Activated Alleles of the *Schizosaccharomyces pombe gpa2*+ Gα Gene Identify Residues Involved in GDP-GTP Exchange

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The *Schizosaccharomyces pombe* glucose/cyclic AMP (cAMP) signaling pathway includes the Gpa2-Git5-Git11 heterotrimeric G protein, whose Gpa2 Gα subunit directly binds to and activates adenylate cyclase in response to signaling from the Git3 G protein-coupled receptor. To study intrinsic and extrinsic regulation of Gpa2, we developed a plasmid-based screen to identify mutationally activated gpa2 alleles that bypass the loss of the Git5-Git11 Gβγ dimer to repress transcription of the glucose-regulated *fbp1*+ gene. Fifteen independently isolated mutations alter 11 different Gpa2 residues, with all but one conferring a receptor-independent activated phenotype upon integration into the gpa2+ chromosomal locus. Biochemical characterization of three activated Gpa2 proteins demonstrated an increased GDP-GTP exchange rate that would explain the mechanism of activation. Interestingly, the amino acid altered in the Gpa2(V90A) exchange rate mutant protein is in a region of Gpa2 with no obvious role in Gα function, thus extending our understanding of Gα protein structure-function relationships.

Heterotrimeric guanine-nucleotide binding proteins (G proteins) relay extracellular signals from surface receptors to intracellular effectors. G proteins are composed of Gα subunits and Gβγ dimers whose interaction depends upon whether Gα is bound to GDP or GTP. The inactive heterotrimer contains a Gα subunit bound to GDP. Ligand binding by seven transmembrane G protein-coupled receptors (GPCRs) leads to GDP release and GTP binding by Gα, resulting in a conformational change that decreases Gα affinity for Gβγ and, depending on the system, promotes effector binding (32). Either or both Gα-GTP and Gβγ are then able to regulate downstream effectors (28). Restoration of the basal state is achieved through hydrolysis of the bound GTP by the Gα and G protein heterotrimer reassociation (28). GTP hydrolysis can be accelerated by protein-protein interactions of Gα with regulator of G protein signaling (40, 50) proteins or through Gα-effector interactions (4).

A variety of approaches, including mutational studies, biochemical analyses of purified G protein subunits, and high-resolution crystallography, inform our current understanding of the mechanisms behind guanine nucleotide binding and release, as well as GTP hydrolysis reactions that govern Gα activation and inactivation cycles. Crystal structure data are available for Gα subunits in the GDP-bound (20, 31) or GTP-bound (5, 29, 42) conformation. Gα subunits possess two domains, a helical domain and a GTPase domain that resembles small GTPases, such as Ras, Rho, and Rheb. Bound guanine nucleotides are buried in a cleft between these domains, which are connected by two linker sequences. Current models suggest that interdomain interactions regulate both receptor-activated and basal GDP release rates (8, 9, 34).

Mutagenesis studies have been used to identify and characterize Gα residues critical for guanine nucleotide binding (1, 33, 38, 39), basal GDP release rates (34, 43), and GTP catalysis (19, 43). Other mutations that alter G protein function involve extrinsic mechanisms of Gα regulation, including altered interactions with receptors [as with *Saccharomyces cerevisiae* Gpa1(N388D) (51)], Gβγ subunits (reviewed in reference 6), effector enzymes (7), or RGS proteins [as with *S. cerevisiae* Gpa1(G184S) (21)].

In the fission yeast *Schizosaccharomyces pombe*, extracellular glucose appears to be detected by the Git3 GPCR that triggers activation of adenylate cyclase via the Gpa2 Gα subunit (11, 12). Gpa2 has been shown to physically interact with an amino-terminal domain in *S. pombe* adenylate cyclase (16), while the Gβγ dimer presumably acts to deliver Gpa2 to the Git3 receptor for activation (22, 23). The ensuing cyclic AMP (cAMP) signal activates protein kinase A (PKA) to repress the transcription of genes involved in gluconeogenesis and sexual development, including *fbp1*+, which encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase.

