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

Roli Budhwar, Ailan Lu,* and Jeanne P. Hirsch

Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY 10029

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***GPB1* and *GPB2* encode kelch repeat-containing proteins that regulate protein kinase A (PKA) in yeast by a cAMP-independent process. Here we show that Gpb1 and Gpb2 stimulate phosphorylation of PKA regulatory subunit Bcy1 in low glucose concentrations, thereby promoting the inhibitory function of Bcy1 when nutrients are scarce and PKA activity is expected to be low. Gpb1 and Gpb2 stimulate Bcy1 phosphorylation at an unknown site, and this modification stabilizes Bcy1 that has been phosphorylated by PKA catalytic subunits at serine-145. The *BCY1*^{S145A} mutation eliminates the effect of *gpb*1Δ *gpb2*Δ on Bcy1 stability but maintains their effect on phosphorylation and signaling, indicating that modulation of PKA activity by Gpb1 and Gpb2 is not solely due to increased levels of Bcy1. Inhibition of PKA catalytic subunits that are ATP analog-sensitive causes increased Bcy1 phosphorylation at the unknown site in high glucose. When PKA is inhibited, *gpb*1Δ *gpb2*Δ mutations have no effect on Bcy1 phosphorylation. Therefore, Gpb1 and Gpb2 oppose PKA activity by blocking the ability of PKA to inhibit Bcy1 phosphorylation at a site other than serine-145. Stimulation of Bcy1 phosphorylation by Gpb1 and Gpb2 produces a form of Bcy1 that is more stable and is a more effective PKA inhibitor.**

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 adenylate 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 activation (Kübler *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 (Kübler *et al.*, 1997; Lorenz and Heitman, 1997; Lorenz *et al.*, 2000; Tamaki *et al.*, 2000). The idea that Gpa2 stimulates adenylate cyclase to produce cAMP, similar to mammalian $G_{\alpha s}$, 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 adenylate cyclase (Peeters *et al.*, 2006). These results demonstrate that one function of Gpa2 is to stimulate adenylate 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

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*Present address: inGenious Targeting Laboratory, Inc., 25 Health Science Drive, LIHTI Building, Suite 118, Stony Brook, NY 11790-3350.

Address correspondence to: Jeanne P. Hirsch (jeanne.hirsch@mssm.edu).

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; Batlle *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 *gpb1*Δ *gpb2*Δ mutations requires the PKA catalytic subunit Tpk2 (Harashima and Heitman, 2002; Batlle *et al.*, 2003). Subsequently, it was shown that deletion of *GPB1* and *GPB2* 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 *GPB1* and *GPB2* 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 *gpb1*Δ *gpb2*Δ 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 becomes 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 (Batlle *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 3.8-kb SmaI 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 PstI site of the *RAS2* gene. The *yak1::LEU2* allele was made by transformation of cells with the 4.3-kb HindIII-SmaI fragment from plasmid pGS136-B (Garrett and Broach, 1989). The *bcy1::URA3* allele was made by transformation of cells with the 5.3-kb BamHI fragment from plasmid pbcy1::URA3 (Toda *et al.*, 1987a).

Table 1. Strains used in this study

Strain	Genotype	Source
SKY762	*MAT***a** *ura3-52 trp1::hisG leu2::hisG his3::hisG*	S. Palecek/ S. Kron
HS182-3B.k2T[a]	*MAT***a** *gpb1::TRP1 gpb2::HIS3*	This study
HS257-12D[a]	*MAT***a** *gpb1::URA3 gpb2::HIS3 tpk1-1::TRP1 tpk2-2::TRP1 tpk3-3::HIS3 yak1::LEU2*	This study
HS260-2B[a]	*MAT***a** *tpk1-1::TRP1 tpk2-2::TRP1 tpk3-3::HIS3 yak1::LEU2*	This study
HS275-1C[a]	*MAT***a** *gpa2::TRP1*	This study
HS275-9B[a]	*MAT***a** *gpa2::TRP1 ras2::HIS3*	This study
HS275-16C[a]	*MAT***a** *ras2::HIS3*	This study
HS278.T-1D[a]	*MAT***a** *gpb1::TRP1 gpb2::HIS3 ras2::HIS3*	This study
HS281-2C[a]	*MAT***a** *gpb1::TRP1 gpb2::HIS3 gpa2::TRP1*	This study
HS282-23C[a]	*MAT***a** *gpb1::TRP1 gpb2::HIS3 gpa2::TRP1 ras2::HIS3*	This study
HS287-2C[a]	*MAT***a** *bcy1::URA3*	This study
HS293-10D[a]	*MAT***a** *gpb1::URA3 gpb2::TRP1 bcy1::URA3*	This study
HS297-2B[a]	*MAT***a** *yak1::LEU2*	This study
HS300-17B[a]	*MAT***a** *gpb1::TRP1 gpb2::TRP1 yak1::LEU2*	This study
HS309-8B[a]	*MAT***a** *bcy1::URA3 yak1::LEU2*	This study
W3031B	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Thomas and Rothstein (1989)
Y3527[b]	*MATα* $tpk1^{M164G}$ $tpk2^{M147G}$ $tpk3^{M165G}$	Yorimitsu *et al.* (2007)
Y3527.k1Tk2U[b]	*MATα* $tpk1^{M164G}$ $tpk2^{M147G}$ $tpk3^{M165G}$ *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 313pBHB1-416 (Griffioen *et al.*, 2000) was kindly provided by Gerard Griffioen (reMYND, Brussels, Belgium). Plasmids pBCS145E-313.3, pBCS145D-313.4, and pBHBSA-313.4 are identical to 313pBHB1-416, 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 $RAS2^{ala18val19}$ allele in vector YCplac111.

