# The git5 Gβ and git11 Gγ Form an Atypical Gβγ Dimer Acting in the Fission Yeast Glucose/cAMP Pathway

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ABSTRACT

Fission yeast adenylate cyclase, like mammalian adenylate cyclases, is regulated by a heterotrimeric G protein. The gpa2 Gα and git5 Gβ are both required for glucose-triggered cAMP signaling. The git5 Gβ is a unique member of the Gβ family in that it lacks an amino-terminal coiled-coil domain shown to be essential for mammalian Gβ folding and interaction with Gγ subunits. Using a git5 bait in a two-hybrid screen, we identified the *git11* Gγ gene. Co-immunoprecipitation studies confirm the composition of this Gβγ dimer. Cells deleted for *git11* are defective in glucose repression of both *fbp1* transcription and sexual development, resembling cells lacking either the gpa2 Gα or the git5 Gβ. Overexpression of the gpa2 Gα partially suppresses loss of either the git5 Gβ or the git11 Gγ, while mutational activation of the Gα fully suppresses loss of either Gβ or Gγ. Deletion of *gpa2* (Gα), *git5* (Gβ), or *git11* (Gγ) confer quantitatively distinct effects on *fbp1* repression, indicating that the gpa2 Gα subunit remains partially active in the absence of the Gβγ dimer and that the git5 Gβ subunit remains partially active in the absence of the git11 Gγ subunit. The addition of the CAAX box from the git11 Gγ to the carboxy-terminus of the git5 Gβ partially suppresses the loss of the Gγ. Thus the Gγ in this system is presumably required for localization of the Gβγ dimer but not for folding of the Gβ subunit. In mammalian cells, the essential roles of the Gβ amino-terminal coiled-coil domains and Gγ partners in Gβ folding may therefore reflect a mechanism used by cells that express multiple forms of both Gβ and Gγ subunits to regulate the composition and activity of its G proteins.

HETEROTRIMERIC G proteins, consisting of α, β, and γ subunits, relay external signals detected by ligand-activated seven-transmembrane receptors to a variety of effector molecules in eukaryotic cells (Simon *et al.* 1991; Neer 1995). In the inactive state, the GDP-bound Gα subunit is associated with the Gβγ dimer to form the heterotrimer. Upon ligand binding, the receptor stimulates GDP release from Gα, allowing Gα to subsequently bind GTP. This nucleotide exchange activates the G protein by triggering a conformational change in Gα and its dissociation from the Gβγ dimer. In the activated state, the Gα subunit and the Gβγ dimer are free to regulate the activity of downstream effectors including adenylate cyclase, phospholipase C, mitogen-activated protein kinase (MAPK) cascades, and ion channels (Gilman 1987; Neer 1995).

Genetic studies and genomic sequencing of the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* show that both organisms possess two Gα genes, but only one Gβ gene. Due to the small size and weak conservation of Gγ subunits, it is harder to identify the Gγ genes *in silico*. In *S. cerevisiae*, the Gpa1 Gα, Ste4 Gβ, and Ste18 Gγ regulate the pheromone response pathway (Dietzel and Kurjan 1987; Whiteway *et al.* 1989), with the Gβγ dimer activating a MAPK pathway (Hirsch and Cross 1992). The Gpa2 Gα, in conjunction with the G-protein-coupled receptor-like protein Gpr1, monitors glucose to activate adenylate cyclase (Nakafuku *et al.* 1988; Colombo *et al.* 1998; Xue *et al.* 1998; Yun *et al.* 1998). There does not appear to be a Gβγ acting in this signaling pathway. In *S. pombe*, the gpa1 Gα is a positive regulator of the pheromone response pathway (Obara *et al.* 1991). As with *S. cerevisiae* Gpa2, gpa1 does not appear to associate with a Gβγ, although one study erroneously concluded that the git5/gpb1 Gβ is a negative regulator of gpa1 (Kim *et al.* 1996). The *S. pombe* gpa2 Gα, in concert with the git5/gbp1 Gβ and the G-protein-coupled receptor-like protein git3, activate adenylate cyclase in a glucose monitoring pathway (Isshiki *et al.* 1992; Nocero *et al.* 1994; Landry *et al.* 2000; Welton and Hoffman 2000).

The *S. pombe gpa2* gene was also identified as *git8* (*g*lucose *i*nsensitive *t*ranscription) in a mutant screen for *git* genes required for glucose repression of transcription of the *fbp1* gene that encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase (Hoffman and Winston 1990; Nocero *et al.* 1994). The *gpa2/git8* gene, along with *git1*, *git3*, *git5*, *git7*, and *git10*, is required for adenylate cyclase (encoded by *git2/cyr1*) activation in response to glucose detection (Hoffman and Winston 1991; Byrne and Hoffman 1993). The *git5* gene, identi-

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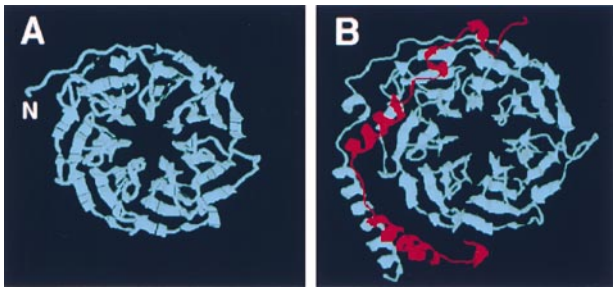


Figure 1.—Protein modeling of the git5 Gβ. (A) The git5 structure was determined by computer modeling of the git5 amino acid sequence (accession no. AAD09020) using the Swiss-Model program (Peitsch 1996) and displayed via RasMol (Sayle and Milner-White 1995). The amino terminus (N) of the git5 protein is indicated. (B) Model of the bovine Gβγ dimer as determined by Sondek *et al.* (1996). The Gβ subunit, possessing an additional 36 amino-terminal residues relative to git5, is in blue and the Gγ subunit is in red.

cal to *gbp1*, encodes a Gβ subunit that positively regulates gpa2 (Landry *et al.* 2000). A *git5* deletion confers the same phenotypes as a *gpa2* deletion, including derepression of *fbp1* transcription and starvation-independent conjugation and sporulation. Strains carrying a *git5* point mutation or deletion display a defect in glucose-triggered cAMP signaling, although basal cAMP levels are unaffected (Byrne and Hoffman 1993; Landry *et al.* 2000). In addition, the *git5* deletion is partially suppressed by multicopy *gpa2*$^{+}$ (Landry *et al.* 2000) and fully suppressed by an activated allele of *gpa2*, *gpa2*$^{R176H}$ (Welton and Hoffman 2000). Finally, as conjugation by git5 deletion strains remains pheromone dependent, git5 does not negatively regulate the gpa1-mediated pheromone response pathway (Landry *et al.* 2000).

