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Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*

Sonia Colombo[1,2], Pingsheng Ma[1], Liesbet Cauwenberg[1], Joris Winderickx[1], Marion Crauwels[1], Aloys Teunissen[1], David Nauwelaers[1], Johannes H.de Winde[1], Marie-Françoise Gorwa[3], Didier Colavizza[3] and Johan M.Thevelein[1,4]

[1]Laboratorium voor Moleculaire Celbiologie, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001 Leuven-Heverlee, Flanders, Belgium, [2]Institut für Mikrobiologie und Genetik, Technische Hochschule Darmstadt, Schnittspahnstrasse 10, D-64287 Darmstadt, Germany and [3]Lesaffre Développement S.A., Rue Gabriel Péri 147, BP 6027, F-59706 Marcq-en-Baroeul, Cedex, France

[4]Corresponding author
e-mail: johan.thevelein@bio.kuleuven.ac.be

S.Colombo and P.Ma contributed equally to this work

Adenylate cyclase activity in *Saccharomyces cerevisiae* is dependent on Ras proteins. Both addition of glucose to glucose-deprived (derepressed) cells and intracellular acidification trigger an increase in the cAMP level *in vivo*. We show that intracellular acidification, but not glucose, causes an increase in the GTP/GDP ratio on the Ras proteins independent of Cdc25 and Sdc25. Deletion of the GTPase-activating proteins Ira1 and Ira2, or expression of the *RAS2*^{val19} allele, causes an enhanced GTP/GDP basal ratio and abolishes the intracellular acidification-induced increase. In the *ira1*Δ *ira2*Δ strain, intracellular acidification still triggers a cAMP increase. Glucose also did not cause an increase in the GTP/GDP ratio in a strain with reduced feedback inhibition of cAMP synthesis. Further investigation indicated that feedback inhibition by cAPK on cAMP synthesis acts independently of changes in the GTP/GDP ratio on Ras. Stimulation by glucose was dependent on the Gα-protein Gpa2, whose deletion confers the typical phenotype associated with a reduced cAMP level: higher heat resistance, a higher level of trehalose and glycogen and elevated expression of STRE-controlled genes. However, the typical fluctuation in these characteristics during diauxic growth on glucose was still present. Overexpression of Ras2^{val19} inhibited both the acidification- and glucose-induced cAMP increase even in a protein kinase A-attenuated strain. Our results suggest that intracellular acidification stimulates cAMP synthesis *in vivo* at least through activation of the Ras proteins, while glucose acts through the Gpa2 protein. Interaction of Ras2^{val19} with adenylate cyclase apparently prevents its activation by both agonists.

Keywords: acidification/cAMP signalling/Gpa2/glucose/Ras

Introduction

In the yeast *Saccharomyces cerevisiae* the cAMP level is controlled by an elaborate regulatory pathway (for reviews, see Broach and Deschenes, 1990; Thevelein, 1991, 1992; Tatchell, 1993). Adenylate cyclase, encoded by the *CYR1/CDC35* gene, is controlled by the Ras1 and Ras2 proteins (Toda *et al*., 1985), which have been thought to act in a similar way to the mammalian G_s proteins. The GTP/GDP ratio on the Ras proteins is controlled by the guanine nucleotide exchange factors Cdc25 (Camonis *et al*., 1986; Martegani *et al*., 1986) and Sdc25 (Damak *et al*., 1991; Boy-Marcotte *et al*., 1996), and by the Ira1 and Ira2 proteins which stimulate Ras-GTPase activity (Tanaka *et al*., 1989, 1990a,b). Also for these components of the Ras-cAMP pathway, close homologues exist in mammalian cells, e.g. for Cdc25 (Martegani *et al*., 1992; Feig, 1994) and for the Ira proteins (Trahey *et al*., 1988; Vogel *et al*., 1988). cAMP activates cAMP-dependent protein kinase (cAPK), which is composed in yeast of catalytic subunits encoded by the genes *TPK1*, *TPK2* and *TPK3* (Toda *et al*., 1987b), and regulatory subunits encoded by the gene *BCY1* (Toda *et al*., 1987a). cAMP is hydrolysed by a low-affinity and a high-affinity phosphodiesterase, respectively encoded by *PDE1* and *PDE2* (Nikawa *et al*., 1987b; Sass *et al*., 1986).

The cAMP pathway is essential for viability. Depletion of cAMP causes a response similar to nutrient starvation. The cells arrest at the start point in the G_1 phase of the cell cycle and accumulate permanently in the stationary phase G_0. In addition, mutants with an overactive cAMP pathway or with reduced activity of the pathway display a pleiotropic phenotype. An overactive pathway causes sensitivity to heat shock and nutrient starvation, low levels of the storage carbohydrates glycogen and trehalose, failure to arrest properly in the G_1 phase of the cell cycle upon nutrient limitation, poor growth on non-fermentable (glycerol, ethanol, acetate) and weakly fermentable carbon sources (galactose, maltose), and failure to sporulate in diploid cells. Reduced activity of the pathway causes enhanced heat resistance, enhanced levels of glycogen and trehalose, constitutive expression of heat shock genes and other genes which are only expressed in stationary phase in wild-type cells, and in diploid cells causes sporulation in rich nutrient media. Part of this pleiotropic phenotype is caused by changes at the transcriptional level in a large number of genes, controlled by so-called STRE elements (consensus: CCCCT) in their promoter. These genes are repressed by high protein kinase A (PKA) activity and derepressed under conditions of low PKA activity (for reviews, see Broach and Deschenes, 1990; Thevelein, 1992, 1994; Tatchell, 1993; Ruis and Schuller, 1995).

A very potent system down-regulating cAMP levels in yeast is feedback inhibition of cAMP synthesis by cAPK

(Nikawa *et al*., 1987a). Several components of the Ras-cAMP pathway have been proposed as possible targets for this feedback inhibition. It has been shown that phosphorylation of the yeast Ras proteins reduces their activating effect on adenylate cyclase *in vitro* (Resnick and Racker, 1988). Cdc25 was shown to be phosphorylated in response to glucose, resulting in reduced accessibility to Ras (Gross *et al*., 1992). A specific mutation in the extreme C-terminus of Cdc25 was shown to result in reduced feedback inhibition after glucose-induced stimulation of cAMP synthesis (Schomerus *et al*., 1990). The Ira proteins have also been proposed as possible targets for feedback inhibition since they also have potential phosphorylation sites for cAPK (Tanaka *et al*., 1989; Tatchell, 1993). The same is true for adenylate cyclase itself for which the distribution between membrane-bound and cytosolic forms was shown to depend on cAPK activity (Engelberg *et al*., 1990; Mitts *et al*., 1990; Tatchell, 1993). However, in all these cases, the physiological significance of feedback inhibition of cAMP synthesis *in vivo* has not been demonstrated and the actual target of the feedback inhibition by cAPK therefore remains unclear.

By analogy with G protein control of adenylate cyclase in mammalian cells, the Ras proteins in yeast are generally thought to mediate agonist-induced signalling towards the cyclase. Two conditions are known that strongly stimulate cAMP accumulation *in vivo* in yeast cells. The addition of glucose or related rapidly fermented sugars to cells growing on a non-fermentable carbon source, to stationary phase cells or ascospores previously deprived of such sugars, triggers a rapid, transient spike in the cAMP level (van der Plaat, 1974; Purwin *et al*., 1982; Tortora *et al*., 1982; Thevelein, 1984b). Conditions which lead to intracellular acidification, such as addition of protonophores at low extracellular pH, cause a much higher and longer lasting increase in the cAMP level (Trevillyan and Pall, 1979). This effect has been observed in many fungi and was thought initially to be due to plasma membrane depolarization (Pall, 1981; Mazon *et al*., 1982; Holzer, 1984). However, later work showed that the increase correlated closely with intracellular acidification rather than plasma membrane depolarization (Caspani *et al*., 1985; Purwin *et al*., 1986; Thevelein *et al*., 1987a). Similarly, the glucose-induced transient increase in the cAMP level initially was considered to be triggered by the transient glucose-induced intracellular acidification which occurs upon glucose addition to derepressed yeast cells (Busa and Nuccitelli, 1984; Caspani *et al*., 1985; Purwin *et al*., 1986). However, elimination of this transient acidification by addition of NH_4Cl at high extracellular pH or by previous addition of a small amount of glucose did not abolish the glucose-induced cAMP increase (Thevelein *et al*., 1987b). These results pointed to a specific signalling effect of glucose rather than an effect caused by one of the metabolic changes resulting from glucose catabolism.

Subsequent work showed that both the Ras and the Cdc25 proteins were essential for glucose-induced activation of cAMP synthesis and that the constitutively activated *RAS2*^{val19} allele was apparently unable to transmit the glucose signal (Mbonyi *et al*., 1988; Munder and Küntzel, 1989; Schomerus *et al*., 1990; Van Aelst *et al*., 1990, 1991; Kim and Powers, 1991). Similar results were obtained for stimulation of cAMP synthesis by intracellular acidification (Mbonyi *et al*., 1988; Van Aelst *et al*., 1991). These studies were complicated by the fact that both the Ras and Cdc25 proteins are not only required for activation of cAMP synthesis but are also essential for basal adenylate cyclase activity and cell viability. Therefore, suppressing mutations or multicopy suppressor genes restoring viability have to be present in cells deleted for Ras or Cdc25. These suppressors might affect cAMP accumulation by causing enhanced feedback inhibition or by other unknown means. Evidence has also been presented against Cdc25 acting as the signal receiver for the glucose-induced cAMP response (Goldberg *et al*., 1994). In this case, many atypical cAMP increases were observed, possibly due to interference with the feedback inhibition mechanism or with intracellular acidification-induced cAMP stimulation.

We have now investigated the possible involvement of the Ras proteins in glucose- and acidification-induced cAMP accumulation in yeast more directly. We have measured the GTP/GDP ratio on the Ras proteins after addition of glucose or a protonophore in a similar way to what has been done previously for agonists causing an increase in the ratio of GTP/GDP bound to the mammalian Ras proteins (Downward *et al*., 1990; Gibbs *et al*., 1990; Satoh *et al*., 1990a,b; Burgering *et al*., 1991; Qiu and Green, 1991). Previous measurements of the GTP/GDP ratio on Ras proteins in yeast cells have only been performed with steady-state cultures. Gibbs *et al*. (1987) demonstrated that in exponential phase glucose-growing cultures nearly all isolated Ras protein was bound to GDP, whereas in strains expressing constitutively active Ras alleles a large part of the guanine nucleotide was recovered as GTP. Tanaka *et al*. (1990a) confirmed and extended these results by demonstrating that deletion of the *IRA1* and/or *IRA2* gene also strongly enhanced the GTP/GDP ratio of guanine nucleotides bound to the Ras proteins. In this work, we show that intracellular acidification, but not glucose, causes a very rapid increase in the ratio of GTP/GDP bound to the Ras proteins.

