Nutrient Control of Yeast PKA Activity Involves Opposing Effects on Phosphorylation of the Bcy1 Regulatory Subunit

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INTRODUCTION

Protein kinase A (PKA) is a component of eukaryotic signaling pathways that is activated by a conserved mechanism that involves binding of cAMP to the PKA regulatory subunit and the resulting release of active catalytic kinase subunits. In the yeast *Saccharomyces cerevisiae*, PKA is essential for viability and is activated by nutrient signals (Dechant and Peter, 2008; Zaman et al., 2008; Rubio-Texeira et al., 2009; Smets et al., 2010). High levels of PKA activity in yeast cause inhibition of stress responses and induction of pseudohyphal and invasive growth. Yeast contain three forms of the PKA catalytic subunit, called Tpk1, Tpk2, and Tpk3, and one form of the regulatory subunit, called Bcy1 (Toda et al., 1987a,b). Although the most well-established mechanism of PKA regulation is its activation by binding of cAMP, studies using yeast mutants that lack adenylyl cyclase have suggested that PKA could be regulated in other ways (Lu and Hirsch, 2005; Peeters et al., 2006). Uncovering an alternative mechanism of PKA regulation in yeast could be relevant to the other systems that employ this common signaling component.

The yeast G protein α-subunit Gpa2 and its coupled receptor Gpr1 activate a signaling pathway that optimizes normal growth, inhibits stress responses, and promotes pseudohyphal growth, similar to the effects of high levels of PKA activity (Kühler et al., 1997; Lorenz and Heitman, 1997; Colombo et al., 1998; Xue et al., 1998). Gpr1 is present on the cell surface and appears to function as a low-affinity glucose receptor that responds to high concentrations of glucose in the extracellular environment (Xue et al., 1998; Kraakman et al., 1999; Rolland et al., 2000; Welton and Hoffman, 2000; Lemaire et al., 2004). GPR1 and GPA2 are not essential genes, and phenotypes resulting from deletion of GPR1 and GPA2 can be suppressed by addition of extracellular cAMP (Kühler et al., 1997; Lorenz and Heitman, 1997; Lorenz et al., 2000; Tamaki et al., 2006). The yeast G protein α-subunit Gpa2 and its coupled receptor Gpr1 activate a signaling pathway that optimizes normal growth, inhibits stress responses, and promotes pseudohyphal growth, similar to the effects of high levels of PKA activity (Kühler et al., 1997; Lorenz and Heitman, 1997; Lorenz et al., 2000; Tamaki et al., 2006). The idea that Gpa2 stimulates adenylyl cyclase to produce cAMP, similar to mammalian G_{ras}, is supported by evidence showing that cells lacking Gpa2 are defective for the spike in cAMP levels that is induced by the addition of glucose (Colombo et al., 1998; Kraakman et al., 1999; Lorenz et al., 2000). Moreover, the GTP-bound form of Gpa2 has been shown to bind preferentially to adenylyl cyclase (Peeters et al., 2006). These results demonstrate that one function of Gpa2 is to stimulate adenylyl cyclase to produce cAMP and activate PKA.

The yeast kelch repeat proteins Gpb1 and Gpb2 were identified based on their ability to interact with Gpa2 in a yeast two-hybrid screen (Harashima and Heitman, 2002; Batlle et al., 2003; Peeters et al., 2006). Analysis of the sequence of the GPB1 (also called KRH2) and GPB2 (also called KRH1) gene products indicates that they contain either six (Batlle et al., 2003) or seven (Harashima and Heitman, 2002) kelch repeats, which are predicted to fold into a β-propeller structure (Adams et al., 2000). Because Gpa2 does not appear to associate with classical G protein β-subunits and the kelch...
repeat proteins are predicted to fold into the same structure as β-subunits, it was originally proposed that Gpb1 and Gpb2 function as G protein β-subunit mimics for Gpa2 (Harashima and Heitman, 2002). However, genetic analysis does not support the idea that the kelch repeat proteins function as β-subunit mimics (for review, see Peeters et al., 2007). Moreover, it has been shown that the domain of Gpa2 that binds to Gpb1 and Gpb2 is not the region of the α-subunit that binds to β-subunits (Niranjan et al., 2007). Recently, another WD repeat protein has been proposed to function as the β-subunit for Gpa2 (Zeller et al., 2007). These results indicate that Gpb1 and Gpb2 are unlikely to function as G protein β-subunit mimics for Gpa2.

Cells that lack Gpb1 and Gpb2 display an increase in pseudohyphal and invasive growth, indicating that the kelch repeat proteins are negative regulators of the signaling pathway (Harashima and Heitman, 2002; Battke et al., 2003). A number of observations support the idea that Gpb1 and Gpb2 negatively regulate signaling by directly inhibiting the activity of PKA. It was initially shown that the increase in pseudohyphal and invasive growth conferred by gpb1Δ gpb2Δ mutations requires the PKA catalytic subunit Tpk2 (Harashima and Heitman, 2002; Battke et al., 2003). Subsequently, it was shown that deletion of GBP1 and GBP2 stimulates the growth of cells that contain a constant level of cAMP, suggesting that Gpb1 and Gpb2 affect the activity of PKA by a process that does not involve changes in cAMP levels (Lu and Hirsch, 2005; Peeters et al., 2006). Moreover, deletion of GBP1 and GBP2 causes an increase in phosphorylation of PKA substrates in vivo, indicating that PKA activity is affected by the presence of Gpb1 and Gpb2 (Lu and Hirsch, 2005). The PKA regulatory subunit Bcy1 is also required for the increase in signaling conferred by gpb1Δ gpb2Δ mutations (Peeters et al., 2006). And finally, Gpb2 has been shown to interact with Tpk1, Tpk2, and Tpk3 (Peeters et al., 2006). These results strongly suggest that the inhibitory function of Gpb1 and Gpb2 acts directly on PKA.