Gβ subunits comprise a highly conserved protein family whose structure includes an amino-terminal coiled-coil followed by a seven-bladed WD repeat β-barrel (Figure 1; Wall *et al.* 1995; Sondek *et al.* 1996). The git5 Gβ is remarkable in that while it is ~43% identical to members of the Gβ family, it lacks the amino-terminal coiled-coil domain that includes 15 residues shown to form contacts with the Gγ subunit in the mammalian Gβγ dimer (Figure 1; Sondek *et al.* 1996; Landry *et al.* 2000). In mammalian systems, this domain appears to be essential for both Gβ folding and assembly of the Gβγ dimer (Garritsen *et al.* 1993; Wall *et al.* 1995; Garcia-Higuera *et al.* 1996; Lambright *et al.* 1996; Sondek *et al.* 1996; Pellegrino *et al.* 1997).

To test whether the *S. pombe* git5 Gβ interacts with a Gγ subunit, we conducted a two-hybrid screen to identify *S. pombe* proteins that physically interact with git5. One clone obtained from this screen encodes a recognizable Gγ subunit possessing several lysine residues and a CAAX-box (Casey 1994) at the carboxy-terminus. Co-immunoprecipitation studies *in vivo* confirm this interaction. Deletion of this gene, designated *git11*, confers phenotypes associated with a defect in glucose detection that, like a deletion of the *git5* Gβ gene, are partially suppressed by overexpression of the *gpa2* Gα gene. These results identify git11 as the functional Gγ partner of git5; thus the git5 Gβ does not require an amino-terminal coiled-coil to assemble into a functional Gβγ dimer.

Additional characterization of the roles of the Gα, Gβ, and Gγ subunits in glucose repression of *fbp1* transcription suggests that the git5-git11 Gβγ dimer is required for activation of the gpa2 Gα subunit and that, contrary to the data from mammalian studies, the git5 Gβ retains some function in the absence of a Gγ partner. Thus, the dependence upon the Gβ amino-terminal coiled-coil and the Gγ subunit for proper folding of mammalian Gβ subunits (Garritsen *et al.* 1993; Garcia-Higuera *et al.* 1996; Pellegrino *et al.* 1997) is not an intrinsic trait of Gβ subunits. These features of mammalian G proteins not observed in *S. pombe* may reflect a mechanism employed by cells that express multiple forms of the both Gβ and Gγ subunits to tightly control the repertoire and activity of Gβγ dimers.

## 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* reporters have been previously described (Hoffman and Winston 1990). Rich medium YEA (Gutz *et al.* 1974) was supplemented with 2% casamino acids. Defined PM media (Watanabe *et al.* 1988) were supplemented with required nutrients at 75 mg/liter, except for leucine, which was at 150 mg/liter. SC solid medium containing 0.4 g/liter 5-fluoroorotic acid (5-FOA) and 8% glucose (Hoffman and Winston 1990) was used to determine 5-FOA sensitivity. Strains were grown at 30° unless otherwise indicated.

**Recombinant DNA methodology:** All DNA manipulations were performed, unless otherwise stated, using reagents and protocols from New England Biolabs (Beverly, MA). *Escherichia coli* transformations were done using XL1-Blue electroporation competent cells (Stratagene, La Jolla, CA). The Expand High Fidelity PCR system (Roche Molecular Biochemicals, Indianapolis) was used for PCR reactions, according to the manufacturer's instructions. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). *S. pombe* plasmid transformations were performed by overnight incubation in a polyethylene glycol-LiOAc-TE buffer as previously described (Dal Santo *et al.* 1996).

**Two-hybrid screening:** A two-hybrid screen was carried out to identify proteins that interact with the git5 Gβ. The *git5* bait was PCR amplified from pSL11 (Landry *et al.* 2000) using oligonucleotides 5-3PTH 5′ GAAGATCTTGAAGCAGTCAAC CTCCTAGAATCGA 3′ and 5-5PTH 5′ CCGGCCATGGAGGC CATGGATTCTGGGTCAAGAGTAAACGT 3′. The PCR product was digested with *Sfi*I and *Bgl*II and ligated into *Sfi*I- and *Bam*HI-digested pAS2 (Harper *et al.* 1993) to form plasmid pSL13 that expresses a Gal4 binding domain (GBD)-git5 fusion protein. Plasmid pSL13 was cotransformed with a *S. pombe* two-hybrid cDNA library in pACT [expressing fusions to the Gal4 activation domain (GAD); Durfee *et al.* 1993] into YRG-2 competent yeast cells (Stratagene) according to the manufacturer's protocol. From ~$3.5 \times 10^5$ transformants, 81 His$^{+}$ candidates were screened for β-galactosidase activity by Xgal filter lift assay (Hoffman and Winston 1990). Ten positive candi-

**TABLE 1**

***S. pombe* strain list**

| Strain | Genotype |
|---|---|
| FWP72 | *h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32* |
| FWP175 | *h⁻ ura4::fbp1-lacZ leu1-32 gpa2-60* |
| CHP439 | *h⁺ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366 gpa2Δ::ura4⁻* |
| CHP463 | *h⁻ ura4::fbp1-lacZ leu1-32 his7-366 git5Δ::his7⁺* |
| CHP477 | *h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git5Δ::his7⁺* |
| SLP17 | *h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 git11Δ::kanMX6* |
| SLP33 | *h⁺ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git5Δ::his7⁺ git11Δ::kanMX6* |
| SLP47 | *h⁺ leu1-32 ura4::fbp1-lacZ leu1-32 his7-366 git11Δ::kanMX6* |
| SLP58 | *h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M216 git11Δ::kanMX6 gpa2$^{R176H}$* |
| RWP4 | *h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M216 gpa2$^{R176H}$* |
| RWP30 | *h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 git5Δ::his7⁺ gpa2$^{R176H}$* |
| CHP362 | *h$^{90}$ leu1-32 ade6-M210 lys1-131* |
| CHP481 | *h$^{90}$ leu1-32 ade6-M216 lys1-131 gpa2Δ::ura4⁺* |
| CHP486 | *h$^{90}$ leu1-32 lys1-131 git5Δ::his7⁺* |
| CHP558 | *h$^{90}$ leu1-32 fbp1::ura4⁺ ade6-M216 git2Δ::LEU2⁺* |
| SLP44 | *h$^{90}$ leu1-32 ade6-M216 lys1-131 git11Δ::kanMX6* |