The *S.cerevisiae GPA2* gene was cloned previously from a yeast genomic DNA library by hybridization using rat brain $G_{i2\alpha}$ and $G_{o\alpha}$ cDNAs as probes. Although evidence was obtained indicating a possible role in control of cAMP levels, no difference was observed in the glucose-induced cAMP signal between wild-type cells and cells carrying a disrupted *GPA2* allele (Nakafuku *et al*., 1988). Another study provided evidence that the *GPA2* product is required for yeast α-mating factor-induced suppression of the glucose-induced cAMP increase (Papasavvas *et al*., 1992). Recently, the possible involvement of Gpa2 in the control of cAMP levels has been re-investigated with respect to the control of pseudohyphal growth (Kübler *et al*., 1997; Lorenz and Heitman, 1997). Although the results reported were also consistent with a role for Gpa2 in controlling adenylate cyclase activity, no direct measurements of cAMP levels were performed. In spite of this, it was concluded that Gpa2 is an element of the nitrogen-sensing machinery that regulates pseudohyphal differentiation by modulating cAMP levels. On the other hand, in *Schizosaccharomyces pombe* (Isshiki *et al*., 1992; Nocero *et al*., 1994) and *Kluyveromyces lactis* (Saviñon-Tejeda *et al*., 1996), Gα proteins appear to be involved in the stimulation of cAMP levels in response to glucose.

We have investigated whether the failure of the previous

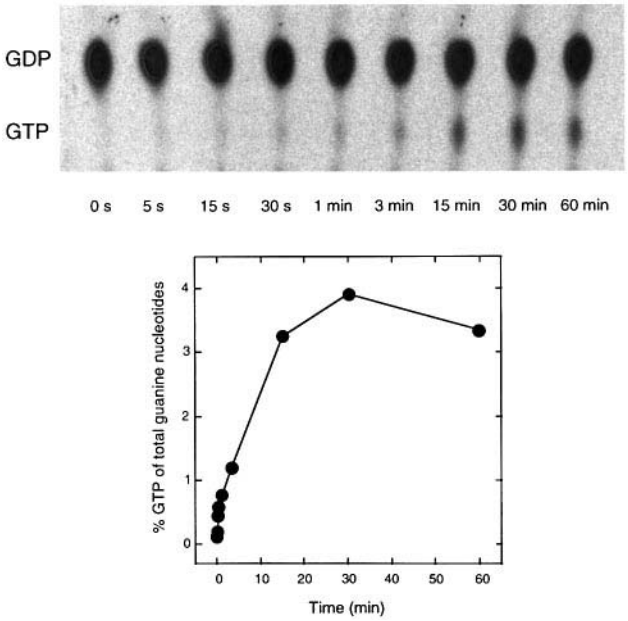

Fig. 1. Amount of GTP and GDP bound to the Ras protein in a wild-type strain + YEp*RAS2* as a function of time after intracellular acidification with 2,4-dinitrophenol at an extracellular pH of 4.5. Quantification of the results is shown as an example.

studies to reveal a requirement for Gpa2 in glucose-induced stimulation of cAMP synthesis in *S.cerevisiae* might be due to interference with the strong stimulating effect of low intracellular pH on cAMP synthesis. Such interference has been observed previously. Yeast cells only respond to addition of glucose with an increase in intracellular cAMP when the cells are derepressed. However, in glucose derepression mutants and occasionally also in wild-type cells, 'artefactual' cAMP increases have been observed upon glucose addition to repressed cells. This is probably due to the low intracellular pH when such cells are suspended in buffer in the absence of a carbon source (Argüelles *et al*., 1990). This interference can be eliminated by pre-addition of a low level of glucose, which raises the ATP level and the intracellular pH, but does not trigger the cAMP spike (Thevelein *et al*., 1987b; Argüelles *et al*., 1990). Re-investigation of the *GPA2* deletion mutant under such conditions, as shown in this study, reveals that absence of Gpa2 completely eliminates glucose-induced cAMP signalling. In addition, cells carrying a disrupted *GPA2* allele display a low cAMP phenotype during growth on glucose.

Results

Intracellular acidification enhances the ratio of GTP/GDP bound to Ras and this increase is independent of Cdc25 and Sdc25

Preliminary work showed that the GTP/GDP ratio on the Ras proteins could only be measured reliably after overexpression of Ras protein, in agreement with results obtained previously (Gibbs *et al*., 1987; Tanaka *et al*., 1990a). Hence, initially all experiments were performed with strains in which the Ras2 protein was overexpressed using the *PGK* promoter. Overexpression of the Ras2 protein did not significantly affect the increase in the

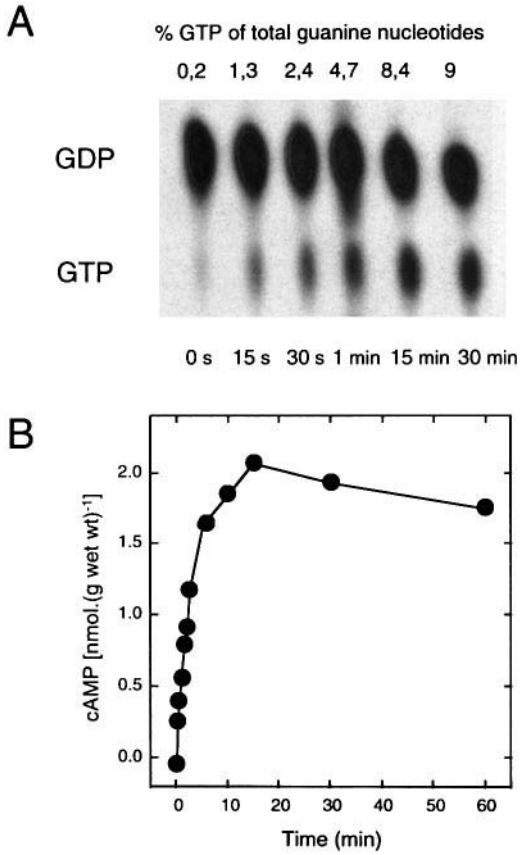

Fig. 2. Intracellular acidification triggered by 2,4-dinitrophenol at an extracellular pH of 4.5 in the *cdc25*Δ *sdc25*Δ + YEp*TPK1* + YEp*RAS2* strain. (**A**) Amount of GTP and GDP bound to the Ras protein as a function of time. (**B**) cAMP content as a function of time.

cAMP level triggered by intracellular acidification or by glucose (results not shown). In later work, it was possible to measure the GTP/GDP ratio in cells of specific strains without overexpression of Ras protein (see below). After labelling the cells *in vivo* with ^{32}Pi, addition of agonist and cell extraction, the Ras proteins were immunoprecipitated with antibodies against the human Ras protein [v-H-ras (259)–agarose conjugate antibodies from Oncogene Science]. Guanine nucleotides were extracted, separated by TLC and quantitated by phosphorimager technology (see Materials and methods). An example of the results after quantification is shown in Figure 1.

Addition of the protonophore 2,4-dinitrophenol to wild-type yeast cells at extracellular pH 4.5 triggered already within a few seconds a clear increase in the GTP/GDP ratio (Figure 1). The increase reached a maximum ~30 min afterwards. When 2,4-dinitrophenol was added at pH 6, no increase in the GTP/GDP ratio could be detected, indicating that the effect is truly dependent on intracellular acidification and not due to the 2,4-dinitrophenol itself (results not shown). A strain deleted for both the *CDC25* and *SDC25* genes and rescued by overexpression of *TPK1* showed an increase in the GTP/GDP ratio comparable with the wild-type strain (Figure 2A). This indicates that the guanine nucleotide exchange proteins of Ras are not required for the acidification-induced cAMP increase. In the same strain, addition of 2,4-dinitrophenol triggered a pronounced increase in the cAMP level (Figure 2B). In the temperature-sensitive mutants *cdc25-5* and *cdc25-1*, a

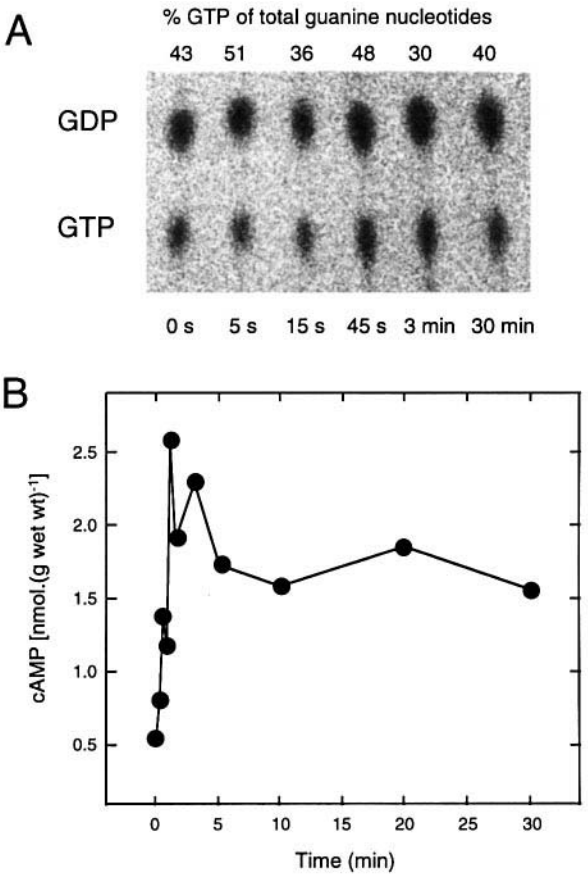

Fig. 3. Intracellular acidification triggered by 2,4-dinitrophenol at an extracellular pH of 4.5 in the *ira1*Δ *ira2*Δ strain. (**A**) Amount of GTP and GDP bound to the Ras protein as a function of time. (**B**) cAMP content as a function of time.

similar increase in the GTP/GDP ratio was observed at permissive and restrictive temperature, confirming that Cdc25 is not required for the increase (results not shown).

Intracellular acidification does not enhance the ratio of GTP/GDP bound to Ras in a strain lacking both Ira1 and Ira2 nor in a strain expressing the RAS2^{val19} allele

In a strain deleted for both genes encoding the Ras-GTPase activating proteins, Ira1 and Ira2, the GTP/GDP ratio could be measured easily both in the absence (Figure 3A) and the presence (results not shown) of Ras2 overexpression. In both cases, the initial GTP content was already very high compared with wild-type cells (~40% versus <1%), but it did not increase after addition of the protonophore (Figure 3A). Under the same conditions, 2,4-dinitrophenol was still able to cause a pronounced increase in the cAMP level (Figure 3B). In strains with overexpression of Ras2 and containing only the wild-type *IRA1* gene (*ira2*Δ) or only the wild-type *IRA2* gene (*ira1*Δ), a normal protonophore-induced increase in the GTP/GDP ratio was observed (results not shown). These results point to the Ira proteins as the targets of the intracellular acidification effect on the Ras GTP/GDP ratio.

