Schizosaccharomyces pombe Git7p, a Member of the Saccharomyces cerevisiae Sgt1p Family, Is Required for Glucose and Cyclic AMP Signaling, Cell Wall Integrity, and Septation

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The Schizosaccharomyces pombe fbp1 gene, encoding fructose-1,6-bisphosphatase, is transcriptionally repressed by glucose. Mutations that confer constitutive fbp1 transcription identify git (glucose-insensitive transcription) genes that encode components of a cyclic AMP (cAMP) signaling pathway required for adenylate cyclase activation. Four of these genes encode the three subunits of a heterotrimeric G protein (gpa2, git5, and git11) and a G protein-coupled receptor (git3). Three additional genes, git1, git7, and git16, act in parallel to or downstream from the G protein genes. Here, we describe the cloning and characterization of the git7 gene. The Git7p protein is a member of the Saccharomyces cerevisiae Sgt1p protein family. In budding yeast, Sgt1p associates with Skp1p and plays an essential role in kinetochore assembly, while in Arabidopsis, a pair of SGT1 proteins have been found to be involved in plant disease resistance through an interaction with RAR1. Like S. cerevisiae Sgt1p, Git7p is essential, but this requirement appears to be due to roles in septation and cell wall integrity, which are unrelated to cAMP signaling, as S. pombe cells lacking either adenylate cyclase or protein kinase A are viable. In addition, git7 mutants are sensitive to the microtubule-destablizing drug benomyl, although they do not display a chromosome stability defect. Two alleles of git7 that are functional for cell growth and septation but defective for glucose-triggered cAMP signaling encode proteins that are altered in the highly conserved carboxy terminus. The S. cerevisiae and human SGT1 genes both suppress git7-93 but not git7-235 for glucose repression of fbp1 transcription and benomyl sensitivity. This allele-specific suppression indicates that the Git7p/Sgt1p proteins may act as multimers, such that Git7-93p but not Git7-235p can deliver the orthologous proteins to species-specific targets. Our studies suggest that members of the Git7p/Sgt1p protein family may play a conserved role in the regulation of adenylate cyclase activation in S. pombe, S. cerevisiae, and humans.

Transcription of the Schizosaccharomyces pombe fbp1 gene is repressed by glucose (23, 48). Previous studies showed that glucose detection triggers a cyclic AMP (cAMP) signal responsible for the activation of cAMP-dependent protein kinase A, which acts to repress fbp1 transcription (11, 20, 26). Glucose starvation leads to the activation of the Spc1p stress-activated mitogen-activated protein kinase pathway, which in turn activates the heterodimeric bZIP transcriptional activator Atf1p-Per1p (27, 44, 45, 51). These two signaling pathways regulate fbp1 transcription via at least two distinct mechanisms, with Atf1p-Per1p acting both directly at one cis-acting element (UAS1) and indirectly at a second element (UAS2) (25). Transcriptional activation from UAS2 appears to be carried out by the Rst2p zinc finger protein (19). Additional regulators of fbp1 transcription include Tup11p and Tup12p, homologs of the Schizosaccharomyces cerevisiae Tup1p global corepressor, which are required for fbp1 repression, and the CCAAT box binding factor, which is required in concert with Atf1p-Per1p for fbp1 derepression (25).

The S. pombe adenylate cyclase (Git2p/Cyr1p) activation mechanism resembles that of the mammalian enzyme (46) in that it involves the activity of a heterotrimeric G protein coupled to a seven-transmembrane domain receptor protein. In S. pombe, the gpa2, git5, and git11 genes encode the Go, Gβ, and Gγ subunits, respectively, while the git3 gene encodes the putative G protein-coupled receptor (GPCR) (13, 19, 20, 23, 36). The role of these four genes is to activate Gpa2p Go, as mutational activation of Gpa2p or overexpression of the wild-type gpa2+ gene bypasses the need for Git5p Gβ, Git11p Gγ, and Git3p GPCR (31, 32, 37, 52). The Git3p, Gpa2p, and Git2p/Cyr1p proteins also display sequence homology to S. cerevisiae Gpr1p GPCR, Gpa2p Gα, and Cyr1p adenylate cyclase, which act in the glucose-triggered cAMP signaling pathway (12, 30, 53), and are involved in the regulation of...
TABLE 1. Strain list