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 100 μg/ml. At various time points after cycloheximide addition (0–120 min), aliquots were removed and centrifuged to pellet the cells. 1.5 ml of cold stop buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaF, and 0.1% NaN_3) was added to the cell pellet, which was centrifuged briefly and snap-frozen in liquid nitrogen. Cell lysates were prepared from the frozen pellets 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).

Immunoblots and Immunoprecipitation Assays

Lysates for immunoblots 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_2HPO_4, pH 7.4, 0.5% TX-100, 25 mM NaF, 100 μM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1× protease inhibitor cocktail [Roche], and 10× phosSTOP 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-phosphoglycerate kinase (PGK) rabbit polyclonal antiserum at a dilution of 1/300,000, and anti-actin rabbit polyclonal antibody (Sigma) at a dilution of 1/1000. Quantification of immunoblots was performed using the infrared dye–labeled secondary antibodies IRDye 800CW goat anti-mouse IgG (Li-Cor, Lincoln, NE) at a dilution of 1/10,000 and IRDye 680 goat anti-rabbit IgG (Li-Cor) at a dilution of 1/10,000. 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× phosSTOP 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 *gpa2*Δ strains (Figure 1A), as described previously (Harashima and Heitman, 2002; Batlle *et al.*, 2003). The same effect is seen in a *ras2*Δ strain (Figure 1A). Quantification of the results showed that there is a six- to sevenfold increase in *FLO11* RNA levels in *gpb1*Δ *gpb2*Δ strains compared with the corresponding *GPB1 GPB2* strains in wild-type and *ras2*Δ backgrounds. Although there is a very little *FLO11* RNA present in a *gpa2*Δ strain, the level of *FLO11* RNA in a *gpa2*Δ *gpb1*Δ *gpb2*Δ strain is just slightly less than that seen in a *GPA2 gpb1*Δ *gpb2*Δ strain, as described previously (Harashima and Heitman, 2002; Batlle *et al.*, 2003). These results demonstrate that the effect of *gpb1*Δ *gpb2*Δ 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 *gpa2*Δ *ras2*Δ 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.