The Ras2^{val19} allele contains a glycine to valine substitution at position 19 of the Ras2 protein. This dominant allele has strongly reduced GTPase activity, is independent of guanine nucleotide exchange factors and insensitive to

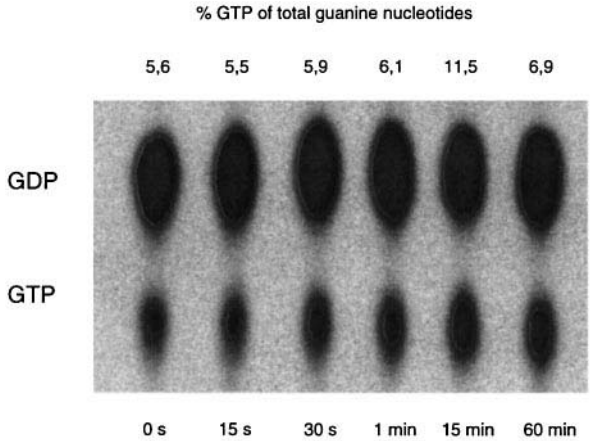

Fig. 4. Amount of GTP and GDP bound to the Ras protein in the W303-1A + YEp*RAS2*^{val19} strain as a function of time after intracellular acidification with 2,4-dinitrophenol at an extracellular pH of 4.5.

the Ira proteins, and as a result causes constitutive activation of adenylate cyclase (Toda *et al*., 1985; Broek *et al*., 1987; Robinson *et al*., 1987; Tanaka *et al*., 1990a, 1992). We have made use of a wild-type strain with a multicopy plasmid carrying the *RAS2*^{val19} allele. Similarly to the *ira1*Δ *ira2*Δ strain, the *RAS2*^{val19} strain showed an elevated GTP content on the Ras proteins already before addition of agonist (Figure 4). Subsequent intracellular acidification by addition of 2,4-dinitrophenol did not cause a further increase in the GTP content (Figure 4). This result is again in agreement with the Ira proteins being targets of the acidification effect. The same results (elevated initial GTP content and no further increase) were obtained with a *tpk1*^{w1} *tpk2*Δ *tpk3*Δ strain in which *RAS2*^{val19} was overexpressed (results not shown). This strain has a deletion in two of the three *TPK* genes encoding the catalytic subunits of cAPK and a partially inactivating point mutation in the third *TPK* gene. As a result, the activity of PKA and, as a consequence, feedback inhibition on cAMP synthesis is very low (Nikawa *et al*., 1987a). Hence, the absence of a further increase in GTP is not due to elevated feedback inhibition caused by the *RAS2*^{val19} allele.

Under the same conditions, the cAMP level only increased after intracellular acidification in the wild-type strain and not in the wild-type strain overexpressing *RAS2*^{val19} (Figure 5A). To investigate whether the absence of the cAMP increase could be due to constitutively high feedback inhibition by the high PKA activity caused by *RAS2*^{val19}, we have again used the *tpk1*^{w1} *tpk2*Δ *tpk3*Δ strain. However, also in such a strain, overexpression of *RAS2*^{val19} abolished the acidification-induced cAMP increase (Figure 5B).

pH-dependent stability in vitro of the ratio of GTP/GDP bound to Ras

We have investigated whether the acidification-induced increase in the GTP/GDP ratio was caused by a direct effect of pH on the binding of GTP and GDP to the Ras proteins. For that purpose, we measured the stability of the GTP/GDP ratio during a period of 60 min with both the wild-type Ras2 and the Ras2^{val19} protein *in vitro* in the pH range 4.5–7.0. In the absence of added guanine

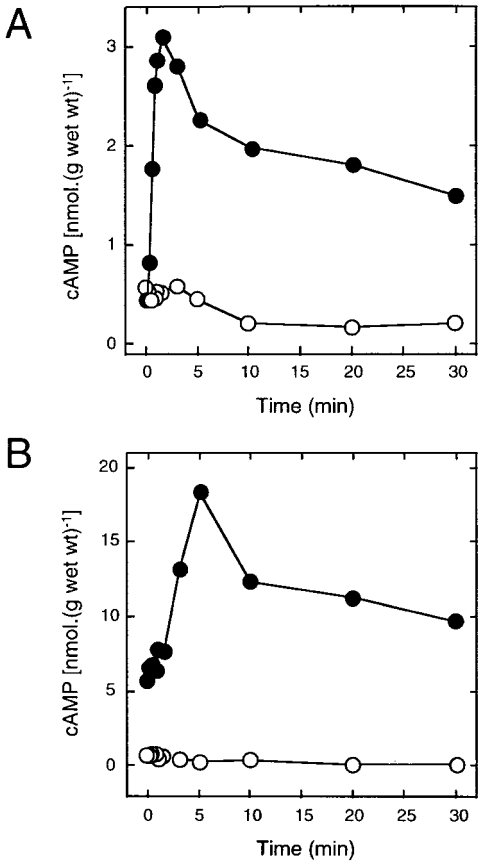

Fig. 5. cAMP content as a function of time after intracellular acidification with 2,4-dinitrophenol at an extracellular pH of 4.5. (**A**) Wild-type strain (●), wild-type strain + YEp*RAS2*^{val19} (○). (**B**) *tpk*^{w1} strain (●), *tpk*^{w1} + YEp*RAS2*^{val19} (○).

nucleotides, no effect at all was observed. In the presence of unlabelled GDP or GTP, a slight decrease in the amount of labelled bound GTP was observed, resulting in a decrease of the GTP/GDP ratio in both cases (results not shown). The physiological relevance of this small effect is unclear and it is not able to explain the acidification-induced increase in the GTP/GDP ratio *in vivo*.

Glucose does not affect the ratio of GTP/GDP bound to Ras

In contrast to intracellular acidification, addition of glucose did not trigger any increase in the GTP/GDP ratio on the Ras protein in a wild-type strain (Figure 6A). This negative result could have been due to the technical difficulty of detecting an increase, since the glucose-induced cAMP signal is very short-lived and much smaller than the increases triggered by intracellular acidification. Therefore, we measured the GTP/GDP ratio on the Ras protein after addition of glucose in a strain with strongly reduced feedback inhibition of cAMP synthesis due to reduced activity of cAPK (*tpk1*^{w1} *tpk2*Δ *tpk3*Δ). Addition of glucose to derepressed cells of such a strain results in a huge, long-lasting increase in the cAMP level (Mbonyi *et al.*, 1990). However, also in such a strain, no glucose-induced increase in the GTP/GDP content on the Ras proteins was observed (Figure 6B). This strongly argues against the Ras proteins as mediators of the glucose effect. In addition, the initial GTP/GDP ratio (Figure 6B) and the response upon intracellular acidification (results not shown) in this strain were similar to those observed in the wild-type strain.

Since previous results had indicated that a Ras2^{val19} strain was unable to display a glucose-induced cAMP signal (Mbonyi *et al.*, 1988), we have investigated whether this might have been due to constitutive feedback inhibition of cAMP synthesis by the high PKA activity. However, overexpression of *RAS2*^{val19} in a *tpk*^{w1} strain (*tpk1*^{w1} *tpk2*Δ *tpk3*Δ) also eliminated the glucose-induced cAMP increase (Figure 7). Hence, Ras2^{val19} does not inhibit the cAMP response through overactivation of PKA. Therefore, both glucose- and acidification-induced stimulation of cAMP synthesis are inhibited by Ras2^{val19} in a way independent of high PKA activity.

Feedback inhibition does not act through the ratio of GTP/GDP bound to Ras

The previous results made it unlikely that the feedback inhibition mechanism acts by changing the ratio of GTP/GDP bound to the Ras proteins. To explore this question further, we also investigated the ratio of GTP/GDP bound to Ras in a strain with enhanced feedback inhibition (*TPK1 tpk2*Δ *tpk3*Δ *bcy1*Δ). Remarkably, in this strain, the initial ratio of GTP/GDP bound to the Ras proteins was consistently higher than in the wild-type strain (Figure 8A). The increase after addition of 2,4-dinitrophenol was comparable with that observed in the wild-type strain (Figure 8A). This result also fits with that shown in Figure 2 where a strain was used for which the lethality caused by deletion of *CDC25* was suppressed by overexpression of *TPK1*. In this case too, the 2,4-dinitrophenol-induced increase in GTP/GDP bound to Ras was similar to that observed in the wild-type strain. However, the initial GTP/GDP ratio was not elevated possibly because of the absence of Cdc25 (Figure 2). On the other hand, in the strain with enhanced feedback inhibition (*TPK1 tpk2*Δ *tpk3*Δ *bcy1*Δ), both the basal cAMP level and the glucose- and acidification-induced cAMP increase were strongly reduced (Figure 8B and C). This indicates a strong inhibitory effect of high PKA activity on basal cAMP synthesis and on the cAMP responses. Overexpression of Ras2 in this strain and in the control strain did not affect the cAMP results (not shown).

Glucose-induced cAMP signalling is absent in a gpa2Δ strain

Investigation of the glucose-induced cAMP signal in the original *gpa2*Δ and overexpression strains constructed by Nakafuku *et al.* (1988) basically confirmed their results and those reported by Papasavvas *et al.* (1992). The glucose-induced cAMP signal in the *gpa2*Δ strain was partially reduced and in the *GPA2* overexpression strain slightly elevated (results not shown). Subsequently, we investigated whether the partial cAMP increase observed in *gpa2*Δ strains upon glucose addition might be due to the intracellular acidification effect. Addition of 5 mM glucose, which raises the ATP level and intracellular pH but only partially raises the cAMP level (Thevelein *et al.*, 1987b; Beullens *et al.*, 1988), abolished a subsequent cAMP signal induced by 100 mM glucose in the *gpa2*Δ strain of Nakafuku *et al.* (1988) (results not shown) and in the *gpa2*Δ strain in the W303-1A background (Figure 9A). The W303-1A wild-type strain was more

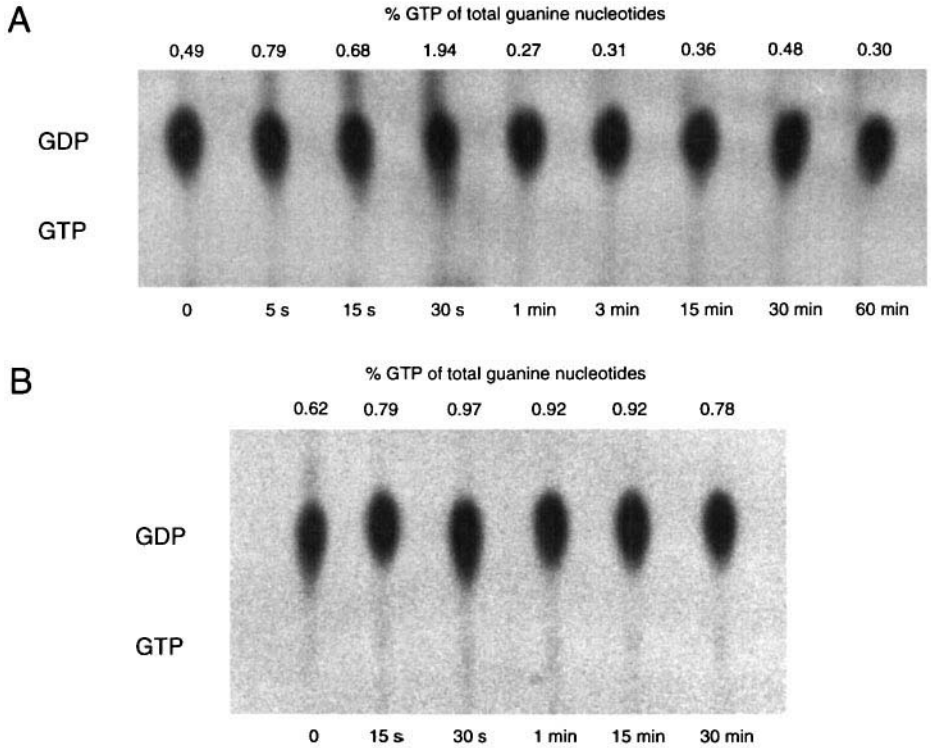

Fig. 6. Amount of GTP and GDP bound to the Ras protein as a function of time after addition of glucose. (**A**) Wild-type strain + YEp*RAS2*. (**B**) *tpk1*^{w1} *tpk2*Δ *tpk3*Δ + YEp*RAS2*.