| Strain | Genotype |
|--------|----------|
| CHP27  | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M210 hist7-366 git7-27 |
| CHP93  | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M210 hist7-366 git7-93 |
| CHP449 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M216 hist7-366 git7-235 |
| CHP556 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M216 hist3-D1 git7-93 |
| CHP558 | h^+ git7::ura4 leu1–32 ade6-M216 git2-1::LEU2 |
| CHP578 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M210 his3-D1 |
| CHP594 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M210 his3-D1 |
| CHP595 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M216 his3-D1 |
| CHP718 | h^+ fbp1::ura4 ura4::D18 leu1–32 ade6-M210 his3-D1 git7-235 |
| CHP758 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M210 git7-GFP::kan skp1::ura4^+ ars1(Mu1):pREP41-skp1-3×HA LEU2^+ |
| CHP767 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M210 hist7-366 git7-GFP::kan |
| CHP792 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M216 hist7-366 |
| CHP795 | h^+ ura4::fbp1-lacZ leu1–32 ade6-M210 skp1::ura4^+ ars1(Mu1):pREP41-skp1-3×HA LEU2^+ |
| CHP803 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M210 hist7-366 |
| FWP72  | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 |
| FWP101 | h^+ ura4::fbp1-lacZ leu1–32 ade6-M210 skp1::ura4^+ |
| FWP145 | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M216 git7-235 |
| KS1    | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M210 his7-366 git7-27 |
| KS5    | h^+ ura4::fbp1-lacZ leu1–32 ade6-M210 git7-GFP::kan (Ch16 ade6-M216 m23::ura4^+ ) |
| KS5    | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 ade6-M210 hist7-366 git7-93 git7-V5::intLEU2^+ |
| PBP1   | h^+ fbp1::ura4 ura4::fbp1-lacZ leu1–32 git7-GFP::kan |

pseudohyphal growth (33, 40). Unlike S. pombe, S. cerevisiae does not appear to express a Giβy dimer that functions in this signaling pathway. Related CAMP signaling pathways are also found in fungal pathogens. For example, the human fungal pathogen Cryptococcus neofor manus uses Gα (Gpa1) (2) and adenylyl cyclase (Cac1) (3), which resemble S. pombe Gpa2p and Git2p, to detect nutrient conditions and to regulate aspects of differentiation and virulence.

Three additional S. pombe genes, git1, git7, and git10, required for glucose-stimulated adenylyl cyclase activation (11, 21), are still required for glucose repression of fbp1 transcription in a strain carrying an activated allele of gpa2 or overexpressing gpa2^+ (37, 52). Thus, Git1p, Git7p, and Git10p presumably regulate adenylyl cyclase activation either downstream from or in parallel to Gpa2p Gα. In addition, git7-235 strains display a temperature-sensitive lethal growth phenotype (21), suggesting an essential role for this gene independent of CAMP signaling, as both adenylyl cyclase and protein kinase A are dispensable in S. pombe (20, 34, 35).

In this article, we describe the cloning and characterization of the S. pombe git7 gene. Sequence analysis shows that Git7p is a member of the Sgt1p family found in many eukaryotes, including the budding yeast S. cerevisiae, plants, and mammals. S. cerevisiae Sgt1p associates with Skp1p and is required for kinetochore assembly (29), while Arabidopsis SGT1a and SGT1b associate with RAR1, playing a role in disease resistance (4, 5). Skp1p is a component of the SCF (Skp1p–Cullin–F-box) E3 ubiquitin ligase responsible for the polyubiquitination of proteins that are subsequently degraded by the proteasome (41); however, there is no evidence that the role of Skp1p and Sgt1p in kinetochore assembly involves ubiquitin ligase activity. Conservation of Sgt1p function in eukaryotes is suggested by the ability of the human ortholog HuSgt1p to restore viability to a strain lacking S. cerevisiae Sgt1p activity (29) and by the ability of the Arabidopsis orthologs to restore temperature-resistant growth to S. cerevisiae sgt1-3 and sgt1-5 mutant strains (5). Our work shows that, like S. cerevisiae Sgt1p, S. pombe Git7p is an essential protein, although cell death in S. pombe is associated with cell lysis along with cell division and septation defects. Two git7 alleles that alter the carboxy terminus of Git7p confer a defect in fbp1 regulation without affecting cell wall integrity and septation, suggesting that Git7p possesses discrete functional domains. Finally, we show that the expression of the human and S. cerevisiae Sgt1p proteins can restore fbp1 transcriptional regulation in a git7-93 mutant but not in a git7-235 mutant. Such allele-specific suppression suggests that the Git7p/Sgt1p proteins form multimeric complexes that play conserved roles in a variety of cellular processes, including the regulation of adenylyl cyclase activity, cell division, septation, and kinetochore assembly.