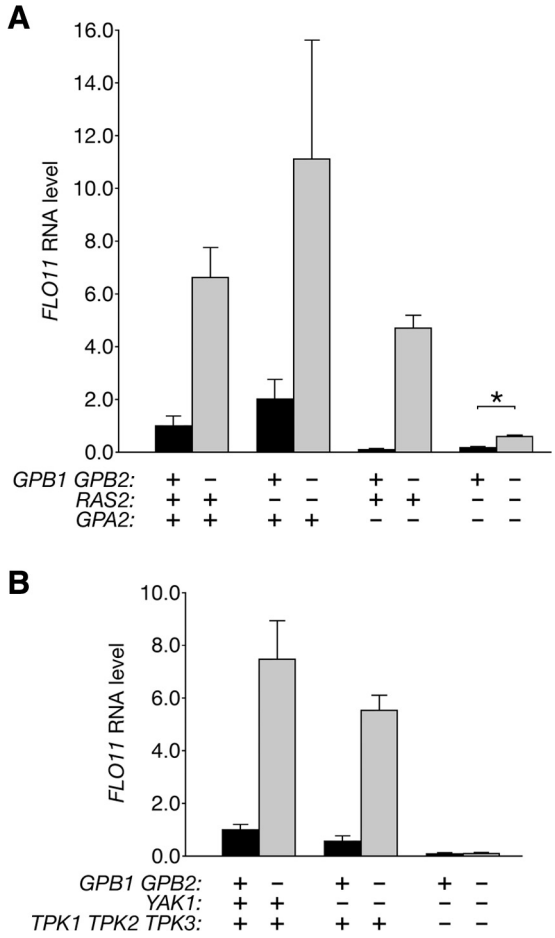

Figure 1. Deletion of *GPB1* and *GPB2* has no effect in cells that lack PKA catalytic subunits. (A) RNA was isolated from the following strains and assayed for the relative amount of *FLO11* and *ACT1* RNA by real-time RT-PCR: wild-type strain SKY762, strain HS182-3B.k2T (*gpb1*Δ *gpb2*Δ), strain HS275-16C (*ras2*Δ), strain HS278.T-1D (*gpb1*Δ *gpb2*Δ *ras2*Δ), strain HS275-1C (*gpa2*Δ), strain HS281-2C (*gpb1*Δ *gpb2*Δ *gpa2*Δ), strain HS275-9B (*gpa2*Δ *ras2*Δ), and strain HS282-23C (*gpb1*Δ *gpb2*Δ *gpa2*Δ *ras2*Δ). Values are plotted as the relative amount of *FLO11* RNA normalized to *ACT1* RNA and are the mean and SD from three independent experiments. *$p < 0.0001$. (B) RNA was isolated from the following strains and assayed for the relative amount of *FLO11* and *ACT1* RNA by real-time RT-PCR as described in A: wild-type strain SKY762, strain HS182-3B.k2T (*gpb1*Δ *gpb2*Δ), strain HS297-2B (*yak1*Δ), strain HS300-17B (*gpb1*Δ *gpb2*Δ *yak1*Δ), strain HS260-2B (*tpk1*Δ *tpk2*Δ *tpk3*Δ *yak1*Δ), and strain HS257-12D (*gpb1*Δ *gpb2*Δ *tpk1*Δ *tpk2*Δ *tpk3*Δ *yak1*Δ).

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 *yak1*Δ mutation (Garrett and Broach, 1989). In a *tpk1*Δ *tpk2*Δ *tpk3*Δ *yak1*Δ 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 *yak1*Δ mutation alone, deletion of *GPB1* and *GPB2* results in a substantial increase in *FLO11* RNA abundance, demonstrating that the *yak1*Δ allele has no effect on the phenotype conferred by the *gpb1*Δ

*gpb2*Δ 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 *gpb1*Δ *gpb2*Δ mutations on the accumulation of reserve carbohydrates (Peeters *et al.*, 2006).

*Phosphorylation and Abundance of Bcy1 Are Affected by gpb1*Δ *gpb2*Δ *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, *gpb1*Δ *gpb2*Δ mutations do not affect signaling (Figure 1; Peeters *et al.*, 2006). However, in cells containing any one of the three PKA subunits, *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ cells in response to lower concentrations of glucose. These results strongly suggest that Gpb1 and Gpb2 play a role in reducing 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 *gpb1*Δ *gpb2*Δ cells. To investigate which effects of *gpb1*Δ *gpb2*Δ mutations on Bcy1 are likely to be indirect, the state of Bcy1 was examined in *gpa2*Δ *ras2*Δ 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 *gpa2*Δ *ras2*Δ cells displays a substantial degree of phosphorylation (Figure 2B). Phosphorylated Bcy1 is present at a much lower level in *gpa2*Δ *ras2*Δ cells that also contain *gpb1*Δ *gpb2*Δ mutations. The abundance of Bcy1 is essentially the same in *gpa2*Δ *ras2*Δ cells that contain either *GPB1 GPB2* or *gpb1*Δ *gpb2*Δ alleles. These results suggest that the primary effect of *gpb1*Δ *gpb2*Δ mutations is to alter the degree of Bcy1 phosphorylation.

