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A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2

Matthias Versele, Johannes H.de Winde and Johan M.Thevelein[1]

Laboratorium voor Moleculaire Celbiologie, Instituut voor Plantkunde en Microbiologie, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001 Leuven-Heverlee, Flanders, Belgium

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

We have characterized a novel member of the recently identified family of regulators of heterotrimeric G protein signalling (RGS) in the yeast *Saccharomyces cerevisiae*. The *YOR107w/RGS2* gene was isolated as a multi-copy suppressor of glucose-induced loss of heat resistance in stationary phase cells. The N-terminal half of the Rgs2 protein consists of a typical RGS domain. Deletion and overexpression of Rgs2, respectively, enhances and reduces glucose-induced accumulation of cAMP. Overexpression of *RGS2* generates phenotypes consistent with low activity of cAMP-dependent protein kinase A (PKA), such as enhanced accumulation of trehalose and glycogen, enhanced heat resistance and elevated expression of STRE-controlled genes. Deletion of *RGS2* causes opposite phenotypes. We demonstrate that Rgs2 functions as a negative regulator of glucose-induced cAMP signalling through direct GTPase activation of the Gs-α protein Gpa2. Rgs2 and Gpa2 constitute the second cognate RGS–G-α protein pair identified in yeast, in addition to the mating pheromone pathway regulators Sst2 and Gpa1. Moreover, Rgs2 and Sst2 exert specific, non-overlapping functions, and deletion mutants in Rgs2 and Sst2 are complemented to some extent by different mammalian RGS proteins.

Keywords: cAMP/G-α/protein kinase A/RGS/yeast

Introduction

In bakers' yeast *Saccharomyces cerevisiae* the cAMP-protein kinase A (PKA) pathway plays a major role in the control of metabolism, stress resistance and cell proliferation (Wigler *et al*., 1988; Broach, 1991; Thevelein, 1994; Thevelein and de Winde, 1999). Activation of the pathway occurs either by addition of fermentable sugars to cells grown on non-fermentable carbon sources or into stationary phase, or by intracellular acidification (Thevelein, 1991). This results in a rapid, transient accumulation of cAMP through activation of adenylate cyclase, followed by hydrolysis of cAMP by the low-affinity phosphodiesterase Pde1 as part of the feedback inhibition by activated PKA (Nikawa *et al*., 1987; Mbonyi *et al*., 1990; Ma *et al*., 1999). Activity of adenylate cyclase requires the action of Ras1 and Ras2 and their guanine nucleotide exchange factors Cdc25 and Sdc25, and is downregulated by the Ras-specific GTPase activators Ira1 and Ira2. cAMP binds to the regulatory subunit Bcy1 of PKA, releasing it from the catalytic subunits Tpk1, Tpk2 and Tpk3, which are thereby activated. Downstream targets of activated PKA include many housekeeping genes and enzymes, as well as proteins important for stress resistance and cell cycle control. High activity of the cAMP-PKA pathway results in low levels of trehalose and glycogen, low stress resistance, constitutive repression of STRE-controlled genes and aberrant G_0 arrest. Low activity of the pathway causes constitutive expression of stationary phase characteristics (high trehalose and glycogen concentration, high stress resistance and derepression of STRE-controlled genes) during exponential growth on glucose.

Saccharomyces cerevisiae contains a G-α protein homologue Gpa2 (Nakafuku *et al*., 1988; Kubler *et al*., 1997; Lorenz and Heitman, 1997) of which the cognate 7-TM receptor Gpr1 (Yun *et al*., 1997; Xue *et al*., 1998; Kraakman *et al*., 1999) has recently been identified. We have shown that glucose-induced activation of the cAMP-PKA pathway during the transition to fermentation is dependent on this G protein coupled receptor (GPCR) system (Colombo *et al*., 1998; Kraakman *et al*., 1999). Deletion of either *GPR1* or *GPA2* causes a significant delay in glucose-induced, PKA-mediated changes in both derepressed and stationary phase cells. The same phenotype has recently been described for an adenylate cyclase mutant, $Cyr1^{K1876M}$, specifically deficient in GTP-dependent activation *in vitro* and cAMP signalling *in vivo* (Vanhalewyn *et al*., 1999). However, the GPCR system responsible for cAMP signalling appears to be required only during the adaptation phase from non-fermentative to fermentative growth, since its deletion does not prevent establishment of typical PKA-controlled properties during exponential growth on glucose. Apart from the effector adenylate cyclase, the identity of putative additional components of this GPCR system, such as cognate G β–γ subunits or other specific regulators has remained elusive.

