DOI: 10.1534/genetics.106.055699

Schizosaccharomyces pombe Git1 Is a C2-Domain Protein Required for Glucose Activation of Adenylate Cyclase

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Manuscript received January 12, 2006
Accepted for publication February 10, 2006

ABSTRACT

Schizosaccharomyces pombe senses environmental glucose through a cAMP-signaling pathway, activating cAMP-dependent protein kinase A (PKA). This requires nine *git* (*g*lucose *i*nsensitive *t*ranscription) genes that encode adenylate cyclase, the PKA catalytic subunit, and seven "upstream" proteins required for glucose-triggered adenylate cyclase activation, including three heterotrimeric G-protein subunits and its associated receptor. We describe here the cloning and characterization of the *git1*$^+$ gene. Git1 is distantly related to a small group of uncharacterized fungal proteins, including a second *S. pombe* protein that is not functionally redundant with Git1, as well as to members of the UNC-13/Munc13 protein family. Mutations in *git1*$^+$ demonstrate functional roles for the two most highly conserved regions of the protein, the C2 domain and the MHD2 Munc homology domain. Cells lacking Git1 are viable, but display phenotypes associated with cAMP-signaling defects, even in strains expressing a mutationally activated Gα-subunit, which activates adenylate cyclase. These cells possess reduced basal cAMP levels and fail to mount a cAMP response to glucose. In addition, Git1 and adenylate cyclase physically interact and partially colocalize in the cell. Thus, Git1 is a critical component of the *S. pombe* glucose/cAMP pathway.

NUTRIENT signaling is essential for unicellular organisms, which must regulate their growth and metabolism in response to the available nutrients in their environment. Glucose is the preferred carbon source for bacteria and fungi, such that glucose-signaling pathways exist to control the expression of genes involved in carbon source metabolism and sexual development for various organisms (Lengeler *et al.* 2000; Thevelein *et al.* 2000; Titgemeyer and Hillen 2002). Yeasts and other fungi also utilize glucose sensing to regulate cellular morphology and growth, which is required for invasive growth and, for some organisms, virulence. For example, glucose is a major component of the dialyzable fraction of serum that induces germ-tube formation, leading to invasive growth in the human pathogen *Candida albicans* (Hudson *et al.* 2004). Similarly, haploid invasive growth in the budding yeast *Saccharomyces cerevisiae* requires both the sensing of a nitrogen-poor environment and an abundant fermentable carbon source (Lengeler *et al.* 2000; Harashima and Heitman 2004). Recently, the fission yeast *Schizosaccharomyces pombe* has also been shown to switch from a unicellular yeast growth mode to a hyphal invasive growth mode in response to nitrogen-poor conditions, but only in strains with an intact cAMP-signaling pathway, which is required for glucose detection (Amoah-Buahin *et al.* 2005). Thus, there is considerable conservation among yeasts and fungi with regard to their biological response to environmental glucose.

S. cerevisiae cells sense glucose through at least three signaling pathways that control different aspects of the biological response to environmental glucose. Extracellular glucose is detected by Snf3 and Rgt2, 12-transmembrane-domain proteins that resemble hexose transporters, leading to the regulation of the Rgt1 transcriptional repressor, which regulates expression of hexose transporter genes (Johnston and Kim 2005). In addition, glucose uptake and metabolism under glucose-rich conditions inhibit the Snf1/AMP kinase, which leads to repression of target genes by the Mig1, Nrg1, and Nrg2 transcriptional repressors (Treitel *et al.* 1998; Kuchin *et al.* 2002). Finally, glucose detection also produces a transient cAMP signal. This involves the Gpr1 G-protein-coupled receptor (GPCR), a 7-transmembrane-domain protein that activates the Gpa2 Gα-subunit protein (Xue *et al.* 1998; Yun *et al.* 1998; Lorenz *et al.* 2000; Lemaire *et al.* 2004). Glucose detection also activates Ras proteins (Colombo *et al.* 2004), which together with Gpa2, stimulate cAMP production by adenylate cyclase (Thevelein and de Winde 1999; Thevelein *et al.* 2005).

Glucose detection in *S. pombe* is largely carried out by a cAMP-signaling pathway to regulate processes such as sexual development and gluconeogenesis (Hoffman 2005a,b). A stress-activated protein kinase (SAPK) pathway is also regulated by glucose starvation; however, in

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the absence of a functioning PKA pathway, the basal level of activity of the Spc1/Sty1 SAPK is sufficient to allow high levels of transcription of genes that are subject to glucose repression (Stettler *et al.* 1996). Conversely, in cells expressing elevated levels of PKA activity, starvation signaling through the SAPK pathway is unable to derepress transcription of glucose-repressed genes (DeVoti *et al.* 1991; Stettler *et al.* 1996; Janoo *et al.* 2001; Stiefel *et al.* 2004).

In *S. pombe*, glucose detection leads to a transient cAMP signal due to the activation of adenylate cyclase (Byrne and Hoffman 1993). The genes required for *S. pombe* adenylate cyclase activation compose at least two functional groups on the basis of genetic interactions involving a mutationally activated form of the $gpa2^{+}$ gene. On the basis of biochemical studies of similarly altered Gα-subunits in other systems, the $gpa2^{R176H}$ allele encodes a protein presumed to be defective in GTPase activity. This defect reduces GTP hydrolysis of the bound guanine nucleotide, causing the Gα-subunit to remain in the active, GTP-bound state longer. The $gpa2^{R176H}$ allele suppresses the loss of the Git3 GPCR, the Git5 Gβ, or the Git11 Gγ (Welton and Hoffman 2000; Landry and Hoffman 2001), consistent with a model in which the Gβγ dimer is required for efficient coupling of the Gα to the receptor to allow Gα activation. On the other hand, the $gpa2^{R176H}$ allele fails to suppress mutations in $git1^{+}$, $git7^{+}$, or $git10^{+}$, suggesting that these genes encode proteins that may activate adenylate cyclase in a G-protein-independent manner. Alternatively, these proteins may be required for the stabilization or localization of Gpa2 or adenylate cyclase, or are somehow otherwise required for Gpa2-mediated activation of adenylate cyclase.

In this study, we describe the cloning and characterization of the $git1^{+}$ gene. Surprisingly, while the other proteins of the *S. pombe* glucose/cAMP pathway have obvious homologs in other eukaryotes, Git1 is only distantly related to a conserved fungal protein family of unknown function as well as the UNC-13/Munc13 protein family involved in synaptic vesicle exocytosis. All of these proteins contain a similar C2 domain near their carboxy-terminus, along with two additional domains known as Munc13-homology domains 1 and 2 (Koch *et al.* 2000). C2 domains are often utilized by proteins to bind phospholipids in a calcium-dependent manner; however, this domain can also function in a calcium-independent manner and mediate interactions with other proteins (Rizo and Sudhof 1998).

We show that Git1 is not essential for *S. pombe* viability, but is required both for maintaining basal cAMP levels and for producing a glucose-triggered cAMP response. The Git1 C2 domain is required for function as well as for the regulation of Git1 protein levels, which are reduced in cells growing in a glucose-rich environment. Additional mutant alleles suggest that the C-terminal MHD2 domain is also necessary for function. Finally, we show that Git1 physically interacts with and colocalizes with the Git2/Cyr1 adenylate cyclase enzyme, although Git1 does not appear to be required for adenylate cyclase stability or its steady-state localization. Thus, we have identified in *S. pombe* a novel regulator of adenylate cyclase activation that plays a critical role in glucose/cAMP sensing.

MATERIALS AND METHODS

***S.pombe* strains and growth media:** *S. pombe* strains used in this study are listed in Table 1. The *fbp1::ura4*$^{+}$ and *ura4::fbp1-lacZ* reporter constructs have been described previously (Hoffman and Winston 1990). Rich, defined, and 5-FOA-containing growth media have been previously described (Hoffman and Winston 1990). The *git1::his3*$^{+}$ allele is an insertion of plasmid pAF1 into codon 608 of $git1^{+}$, while *git1::LEU2*$^{+}$ (*git1*Δ) is a deletion of the entire $git1^{+}$ ORF.

