Activation State of the Ras2 Protein and Glucose-induced Signaling in Saccharomyces cerevisiae*

Sonia Colombo‡, Daniela Ronchetti‡, Johan M. Thevelein§, Joris Winderickx¶, and Enzo Martegani‡∥

From the ‡Dipartimento di Biotecnologie e Bioscienze, Università Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy, §Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, Katholieke Universiteit Leuven and Department of Molecular Microbiology, Flemish Interuniversity Institute of Biotechnology (VIB), Kasteelpark Arenberg 31, B-3001 Leuven-Heverlee, Flanders, Belgium, and †Laboratorium voor Functionele Biologie, Katholieke Universiteit Leuven, Instituut voor Plantkunde en Microbiologie, Kasteelpark Arenberg 31, B-3001 Leuven-Heverlee, Flanders, Belgium

The activity of adenylate cyclase in the yeast Saccharomyces cerevisiae is controlled by two G-protein systems, the Ras proteins and the Ga protein Gpa2. Glucose activation of cAMP synthesis is thought to be mediated by Gpa2 and its G-protein-coupled receptor Gpr1. Using a sensitive GTP-loading assay for Ras2 we demonstrate that glucose addition also triggers a fast increase in the GTP loading state of Ras2 concomitant with the glucose-induced increase in cAMP. This increase is severely delayed in a strain lacking Cdc25, the guanine nucleotide exchange factor for Ras proteins. Deletion of the Ras-GAPs IRA2 (alone or with IRA1) or the presence of RAS2Val19 allele causes constitutively high Ras GTP loading that no longer increases upon glucose addition. The glucose-induced increase in Ras2 GTP-loading is not dependent on Gpr1 or Gpa2. Deletion of these proteins causes higher GTP loading indicating that the two G-protein systems might directly or indirectly interact. Because deletion of GPR1 or GPA2 reduces the glucose-induced cAMP increase the observed enhancement of Ras2 GTP loading is not sufficient for full stimulation of cAMP synthesis. Glucose phosphorylation by glucokinase or the hexokinases is required for glucose-induced Ras2 GTP loading. These results indicate that glucose phosphorylation might sustain activation of cAMP synthesis by enhancing Ras2 GTP loading likely through inhibition of the Ira proteins. Strains with reduced feedback inhibition on cAMP synthesis also display elevated basal and induced Ras2 GTP loading consistent with the Ras2 protein acting as a target of the feedback-inhibition mechanism.

In Saccharomyces cerevisiae the addition of glucose or other readily fermentable sugars to derepressed cells (carbon-starved or growing on a non-fermentable carbon source) triggers a remarkable variety of regulatory phenomena, including many rapid changes at the post-translational and transcriptional level. Several signaling pathways are activated by glucose. One of the best studied pathways is the cAMP/PKA pathway. The main component of this signaling transduction pathway is adenylate cyclase, which catalyzes the synthesis of cAMP. In S. cerevisiae adenylate cyclase activity is controlled by the Ras proteins. These proteins are members of the small GTPase superfamily, which are active in the GDP-bound form and inactive in the GTP-bound form. Recently it has been demonstrated that in S. cerevisiae adenylate cyclase activity is also controlled by a heterotrimeric Ga-protein, Gpa2 (1). A G-protein-coupled receptor, Gpr1, has been identified to be responsible for activation of the Gpa2 protein (2–4). Two triggers are known to activate the cAMP/PKA pathway: the addition of glucose to derepressed cells and intracellular acidification. The Gpr1/Gpa2 G-protein-coupled receptor system is only required for glucose activation of cAMP synthesis (2, 5). The results reported in the literature about the role played by the Ras proteins in activation of cAMP synthesis are in part contradictory. Colombo et al. (5) showed that intracellular acidification (but not glucose) caused an increase in the GTP/GDP ratio on the Ras proteins, suggesting that only intracellular acidification would stimulate CAMP synthesis through activation of the Ras proteins. Other data reported by Rudoni et al. (6) using another assay show that the addition of glucose to glucose-starved cells also causes a fast increase of the Ras2-GTP level. Two different assays have been used to analyze quantitatively the guanine nucleotides bound to the Ras proteins in vivo. The first assay was developed by Gibbs et al. (7) and used by Colombo et al. (5). After labeling the cells in vivo with 32P]orthophosphate, the Ras proteins were immunoprecipitated with antibodies against the human Ras protein (+/H-ras 258). Guanine nucleotides were extracted, separated by TLC, and quantitated by phosphorimager technology. With this assay, the GTP/GDP ratio on the yeast Ras proteins could be measured only after overexpression of Ras2 protein indicating that the sensitivity of this assay is probably not very high. A more recent assay to analyze quantitatively the guanine nucleotides bound to the Ras proteins in vivo was described by Taylor and Shalloway (8) and exploits the known specificity of the interaction between Ras-GTP and the Ras-binding domain (RBD) of Raf-1 to detect activated Ras. Because there is a high degree of homology between yeast and mammalian Ras proteins, the yeast Ras proteins are able to interact with the RBD of Raf-1. As a result the assay could be used successfully by Rudoni et al. (6) to measure the GTP loading on yeast Ras2 without overexpression of this protein. In this work we have used the highly sensitive non-radioisotopic pull-down assay for Ras2 GTP loading to study the requirements for the rapid glucose enhancement of GTP loading. We show that it is dependent on Cdc25 and that deletion of the Ira proteins causes...
constitutively high GTP loading. Remarkably, also deletion of GPR1 or GPA2 enhances basal and induced Ras2 GTP loading. It is known that glucose phosphorylation by glucokinase or the hexokinases is required for glucose-induced cAMP signaling (9, 10). This glucose phosphorylation requirement can also be fulfilled in a glucose transport deficient strain by provision of intracellular maltose (11). We show that glucose-induced Ras2 GTP loading is dependent on precisely the same requirements indicating that glucose phosphorylation probably acts through Ras to sustain cAMP signaling. Finally we provide evidence that the Ras proteins might be a target for the stringent feedback inhibition of PKA on cAMP synthesis.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—The yeast strains used in this study are indicated in Table I. Cells were grown in 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone (YP) supplemented with either 3% (w/v) glycerol, 0.1% (w/v) glucose and 50 mg/liter adenine, or 2% (w/v) glucose and 50 mg/liter adenine (YPDA). Strains carrying a plasmid were grown in 0.1% (w/v) glucose and 50 mg/liter adenine, or 2% (w/v) glucose and 50 mg/liter adenine (YPDA). Strains carrying a plasmid were grown in 0.1% (w/v) glucose and 50 mg/liter adenine, or 2% (w/v) glucose and 50 mg/liter adenine (YPDA).