dates were rescued into *E. coli* (Hoffman and Winston 1987), and the inserts were sequenced by Bioserve Biotechnologies, (Laurel, MD) using primer TH5-F1 5′ CGTTTGGAATCACTA CAGGG 3′. Of these 10 plasmids, only pSL20 displayed a bait-specific interaction. Plasmid pSL20 contains the entire *git11* coding region with the exception of the start codon (see results). Specificity of the interaction was determined by co-transformation of bait plasmid pSL13 and prey plasmid pSL20 in appropriate combinations with pSE1112 (GBD-Snf1) or pSE1111 (GAD-Snf4; Fields and Song 1989) followed by testing for growth on SC-Trp-Leu-His medium containing 25 mm 3-aminotriazole (3AT) and for β-galactosidase production.

**Construction of functional *git11* clones:** Functional clones of the *git11* gene were constructed as follows. Plasmid pSL24, expressing an HA-tagged form of *git11* on a *URA3*⁺-based vector, was created by PCR amplifying *git11* from plasmid pSL20 using oligonucleotides git11-HA-for 5′ CTACTAGCTAGCATG GAAACAGAGGCTTTATTGAATG 3′ (that restores the START codon) and git11-HA-rev 5′ CGGGGTACCTTAGGAAATAGT ACAGCATTTGGTAGTGGC 3′. The PCR product was gel purified, digested with *Nhe*I and *Kpn*I, and ligated with *Nhe*I- and *Kpn*I-treated plasmid pALU (Chang *et al.* 1994). Plasmid pSL25 was created by replacing the 1.8-kb *Hin*dIII fragment containing the *URA3*⁺ selectable marker in pSL24 with a 2.2-kb *Hin*dIII fragment carrying the *LEU2*⁺ selectable marker from pARTCM (Chang *et al.* 1994).

***git5* plasmid constructions:** Three plasmids expressing git5 Gβ derivatives from the *S. pombe nmt41* promoter were constructed by the insertion of PCR products into the pNMT41-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. *Kpn*I-linearized plasmid pSL12 (Landry *et al.* 2000) was used as the template DNA for the PCR reactions. The insert in plasmid pSL27, which expresses the wild-type git5 protein, was generated using primers git5-for-topo 5′ ATGGATTCTGGGTCAAGAGTA 3′ and git5-rev-topo 5′ TTACCCTGACGAAGACCAGAGAC 3′. The insert in plasmid pSL28, which expresses a functional git5-V5 tagged protein [the V5 tag (Southern *et al.* 1991) is contributed by vector sequences], was generated using primers git5-for-topo 5′ ATGGATTCTGGGTCAAGAGTA 3′ and git5V5-rev 5′ CCC TGACGAAGACCAGAGAC 3′. The insert in plasmid pSL29, which expresses the 305-amino-acid git5 Gβ protein fused to the carboxy-terminal nine amino acids of the git11 Gγ protein, was generated using primers git5-for-topo 5′ ATGGATTCTGG GTCAAGAGTA 3′ and git5-CAAX-rev 5′ TTAGGAAATAGTA CAGCATTTGGTAGTGGCCCCTGACGAAGACCAGAGAC-3′.

**Deletion of the *git11* gene:** Strains deleted for the *git11* gene were constructed using the PCR-based gene targeting method of Bahler *et al.* (1998). Oligonucleotides git11-deltafor 5′ TACTAGGTGAGCACAGACGGTAGGAAGTGCACGT AAGATGCTTAAACAACGTTCCACAAAACACGGATCCCC GGGTTAATTAA 3′ and git11-deltarev 5′ CAAGGCTATAATT TTACTTAACAGGCATTACTTATTGAAATTGTAGTT GATCGGTCCTTAAACAAGAATTCGAGCTCGTTTAAAC 3′ were used to PCR amplify the kanMX6 cassette from pFA6a-GFP(S65T)-kanMX6 (Wach *et al.* 1997) such that the product was flanked with sequences from either side of the *git11* open reading frame (ORF). The PCR product was used to transform strain FWP72 to G418 resistance. The *git11* deletion was confirmed by PCR using oligonucleotides git11-test 5′ CCAAGCA AAATCGCATCTA 3′ and intKANtest 5′ CATCCTATGGAACT GCCTCGG 3′.

**Multicopy suppression analyses:** *S. pombe* strains FWP72 (wild type), CHP439 (*gpa2*Δ), CP477 (*git5*Δ), and SLP17 (*git11*Δ) were transformed to Leu⁺ with plasmids expressing *gpa2* (pRW7; expressing a *myc-gpa2* fusion, R. M. Welton and C. S. Hoffman, unpublished results), *git5* (pSL26; a derivative of pSL11, Landry *et al.* 2000, expressing a 6his-tagged *git5* gene), or *git11* (pSL25), as well as with the pART1 empty vector control (McLeod *et al.* 1987). β-Galactosidase activity was determined from two independent transformants for each host and plasmid combination as previously described (Nocero *et al.* 1994). The values given are the average specific activity ± standard error from three separate cultures of each transformant grown to exponential phase in PM medium containing 8% glucose (repressing conditions).

**Mating assays:** Homothallic (*h*$^{90}$) strains CHP362 (wild type), CHP481 (*gpa2*Δ), CHP486 (*git5*Δ), CHP558 (*git2*Δ), and SLP44 (*git11*Δ) were grown to exponential phase in PM liquid medium (at 37° to inhibit conjugation) and diluted to $10^6$ cells/ml in PM liquid medium with or without 5 mm cAMP. Cultures were incubated 30 hr at 30° without shaking and photographed.