Recombinant DNA methods: *Escherichia coli* transformations were carried out using ElectroTen-Blue or XL1-Blue electroporation-competent cells (Stratagene, La Jolla, CA) or TOP 10 chemically competent cells (Invitrogen, San Diego). Yeast transformations were carried out as previously described (Bähler *et al.* 1998). The Failsafe PCR system (Epicentre Technologies, Madison, WI), AccuPrime *Taq* DNA polymerase system (Invitrogen), and *PfuTurbo* DNA polymerase (Stratagene) were used for PCR according to the manufacturers' protocols. Oligonucleotides were from Integrated DNA Technologies. Isolation of DNA from *S. pombe* was achieved by the Smash and Grab method (Hoffman and Winston 1987). DNA sequencing was performed using custom oligonucleotides and the CEQ DTCS-Quick Start kit (Beckman Coulter) according to the manufacturer's directions.

Identification of the $git1^{+}$ gene: A nonhomologous plasmid integration and rescue method was used to identify $git1^{+}$ (Hoffman and Welton 2000). *S. pombe* strain CHP578 was transformed to His^{+} with *Kpn*I or *Ngo*MIV-linearized plasmid pAF1 ($his3^{+}$) DNA. Pools of His^{+} colonies were collected after 5 days growth from each transformation plate. Approximately 10^{6} cells were spread onto SC–ura, and colonies that formed were further tested for elevated β-galactosidase activity. Integrated plasmids were rescued into *E. coli* by *Eco*RI digestion of genomic DNA and ligation to circularize the DNA. Rescued plasmids were sequenced using primer BS250 (5′-TTTTTGGGGTCGAGGTGC-3′) to determine the genomic site of plasmid insertion.

Plasmid constructions: Oligonucleotides git1-for (5′-ATGG GATTCACTAGTGTTGAACC-3′) and git1STOP-rev (5′-CTAA CTACTCACTCTTTTGACATCCTTAG-3′) were used in a PCR reaction on wild-type *S. pombe* genomic DNA to amplify the $git1^{+}$ ORF, which was cloned into vector pNMT41 (Invitrogen) to create plasmid pRSK3. A second PCR reaction using oligonucleotide git1-for with git1V5-rev (5′-ACTACTCACTCTTTTGA CATCCTTAGAC-3′) produced a PCR product lacking the $git1^{+}$ STOP codon, such that cloning into pNMT41 to create plasmid pRSK4 results in a translational fusion to a C-terminal V5 tag (Southern *et al.* 1991) followed by a hexahistidine tag (*git1-V5his6*). Fourteen codons within the C2 domain of $git1^{+}$ were removed by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) and oligonucleotides git1ΔC2-for (5′-CTGAGTCGGAATTTTGTGTACATACGTATC AGATGGAGATTAC-3′) and git1ΔC2-rev (5′-GTAATCTCCATC TGATACGTATGTACACAAAATTCCGACTCAG-3′) on pRSK3 and pRSK4 to create pRSK8 (*git1ΔC2*) and pRSK9 (*git1ΔC2-V5his6*), respectively.

TABLE 1

Strain list

Strain	Genotype
972	*h*$^-$
CHP7	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git2-7*
CHP9	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git1-45*
CHP45	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git1-9*
CHP46	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git1-9*
CHP61	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git2-61*
CHP79	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git1-79*
CHP99	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git1-99*
CHP216	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366 git2-216*
CHP287	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366 git1-287*
CHP568	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his3-D1 git1-1*
CHP569	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216 his3-D1 lys1-131 git1-1*
CHP578	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his3-D1*
CHP795	*h*^{90} *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366*
CHP856	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his3-D1 git1::his3*
CHP857	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his3-D1 git1::his3*$^+$
CHP861	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 his3-D1 gpa2::his3*$^+$
CHP889	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216 his3-D1 gpa2*^{R176H}
CHP896	*h*^{90} *ura4::fbp1-lacZ leu1-32 ade6-M210 git1*Δ*::LEU2*$^+$
CHP954	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 git1*Δ*::LEU2*$^+$
CHP973	*h*$^-$ *leu1-32 git1*Δ*::LEU2*$^+$
DIP59	*h*$^+$ *ura4::fbp1-lacZ leu1-32 git2-13myc::kan*
FWP72	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32*
FWP77	*h*$^+$ *ura4::fbp1-lacZ leu1-32*
FWP79	*h*$^-$ *ura4::fbp1-lacZ leu1-32 ade6-M216*
FWP87	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32*
FWP89	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216*
FWP95	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32*
FWP96	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210*
FWP111	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366 git1-1*
FWP112	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366*
FWP134	*h*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M210 git1-1*
FWP188	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366 git2-1::LEU2*$^+$
RKP2	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 git1*Δ*::LEU2*$^+$
RKP9	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 his3-D1 git1*Δ*::LEU2*$^+$ *gpa2*^{R176H}
RKP15	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 SPAC11E3.02c*Δ*::LEU2*$^+$
RKP19	*h*$^-$ *leu1-32 git1-V5his6::LEU2*$^+$ *his3*$^+$
RKP29	*h*$^+$ *ura4::fbp1-lacZ leu1-32 git1-V5his6::LEU2*$^+$ *his3*$^+$ *git2-13myc::kan*
RKP44	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 git1*Δ*::LEU2*$^+$ *git2-13myc::kan*
RKP45	*h*$^-$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 git1*Δ*::LEU2*$^+$ *git2-13myc::kan*
RKP62	*h*$^+$ *fbp1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 git1*Δ*::LEU2*$^+$ *SPAC11E3.02c*Δ*::LEU2*$^+$

Epitope tagging of *git1*$^+$ and *git2*$^+$ chromosomal loci: The *git1-V5his6* allele was introduced into the *git1*$^+$ genomic locus so that it is expressed from the *git1*$^+$ promoter by linearizing plasmid pRSK4 (*git1-V5his6*) with *Bsp*EI at nucleotide 711 of the *git1*$^+$ ORF and by transforming CHP857 (*git1::his3*$^+$) to Leu$^+$. Homologous insertion of the plasmid was confirmed by PCR. The *git2-13myc* allele was constructed by the PCR-based method of Bähler *et al.* (1998). Homologous insertion of the PCR product into the *git2*$^+$ locus of strain FWP77 was confirmed by PCR. The tagged protein was shown to be functional by β-galactosidase assay of *fbp1-lacZ* expression in glucose-grown cells.

β-Galactosidase assays of *fbp1-lacZ* expression: Cells were cultured overnight under repressing conditions (8% glucose) in yeast extract (YEL) or *S. pombe* minimal (PM; for transformants) liquid medium. Subcultures were grown for 24 hr to a final density of ~1×10^7 cells/ml. Protein lysates were prepared and assayed as previously described (Nocero *et al.* 1994).

Deletion of *git1*$^+$ and SPAC11E3.02c: The *git1*$^+$ and SPAC11E3.02c genes were deleted by a two-step PCR approach (Wang *et al.* 2004) and confirmed by PCR.

Protein isolation and immunoblotting: Strains were cultured in appropriate liquid media to ~1×10^7 cells/ml. Total protein extracts were prepared by TCA precipitation as previously described (Volland *et al.* 1994). Protein extracts were separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane. Immunodetection of *myc*-tagged and V5-tagged proteins was carried out using mouse α-*myc* (Santa Cruz Biotechnology) and mouse α-V5 (Invitrogen) primary antibodies and peroxidase-labeled goat α-mouse IgG secondary antibody (Kirkegaard & Perry Laboratories). Visualization was carried out by using LumiGLO chemiluminescence (Kirkegaard & Perry Laboratories). Cdc2 was detected using rabbit polyclonal IgG against Cdc2 p34 PSTAIRE (Santa Cruz Biotechnology) in combination with horseradish-peroxidase-conjugated secondary antibody.