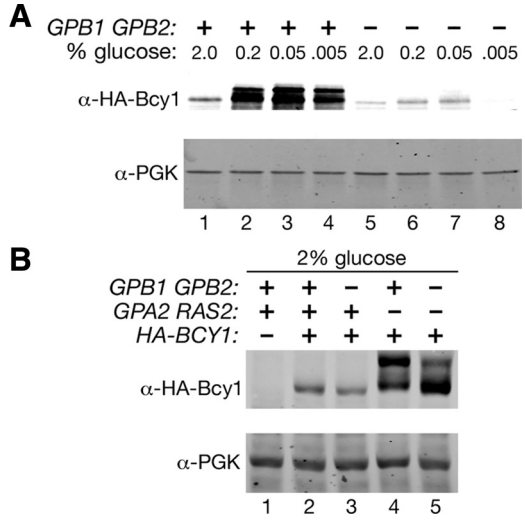

Figure 2. Deletion of *GPB1* and *GPB2* affects phosphorylation and abundance of Bcy1. (A) Cell lysates were prepared from strains HS287-2C (*bcy1*Δ) and HS293-10D (*gpb1*Δ *gpb2*Δ *bcy1*Δ) carrying plasmid 313pBHB$_{1\text{-}416}$ that were grown in 2% glucose to log phase and then switched to the indicated concentrations of glucose for 3 h. Lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA and anti-PGK antibodies. (B) Cell lysates were prepared from wild-type strain SKY762 carrying vector YCplac33 and from the following strains carrying plasmid 313pBHB$_{1\text{-}416}$: wild-type strain SKY762, strain HS182-3B.k2T (*gpb1*Δ *gpb2*Δ), strain HS275-9B (*gpa2*Δ *ras2*Δ), and strain HS282-23C (*gpb1*Δ *gpb2*Δ *gpa2*Δ *ras2*Δ). Cells were grown in 2.0% glucose to log phase, and lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA and anti-PGK antibodies.

*BCY1^{S145A} Mutation Eliminates the Effect of gpb1*Δ *gpb2*Δ *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 immunoblots 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