To study the regulation of Gpa2-mediated signaling, we developed a genetic screen to identify activated alleles of *gpa2* that repress transcription of an *fbp1-ura4* reporter, which has been used previously to identify mutations that reduce or elevate PKA activity (13, 41, 46). In this study, we identified 15 candidate activated *gpa2* alleles representing 12 distinct mutations. All but one bypass the loss of the Git3 receptor when introduced into the *gpa2* chromosomal locus. We have expressed and purified three of these activated Gpa2 proteins from *Escherichia coli*. All three activated proteins display an increased GDP-GTP exchange rate relative to wild-type Gpa2, which would account for the mechanism of activation. Surprisingly, one of the affected residues (V90) is in a portion of the helical domain that is unlikely to be directly involved in either

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nucleotide binding or interdomain interactions. Thus, our current understanding of G protein activation cannot explain why the gpa2(V90A) mutation, as well as the nearby gpa2(S81F) mutation, leads to receptor-independent activation of Gpa2.

MATERIALS AND METHODS

Strains, media, and recombinant-DNA techniques. The yeast strains used in this study are listed in Table 1. The yeasts were grown at 30°C on pombe medium (PM) (48) supplemented with 3% glucose, required nutrients (at 75 mg/liter, except for leucine, which was present at 150 mg/liter), and 10 μg/ml thiamine where indicated or on 5-FOA medium (based on synthetic complete [SC] medium) containing 8% glucose, 50 mg/liter uracil, and 0.4 g/liter 5-fluoroorotic acid (5-FOA) (13). Yeast transformations were performed according to the protocol of Bähler et al. (2). When candidate activated alleles were integrated into the gpa2 locus (replacing a ura4+–marked deletion of gpa2), cells were grown in yeast extract liquid medium (10) for 20 h before being plated onto 5-FOA medium to select for loss of the ura4+ marker. DNA-sequencing reactions were performed using the CEQ Dye Terminator Quick Start Kit (Beckman Coulter) and analyzed on an ABI Prism 6800 using the CEQ 800 Genetic Analysis System (Beckman Coulter).

Two-hybrid interactions. S. cerevisiae strain YRG2 (Stratagene) was used to characterize two-hybrid interactions, as previously described (22). Bait plasmids expressing Gpa2* or mutationally activated Gpa2(R716H) have been described previously (16). The prey plasmid was a derivative of pACT2 that expressed the third cytoplasmic loop through to the carboxy terminus of Git3 (residues 207 to 466). The facilitating plasmids were either pGBS10 (35) as a negative control or a pGBS10 derivative in which the lambda cI repressor coding region was replaced by the Git5 Gii coding region via gap repair transformation. For this, the Git5 Gii coding region was PCR amplified using the oligonucleotides Git5afacF (5'-CAAGCTTATACCAAGCATACATACCTAAAGCTGTACTACGTTGCAGGTTAACCG-3') and Git5afacR (5'-AACATATATAACTCAAAAGAAT TGCCCGGAATTGGTCGTCAGTTACCTGACGAGAACAGC-3') with the bait, and preying plasmids were used to transform YRG2 to Trp', Leu', and G418', respectively. Bait-prey interactions were scored by growth on SC medium lacking Trp, Leu, and His (SC-TRP-Leu, His) with or without 3-aminoanthrazone (3AT) to detect HIS3 reporter expression and by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) filter lift to detect lacZ reporter expression (13).