Co-immunoprecipitations: *S. pombe* strains DIP59, RKP19, RKP29, and FWP72 were grown to exponential phase and broken in NP-40 buffer [6 mM Na_2HPO_4, 4 mM NaH_2PO_4 H_2O, 1% NONIDET P-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 16.7 μM PMSF, and 1 tablet of protease inhibitor (Boehringer Mannheim, Indianapolis) in 50 ml of buffer] using acid-washed glass beads in a Bead Beater. A total of 500 μl of cell lysate was incubated with 2.5 μg of α-V5 or α-Myc antibody for >1 hr at 4° on a rotator. Fifty microliters of Protein G Sepharose 4 Fast Flow was added in a 1:1 ratio with NP-40 buffer and incubated on a rotator for 1 hr or more. Precipitated immune complexes were isolated by microcentrifugation for 20 sec. The pellets were washed four times with NP-40 buffer and once with 50 mM Tris pH 8. Pellets were resuspended in 30 μl Laemmli buffer and heated for 3 min at 95°. Beads were pelleted by microcentrifugation for 20 sec, and the supernatants were removed for analysis.

Immunofluorescence microscopy: Strains were grown in appropriate liquid media to 2–4 × 10^6 cells/ml before fixing with paraformaldehyde as previously described (Hagan and Hyams 1988) with slight modifications. Cell walls were digested with 0.5 mg/ml 100T Zymolyase. V5-tagged proteins were detected using α-V5 primary antibody diluted 1:100 in PEMBAL and visualized by incubating with Alexa Fluor 488-labeled goat α-mouse secondary antibody diluted 1:50 in PEMBAL overnight. Rabbit polyclonal IgG against c-Myc was used together with Alexa Fluor 594-labeled goat α-rabbit IgG to visualize the 13Myc-tagged protein. Images were visualized and captured using a Zeiss Axioplan 2 microscope with an Orca-ER CCD camera and Openlab software.

RESULTS

Cloning of the *S.pombe git1*$^+$ gene: The *S. pombe git1*$^+$ gene was identified and cloned using a plasmid insertional mutagenesis screen developed to overcome problems associated with the high background of multicopy suppressors encountered during library screens in fission yeast (Hoffman and Welton 2000). This screen involves the random insertion of linearized plasmid pAF1 (*his3*$^+$) into strain CHP578 (*his3*Δ), and a subsequent screen of these His$^+$ transformants for Git$^-$ strains that are able to form colonies on glucose-rich medium lacking uracil due to the loss of glucose repression of an *fbp1-ura4*$^+$ reporter gene (see materials and methods). Two independent Git$^-$ mutants were identified and the integrated plasmid in each strain was rescued into *E. coli* such that some genomic DNA from the site of insertion was cloned. Sequence analysis of the rescued plasmids showed that one insertion is in the Gα-encoding *gpa2*$^+$ gene (Isshiki *et al.* 1992; Nocero *et al.* 1994) while the second insertion is in the gene designated SPBC21C3.20c in the *S. pombe* genomic sequence (Wood *et al.* 2002). An earlier genetic screen had identified spontaneous Git$^-$ mutant strains carrying mutations affecting a collection of genes, *git1*$^+$–*git10*$^+$, which confer the ability to form colonies on SC–ura medium as seen in these insertion mutant strains. Git$^-$ mutant strains also display 5-FOA-sensitive (5-FOA^S) growth (Hoffman and Winston 1990), unlike the 5-FOA-resistant (5-FOA^R) phenotype of cells able to glucose repress expression of the *fbp1-ura4*$^+$ reporter.

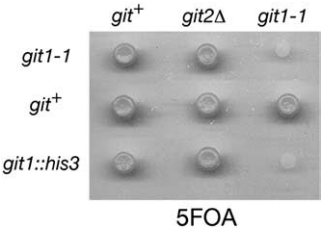
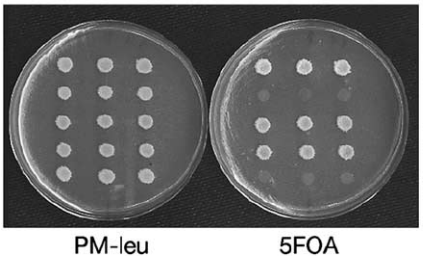


Figure 1.—Plasmid pAF1 (*his3*$^+$) insertion mutation is in the *git1*$^+$ complementation group. Diploid strains were constructed using intragenically complementing *ade6-M210* and *ade6-M216* mutant alleles to form Ade$^+$ diploids. Diploid strains were spotted onto 5-FOA medium and grown for 3 days at 30°. 5-FOA^R growth reflects the wild-type ability to glucose repress transcription of an *fbp1-ura4*$^+$ reporter gene.

Eight of these *git* genes play a significant role in *fbp1*$^+$ repression, with only *git1*$^+$ and *git10*$^+$ remaining to be cloned. We therefore carried out linkage and complementation tests to determine whether SPBC21C3.20c represents either *git1*$^+$ or *git10*$^+$. The pAF1 insertion allele of SPBC21C3.20c fails to complement a *git1-1* mutant allele in the diploid strains while complementing a *git2* deletion (Figure 1) and a *git10-201* mutant allele (data not shown). Consistent with this, all 56 tetrads produced from a cross of a SPBC21C3.20c-insertion mutant (CHP857) with a *git1-1* mutant (CHP569) were parental ditypes (0:4 5-FOA^R:5-FOA^S progeny), indicating that the insertion occurred within 0.9 cM of the *git1-1* mutation. We conclude that *git1*$^+$ is SPBC21C3.20c.

Using sequence information from the *S. pombe* genome project (Wood *et al.* 2002), we designed PCR primers to amplify and clone *git1*$^+$, creating plasmid pRSK3. As expected, plasmid pRSK3 restores glucose repression of *fbp1-ura4* expression to strain CHP568 (*git1-1*; Figure 2), conferring 5-FOA^R growth. These transformants also glucose repress *fbp1-lacZ* expression as judged by β-galactosidase assays (Table 2). Similar



Host genotype	Plasmid product
git1$^+$	none
git1-1	none
git1-1	Git1$^+$
git1-1	Git1$^+$-V5
git1-1	Git1ΔC2

Figure 2.—Complementation of a *git1-1* mutation by plasmid-expressed *git1*$^+$. Strains FWP72 (*git1*$^+$) and CHP568 (*git1-1*) were transformed to Leu$^+$ with pNMT41 (empty vector), pRSK3 (*git1*$^+$), pRSK4 (*git1-V5his6*), or pRSK8 (*git1ΔC2*). Three independent transformants of each host strain and plasmid combination indicated in the figure were spotted to PM–leu and then replica plated after 2 days to either PM–leu or 5-FOA media. Plates were photographed after 3 days at 30°.

TABLE 2

Glucose repression of *fbp1-lacZ* expression in transformants

Strain (relevant genotype)	Plasmid (expressed gene)	β-Gal activity
FWP72 (*git1*$^+$)	pNMT41 (none)	10 ± 1
CHP568 (*git1-1*)	pNMT41 (none)	1313 ± 16
CHP568 (*git1-1*)	pRSK3 (*git1*$^+$)	37 ± 6
CHP568 (*git1-1*)	pRSK4 (*git1-V5his6*)	31 ± 4
CHP568 (*git1-1*)	pRSK8 (*git1*Δ*C2*)	729 ± 32
FWP72 (*git1*$^+$)	pRSK8 (*git1*Δ*C2*)	47 ± 10

β-Galactosidase activity was determined from at least two independent transformants as described in MATERIALS AND METHODS. The values represent the average ± standard deviation and are given as specific activity per milligram of soluble protein.