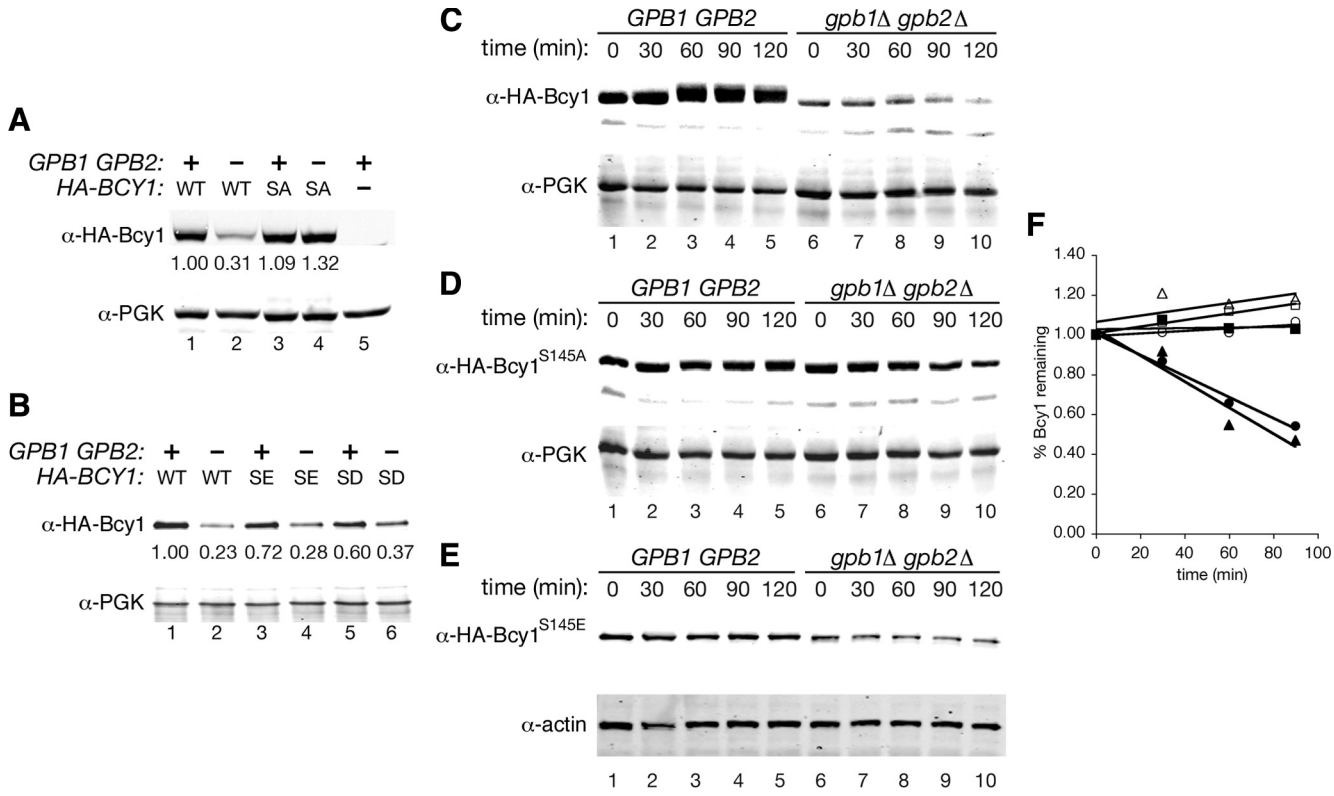

Figure 3. The *BCY1*^{S145A} mutation eliminates the effect of *gpb1*Δ *gpb2*Δ mutations on Bcy1 stability. (A) Cell lysates were prepared from the following strains: strain HS287-2C (*bcy1*Δ) carrying plasmid 313pBHB$_{1-416}$ (*HA-BCY1*, WT), strain HS293-10D (*gpb1*Δ *gpb2*Δ *bcy1*Δ) carrying plasmid 313pBHB$_{1-416}$, strain HS287-2C carrying plasmid pBHBSA-313.4 (*HA-BCY1*, SA), strain HS293-10D carrying plasmid pBHBSA-313.4, and wild-type strain SKY762 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 (*bcy1*Δ) carrying plasmid 313pBHB$_{1-416}$ (*HA-BCY1*, WT), strain HS293-10D (*gpb1*Δ *gpb2*Δ *bcy1*Δ) carrying plasmid 313pBHB$_{1-416}$, 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 (*bcy1*Δ) and HS293-10D (*gpb1*Δ *gpb2*Δ *bcy1*Δ) carrying plasmid 313pBHB$_{1-416}$ 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 (*bcy1*Δ) and HS293-10D (*gpb1*Δ *gpb2*Δ *bcy1*Δ) carrying plasmid pBHBSA-313.4 were treated as described in C. (E) Strains HS287-2C (*bcy1*Δ) and HS293-10D (*gpb1*Δ *gpb2*Δ *bcy1*Δ) 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: △, 313pBHB$_{1-416}$ in HS287-2C; ▲, 313pBHB$_{1-416}$ 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 *gpb1*Δ *gpb2*Δ cells (Figures 3A, lanes 1 and 2). However, the Bcy1^{S145A} variant is present at the same level in both *GPB1 GPB2* and *gpb1*Δ *gpb2*Δ cells (Figure 3A, lanes 3 and 4). Thus, changing the PKA phosphorylation site in Bcy1 to a site that cannot be phosphorylated eliminates the effect of the *gpb1*Δ *gpb2*Δ mutations on Bcy1 abundance. The phenotype of cells containing the Bcy1^{S145A} variant is slow growth (Werner-Washburne *et al.*, 1991), which probably results from the failure of PKA to become fully activated due to excess levels of the altered regulatory subunit.

To determine the effect of 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 Bcy1^{S145E} and Bcy1^{S145D} variants are present at a lower level in *gpb1*Δ *gpb2*Δ 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 Bcy1^{S145E} and Bcy1^{S145D} in wild-type cells. However, the results clearly show that the effect of *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ cells is ~80–85 min. In contrast, the level of Bcy1^{S145A} remains constant throughout the time course of the experiment in both *GPB1 GPB2* and *gpb1*Δ *gpb2*Δ cells (Figure 3D). The Bcy1^{S145E} variant displays levels that are constant in wild-type cells, but decrease in *gpb1*Δ *gpb2*Δ cells

in a manner similar to wild-type Bcy1 (Figure 3E). Quantification of these results showed that whereas wild-type Bcy1 and Bcy1^{S145E} decay with similar kinetics in *gpb1*Δ *gpb2*Δ cells, Bcy1^{S145A} levels are unchanged during the time course in *gpb1*Δ *gpb2*Δ 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 gpb1*Δ *gpb2*Δ *Mutations on Bcy1 Phosphorylation and PKA Activity Are Not Eliminated by BCY1^{S145A}*