MATERIALS AND METHODS

S. pombe strains and growth media. The S. pombe strains used in this study are listed in Table 1. The fbp1::ura4^+ and ura4::fbp1-lacZ reporters (21) are transcriptional fusions integrated in single copies at the fbp1 and ura4 loci, respectively. Rich media YEA and YEL (16) were supplemented with 2% Casamino Acids. Defined medium PM (50) was supplemented with required nutrients at 75 mg/liter, except for l-leucine, which was used at 150 mg/liter. Solid medium SC containing 0.4 g of 5-fluoro-orotic acid (5-FOA)/liter and 8% glucose was used to determine 5-FOA resistance (21), which is a reflection of glucose repression of the fbp1::ura4 reporter. Strains were grown at 30°C unless otherwise indicated.

Recombinant DNA Methods. All DNA manipulations were performed, unless otherwise stated, by using reagents and protocols from New England Biolabs. E. coli K12 strains were transformed by electroporation, and S. pombe plasmid transformations were performed by overnight incubation with polyethylene glycol–liithium acetate–Tri-EDTA buffer (13) or as described byBähler et al. (6).

Cloning of the git7 gene. A pBR322-based S. pombe genomic DNA library (38) was screened for clones that confer 5-FOA resistance (5-FOA') to host strain CHP556 (git7-93), indicating the restoration of glucose repression of the fbp1::ura4 reporter. From 33,000 transformants, 37 5-FOA' candidates were identified. Plasmids from these strains were rescued in E. coli (22) and screened for their
ability to restore growth at 37°C to S. pombe strain CHP718, which is temperature sensitive. CHP718 is a temperature-sensitive derivative of the CHA10 strain. Two of the 27 plasmid candidates conferred temperature-resistant growth to strain CHP718. These two plasmids, one of which was designated pPH1, contained identical inserts. Plasmid pPH1 was linearized in the insert with NheI and used to transform strain CHP718 (his3-D1 gly7-235) to His+1. A stable integrant was identified and determined to be temperature resistant and 5-FOA+, indicating that integrated plasmid pPH1 suppresses the gly7-235 mutation. This strain was then crossed with strain CHP95 (his3-D1 gly7+). All 13 tetrads examined displayed a parental ditypic pattern (4 5-FOA−/5-FOA− [5-FOA sensitive] progeny), indicating that plasmid pPH1 had integrated in or near the gly7 locus. DNA sequence analysis of the two ends of the insert DNA from plasmid pPH1 revealed that the insert contains the sequence from positions 30,876 to 35,540 of cosmide c6 (GenBank accession number AL023589), which carries a portion of S. pombe chromosome 2, including two candidate open reading frames. Subcloning analyses demonstrated that the gly7 gene is open reading frame SPBC3612c.

Disruption and green fluorescent protein (GFP) tagging of gly7. The gly7 gene was disrupted by using a PCR-based approach as described by lhler et al. (6). Oligonucleotides gly7Δ5′ (5′-TCGTGCAGATAATGCCGCGCTCAGCTCTCATGCTGGAGGAGCCTTGTGACCTTGATCATCCTGCGTATTAATA-3′) and gly7Δ3′ (5′-TTGCGACAACTTTCCGGGAGGACGGCACACATATCGATCTTTGCAGGTATTTAATG-3′) were used to PCR amplify a kanMX6-containing fragment from pFA6a-GFP-kanMX6. The amplified fragment was used to transform a diploid S. pombe strain, constructed by mating CHP556 (gly7-93) with CHP94 (gly7+), to G418 resistance, replacing the wild-type gly7+ allele with a kanMX6-marked disruption allele. Homologous recombination at the gly7 locus was confirmed by Southern blot analysis. Asyntotic ascii, dissected on YEA medium, produced tetrads with only two viable progeny that were 5-FOA−/G418 sensitive, indicating that the gly7 deletion strain was nonviable. Progeny carrying the gly7+ disruption could be rescued by transforming the diploid strain with plasmid pPH1 (gly7+) prior to tetrad dissection.

A gly7−GFP fusion was created at the gly7 locus in strain FWP72 by homologous recombination with a PCR product made by PCR amplification of plasmid pFA6a-GFP-kanMX6 with primers gly7T7G1 (5′-CAACTAAATGCGGAAAGGTTGGATCTATGGAACGGTCTCGAAGCTTGGCAGCTTCGAGTACACAAAGAGTTTGTGCCGGTGATTAATAA-3′) and gly7T7G2 (5′-TTGGCACCAATTCCAGGAAGGTTGGATCTATGGAACGGTCTCGAAGCTTGGCAGCTTCGAGTACACAAAGAGTTTGTGCCGGTGATTAATAA-3′). The resulting strain, BPH1, was defective in growth on YEP medium but grew very slowly on minimal medium. The disruption of the gly7 gene was confirmed by PCR amplification of the gly7 coding sequence from the disrupted strain and sequencing of the complementary strand.