**TABLE I**

| Strain | Genotype | Source/Ref. |
|--------|----------|-------------|
| W303–1A | MATa leu2–3, 112 ura3–1 trp1–92 his3–11, 15 ade2–1 can1–100 GAL SUC mal | |
| W303–1A (YEgRAS2) | MATa leu2–3, 112 ura3–1 trp1–92 his3–11, 15 ade2–1 can1–100 GAL SUC mal | |
| W303–1A (YEgTPK1) | MATa leu2–3, 112 ura3–1 trp1–92 his3–11, 15 ade2–1 can1–100 GAL SUC mal | |
| W303–1A (YCpRAS2) | MATa leu2–3, 112 ura3–1 trp1–92 his3–11, 15 ade2–1 can1–100 GAL SUC mal | |
| W303–1A (YCpgRAS2<sub>nosp</sub>) | MATa leu2–3, 112 ura3–1 trp1–92 his3–11, 15 ade2–1 can1–100 GAL SUC mal | |

a. M. Wigler (Cold Spring Harbor Laboratory).
b. L. Caunwenberg (Katholieke Universiteit Leuven).
c. P. Ma (Katholieke Universiteit Leuven).
d. J. Winderickx (Katholieke Universiteit Leuven).

e. This work.

All experiments were done in triplicate, and error bars are reported in the figures. In our experimental conditions anti-Ras2 antibodies recognize in a specific way yeast Ras2 protein as shown in Fig. 1A because no signal was detected in strains bearing a RAS2 deletion. For evaluation of the reliability of the assay we set up conditions to load purified Ras2 protein specifically either with GTP or with GDP. For this purpose 1 μg of purified Ras2 protein (10) was incubated at room temperature for 30 min in 150 μl of phosphate-buffered saline 1×, 1% (w/v) Triton X-100, 100% (w/v) glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium vanadate, one tablet of Protease Inhibitor Mixture from Roche Applied Science in 50 ml of this solution. Bound proteins were eluted with 2× SDS-sample buffer (100 mM diithiothreitol, pH 6.8, 2% w/v mercaptoethanol, 4% (w/v) SDS, 0.2% (w/v) bromphenol blue, 20% (w/v) glycerol), separated by SDS-PAGE, blotted onto nitrocellulose, immunodecorated with anti-Ras2 polyclonal antibodies (SC-6759, Santa Cruz Biotechnology), and revealed with an ECL Western blotting analysis system (Amersham Biosciences). Total Ras2 protein was detected in cleared lysate by Western blotting using the same anti-Ras2 antibodies. The ratios of Ras2-GTP/total Ras2 were determined by densitometric analysis (Scion-Image software).