We have developed a screening procedure to identify components specifically involved in glucose activation of the yeast cAMP-PKA pathway. This method is based on the rapid loss of heat stress resistance in stationary phase cells following the addition of glucose (Thevelein and de Winde, 1999). Mutant alleles of adenylate cyclase (*fil1*, deficient in fermentation-induced loss of stress resistance; P.Van Dijck, P.Ma, M.Versele, M.-F.Gorwa, S.Colombo, K.Lemaire, A.Loïez and J.M.Thevelein, manuscript in preparation) and *GPR1* (*fil2*; Kraakman *et al*., 1999) have been isolated in a screen for mutants that display a reduced rate of glucose-induced loss of heat stress resistance. The resistance of yeast cells to heat stress depends to a large extent on the heat shock protein Hsp104 (Sanchez *et al*.,

1992) and the intracellular content of trehalose (Hottiger *et al*., 1987; Elliott *et al*., 1996). A strain deficient in both trehalose synthesis and expression of *HSP104* is very sensitive to heat stress but still displays appreciable heat resistance in the absence of glucose. We have now used such a strain, deleted for both the trehalose-6-phosphate synthase gene *TPS1* and *HSP104* to isolate multi-copy suppressors of the glucose-induced loss of heat resistance. Such suppressors should in principle function antagonistically to components required for glucose activation of the cAMP-PKA pathway.

In this paper, we describe the isolation of the *YOR107w* gene as a multi-copy suppressor of glucose-induced loss of heat resistance, using this novel approach. Protein sequence comparison and functional analysis show that the *YOR107w* gene product is a novel member of the recently characterized family of regulators of heterotrimeric G protein signalling (RGS) and that its function is to regulate glucose-activation of the cAMP pathway negatively through direct inhibition of Gpa2. Hence, we renamed the gene *RGS2*. RGS proteins function as GTPase activators of their cognate G-α protein, and Rgs2 and Gpa2 constitute the second RGS–G-α protein pair identified in *S.cerevisiae*. The previously characterized Sst2 functions as an RGS protein for Gpa1, the G-α subunit of the mating pheromone response G protein (Dohlman *et al*., 1996; Apanovitch *et al*., 1998). We present evidence that Rgs2 and Sst2 fulfil specific regulatory functions in heterotrimeric G-protein-mediated signal transduction in yeast.

Results

Genetic identification of YOR107w

We have performed a novel genetic screen for multi-copy suppressors of the glucose-induced loss of heat resistance in stationary phase cells of the stress-sensitive *hxk2*Δ *tps1*Δ *hsp104*Δ strain. This strain does not accumulate trehalose, due to the absence of the trehalose synthase subunit Tps1 (Van Aelst *et al*., 1993), and it lacks the heat shock protein Hsp104 (Sanchez *et al*., 1992). In addition, *HXK2* encoding hexokinase PII is deleted to suppress the growth deficiency on glucose associated with a deletion of *TPS1* (Hohmann *et al*., 1993). Details of this novel genetic screening procedure are given in Materials and methods. One specific genomic library fragment was obtained several times and contained both the syntaxin homologue *YOR106w*/*VAM3* (Darsow *et al*., 1997) and the previously uncharacterized *YOR107w* gene. Truncation and complementation analysis of the original fragment showed that the *YOR107w* gene was responsible for the heat stress resistant suppressor phenotype (see Materials and methods; results not shown).

