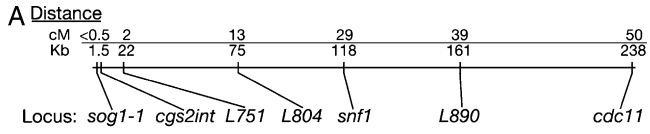


FIGURE 2.—Multicopy suppression of *git2*$^-$ and *pka1*$^-$ mutations by overexpression of *esc1*$^+$. His$^+$ transformants carrying either the pEA500 empty vector (APOLINARIO *et al.* 1993) or pYZ3 (*esc1*$^+$) were pregrown on PM-his medium before replica plating to PM-his and to 5-FOA media. Plates were photographed after 3 days.

A series of tetrad dissections involving strains with various chromosome 3 markers led us to the *cgs2*$^+$ cAMP phosphodiesterase gene (Figure 3; additional crosses involving markers that are unlinked to the *sog1-1* mutant allele are not shown). These mapping experiments involved the use of available chromosome 3 markers, along with engineered markers created by homologous integration of a *LEU2*-marked plasmid at various sites along the chromosome (see MATERIALS AND METHODS). One such marker, integrated to within 1.5 kb of the *cgs2*$^+$ translational start site, maps to within 0.45 cM of *sog1-1*. Thus, it appears that *sog1-1* is either an allele of *cgs2*$^+$ or a gene adjacent to *cgs2*$^+$.

***sog1-1* and *sog1-4* are alleles of the *cgs2*$^+$ cAMP phosphodiesterase gene:** To determine whether *sog1-1* and *sog1-4* are alleles of *cgs2*, we cloned and sequenced *cgs2* from *sog1-1* and *sog1-4* strains, as well as from *cgs2*$^+$



B

Locus	PD	TT	NPD	Map distance (cM)	Kb/cM
cdc11	25	15	5	50	4.8
L890	53	50	6	39	4.1
snf1	33	32	1	29	4.1
L804	36	12	0	13	5.8
L751	115	5	0	2	11.0
cgs2int	111	0	0	<0.5	>3.0

FIGURE 3.—Genetic mapping of *sog1-1* on chromosome 3. (A) Schematic of a portion of chromosome 3 displaying the relative locations of the *sog1-1* allele, the *snf1*$^+$/*ssp2*$^+$ gene (systematic name SPCC74.03c), the *cdc11*$^+$ gene, and four *LEU2*$^+$-marked plasmid integrations (see MATERIALS AND METHODS). The *cgs2int* integration site is within 1.5 kb of the translational start of the *cgs2*$^+$ gene, while the *L751*, *L804*, and *L890* integration sites are at approximately positions 751,000, 804,000, and 890,000, respectively, of contig c1676. Distances from *sog1-1* are given both in kilobase pairs and in centimorgans (as determined from our genetic mapping experiments). (B) Linkage data used to determine genetic map distances from *sog1-1*.