Gpb1 and Gpb2 affect both phosphorylation and stability of Bcy1. Because the *BCY1^{S145A}* mutation eliminates the effect of *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ cells containing wild-type *HA-BCY1*, *HA-BCY1^{S145A}*, and *HA-BCY1^{S145E}* were grown to log phase in 0.05% glucose, and HA-Bcy1 was detected by immunoblot. For wild-type *BCY1* and the *BCY1^{S145E}* allele, the abundance and phosphorylation level of Bcy1 were decreased in *gpb1*Δ *gpb2*Δ cells (Figure 4A, lanes 1, 2, 5, and 6). For the *BCY1^{S145A}* allele, the abundance Bcy1 was unaffected by *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ 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-BCY1^{S145A}*, and *HA-BCY1^{S145E}* 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 *gpb1*Δ *gpb2*Δ cells containing *BCY1^{S145A}* compared with the same cells containing wild-type *BCY1*. This decrease in signaling output is probably due to the increase in stability of the Bcy1^{S145A} variant. However, in cells containing *BCY1^{S145A}*, the presence of Gpb1 and Gpb2 causes about a twofold decrease in *FLO11* RNA expression. Wild-type cells containing *BCY1^{S145A}* display a level of *FLO11* RNA that is ~75% of the level seen when these cells contain *BCY1*. Thus, the *BCY1^{S145A}* mutation causes a decrease in the level of signaling in both wild-type and *gpb1*Δ *gpb2*Δ cells, but it does not eliminate the effect of *gpb1*Δ *gpb2*Δ mutations on signaling. In wild-type cells containing *BCY1^{S145E}*, *FLO11* RNA is present at a slightly higher level than in the same cells containing *BCY1*. Deletion of *GPB1* and *GPB2* in cells containing *BCY1^{S145E}* 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 *gpb1*Δ *gpb2*Δ

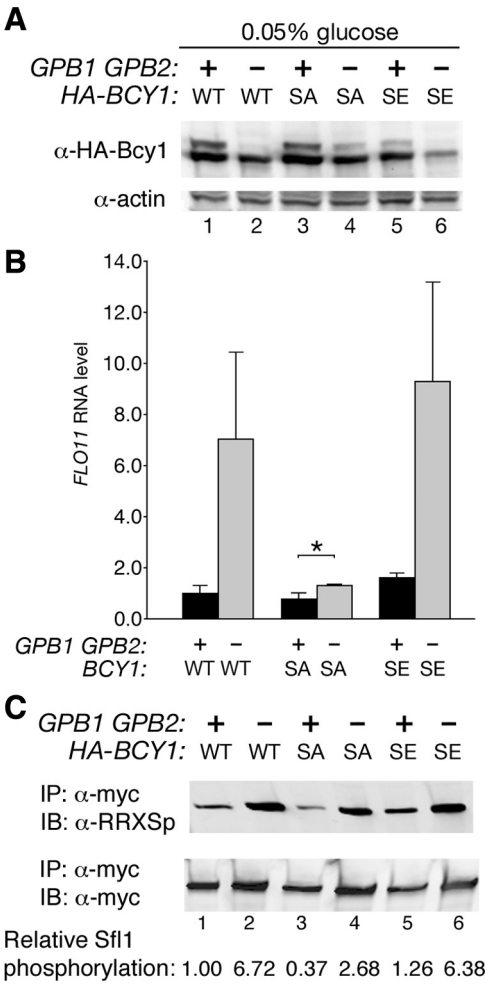

Figure 4. Bcy1^{S145A} retains the effect of *gpb1*Δ *gpb2*Δ mutations on Bcy1 phosphorylation and PKA activity. (A) Cell lysates were prepared from the following strains grown in 0.05% glucose to log phase: strain HS287-2C (*bcy1*Δ) carrying plasmid 313pBHB$_{1\text{-}416}$ (*HA-BCY1*, WT), strain HS293-10D (*gpb1*Δ *gpb2*Δ *bcy1*Δ) carrying plasmid 313pBHB$_{1\text{-}416}$, strain HS287-2C carrying plasmid pBHBSA-313.4 (*HA-BCY1*, SA), strain HS293-10D carrying plasmid pBHBSA-313.4, strain HS287-2C carrying plasmid pBCS145E-313.3 (*HA-BCY1*, SE), and strain HS293-10D carrying plasmid pBCS145E-313.3. Lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA and anti-actin antibodies. (B) RNA isolated from the strains described in A was assayed for the relative amount of *FLO11* and *ACT1* RNA by real-time RT-PCR. Values are plotted as the relative amount of *FLO11* RNA normalized to *ACT1* RNA and are the mean and SD from three independent experiments. Asterisk indicates $p<0.0001$. (C) Sfl1-myc was immunoprecipitated with anti-myc 9E10 mAb from the strains described in A carrying plasmid pXP181. Immunoprecipitated protein was analyzed by SDS-PAGE and immunoblotting with anti-phospho-PKA substrate (RRXSp) and anti-myc antibodies. Protein levels were quantified by infrared imaging, and relative Sfl1 phosphorylation was calculated as the amount of phosphorylated Sfl1 divided by the amount of total Sfl1, normalized to the value obtained in strain HS287-2C carrying plasmid 313pBHB$_{1\text{-}416}$.