Construction of a transcriptionally regulated allele of skp1. The S. pombe skp1+ gene (GenBank accession number AF071066) was precisely replaced with a polyvinylidene difluoride membrane. Immunodetection was carried out by using an anti-V5-horseradish peroxidase antibody (Invitrogen) according to the manufacturer’s instructions, and visualization was carried out by using LumiGLO chemiluminescence (Kirkegaard & Perry Laboratories).

Immunofluorescence microscopy. Strains were cultured overnight in PM liquid medium and grown to a final cell density of approximately 107 cells/ml. Cells were fixed by using paraformaldehyde for 30 min and prepared for immunofluorescence microscopy as described by hagan and hyams (17). Git7-V5 was localized by using an anti-V5 antibody (Invitrogen) according to the manufacturer’s instructions and visualized by using Alex Fluor 488-labeled immunoglobulin G as a secondary antibody (Molecular Probes) at 10 μg/ml for 1 h. Cells were resuspended in mounting medium containing 1 μg/ml of Hoechst 33342.

RESULTS

Cloning of the S. pombe gly7+ gene. The gly7+ genes, including gly7, were originally identified in a genetic screen for mutations that confer a defect in the glucose repression of both fbp1-ura4 and fbp1-lacZ reporters in S. pombe (21). Thus, while wild-type (gly7−) strains carrying these reporters are 5-FOA− in a glucose-rich medium, gly7 mutant strains are 5-FOA+. By screening plasmid libraries for clones that restore a 5-FOA− phenotype to gly7 mutant hosts, we have been able to clone gly7+ genes as well as multicityc suppressors (13, 20, 26, 32). To clone gly7+, strain CHP566 (gly7-93) was transformed with his+ and a pBG2-based S. pombe genomic DNA library (38). From 33,000 transformants, we identified 37 5-FOA+ candidates. These candidate plasmids were subsequently screened for their ability to sup-
press the temperature-sensitive growth of strain CHP718 conferred by the git7-235 allele. The original library screening was not carried out with this strain due to its poor transformation efficiency. Two of the 37 rescued plasmid candidates conferred temperature-resistant growth to strain CHP718. These two plasmids possess identical inserts, and one was designated pHF1 (Fig. 1). Sequence analysis across the two ends of the insert showed that pHF1 carries a portion of chromosome 2 from bp 30,876 to 35,540 of cosmid c36. Subclones from pHF1 insertion showed that pHF1 carries a portion of chromosome 2 in the host genome by homologous recombination followed by genetic linkage analysis indicated that pHF1 carries the git7 gene and not a multicopy suppressor (see Materials and Methods). In addition, plasmid pHF1 failed to act as a multicopy suppressor when introduced into strains carrying mutations in any of five git genes required for adenylate cyclase activation: git1, git3 (GPCR), git5 (GB), gpa2 (Ga), and git10 (data not shown).

Git7p is a member of the Saccharomyces Sgt1p protein family. A BLASTP analysis shows that Git7p is a member of a small family of proteins related to the S. cerevisiae Sgt1p protein (Fig. 2). Members of this family possess three distinct domains: a weakly conserved amino-terminal domain that appears to be a tetratricopeptide domain (5); a moderately conserved central domain referred to as the CS domain, due to its presence in some cytochrome- and histidine-rich domain-containing proteins (42); and a highly conserved carboxy-terminal domain (Fig. 2). During our subcloning analysis of git7, we constructed plasmid pHF4, which lacks exon 1 and part of exon 2 and presumably expresses only the carboxy-terminal 216 residues of Git7p. This plasmid partially restores both fbp1 regulation in a git7-93 strain (Fig. 1, plasmid pHF4) and 37°C growth in a git7-235 strain (data not shown).

The Git7p protein was originally predicted to be 444 residues long due to the fact that exon 1 potentially includes 150 codons. However, a Clustal W alignment (47) of the putative 444-residue Git7p protein and other members of the family suggested that Git7p possesses a 50-residue amino-terminal domain not found in the other proteins (data not shown). The identity of the git7 translational start site was brought into question when we cloned this presumed open reading frame into an nmt81-driven expression vector (nmt stands for “no message on thiamine”; see Materials and Methods). Plasmid pKS1 expresses a smaller-than-expected Git7p-V5 product whose activity is not repressed by thiamine (Fig. 3; CHP93/pKS1 cells express 12 ± 7 U of β-galactosidase activity after 48 h of thiamine repression, indicating plasmid suppression of the host git7-93 mutation). Thus, it appears that the 5’ end of the cloned sequence includes the git7 promoter. The most likely candidate TATA box is immediately upstream of the second putative ATG start codon, too close to allow transcription of this ATG. As translation from the third available start codon would produce a 379-residue protein, a size similar to those of other members of the Sgt1p/Git7p family (Fig. 2), we cloned the git7 open reading frame starting with this third potential start codon into the nmt81-driven vector. Plasmid pKS2 expresses a Git7p-V5 protein with the same mobility as that of plasmid pKS1; however, this activity is now thiamine repressible (Fig. 3; CHP93/pKS2 cells express 1,884 ± 184 U of β-galactosidase activity after 48 h of thiamine repression, indicating a loss of suppression of the host git7-93 mutation). As cells expressing Git7p-V5 from the git7 genomic locus (see Materials and Methods) produce a protein with the same mobility as those of the plasmid-expressed constructs (Fig. 3), the authentic Git7p protein appears to be 379 residues long.