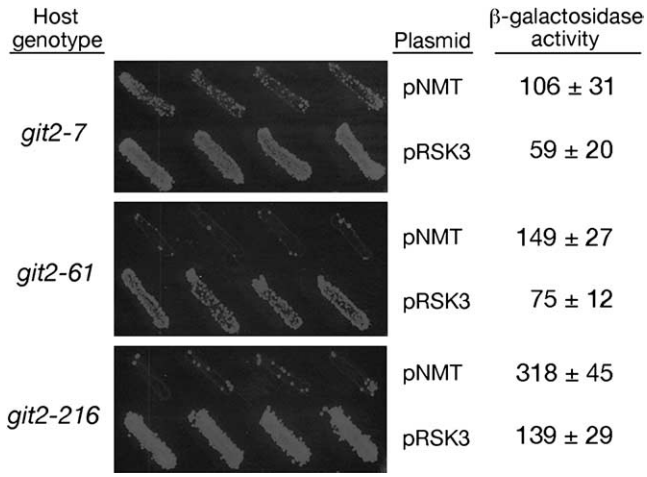


FIGURE 3.—Multicopy suppression of *git2*$^-$ mutations by plasmid-expressed *git1*$^+$. Host strains CHP7 (*git2-7*), CHP61 (*git2-61*), and CHP216 (*git2-216*) were transformed to Leu$^+$ with pNMT41 (empty vector) or pRSK3 (*git1*$^+$). Four independent transformants of each host strain and plasmid combination indicated in the figure were plated onto EMM–leu and then replica plated after 1 day to either EMM–leu or 5-FOA media. 5-FOA plates were photographed after 5 days at 30°. All transformants grew equally well on EMM–leu medium (not shown). β-Galactosidase activity was determined as described in MATERIALS AND METHODS. The values given represent specific activity ± standard deviation from three independent transformants.

results were observed when the plasmid-expressed Git1 protein contained a C-terminal V5-6his tag, indicating that the Git1$^+$-V5his6 protein expressed by plasmid pRSK4 is functional (Table 2, Figure 2). Plasmid pRSK3 was also introduced into strains carrying mutations in *git3*$^+$ (GPCR), *gpa2*$^+$ (Gα), *git7*$^+$ (Sgt1 family member), *git10*$^+$, or *pka1*$^+$ (catalytic subunit of PKA). These transformants remain 5-FOA sensitive, indicating that *git1*$^+$ cannot act as a multicopy suppressor of mutations in any of these genes (data not shown). In contrast, plasmid pRSK3 partially suppresses certain mutations in the *git2*$^+$/*cyr1*$^+$ adenylate cyclase gene. The *git2-7* and *git2-216* mutations previously have been shown to intragenically complement the *git2-61* mutation (HOFFMAN and WINSTON 1990, 1991). Surprisingly, multicopy *git1*$^+$ restores some degree of 5-FOA^R growth to host strains carrying any of these three mutations (Figure 3) and reduces *fbp1-lacZ* expression, but does not fully restore it to the glucose-repressed levels seen in wild-type transformants. [β-Galactosidase values for FWP112 (*git*$^+$) transformants carrying the pNMT empty vector were 21 ± 3 units, while transformants carrying pRSK3 were 15 ± 5.] The *git2-216* transformants carrying pRSK3 display an apparent contradiction in terms of their 5-FOA^R growth, yet relatively high β-galactosidase activity (Figure 3). This may be due to greater heterogeneity in these transformants with regard to *fbp1*-driven expression. A subset of cells may repress *fbp1-ura4*$^+$ expression to a level permitting growth on 5-FOA, while other cells of the same transformant may express the *fbp1-lacZ* reporter at a level high enough to produce the observed average β-galactosidase value for the culture that is similar to or greater than that seen in some 5-FOA^S transformants.

Git1$^+$ is a C2 domain-containing protein: The *git1*$^+$ gene encodes a 1098-amino-acid protein with only modest sequence similarity to other proteins in the GenBank database (Figure 4A). A BLASTP search (ALTSCHUL *et al.* 1990) using the predicted Git1 protein as the query sequence (with no filter) reveals a group of eight fungal proteins that align with *E*-values in the range of 10^{-23}–10^{-12}. However, these proteins align to each other with *E*-values $\leq 10^{-52}$ (Figure 4B), suggesting that Git1 function may be distinct from that of these other proteins. One of these proteins, SPAC11E3.02c, is also from *S. pombe*, while the remaining seven proteins are from various fungi. A BLASTP search using SPAC11E3.02c as the query sequence reveals a somewhat larger family of proteins that includes *S. cerevisiae* Yor296w (*E*-value 5×10^{-37}), a protein of unknown function. Yor296w is not returned in a BLASTP search using Git1 as the query protein, consistent with the impression that Git1 is only distantly related to the SPAC11E3.02c/Yor296w protein family. Finally, the SPAC11E3.02c-based search reveals that the UNC-13/Munc13 protein family is very distantly related to these fungal proteins (*E*-values of 10^{-4}–10^{-6}). In fact, SPAC11E3.02c and Yor296w had been previously identified as possessing both a C2 domain related to that of the UNC-13/Munc13 protein family and two conserved regions referred to as Munc13-homology domains 1 and 2 (MHD1 and MHD2; KOCH *et al.* 2000). MHD1 and MHD2 are also moderately conserved in Git1, representing residues 585–700 and 980–1098, respectively (Figure 4A).

To determine the relative roles of *git1*$^+$ and SPAC11E3.02c in glucose signaling, we deleted each gene from strain FWP72 and examined the effect on the *fbp1*-driven reporter constructs. The *git1* deletion