mutations on PKA activity, immunoprecipitated Sfl1-myc was probed with an antibody that recognizes phosphorylated PKA sites. In cells containing wild-type Bcy1, Bcy1^{S145A}, and Bcy1^{S145E}, 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 *gpb1*Δ *gpb2*Δ cells containing the Bcy1^{S145A} variant is 40% lower than in the corresponding strains containing wild-type Bcy1. These results establish that the *BCY1^{S145A}* mutation does not eliminate the effect of *gpb1*Δ *gpb2*Δ mutations on in vivo PKA activity. The *BCY1^{S145A}* mutation does, however, decrease the level of PKA activity in both in *GPB1 GPB2* and *gpb1*Δ *gpb2*Δ cells, most likely due to increased stability of the Bcy1^{S145A} 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 *yak1*Δ 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 *yak1*Δ mutation do not display phenotypes associated with high PKA activity, such as increased *FLO11* expression (Figure 1B).

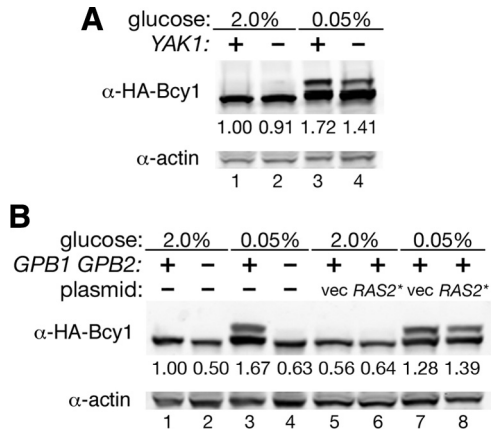

Figure 5. Bcy1 phosphorylation and abundance are not affected by a *yak1*Δ mutation or activated Ras2. (A) Cell lysates were prepared from the following strains grown to log phase in the indicated concentrations of glucose: strain HS287-2C (*bcy1*Δ) carrying plasmid 313pBHB$_{1-416}$ and strain HS309-8B (*bcy1*Δ *yak1*Δ) carrying plasmid 313pBHB$_{1-416}$. Lysates were analyzed by SDS-PAGE and immunoblotting with anti-HA and anti-actin antibodies. Protein levels were quantified by infrared imaging, and numbers under the lanes are the relative amount of HA-Bcy1 normalized to actin. (B) Cell lysates were prepared from the following strains grown to log phase in the indicated concentrations of glucose: strain HS287-2C (*bcy1*Δ) carrying plasmid 313pBHB$_{1-416}$, strain HS293-10D (*gpb1*Δ *gpb2*Δ *bcy1*Δ) carrying plasmid 313pBHB$_{1-416}$, strain HS287-2C carrying plasmid 313pBHB$_{1-416}$ and vector YCplac111 (vec), and strain HS287-2C carrying plasmids 313pBHB$_{1-416}$ and pRAS2AV-111.9 (*RAS2**). Lysates were analyzed as described in A.

Bcy1 Phosphorylation and Abundance Are Not Affected by Activated Ras2

Cells containing *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ mutations display comparable levels of increased PKA activity, but the effect on Bcy1 abundance and phosphorylation is specific to *gpb1*Δ *gpb2*Δ mutations. These results demonstrate that the effects of *gpb1*Δ *gpb2*Δ mutations on Bcy1 are not common to all mutations that cause PKA activation.

Gpb1 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 *gpb1*Δ *gpb2*Δ 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–sensitive (Tpk^{as}) cells containing *HA-BCY1* and *HA-BCY1^{S145A}* 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 Tpk^{as} 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 Tpk^{as} cells containing the Bcy1^{S145A} 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 *gpb1*Δ *gpb2*Δ cells that also contain Tpk^{as} 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 *gpb1*Δ *gpb2*Δ mutations (Figure 6B, lanes 1 and 2), as described above. Treat-