S. pombe Skp1p does not appear to regulate fbp1-lacZ expression. It has been shown that the S. cerevisiae Sgt1p protein interacts with Skp1p of the SCF E3 ubiquitin ligase. We therefore set out to determine whether the S. pombe homolog of Skp1p plays a role in fbp1 regulation and cAMP signaling, as does Git7p. Surprisingly, thiamine repression of nmt41-HA-skp1 expression in a git7 strain (CHP803) had almost no effect on fbp1-lacZ expression. Cells continued to display glucose-repressed β-galactosidase levels (25 ± 8 U) after 16 h in the presence of thiamine. After 48 h of thiamine repression, cells showed arrested growth, yet β-galactosidase levels were only 207 ± 64 U. As we have observed a higher level of derepression of fbp1-lacZ expression in cells grown to stationary phase under glucose-rich conditions, this small degree of derepression does not suggest a role for S. pombe Skp1p in fbp1 regulation. Therefore, it appears that the role of Git7p in cAMP signaling is Skp1p independent.

The git7 gene is essential for cell wall integrity and septa-
We constructed a git7-null allele (git7Δ) by homologous recombination (6) in an effort to study the role of git7 in cAMP signaling and other processes. The sporulation of a diploid strain carrying the git7Δ/H9004 allele showed that git7 is essential for viability. git7Δ/H9004 spores germinated to produce microcolonies of approximately 200 to 300 cells, at which point all the cells appeared to undergo lysis (data not shown). git7Δ/H9004 viability was rescued by either plasmid pHF1 or plasmid pHF4 (Fig. 1). Microscopic examination of cells from such transformants revealed the presence of both lysed cells and multinucleate cells, which likely resulted from plasmid loss (data not shown). Thus, Git7p seems to be required for both cell wall integrity and septation.

The original collection of git mutant strains included three independent git7 mutants (21). At the time, it was noted that git7-235 strains were temperature sensitive for growth. We therefore reexamined strains carrying the git7-27, git7-93, or git7-235 allele for defects in growth and septation at 30 and 37°C. As shown in Fig. 4, most git7-235 cells underwent lysis or failed to septate after 24 h at 37°C on solid medium. Even at 30°C, these defects were evident in git7-235 cells. Strains carrying the git7-27 allele displayed lysis and septation defects similar to those of git7-235 strains (Fig. 4), although to a lesser degree. Strains carrying the git7-93 allele appeared to have no growth defects, although they resembled other cAMP pathway mutants in that they divided at a slightly reduced cell length (26). Surprisingly, of the three original mutant alleles, the git7-93 allele conferred the most severe defect with regard to glucose repression of an fbp1-lacZ reporter (Table 2). Thus, Git7p has distinct roles in cAMP signaling and in cell wall integrity and septation.

To better characterize the growth defects conferred by the git7-235 allele, a temperature shift experiment was performed with two git7-235 strains growing in liquid medium. Strains FWP145 and CHP449 differ by the presence of a his7-366 allele in the latter strain. We have noted in the past that the his7-366 mutant allele enhances growth defects that are conferred by mutations in other genes (unpublished data). Consistent with this observation, a greater percentage of cells in the CHP449 culture than in the FWP145 culture exhibited one of three growth defects at all time points (Table 3). These included multinucleate cells (cells with four or more nuclei; this phenotype was more common in cells growing on solid medium than in liquid medium), binucleate cells that appeared to be arrested during cell division, and lysed cells (Table 3 and Fig. 5). In addition, some cells that did form septa appeared to
fail to complete cytokinesis (Fig. 5C). Further examination of the CHP449 cells failed to show any actin delocalization (data not shown). These results confirm a role for Git7p in cell wall integrity, cell division, and septation.