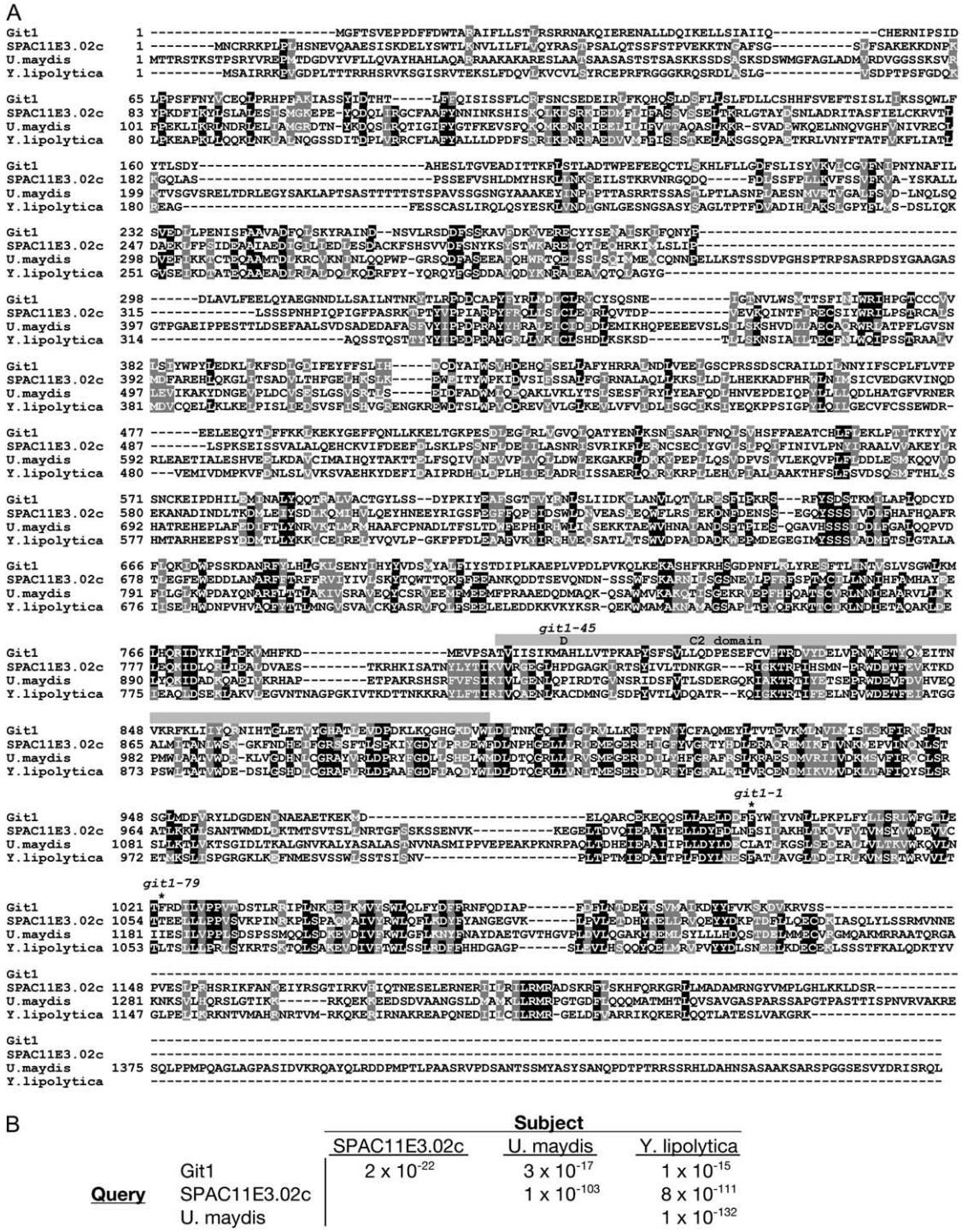


FIGURE 4.—Alignment of Git1 with SPAC11E3.02c and related proteins from *Ustilago maydis* and *Yarrowia lipolytica*. (A) Alignment of *S. pombe* proteins Git1 (NP_596600) and SPAC11E3.02c (NP_594926) with related proteins from *U. maydis* (XP_400071) and *Y. lipolytica* (XP_501312) using the ClustalW sequence alignment program (THOMPSON *et al.* 1994) and displayed using BOXSHADE. Identities are shown against a solid background and similarities are shown against a shaded background. A shaded box above the sequences denotes the predicted C2 domain of Git1. The alanine-to-aspartic-acid missense mutation in *git1-45* is shown above residue 799 of the Git1 sequence. Asterisks above the Git1 sequence at residues 995 and 1022 denote the last correctly translated residues expressed from alleles *git1-1* and *git1-79*, respectively. (B) *E*-values associated with alignments from unfiltered BLASTP searches involving the proteins shown in A.

TABLE 3

Defect in glucose repression of *fbp1-lacZ* expression in mutant strains

Strain	Relevant genotype	β-Galactosidase activity: 8% glucose	β-Galactosidase activity: 8% glucose + cAMP
FWP72	Wild type	15 ± 8	ND
RKP2	*git1*Δ	834 ± 128	12 ± 2
RKP15	SPAC11E3.02cΔ	13 ± 5	ND
RKP62[a]	*git1*Δ SPAC11E3.02cΔ	776 ± 81	ND
CHP889	*gpa2*^{R176H}	18 ± 10	ND
RKP9	*git1*Δ *gpa2*^{R176H}	705 ± 123	ND

Cells were grown in YEL to exponential phase under repressing conditions (8% glucose) and assayed as described in MATERIALS AND METHODS. The values given represent specific activity ± standard deviation from three or four independent cultures of each strain. ND, not determined.

[a] RKP62 values are based on an average of assays of strains RKP62, RKP66, and RKP67, which are genetically identical.

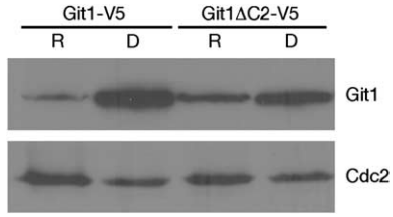


FIGURE 5.—Git1 protein levels are regulated by glucose conditions. Western blot analysis of protein extracts from FWP72 (Git$^+$) transformants carrying plasmid pRSK4 (Git1$^+$-V5) or plasmid pRSK9 (Git1ΔC2-V5). Cells were grown under glucose-repressing (R) or derepressing (D; glucose-starved) conditions. Immunoblots were carried out using either α-V5 or α-Cdc2 (as a loading control) antibody as described in MATERIALS AND METHODS.

(*git1*Δ) confers 5-FOA^S growth, similar to that seen in Figure 1, and elevated β-galactosidase expression (Table 3) in glucose-grown cells due to the constitutive expression of the *fbp1-ura4* and *fbp1-lacZ* reporters. Conversely, the SPAC11E3.02c deletion has no effect on expression of either reporter and does not exacerbate the defect in *fbp1* regulation in a *git1*Δ strain (Table 3 and data not shown). Thus, SPAC11E3.02c is not functionally redundant with Git1, and we assume that the proteins closely related to SPAC11E3.02c are not functional homologs of Git1$^+$.

Both the Git1 C2 domain and MHD2 are required for function: As described above, Git1 contains a putative C2 domain (amino acids 791–889) as well as two potential Munc homology domains (Figure 4A). C2 domains are found in proteins involved in signal transduction such as protein kinase C where they bind phospholipids in a calcium-dependent manner. However, some C2 domains in other proteins neither are calcium dependent nor bind phospholipids (RIZO and SUDHOF 1998). To determine if the Git1 C2 domain is required for Git1 function, we used site-directed mutagenesis to delete codons 826–839, predicted to encode a pair of antiparallel β-strands from the *git1*$^+$ ORF and tested whether this protein can provide Git1 function when expressed from a plasmid. As shown in Figure 2 and Table 2, the Git1ΔC2 protein is largely nonfunctional, failing to restore 5-FOA^R growth and glucose repression of *fbp1-lacZ* expression to a *git1-1* host strain, although a modest reduction of *fbp1-lacZ* expression is observed. In addition, Git1ΔC2 does not confer a dominant-negative phenotype when expressed in FWP72 (*git1*$^+$) cells (Table 2). To determine whether the loss of function is due to destabilization of the protein, we expressed both Git1$^+$-V5 and Git1ΔC2-V5 proteins from plasmids pRSK4 and pRSK9 in FWP72 (*git1*$^+$) cells. We observe that the Git1$^+$-V5 protein level is regulated by the carbon source, with 11.5-fold less protein present in glucose-grown cells than in glucose-starved cells (Figure 5). While the Git1ΔC2-V5 protein level is also regulated by glucose, there is only a threefold reduction in glucose-grown cells (Figure 5). As Git1ΔC2-V5 expressed from plasmid pRSK9 is readily detectable, the loss of the C2 domain does not simply destabilize the protein, suggesting a functional role for the Git1 C2 domain. In addition, the presence of the C2 domain appears to be important for the post-translational regulation controlling the level of Git1 in the cell as a function of glucose signaling.

The *git1*$^+$ gene was notable in our original selection for spontaneous mutations that conferred constitutive *fbp1*$^+$ transcription (HOFFMAN and WINSTON 1990) in that almost 40% of the independently isolated mutations were in *git1*$^+$. While Git1 is a moderately large protein of 1098 residues, this cannot fully account for the number of *git1*$^-$ mutants identified. A DNA sequence analysis of these strains explains the high frequency of mutations and supports the idea that the C2 domain and the MHD2 domain are important for Git1 function. The *git1-1* allele contains a +1 frameshift in which a sequence of eight thymidine nucleotides in the coding strand acquires an additional thymidine, leading to the loss of the C-terminal 103 residues of the protein (Figure 4A). This suggests a functional role for the MHD2 domain, although we have not determined whether the truncated protein is stable. In addition, PCR analysis of the original 73 *git1* mutant alleles found that 57 alleles possess an additional base pair in a 64-bp region that includes the *git1-1* mutation, while direct sequencing of *git1-9*, *git1-46*, and *git1-99* confirms that they possess the same frameshift as in *git1-1* (data not shown). As these mutant strains were isolated independently, this frameshift is a high-frequency event relative to other spontaneous mutations, explaining the large number of *git1*$^-$ strains isolated in the original study. An additional allele, *git1-287*, contains a −1 frameshift in this same run of thymidine residues. It is worth noting