**Co-immunoprecipitation studies:** *S. pombe* strain CHP463 (*git5*Δ) was cotransformed to Leu⁺, Ura⁺ with either plasmids pSL28 (git5-V5) and pSL24 (HA-git11), pSL28 (git5-V5) and

pALU (empty vector control), or pNMT41-TOPO (empty vector control) and pSL24 (HA-git11). Cultures were grown to exponential phase in PM-ura-leu (0.1% glucose, 3% glycerol), harvested, washed twice with chilled distilled water, and resuspended at a concentration of 1000:1 in chilled lysis buffer [50 mM HEPES, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals)]. Cell lysates were made by glass bead lysis, vortexing five times in a mini-beadbeater (BioSpec Products, Bartlesville, OK) at 4° for 1 min at maximum speed with 1-min intervals on ice. Protein extracts were clarified and quantitated with the bicinchonic acid (BCA) kit (Pierce Chemical Co., Rockford, IL). Co-immunoprecipitations were performed as described by Celenza *et al.* (1989) with three modifications. The immunoprecipitation buffer contained 0.5 mg/ml of BSA instead of ovalbumin. The extracts were precleared with 0.5 μg of normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and Protein A beads (Sigma, St. Louis) for 1 hr. Immunoprecipitation of HA-git11 was carried out by incubating extracts with 1 μg of α-HA (Roche Molecular Biochemicals) for 2 hr followed by the addition of Protein A beads for 1.5 hr. Whole-cell extracts and α-HA precipitated proteins were resolved on a 15% SDS polyacrylamide gel and transferred to Immobilon P (Millipore Corp., Bedford, MA). The filter was probed with HRP-conjugated α-HA (Roche Molecular Biochemicals) and HRP-conjugated α-V5 (Invitrogen) antibodies and visualized with HRP-conjugated goat α-mouse antibody and LumiGlo Chemiluminescent Substrate Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

## RESULTS

**A two-hybrid screen identifies a potential Gγ partner for the git5 Gβ:** To investigate whether the git5 Gβ interacts with a Gγ partner, we conducted a two-hybrid screen for *S. pombe* genes whose products physically interact with git5. The bait in the screen was the *S. cerevisiae* GBD fused to the 305-residue git5 protein. Candidate plasmids from the *S. pombe* two-hybrid library, whose products interact with git5, trigger the expression of *HIS3* and *lacZ* reporter genes in the host strain YRG-2, resulting in $His^+$ (3AT-resistant) growth and the production of β-galactosidase that causes colonies to stain blue in an Xgal filter lift. Plasmid pSL20, identified in this screen, displays a bait-specific interaction (Figure 2A), as indicated by the fact that only the combinations of the GBD-git5 bait and the pSL20-encoded GAD-git11 prey, or the control GBD-Snf1 bait and GAD-Snf4 prey (Fields and Song 1989), produce 3AT-resistant, Xgal-blue transformants.

DNA sequence analysis of the insert in pSL20 reveals a 71-codon open reading frame whose product bears key signatures of a Gγ subunit. This gene is present on cosmid c215 (accession no. AL033534; open reading frame 4) from the *S. pombe* genome database (http://www.sanger.ac.uk/Projects/S_pombe/index.shtml), which carries a portion of chromosome 2. The sequence of the pSL20 cDNA insert, identical to base pairs 6367 to 6419 and 6509 to 7006 of cosmid c215, confirms the predicted splice junction. The genomic sequence indicates that only the start codon of this gene, designated *git11*, is missing from the cDNA clone. The 72-amino-acid git11 protein (accession no. CAA22118) is similar in length to mammalian Gγ subunits and resembles the human Gγ1 and Gγ4 subunits (Figure 2B). Critical features shared by these proteins include lysine residues in the carboxy-terminal region of the proteins and a carboxy-terminal CAAX box, the site of prenylation needed to associate the Gβγ dimer with the peripheral membrane (Casey 1994). Thus, git11 appears to be a viable candidate for the Gγ partner to the git5 Gβ.

**Co-immunopreciptation of git5 Gβ and git11 Gγ:** We confirmed that the git5 and git11 proteins physically interact in *S. pombe* by co-immunoprecipitation. Protein extracts from cells expressing either a functional git5-V5 tagged Gβ, a functional HA-git11 tagged Gγ, or both tagged proteins were subjected to immunoprecipitation using anti-HA antibody. The precipitated proteins were examined by Western blot analysis for the presence of the git5-V5 Gβ (see materials and methods). Immunoblotting to detect the git5-V5 (Figure 3, top) or HA-git11 (Figure 3, bottom) proteins in whole-cell extracts revealed that the relative amount of git5-V5 was reduced in cells not overexpressing HA-git11 [Figure 3, lanes 1 and 2; the host strain carries a *git5* deletion while transcription of the endogenous *git11* gene is very low (data not shown) compared to that of the *adh*-driven *HA-git11* construct]. More importantly, the git5-V5 protein was detected only in the precipitated material from the extract from cells expressing HA-git11 (Figure 3, lanes 4–6). A similar interaction was seen using HA-git11 together with a git5-GFP tagged protein expressed from the *git5* locus (data not shown). The git5-V5 tagged Gβ expressed from plasmid pSL28 contains only the 305-codon git5 open reading frame fused to sequences encoding the V5 tag (Southern *et al.* 1991); thus the 305-amino-acid git5 Gβ that lacks an amino-terminal coiled-coil domain physically interacts with the git11 Gγ *in vivo*.

**Deletion of the *git11* gene confers phenotypes associated with defects in the glucose/cAMP pathway:** To test whether the putative git11 Gγ acts in the glucose/cAMP pathway, we constructed *git11*Δ deletion strains and characterized them with regard to transcriptional regulation of the glucose-repressed *fbp1* gene and regulation of conjugation and sporulation. Deletion of the *git11* gene from strain FWP72 to create strain SLP17 causes a significant increase in β-galactosidase expression from an integrated *fbp1-lacZ* reporter (Hoffman and Winston 1990; Tables 2 and 3). SLP17 (*git11*Δ) cells also display increased expression of an integrated *fbp1-ura4*$^+$ reporter resulting in $Ura^+$ and 5-FOA-sensitive growth similar to that of *gpa2*Δ and *git5*Δ strains, while FWP72 (*git*$^+$) cells are $Ura^-$ and 5-FOA resistant due to glucose repression of transcription from the *fbp1* promoter (Figure 4; see empty vector control transformants). These growth phenotypes represent the criteria on which the original collection of *git* mutants, including *gpa2/git8*