A Git7p-GFP fusion is defective in cAMP signaling. In an effort to study the localization of Git7p in the cell, we constructed a git7-GFP fusion at the git7 locus by homologous recombination (see Materials and Methods) (6). Cells expressing this fusion as the sole source of Git7p activity were viable and showed no growth defects (data not shown); however, these cells were defective in the glucose repression of fbp1-lacZ, indicating a defect in cAMP signaling (Table 2). Thus, like the git7-93 allele, the git7-GFP fusion allele confers a defect in the glucose/cAMP signaling pathway but has no effect on cell wall integrity or on septation.

Git7p is required for nutrient regulation of mating. S. pombe cells normally require either a glucose or a nitrogen starvation signal to initiate mating and meiotic entry (44). Therefore, cells carrying mutations in genes required for the glucose-triggered cAMP signal will mate and sporulate in nutrient-rich media (24, 31, 32, 34, 52). Consistent with the defect in the glucose repression of fbp1-lacZ expression (Table 2), the git7-GFP allele allows homothallic (h<sup>+</sup>) cells to mate in a glucose-rich medium (Fig. 6). This starvation-independent mating is similar to that conferred by a deletion of the git2 adenylate cyclase gene (git2Δ), as the addition of 5 mM cAMP to the medium prevented conjugation in both git7-GFP and git2Δ cells (Fig. 6). Thus, git7 is required for the cAMP-dependent regulation of conjugation as well as fbp1 transcriptional regulation.

The carboxy-terminal domain of Git7p is specifically involved in cAMP signaling. Both the git7-GFP and the git7-93 alleles confer a defect in fbp1 regulation but not in cell growth and septation (Table 2, Fig. 4, and data not shown). We therefore cloned all three spontaneous git7 alleles by gap repair of EcoRV-linearized plasmid pHF5 (Fig. 1). The git7-93 allele contains a 54-bp duplication of codons 345 to 363, a sequence that is flanked in the wild-type allele by an 8-bp direct repeat. This leads to a duplication of 18 amino acids (YTESNGLALVSKKS; Fig. 2) in the carboxy-terminal domain of Git7p; this domain is highly conserved in other members of this protein family. Thus, both the git7-93 and the git7-GFP alleles encode proteins that are altered at their carboxy termini. In contrast, both the git7-27 and the git7-235 alleles contain single missense mutations in the region encoding the amino terminus of Git7p. The mutation in git7-27 changes a histidine to an asparatic acid at residue 42, while the mutation in git7-235 changes an alanine to a glutamic acid at residue 69 (Fig. 2). As these two mutant alleles confer temperature-sensitive lethality, it is possible that these mutations result in a general instability of the Git7p protein. Alternatively, they may identify a domain required for all Git7p functions, including cell wall integrity and septation.

git7 mutants do not display a kinetochore assembly defect. As S. cerevisiae Sbt1p is required for kinetochore assembly (29), we tested whether any phenotypes associated with git7 mutations would suggest a similar role for Git7p in S. pombe. We therefore examined the effect of the microtubule-destabilizing drug benomyl on strains carrying various git7 alleles. Strains carrying any of the three spontaneous mutant alleles or the git7-GFP allele were benomyl sensitive (Fig. 7). The intro-
duction of plasmid pKS1 (git7-V5) into a git7-93 strain restored benomyl-resistant growth, providing further evidence that Git7p-V5 is fully functional for all Git7p activities of which we are aware, unlike Git7p-GFP. However, when assayed for mitotic stability of the Ch16 minichromosome (1), strain KSP2 (git7-GFP) did not display a chromosome stability defect (data not shown). Thus, the benomyl sensitivity is not likely due to a defect in kinetochore assembly, as is seen in S. cerevisiae sgt1 mutants (29), but may reflect other interactions with spindle or cytoplasmic microtubules. To test whether Git7p plays a role in spindle assembly, we constructed a double mutant carrying git7-235 and a deletion of the mad2 spindle checkpoint gene (mad2Δ) (18). The git7-235 mad2Δ double mutant failed to display any synthetic growth defects that would be expected if Git7p were required for spindle assembly (data not shown).

**Localization of a Git7p-V5 fusion.** Because the Git7p-GFP fusion protein is not functional in the glucose-cAMP pathway, we examined the localization of the fully functional Git7p-V5 protein (Fig. 3 and Table 4) expressed from the git7 promoter in a fusion integrated in a single copy (see Materials and Methods). The Git7p-V5 protein is seen as punctate staining in both the nucleus and the cytoplasm of cells (Fig. 8), while little or no staining is seen in untagged cells. A similar localization pattern has been observed for S. cerevisiae Sgt1p (C. Dubacq and C. Mann, personal communication).