Random mutagenesis of gpa2* and screen for activated gpa2 alleles. Wild-type S. pombe gpa2* was amplified from XhoI-linearized SLE1-cDNA library DNA (17) by PCR using the FailSafe PCR System (buffer G; Epicentre Biotechnologies; pooling two separate reactions carried out using buffers A and K), Taq polymerase (New England Biolabs), or Pfu polymerase (Stratagene). Plasmid pDII was the template DNA, and the PCR primers annealed to regions that flank the cloning site in the pNMT vector series to amplify gpa2 and flanking vector sequences. The PCR products were cloned via gap repair transformation into EcoRI-linearized pNMT1 (a derivative of the TOPO pNMT1 isolated from E. coli that does not contain insert DNA) (18, 47). The screen to detect activated alleles of gpa2 was carried out as follows. Strain SLP29, lacking all three genes encoding the three subunits of the S. pombe glucose/cAMP pathway heterotrimERIC protein, was used as the host strain for gap repair transformation and detection of gpa2 activated alleles, which bypass the defect in glucose signaling to repress fbpl-ura4 transcription and confer 5-FOA resistance (5-FOA') growth (22). The cells were transformed to Leu+ using EcoRI-digested pNMT1 and PCR-amplified gpa2 DNA and plated onto PM-Leu medium (containing thiamine) to select for transformants. Leu+ colonies were allowed to grow for 3 days at 30°C and then were replica plated onto 5-FOA medium and incubated at 30°C for 2 days to screen for 5-FOA' growth. 5-FOA' colonies were grown on PM-Leu medium, and plasmids were rescued into E. coli as previously described (14). To confirm that 5-FOA' growth was plasmid conferred, strain SLP29 (gpa2Δ gpa5 gpa7) was reconstructed with the rescued plasmids and rescreened as described above. The gpa2 open reading frames from plasmids that conferred 5-FOA' growth upon retransformation were sequenced with custom primers to cover the entire gpa2 open reading frame on both strands.

Integration of gpa2 activated alleles into the gpa2 chromosomal locus. Site-directed mutagenesis was performed using QuickChange PCR (Stratagene) to introduce the 12 gpa2 mutations identified in this study, along with gpa2(R716H), into a genomic clone of gpa2* (unlike the form used for detection of activated gpa2 alleles, this clone contains the gpa2 promoter and an intron). Integration of these alleles, as well as gpa2*, into the chromosomal gpa2 locus was detected by 5-FOA counterselection due to replacement of a gpa2Δ ura4+ allele in host strain MZP2. Candidate transformants were verified by PCR and DNA sequence analyses. Mutational activation of gpa2 was assessed by the ability of the introduced allele to suppress a deletion of the git3+ GPCR gene (git3Δ) that confers a glucose-sensing defect to elevate fbpl-lacZ expression in glucose-grown cells, as reported for gpa2(R716H) (49). β-Galactosidase activity was measured in glucose-grown cultures of these strains as previously described (30).

Expression and purification of Gpa2 variants. cDNA clones of gpa2*, gpa2(K720E), gpa2(L727P), and gpa2(V90A) were subcloned into the E. coli expression vector pET15b (Novagen), which provides an N-terminal hexahistidine tag to facilitate protein purification (24). E. coli BL21(DE3) Star cells (Invitrogen) were transformed to AmpR by these plasmids and grown in 2 liters of 2× yeast tryptone (YT) at 30°C to an optical density at 600 nm (OD600) of 0.4 to 0.6. Gpa2 protein expression was induced with 30 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 16°C for 20 h (36). Cells were collected, and the pellets were resuspended in 20 volumes of ice-cold lysis buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 4 mM 2-mercaptoethanol, 200 mM methyl-mercaptan, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 50 mM GDP, 10 mM leupeptin, 1 mM pepstatin, 10 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 100 mg/mL chicken egg white lysozyme) (51) and lysed by ultrasonication at 4°C. The lysates were centrifuged at 150,000 × g for 30 min at 4°C. The resulting soluble fractions