Several different functions for Gpb1 and Gpb2 have been proposed by others to explain their effects on signal transduction. The proposed mechanisms include a role for Gpb1 and Gpb2 in inhibition of receptor-G protein coupling (Harashima and Heitman, 2005), positive regulation of Ras GTPase activating proteins (Harashima et al., 2006), and stimulation of the association between PKA regulatory and catalytic subunits (Peeters et al., 2006). The results presented here support the idea that Gpb1 and Gpb2 promote the association between PKA regulatory and catalytic subunits by stimulating Bcy1 phosphorylation and stabilization in opposition to the activity of PKA catalytic subunits. In cells that lack Gpb1 and Gpb2, Bcy1 phosphorylation is unstable, which results in a higher proportion of free active catalytic subunits and an increase in PKA activity.

MATERIALS AND METHODS

Strains and Media

Strains used in this study are listed in Table 1. Construction of strains containing the gpb1::URA3 and gpb2::HIS3 alleles (Battke et al., 2003), the gpa2::TRP1 allele (Xue et al., 1998), and the gpb2::TRP1, tpk1-1::TRP1, tpk2-2::TRP1, and tpk3-3::HIS3 alleles (Lu and Hirsch, 2005) were described previously. The gpb1::TRP1 allele was made by transformation of a gpb1::URA3 strain with a 3.6-kb Smal fragment from marker swap plasmid pUT11 (Cross, 1997). The ras2::HIS3 allele was made by transformation of cells with the 6.1-kb EcoRI fragment from plasmid pras2::HIS3, which contains the HIS3 gene inserted into the Psal site of the RAS2 gene. The gpb1::LEU2 allele was made by transformation of cells with the 5.3-kb BamHI fragment from plasmid pbcy1::URA3 (Toda et al., 1997a).

Table 1. Strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| SKY762 | MATa ura3-52 trp1::hisG leu2::his3::hisG | S. Palecek/S. Kron |
| HS182-3.K27a | MATa gpb1::TRP1 gpb2::HIS3 | This study |
| HS257-12Da | MATa gpb1::URA3 ras2::HIS3 gpb2::HIS3 | This study |
| HS260-2Ba | MATa tpk1-1::TRP1 tpk2-2::TRP1 tpk3-3::HIS3 yak1::LEU2 | This study |
| HS275-1Ca | MATa gpb2::HIS3 | This study |
| HS275-9Ba | MATa gpb2::TRP1 | This study |
| HS275-16Ca | MATa ras2::HIS3 | This study |
| HS278-1Dc | MATa gpb1::TRP1 gpb2::HIS3 ras2::HIS3 | This study |
| HS281-2Ca | MATa gpb1::TRP1 gpb2::HIS3 | This study |
| HS282-23Ca | MATa gpb1::TRP1 gpb2::HIS3 | This study |
| HS287-2Ca | MATa bcyl::URA3 | This study |
| HS293-10Da | MATa gpb1::URA3 | This study |
| HS297-2Bb | MATa yak1::LEU2 | This study |
| HS300-17Bb | MATa gpb1::TRP1 gpb2::TRP1 yak1::LEU2 | This study |
| HS309-8Bb | MATa bcyl::URA3 | This study |
| W3031B | MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 | Thomas and Rothstein (1989) |
| Y352bb | MATa tpk1M164G tpk2M147G tpk3M165G | Yorimitsu et al. (2007) |
| Y3527 k1Tk2Ub | MATa tpk1M164G tpk2M147G tpk3M165G gpb1::URA3 gpb2::TRP1 | This study |

a SKY762 background; SKY762 is derived from Σ1278b.
b W303 background.

Yeast cells were grown on YEPD (2% glucose), and strains under selection were grown on synthetic dropout media, as described (Xue et al., 1998).

Plasmid Construction

Plasmid 313pBHBI-416 (Griffioen et al., 2000) was kindly provided by Gerard Griffioen (reMYND, Brussels, Belgium). Plasmids pBCS145E-313.3, pBCS145D-313.3 and pBCS2020-313.3 are identical to 31pBHBI-416, except that codon 145 of BCY1 was changed from TCT to GAG, GAT, or GCT, respectively, by site-directed mutagenesis. Plasmid pRAS2AV-111.9 contains the activated RAS2M147G allele in vector YCplac11.

Yeast Methods and RNA Isolation

To test the effect of low glucose concentrations, cells were grown to early log phase, washed once, and resuspended in the medium lacking glucose. Glucose was added to a final concentration of 2, 0.2, 0.05, or 0.005%, cells were incubated for 3 h at 30°C with shaking, and cells lysates were prepared. Alternatively, cells were grown to early log phase overnight in 2, 0.2, or 0.05% glucose.

For Bcy1 half-life determination, cycloheximide (Sigma, St. Louis, MO) was added to cells growing in log phase to a final concentration of 1 or 5 μM. Cells were incubated for 90 min with shaking, and cell lysates were prepared as described below.

For inhibition of PKA, 1NM-PP1 (VWR Scientific, West Chester, PA) was added to cells growing in log phase to a final concentration of 1 or 5 μM. Cells were incubated for 90 min with shaking, and cell lysates were prepared as described below.
Yeast RNA extraction and measurement of FLO11 and ACT1 RNA by real time RT-PCR was performed on a LightCycler version 3.5 instrument (Roche, Indianapolis, IN), as described previously (Niranjan et al., 2007).