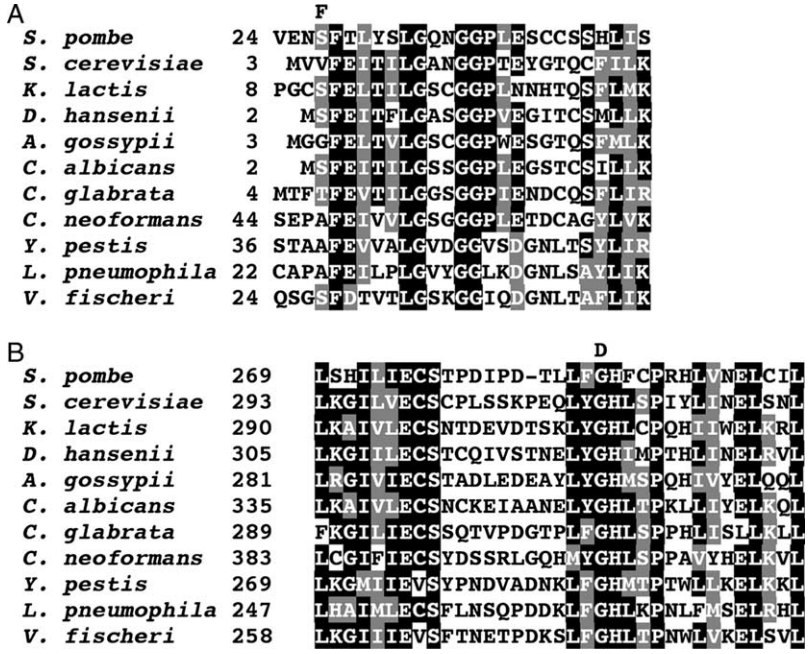


FIGURE 4.—*cgs2-s1* and *cgs2-s4* mutations alter residues in highly conserved domains within the Cgs2 protein. (A) Alignment of N-terminal domains that include the residue affected by the *cgs2-s1* mutation from phosphodiesterases from *S. pombe* (NP_588337), *S. cerevisiae* (NP_011266), *K. lactis* (XP_453575), *D. hansenii* (XP_460986), *C. albicans* (P32782), *C. glabrata* (XP_447131), *C. neoformans* (XP_568076), *A. gossypii* (NP_985806), *Y. pestis* (NP_406214), *L. pneumophila* (YP_095961), and *V. fischeri* (Q56686). The number preceding the protein sequence indicates the position of the residue that aligns with serine 24 of Cgs2, which is changed to phenylalanine (this position is shown above the alignment) in the Cgs2-s1 protein. (B) Alignment of the conserved domain containing glycine 269 of Cgs2 with the same phosphodiesterases as in A. The number preceding the protein sequence indicates the position of the residue that aligns with glycine 269 of Cgs2, which is changed to aspartic acid (this position is shown above the alignment) in the Cgs2-s4 protein. Note that the *cgs2-2* mutation is a frameshift in codon 270.

and *cgs2-2* strains. These alleles were cloned by gap repair to avoid PCR amplification errors (see MATERIALS AND METHODS). DNA sequence analyses of the cloned alleles showed that the *cgs2-2* mutation is a +1 frameshift in codon 270 (a duplication of the T at nucleotide 1366 in the genomic sequence; the *cgs2*$^+$ gene contains three introns with a total length of 559 nucleotides), leading to a truncated product of 273 residues. The *cgs2* allele from a *sog1-1* strain contains a single-point mutation (C to T) at nucleotide 71, changing residue 24 from serine to phenylalanine (Figure 4A). The *cgs2* allele from a *sog1-4* strain has a single-point mutation (G to A) at nucleotide 1365, changing residue 269 from glycine to aspartic acid (Figure 4B). These sequence changes were confirmed by PCR amplification and direct sequencing of the PCR products from strains KGP5 (*git2-7 sog1-1*) and KGP6 (*git2-216 sog1-4*). As shown in Figure 4, the residues affected by these mutations are found within two highly conserved sequences present in phosphodiesterases from a wide variety of organisms, including other yeasts and fungi (*S. cerevisiae*, *Kluyveromyces lactis*, *Debaryomyces hansenii*), human and plant fungal pathogens (*Candida albicans*, *C. glabrata*, *Cryptococcus neoformans*, *Ashbya gossypii*), and bacterial pathogens (*Yersinia pestis*, *Legionella pneumophila*, *Vibrio fischeri*). In a complete alignment of these 11 enzymes, only 22 residues are perfectly conserved (data not shown). Thus the presence of 12 of these conserved residues in these two small regions of the proteins suggests that we have identified two conserved domains that are important for phosphodiesterase function and/or regulation and that the *sog1-1* and *sog1-4* mutations reduce phosphodiesterase activity to suppress defects in the glucose/cAMP pathway. Thus *sog1-1* and *sog1-4* are two mutant alleles of *cgs2* and have been renamed *cgs2-s1* and *cgs2-s4*.