YOR107w/RGS2 encodes a putative RGS protein

Screening the predicted protein sequence of Yor107w with the SMART domain alignment tool (Schultz *et al*., 1998) revealed that the protein contained a putative RGS domain. Detailed sequence comparison against the Swissprot database using BLAST2.0 and PSI-BLAST algorithms (Altschul *et al*., 1997), and multiple alignment using CLUSTALw (Thompson *et al*., 1994) and DIALIGN (Morgenstern *et al*., 1996) algorithms, confirmed that the Yor107w protein is a putative member of the family of eukaryotic regulators of G protein signalling (Koelle, 1997; Figure 1A). This would be the second RGS protein identified in *S.cerevisiae*, the first one being Sst2 (Dohlman *et al*., 1996). Whereas Sst2 bears pronounced differences with respect to the domain structure of most members of the RGS family (Figure 1B), Yor107w fits better into the general structural scheme of this family. The RGS domain comprises most of the N-terminal half of the 309 amino acids in the protein. The C-terminal half does not exhibit any apparent structural features, except for a putative direct repeat region in the extreme C-terminus (Figure 1C). A potential PEST sequence (Rogers *et al*., 1986) is present between amino acids 230 and 246. We tentatively renamed the *YOR107w* gene *RGS2*, and undertook a series of experiments to investigate the functional role of the Rgs2 protein.

Overexpression of RGS2 causes a 'low PKA activity' phenotype, deletion causes the opposite effect

Since *RGS2* had been identified as a multi-copy suppressor of the glucose-induced loss of heat resistance in an intrinsically heat stress-sensitive strain (see above), we first checked whether Rgs2 would confer a comparable phenotype in a wild-type strain. As shown in Figure 2A, *RGS2* overexpression indeed caused a significant increase in heat resistance in the wild-type strain both before and after the addition of glucose, whereas the *rgs2*Δ mutant was more sensitive to heat stress. High general stress resistance is a typical phenotype of yeast cells with low activity of PKA, whereas high stress sensitivity is indicative of high PKA activity (de Winde *et al*., 1997). Therefore, we tested several other phenotypical characteristics correlated with PKA activity in strains overexpressing or lacking *RGS2*. The reserve and stress protection carbohydrate trehalose accumulated to higher concentrations, and mobilization upon addition of glucose to stationary phase cells was delayed when *RGS2 was* overexpressed (Figure 2B). In contrast, trehalose levels were always lower in an *rgs2*Δ strain. Mobilization of glycogen was affected similarly to trehalose (Figure 2C). Transcriptional repression of many genes controlled through *cis*-acting STRE elements in their promoters has been correlated with high PKA activity (Ruis and Schuller, 1995; Varela *et al*., 1995; de Winde *et al*., 1997). Accordingly, expression of *HSP12* (Figure 2D) and *SSA3* (not shown) was rapidly repressed after addition of glucose to stationary phase cells. This glucose-induced repression was clearly delayed in a strain overexpressing *RGS2*, suggesting that glucose activation of the cAMP-PKA pathway was compromised. In contrast, deletion of *RGS2* resulted in an overall decrease in *HSP12* transcript levels. These results are consistent with overexpression of *RGS2* inhibiting PKA-dependent signal transduction, whereas deletion of *RGS2* enhances PKA-dependent effects on the various targets.

Effects of RGS2 deletion and overexpression are dependent on cAMP

Since the role of Rgs2 appeared to be correlated with PKA-dependent phenotypic characteristics, we investigated whether effects of Rgs2 require cAMP control of