**Immunobots and Immunoprecipitation Assays**

Lysates for immunobots and immunoprecipitation assays were prepared from cells growing in log phase (OD$_{600}$ = 0.3–0.85) as described previously (Lu and Hirsch, 2005), with the modification that cells were lysed in 100–200 µl lysis buffer (20 mM K$_2$HPO$_4$, pH 7.4, 0.5% TX-100, 25 mM NaF, 100 µM Na$_3$VO$_4$, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1× protease inhibitor cocktail [Roche], and 10× phenSTOP phosphatase inhibitor cocktail [Roche]). An equal amount of total protein from each lysate was loaded and resolved by 8% SDS-PAGE or 4–15% gradient SDS-PAGE and analyzed by immunoblotting. Blots were probed with the following antibodies: anti-hemagglutinin (HA) 12CA5 mAb at a dilution of 1/1000, anti-myc 9E10 mAb at a dilution of 1/1000, anti-phospho-PKA substrate (RRXS/T) rabbit mAb (Cell Signaling, Beverly, MA) at a dilution of 1/1000, anti-phosphorylase kinase (PGK) rabbit polyclonal antiserum at a dilution of 1/300,000, and anti-actin rabbit polyclonal antibody (Sigma) at a dilution of 1/100,000. The infrared signal was scanned using the Odyssey Infrared Imaging System (Cincinnati, OH).

For immunoprecipitations, samples containing 500 µg of total protein were incubated with 3–4 µg of anti-myc 9E10 antibody for at least 2 h at 4°C with constant mixing. The samples were then incubated with 50 µl of 50% protein G-agarose slurry (Roche) for at least three hours or overnight at 4°C. The samples were washed three times with cold wash buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1× protease inhibitor cocktail [Roche], and 10× phenSTOP phosphatase inhibitor cocktail [Roche]), and proteins were resolved by SDS-PAGE and analyzed by immunoblotting.

**RESULTS**

**Effect of gpb1Δ gpb2Δ Mutations on Signaling Requires PKA Catalytic Subunits**

The kelch repeat proteins Gpb1 and Gpb2 control PKA activity by negatively regulating one or more components of the PKA/cAMP pathway. Potential targets of negative regulation by Gpb1 and Gpb2 include Gpa2 (Harashima and Heitman, 2002; Batlle et al., 2003), Ras GTPase-activating proteins (GAPs; Harashima et al., 2006), and the cAMP-dependent kinase PKA (Peeters et al., 2006). To investigate the effect of GPB1 and GPB2 on these signaling components, a real time RT-PCR assay for FLO11 RNA abundance was performed in various mutant strains. The FLO11 gene, which encodes a cell surface flocculin, is induced in response to increases in cAMP levels in strains that are capable of undergoing pseudohyphal and invasive growth (Rupp et al., 1999).

Deletion of GPB1 and GPB2 results in a substantial increase in FLO11 RNA abundance in both wild-type and gpa2Δ strains (Figure 1A), as described previously (Harashima and Heitman, 2002; Batlle et al., 2003). The same effect is seen in a ras2Δ strain (Figure 1A). Quantification of the results showed that there is a six- to sevenfold increase in FLO11 RNA levels in gpb1Δ gpb2Δ strains compared with the corresponding GPB1 GPB2 strains in wild-type and ras2Δ backgrounds. Although there is a very little FLO11 RNA present in a gpa2Δ strain, the level of FLO11 RNA in a gpb1Δ gpb2Δ strain is just slightly less than that seen in a GPA2 gpb1Δ gpb2Δ strain, as described previously (Harashima and Heitman, 2002; Batlle et al., 2003). These results demonstrate that the effect of gpb1Δ gpb2Δ mutations is not solely dependent on either RAS2 or GPA2. To test whether Gpa2 and Ras GAPs comprise all the targets of Gpb1 and Gpb2, FLO11 RNA levels were measured in strains that lack both Gpa2 and Ras2. In a gpa2Δ ras2Δ strain, FLO11 RNA is present at a very low level (Figure 1A). However, deletion of GPB1 and GPB2 in such a strain confers a significant threefold increase in the amount of FLO11 RNA over the basal level. This result indicates that at least one target of Gpb1 and Gpb2 regulation is capable of transmitting a signal in a strain that lacks both Gpa2 and Ras2.

To test the possibility that PKA is a major target of Gpb1 and Gpb2, FLO11 RNA levels were measured in strains containing deletions of the PKA catalytic subunit genes. Because the triple deletion of TPK1, TPK2, and TPK3 produces a lethal phenotype, the viability of this strain was maintained by a yak1Δ mutation (Garrett and Broach, 1989). In a tpk1Δ tpk2Δ tpk3Δ yak1Δ strain, FLO11 RNA is present at a very low level (Figure 1B). Deletion of GPB1 and GPB2 in such a strain does not cause an increase in the amount of FLO11 RNA. In cells containing a yak1Δ mutation alone, deletion of GPB1 and GPB2 results in a substantial increase in FLO11 RNA abundance, demonstrating that the yak1Δ allele has no effect on the phenotype conferred by the gpb1Δ.
gpbΔ mutations. These results indicate that PKA catalytic subunits are required for the effect of Gpb1 and Gpb2 on signaling.

Previous results have shown that Gpb1 and Gpb2 do not function by regulating adenylate cyclase, because deletion of GPB1 and GPB2 has an effect in cells containing a deletion of the adenylate cyclase gene CYR1 (Lu and Hirsch, 2005; Peeters et al., 2006). Therefore, a reasonable interpretation of these findings is that Gpb1 and Gpb2 act directly on PKA to inhibit its activity. This idea is consistent with the previous observation that both the catalytic and regulatory subunits of PKA are required for the effect of gpb1Δ gpb2Δ mutations on the accumulation of reserve carbohydrates (Peeters et al., 2006).