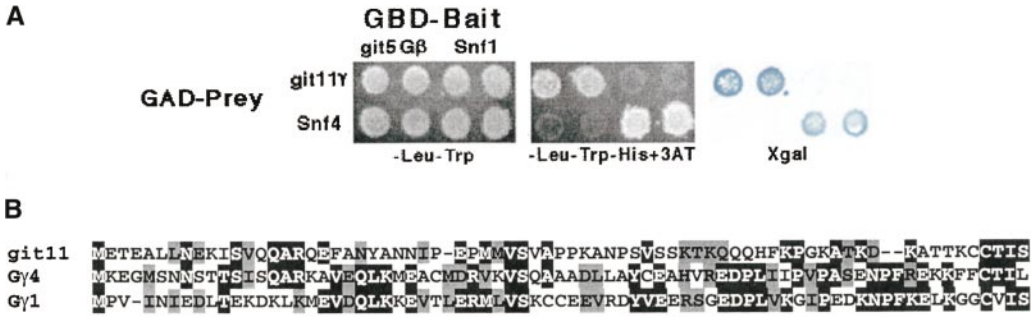


FIGURE 2.—Identification of the git11 Gγ. (A) Two-hybrid screening strain YRG-2 was cotransformed with bait plasmids expressing either GBD-git5 or GBD-Snf1 and prey plasmids expressing either GAD-git11 or GAD-Snf4. Duplicate transformants were tested for growth in the absence or presence of 25 mM 3AT and for β-galactosidase activity by Xgal filter lift. The results suggest that git5 and git11 physically interact, as do Snf1 and Snf4. (B) The amino acid sequence alignment of the predicted git11 protein and two human Gγ subunits. The git11 sequence (accession no. CAA22118) was aligned with the human gamma 1 (accession no. Q08447) and human gamma 4 (accession no. NP_004476) protein sequences using the Clustal W (version 1.8) sequence alignment program (THOMPSON *et al.* 1994) and displayed using BOXSHADE. Identical residues are shaded in black with white letters, while conserved residues are shaded in gray with black letters.

and *git5* mutants, was identified (HOFFMAN and WINSTON 1990). In addition and similar to our previous observations using strains carrying a point mutation or deletion of the *git5* Gβ gene (BYRNE and HOFFMAN 1993; LANDRY *et al.* 2000), SLP47 (*git11*Δ) cells possess wild-type basal levels of intracellular cAMP but fail to increase intracellular cAMP in response to glucose (data not shown).

The *git11*Δ allele was introduced into a homothallic ($h^{90}$) strain to determine the effect of this deletion on the regulation of sexual development. Homothallic strains undergo mating-type switching to produce mating partners in a purified population. Wild-type cells growing in a nutrient-rich medium show little or no mating (Figure 5), whereas strains defective in glucose signaling due to the loss of gpa2 (Gα), git5 (Gβ), or git2 (adenylate cyclase) readily mate and sporulate to produce asci (Figure 5; MAEDA *et al.* 1990; ISSHIKI *et al.* 1992; LANDRY *et al.* 2000). The $h^{90}$ *git11*Δ cells also display starvation-independent sexual development. This unregulated conjugation and sporulation is suppressed in all four mutant strains by the addition of 5 mM cAMP to the growth medium (Figure 5), indicating that the defect in these mutant strains is in cAMP signaling.

**The *git5* Gβ and *git11* Gγ genes display the same genetic relationship to the *gpa2* gene:** We previously showed that multicopy $gpa2^+$ partially suppresses a mutation or deletion of the *git5* Gβ gene, while multicopy $git5^+$ has no effect on a *gpa2* deletion (NOCERO *et al.* 1994; LANDRY *et al.* 2000). We have now extended this analysis to include the *git11* gene. Overexpression of gpa2 (by expression of a functional *myc-gpa2* fusion from the *adh* promoter) completely suppresses a *gpa2* deletion and partially suppresses *git5* and *git11* deletions with regard to glucose repression of an *fbp1-lacZ* reporter. Multicopy *git5* and multicopy *git11* only affect *fbp1-lacZ*

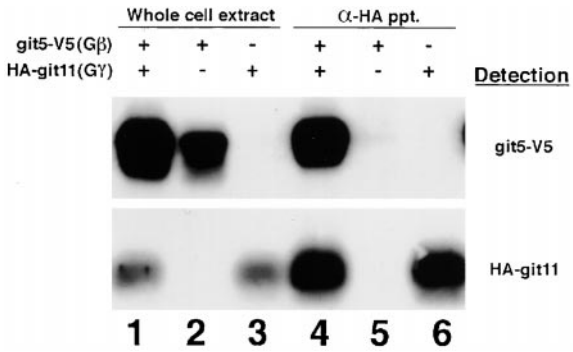


FIGURE 3.—Co-immunoprecipitation of the git5 Gβ with the git11 Gγ. Protein extracts were prepared from strain CHP463 (*git5*Δ) transformed with either pSL28 (git5-V5) and pSL24 (HA-git11; lanes 1 and 4), pSL28 (git5-V5) and pALU (empty vector control; lanes 2 and 5), or pNMT41-TOPO (empty vector control) and pSL24 (HA-git11; lanes 3 and 6). Immunoblots were performed to detect git5-V5 (top) and HA-git11 (bottom) from whole-cell extracts (lanes 1–3). The HA-git11 protein was immunoprecipitated using α-HA antibody (see MATERIALS AND METHODS) and the precipitated proteins were examined by immunoblot (lanes 4–6).

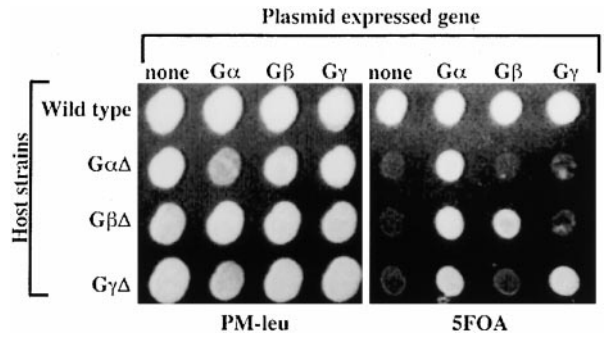


FIGURE 4.—Genetic analysis of *gpa2*, *git5*, and *git11* deletions and multicopy expression on *fbp1-ura4* expression. FWP72 (wild type), CHP439 (*gpa2*Δ), CHP477(*git5*Δ), and SLP17 (*git11*Δ) strains were transformed with either an empty vector control (pARTCM; 6) or plasmids expressing *gpa2* (pRW7), *git5* (pSL26), or *git11* (pSL25). Glucose repression of an integrated *fbp1-ura4* reporter gene in these strains results in 5-FOA-resistant growth, while constitutive *fbp1-ura4* expression results in 5-FOA sensitivity.