All experiments were done in triplicate, and error bars are reported in the figures. In our experimental conditions anti-Ras2 antibodies recognize in a specific way yeast Ras2 protein as shown in Fig. 1A because no signal was detected in strains bearing a RAS2 deletion.

For evaluation of the reliability of the assay we set up conditions to load purified Ras2 protein specifically either with GTP or with GDP. For this purpose 1 μg of purified Ras2 protein (10) was incubated at room temperature for 30 min in 150 μl of phosphate-buffered saline 1× (containing one tablet of Protease Inhibitor Mixture in 50 ml of buffer) in the presence of 5 mM EDTA and either 1 μM GTP or 1 μM GDP. The removal of Mg<sup>2+</sup> by EDTA partially unfolds the protein and induces an open conformation enhancing the binding of either GTP or GDP. Immediately after incubation, an excess amount of MgCl<sub>2</sub> (20 mM) was added to refold the Ras2 protein so that the nucleotide was locked in the open conformation enhancing the binding of either GTP or GDP. Immediately after incubation, an excess amount of MgCl<sub>2</sub> (20 mM) was added to refold the Ras2 protein so that the nucleotide was locked in the open conformation enhancing the binding of either GTP or GDP.
Biosciences). Total Ras2 protein (bound either to GTP or to GDP) was detected by Western blotting using the same anti-Ras2 antibodies. Only the activated form of Ras2 was able to bind to the resin (Fig. 1B) indicating that the assay is specific for Ras2-GTP.

**RESULTS**

Overexpression of Ras2 Abolishes Glucose-induced GTP Loading on Ras2—We previously reported an increase in the GTP-loading state of Ras after intracellular acidification with the protonophore 2,4 dinitrophenol using a method based on in vivo labeling of strains overexpressing Ras2. This method, however, did not reveal a glucose-induced increase in Ras GTP loading (5). More recently, we adopted the more sensitive Ras-GTP/Raf interaction assay, and this allowed us to demonstrate a rapid activation of Ras2 upon the addition of glucose to glucose-starved cells (6). We now show, using the more sensitive assay, that the overexpression of Ras2 abolished the glucose-induced increase in Ras2-GTP and delays the increase triggered by intracellular acidification (Fig. 2B), whereas a fast increase was obtained in a wild-type W303–1A strain after the addition of glucose (Fig. 2A). This provides an explanation for the discrepancy between the results of our two previous studies and confirms the results reported by Rudoni *et al.* (6) performed with a different wild type strain. The inset of Fig. 2B shows a Western blot giving a rough evaluation of the Ras2 overexpression in W303 (YepRAS2); from this data a 15-fold increase was calculated in comparison with the wild type W303–1A.

Addition of Glucose Enhances the Ras2-GTP Level and the Rapid Increase Is Dependent on Cdc25—Because the addition of glucose triggered a rapid increase in the Ras2-GTP level we have evaluated whether this increase was dependent on Cdc25, the main GDP/GTP exchange factor for Ras in yeast. The addition of 100 mM glucose to derepressed cells of a strain deleted for *CDC25* and rescued by overexpression of *TPK1* did not show the rapid increase in the Ras2-GTP level (Fig. 3). The Ras2-GTP loading state increased slowly to a level similar to that of the wild type strain. Hence, Cdc25 is required for the rapid increase. It is also evident that *TPK1* overexpression has a negative effect on Ras2-GTP loading. This could be due to a feedback caused by high PKA activity and suggests (as...
reported later) that Ras2 is a possible target of the feedback inhibition.

Ira1 and Ira2 Negatively Regulate the Ras2 Protein Activation State and Are Required for the Glucose-induced Increase in Ras2 GTP Loading—Tanaka et al. (14) reported that in ira mutants the Ras2 proteins accumulate in the GTP-bound form, whereas in the wild type strain these proteins were found mostly in the GDP-bound form, indicating that Ira1 and Ira2 negatively regulate the level of Ras-GTP. Our results with the new assay confirm these conclusions. In a strain containing only the wild type IRA2 gene (ira1Δ) the initial Ras2-GTP/total Ras2 ratio was about 3 times higher compared with wild type cells. However, the ratio still increased after the addition of glucose (Fig. 4A). In a strain containing only the IRA1 gene (ira2Δ) the initial ratio was about 6 times higher than in the wild type strain indicating that Ira2 plays a prominent role in controlling the basal Ras2 GTP loading state. In addition, this strain did not show any further glucose-induced increase in Ras2 GTP loading (Fig. 4A). A similar result was obtained for the ira1Δ ira2Δ strain indicating that the Ira proteins (and mainly Ira2) are essential to obtain a normal glucose-induced increase in Ras2 GTP loading probably because the inhibition or deletion of these GTPases already triggers a maximal GTP loading before the addition of the sugar.