| Strain          | Genotype                      |
|-----------------|-------------------------------|
| CHP468          | ade6-M216 his7-32 ura4::fbpl1-lacZ fbpl1::ura4* gpa2Δ::ura4* |
| SLP29           | ade6-M216 his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::his7 git11Δ::kan gpa2Δ::ura4* |
| DIP10           | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2::ura4* |
| MZP2            | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2::ura4* |
| MZP5            | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(R716H) |
| MZP6            | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(F62S) |
| MZP7            | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(K270E) |
| MZP8            | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(L57P) |
| MZP9            | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(K270N) |
| MZP10           | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(S149F) |
| MZP12           | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(T325A) |
| MZP13           | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(K904A) |
| MZP14           | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(V237A) |
| MZP15           | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(T94-A) |
| MZP18           | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(V90A) |
| FYP1            | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(I56S) |
| FYP2            | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(S81F) |
| FYP3            | his7-366 leu1-32 ura4::fbpl1-lacZ fbpl1::ura4* git3Δ::kan gpa2(E184D) |
were collected and adjusted to 350 mM NaCl and 12.5 mM imidazole by adding 8 × binding buffer (160 mM HEPES, pH 7.5, 2.8 M NaCl, 100 mM imidazole) (similar to that described in reference 37) before loading them onto a preequilibrated 1-ml anion-exchange HiTrap Q-Sepharose fast protein liquid chromatography (FPLC) column to remove contaminating proteins (Gpa2 does not bind to Q-Sepharose). Purified Gpa2 protein preparations were immediately assayed for [35S]GTPγS binding as previously described (51).

RESULTS

The Git5 Gβ enhances the interaction of the Gpa2 Gα and the Git3 GPCR. Unlike the Saccharomyces cerevisiae pheromone signaling pathway, in which the primary function of the Gpa1p Gα is to prevent signaling by the Ste4p-Ste18p Gβγ dimer in the absence of a pheromone signal, all three subunits of the S. pombe glucose/cAMP signaling pathway G protein act to promote signaling (11). Thus, the Git5-Git11 Gβγ dimer presumably facilitates the interaction between the Gpa2 Gα subunit and the Git3 GPCR. To test this hypothesis, we examined whether expression of the Git5 Gβ would enhance a two-hybrid interaction between the Gpa2 Gα and the region of the Git3 GPCR that was expected to interact with Gpa2 (from the third cytoplasmic loop to the carboxy terminus). Wild-type Gpa2+ or mutational activated Gpa2(R176H) bait plasmids (16) were introduced together with a prey plasmid that expressed amino acids 207 to 466 of Git3, as well as a third plasmid that expressed either the Git5 Gβ (+Gβ) or the lambda cI repressor protein (−Gβ, as a negative control) (Fig. 1). Consistent with our model, there was a weak interaction between Gpa2+ and Git3 that was strengthened by the coexpression of the Git5 Gβ. This was demonstrated by the enhanced growth in medium lacking histidine and containing 3AT (Fig. 1C), as well as an increase in expression of a lacZ reporter (Fig. 1D).

Development of a genetic screen for constitutively activated gpa2 alleles. In an effort to identify and characterize activated alleles of gpa2, we developed a plasmid-based screen to detect gpa2 alleles that bypassed the glucose/cAMP signaling defect conferred by the loss of the Git5 Gβ and Git11 Gγ subunits (22, 23). As overexpression of the wild-type gpa2+ gene bypassed the loss of Gβγ (22, 30), we had to first identify a level of expression that would distinguish between wild-type and activated alleles of gpa2. Therefore, various expression levels of wild-type Gpa2+ and the known constitutively activated Gpa2(R176H) were compared using the no message on thiamine (pNMT) expression vector series (Invitrogen) that allows for various levels of gene expression when used in combination with exogenous thiamine (3, 27). The vectors pNMT1 (carrying the full-strength nmt1 promoter) and pNMT41 (carrying the moderate-strength nmt41 promoter) expressing either gpa2(R176H) or gpa2+ were used to transform CHP468 (gpa2Δ) and SLP29 (gpa2Δ git5Δ git11Δ) were transformed to Leu+ with pNMT41-1 (empty vector) (columns A), pDI2 (pNMT41-gpa2+), pDI3/pNMT41-gpa2(R176H) (columns B), pDI28 (pNMT41-myc-gpa2+) (columns D). Transformants were pregrown on PM-Leu medium with and without 10 μg/ml thiamine before being replica plated onto 5-FOA medium. The plates were incubated for 2 days at 30°C before being photographed.