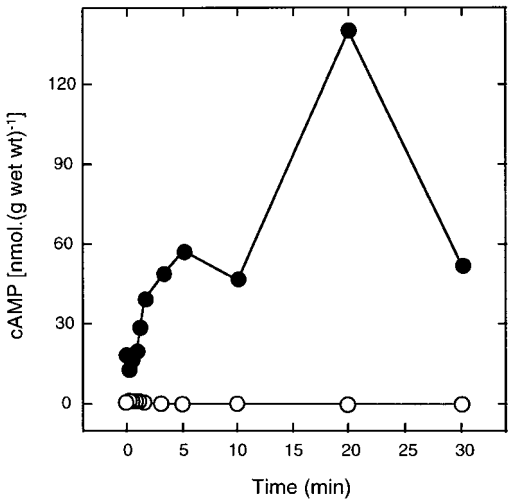

Fig. 7. cAMP content as a function of time after addition of glucose. *tpk*^{w1} strain (●), *tpk*^{w1} + YEp*RAS2*^{val19} (○).

sensitive to low glucose levels than the other strains used previously (Beullens *et al*., 1988; Argüelles *et al*., 1990). However, it showed a clear second cAMP signal with 100 mM glucose. In a strain overexpressing Gpa2, the cAMP signal triggered by 100 mM glucose also appeared somewhat elevated compared with the signal in the wild-type strain (Figure 9A). Deletion or overexpression of Gpa2 had no significant effect on the initial cAMP increase triggered by 5 mM glucose. Similar results were obtained with *gpa2*Δ and *GPA2* overexpression strains in the SP1 background (results not shown). When intracellular acidification was triggered by addition of 2 mM 2,4-dinitrophenol, after addition of 5 mM glucose the same cAMP increase was observed in the wild-type strain, the *gpa2*Δ strain and the *GPA2* overexpression strain (W303-1A background) (Figure 9B). Similar results were obtained with *gpa2*Δ and *GPA2* overexpression strains in the SP1 background (results not shown). These results indicate that Gpa2 is essential for true glucose-induced activation of cAMP synthesis and that it is not involved in stimulation of cAMP synthesis by intracellular acidification.

cAMP pathway targets are down-regulated in a gpa2Δ strain

To gain further evidence for a role of Gpa2 in controlling the cAMP level, we investigated several well-known targets of the cAMP pathway during growth on SDglucose medium (Figure 10). Under the growth conditions used, the growth rate started declining after ~10 h and stationary phase was reached at 34 h, while the glucose level in the medium (100 mM) was only exhausted after 48 h. There was no significant difference in the growth rate and the rate of glucose exhaustion in the medium between the *gpa2*Δ strain and the wild-type strain (Figure 10). Trehalase activity was lower in the *gpa2*Δ strain compared with the wild-type strain. Consistent with the reduced trehalase activity, the trehalose level was higher in the *gpa2*Δ strain. The glycogen content was also elevated in the *gpa2*Δ strain.

The trehalose and glycogen levels were never affected to such an extent that the gradual increase observed as a function of time in the wild-type strain was abolished or even significantly affected. In all strains, there was a clear increase in trehalose and glycogen as a function of time in SDglucose medium. Figure 10 shows the results for trehalase activity, trehalose content and glycogen content for up to 24 h after addition of glucose. Samples were also taken at 48 h, which confirmed the trends observed for the 24 h period (results not shown). Similar results

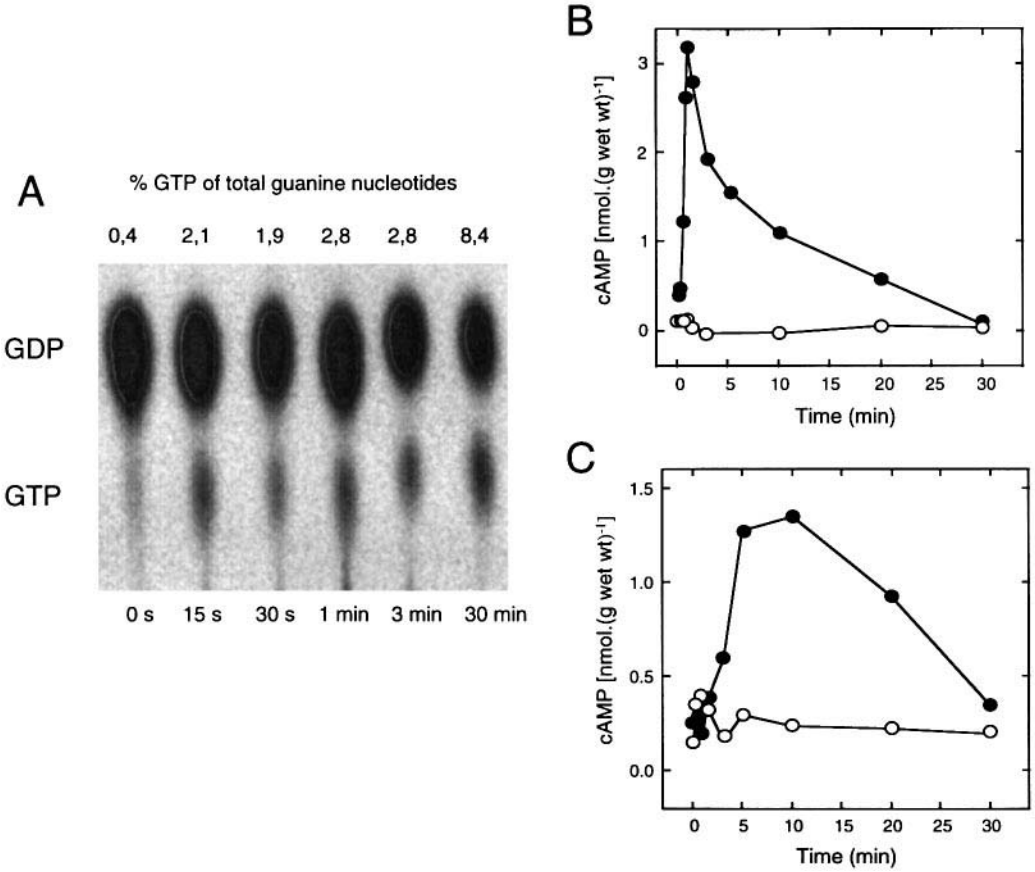

Fig. 8. Effect of constitutively elevated protein kinase A activity on the Ras activity status and on cAMP levels. (**A**) Amount of GTP and GDP bound to the Ras protein as a function of time after intracellular acidification by 2,4-dinitrophenol in a *TPK1 bcy1*Δ strain. cAMP content as a function of time after addition of glucose (**B**) or 2,4-dinitrophenol (**C**) in a *TPK1 bcy1*Δ strain (○) and in the control strain (*TPK1 BCY1*) (●).

were obtained with a *gpa2*Δ strain in the SP1 background (results not shown).

The expression of several genes with STRE elements in their promoter, such as *CTT1*, *SSA3* and *HSP12*, was measured in the same SDglucose cultures. Figure 11 shows for the strains in the SP1 background that the expression of STRE-controlled genes was clearly elevated in the *gpa2*Δ strain. In the wild-type strain, the level of these transcripts was very low during exponential growth on glucose (30–180 min) while they all significantly increased at different time points (from 240 min onwards) when the glucose level in the medium dropped. In the *gpa2*Δ strain, the initial increase in particular was more pronounced, and for CTT1 and HSP12 the maximum level obtained was also elevated. Figure 11 also shows that the expression of the *SUC2* gene, encoding invertase, is not significantly affected by deletion of *GPA2*. The *SUC2* gene does not contain STRE elements in its promoter and is controlled by the main glucose repression pathway (Johnston and Carlson, 1992). The level of the *GPA2* messenger itself drops by ~50% during the initiation of growth on glucose but then increases to a level similar to the initial level. This expression pattern resembles that of *TPS1* of which the messenger level also transiently drops after addition of glucose (Winderickx *et al*., 1996). Both *TPS1* and *GPA2* (at position –134 to –130, –243 to –239, and –581 to –577) contain several STRE elements (consensus: CCCCT) in their promoter. Apparently, expression control by these elements is overruled by other controls during growth on glucose. As observed for trehalose and glycogen content, the expression regulation of the STRE-controlled genes was only affected in a quantitative way, not in a qualitative way. For all three genes, the transient drop in the mRNA level during the initiation of growth on glucose remained present. It was only shorter and less pronounced in the *gpa2*Δ strain (Figure 11). Similar results were obtained with a *gpa2*Δ strain in the W303-1A background (results not shown).

Heat resistance in yeast cells is determined by several factors such as the trehalose content (Wiemken, 1990), the level of heat shock proteins, of which some, like Hsp104, are controlled by STRE elements in their promoter (Sanchez *et al*., 1992), and possibly other factors. Therefore, heat resistance as a whole might be more affected than the individual parameters that are responsible for it. Indeed, heat resistance as measured by survival after a heat treatment of 20 min (Figure 10, W303-1A background) or 30 min (results not shown) at 52°C was strongly enhanced in the *gpa2*Δ strain. Figure 10 shows the results for heat resistance for up to 24 h after addition of glucose. Samples were also taken at 48 h, which confirmed the trends observed for the 24 h period (results not shown). Similar results were obtained with a *gpa2*Δ strain in the SP1 background (results not shown).