To confirm the importance of Ras-GTPase activating capacity and/or guanine nucleotide exchange capacity for the glucose-induced increase in Ras-GTP loading we used a strain expressing the RAS2Val19GDP dominant allele. This allele has strongly reduced GTPase activity, is insensitive to the Ira proteins, and is also independent of the guanine nucleotide exchange factors Cdc25 and Sdc25. The results obtained were similar to those for the ira1Δ ira2Δ strain. The basal level of Ras2 GTP loading was high, and there was no further glucose-induced increase (Fig. 4B). This effect was specific for RAS2Val19 because a similar experiment with a strain bearing the normal RAS2 gene in the same centromeric vector (YCPRAS2) gave a response practically identical to a wild type (Fig. 4B); however in this case the level of Ras2 overexpression was about 2-fold as shown also in Fig. 1A, lane 2).

Role of the Gpr1-Gpa2 G-protein-coupled Receptor (GPCR) System in Regulation of the Ras2 Protein Activation State—Because the G-protein-coupled receptor Gpr1 and its Ga protein Gpa2 are required for the stimulation of cAMP synthesis by high glucose levels (2, 3, 5, 11), we have investigated whether they are also required for the glucose-induced increase in Ras2 GTP loading. Both in a gpr1Δ and in a gpa2Δ strain the glucose-induced increase was still present (Fig. 5A). Remarkably, however, the GTP loading state of Ras2 was enhanced in both strains. This indicates that the GPCR system in some way negatively interferes with the activation of Ras2.

It has been demonstrated that the glucose-induced increase in cAMP is dependent on two mechanisms: a glucose-phosphorylation dependent mechanism, which is already stimulated by low glucose levels of a few millimolars, and the GPCR-dependent mechanism, which is only triggered by high glucose levels (20–100 mM) (11). To determine the effect of the glucose concentration on Ras2 GTP loading, we have measured the Ras2 activation state during sequential addition of a low (5 mM) glucose concentration followed 3.5 min later by a high (100 mM) glucose concentration. Fig. 5B shows that addition of 5 mM glucose did not trigger a detectable increase in Ras2-GTP in the wild type strain. However, in the gpr1Δ and gpa2Δ strains an increase was observed (Fig. 5B), consistent with the previous observation that the absence of Gpr1 or Gpa2 facilitates Ras activation. Subsequent addition of 100 mM glucose triggered in all strains a further increase in Ras2 GTP; this increase was again more pronounced in gpr1Δ and gpa2Δ strains.
whereas specifically one of the two hexokinases is required for fructose-induced cAMP signaling (9, 10). We have investigated the requirement for glucose phosphorylation using a strain lacking the three sugar kinases (hxk1/H9004, hxk2/H9004, glk1/H9004) or the two hexokinases (hxk1/H9004, hxk2/H9004). In the latter strain only the glucokinase Glk1 is active. In the hxk1/H9004, hxk2/H9004, glk1/H9004 strain the glucose-induced increase in Ras2-GTP was absent, whereas in the hxk1Δ, hxk2Δ strain the glucose-induced increase in Ras2-GTP was present but somewhat lower than in the wild type strain (Fig. 6). These results indicate that glucose phosphorylation is required for the glucose-induced increase in Ras2-GTP and that glucokinase alone can sustain the increase. Moreover when we tested the Ras2-GTP increase in a hxk1Δ, hxk2Δ, glk1Δ, ira2Δ strain we obtained a result comparable with that observed for ira2Δ strain (see Fig. 4A), a high basal level of Ras2-GTP that did not increase after glucose addition. This result suggests that glucose induced Ras2-GTP loading operates likely through an inhibition of the Ira2 protein.

Glucose-induced Activation of Ras2 in a Glucose Transport-deficient Strain—Recent data have shown that the two essential requirements for glucose-induced activation of cAMP synthesis can be fulfilled separately: the extracellular glucose detection by the GPCR system and the intracellular sugar-sensing process requiring the hexose kinases (10, 11). Addition of a low concentration of maltose (0.7 mM), which provides intracellular glucose in the absence of glucose transport, to cells of a strain lacking all physiologically important glucose carriers (HXT1–7, GAL2) did not increase the cAMP level by itself. However, it provided enough glucose phosphorylation to sustain the activation of cAMP synthesis by a high external glucose level through the Gpr1/Gpa2 GPCR system. Fig. 7 shows that in a glucose-transport deficient strain the rapid glucose-induced increase in Ras2-GTP was largely absent. This is in agreement with the requirement for active glucose phosphorylation because the latter normally requires glucose transport. The small remaining increase in Ras2-GTP is consistent with the small remaining increase in glucose 6-phosphate previously observed in the glucose transport deficient strain (10, 22).