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repression of the nmt41 promoter reduced gpa2+ expression to a level that conferred 5-FOA' growth on CHP468 (gpa2Δ) transformants, but not SLP29 (gpa2Δ git3Δ git11Δ) transformants (Fig. 2, right plate, column B). Thus, at this level of Gpa2 expression, the Gβγ dimer was still required for glucose-mediated repression of fbp1 transcription. However, this level of expression of the GTPase-defective Gpa2(R176H) subunit bypassed the Gβγ requirement for signaling to confer 5-FOA' growth on SLP29 (gpa2Δ git5Δ git11Δ) transformants (Fig. 2, right plate, column C). Based on these results, we subsequently screened for novel mutationally activated alleles of gpa2 by cloning PCR-generated copies of the gpa2 cDNA into the pNMT41 expression vector and pregrowing SLP29 transformants in the presence of thiamine before replica plating them onto 5-FOA medium. Under these conditions, only activated alleles of gpa2 were expected to confer 5-FOA' growth.

Isolation of activated gpa2 alleles. Activated gpa2 alleles were generated by PCR amplification, cloned by gap repair transformation, and detected using the screen described above. The initial PCR conditions utilized Taq polymerase under nonmutagenic conditions, as multiple mutations from mutagenic PCR would likely lead to inactive Gpa2 Go subunits rather than activated ones. As indicated in Table 2, 6.5% of the Leu' transformants carrying Taq-generated inserts displayed 5-FOA' growth. However, only 25% of these candidate clones (8 of 32 sequenced plasmids) carried mutations within the gpa2 ORF that could account for the 5-FOA' growth. In an effort to optimize the screen, we compared two other methods of PCR amplification. As expected, only 1.6% of transformants carrying inserts made by the high-fidelity Pfu DNA polymerase displayed 5-FOA' growth (Table 2). No further effort was made to determine how many of these carried activated gpa2 alleles. PCR products were also generated by amplification using the FailSafe PCR system (pooling two reactions, one with buffer A and one with buffer K). Remarkably, there was a significant increase relative to the Taq-mediated amplification in both the initial frequency of 5-FOA' colonies and the frequency of recovered plasmids that carried missense mutations in the gpa2 ORF (Table 2) (7 of 16 sequenced plasmids carried gpa2 ORF mutations). In addition, both transformations and transversions were observed among the seven FailSafe-generated alleles, while all eight Taq-generated mutations were transitions.

Three mutations [gpa2(T49I), gpa2(L57P), and gpa2(K270E)] were identified twice from independent PCRs and screens. Finally, only one of these 15 alleles carried two mutations (one of which was a silent mutation); thus, further deconvolution of the candidate alleles into single mutations was not required.

As part of the screening process, candidate plasmids were rescued into E. coli for purification and transformation of SLP29 to determine whether the 5-FOA' phenotype is plasmid conferred. This was true for all 15 candidate plasmids included in Table 2. In addition, strain DIP10 (gpa2Δ git3Δ) was transformed with these plasmids, and all transformants displayed 5-FOA' growth (data not shown). Therefore, these mutations did not simply allow signaling between Gpa2 and Git3 in the absence of the Gβγ dimer, they bypassed glucose signaling through the Git3 GPCR.

Chromosomal integration of gpa2 alleles to confirm activation. Our screen identified plasmids containing mutant alleles of gpa2; however, we could not yet conclude that each missense mutation was responsible for the 5-FOA' growth of the transformants. Many plasmids recovered from other 5-FOA' colonies did not contain any sequence change in the gpa2 ORF (Table 2) and yet transformed SLP29 to 5-FOA'. These plasmids may carry mutations that alter the plasmid copy number or the level of transcription from the nmt41 promoter. Similar mutations may be present in plasmids that contain a missense mutation, leading to the incorrect assignment of a particular Gpa2 residue as having a role in Gα regulation. Alternatively, a mutation could weakly activate Gpa2 so that it is detectable only when expressed from the plasmid and not from the gpa2+ chromosomal locus. Therefore, to identify false positives among these mutations, each mutation was reintroduced in single copy into the gpa2+ locus. This was accomplished by performing site-directed mutagenesis on a genomic clone of gpa2 (the original screen was carried out using a plasmid lacking the single gpa2 intron) and knocking the mutant allele into the gpa2 locus in strain MZP2 bearing a ura4Δ::LEU2 integrated disruption of the gpa2 gene (see Materials and Methods). As controls, both the wild-type gpa2+ and the well-characterized GTPase-defective gpa2(R176H) alleles were also introduced into MZP2. This strain also carries a deletion of the git3+ GPCR gene and an fbp1-lacZ reporter, so that β-galactosidase assays can be used to measure the ability of the gpa2 allele to suppress the loss of the Git3 GPCR.