Phosphorylation and Abundance of Bcy1 Are Affected by gpbΔ gpbΔ Mutations When Glucose Concentrations Are Low

In yeast, PKA is involved in the response to extracellular glucose through a complex network of nutrient signaling pathways (Dechant and Peter, 2008; Zaman et al., 2008; Rubio-Texeira et al., 2009; Smets et al., 2010). Cells that contain high PKA activity do not respond appropriately to decreased glucose concentration. Given that Gpb1 and Gpb2 are negative regulators of PKA activity, it is possible that the physiological role of these proteins is to inhibit PKA activity under conditions of low glucose. In cells lacking all PKA catalytic subunits, gpb1Δ gpb2Δ mutations do not affect signaling (Figure 1; Peeters et al., 2006). However, in cells containing any one of the three PKA subunits, gpb1Δ gpb2Δ mutations confer an increase in signaling (Lu and Hirsch, 2005). One way in which Gpb1 and Gpb2 could control the activity of all PKA catalytic subunits is by affecting the sole PKA regulatory subunit, Bcy1. As glucose is depleted from the medium, Bcy1 increases in abundance and becomes phosphorylated at serine-145, the autocatalytic site, and at multiple unknown sites (Werner-Washburne et al., 1991; Griffioen et al., 2001). These changes are thought to contribute to the decrease in PKA activity under these conditions. These observations raise the possibility that Gpb1 and Gpb2 inhibit PKA activity by affecting the abundance or modification of Bcy1 under conditions of low glucose.

To determine whether Gpb1 and Gpb2 are involved in the response to low glucose, wild-type and gpb1Δ gpb2Δ cells grown to log phase in 2% glucose were transferred to medium containing lower concentrations of glucose, and protein levels of HA-Bcy1 were measured by immunoblot. For the wild-type strain, transferring cells to 0.2, 0.05, or 0.005% glucose caused a substantial increase in Bcy1 levels (Figure 2A, lanes 1–4). The degree of Bcy1 phosphorylation also increased in response to lower concentrations of glucose. For the gpb1Δ gpb2Δ strain, transferring cells to low glucose concentrations resulted in Bcy1 levels that were more than 10-fold lower than the levels present in wild-type cells grown at the same glucose concentrations (Figure 2A, lanes 5–8). Moreover, the degree of Bcy1 phosphorylation was not substantially changed in gpb1Δ gpb2Δ cells in response to lower concentrations of glucose. These results strongly suggest that Gpb1 and Gpb2 act in parallel to reduce PKA activity in response to low levels of extracellular glucose.

The results shown above indicate that deletion of GPB1 and GPB2 has multiple effects on Bcy1 in response to glucose concentration. These effects could be due to changes in transcription of the BCY1 gene, changes in stability of the Bcy1 protein, or modification of Bcy1. PKA activity is subject to feedback regulation through regulation of cAMP breakdown (Nikawa et al., 1987; Mbonyi et al., 1990; Ma et al., 1999), which suggests that some of these effects could be indirect consequences of high PKA activity in gpb1Δ gpb2Δ cells. To investigate which effects of gpb1Δ gpb2Δ mutations on Bcy1 are likely to be indirect, the state of Bcy1 was examined in gpa2Δ ras2Δ cells, which contain a very low level of PKA activity. Whereas Bcy1 in wild-type cells grown in high glucose is not detectably phosphorylated, Bcy1 in gpa2Δ ras2Δ cells displays a substantial degree of phosphorylation (Figure 2B). Phosphorylated Bcy1 is present at a much lower level in gpa2Δ ras2Δ cells that also contain gpb1Δ gpb2Δ mutations. The abundance of Bcy1 is essentially the same in gpa2Δ ras2Δ cells that contain either GPB1 GPB2 or gpb1Δ gpb2Δ alleles. These results suggest that the primary effect of gpb1Δ gpb2Δ mutations is to alter the degree of Bcy1 phosphorylation.

BCY1S145A Mutation Eliminates the Effect of gpb1Δ gpb2Δ Mutations on Bcy1 Stability

Bcy1 is known to be phosphorylated by several kinases, including the catalytic subunits of PKA itself (Kuret et al., 1988; Werner-Washburne et al., 1991; Griffioen et al., 2001, 2003). PKA catalytic subunits phosphorylate Bcy1 at a single site, serine-145 (Kuret et al., 1988; Werner-Washburne et al., 1991). To investigate whether phosphorylation at this site plays a role in the regulation of PKA by Gpb1 and Gpb2, codon 145 of BCY1 was changed from serine to alanine. Protein levels were measured using immunoblotting incubated with fluorescently labeled secondary antibodies, which were detected with an infrared imaging system to allow accurate quantification.

In wild-type cells grown in a high concentration of glucose, Bcy1 is present at an approximately three- to fourfold...
Nutrient Control of Bcy1 Phosphorylation

Figure 3. The BCY1S145A mutation eliminates the effect of gpb1Δ gpb2Δ mutations on Bcy1 stability. (A) Cell lysates were prepared from the following strains: strain HS287-2C (bcy1Δ) carrying plasmid 313pBH1-416 (HA-BCY1, WT), strain HS293-10D (gpb1Δ gpb2Δ bcy1Δ) carrying plasmid 313pBH1-416, strain HS287-2C carrying plasmid pBHBSA-313.4 (HA-BCY1, SA), strain HS293-10D carrying plasmid pBHBSA-313.4, and wild-type strain SKY763 carrying vector pRS313. Lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA and anti-PGK antibodies. Protein levels were quantified by infrared imaging, and numbers under the lanes are the relative amount of HA-Bcy1 normalized to PGK. (B) Cell lysates from the following strains were analyzed as described in A: strain HS287-2C (bcy1Δ) carrying plasmid 313pBH1-416 (HA-BCY1, WT), strain HS293-10D (gpb1Δ gpb2Δ bcy1Δ) carrying plasmid 313pBH1-416, strain HS287-2C carrying plasmid pBCS145E-313.3 (HA-BCY1, SE), strain HS293-10D carrying plasmid pBCS145E-313.3, strain HS287-2C carrying plasmid pBCS145D-313.4 (HA-BCY1, SD), and strain HS293-10D carrying plasmid pBCS145D-313.4. (C) Strains HS287-2C (bcy1Δ) and HS293-10D (gpb1Δ gpb2Δ bcy1Δ) carrying plasmid pBHBSA-313.4 were treated with cycloheximide (100 μg/ml) at time 0, and lysates were made from samples removed at the indicated times. Lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA and anti-PGK antibodies. (D) Strains HS287-2C (bcy1Δ) and HS293-10D (gpb1Δ gpb2Δ bcy1Δ) carrying plasmid pBHBSA-313.4 were treated as described in C. (E) Strains HS287-2C (bcy1Δ) and HS293-10D (gpb1Δ gpb2Δ bcy1Δ) carrying plasmid pBCS145E-313.3 were treated as described in C. (F) Protein levels from the blots shown in C–E were quantified by infrared imaging, and the relative amount of HA-Bcy1 normalized to PGK was plotted as the percent remaining over time, using the following symbols: △, 313pBH1-416 in HS287-2C; ○, 313pBH1-416 in HS293-10D; ■, pBHBSA-313.4 in HS287-2C; ♦, pBHBSA-313.4 in HS293-10D; ◊, pBCS145E-313.3 in HS287-2C; □, pBCS145E-313.3 in HS293-10D.