It is well known that enhanced cAMP pathway activity negatively affects the growth rate on media containing a non-fermentable carbon source (Broach and Deschenes, 1990; Thevelein, 1992; Tatchell, 1993). The *gpa2*Δ strain

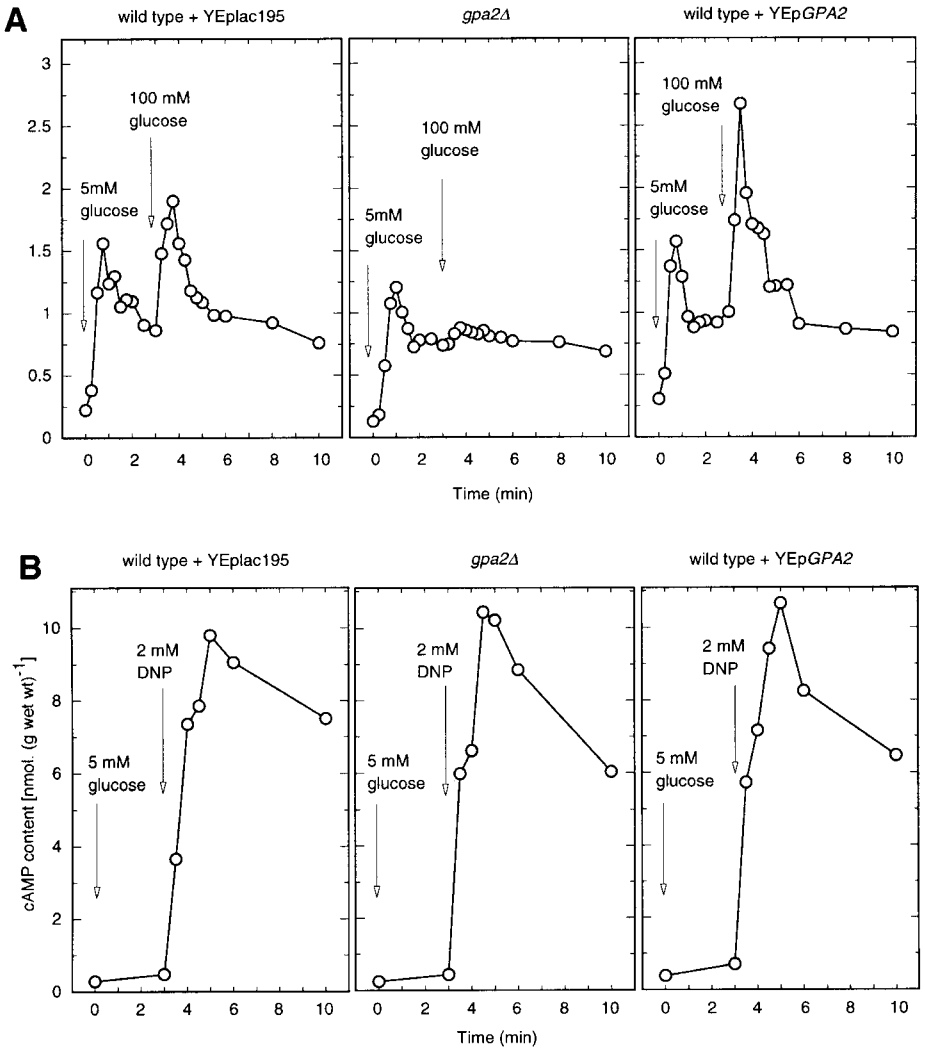

Fig. 9. Intracellular cAMP level as a function of time after addition of 5 mM glucose and subsequent addition of 100 mM glucose (**A**) or 2 mM 2,4-dinitrophenol (**B**) in the wild-type strain W303-1A + YEplac195, the *gpa2*Δ strain and the *GPA2* overexpression strain.

grew significantly faster on a glycerol-containing medium than the wild-type strain with the empty vector (results not shown). The difference cannot be a marker gene effect, since both strains contained the *URA3* marker gene.

Deletion of GPA2 does not affect the expression of STRE-controlled genes in a bcy1 strain

cAMP pathway targets can be affected by interference with the cAMP level or with cAPK activity. If Gpa2 only controls adenylate cyclase activity, deletion of *GPA2* should not affect the phenotype of a strain lacking the regulatory subunit (Bcy1) of cAPK. Previous work has shown that in such strains the STRE-controlled genes are strongly repressed (Bissinger *et al*., 1989; Winderickx *et al*., 1996). Therefore we deleted *GPA2* in the *TPK1 tpk2*Δ *tpk3*Δ *bcy1*Δ strain and the isogenic *TPK1 tpk2*Δ *tpk3*Δ *BCY1* strain (SP1 background) and we measured glucose-triggered repression of the STRE-controlled genes *CTT1*, *SSA3* and *HSP12*. Figure 12 shows that in the strain with regulated cAPK activity (expressing the Bcy1 regulatory subunit), deletion of *GPA2* caused clearly elevated expression of the STRE-controlled genes *CTT1*, *SSA3* and *HSP12*, although in all cases a transient drop in the messenger level upon addition of glucose remained present. On the other hand, in the strain with high, unbridled activity of PKA (*bcy1*Δ), the expression of all messengers was virtually undetectable and the same was true in the *bcy1*Δ *gpa2*Δ strain (results not shown). Figure 12 also shows that glucose induction of the ribosomal protein gene *RPL25* was still present in the *gpa2*Δ strain.

Discussion

Intracellular acidification enhances the ratio of GTP/GDP bound to Ras

Our results show that intracellular acidification in yeast cells triggers within seconds an increase in the ratio of GTP to GDP bound to the Ras proteins. Several arguments indicate that this is not due to a differential effect of pH on the binding of GTP and GDP to the Ras proteins. No further increase of the GTP/GDP ratio was observed in the *ira1*Δ *ira2*Δ and *RAS2*^{val19} strains upon intracellular acidification. In addition, incubation of Ras proteins *in vitro* at different pH values either in the absence or in the presence of guanine nuleotides did not result in a

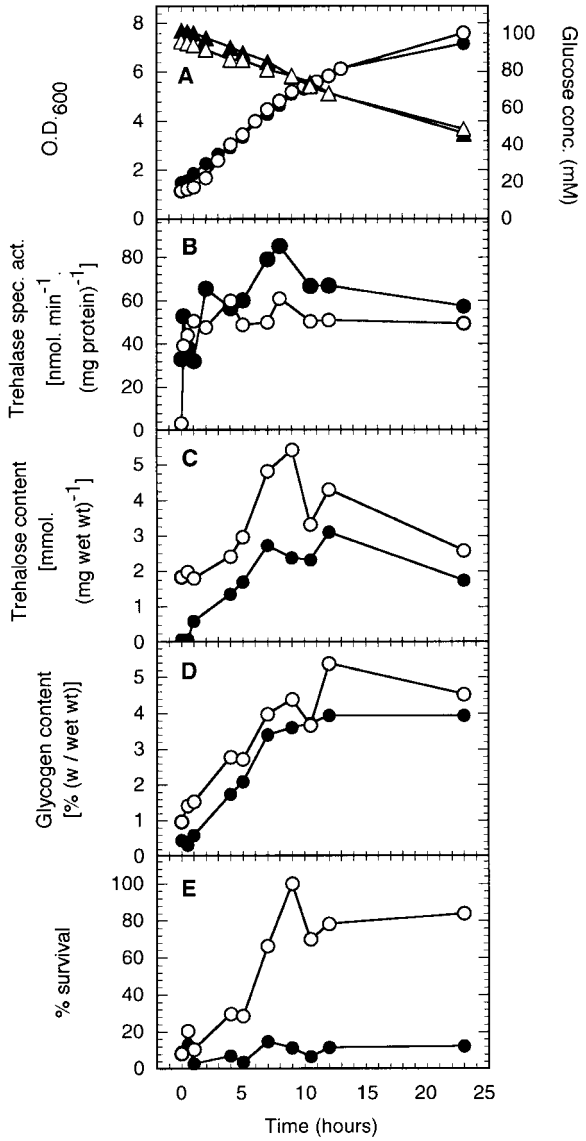

Fig. 10. Effect of *GPA2* deletion on PKA-controlled characteristics during growth on glucose. (**A**) OD_{600} (●, ○) and glucose level in the medium (▲, △). (**B**) Trehalase activity. (**C**) Trehalose content. (**D**) Glycogen content. (**E**) Heat resistance (percentage survival after a heat shock of 20 min at 52°C). Wild-type strain W303-1A + YEplac195 (●, ▲), *gpa2*Δ strain (○, △).

consistent change in the GTP/GDP ratio. A systematic investigation of the possible involvement of the known regulators of the Ras proteins points to the Ira proteins as the most likely candidates. Deletion of both *CDC25* and *SDC25* did not affect the acidification-induced increase in the GTP/GDP ratio. Deletion of the *IRA* genes separately also did not affect the increase, but double deletion of both *IRA1* and *IRA2* completely abolished the increase in the GTP/GDP ratio. In such a strain, the initial ratio was already elevated, but it was still not higher than 40% so that one would expect a further increase to be possible. In addition, in the single *ira1*Δ and *ira2*Δ strains, the initial GTP level is also elevated to some extent (Tanaka *et al.*, 1990a; results not shown) and there is still a further and pronounced increase in GTP upon intracellular acidification. In a *RAS2*^{val19} strain, the initial ratio was also elevated and no further increase was observed. The Ras2^{val19} allele is constitutively active and insensitive to the Ras-GTPase-activating capacity of the Ira proteins. Therefore, this result fits with the idea that the Ira proteins constitute a target of the intracellular acidification effect. On the other hand, we cannot completely exclude that there is an increase in GTP in the *ira1*Δ *ira2*Δ strain that for some reason we cannot detect. However, since we have shown that neither Cdc25 nor Sdc25 are required for the acidification-induced increase in GTP, it is unclear what other protein then would be responsible for triggering the increase in the *ira1*Δ *ira2*Δ strain. Hence, although we have no definite proof yet that the Ira proteins are the targets for the activation of Ras by intracellular acidification, they appear to be the most likely candidates at present.

Inhibition of the Ira proteins leads to reduced Ras-GTPase activity and therefore a higher GTP content bound to the Ras proteins, which causes enhanced stimulation of the effector adenylate cyclase. This sequence of events provides a partial explanation for the long-standing observation that protonophores and other agents that lower the intracellular pH in yeast cause a dramatic increase in the cellular cAMP level (Trevillyan and Pall, 1979; Mazon *et al.*, 1982; Caspani *et al.*, 1985; Purwin *et al.*, 1986; Thevelein *et al.*, 1987a). Such agents trigger the same effect in many other fungal species (Pall, 1977, 1981; Trevillyan and Pall, 1979). However, in some fungi, such as *S.pombe*, the Ras proteins apparently do not act as regulators of adenylate cyclase (Fukui *et al.*, 1986). It remains to be investigated whether in such fungi intracellular acidification is also able to trigger an increase in the cAMP level and if so what the underlying mechanism is. On the other hand, in *S.cerevisiae* there also appears to be an additional target for acidification-induced stimulation of cAMP synthesis.