Comparison of *cgs2-s1* and *cgs2-2* mutations: The demonstration that the *sog1-1* and *sog1-4* suppressor mutations are alleles of the *cgs2*$^+$ cAMP phosphodiesterase gene was unexpected for two reasons. We expected to obtain *cgs2* loss-of-function alleles among our suppressors of the adenylate cyclase defect, as the loss of phosphodiesterase activity would elevate cAMP levels; however, we assumed that such alleles would be recessive to wild type. In addition, we assumed that such *cgs2*$^-$ mutations would be able to suppress *gpa2*$^-$ Gα mutations, as *gpa2*$^-$ mutants display a measurable basal cAMP level (ISSHIKI *et al.* 1992; BYRNE and HOFFMAN 1993). We therefore revisited these observations by comparing *cgs2-s1* with the previously identified *cgs2-2* allele both for dominance and for suppression of *gpa2*$^-$ mutations. Both *cgs2-s1* and *cgs2-2* behave as dominant suppressors of a *git3* deletion (WELTON and HOFFMAN 2000) as judged by the 5-FOA^R growth of diploid strains that are homozygous *git3*Δ and heterozygous at the *cgs2* locus (Figure 5). In fact, the *cgs2-2/cgs2*$^+$ diploid appears to be more resistant to 5-FOA than the *cgs2-s1/cgs2*$^+$ diploid. A quantitative assessment of suppression by measuring β-galactosidase activity expressed from the *fbp1-lacZ* reporter confirms this observation (Table 3). In haploid *git3*Δ strains, the *cgs2-2* allele completely restores glucose-repressed levels under repressing conditions and confers a significant defect in derepression under glucose-starved conditions. The *cgs2-s1* allele significantly reduces *fbp1-lacZ* expression under repressing conditions, but has only a modest effect on derepressed levels of expression. Diploid *cgs2-2/cgs2*$^+$ cells express the *fbp1-lacZ* reporter at the same levels as the *cgs2-2* haploid strain does; thus the *cgs2-2* allele is dominant to the wild-type allele with respect to the regulation of *fbp1-lacZ* and *fbp1-ura4* transcription. Similar to its ability to confer 5-FOA^R growth

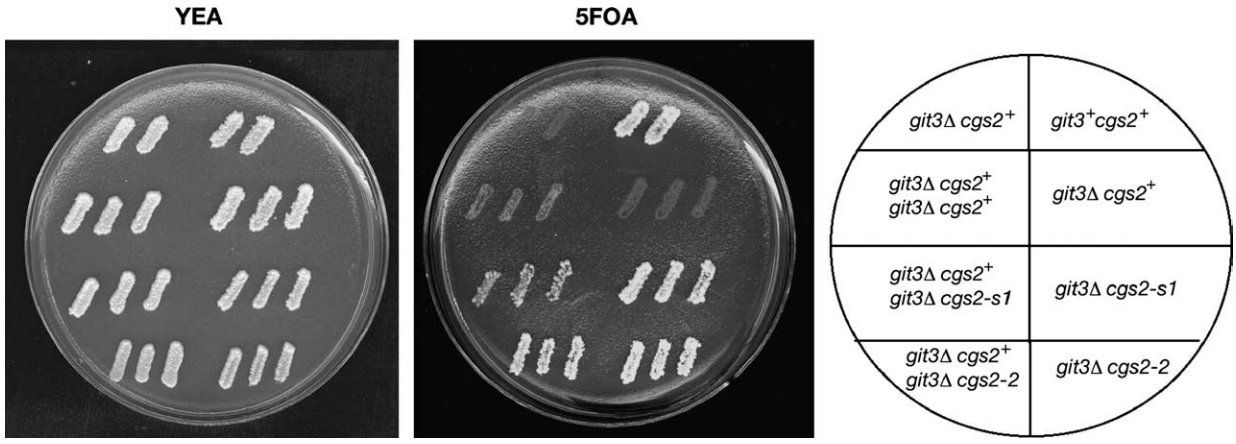


FIGURE 5.—*cgs2-s1* and *cgs2-2* mutations are dominant suppressors of a deletion of the *git3*$^+$ GPCR gene. Haploid and diploid strains were pregrown on YEA medium overnight at 30° before replica plating to YEA and 5-FOA media. Plates were photographed after 3 days.