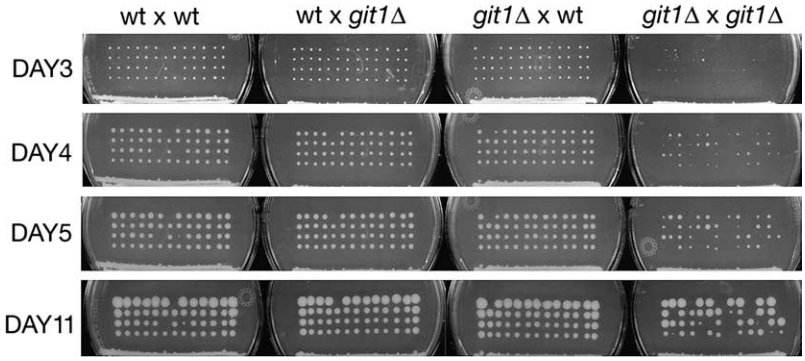


FIGURE 6.—Spore germination delay in *git1*Δ by *git1*Δ cross. Thirteen tetrads from each cross were dissected on yeast extract agar plates and incubated at 30°. Photographs were taken on days 3, 4, 5, and 11. Crosses (from left to right) are of strains FWP95 (*git1*$^+$) × FWP87 (*git1*$^+$), FWP95 × CHP954 (*git1*Δ), RKP2 (*git1*Δ) × FWP87, and RKP2 × CHP954.

that high-frequency frameshift mutations have also been identified in A-T-rich sequences within the *S. cerevisiae IRA1* and *IRA2* genes (HALME *et al.* 2004).

Of the remaining *git1*$^-$ mutant alleles, *git1-79* and *git1-82* contain a nonsense mutation causing the loss of the C-terminal 76 residues of the protein, further supporting the notion that the MHD2 domain is important for Git1 function. Several other frameshift or nonsense mutations cause larger truncations of the protein and are thus uninformative. Only the *git1-45* allele contains a missense mutation, changing an alanine to an aspartic acid at residue 799 within the C2 domain. β-Galactosidase assays of *git1-79* and *git1-45* mutant strains demonstrate that *fbp1-lacZ* expression is derepressed to a similar degree in a *git1*Δ strain (data not shown), indicating that both the C2 and the MHD2 domains are critical for Git1 function.

Analysis of *git1*Δ strains: To fully assess the role of Git1 in *fbp1*$^+$ transcriptional regulation and glucose detection, we constructed a complete deletion of the *git1*$^+$ gene (*git1*Δ) and characterized the effect of this deletion on several cAMP-regulated processes. As the loss of several genes in the PKA pathway results in a spore germination delay (WELTON and HOFFMAN 2000), we examined whether *git1*Δ spores display a similar delay. As shown in Figure 6, in *git1*$^+$ × *git1*Δ crosses both *git1*$^+$ and *git1*Δ spores germinate to form clearly visible colonies by 3 days. However, in a *git1*Δ × *git1*Δ cross, spore germination is more stochastic with relatively few colonies forming within 3 days. Most colonies grew to a visible size within 4–5 days, while others took over a week to form (Figure 6). These differences are due to delays in germination rather than reduced growth rates as judged by daily microscopic examination of the spores and colonies on the dissection plates. In addition, exponential phase cells of strain 972 (*git1*$^+$) display a doubling time of 2.47 ± 0.03 hr in YEL medium and 3.13 ± 0.13 hr in EMM medium, while CHP973 (*git1*Δ) cells display a doubling time of 2.62 ± 0.08 hr in YEL medium and 4.32 ± 0.79 hr in EMM medium. The lack of a germination delay in *git1*Δ spores from a *git1*$^+$ × *git1*Δ cross suggests either that sufficient Git1 for germination is packaged in the spore during sporulation or that Git1 carries out its function on a target that is then packaged into the spore.

The *git1*Δ strains were examined for other phenotypes associated with defects in the cAMP pathway. These include a reduced cell length at the time of septation (JIN *et al.* 1995) and the acquisition of starvation-independent conjugation and sporulation (DAL SANTO *et al.* 1996; LANDRY *et al.* 2000; WELTON and HOFFMAN 2000; LANDRY and HOFFMAN 2001; SCHADICK *et al.* 2002), along with the previously described loss of glucose repression of transcription of an *fbp1-lacZ* reporter (HOFFMAN and WINSTON 1990, 1991). As shown in Figure 7A, homothallic *git1*Δ cells conjugate and sporulate in nutrient-rich medium. Addition of 5 mM cAMP suppresses the conjugation phenotype (Figure 7A), consistent with ability of exogenous cAMP to suppress the constitutive *fbp1-lacZ* expression in RKP2 (*git1*Δ) cells (Table 2). We also measured the cell length of wild-type and *git1*Δ cells that display septa in exponential-phase cultures. By measuring the first 10 septated cells observed by microscopy (see Figure 7B for examples of such cells), we found that strain 972 (*git1*$^+$) cells produce septa at an average length of 15.6 ± 1.6 μm, while strain CHP973 (*git1*Δ) cells produce septa at an average length of 11.7 ± 0.8 μm. Thus, loss of Git1 leads to a significant reduction in the length of septated cells.

To directly test for a defect in cAMP signaling, we assayed the cAMP response to glucose in wild-type and *git1*Δ cells (Table 4). Relative to wild-type cells, the *git1*Δ cells display both a modest reduction in basal cAMP levels under glucose-starvation conditions and a total loss of a cAMP response to glucose addition. Therefore, Git1 is essential to glucose/cAMP signaling in *S. pombe.*

Git1 physically interacts with adenylate cyclase, but is not required for adenylate cyclase levels or localization: To investigate of the role of Git1 in the glucose/cAMP pathway, we examined whether or not Git1 binds the Git2 adenylate cyclase enzyme. Protein extracts from cells expressing *myc*-tagged Git2 and V5-tagged Git1 were subjected to co-immunoprecipitations. In reciprocal experiments, we found that immunoprecipitation of Git1-V5 leads to coprecipitation of Git2-*myc* (Figure 8A) and that immunoprecipitation of Git2-*myc* leads to

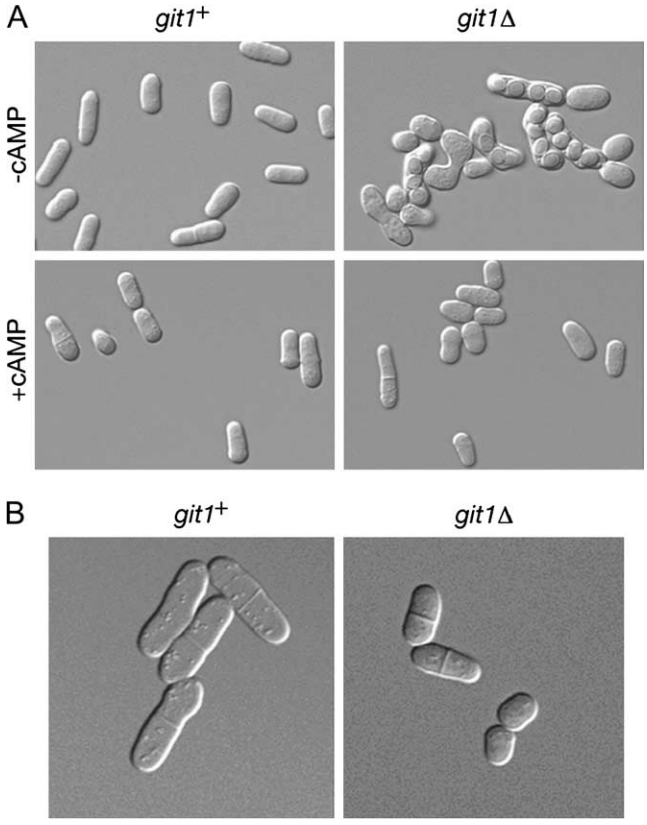


FIGURE 7.—*git1*Δ cells display defects associated with the loss of cAMP signaling. (A) Homothallic (h^{90}) strains CHP795 (*git1*$^+$) and CHP896 (*git1*Δ) were grown to exponential phase in PM liquid medium (at 37° to inhibit conjugation) and then diluted to 10^6 cells/ml in PM liquid medium in the presence or absence of 5 mM cAMP. These cells were incubated for 24 hr at 30° without shaking and photographed. (B) Strains 972 (*git1*$^+$) and CHP973 (*git1*Δ) were grown to exponential phase in YEL. Cells displaying septa were measured and photographed.

coprecipitation of Git1-V5 (Figure 8B). Consistent with a physical interaction between Git1 and adenylate cyclase, indirect immunofluorescence of Git1-V5 and Git2-*myc* demonstrates that both proteins display a punctate cytoplasmic staining pattern and partially colocalize within cells (Figure 9).