**Fig. 5.** Effect of **GPR1** and **GPA2** gene deletions on glucose-induced Ras2-GTP loading. A, GTP content of Ras2 after the addition of 100 mM glucose to cells of wild type W303–1A (●), W303–1A gpa2Δ (▲), and W303–1A gpr1Δ (○). B, GTP content of Ras2 after the pre-addition of 5 mM glucose followed 3 min and 30 s later by the addition of 100 mM glucose to W303–1A (black bars), W303–1A gpa2Δ (gray bars), and W303–1A gpr1Δ (white bars). Treatment of cells and determination of the Ras2-GTP content were as described in the legend for Fig. 2.

**Fig. 6.** Glucose kinases are required for glucose-induced Ras2-GTP loading. GTP content of Ras2 after the addition of 100 mM glucose to cells of wild type W303–1A (●), hxk1Δ hxk2Δ (△), hxk1Δ hxk2Δ glk1Δ (●), and hxk1Δ hxk2Δ glk1Δ ira2Δ (○) strains. Treatment of cells and determination of the Ras2-GTP content were as described in the legend for Fig. 2.
Fig. 7. Kinetics of glucose-induced Ras2-GTP loading in strains lacking glucose transporters. GTP content of Ras2 after the addition of 100 mM glucose to wild type MC996A (□), hxt1-7a gal2Δ (●), hxt1-7a gal2Δ gpr1Δ (○), and hxt1-7a gal2Δ gpa2Δ (▲) strains. The kinetics of Ras2-GTP in MC996A is different from that usually observed in other wild type strains. Treatment of cells and determination of the Ras2-GTP content were as described in the legend for Fig. 2.

Fig. 8. Ras2 activation is overstimulated in strains with reduced feedback inhibition. GTP content of Ras2 after the addition of 100 mM glucose to cells of wild type SP1 (■), SP1 tpk1Δ tpk2Δ (▲), and SP1 tpk1Δ gpa2Δ (○) strains. Treatment of cells and determination of the Ras2-GTP content were as described in the legend for Fig. 2.

It is apparently sustained by the very low level of residual glucose transport in this strain, but it is unable to support cAMP signaling. Deletion of GPA1 or GPA2 in the glucose transport-deficient strain also enhanced the basal level of Ras2-GTP in agreement with the previous observation that the GPCR system negatively affects Ras2 GTP loading. There was no further glucose-induced increase in the Ras2-GTP level, which can be explained by the absence of glucose transport and sufficient glucose phosphorylation.

Ras2 Is a Possible Target of the Feedback-inhibition Mechanism—cAMP accumulation in yeast is under strong feedback inhibition by protein kinase A (17, 18). The only target conclusively identified so far is the low-affinity cAMP phosphodiesterase Pde1 (19). To evaluate whether the feedback-inhibition mechanism on cAMP accumulation by PKA acts by changing the Ras2 protein activation state, we measured the Ras2-GTP/total Ras2 ratio in a strain with reduced feedback inhibition (tpk1Δ tpk2Δ tpk3Δ). The initial Ras2-GTP level was about 3 times higher compared with that in wild type cells, and the Ras2-GTP level further increased after the addition of glucose (Fig. 8). These results indicate that PKA down-regulates the Ras2-GTP-loading state. Deletion of GPA2 gene did not prevent the increase in the basal level of Ras2-GTP or the glucose-induced increase. This confirms that the glucose-induced increase in Ras2-GTP does not require the GPCR system and is apparently only triggered by the glucose phosphorylation-dependent system.