(Figure 5), the *cgs2-s1* allele restores repression of *fbp1-lacZ* expression even in the presence of the wild-type *cgs2*$^+$ allele, although not to the same extent as the *cgs2-2* allele (Table 3).

We next examined the ability of *cgs2-2* and *cgs2-s1* alleles to suppress mutations in the *gpa2*$^+$ gene that encodes a Gα subunit, which we have shown to directly bind to an N-terminal domain of adenylate cyclase (IVEY and HOFFMAN 2005). Using two different marked disruptions of *gpa2*$^+$ that result in similar levels of *fbp1-lacZ* expression in glucose-grown cells (Table 4), we show that *cgs2-2* completely restores glucose-repressed levels of expression while *cgs2-s1* has only a modest effect on expression (Table 4). These results are similar to observations using spontaneous *gpa2*$^-$ mutant alleles with regard to the 5-FOA growth phenotype, which reflects transcription of the *fbp1-ura4* reporter. We observe that the *cgs2-2* allele restores 5-FOA^R growth while the *cgs2-s1* allele does not (data not shown).

To investigate the roles of Cgs2 and Gpa2 in the glucose/cAMP pathway further, we examined *fbp1-lacZ* expression in double-mutant strains carrying either *cgs2-2* or *cgs2-s1* in combination with *gpa2*^{R176H}, an allele activated due to a loss of GTPase activity. We had previously shown that *gpa2*^{R176H} suppresses the loss of the Git3 GPCR, the Git5 Gβ subunit, or the Git11 Gγ subunit, indicating that the role of the Gβγ dimer is to couple the Gpa2 Gα to the Git3 GPCR, so that, upon glucose detection, Git3 could promote the activation of Gpa2 (WELTON and HOFFMAN 2000). We had also noted that the *gpa2*^{R176H} allele confers only a partial defect in *fbp1-lacZ* derepression. The *cgs2-s1* or *gpa2*^{R176H} single-mutant strains display a moderate reduction in *fbp1-lacZ* derepression, while the *cgs2-s1 gpa2*^{R176H} double mutant displays an almost total loss of derepression (Table 5). On the other hand, the *cgs2-2* allele is sufficient to confer a severe defect in *fbp1-lacZ* derepression. The synthetic interaction between the *cgs2-s1* or *gpa2*^{R176H} alleles supports a model in which cAMP phosphodiesterase activity serves as a counterbalance to Gpa2-mediated adenylate cyclase activation.

***cgs2-s1* and *cgs2-s4* strains display distinct cAMP responses to glucose:** To examine the effect of the *cgs2-s1* and *cgs2-s4* mutations on cAMP signaling, we assayed the glucose-triggered cAMP response in wild-type cells

TABLE 3

Suppression of *git3*Δ by *cgs2-s1* and *cgs2-2* in haploid and diploid strains

		β-Galactosidase activity	
Strain	Genotype	Repressed	Derepressed
FWP72	*git3*$^+$ *cgs2*$^+$	3 ± 0	1369 ± 142
RWP9	*git3*Δ *cgs2*$^+$	925 ± 10	1282 ± 10
LWP167	*git3*Δ *cgs2-s1*	27 ± 10	527 ± 56
LWP161	*git3*Δ *cgs2-2*	4 ± 0	52 ± 20
LWP127 × LWP110	*git3*$^+$/*git3*$^+$ *cgs2*$^+$/*cgs2*$^+$	6 ± 1	1754 ± 32
LWP156 × CHP934	*git3*Δ/*git3*Δ *cgs2*$^+$/*cgs2*$^+$	513 ± 130	1573 ± 32
LWP156 × CHP769	*git3*Δ/*git3*Δ *cgs2*$^+$/*cgs2-s1*	68 ± 15	970 ± 234
LWP156 × LWP159	*git3*Δ/*git3*Δ *cgs2*$^+$/*cgs2-2*	4 ± 1	43 ± 22

β-Galactosidase activity was determined from three independent cultures as described in MATERIALS AND METHODS. The average ±SE represents specific activity per milligram of soluble protein.