TABLE 4

cAMP response to glucose in *git1*$^+$ and *git1*Δ cells

Strain	Genotype	cAMP (pmol)/mg protein			
		T = 0′	T = 1′	T = 5′	T = 10′
FWP79	*git1*$^+$	3.6 ± 0.6	13.9 ± 1.4	16.2 ± 5.1	16.8 ± 3.9
CHP973	*git1*Δ	2.7 ± 0.6	2.6 ± 0.4	2.6 ± 0.6	2.7 ± 0.7

Assays were carried out on glucose-starved cultures (T = 0′), as well as on the same cells, 1, 5, and 10 min after the addition of glucose to a final concentration of 100 mM as previously described (BYRNE and HOFFMAN 1993) using the cAMP [^{125}I]Biotrak Assay System (Amersham Biosciences).

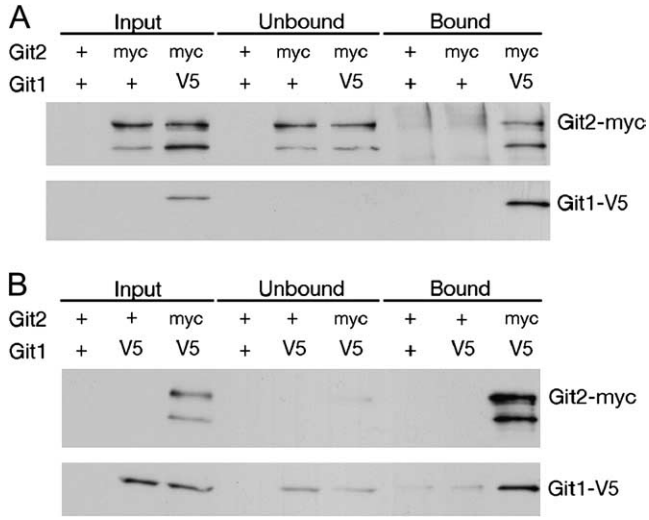


FIGURE 8.—Git1 and Git2 (adenylate cyclase) physically interact. (A) Immunoblot of protein extracts from α-V5 immunoprecipitation. Protein extracts were prepared from strains expressing tagged (Git2-*myc* and Git1-V5) or untagged proteins (+) as indicated. Crude extracts, along with fractions that bound or failed to bind α-V5 antibodies, were probed with α-*myc* to detect Git2-*myc* and with α-V5 to detect Git1-V5. (B) Immunoblot of protein extracts from α-*myc* immunoprecipitation. Protein extracts were prepared from strains expressing tagged or untagged proteins as indicated. Crude extracts, along with fractions that bound or failed to bind α-*myc* antibodies were probed with α-V5 to detect Git1-V5 and with α-*myc* to detect Git2-*myc*.

As Git1 is required for glucose/cAMP signaling even in cells expressing the mutationally activated Gpa2^{R176H} Gα-subunit (Table 3), we examined whether Git1 controls either adenylate cyclase stability or localization. Indirect immunofluorescence of Git2-*myc* produces a punctate staining pattern in a *git1*Δ strain that is indistinguishable from that seen in *git1*$^+$ cells with regard to localization and intensity (data not shown). Similarly, immunoblots of extracts made from *git1*Δ cells expressing Git2-*myc* show little or no change in the level of Git2-*myc* in both glucose-grown and glucose-starved cells (data not shown). Therefore, the loss of Git1 does not lead to the destabilization of adenylate cyclase or to a gross change in adenylate cyclase localization within the cell. As discussed below, it remains possible that Git1 is involved in a transient localization event required for adenylate cyclase activation.

DISCUSSION

The fission yeast *S. pombe* utilizes a cAMP-signaling pathway as the primary mechanism for sensing environmental glucose to regulate transcription of genes involved in gluconeogenesis and sexual development. In this study, we describe the cloning and characterization of the *git1*$^+$ gene, which is required for glucose-triggered adenylate cyclase activation.

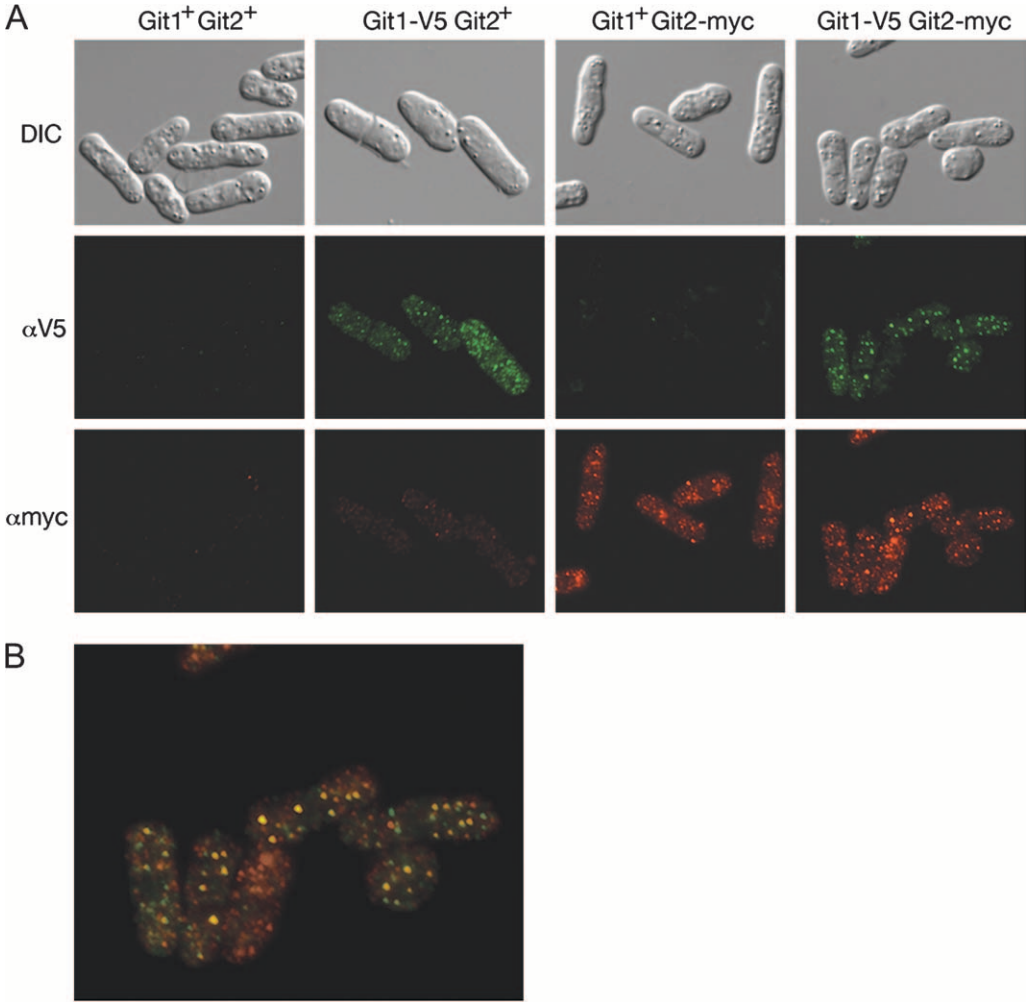


FIGURE 9.—Partial colocalization of Git1 and Git2. (A) DIC and fluorescent images of cells expressing tagged and untagged forms of Git1 and Git2 (adenylate cyclase) as indicated. Fluorescent images were taken with the same exposure time for each sample. (B) Merged fluorescent image from A of cells expressing Git1-V5 and Git2-*myc*.