higher level in GPB1 GPB2 cells compared with gpb1Δ gpb2Δ cells (Figures 3A, lanes 1 and 2). However, the Bcy1S145A variant is present at the same level in both GPB1 GPB2 and gpb1Δ gpb2Δ cells (Figure 3A, lanes 3 and 4). Thus, changing the PKA phosphorylation site in Bcy1 to amino acids that mimic a phosphorylated residue, codon 145 of Bcy1 was changed from serine to either glutamic acid or aspartic acid. The Bcy1S145E and Bcy1S145D variants are present at a lower level in gpb1Δ gpb2Δ cells than in GPB1 GPB2 cells, although the difference is not as substantial as that seen with wild-type Bcy1 (Figure 3B). Repeat experiments have confirmed that this is due to a slight decrease in the abundance of Bcy1S145E and Bcy1S145D in wild-type cells. However, the results clearly show that the effect of gpb1Δ gpb2Δ mutations on Bcy1 abundance is retained by forms of Bcy1 in which serine-145 is replaced by amino acids that mimic a phosphorylated residue.

The observation that deleting GPB1 and GPB2 causes a three- to fourfold decrease in the steady-state level of Bcy1 suggests that the function of Gpb1 and Gpb2 could involve regulating the half-life of the Bcy1 protein. To test this idea, cells were treated with cycloheximide to arrest protein translation, and the amount of Bcy1 remaining was examined at increasing times after blocking its synthesis. In wild-type cells, the level of Bcy1 remains constant throughout the time course of the experiment, indicating that it is extremely stable (Figure 3C, lanes 1–5). In gpb1Δ gpb2Δ cells, the level of Bcy1p decreases during the time course (Figure 3C, lanes 6–10). Multiple trials of this experiment and quantification of the protein levels demonstrated that the half-life of Bcy1p in gpb1Δ gpb2Δ cells is ~80–85 min. In contrast, the level of Bcy1S145A remains constant throughout the time course of the experiment in both GPB1 GPB2 and gpb1Δ gpb2Δ cells (Figure 3D). The Bcy1S145E variant displays levels that are constant in wild-type cells, but decrease in gpb1Δ gpb2Δ cells
in a manner similar to wild-type Bcy1 (Figure 3E). Quantification of these results showed that whereas wild-type Bcy1 and Bcy1S145E decay with similar kinetics in gpb1Δ gpb2Δ cells, Bcy1S145A levels are unchanged during the time course in gpb1Δ gpb2Δ cells (Figure 3F). These results indicate that the absence of Gpb1 and Gpb2 causes a decrease in the half-life of Bcy1. Moreover, the results show that the decreased half-life of Bcy1 depends on its ability to be phosphorylated on serine-145.

**Effects of gpb1Δ gpb2Δ Mutations on Bcy1 Phosphorylation and PKA Activity Are Not Eliminated by BCY1S145A**

Gpb1 and Gpb2 affect both phosphorylation and stability of Bcy1. Because the BCY1S145A mutation eliminates the effect of gpb1Δ gpb2Δ mutations on Bcy1 stability, it was possible to use this mutation to determine whether the effect of Gpb1 and Gpb2 on phosphorylation is dependent on their effect on stability. To test this idea, wild-type and gpb1Δ gpb2Δ cells containing wild-type HA-BCY1, HA-BCY1S145A, and HA-BCY1S145E were grown to log phase in 0.05% glucose, and HA-Bcy1 was detected by immunoblot. For wild-type BCY1 and the BCY1S145E allele, the abundance and phosphorylation level of Bcy1 were decreased in gpb1Δ gpb2Δ cells (Figure 4A, lanes 1, 2, 5, and 6). For the BCY1S145A allele, the abundance Bcy1 was unaffected by gpb1Δ gpb2Δ mutations, but the level of phosphorylation was decreased (Figure 4A, lanes 3 and 4). These findings indicate that Gpb1 and Gpb2 can have an effect on Bcy1 phosphorylation in the absence of an effect on its abundance. This conclusion provides further support for the idea that the primary effect of gpb1Δ gpb2Δ mutations is to alter the degree of Bcy1 phosphorylation. The results also show that a form of Bcy1 that cannot be phosphorylated at the PKA site retains its ability to be differentially phosphorylated at another site in a manner dependent on Gpb1 and Gpb2.