TABLE 4

Differential suppression of a *gpa2* deletion by *cgs2-s1* and *cgs2-2*

Strain	Genotype	β-Galactosidase repressed
LWP99	*gpa2*$^+$ *cgs2*$^+$	7 ± 1
CHP863	*gpa2::his3*$^+$ *cgs2*$^+$	1351 ± 48
LWP181	*gpa2::ura4*$^+$ *cgs2*$^+$	1221 ± 55
CHP733	*gpa2*$^+$ *cgs2-s1*	4 ± 0
LWP96	*gpa2*$^+$ *cgs2-2*	7 ± 1
LWP191	*gpa2::ura4*$^+$ *cgs2-s1*	482 ± 71
LWP98	*gpa2::his3*$^+$ *cgs2-2*	10 ± 1

β-Galactosidase activity was determined from three independent cultures as described in MATERIALS AND METHODS. The average ±SE represents specific activity per milligram of soluble protein. The *gpa2::ura4*$^+$ disruption allele was previously described (ISSHIKI *et al.* 1992), while the *gpa2::his3*$^+$ allele was created by the nonhomologous integration of plasmid pAF1 into codon 329 of the 354-codon open reading frame (HOFFMAN and WELTON 2000).

and in various mutant strains (Figure 6). Similar to our previous observations (BYRNE and HOFFMAN 1993), wild-type *S. pombe* cells respond to glucose exposure with a four- to sixfold increase in cAMP levels, while *cgs2-2* cells display an elevated basal cAMP level and a similar-fold increase in cAMP levels after glucose addition (Figure 6). The *cgs2-s4* cells display an intermediate result with cAMP levels that are elevated relative to wild-type cells, but not to the same degree as *cgs2-2* cells are, at each time point. Overall, the *cgs2-s4* response resembles that of the *cgs2-2* cells in that cAMP levels accumulate at a somewhat linear rate over the course of the 10-min assay (this includes data from a 5-min time point; data not shown). Remarkably, the *cgs2-s1* cells possess a basal cAMP level similar to that of wild-type cells; however, within 1 min of glucose exposure, the cAMP levels reach that observed in the glucose-stimulated *cgs2-2* cells. More importantly, whereas the rate of cAMP accumulation in wild-type cells decreases significantly after the 1-min time point, the rate of cAMP accumulation in

TABLE 5

Synthetic defect in *fbp1-lacZ* derepression by *cgs2-s1* and *gpa2*^{R176H}

Strain	Genotype	β-Galactosidase activity	
		Repressed	Derepressed
FWP72	*gpa2*$^+$ *cgs2*$^+$	3 ± 0	1369 ± 142
CHP733	*gpa2*$^+$ *cgs2-s1*	4 ± 0	263 ± 12
RWP1	*gpa2*^{R176H} *cgs2*$^+$	2 ± 0	483 ± 5
CHP732	*gpa2*^{R176H} *cgs2-s1*	3 ± 0	12 ± 3
CHP384	*gpa2*$^+$ *cgs2-2*	3 ± 0	24 ± 2

Cells were grown in PM complete medium and β-galactosidase activity was determined from three independent cultures as described in MATERIALS AND METHODS. The average ±SE represents specific activity per milligram of soluble protein.