**Complementation of git7-93 by plasmid-expressed S. cerevisiae and human Sgt1p proteins.** We tested whether the human Sgt1p (Sgt1p) or S. cerevisiae Sgt1p proteins could restore transcriptional regulation of the fbp1-lacZ reporter when expressed in git7 mutant strains. Indeed, the expression of either the human or the S. cerevisiae SGT1 gene from the nmt81 promoter completely suppressed the constitutive fbp1-lacZ expression observed in a git7-93 mutant strain (Table 4). However, the expression of these genes in a git7-235 strain had little or no effect on the constitutive expression of the fbp1-lacZ reporter. As expected, the expression of the Git7p-V5 fusion from the same vector suppressed both git7-93 and git7-235 mutations (Table 4). Consistent with this allele-specific suppression, we observed that the git7-93 transformants were benomyl resistant, while the git7-235 transformants remained benomyl sensitive (data not shown). Therefore, the ability of the human and budding yeast proteins to restore Git7p functions to S. pombe depends upon the expression of the Git7-93p mutant protein.

**DISCUSSION**

In this study, we have cloned and carried out an initial characterization of the S. pombe git7 gene, which is required for the glucose repression of fbp1 transcription as part of the glucose-triggered cAMP signaling pathway. In previous studies, it was shown that git7 is required for both the maintenance of basal cAMP levels and the generation of the glucose-stimulated cAMP.
ultated cAMP signal (11). It was also shown that while muta-
tional activation of Gpa2p Go bypasses the requirement for
the Git3p GPCR or Git5p-Git11p Gγ, it fails to suppress
mutations in git1, git7, or git10 (52). Therefore, while the role of
the Git3p GPCR and Git5p-Git11p Gγ is to activate Gpa2p
Go, Git1p, Git7p, and Git10p appear to function either down-
stream from or in parallel to Gpa2p Go.

It was surprising to discover that git7 is homologous to the S.
cerevisiae SGT1 gene, whose best-characterized role had been
in kinetochore assembly (29). However, SGT1 must play mul-
tiple roles in S. cerevisiae, as different temperature-sensitive
git alleles result in either G1 or G2 arrest. In addition, while
Sgt1p physically interacts with Skp1p of the SCF E3 ubiquitin
ligase, Sgt1p may carry out at least one Skp1p-independent
function in G1. The S. cerevisiae skp1-11 allele causes G1 arrest
due to the accumulation of the Sic1p inhibitor of the Cdc28p-
Clb kinase required for G1 exit (7). As such, the arrest point for
skp1-11 cells is after the pheromone arrest point in G1. Mean-
while, the arrest point for Sgt1p in G1 precedes the phero-
omone arrest point (29), indicating a role for Sgt1p distinct
from that of Skp1p in G1. Our studies show little or no con-
nection between Git7p and Skp1p in S. pombe. While both
Git7p and Skp1p are essential in S. pombe, cells depleted of
Skp1p do not show the same phenotypes as git7 mutants. Thi-
amine repression of nmt41-HA-skp1 cells causes cell growth
arrest but not a defect in septation or an increase in cell lysis
(data not shown). In addition, fbp1-lacZ expression remains
glucose repressed upon Skp1p depletion, with only partial de-
pression as cell growth is arrested, indicating that S. pombe
Skp1p is not involved in the glucose-triggered cAMP signaling
pathway.

Our characterization of a git7 deletion allele and other mu-
ant alleles revealed a large number of mutant phenotypes,
including constitutive fbp1 transcription, starvation-indepen-
dent conjugation and sporulation, cell lysis, a septation defect,
a cell division defect, and sensitivity to the microtubule-desta-
bilizing drug benomyl. These phenotypes suggest at least three
distinct Git7p functions. The inability to repress fbp1 transcrip-
tion and conjugation is due to a defect in the cAMP signaling
pathway, as has been shown for other git genes (26, 31, 32, 34,
52). The septation and lysis defects are most likely related,
since lysis appears to occur at the septum during cytokinesis
(data not shown). Along with a failure to form septa, git7-27
and git7-235 cells also form defective septa that lead to either
cell lysis during cytokinesis or a failure to undergo cytokinesis,
as shown in Fig. 5C. Finally, the cell division defect and beno-
myl sensitivity may be related, since microtubules are involved
in the migration and positioning of the nuclei during mitosis in
S. pombe (17). The lack of a mitotic chromosome stability de-
fect in git7 mutants or synthetic growth defects in a git7-235
expression by human Sgt1p and S. cerevisiae Sgt1p in git7-93 but not git7-235 cells

| Plasmid-expressed protein | β-Galactosidase activity in the following host: |
|---------------------------|------------------------------------------------|
|                           | git7-93 | git7-235 |
| None                      | 2,428 ± 488 | 1,581 ± 198 |
| Git7p-V5                  | 21 ± 3  | 17 ± 4  |
| Human Sgt1p               | 21 ± 9  | 1,919 ± 214 |
| S. cerevisiae Sgt1p       | 11 ± 3  | 1,176 ± 37 |

*β-Galactosidase activity was assayed in CHP93 (git7-93) and CHP49 (git7-235) transformants grown in PM medium lacking thiamine (to derepress expres-
sion of the nmt81 promoter in the vector) under glucose-rich conditions. The plas-
mods used were pNMT81-TOPO (none; empty vector control), pKS2 (Git7p-V5), pKS3 (human Sgt1p), and pKS4 (S. cerevisiae Sgt1p). Values represent
mean specific activities and standard errors from two to four independent cult-
ures.