As shown in Table 3, 11 of the 12 gpa2 alleles [with the exception of gpa2(E184D)] identified in these screens, as well as gpa2(R176H), restored repression of the fbp1-lacZ reporter in a strain lacking the Git3 GPCR. Conversely, loss of Gpa2 activity further elevated fbp1-lacZ expression. Therefore, most of these strains carry activated alleles of gpa2. As described in the introduction, Gα subunits possess two discrete structural domains, an alpha-helical domain and a GTPase domain, that are connected by a pair of linkers. The 10 residues affected by these mutations (lysine 270 is changed to a glutamic acid and to an asparagine in different alleles) are found in both domains, as well as the linker 1 region (Table 3 and Fig. 3 and 4).
Gpa2(L57P), Gpa2(V90A), and Gpa2(K270E) variants are GDP-GTP exchange mutants. To initiate a biochemical analysis of these activated forms of Gpa2, we expressed three Gpa2 variants, along with wild-type Gpa2, in E. coli and purified the proteins to near homogeneity (see Materials and Methods). Coomassie staining of SDS-PAGE gels detected most of the protein as a single band migrating at 41 kDa, the predicted molecular mass of His6-Gpa2 (data not shown). The three mutant Gpa2 proteins were chosen to represent the three different regions of Gpa2 that were altered in our mutant collection; Gpa2(K270E) is altered in a residue of the GTPase domain that directly contacts the guanine nucleotide (red in Fig. 3A and B), Gpa2(L57P) is altered in a residue in the linker 1 region (green in Fig. 3C), and Gpa2(V90A) is altered in a residue in the helical domain (green in Fig. 3D). All three mutations confer potent activation of Gpa2 (Table 3) to repress fbp1-lacZ expression in a strain lacking the Git3 GPCR.

After purification in the presence of GDP, the Gpa2 proteins were immediately assayed for GTP binding, reflecting the rate of GDP release that is required to allow GTP binding. Wild-type Gpa2 displayed a binding rate of 3.0 ± 1.5 fmol GTP/min/mg, while significantly higher binding rates were observed for Gpa2(K270E) (27.8 ± 3.5 fmol GTP/min/mg) versus Gpa2(L57P) (68.9 ± 18.5 fmol GTP/min/mg) and

| gpa2 allele | Domain | β-Gal activity |
|-------------|--------|----------------|
| gpa2Δ       |        | 1,240 ± 166    |
| gpa2*       |        | 390 ± 101      |
| gpa2(L57P)  | Linker 1 | 15 ± 13        |
| gpa2(L57P)  | Linker 1 | 7 ± 1          |
| gpa2(V90A)  | Helical | 13 ± 8         |
| gpa2(V90A)  | Helical | 8 ± 3          |
| gpa2(3149F) | Helical | 7 ± 1          |
| gpa2(T49A)  | GTPase  | 8 ± 1          |
| gpa2(R176H) | GTPase  | 5 ± 0          |
| gpa2(E184D) | GTPase  | 658 ± 90       |
| gpa2(K270E) | GTPase  | 7 ± 1          |
| gpa2(K270N) | GTPase  | 5 ± 3          |
| gpa2(T325A) | GTPase  | 37 ± 20        |
| gpa2(V327A) | GTPase  | 11 ± 4         |

β-Galactosidase activity was determined from three or four independent cultures grown overnight in yeast extract rich medium containing 8% glucose to a final density of ~1 × 10^7 cells/ml. The assays were carried out as previously described (30). All strains carry a deletion of the git3 GPCR gene, which can be suppressed by mutational activation of Gpa2 to restore repression of fbp1-lacZ expression. The values represent the mean specific activities per milligram of soluble protein ± standard errors.