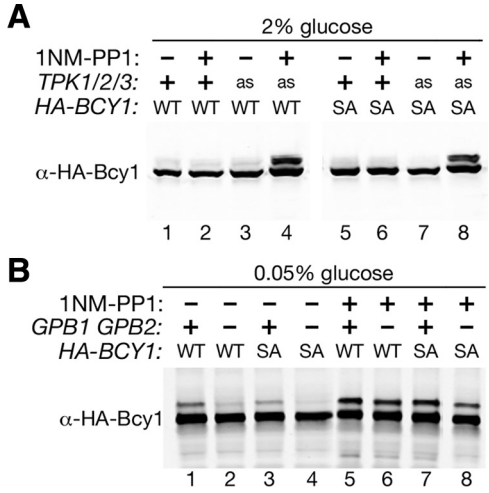

Figure 6. Inhibition of PKA results in phosphorylation of Bcy1. (A) Cell lysates were prepared from the following strains grown in 2.0% glucose that were untreated or treated with 5 μM 1NM-PP1 for 90 min: strain W3031B carrying plasmid 313pBHB$_{1\text{-}416}$ (*TPK1/2/3*, +; *HA-BCY1*, WT), strain Y3527 (*tpk1^{as} tpk2 ^{as} tpk3 ^{as}*) carrying plasmid 313pBHB$_{1\text{-}416}$ (*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 1 μM 1NM-PP1 for 90 min: strain Y3527 (*tpk1^{as} tpk2^{as} tpk3^{as}*) carrying plasmid 313pBHB$_{1\text{-}416}$ (*HA-BCY1*, WT), strain Y3527.k1Tk2U (*tpk1^{as} tpk2 ^{as} tpk3 ^{as} gpb1*Δ *gpb2*Δ) carrying plasmid 313pBHB$_{1\text{-}416}$, 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.

ment with 1NM-PP1 causes a substantial increase in phosphorylated Bcy1 that is essentially the same in *GPB1 GPB2* and *gpb1*Δ *gpb2*Δ cells (Figure 6B, lanes 5 and 6). Similar effects are seen with the Bcy1^{S145A} variant (Figure 6B, lanes 3, 4, 7, and 8). Therefore, the effect of *gpb1*Δ *gpb2*Δ mutations on Bcy1 phosphorylation is eliminated when PKA activity is inhibited. These results suggest that Gpb1 and Gpb2 antagonize the effect of PKA activity on Bcy1 phosphorylation at a site other than S145.

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 Hirsch, 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 Hirsch, 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 *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ 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. The effect of Gpb1 and Gpb2 on Bcy1 phosphorylation increases as the concentration of extracellular glucose decreases, indicating that glucose blocks the function of Gpb1 and Gpb2. Loss of Gpb1

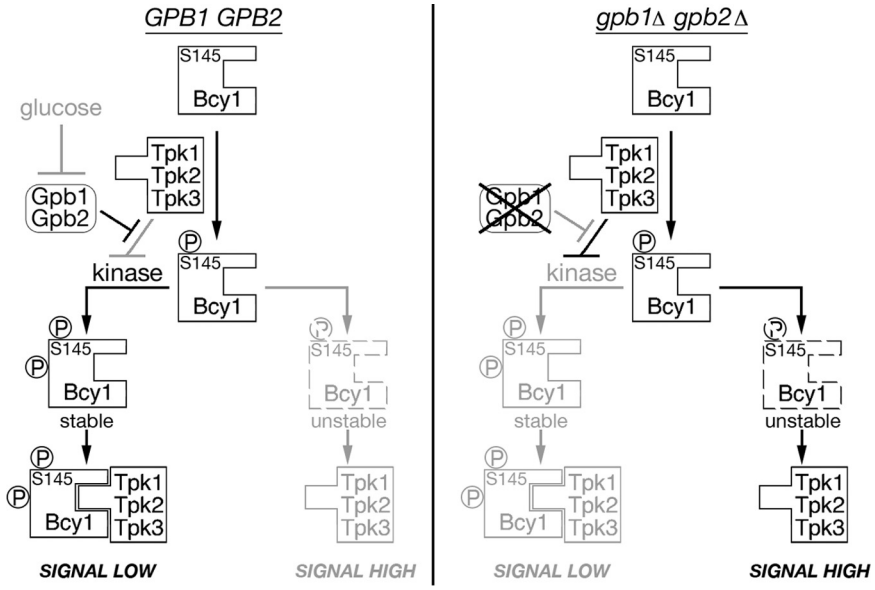

Figure 7. Model for control of PKA activity by Gpb1 and Gpb2. The PKA regulatory subunit Bcy1 is phosphorylated on an unknown site by a kinase that is inhibited by PKA catalytic activity. This phosphorylation stabilizes Bcy1 that has been phosphorylated at serine-145 by PKA catalytic subunits and causes 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 $Bcy1^{S145A}$ (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 $Bcy1^{S145A}$ 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 *gpb1*Δ *gpb2*Δ 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 *gpb1*Δ *gpb2*Δ 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_α 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 *gpa2*Δ *ras2*Δ 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 *gpa2*Δ 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-β5 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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