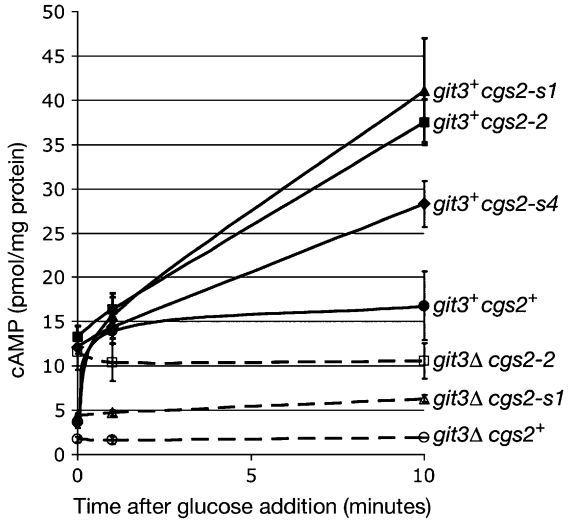


FIGURE 6.—cAMP response curves in wild-type and mutant strains. Cells were cultured for 24 hr in medium containing 0.1% glucose and 3% glycerol. cAMP levels were measured as previously described (BYRNE and HOFFMAN 1993) immediately prior to glucose addition to the cultures, as well as 1 min and 10 min after glucose addition. Strains carried either the wild-type *git3*$^+$ gene (solid lines) or a *git3*Δ deletion allele (dashed lines), as well as the wild-type *cgs2*$^+$ gene (circles), the *cgs2-s1* allele (triangles), the *cgs2-s4* allele (diamonds), or the *cgs2-2* allele (squares).

cgs2-s1 cells is only moderately reduced, leading to a total accumulation at the 10-min time point exceeding that of the *cgs2-2* cells. However, the *cgs2-s1* response profile does resemble that of the *cgs2*$^+$ cells in that it is biphasic with a rapid increase in cAMP levels in the first minute, followed by a reduced rate of cAMP accumulation during the remaining 9 min. The significance of the linear *vs.* the biphasic response curves is discussed below.

We carried out parallel cAMP assays on *git3*Δ strains to better understand how the *cgs2-s1* mutation suppresses the loss of the Git3 receptor protein. The *git3*Δ *cgs2*$^+$ cells display both a reduced basal cAMP level and a loss of the glucose-triggered cAMP response (Figure 6). The *git3*Δ *cgs2-s1* cells possess a basal cAMP level similar to that of wild-type cells, but show little or no response to glucose. The *git3*Δ *cgs2-2* cells possess significantly elevated basal cAMP levels that are largely unaffected by glucose addition. These assays demonstrate that the *cgs2-s1* mutation does not suppress the *git3*Δ allele by restoring adenylate cyclase activation, but does increase basal cAMP levels enough to restore glucose repression of *fbp1*$^+$ transcription.

DISCUSSION

The original goal of this study was to identify a direct activator of *S. pombe* Git2/Cyr1 adenylate cyclase enzyme by isolating allele-specific suppressors of *git2-7* and *git2-216* alleles that produce activation-defective enzymes. We assumed that these suppressor mutations would

restore an interaction between adenylate cyclase and the activator protein and would thus be dominant. We unexpectedly found that loss-of-function alleles of the *cgs2*$^+$ cAMP phosphodiesterase gene are dominant for suppression of the *git2* mutant alleles (Figure 5, Table 3). This dominant-negative phenotype could be due to either haplo-insufficiency or a protein-poisoning effect of the defective protein by forming inactive multimeric complexes with wild-type subunits.