FIG. 3. Crystal structure of transducin Gα bound to GDP, showing the locations of Gpa2 residues altered by activating mutations. The structure (Protein Data Base [PDB] ID 1TAG) was generated using RasMol. GDP is displayed in white using the spacefill format. (A and B) The 5 residues (T49 [blue], S149 [cyan], K270 [red], T325 [yellow], and V327 [green]) that contact the guanine nucleotide are displayed in the spacefill format. Two orientations of the structure are provided to show the contacts with GDP. (C) The 3 residues (I56 [blue], L57 [green], and F62 [red]) located in the linker 1 region are displayed in the spacefill format. (D) The 2 residues (S81 [blue] and V90 [green]) located in the αA helix of the helical domain are displayed in the spacefill format.
Gpa2(V90A) (66.0 ± 8.5 fmol GTP/S/min/mg). Thus, all three mutations conferred an enhanced GDP-GTP exchange rate that could result in receptor-independent Gpa2 activation.

**DISCUSSION**

We carried out a genetic screen to identify an unbiased collection of activated gpa2 alleles encoding Go subunits that signal to adenylate cyclase in the absence of either the Gβγ dimer or the GPCR. This collection includes 15 independently isolated mutations that represent 12 distinct missense mutations affecting 11 different Gpa2 residues. While isolated in a plasmid-based screen, all but one mutation conferred an activated phenotype when introduced in single copy into the gpa2 genomic locus (Table 3).

Though it was not the central focus of this study, we determined that the FailSafe PCR system provides a method for random mutagenesis superior to the use of Taq polymerase (Table 2). Of note, we recovered both transitions and transversions from FailSafe-generated mutant alleles, while only

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**FIG. 4.** Alignment of Go subunits and *S. pombe* Rheb protein, together with identification of altered residues in activated *S. pombe* Gpa2 proteins. *S. pombe* (Sp) Gpa2 (CAB16244), *S. cerevisiae* (Sc) Gpa2 (NP 010937), human transducin α subunit (NP 000163), *S. pombe* Gpa1 (CAA21150; Go of the pheromone pathway), and *S. pombe* Rhb1 (CAA22291) were aligned using Clustal W (44) and displayed using BOXSHADE 3.21. Identical residues are shaded in black. Conserved residues are shaded in gray. Dashes were introduced by the alignment software. The locations of altered residues that activate *S. pombe* Gpa2 (Table 3) are indicated above the alignment, while the locations of two altered residues that activate *S. pombe* Rhb1 (45) are indicated below the alignment.
transitions were found among 

**Taq-generated mutant alleles.**

Additional studies of the products made in PCRs using the 12 individual FailSafe buffers may lead to further optimization of random-mutagenesis protocols.

The 11 activating amino acid substitutions can be placed into three groups based on the physical locations of the affected residues. The first group includes T49A, S149F, K270E, K270N, T325A, and V327A (Fig. 4), residues that all contact the guanine nucleotide (Fig. 3A and B). Consequently, these GDP/GTP-binding pocket substitutions most likely either reduce GTP hydrolysis or increase GDP dissociation. Consistent with this, we observed a nearly 9-fold increase in the GDP-GTP exchange rate in the Gpa2(K270E) protein. Four of the five residues in this group, or adjacent residues, have been previously identified in Gα or small GTPase nucleotide exchange mutants. The T49A substitution is equivalent to the K120R substitution in Rhb1 (Fig. 4) (45). Rhb1 protein, while the K270E and K270N substitutions in Schizosaccharomyces pombe (Fig. 3C) do not suggest a role in nucleotide binding or in dinucleotide binding. The J. Biol. Chem. 274:19639–19643.

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