DISCUSSION

Involvement of Ras in Glucose-induced cAMP Signaling—The first evidence for involvement of the Ras proteins in cAMP signaling was provided by Broek et al. (20), Toda et al. (21), and Mbonyi et al. (22). Subsequent work also supported a requirement of CDC25 in glucose-induced cAMP signaling (23–26). The involvement of Ras in glucose-induced cAMP signaling was further supported by the inability of a Ras mutant allele that lacked lipid modification and therefore plasma membrane attachment to mediate glucose-induced cAMP signaling although the allele could support a basal cAMP level high enough for growth (27, 28). After Colombo et al. (5) showed that only intracellular acidification and not glucose caused an increase in the GTP content on the Ras proteins, attention re-focused on the Gpa2 protein and its G-protein-coupled receptor Gpr1 as a glucose-sensing module required for activation of cAMP synthesis. The latter, however, did not explain the many previous results supporting a role for the Cdc25-Ras module in glucose-induced cAMP signaling. Moreover, Rudoni et al. (6) have recently been able to detect a glucose-induced increase in the Ras2-GTP content with a more sensitive assay. Our present results reconcile the discrepancies between the previous findings. Using the more sensitive assay for the Ras2 GTP detection we show that glucose addition rapidly enhances Ras2-GTP. We confirm that intracellular acidification enhances the GTP loading on Ras. Moreover we show that overexpression of Ras2 delays the increase caused by intracellular acidification and abolishes the increase triggered by glucose. This explains the inability of Colombo et al. (5) to detect an increase in the GTP/GDP ratio on Ras because they necessarily had to use Ras2 overexpression strains to be able to measure the GTP and GDP load on Ras because of the lower sensitivity of the method. The strong Ras2 overexpression (up to 15-fold) could impair the glucose responding mechanism in different ways. Whereas the ratio Ras2-GTP/total Ras2 was comparable with that found in the wild type strain, the absolute amount of Ras2-GTP was very high, with a 10–15-fold increase; therefore the pathway is overstimulated and no additional increase of Ras2-GTP can be induced by glucose, or the high amount of Ras2-GTP can inhibit any additional Ras2-GTP increase. Alternatively the overexpression of Ras2 dilutes the amount available for stimulation and therefore reduces the glucose-induced Ras2-GTP increase to below the detection limit. Another explanation is that overexpression of Ras2 causes mislocalization of the protein with inaccessibility to the activation mechanism as a result. Immunofluorescence staining, however, showed that in the strain overexpressing Ras2 the protein was localized mainly at the plasma membrane (results not shown). In conclusion, the glucose-induced stimulation of cAMP synthesis is apparently mediated by the two G-proteins, Ras2 and Gpa2, raising the question as to the mechanisms involved in glucose activation of Ras.

Mechanism of Glucose Activation of Ras—Yeast Ras activation is controlled by the guanine nucleotide exchange proteins Cdc25 and Sdc25 of which the former plays a major role (29). We show that deletion of CDC25 severely delays the glucose-induced increase in Ras2-GTP consistent with a requirement of Cdc25. The slow residual increase might be mediated by Sdc25. This result opens the possibility that Cdc25 would act as a signal mediator for glucose-induced activation of Ras. On the other hand, also in case the glucose signal would arrive on the Ira proteins, causing their inhibition, a requirement for Cdc25 as GDP/GTP exchange stimulator could be expected. Deletion of the IRA genes, and in particular the IRA2 gene, caused a
conspicuous increase in the basal level of Ras2-GTP. This is consistent with previous results obtained by Tanaka et al. (14) revealing a role for the Ira proteins as Ras-GTPase activating proteins. As opposed to the results of Tanaka et al. (14), however, which were obtained with strains overexpressing Ras2, our results were obtained with strains displaying regular Ras2 expression. More importantly, the ira2Δ and ira1Δ ira2Δ strains did not show a further glucose-induced increase in Ras2-GTP. The level of Ras2-GTP in these strains was higher in comparison to the maximal levels observed in wild type strains, but because Ras2-GTP was about 20% of the total Ras2 content one would expect a further increase to be possible. The results indicate that the glucose-induced rise in Ras2-GTP might be mediated by inhibition of the Ira proteins. As mentioned previously, in this scenario deletion of CDC25 would also be expected to cause a delay of the glucose-induced increase in Ras2-GTP because Cdc25 is required to load Ras with GTP. On the other hand, if Cdc25 would be the mediator of glucose signaling, one would expect a further glucose-induced increase in the ira2Δ and ira1Δ ira2Δ strains. The inability of Colombo et al. (5) to detect a glucose-induced increase in GTP on Ras focused the attention on Gpa2 and led to the discovery of the Gpr1-Gpa2-Rgs2 GPCR module as the glucose-sensing system for activation of the cAMP pathway (2, 3, 5, 11, 30–32). Our results now show that this module is not required for glucose activation of Ras2. Deletion of GPR1 or GPA2 did not prevent the glucose-induced increase in Ras2-GTP. Unexpectedly, deletion of GPR1 and especially of GPA2 also enhanced the levels of Ras2-GTP. This points to a competition between the two G-protein systems. One possibility is that the two G-proteins compete for interaction with adenylyl cyclase. Inactivation of the Gpr1-Gpa2 module might facilitate interaction of Ras with adenylyl cyclase, and if this interaction would decrease the accessibility of Ras to the Ira proteins a more persistent Ras2-GTP load could be the final result.