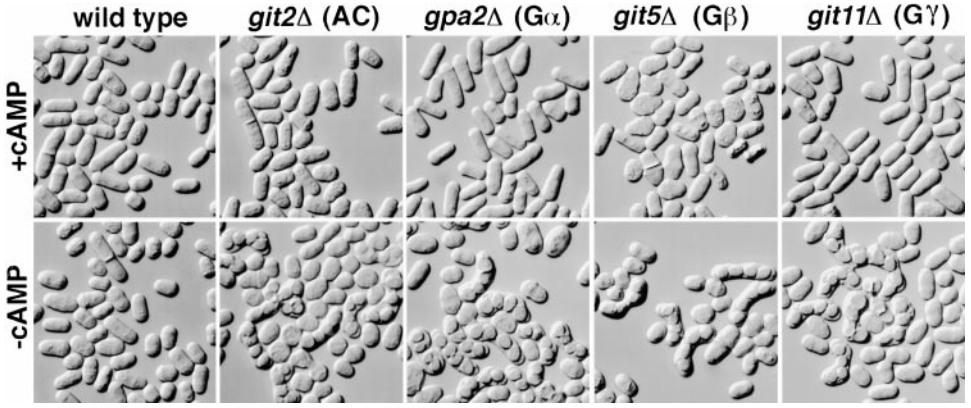


FIGURE 5.—Starvation-independent sexual development in *git2*Δ (adenlyate cyclase), *gpa2*Δ (Gα), *git5*Δ (Gβ), and *git11*Δ (Gγ) homothallic strains. Cells of homothallic strains CHP362 (wild type), CHP481 (*gpa2*Δ), CHP486 (*git5*Δ), CHP558 (*git2*Δ), and SLP44 (*git11*Δ) were pregrown at 37° to inhibit conjugation and then grown overnight in glucose- and nitrogen-rich medium (in the presence or absence of 5 mM cAMP) at 30° before photographing.

expression in *git5* and *git11* deletion strains, respectively (Table 2). The same suppression pattern is observed when examining the ability of these plasmids to restore glucose repression of an *fbp1-ura4* reporter resulting in 5-FOA$^R$ growth (Figure 4). Meanwhile, deletion of either *git5* or *git11* is completely suppressed by the *gpa2*$^{R176H}$ "activated" allele that carries a mutation in the coding region of the GTPase domain of the Gα subunit (Table 3; ISSHIKI *et al.* 1992; WELTON and HOFFMAN 2000). We previously showed that the *gpa2*$^{R176H}$ allele suppresses mutations in *git5* (Gβ) and *git3* (putative G-protein-coupled glucose receptor), but not in *git1*, *git7*, or *git10* (WELTON and HOFFMAN 2000). Thus, *git5* and *git11* display the same genetic relationship to *gpa2*, consistent with a model in which these genes encode the two subunits of the Gβγ dimer acting in the *S. pombe* glucose/cAMP pathway.

**Deletion of the *gpa2* Gα, *git5* Gβ, and *git11* Gγ genes produce quantitatively distinct effects on *fbp1-lacZ* expression:** To characterize the relative roles in cAMP signaling, we measured the effects of *gpa2* (Gα), *git5* (Gβ), and *git11* (Gγ) deletions on *fbp1-lacZ* expression (Table 3). The effect of each deletion on *fbp1-lacZ* expression is distinguishable with the *gpa2* deletion causing a 250-fold increase, the git5 deletion causing a >100-fold increase, and the git11 deletion causing a >30-fold increase (Table 3). These results suggest that the Gα subunit remains partially active in the absence of the Gβγ dimer. They also indicate that either the Gβ subunit remains partially active in the absence of the Gγ subunit or that git11 is not the only Gγ partner for the git5 Gβ.

To investigate whether the git5-git11 Gβγ carries out any additional role in glucose monitoring other than to facilitate the activation of the gpa2 Gα, we co-overexpressed git5 and git11 in *gpa2* mutant strains. This overexpression failed to reduce *fbp1-lacZ* expression in a *gpa2-60* (reduction in function allele; HOFFMAN and WINSTON 1990; NOCERO *et al.* 1994) strain (FWP175) grown under glucose-rich conditions, which possessed 458 ± 38 units of β-galactosidase activity relative to 518 ± 14 units detected in a control transformant carrying empty vectors pART1 and pALU. It therefore appears that the only role of the Gβγ dimer is to regulate Gα activity.

**Bypass of a *git11* deletion by the addition of the git11 CAAX box to git5:** The quantitative difference between a *git5* deletion (Gβ) and a *git11* deletion (Gγ; Tables 2 and 3) indicates that either the Gβ is partially active in the absence of Gγ or that git11 is not the only Gγ partner for git5. The suggestion that a Gβ subunit can remain partially functional in the absence of a Gγ part-

**TABLE 2**

**Multicopy effects of *gpa2*, *git5*, and *git11* on *fbp1-lacZ* expression**

| | | β-Galactosidase activity: Plasmid-expressed gene | | | |
|---|---|---|---|---|---|
| Strain | Relevant genotype | None | *gpa2* Gα | *git5* Gβ | *git11* Gγ |
| FWP72 | Wild type | 18 ± 2 | 17 ± 5 | 24 ± 4 | 15 ± 3 |
| CHP439 | *gpa2*Δ | 2542 ± 181 | 23 ± 5 | 2300 ± 200 | 2626 ± 357 |
| CHP477 | *git5*Δ | 1600 ± 245 | 227 ± 44 | 54 ± 7 | 1183 ± 104 |
| SLP17 | *git11*Δ | 410 ± 14 | 147 ± 32 | 329 ± 22 | 15 ± 3 |

β-Galactosidase activity was assayed in transformants grown under glucose-repressed conditions that carry plasmids pART1 (none; empty vector control), pRW7 (*gpa2*), pSL11 (*git5*), or pSL25 (*git11*) as described in the MATERIALS AND METHODS. Values represent specific activity ± standard error from three independent cultures of two transformants for each combination of host and plasmid.