As *cgs2-s1* and *cgs2-s4* are dominant suppressor mutations, we tried to clone *cgs2-s1* from a genomic library containing insert DNA from KGP5 (*git2-7 cgs2-s1*) cells. While failing to identify *cgs2-s1*, our screen identified several interesting multicopy suppressors (Table 2). We repeatedly cloned *pka1*$^+$, recovering seven distinct clones whose inserts ranged from 3.4 to 8.4 kb, demonstrating the high quality of this *his7*$^+$-marked library. The remaining multicopy suppressors encode proteins that fall into three groups. The Sck2 kinase (Fujita and Yamamoto 1998) is 42% identical to *S. pombe* Sck1, which we previously identified as a multicopy suppressor of PKA pathway mutations (Jin *et al.* 1995). Mcs4, Pyp1, and Spc1 are components of a stress-activated MAP kinase (SAPK) pathway, required for *fbp1*$^+$ transcription. The Pyp1 protein tyrosine phosphatase dephosphorylates and inactivates the Spc1 SAPK (Millar *et al.* 1995; Shiozaki and Russell 1995). We previously showed that *pyp1*$^+$ and the closely related *pyp2*$^+$ gene are multicopy suppressors of PKA pathway mutants (Dal Santo *et al.* 1996). It is less obvious why Spc1 or the Mcs4 response regulator were identified in this screen. Spc1 overexpression may create a pool of inactive protein that inhibits activation of a target protein required for *fbp1*$^+$ transcription such as the Atf1 bZIP transcriptional activator (Shiozaki and Russell 1996; Gaits *et al.* 1998). Similarly, Mcs4 overexpression may produce partially assembled signaling complexes that reduce Spc1 activation. This is not the first instance in which positive and negative regulators of a *S. pombe* MAP kinase (MAPK) pathway have been found to confer the same multicopy suppressor phenotype. The Pek1 MAPK kinase and the Pmp1 MAPK phosphatase, which antagonistically regulate the Pmk1 cell wall integrity MAPK, are multicopy suppressors of the chloride-sensitive growth conferred by disruption of the *ppb1*$^+$ calcineurin gene (Sugiura *et al.* 1998, 1999). As signaling from both MAPK pathways is reduced by overexpression of positive regulators, the proper stoichiometry of pathway components appears to be important for signaling. Finally, the Esc1 helix-turn helix protein was previously identified as a multicopy suppressor of a temperature-sensitive lethal mutation in *pat1*$^+$ (*pat1-114*), which encodes a protein kinase that regulates meiotic entry (Benton *et al.* 1993). Interestingly, increased PKA activity due to loss of the Cgs1 PKA regulatory subunit or the Cgs2 PDE also suppresses *pat1-114* (DeVoti *et al.* 1991), as does loss of SAPK activation (Stettler *et al.* 1996). Therefore, overexpression of Esc1 may mimic either a "high-PKA-activity" or a "low-SAPK-activity" phenotype.

Prior to this study, we assumed that the cAMP response to glucose was a function of adenylate cyclase regulation while Cgs2 PDE activity remained constant. This was due to the observation that *cgs2-2* cells display a similar-fold increase in cAMP levels as seen in wild-type cells, although the increase in absolute cAMP levels is significantly higher in *cgs2-2* cells (Byrne and Hoffman 1993). However, the cAMP response data in Figure 6 challenge this model. The rate of cAMP accumulation in both *cgs2-2* and *cgs2-s4* cells is relatively constant over the course of the 10-min assay (this is further supported by 5-min time points taken for the *cgs2-s4* cells; data not shown). In contrast, the rate of cAMP accumulation drops off significantly after the first minute in both *cgs2*$^+$ and *cgs2-s1* cells. These results are consistent with our previous assays of *cgs2*$^+$ and *cgs2-2* strains, which were carried out over a 2-hr time course. In these assays, cAMP accumulation in *cgs2-2* cells is constant for the first 20 min after glucose addition, while the rate of cAMP accumulation in *cgs2*$^+$ cells falls 10-fold after the first minute (Byrne and Hoffman 1993). The *cgs2-2* mutation is a frameshift that removes the carboxy-terminal 20% of Cgs2, most likely inactivating the enzyme. The resulting linear response to glucose seen in Figure 6, as compared with the biphasic response seen for the wild-type cells, suggests that in wild-type cells, PDE becomes activated shortly after adenylate cyclase. The linear response and intermediate cAMP values observed in *cgs2-s4* cells is consistent with a severe reduction, but not a total loss, of PDE activity. Meanwhile, the basal cAMP level in *cgs2-s1* cells is similar to that of wild-type cells, while the glucose-stimulated level resembles that of *cgs2-2* cells. The serine-to-phenylalanine substitution at residue 24 in the Cgs2-s1 protein occurs in one of the most highly conserved regions of this family of phosphodiesterases (Figure 4A). This domain may be required for PDE activation by binding cyclic nucleotides to allosterically regulate PDE activity or through a post-translational modification, similar to the *S. cerevisiae* Pde1 enzyme, which appears to be activated by PKA phosphorylation of serine 252 (Ma *et al.* 1999).