Despite the discovery of the Gpr1-Gpa2 glucose-sensing GPCR module, it has been firmly established that glucose activation by this module is also strictly dependent on glucose phosphorylation (10, 11). It is awkward that a ligand of a GPCR system has to be transported and modified in metabolism before the same ligand can activate the effector system through activation of Ras, but detection of the latter would require a stabilization of GTP-loaded Ras2 as apparently happens in the gpr1Δ and gpa2Δ strains. Therefore, the increase in Ras2-GTP in the gpr1Δ and gpa2Δ strains with a low glucose concentration supports the idea that glucose phosphorylation acts through the Ras proteins. Obviously, higher external glucose concentrations will lead to faster transport and faster intracellular glucose phosphorylation, consistent with the further increase in Ras2-GTP upon the addition of 100 mM glucose. In conclusion, the most appealing hypothesis concerning the mechanism by which glucose increases the GTP-loading on the Ras2 protein is that the glucose phosphorylation-dependent mechanism causes inhibition of the Ira proteins. This switches the equilibrium between Cdc25 stimulated exchange of GDP for GTP and GTP hydrolysis by the Ira-stimulated GTPase activity of the Ras2 protein to GTP-loading resulting in a rapid increase in Ras2-GTP. This hypothesis is also in agreement with the observation that deletion of IRA2 in a triple glucose kinases deleted strain produced a high level of Ras2-GTP. Although further work is required to substantiate this hypothesis it provides an elegant explanation for the dual requirement in glucose activation of cAMP synthesis. It also reconciles the former results indicating involvement of the Cdc25-Ras module and the more recent results on glucose sensing by the GPCR module. Possibly, the double G-protein control of adenylyl cyclase serves to integrate sensing of extracellular glucose with the sensing of intracellular glucose.

**Feedback Inhibition of Adenylyl Cyclase**—Colombo et al. (5) were unable to detect an increase in the GTP content on Ras in a strain with reduced feedback inhibition. This might be because of the same technical problem of the assay as discussed previously because with the current assay a significant increase in the basal level of Ras2-GTP was detected in a PKA-attenuated strain. Importantly, also the glucose-induced increase in Ras2-GTP was prominently present making it unlikely that the latter is caused by reduction of the feedback inhibition. Deletion of GPA2 did not affect the basal level of Ras2-GTP nor the glucose-induced increase indicating that the feedback inhibition proceeds independently from the GPCR module. Although the Ras proteins have been suggested as a target of the feedback inhibition the proposed mechanism, phosphorylation-induced reduction of adenylyl cyclase interaction, did not involve changes in the GTP/GDP content of Ras (33). Cdc25 has also been proposed as a target of the feedback inhibition mechanism. It is phosphorylated in response to glucose, and this reduces its accessibility to Ras (34). A mutation in the Cdc25 C terminus was identified that reduces feedback inhibition after glucose-induced stimulation of cAMP synthesis (25). The Ira proteins have also been suggested as possible targets of the feedback inhibition mechanism (35), but this has not been substantiated further. Although the Cdc25 and Ira proteins are the most likely candidates for the feedback-inhibition mechanism to explain the increased Ras2-GTP content in a PKA-attenuated strain, it cannot be excluded that modification of adenylyl cyclase would affect the Ras2-GTP loading.
state. Actually, deletion of GPA2 or GPR1 also enhanced the Ras2-GTP level although not as pronounced as in the PKA-attenuated strain. Because deletion of GPA2 in the PKA-attenuated strain did not further enhance the basal Ras2-GTP content its effect on the Ras2-GTP level might be related to the feedback-inhibition mechanism.

Acknowledgments—We thank A. Wittinghofer for providing the expression vector for the production of GST-RBD and Katrien Pardons (Katholieke Universiteit Leuven) for providing strain glk1::LEU2 his3::LEU2 ira2::URA3.