To test whether the change in Bcy1 abundance caused by the S145A mutation has an effect on signaling, cells containing HA-BCY1, HA-BCY1S145A, and HA-BCY1S145E as their only copy of BCY1 were assayed for FLO11 RNA expression. In cells containing wild-type BCY1, deletion of GPB1 and GPB2 causes a sevenfold increase in FLO11 RNA levels, as expected (Figure 4B). The level of FLO11 RNA is dramatically reduced in gpb1Δ gpb2Δ cells compared with the same cells containing wild-type BCY1. This decrease in signaling output is probably due to the increase in stability of the Bcy1S145A variant. However, in cells containing BCY1S145A, the presence of Gpb1 and Gpb2 causes a twofold decrease in FLO11 RNA expression. Wild-type cells containing BCY1S145A display a level of FLO11 RNA that is ~75% of the level seen when these cells contain BCY1. Thus, the BCY1S145A mutation causes a decrease in the level of signaling in both wild-type and gpb1Δ gpb2Δ cells, but it does not eliminate the effect of gpb1Δ gpb2Δ mutations on signaling. In wild-type cells containing BCY1S145E, FLO11 RNA is present at a slightly higher level than in the same cells containing BCY1. Deletion of GPB1 and GPB2 in cells containing BCY1S145E causes a sixfold increase in FLO11 RNA levels. These results reveal an inverse correlation between Bcy1 stability and signaling through the PKA pathway, as would be expected for a negative regulator of PKA activity.

To provide more support for the idea that PKA activity is controlled by the effects of Gpb1 and Gpb2 on Bcy1, the degree of phosphorylation of a known PKA substrate was measured. The transcriptional repressor Sfl1 negatively regulates expression of the FLO11 gene (Robertson and Fink, 1998; Conlan and Tzamarias, 2001; Pan and Heitman, 2002). Phosphorylation of Sfl1 by PKA prevents its binding to the FLO11 promoter, resulting in increased FLO11 expression (Conlan and Tzamarias, 2001; Pan and Heitman, 2002). Sfl1 contains two sites with the PKA consensus phosphorylation sequence, RRXS. To determine the effect of gpb1Δ gpb2Δ
mutations on PKA activity, immunoprecipitated Sfl1-myc was probed with an antibody that recognizes phosphorylated PKA sites. In cells containing wild-type Bcy1, Bcy1S145A, and Bcy1S45E, deletion of GPB1 and GPB2 causes a five- to sevenfold increase in the level of Sfl1 phosphorylation at the PKA sites (Figure 4C). However, the level of Sfl1 phosphorylation in GPB1 GPB2 and gpblΔ gpblΔ cells containing the Bcy1S145A variant is 40% lower than in the corresponding strains containing wild-type Bcy1. These results establish that the BCY1S145A mutation does not eliminate the effect of gpblΔ gpblΔ mutations on in vivo PKA activity. The BCY1S145A mutation does, however, decrease the level of PKA activity in both in GPB1 GPB2 and gpblΔ gpblΔ cells, most likely due to increased stability of the Bcy1S145A variant.

Yak1 Kinase Is Not Required for Phosphorylation of Bcy1 in a Gpb-dependent Manner

In addition to PKA catalytic subunits, another kinase that phosphorylates Bcy1 is Yak1 (Werner-Washburne et al., 1991; Griffioen et al., 2001). Yak1 is a growth inhibitor that acts in opposition to PKA (Garrett and Broach, 1989). Cells containing a yak1Δ mutation lack a phosphorylated form of Bcy1 that increases in abundance when glucose is depleted from the medium (Werner-Washburne et al., 1991; Griffioen et al., 2001). Therefore, Yak1 is a potential candidate for the kinase that phosphorylates Bcy1 in a manner stimulated by Gpb1 and Gpb2. However, an experiment designed to test this possibility showed that deletion of YAK1 has no effect on abundance or phosphorylation of Bcy1 in cells grown in high or low concentrations of glucose (Figure 5A). These results show that Yak1 is not the kinase responsible for phosphorylation of Bcy1 at the site stimulated by Gpb1 and Gpb2. This finding is consistent with the observation that cells containing a yak1Δ mutation do not display phenotypes associated with high PKA activity, such as increased FLO11 expression (Figure 1B).

**Bcy1 Phosphorylation and Abundance Are Not Affected by Activated Ras2**

Cells containing gpblΔ gpblΔ mutations display a high level of PKA activity that is associated with decreased Bcy1 abundance and phosphorylation. It was therefore of interest to determine whether these effects on Bcy1 are specific to gpblΔ gpblΔ mutations or are common to other mutations that cause PKA activation. To address this question, the effect of constitutively active Ras2 on Bcy1 was investigated in cells grown in different concentrations of glucose. As described above, deletion of GPB1 and GPB2 causes a decrease in Bcy1 abundance in cells grown in 2% glucose and eliminates the appearance of a phosphorylated form of Bcy1 in cells grown in 0.05% glucose (Figure 5B, lanes 1–4). In contrast, expression of constitutively active Ras2 has no effect on Bcy1 abundance or phosphorylation in cells grown in high or low concentrations of glucose (Figure 5B, lanes 5–8). This constitutively active allele of RAS2 has been shown to cause an increase in PKA phosphorylation of the transcription factor Msn2 that is similar to the increase caused by deletion of GPB1 and GPB2 (Lu and Hirsch, 2005). Therefore, cells containing constitutively active Ras2 and gpblΔ gpblΔ mutations display comparable levels of increased PKA activity, but the effect on Bcy1 abundance and phosphorylation is specific to gpblΔ gpblΔ mutations. These results demonstrate that the effects of gpblΔ gpblΔ mutations on Bcy1 are not common to all mutations that cause PKA activation.

**Gpbl1 and Gpb2 Antagonize the Effects of PKA on Bcy1 Phosphorylation**

The results presented above suggest that the Tpk proteins and the kelch repeat proteins have antagonistic effects on the function of Bcy1. Whereas PKA phosphorylation of Bcy1 is associated with increased PKA activity, the effect of Gpb1 and Gpb2 on Bcy1 is associated with decreased PKA activity. To explore this idea further, the catalytic activity of PKA was inhibited, and the effect of this inhibition on Bcy1 was determined in GPB1 GPB2 and gpblΔ gpblΔ cells.