**TABLE 3**

***fbp1-lacZ* expression in *gpa2*, *git5*, and *git11* mutant strains**

| Strain | Relevant genotype | β-Galactosidase activity |
|---|---|---|
| FWP72 | Wild type | 9 ± 3 |
| CHP439 | *gpa2*Δ | 2262 ± 81 |
| CHP477 | *git5*Δ | 937 ± 50 |
| SLP17 | *git11*Δ | 306 ± 30 |
| RWP4 | $gpa2^{R176H}$ | 6 ± 0 |
| RWP30 | $gpa2^{R176H}$ *git5*Δ | 8 ± 1 |
| SLP58 | $gpa2^{R176H}$ *git11*Δ | 7 ± 1 |

β-Galactosidase activity was assayed in strains grown under glucose-repressed conditions. Values represent specific activity ± standard error from three independent cultures.

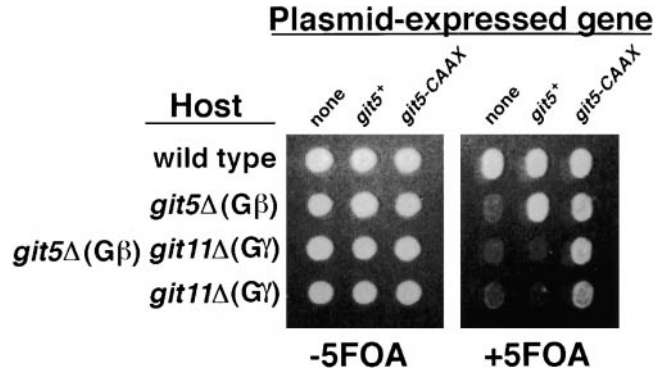


FIGURE 6.—Functional testing of the git5-CAAX chimeric protein. Strains FWP72 (wild type), CHP477 (*git5*Δ), SLP33 (*git5*Δ *git11*Δ), and SLP17 (*git11*Δ) were transformed with either pNMT41-TOPO (empty vector control), pSL27 (*nmt41*-driven *git5*$^+$), or pSL29 (*nmt41*-driven *git5-CAAX*; *git5* fused to the carboxy-terminal nine codons of *git11*). Purified transformants were pregrown on PM-leu (8% glucose) medium. Equal numbers of cells were spotted to either PM-leu (−5-FOA) or 5-FOA-containing (+5-FOA) medium and grown for 2 days before photographing. Glucose repression of an integrated *fbp1-ura4* reporter gene in these strains results in 5-FOA-resistant growth, while constitutive *fbp1-ura4* expression results in 5-FOA sensitivity.

ner is unprecedented. This would imply that the Gβ subunit can properly fold in the absence of Gγ and may require Gγ only for localization of Gβ to the peripheral membrane. If true, the addition of a CAAX box to the git5 Gβ subunit might partially or fully bypass the need for an intact git11 Gγ subunit. However, if there are multiple Gγ subunits, and Gβγ dimer formation is required for function, the addition of a CAAX box to the Gβ subunit would most likely inhibit dimer formation and G-protein activity. To distinguish between these two hypotheses, we fused the coding region for the carboxy-terminal nine residues from git11 to the git5 ORF and tested the ability of this construct to suppress either or both *git5*Δ and *git11*Δ deletions. The git5-CAAX chimeric protein, expressed from the *nmt41* promoter on plasmid pSL29, suppressed the 5-FOA$^S$ growth (indicating a restoration of glucose repression of the *fbp1-ura4* reporter gene) to strains carrying either a *git5*Δ or *git11*Δ single deletion or a *git5*Δ *git11*Δ double deletion (Figure 6). Thus the addition of the CAAX box increases the function of git5 in the absence of git11, since expression of the wild-type git5 protein from the same promoter on plasmid pSL27 only suppressed the growth defect in the *git5*Δ single deletion strain (Figure 6). A quantitative analysis of the effect of these plasmids on expression of the *fbp1-lacZ* reporter in these same transformants shows that the git5-CAAX protein is only partially better than wild-type git5 in restoring glucose repression to a *git5*Δ *git11*Δ double deletion strain (Table 4). However, this analysis is complicated by the fact that the git5-CAAX protein is less functional than the wild-type git5 protein in a strain that expresses a functional git11 protein (strain CHP477, Table 4).

## DISCUSSION

The cloning and characterization of the *S. pombe git11* gene suggest that its product is the Gγ subunit of the gpa2-git5-git11 heterotrimeric G protein responsible for adenylate cyclase activation in response to glucose detection. While git11 is a conventional Gγ subunit, its git5 Gβ partner lacks the amino-terminal coiled-coil domain that has been suggested by both structural and functional studies to be critical to the assembly of the Gβγ dimer. Our previous observation that the *S. pombe* git5 Gβ lacks this domain (LANDRY *et al.* 2000) brought this system into conflict with one of two well-established paradigms of G-protein research. The first is that G proteins of this type, as opposed to the Ras-like monomeric G proteins, are heterotrimeric. This principle is already challenged by the fact that the *S. cerevisiae* Gpa2 and *S. pombe* gpa1 Gα subunits appear to function in the absence of a Gβγ dimer. Thus it seemed equally plausible that a heterodimeric G protein lacking a Gγ subunit could exist. The second paradigm is that the Gβ amino-terminal coiled-coil is essential for both the folding of the Gβ subunit and its assembly with Gγ (GARRITSEN *et al.* 1993; GARCIA-HIGUERA *et al.* 1996; PELLEGRINO *et al.* 1997). As we have demonstrated here, the interaction between the git5 Gβ and the git11 Gγ indicates that the WD repeat β-barrel of the Gβ subunit can be sufficient to allow Gβγ dimer assembly.