REFERENCES
1. Nakafuku, M., Obara, T., Kaibuchi, K., Miyajima, I., Miyajima, A., Itsh, H., Nakamura, S., Arai, K., Matsumoto, K., and Kaziro, Y. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1374–1378
2. Kraakman, L., Lemaire, K., Ma, P., Teunissen, A. W., Donaton, M. C., Van Dijck, P., Winderickx, J., de Winde, J. H., and Thevelein, J. M. (1999) Mol. Microbiol. 32, 1002–1012A. W.
3. Xue, Y., Montserrat, B., and Hirsch, J. P. (1998) EMBO J. 17, 1996–2007
4. Yun, C. W., Tamaki, H., Nakayama, R., Yamamoto, K., and Kumagai, H. (1997) Biochem. Biophys. Res. Commun. 240, 287–292
5. Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Cauwenberg, M., Teunissen, A., Nauwelaers, D., de Winde, J. H., Gorwa, M. F., Colavizza, D., and Thevelein, J. M. (1998) EMBO J. 17, 3326–3341
6. Rudoni, S., Colombo, S., Cocetti, P., and Martegani, E. (2001) Biochim. Biophys. Acta 1538, 181–189
7. Gibbs, J. B., Schaber, M. D., Marshall, M. S., Scolnick, E. M., and Sigal, I. S. (1987) J. Biol. Chem. 262, 10426–10429
8. Taylor, S., and Shalloway, D. (1996) Curr. Biol. 6, 1621–1627
9. Beullens, M., Mbuyi, K., Geerts, L., Gladines, D., Detremere, K., Jans, A. W. H., and Thevelein, J. M. (1988) Eur. J. Biochem. 172, 227–231
10. Rolland, F., de Winde, J. H., Lemaire, K., Boles, E., Thevelein, J. M., and Winderickx, J. (2000) Mol. Microbiol. 38, 348–358
11. Rolland, F., Wanke, V., Cauwenberg, L., Ma, P., Boles, E., Vanoni, M., de Winde, J. H., Thevelein, J. M., and Winderickx, J. (2001) FEBS Yeast Res. 1403, 1–13
12. Robinson, L. A., Gibbs, J. B., Marshall, M. S., Sigal, I. S., and Tatchell, K. (1987) Science 235, 1218–1221
13. Jacquet, E., Parrini, M. C., Bernardi, E., Martegani, E., and Parmeggiani, A. (1994) Biochem. Biophys. Res. Commun. 199, 497–503
14. Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M. S., Gibbs, J. B., Matsumoto, K., Kaziro, Y., and Tob-e, A. (1990) Cell 60, 893–897
15. Lobo, Z., and Maitra, P. K. (1977) Genetics 86, 727–744
16. Maitra, P. K., and Lobo, Z. (1983) Genetics 105, 501–515
17. Nikowa, J., Cameron, S., Toda, T., Ferguson, K. W., and Wigler, M. (1987) Genes Dev. 1, 931–937
18. Mbonyi, K., Van Aelst, L., Arguelles, J. C., Jans A. W. H., and Thevelein, J. M. (1990) Mol. Cell. Biol. 10, 4518–4523
19. Ma, P., Wera, S., Van Dijck, P., and Thevelein, J. M. (1999) Mol. Biol. Cell 10, 91–104
20. Broek, D., Samlly, N., Fasano, O., Fujiyama, A., Tamanou, F., Northup, J., and Wigler, M. (1985) Cell 41, 763–769
21. Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985) Cell 40, 27–36
22. Mbonyi, K., Beullens, M., Detremere, K., Geerts, L., and Thevelein, J. M. (1988) Mol. Cell. Biol. 8, 3051–3057
23. Munder, T., and Kuntzel, H., (1989) FEBS Lett. 242, 341–354
24. Van Aelst, L., Boy-Marcotte, E., Camonis, J. H., Thevelein, J. M., and Jacquet, M. (1990) Eur. J. Biochem. 193, 675–680
25. Schomerus, C., Munder, T., and Kuntzel, H., (1999) Mol. Gen. Genet. 223, 426–432
26. Van Aelst, L., Jans, A. W. H., and Thevelein, J. M. (1991) J. Gen. Microbiol. 137, 341–349
27. Bhattacharyya, S., Chen, L., Broach, J. R., and Powers, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2984–2988
28. Jiang, Y., Davis, C., and Broach, J. R. (1998) EMBO J. 17, 6942–6951
29. Boy-Marcotte, E., Ikonomi, P., and Jacquet, M. (1996) Mol. Biol. Cell, 7, 529–539
30. Thevelein, J. M., and de Winde, J. H. (1999) Mol. Microbiol. 33, 904–918
31. Lorenz, M. C., Pan, X., Harashima, T., Cardenas, M. E., Xue, Y., Hirsch, J. P., and Heitman, J. (2000) Genetics 154, 609–622M. C.
32. Versele, M., Lemaire K., and Thevelein, J. M. (2001) EMBO Rep. 2, 574–579
33. Resnick, R. J., and Racker, E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2474–2478
34. Gross, E., Goldberg, D., and Levitzki, A. (1992) Nature 360, 762–765
35. Tanaka, K., Matsumoto, K., and Tob-e, A. (1989) Mol. Cell. Biol. 9, 757–768
36. Thomas, B. J., and Rothstein, R. (1989) Cell 56, 619–630
37. de Winde, J. H., Cauwenbergs, M., Hohmann, S., Thevelein, J. M., and Winderickx, J. (1996) Eur. J. Biochem. 241, 633–643H.
38. Reifenberger, E., Freidel, K., and Ciriacy, M. (1995) Mol. Microbiol. 16, 157–167
Activation State of the Ras2 Protein and Glucose-induced Signaling in *Saccharomyces cerevisiae*

Sonia Colombo, Daniela Ronchetti, Johan M. Thevelein, Joris Winderickx and Enzo Martegani

*J. Biol. Chem. 2004, 279:46715-46722.*  
doi: 10.1074/jbc.M405136200 originally published online August 31, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405136200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 17 of which can be accessed free at [http://www.jbc.org/content/279/45/46715.full.html#ref-list-1](http://www.jbc.org/content/279/45/46715.full.html#ref-list-1)