Alternatively, our original model, in which only adenylate cyclase regulation controls the cAMP response, could be correct if activation and feedback mechanisms independently regulate adenylate cyclase activity. In wild-type cells, glucose signaling through the Git3 GPCR would lead to Gpa2-mediated activation of adenylate cyclase (Welton and Hoffman 2000; Ivey and Hoffman 2005), while increased cAMP levels would trigger a feedback response to downregulate adenylate cyclase activity. In *cgs2-2* cells, high basal cAMP levels would trigger the feedback mechanism without preventing glucose-mediated activation, allowing adenylate cyclase to be activated by glucose, but to a lesser extent than in wild-type cells. This would produce a linear increase in

cAMP levels, as the feedback regulatory event had occurred prior to glucose detection. In this model, the Csg2-s1 PDE activity is high enough to control basal cAMP levels in glucose-starved cells, but insufficient to limit the cAMP response in glucose-stimulated cells. However, we do not favor this model since PKA activity negatively regulates the level of cAMP pathway components, as we previously showed for *cgs1*$^+$ and *pka1*$^+$ transcription (Stiefel *et al.* 2004). Both the Git3 GPCR and adenylate cyclase protein levels are significantly lower in a PKA-dependent manner in glucose-grown cells than in glucose-starved cells (D. Chandler-Militello, L. Wang and C. S. Hoffman, unpublished results). The cAMP assays are carried out after a 24-hr glucose starvation period to derepress the glucose detection apparatus. However, such derepression should not occur in *cgs2-s4* and *cgs2-2* cells due to their high basal cAMP levels. Therefore, the reduced rate of cAMP accumulation in these strains is most likely due to a lower concentration of adenylate cyclase at the time of glucose addition, rather than to a reduction in the degree of stimulation of the enzyme.

While characterizing genetic interactions between *cgs2-s1* and mutations in other glucose/cAMP pathway genes, we uncovered a paradox with regard to the relative importance of these genes in the pathway. *cgs2-s1* fails to suppress a *gpa2* deletion (Table 4), but is able to suppress *git1*, *git7*, and *git10* mutations, suggesting that the Gpa2 Gα subunit is more important in cAMP signaling than Git1, Git7, and Git10. However, we previously showed that the activated *gpa2*^{R176H} allele cannot suppress *git1*, *git7*, or *git10* mutations, suggesting that Git1, Git7, and Git10 are required for adenylate cyclase activation even when Gpa2 is mutationally activated (Welton and Hoffman 2000). In addition, *git1* mutations confer a higher level of *fbp1-lacZ* expression in glucose-grown cells than do *gpa2* mutations (Hoffman and Winston 1990, 1991; Nocero *et al.* 1994), suggesting that Git1 plays a more important role in cAMP signaling than Gpa2. These conflicting results may reflect differences in the roles of these genes in maintaining basal cAMP levels *vs.* activating adenylate cyclase or may point to an as-yet-unidentified interaction between Gpa2 and Cgs2 to regulate the production and turnover of cAMP. Such a regulatory interaction would be consistent with the observation that both *gpa2*^{R176H} and *cgs2-s1* strains retain some ability to regulate *fbp1-lacZ* expression, while the *gpa2*^{R176H} *cgs2-s1* double mutant is completely defective in derepression (Table 5). The Cgs2$^+$ PDE may be able to compensate for the presumed increased adenylate cyclase activation by the Gpa2^{R176H} subunit, while the Cgs2-s1 enzyme cannot. Further analysis of the function of the N-terminal domain of Cgs2 that is altered in the Cgs2-s1 protein may help to determine the role of this domain and whether there is a direct regulatory interaction between Cgs2 and Gpa2 to control cAMP signaling in *S. pombe*.

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