Intracellular acidification still caused an increase in the cAMP level in an *ira1*Δ *ira2*Δ strain. In addition, 2,4-dinitrophenol did not cause a detectable increase in the GTP/GDP ratio at pH 6 whereas it enhanced the cAMP level under these conditions. This indicates that at least one other target of intracellular acidification exists for stimulation of cAMP synthesis, possibly adenylate cyclase itself. The pH optimum of adenylate cyclase (pH 6) is lower than the intracellular pH (±6.5–7.2) and much lower than the pH optimum of the cAMP phosphodiesterases (pH 8). Hence, in the physiological pH range, yeast adenylate cyclase activity decreases with increasing pH while phosphodiesterase activity increases (Londesborough and Nurminen, 1972; Londesborough, 1977; Busa and Nuccitelli, 1984). However, the intracellular acidification effect might be more specific than just a pH optimum effect since it is blocked by overexpression of *RAS2*^{val19} (Figure 5). Besides, since intracellular acidification still caused an increase in the cAMP level in an *ira1*Δ *ira2*Δ strain as opposed to a Ras2^{val19} overexpression strain, the Ras2^{val19} protein seems to have an additional type of interaction with adenylate cyclase compared with the wild-type GTP-bound Ras2 protein, preventing the cAMP increase.

The two strains, *ira1*Δ *ira2*Δ and *RAS2*^{val19}, that lack the acidification-induced increase in the GTP/GDP ratio both display constitutively high PKA activity and therefore constitutively high feedback inhibition on cAMP synthesis.

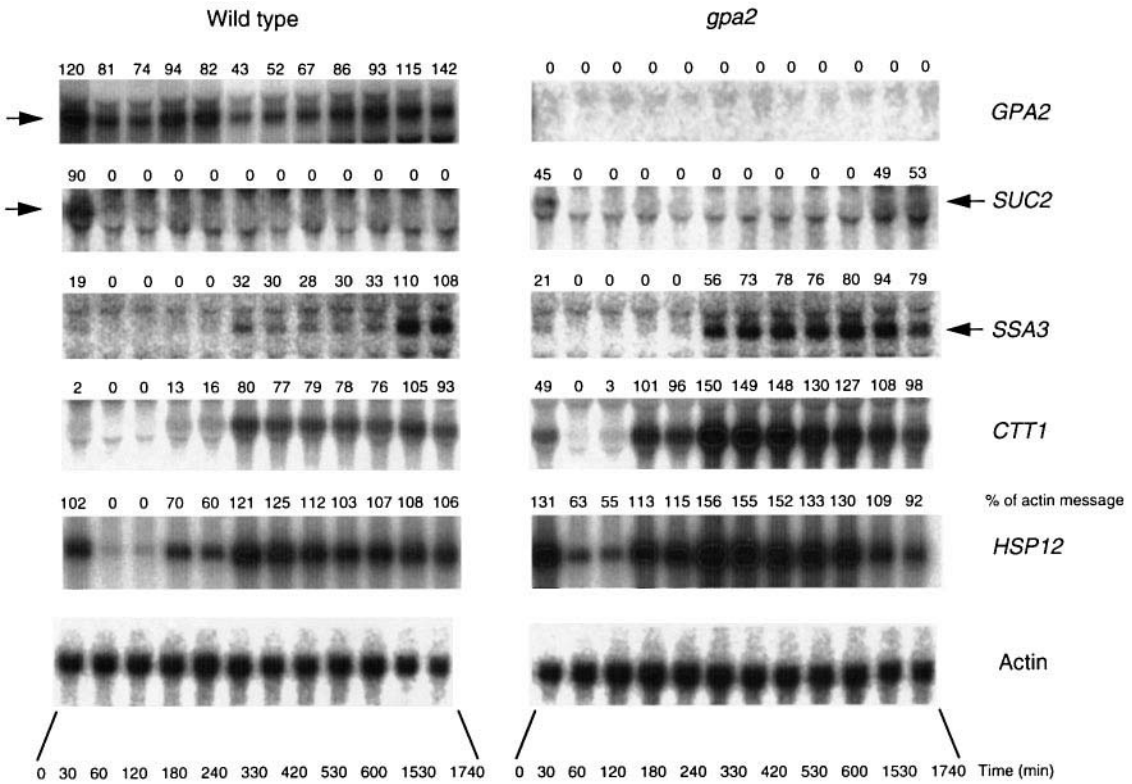

Fig. 11. Northern blot analysis of the expression of the STRE-controlled genes *SSA3*, *CTT1* and *HSP12* in the wild-type strain SP1 + YEplac195 and the *gpa2*Δ strain as a function of time during growth on glucose (see Figure 10). For comparison, the mRNA levels of *SUC2* (invertase), which is regulated by the main glucose repression pathway, and *GPA2* itself are also shown. Actin messenger levels were used as control (they decline slightly at the initiation of stationary phase).

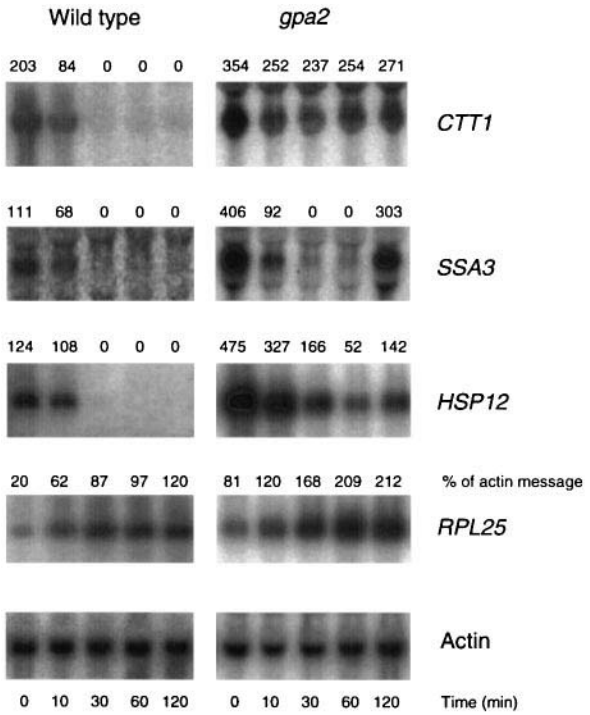

Fig. 12. Northern blot analysis of the expression of the STRE-controlled genes *CTT1*, *SSA3* and *HSP12*, and the ribosomal protein gene *RPL25* in the wild-type and *gpa2*Δ strains (SP1 background) as a function of time after addition of glucose to cells growing on ethanol/glycerol. Actin messenger levels are shown as control.

This might have been the reason why these two strains specifically lack the acidification-induced increase in the GTP/GDP ratio. However, several arguments contradict this interpretation. First, the *bcy1* strain which also displays constitutively elevated PKA activity and feedback inhibition still shows an acidification-induced increase in the GTP/GDP ratio (Figure 8). In addition, expression of the $Ras2^{val19}$ allele in the Tpk-attenuated strain, which has reduced PKA activity and feedback inhibition, also abolished the acidification-induced increase in the GTP/GDP ratio. These results show that enhanced feedback inhibition cannot be the cause of the absence of the acidification-induced increase in the GTP/GDP ratio in the *ira1*Δ *ira2*Δ and $RAS2^{val19}$ strains.

The activation of the yeast Ras proteins by intracellular acidification, possibly through inhibition of the Ras-GTPase activating proteins Ira1 and Ira2, represents a novel control for Ras. It remains to be investigated whether a similar mechanism operates on the Ras proteins in other organisms. The physiological role of the stimulation of cAMP synthesis by intracellular acidification has always remained rather enigmatic. It might constitute a link between ATP shortage and ATP regeneration during carbon starvation. Under such conditions, the intracellular pH in yeast cells drops. The resulting increase in the cAMP level may cause mobilization of storage carbohydrates with an increase in ATP generation as a consequence. In yeast cells, trehalose and glycogen are well known to be consumed during starvation (Lillie and Pringle, 1980), and intracellular acidification is known to cause mobilization of trehalose and enhancement of endogenous fermentation

(Stickland, 1956; Berke and Rothstein, 1957; Brady *et al*., 1961), presumably due to cAMP-triggered activation of trehalase (Thevelein, 1984a,c).

Glucose does not affect the ratio of GTP/GDP bound to Ras

The rapid, transient spike in the cAMP level which occurs upon addition of glucose to glucose-deprived, derepressed yeast cells has also been ascribed to transient intracellular acidification (Busa and Nuccitelli, 1984; Caspani *et al*., 1985; Purwin *et al*., 1986). We have shown previously that the pH drop can be eliminated in different ways without abolishing the cAMP spike (Thevelein *et al*., 1987b). In addition, mutants with reduced feedback inhibition of cAMP synthesis show a continuous increase in cAMP after addition of glucose over a period of several minutes (Mbonyi *et al*., 1990), while they show a normal initiation of glucose metabolism without an aberrant pH drop (Ma *et al*., 1997). Our present results confirm that glucose and acidification act in different ways. Acidification causes an increase in the GTP/GDP ratio on the Ras proteins whereas glucose does not. Because the glucose effect on cAMP in wild-type cells is transient and smaller than the acidification effect, the increase in the GTP/GDP ratio could be too small and/or too rapid to detect with our method. Therefore, we have also investigated the effect of glucose in a strain with attenuated activity of cAPK, in which glucose addition causes a huge and long-lasting increase in cAMP (Mbonyi *et al*., 1990). However, in this case too there was no effect of glucose at all on the ratio of GTP/GDP bound to the Ras proteins.

Genetic experiments with mutants in the *RAS* genes (Mbonyi *et al*., 1988; Kim and Powers, 1991) and in the *CDC25* gene (Munder and Küntzel, 1989; Schomerus *et al*., 1990; Van Aelst *et al*., 1990, 1991) had indicated that these gene products were required for the glucose-induced cAMP signal. However, they are both also required for basal cAMP synthesis. Since cAMP depletion is lethal in yeast, suppressing mutations or multicopy suppressors had to be used to maintain viability in the *ras* and *cdc25* mutants. Maybe these suppressors have affected the cAMP signal through interference with the feedback inhibition mechanism or through other mechanisms. It has already been proposed that the Ras/GTP–adenylate cyclase complex, and not the Cdc25 protein, is the receiver for the glucose-induced cAMP signal (Goldberg *et al*., 1994).

It can also not be excluded that the Ras and Cdc25 proteins are truly required for the glucose-induced cAMP increase, and not only for basal adenylate cyclase activity, but for reasons other than for activation of adenylate cyclase by GTP loading of Ras. Evidence has been presented for a close interaction between Ira1 and adenylate cyclase (Mitts *et al*., 1991) and between Cdc25 and adenylate cyclase (Pardo *et al*., 1993). It has also been shown that Ras membrane targeting is essential for glucose-induced cAMP signalling but not for viability and therefore provision of a basal cAMP level (Bhattacharya *et al*., 1995). This result tends to indicate a specific function for Ras in transduction of the glucose-induced signal. However, an alternative explanation is that the absence of membrane localization of Ras reduces the activity of adenylate cyclase to such an extent that it is no longer able to support the rapid cAMP increases but that it is still high enough to maintain a basal cAMP level adequate for viability. This possibility could be checked by measuring the acidification-induced cAMP increase in the Ras mislocalization mutant.