The most surprising aspect of this work is the identity of *git1*$^+$ itself, as it lacks any obvious functional homologs in other organisms. Prior to this study, work in *S. cerevisiae* and *S. pombe* glucose/cAMP signaling had uncovered pathways whose components display considerable conservation, although several key differences exist (see review by HOFFMAN 2005a and references therein). Both yeasts express seven-transmembrane GPCRs that activate G-proteins, which are involved in adenylate cyclase activation. However, the *S. cerevisiae* Gpr1 GPCR is coupled to the Gpa2 Gα-subunit that lacks a traditional Gβγ dimer, interacting instead with a Gβγ mimic that negatively regulates the pathway (HARASHIMA and HEITMAN 2002; BATLLE *et al.* 2003). In addition, glucose signaling through Ras proteins occurs in concert with Gpa2-mediated signaling to play a central role in *S. cerevisiae* adenylate cyclase activation. In contrast, the *S. pombe* Git3 GPCR functions together with a heterotrimeric G-protein, whose Gpa2 Gα-subunit directly binds an N-terminal region of adenylate cyclase (IVEY and HOFFMAN 2005) that is distinct from the Ras-activation domain in the *S. cerevisiae* enzyme (SUZUKI *et al.* 1993), although another study suggests that *S. pombe* Gpa2 acts through an adenylate cyclase domain that is homologous to the Ras-activation domain (OGIHARA *et al.* 2004). In addition, the only Ras homolog in *S. pombe* plays no role in adenylate cyclase activation. Finally, *S. cerevisiae* Sgt1 and *S. pombe* Git7 are homologous cochaperone proteins involved in cAMP signaling (DUBACQ *et al.* 2002; SCHADICK *et al.* 2002), while carrying out other essential functions as well. The Git1 protein is unusual in that while it is critical for glucose-triggered adenylate cyclase activation in *S. pombe*, it may be functionally unique to fission yeast.

As seen in Figure 4A, Git1 is distantly related to a small set of proteins that includes a second *S. pombe* protein, SPAC11E3.02c. Deletion of the SPAC11E3.02c gene has no effect on *fbp1-lacZ* expression and no additive effect when combined with a *git1*Δ allele; therefore this gene does not function in the glucose/cAMP pathway. As the remaining proteins of this family are much more closely related to SPAC11E3.02c than to Git1$^+$ (Figure 4B), we assume that they represent functional homologs of SPAC11E3.02c and not Git1. The requirement for a novel protein such as Git1 by *S. pombe* adenylate cyclase is unexpected due to the high degree of structural

conservation among yeast and fungal adenylate cyclase enzymes and may point to a unique regulatory mechanism in fission yeast adenylate cyclase. Alternatively, the Git1 function may be carried out in other fungi by a protein with little sequence similarity to Git1.

While the 1098-amino-acid Git1 protein displays some similarity to a family of large fungal proteins, ranging from 1185 to 1472 residues, the majority of the conserved sequences are found in the C-terminal half of Git1. This includes a 100-amino-acid C2 domain (Rizo and Sudhof 1998), which may promote an interaction with phospholipids, flanked by two additional regions of conservation. Interestingly, two proteins in this family, *S. pombe* SPAC11E3.02c and *S. cerevisiae* Yor296w, were previously cited in a study identifying a family of proteins related to the Munc13 protein, which is involved in vesicle priming for neurotransmitter secretion (Koch *et al.* 2000). Along with the C2 domain, these proteins were defined by the presence of MHD1 and MHD2, which correspond to the sequences before and after the C2 domain in the fungal proteins. Recently, it has been shown that the MHD1–MHD2–C2 domain region (the MHD2 domain precedes the C2 domain in the Munc13 proteins) is sufficient for function of both *Caenorhabditis elegans* UNC-13 (Madison *et al.* 2005) and mouse Munc13 (Stevens *et al.* 2005). Sequence analysis of *git1*$^-$ mutant alleles, along with site-directed mutagenesis of the C2 domain, demonstrates that the C2 and MHD2 domains are required for Git1 function. Therefore, while Git1 may be functionally distinct from these fungal and metazoan proteins, Git1 and these other proteins may act through a similar biochemical mechanism.

The level of Git1 protein is lower in glucose-grown cells than in glucose-starved cells (Figure 5), which resembles the glucose-mediated regulation of other components of the pathway. We previously showed that transcription of *cgs1*$^+$ (PKA regulatory subunit) and *pka1*$^+$ (PKA catalytic subunit) is repressed by glucose in a PKA-dependent manner (Stiefel *et al.* 2004). In addition, there is significantly less Git3 GPCR and adenylate cyclase protein in glucose-grown cells than in glucose-starved cells (D. Chandler-Militello, L. Wang and C. S. Hoffman, unpublished results). Removal of the C2 domain in the Git1ΔC2-V5 protein abolishes much of this regulation, even in a wild-type strain that retains a functional cAMP pathway (Figure 5). Thus, the C2 domain may be required to assemble Git1 into a complex whose abundance is the target of feedback regulation by PKA.

Co-immunoprecipitation experiments using *myc*-tagged adenylate cyclase and the Git1-V5 protein demonstrate that these proteins either directly or indirectly interact (Figure 8). In contrast, Git7 (Schadick *et al.* 2002) and Git1 do not co-immunoprecipitate, suggesting that these two proteins carry out distinct functions in the cAMP pathway (M. Alamery and C. S. Hoffman, unpublished results). Consistent with the co-precipitation results, indirect immunofluorescence detection of Git1 and Git2 results in a punctate staining pattern with some colocalization (Figure 9). Thus, Git1 appears to be present in a complex with adenylate cyclase, playing a direct role in adenylate cyclase activation. Such a direct role for Git1 is consistent with the reduction in basal cAMP levels and the total absence of a cAMP response observed in *git1*Δ cells (Table 4). This is also consistent with the observation that Git1 is required for *fbp1* repression, even in a strain expressing a mutationally activated form of the Gpa2 Gα, which also binds adenylate cyclase (Table 3). A direct role for Git1 in adenylate cyclase activation is also supported by the ability of Git1 overexpression to partially suppress mutations in the *git2*$^+$ adenylate cyclase gene (Figure 3). Prompted by the physical and genetic interactions between Git1 and adenylate cyclase, we examined whether Git1 is required either for adenylate cyclase localization or for stability, but found no significant change in adenylate cyclase levels or localization in a *git1*Δ strain (data not shown). However, our localization experiments show little or no adenylate cyclase associated with the plasma membrane (Figure 9), which is where the Git3 GPCR is localized (D. Chandler-Militello, D. A. Kelly and C. S. Hoffman, unpublished results). While this appears to differ from the situation in budding yeast, biochemical studies of adenylate cyclase in *S. cerevisiae* show that ~50% of the protein is soluble (Mitts *et al.* 1990). There has not been a similar analysis of budding yeast adenylate cyclase localization by microscopy, presumably due to the low abundance of the protein. In fact, we were able to detect our tagged protein due to the use of a 13myc epitope (GFP fusions expressed from the genomic locus do not produce a visible signal; K. Cummings, L. Wang, and C. S. Hoffman, unpublished results). It remains possible that Git1 is required to shuttle adenylate cyclase to the plasma membrane to allow Gpa2-mediated activation, which could be related to the Munc13 role in priming vesicles for membrane fusion. Future experiments will address the mechanism by which Git1 facilitates adenylate cyclase activation in response to glucose detection in fission yeast.

We thank David Burgess for advice on microscopy and members of the Hoffman lab for insightful discussions and critical evaluation of the manuscript. This work was supported by National Institutes of Health grant R01 GM46226 to C.S.H.

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Communicating editor: P. Russell