Inhibition of PKA activity was accomplished by using a strain in which the TPK1, TPK2, and TPK3 genes have been replaced by alleles encoding kinases with enzymatic activity that is sensitive to the ATP analog 1NM-PP1 (Yorimitsu et al., 2007). Wild-type and Tpk analog-sensitiv (Tpkae) cells containing HA-BCY1 and HA-BCY1S145A were grown to log phase in 2.0% glucose, and HA-Bcy1 was detected by immunoblot. Treatment of wild-type cells with 1NM-PP1 has no effect on Bcy1, as expected (Figure 6A, lanes 1 and 2). However, treatment of Tpkae cells with 1NM-PP1 results in the appearance of phosphorylated Bcy1 in cells grown in high glucose (Figure 6A, lanes 3 and 4). The same effect is observed in Tpkae cells containing the Bcy1S145A variant (Figure 6A, lanes 7 and 8). These results indicate that, when glucose concentrations are high, PKA activity prevents phosphorylation of Bcy1 on a site other than S145.

To determine whether the function of Gpb1 and Gpb2 is affected by inhibition of PKA activity, GPB1 GPB2 and gpblΔ gpblΔ cells that also contain Tpkae alleles were grown to log phase in 0.05% glucose, and HA-Bcy1 was detected by immunoblot. In the absence of 1NM-PP1 treatment, there is less phosphorylated Bcy1 in cells containing gpblΔ gpblΔ mutations (Figure 6B, lanes 1 and 2), as described above. Treat-
ment with 1NM-PP1 causes a substantial increase in phosphorylated Bcy1 that is essentially the same in 1NM-PP1, strain Y3527 carrying plasmid 313pBHB1-416 (HA-BCY1), SA, and strain Y3527.k1Tk2U carrying plasmid pBHBSA-313.4 (HA-BCY1, SA), Lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA antibody.

**Figure 6.** Inhibition of PKA results in phosphorylation of Bcy1. (A) Cell lysates were prepared from the following strains grown in 2.0% glucose that were untreated or treated with 5 μM 1NM-PP1 for 90 min: strain W3031B carrying plasmid 313pBHB1-416 (TPK1/2/3, +; HA-BCY1, WT), strain Y3527 (tpk1Δ tpk2Δ tpk3Δ) carrying plasmid 313pBHB1-416 (TPK1/2/3, as; HA-BCY1, WT), strain W3031B carrying plasmid pBHBSA-313.4 (TPK1/2/3, +; HA-BCY1, SA), and strain Y3527 carrying plasmid pBHBSA-313.4 (TPK1/2/3, as; HA-BCY1, SA). Lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA antibody. (B) Cell lysates were prepared from the following strains grown in 0.05% glucose that were untreated or treated with 1NM-PP1 for 90 min: strain Y3527 (tpk1Δ tpk2Δ tpk3Δ) carrying plasmid 313pBHB1-416 (HA-BCY1, WT), strain Y3527.k1Tk2U (tpk1Δ tpk2Δ tpk3Δ gpb1Δ gpb2Δ) carrying plasmid 313pBHB1-416, strain Y3527 carrying plasmid pBHBSA-313.4 (HA-BCY1, SA), and strain Y3527.k1Tk2U carrying plasmid pBHBSA-313.4. Lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA antibody.

**DISCUSSION**

Deletion of GPB1 and GPB2 results in phenotypes that are characteristic of increased activation of the cAMP-dependent kinase PKA (Harashima and Heitman, 2002; Batlle et al., 2003). Cells lacking Gpb1 and Gpb2 also display increased phosphorylation of PKA substrates, such as the stress-responsive transcription factor Msn2 (Lu and Hirsh, 2005). However, Gpb1 and Gpb2 do not appear to function by regulating the synthesis or degradation of cAMP, because deleting GPB1 and GPB2 has effects in cells that lack adenylate cyclase and cAMP phosphodiesterases (Lu and Hirsh, 2005; Peeters et al., 2006). These results suggested that Gpb1 and Gpb2 could directly control the activity of PKA. Here we present evidence that is consistent with a model in which Gpb1 and Gpb2 promote phosphorylation and stabilization of Bcy1 in opposition to the effects of PKA catalytic subunits.

The model shown in Figure 7 incorporates the following findings. In gpb1Δ gpb2Δ mutants, Bcy1 abundance is low because its rate of degradation is increased (Figure 3). When the abundance of Bcy1 is low, the amount of free, active PKA catalytic subunits is expected to be high. The effect of gpb1Δ gpb2Δ mutations on Bcy1 stability requires either serine or a negatively charged amino acid at position 145 of Bcy1. Serine-145 is the site at which Bcy1 is phosphorylated by Tpk proteins (Kuret et al., 1988; Werner-Washburne et al., 1991). Therefore, these observations suggest that phosphorylation of Bcy1 by Tpk proteins targets it for degradation. Phosphorylated Bcy1 could be recognized by F-box subunits of the SCF E3 ubiquitin ligase, which target proteins that contain a phosphorylated motif (Vodermaier, 2004).

In wild-type cells, Gpb1 and Gpb2 promote the phosphorylation of Bcy1 at a site other than S145. Therefore, these observations suggest that phosphorylation of Bcy1 in opposition to the effects of PKA catalytic subunits.