The functional relationship of the G-protein subunits in the *S. pombe* glucose/cAMP pathway is clearly different from that of the *S. cerevisiae* pheromone response pathway in which the Gβγ dimer activates a downstream MAPK pathway. Due to this functional relationship of the G-protein subunits in *S. cerevisiae*, mutations in the *GPA1* Gα gene confer the opposite phenotypes to those associated with mutations in the *STE4* Gβ gene or the *STE18* Gγ gene (DIETZEL and KURJAN 1987; WHITEWAY *et al.* 1989; HIRSCH and CROSS 1992). In the *S. pombe*

**TABLE 4**

**Partial bypass of the loss of the git11 Gγ by a git5-CAAX chimeric protein**

| Strain | Relevant genotype | β-Galactosidase activity: Plasmid-expressed gene | | |
|---|---|---|---|---|
| | | None | *git5*$^+$ | *git5-CAAX* |
| FWP72 | Wild type | 15 ± 2 | 36 ± 6 | 26 ± 6 |
| CHP477 | *git5*Δ | 1149 ± 89 | 78 ± 33 | 268 ± 90 |
| SLP33 | *git5*Δ *git11*Δ | 1024 ± 73 | 703 ± 6 | 374 ± 39 |
| SLP17 | *git11*Δ | 569 ± 9 | 407 ± 53 | 395 ± 40 |

β-Galactosidase activity was assayed in transformants grown under glucose-repressed conditions that carry plasmids pNMT41-TOPO (none; empty vector control), pSL27 (*git5*), or pSL29 (*git5-CAAX*) as described in MATERIALS AND METHODS. Values represent specific activity ± standard error from one to two cultures of two independent transformants.

glucose/cAMP pathway, the *gpa2* (Gα), *git5* (Gβ), and *git11* (Gγ) genes function cooperatively, as evidenced by the similar defects in *fbp1* transcriptional regulation (Figure 4, Table 3) and nutrient regulation of sexual development (Figure 5) observed in *gpa2*, *git5*, and *git11* mutants. These results, along with the multicopy suppression studies (Table 2), indicate that the gpa2 Gα is the key activator of adenylate cyclase in response to glucose detection and that the git5-git11 Gβγ is a positive regulator of Gα. Consistent with this model, the loss of the gpa2 Gα has a greater effect on glucose repression of *fbp1* transcription than does the loss of the git5 Gβ or the git11 Gγ (Table 3). Conversely, mutational activation of gpa2 fully suppresses the loss of either git5 or git11 (Table 3). For proper glucose signaling to occur, the Gβγ dimer may be required to promote an efficient interaction between the Gα subunit and git3, the likely glucose receptor (WELTON and HOFFMAN 2000). A similar role has been observed for the *S. cerevisiae* Ste4-Ste18 Gβγ in coupling of the Gpa1 Gα to the Ste2 pheromone receptor (BLUMER and THORNER 1990). As overexpression of both the Gβ and the Gγ subunit has no effect on *fbp1-lacZ* expression in a *gpa2* mutant strain, we conclude that the Gβγ dimer does not have any Gα-independent role in glucose monitoring.

Deletion of the *git5* Gβ gene confers a two- to fourfold greater increase in *fbp1-lacZ* expression than does the deletion of the *git11* Gγ gene (Tables 2–4); therefore it appears that the git5 Gβ retains some function in the absence of its Gγ partner. Consistent with this suggestion, the addition of a CAAX box to the carboxy-terminus of the git5 Gβ increases the function of Gβ in cells lacking Gγ (Figure 6, Table 4). As mentioned above, this result does not support the alternative hypothesis that *S. pombe* expresses multiple Gγ subunits. Thus the git11 Gγ appears to act to localize the git5 Gβ to the peripheral membrane but is not required for proper folding of the Gβ subunit. While the git5-CAAX protein clearly bypasses the need for a git11 Gγ as judged by the 5-FOA growth test (Figure 6), results from β-galactosidase assays measuring the effect of these constructs on expression of the *fbp1-lacZ* reporter (Table 4) are not as convincing. We have observed similar discrepancies between 5-FOA growth results and β-galactosidase activity while studying suppression of mutations affecting the protein kinase A pathway by multicopy *pyp1* (DAL SANTO *et al.* 1996; only a partial reduction in β-galactosidase activity was observed) and *sck1* (JIN *et al.* 1995; no reduction in β-galactosidase activity was observed). We believe that these discrepancies represent heterogeneity in the population of plasmid-containing cells. If a subpopulation of cells establishes glucose repression of the *fbp1-ura4* reporter due to a particular level of expression of the git5-CAAX fusion protein, these cells will grow on the 5-FOA medium to produce a 5-FOA$^R$ patch. However, if the majority of the cells fail to establish glucose repression of the *fbp1-lacZ* reporter due to over- or underexpression of the git5-CAAX fusion protein, the β-galactosidase activity in the overall culture will show little or no change from control cultures.

Our results stand in striking contrast to data from studies showing that mammalian Gβ subunits are unable to fold in the absence of Gγ partners (GARCIA-HIGUERA *et al.* 1996) and that the Gβ amino-terminal coiled-coil is essential for dimer assembly (GARRITSEN *et al.* 1993). The apparent conflict between those studies and ours may point to an inherent difference in the biology of cells that express a single Gβ and Gγ subunit *vs.* ones that express multiple forms of each subunit. In mammalian cells, the requirement of the Gβ amino-terminal coiled-coil for Gβγ association and thus the proper folding of the Gβ subunit may allow cells to tightly regulate the combinations of Gβγ dimers assembled. If mammalian Gβ subunits could fold into β-barrels in the absence of Gγ subunits, it might reduce the stringency of the dimer interaction and allow the subsequent assembly of a broader range of Gβγ dimers. Alternatively, these monomeric Gβ subunits might either promote or interfere with signaling in pathways other than the ones in which they normally act. These issues do not arise in *S.*

*pombe*, which expresses a single species of Gβ and Gγ subunit. Even so, we detect lower levels of the Gβ subunit in extracts from cells overexpressing the tagged git5-V5 Gβ subunit alone relative to those of cells overexpressing both git5-V5 and HA-git11 Gγ (Figure 3, lanes 1 and 2). Thus, stability of the git5 Gβ subunit may be partially dependent upon assembly of a Gβγ dimer, showing that the behavior of the fission yeast Gβγ is not wholly unlike that of the mammalian dimer. In the same vein, it is possible that some as yet untested mammalian Gβ subunits may behave more like the git5 Gβ with respect to folding in the absence of a Gγ partner. The continued study of the structure and function of G proteins may therefore identify a broad spectrum of structural tendencies rather than a few strictly followed rules.

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