FIG. 7. Benomyl sensitivity in git7 mutant strains. Serial dilutions (1:5) of strains FWP101 (git7+), CHP27 (git7-27), CHP93 (git7-93), CHP449 (git7-235), CHP758 (git7-GFP), and CHP93 carrying plasmid pKS1 (git7-93 + git7-V5) were spotted on YEA plates containing 75 μg of adenine/ml and either 0 or 5 μg of benomyl/ml. Growth was recorded after 3 days at 25°C.

FIG. 8. Localization of Git7p-V5. Indirect immunofluorescence was used to detect Git7p-V5 localization in KSP5 cells (carrying an integrated git7-V5 fusion) and in FWP101 cells (untagged git7+). Cells were stained with Hoechst 33342 to detect DNA prior to microscopy. KSP5 cells display a punctate signal throughout the nucleus and the cytoplasm. DIC, differential interference contrast microscopy.
**mad2Δ** strain indicates that, unlike *S. cerevisiae* Sgt1p (29), Git7p plays no role in kinetochore assembly. However, as only a subset of *S. cerevisiae* Sgt1 mutants display a kinetochore assembly defect, our failure to observe a similar defect in git7 mutants does not rule out a similar role for Git7p. The characteristics described here represent an initial investigation of a protein that is involved in at least three distinct processes, and further detailed studies are required in each area.

Protein modeling work carried out on members of the Sgt1p protein family by Azevedo et al. (5) suggests that there are three distinct domains, with the poorly conserved amino-terminal domain acting as a tetratricopeptide domain (9). Our studies show that alteration of the highly conserved carboxy terminus by a short duplication in the git7-93 allele (Fig. 2) or by the addition of a large GFP tag to the carboxy terminus leads to defects in the glucose-triggered cAMP signaling pathway (Table 2) and benomyl sensitivity (Fig. 7) but has no effect on either septation or cell wall integrity (Fig. 4). Meanwhile, the Git7-27p and Git7-235p proteins both have alterations in the amino-terminal domain and share similar phenotypes that include cell lysis and septation defects when grown at 37°C (Fig. 4 and 5), in addition to the cAMP signaling and benomyl sensitivity phenotypes. These results indicate that Git7p is composed of distinct functional domains that act in a modular fashion such that the git7-93 mutation that interferes with cAMP signaling does not affect other essential Git7p functions.

In support of this notion, we observed allele-specific suppression of the git7-93 mutation by the expression of human or budding yeast SGT1 genes (Table 4). Because such expression fails to suppress the same constitutive fbp1-lacZ expression or benomyl-sensitive growth conferred by a git7-235 allele, it appears that Git7-93p provides a function that is defective in Git7-235p and that is needed to allow human or *S. cerevisiae* Sgt1p to act in *S. pombe*. Thus, Git7-235p may be defective in forming complexes with either Sgt1p or other components of a protein complex, while Git7-93p can interact with Sgt1p, which then provides a functional carboxy-terminal domain to the complex. However, since we have not observed intragenic complementation between git7-93 and git7-235, it is possible that Git7-235p cannot interact with human or budding yeast Sgt1p because it is unstable.

The ability of human and *S. cerevisiae* SGT1 genes to suppress a git7-93 mutation suggests that this gene family may have a conserved role in cAMP signaling, although it need not be as a function of glucose detection. Indeed, *S. cerevisiae* Sgt1p has also been shown to interact both physically and genetically with adenylyl cyclase in budding yeast (Dubacq and Mann, personal communication), supporting the idea that Sgt1p is involved in cAMP signaling. However, it remains to be seen whether or not members of the Git7p/Sgt1p family act to regulate adenylyl cyclase in mammalian cells, even though mammalian Ras proteins are able to replace *S. cerevisiae* Ras proteins in the budding yeast cAMP signaling pathway (10, 28), they do not appear to regulate cAMP signaling in mammalian cells (8). Thus, additional studies are needed to determine whether members of the Git7p/Sgt1p family are universally involved in cAMP signaling in yeasts, plants, and animals.

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