In a strain containing the constitutively activated $Ras2^{val19}$ allele, glucose was unable to trigger a cAMP signal (Mbonyi *et al*., 1988). Since no other G proteins able to regulate adenylate cyclase had been identified at that time, this result was interpreted as indicating that the $RAS2^{val19}$ gene product is unable to transmit the glucose-induced cAMP signal. However, the constitutively activated $Ras2^{val19}$ allele might prevent activation of adenylate cyclase by another G protein (e.g. Gpa2, see below). Interestingly, also in a Tpk-attenuated strain, expression of $Ras2^{val19}$ inhibited the glucose-induced cAMP increase. This indicates that $Ras2^{val19}$ prevents glucose-induced cAMP signalling independently of the feedback inhibition mechanism. It also indicates that if a Ras/GTP–adenylate cyclase complex is the receiver of the glucose-derived signal, as suggested by Goldberg *et al*. (1994), the $Ras2^{val19}$/GTP–adenylate cyclase complex would be unable to respond to this signal.

Although overexpression of the Ras proteins was required for measurement of the GTP/GDP ratio on Ras in most strains, we feel that this is not likely to influence the conclusions in a substantial way. It is unclear how overexpression of the Ras proteins could lead to the observation of acidification-induced activation of Ras. Hence, we feel that this observation reflects a mechanism truly existing *in vivo*. It is possible, however, that the extent of the activation would be larger in cells with a smaller amount of Ras proteins. This can only enhance the significance of this effect. Following the same reasoning, glucose might cause a slight increase in the GTP content on the Ras proteins in cells without overexpression of Ras, which then would become undetectably small in cells with overexpression of Ras. Although this cannot be excluded formally, the inability to detect a glucose-induced activation of Ras in any other strain and the very clear effect of Gpa2 deletion on glucose activation of cAMP synthesis make it most likely that Gpa2 rather than Ras is the mediator of the glucose effect. Future work on the precise mechanisms involved should be able to establish whether the presence of Ras itself is actually required for Gpa2-dependent glucose activation of cAMP synthesis.

Feedback inhibition of cAMP synthesis by cAPK does not act through the ratio of GTP/GDP bound to Ras

Our results show that strong reduction of cAPK activity does not affect the ratio of GTP/GDP bound to the Ras proteins, as opposed to the strong effects observed on cAMP accumulation. Remarkably, in a strain with constitutively elevated PKA activity, the ratio of GTP/GDP bound to the Ras proteins was enhanced in spite of a clear reduction in the basal cAMP level and a strong inhibition of the cAMP responses (Figure 8). This observation contradicts the hypothesis that PKA would inhibit cAMP synthesis by reducing the GTP content on the Ras proteins. The actual increase in GTP is puzzling. It might be caused by uncoupling of the Ras–adenylate cyclase interaction. If interaction of Ras with its effector would in some way

stimulate its inactivation (i.e. hydrolysis of bound GTP), uncoupling of the interaction might lead to a higher GTP level.

Our results appear to exclude any mechanism for the feedback inhibition of cAPK on cAMP synthesis that involves down-regulation of the Ras proteins by changing their content of bound guanine nucleotides. They contradict involvement of Cdc25, Ira1, Ira2 and also Ras itself, at least if in the latter case the ratio of GTP/GDP is affected. Our results do not exclude involvement of the Ras proteins in feedback inhibition by mechanisms other than alteration of the GTP/GDP ratio. On the other hand, because of the very potent character of this feedback inhibition and its presence also in strains lacking both phosphodiesterases, we feel that adenylate cyclase itself is the most likely candidate for the (major) target of the feedback inhibition mechanism.

Gpa2 is required for glucose- but not for acidification-induced cAMP responses

Our results show that the G_α protein encoded by *GPA2* is essential for the stimulation of cAMP accumulation *in vivo* by glucose but not by intracellular acidification. This can explain why previous work (Nakafuku *et al.*, 1988) failed to reveal the requirement for Gpa2 for the glucose-induced cAMP signal. Intracellular acidification is a much more potent stimulator of cAMP accumulation than is glucose (Thevelein *et al.*, 1987a). Normally, to measure glucose activation of cAMP accumulation, yeast cells are pre-grown on a non-fermentable carbon source. As a result, they are glucose derepressed and display active respiration. When such cells are suspended in buffer, they can respire to some extent endogenous carbon reserves and in this way maintain a proper intracellular ATP level and pH (den Hollander *et al.*, 1981; Thevelein *et al.*, 1987b). However, this capacity appears to be dependent on the genetic background of the strain and the precise incubation conditions, in particular the provision of oxygen. When the ATP level drops, the intracellular pH will inevitably also drop. The synthesis of cAMP under these conditions will be limited by the low ATP level, probably because the K_m of adenylate cyclase is relatively high (2–5 mM in the physiological pH range of 6.4–7.5) (Varimo and Londesborough, 1982). Evidence has been provided for this possibility previously with the demonstration that glucose-repressed cells suspended in buffer were unable to increase their cAMP level when challenged with a protonophore causing intracellular acidification, but rapidly responded when a small amount of glucose was added just before the protonophore (Argüelles *et al.*, 1990). Addition of glucose to ATP-compromised cells causes an increase in the ATP level. Subsequently, a high intracellular pH is recovered via the stimulation of proton efflux by the plasma membrane H^+-ATPase. During this sequence of events, there will be an interval where the ATP level has increased but the intracellular pH is still low. We suggest that the glucose-induced cAMP increases occasionally observed in glucose-repressed wild-type cells and in derepressed cells of certain mutant strains are triggered by this transient, short-lived co-existence of an elevated ATP level and a low intracellular pH.

Since acidification-induced cAMP accumulation is not significantly affected in the *gpa2*Δ strain, deletion of Gpa2 apparently does not cause a drastic decrease in adenylate cyclase activity *in vivo*. This is supported further by the observation that deletion of Gpa2 has only a limited effect on PKA-controlled phenotypic properties. Hence, the absence of the glucose-induced cAMP signal in the *gpa2*Δ strain cannot be caused by a strong reduction in adenylate cyclase activity *in vivo*.

The requirement for Gpa2 for glucose-induced activation of cAMP synthesis but not for activation by intracellular acidification fits with the observations that intracellular acidification but not glucose triggers an increase in the ratio of GTP to GDP bound to the Ras proteins. These results tend to indicate that intracellular acidification stimulates adenylate cyclase at least through the Ras proteins, while glucose stimulates adenylate cyclase through the Gpa2 protein. This further supports the finding that glucose does not stimulate cAMP synthesis through intracellular acidification. Our results also fit with results obtained in the other yeast species *S.pombe* (Isshiki *et al.*, 1992; Nocero *et al.*, 1994) and *K.lactis* (Saviñon-Tejeda *et al.*, 1996) where a *GPA2* homologue was found to be required for glucose-induced stimulation of cAMP synthesis. Previously, the inability of a $Ras2^{val19}$ mutant to mediate glucose-induced cAMP signalling was interpreted as indicating a role for the Ras proteins in transmission of the glucose signal (Mbonyi *et al.*, 1988). However, our results now show that the constitutively active $Ras2^{val19}$ allele in some way blocks glucose-induced cAMP signalling even in a strain with reduced PKA activity and, as a result, reduced feedback inhibition of PKA on cAMP synthesis. Possibly the Ras proteins compete with Gpa2 for binding to the same site on adenylate cyclase, and the constitutively active $Ras2^{val19}$ allele binds in such a way that it prevents any further interaction of Gpa2 with adenylate cyclase.

Gpa2 plays only a minor role in the control of the protein kinase A pathway during growth on glucose

Our results indicate a strong requirement for Gpa2 for glucose-induced activation of cAMP synthesis but only a minor effect on basal adenylate cyclase activity. Intracellular acidification-induced cAMP accumulation is not affected detectably (Figure 9) and, although the effect of *GPA2* deletion is clear for all cAMP pathway targets studied, in most cases it is not very pronounced (Figure 10). Only for heat resistance is there a dramatic increase in the *gpa2*Δ strain. This might be due to the fact that several targets of the cAMP pathway are involved in determining heat resistance in a synergistic way, such as reported for trehalose and Hsp104 (De Virgilio *et al.*, 1991; Elliott *et al.*, 1996). For all cAMP pathway targets, the gradual increase observed as a function of time during diauxic growth in the wild-type strain was also observed in the *gpa2*Δ strain. Therefore, detection of the diminishing glucose level in the medium by Gpa2 cannot be responsible for this increase. The function of Gpa2 appears limited mainly to the stimulation of cAMP synthesis during the transition from respirative growth on a non-fermentable carbon source to fermentative growth on glucose. An alternative glucose-sensing pathway, which we previously called the 'fermentable-growth-medium'-induced pathway (FGM pathway) (Thevelein, 1994), seems to be responsible

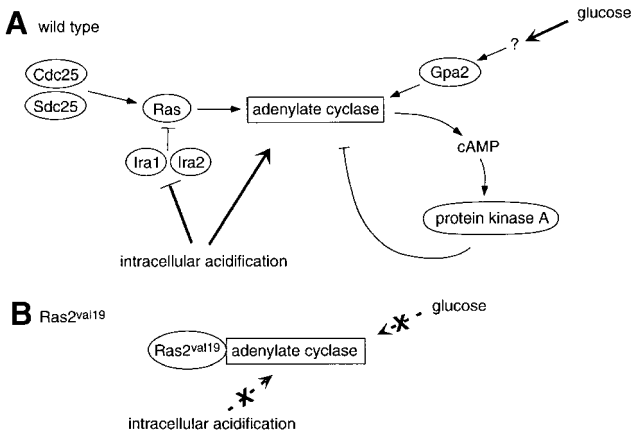

Fig. 13. Scheme of the connections proposed between the Ras–adenylate cyclase pathway and intracellular acidification, glucose and feedback inhibition by protein kinase A. The cAMP responses triggered by intracellular acidification and glucose are both inhibited by Ras2^{val19}.

for the maintenance of the high PKA phenotype during exponential growth on glucose. This fits with previous observations that the presence of the glucose-induced cAMP spike correlates with the glucose repression state of the cells (Beullens *et al*., 1988; Argüelles *et al*., 1990; Dumortier *et al*., 1995) and that during growth on glucose the cytosolic cAMP level is not determined by the diminishing glucose level in the medium (Ma *et al*., 1997).

Conclusions

Our results have identified intracellular acidification as a stimulator of the Ras proteins in yeast by interference with the ratio of bound guanine nucleotides. The Ras proteins do not appear to be involved as signal transducers in glucose-induced activation of adenylate cyclase, whereas the Gpa2 protein is required specifically for this process. Stimulation of Ras by acidification is independent of the guanine nucleotide exchange proteins Cdc25 and Sdc25 and dependent on the Ras-GTPase-activating proteins Ira1 and Ira2. However, for intracellular acidification-induced stimulation of cAMP synthesis there has to be another target in addition to the Ira proteins. The *RAS2*^{val19} oncogene product inhibits the stimulation of cAMP synthesis both by intracellular acidification and by glucose. The potent feedback inhibition mechanism of cAPK on cAMP synthesis in yeast does not act through a change in the ratio of GTP/GDP bound to the Ras proteins. These conclusions are summarized in Figure 13. Deletion of Gpa2 affects several PKA-controlled properties in a way consistent with a reduction in the activity of the cAMP pathway, but it does not abolish the typical fluctuation in these properties during diauxic growth on glucose. In addition to control by Ras, *S.cerevisiae* can also be used as a model for the study of G-protein regulation of adenylate cyclase.