**Figure 7.** Model for control of PKA activity by Gpb1 and Gpb2. The PKA regulatory subunit Bcy1 is phosphorylated on an unknown site by a kinase that is inhibited by PKA catalytic activity. This phosphorylation stabilizes Bcy1 that has been phosphorylated at serine-145 by PKA catalytic subunits and causes Bcy1 to be a more efficient inhibitor of Tpk1, Tpk2, and Tpk3. In low glucose, Gpb1 and Gpb2 block the inhibitory effect of PKA on the kinase that phosphorylates Bcy1. Under these conditions, more Bcy1 is bound to PKA catalytic subunits and the signal output is low. In the absence of Gpb1 and Gpb2, PKA inhibits the kinase that phosphorylates Bcy1, and Bcy1 that is phosphorylated at serine-145 becomes unstable. Under these conditions, less Bcy1 is bound to PKA catalytic subunits and the signal output is high.
and Gpb2 causes destabilization of wild-type Bcy1 but does not affect the stability of Bcy1S145A (Figure 3), suggesting that Gpb-dependent phosphorylation stabilizes Bcy1 that is phosphorylated at serine-145. However, Bcy1 that is not phosphorylated at S145 can be phosphorylated at the Gpb-dependent site, and this modification has functional consequences, as shown by the observation that Bcy1S145A is subject to regulation by Gpb1 and Gpb2 (Figure 4). Therefore, the decrease in PKA activity that is mediated by Gpb1 and Gpb2 probably results from both increased abundance of Bcy1 combined with modifications that promote its binding to Tpk subunits. This model is supported by previous results showing that there is less Bcy1 bound to the PKA catalytic subunit Tpk1 in gpb1Δ gpb2Δ cells (Peeters et al., 2006).

PKA catalytic subunits play a dual role in this regulatory network. First, Tpk proteins phosphorylate Bcy1 at S145, which causes it to be destabilized. Second, Tpk proteins inhibit phosphorylation of Bcy1 by another kinase (Figure 6). The role of Gpb1 and Gpb2 is to prevent inhibition of this kinase by Tpk proteins. This idea is based on the observation that gpb1Δ gpb2Δ mutations do not affect Bcy1 phosphorylation in the absence of PKA activity. Therefore, the only role of Gpb1 and Gpb2 is to antagonize the effect of PKA on the Bcy1 kinase. Given that Tpk proteins have been shown to interact with Gpb1 and Gpb2 (Peeters et al., 2006; Lu and Hirsch, unpublished results), one possible mechanism to explain these results is that binding of Gpb1 and Gpb2 to Tpk proteins specifically blocks their interaction with the kinase that phosphorylates Bcy1.

A different way in which Gpb1 and Gpb2 have been proposed to function is by stabilizing Ira1 and Ira2, the GAPs of yeast Ras (Harashima et al., 2006). These authors presented a model in which the only two cellular targets of Gpb1 and Gpb2 are the G<sub>G</sub> subunit Gpa2 and Ras. In this model, Gpb1 and Gpb2 regulate Gpa2 by inhibiting receptor-G protein coupling, and they regulate Ras by stabilization of RasGAPs. The results presented here do not support the idea that Gpb1 and Gpb2 function solely as upstream inhibitors of Gpa2 and Ras, because deletion of Gpb1 and Gpb2 in a gpa2Δ ras2Δ strain has effects on FLO11 RNA expression and Bcy1 phosphorylation. Instead, our results support the conclusion that Bcy1 is a major target of Gpb1 and Gpb2, consistent with previous results showing that Gpb1 and Gpb2 act downstream of adenylate cyclase (Lu and Hirsch, 2005; Peeters et al., 2006). These findings do not rule out the possibility that Ira1 and Ira2 are also regulated by Gpb1 and Gpb2. However, a recent report showed that Gpb1 promotes degradation of Ira2 (Phan et al., 2010), which is the opposite effect of the one observed by Harashima et al. (2006). Therefore, additional studies will be necessary to resolve the question of how Gpb1 and Gpb2 regulate RasGAP proteins.

Several lines of evidence indicate that adenylate cyclase functions as a downstream effector of Gpa2 (Peeters et al., 2007). However, this does not preclude the possibility that Gpb1 and Gpb2 also function as Gpa2 effectors, as has been proposed previously (Peeters et al., 2007). In this model, binding of glucose to the cell surface receptor Gpr1 and concomitant activation of Gpa2 would result in blocking the inhibitory effect of Gpb1 and Gpb2 on downstream signaling components. Support for this idea includes the observation that deletion of Gpb1 and Gpb2 largely eliminates the negative effects of a gpa2Δ mutation, indicating that Gpb1 and Gpb2 could function downstream of Gpa2 (Harashima and Heitman, 2002; Batlle et al., 2003). Gpa2 and PKA catalytic subunits bind to different domains of Gpb1 and Gpb2, suggesting that the kelch repeat proteins could function as scaffolding proteins that bring signaling components into proximity with each other (Peeters et al., 2006). However, binding of Gpb2 to Gpa2 is not dramatically increased when Gpa2 is in the GTP-bound form compared with the GDP-bound form (Peeters et al., 2006). Moreover, Gpb2 does not interact with the region of Gpa2 that would be expected to contact an effector (Niranjan et al., 2007), which includes the Switch II region (α2-helix) and the α3-β3 and α4-β6 loops of the α-subunit (Tesmer et al., 1997). Therefore, it is not clear whether the relationship between Gpa2 and the kelch repeat proteins represents a classical G protein-effector interaction. Although the results presented here do not address the question of whether Gpb1 and Gpb2 directly couple Gpa2 to PKA, this model presents an attractive possibility for the way in which the function of Gpb1 and Gpb2 could be controlled by extracellular glucose concentration.

Processes involving signaling components are often highly conserved, and it is likely that regulation of PKA by kelch repeat proteins in response to nutritional conditions occurs in other organisms. Kelch repeat proteins with functions that have not been characterized are present in all eukaryotic organisms. Evidence to support a conserved role for Gpb1 and Gpb2 comes from the finding that their presence causes increased binding of the mammalian PKA regulatory and catalytic subunits (Peeters et al., 2006). Further experiments will be necessary to establish whether regulation of PKA regulatory subunit phosphorylation and stability by kelch repeat proteins in response to nutritional conditions is a widely conserved mechanism.

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