Materials and methods

Plasmids, strains and growth conditions

The plasmids for overexpression of Ras2 and Ras2^{val19} were constructed by inserting the *Hpa*I fragment of YCp50 plasmids containing these genes into the *Bgl*II site of pXKL1. The pXKL1 plasmid was constructed by insertion of the *PGK* promoter and terminater sequences, linked by a synthetic linker with an unique *Bgl*II site, into the multicopy vector YEplac195. These plasmids contained *URA3* as selectable marker. A plasmid containing *LEU2* as selectable marker and which was also used for overexpression of Ras2, was constructed by inserting the *Hpa*I fragment of a YCp50 plasmid containing *RAS2* into the *Bgl*II site of pXKL2. The pXKL2 plasmid was constructed in the same way as pXKL1 but using the multicopy vector YEplac181 (*LEU2*).

Yeast strains used in this study were: W303-1A (*MAT***a** *leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100 GAL SUC mal*) (Thomas and Rothstein, 1989), FDL31-21A (*MAT***a** *sdc25::HIS3 cdc25::HIS3 his3 trp1 leu2 lys1 ade2 ade8? ura3* + YEp*RAS2*^{Ile152}-*URA3*) kindly provided by E.Boy-Marcotte and M.Jacquet (Orsay), S18-1D (*Mat*α *his3 leu2 ura3 trp1 ade8 tpk1*^{w1} *tpk2::HIS3 tpk3::TRP1*), S7-7A (*MAT***a** *his3 leu2 ura3 trp1 ade8 TPK1 tpk2::HIS3 tpk3::TRP1 BCY1*) and S13-3A (*MAT***a** *his3 leu2 ura3 trp1 ade8 TPK1 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*) (Nikawa *et al*., 1987a). The strain FDL31-21A is viable because of the presence of the YEp-*RAS2*^{Ileu152} plasmid. This strain was transformed with the plasmid YEp-*TPK1*, which contains a *LEU2* marker, and grown for several generations in uracil-containing medium to allow loss of the *URA3*-containing YEp-*RAS2*^{Ileu152} plasmid. All strains were transformed with the *RAS2* or *RAS2*^{val19} overexpression plasmid as indicated. Temperature-sensitive *cdc25* mutants were LRA66 (*MAT***a** *leu2 ura3-52 his4 cdc25-1*) + YEp*RAS2*, LRA67 (*MAT***a** *leu2 ura3-52 his4 cdc25-5*) + YEp*RAS2* and OL97-1-11BΔira2 (*MAT*a *cdc25-5 leu2 ura3 his3 his7 ira2::LEU2*) + YEp*RAS2*. The following strains were constructed by disruption of *IRA1* with *URA3* and/or *IRA2* with *LEU2* in strain W303-1A, using disruption constructs kindly provided by E.Boy-Marcotte and M.Jacquet (Orsay), SC7 (*MAT***a** *ira1::URA3 leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100 GAL SUC mal*) and SC8 (*MAT***a** *ira2::LEU2 leu2-3,112 ura3-1 trp1-92 his3-11/15 ade2-1 can1-100 GAL SUC mal*). These strains were transformed with the *RAS2* overexpression plasmid as indicated. Strain PM903 (*MAT***a** *ira1::LEU2 ira2::URA3 leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2 mal*) was constructed by partial deletion of *IRA1* (from the *Xho*I site at position 3611 to the *Xba*I site at 4832) with *LEU2* and partial deletion of *IRA2* (from the *Hil*cII site at position 3644 to the *Bgl*II site at 4351) with *URA3* in strain W303-1A. Strain PM903 was transformed with a *RAS2* overexpression plasmid, constructed by insertion of the *PGK* promoter, terminater and *RAS2* gene into the vector YEplac112 (*TRP1*).

Gpa2 involvement was studied using the following strains: wild-type strain W303-1A + YEplac195, and isogenic strains PM731 (*Mat***a** *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2 gpa2::URA3*) and PM732 (*Mat***a** *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2* + YEp*GPA2*), all results shown in Figures 9 and 10 were obtained with this set of strains; wild-type strain DC124 (isogenic to SP1 wild-type strain) (*Mat*α *his4 leu2 ura3 trp1 ade8 can1*) + YEplac195, and isogenic strains PM191 (*Mat*α *his4 leu2 ura3 trp1 ade8 can1 gpa2::URA3*) and PM192 (*Mat*α *his4 leu2 ura3 trp1 ade8 can1* + YEp*GPA2*), all results shown in Figures 11 and 12 were obtained with this set of strains; wild-type strains JRY28 (*Mat***a** *leu2 his3 trp1 ura3 lys2*), JRY79 (*Mat***a** *leu2 his3 trp1 ura3 lys2 gpa2::HIS3*) and JRY137 (=JRY128) (JRY79 + YEpMN5-*GPA2*, *URA3*) (Nakafuku *et al*., 1988). The following strains are isogenic to SP1: S13-3A (*Mat*α *his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*) (Nikawa *et al*., 1987a) and MC09 (*Mat*α *his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 gpa2::URA*).

Cells were grown in minimal media that contained 0.67% of yeast nitrogen base without amino acids, 2% glycerol, 0.1% glucose and appropriate amounts of amino acids depending on the type of plasmid selection. Low phosphate minimal media were prepared as described by Rubin (1975).

Determination of GTP/GDP ratio on Ras

The determination of the GTP/GDP ratio on the Ras proteins was performed essentially as described by Tanaka *et al*. (1990a), with some modifications. Cells were grown to an early logarithmic phase in the appropriate medium at 30 or 23°C for temperature-sensitive strains. The cells were harvested by centrifugation, washed and re-inoculated into a medium with reduced phosphate content for 40 min before addition of [^{32}P]orthophosphate to a final activity of 75 μCi/ml. After shaking for ~3 h (during which the extracellular pH dropped to ±4.5), the labeled cells were treated with 2 mM 2,4-dinitrophenol or 100 mM glucose. Samples were taken at specific times and added to ice-cold water. The cells were precipitated by centrifugation and, after addition of ice-cold extraction buffer, they were disrupted with glass beads in a Fastprep instrument (Savant). The extraction buffer contained 50 mM Tris–HCl, pH 7.4, 20 mM $MgCl_2$, 100 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol

(DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1.4 µg/ml pepstatin and 0.025% aprotinin. Cleared supernatant (40 min at 100 000 *g*) was incubated for 1 h at 4°C with v-H-ras (259) agarose conjugate antibodies (Oncogene Science). After washing five times with extraction buffer (without aprotinin) and twice with washing buffer (extraction buffer without NP-40), nucleotides bound to Ras proteins were eluted by heating the immune complex in a solution containing 25 mM Tris–HCl pH 7.4, 2% SDS, 20 mM EDTA, 2 mM GTP and 2 mM GDP for 5 min at 70°C. The supernatant was spotted onto PEI-cellulose plates and the developing solvent was 1 M KH_2PO_4 pH 4.0. The radioactive spots on the plates corresponding to GTP and GDP were quantitated using phosphorimager technology (Fuji, BAS-1000; Software, PCBAS 2.0). Ratios of GTP/GDP were calculated by assuming uniform ^{32}P labelling of all phosphate groups; a factor of 1/2 was applied to the counts obtained for GDP and a factor of 1/3 to those obtained for GTP to correct for their respective phosphate content.

Biochemical determinations

cAMP levels were determined in cell extracts prepared by the rapid quenching method of de Koning and van Dam (1992). Cells were incubated at a density of 37.5 mg (wet weight) per ml at 30°C in low phosphate minimal medium for 3 h or in 25 mM MES buffer (pH 6) for 10 min (*gpa2*Δ experiments) before addition of 2 mM 2,4-dinitrophenol (from a stock solution of 80 mM in ethanol), 5 or 100 mM glucose, as indicated. Samples containing 75 mg of cells were quenched in 10 ml of 60% methanol at –40°C. The cells subsequently were centrifuged at –19°C (5 min at 3000 r.p.m.). Then 1 ml of ice-cold perchloric acid and 1.5 g of glass beads (0.75 mm Ø) were added and the cells were broken by vibration on a vortex in the cold room. The extracts were used to determine cAMP as described previously (Thevelein *et al*., 1987a).

Trehalase activity was determined in crude cell extracts as described previously (Pernambuco *et al*., 1996). The specific activity of trehalase is expressed as nmoles of glucose liberated per min per mg of protein. For trehalose and glycogen determination, cells were collected by filtration, washed once with cold water, weighed and frozen in liquid nitrogen. The pellets were resuspended in 0.5 ml of 0.25 M Na_2CO_3 per 50 mg of cells and boiled at 95°C for 20 min. Samples were taken for trehalose determination and the remainder was boiled for another 60 min. The samples for trehalose determination were spun down and 10 µl of the clear supernatant was used. The samples for glycogen determination were mixed well and used directly (10 µl). All samples were neutralized by addition of 5 µl of 1 M HAc. For trehalose determination, 5 µl of buffer (300 mM NaAc, 30 mM $CaCl_2$, pH 5.4) and 20 µl of *Humicola* trehalase (360 U/ml) (Neves *et al*., 1994) were added and incubated for 45 min at 40°C. For glycogen determination, 5 µl of buffer (400 mM NaAc, pH 4.7) and 20 µl of amyloglucosidase (0.25 U) from *Aspergillus niger* (Boehringer Mannheim) were added and incubated for 2 h at 37°C. For all samples, the glucose liberated was measured in 30 µl of cleared supernatant using the glucose oxidase/peroxidase method.

RNA extraction and Northern blot analysis

Culture samples for Northern blot experiments were cooled immediately by addition of ice-cold water. The cells were collected by centrifugation at 4°C. The cell sediment was washed once with ice-cold water and stored at –70°C. Total RNA was isolated by phenol extraction as described previously (Pernambuco *et al*., 1996). The RNA was separated on formaldehyde-containing agarose gels, transferred to nylon membranes and hybridized as described previously (Crauwels *et al*., 1997). Probes used were ^{32}P-labelled PCR fragments for *GPA2*, *CTT1*, *HSP12*, *SSA3*, *RPL25*, *SUC2* and actin. Actin mRNA levels were used as loading standards. Northern blots were analysed using phosphorimager technology (Fuji, BAS-1000; software, PCBAS 2.0). All signals were quantitated and expressed as a percentage of the actin messenger level.

Determination of heat shock resistance

For determination of heat shock resistance, samples were taken from the culture at the indicated time points and heated for 20, 30 or 40 min at 52°C. After cooling, aliquots were spread on nutrient plates and colonies counted after 4 days of growth at 30°C.

Reproducibility of the results

All experiments were repeated at least twice. Representative results are shown.

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