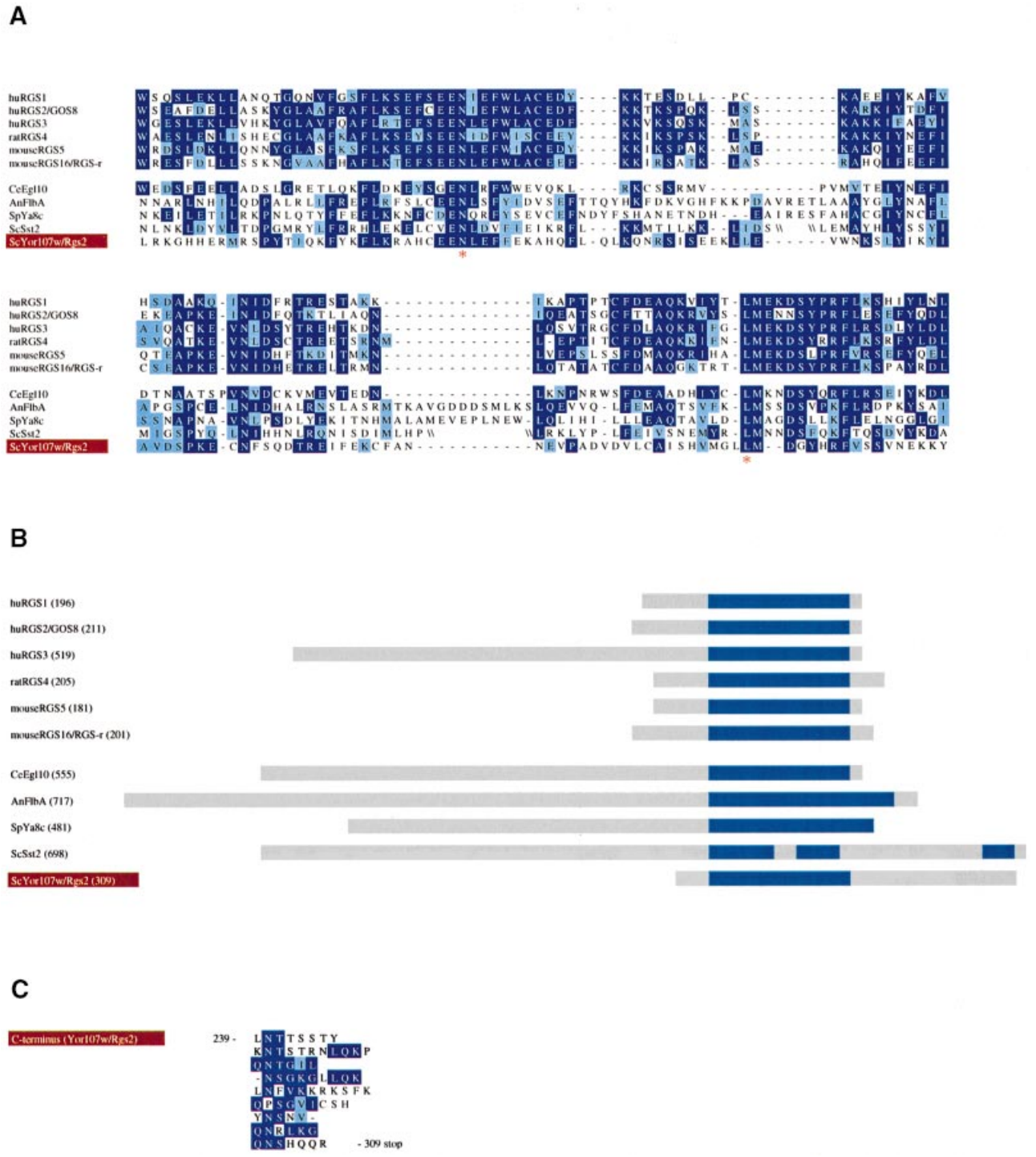

Fig. 1. Alignment of the amino acid sequence and protein domain structure of Yor107w/Rgs2 with other members of the RGS protein family. (**A**) Alignment of the RGS domain; identical residues are indicated in dark blue, similar residues in light blue. (**B**) Alignment of the protein domain structure (the start of the RGS domain is aligned and indicated in blue; other protein domains are not indicated). (**C**) Part of the C-terminus of Yor107w/Rgs2 possibly originates from multiple repetition of a sequence element.

PKA activity. We therefore deleted and overexpressed *RGS2 in* a strain with low, constitutive (i.e. cAMP-independent) PKA activity, *tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ (Nikawa *et al*., 1987). As shown in Figure 3, in the absence of the PKA regulatory subunit Bcy1, neither deletion nor overexpression of *RGS2* had any effect on the glucose-induced loss of heat resistance or on trehalose mobilization. This showed that the function of Rgs2 is dependent on Bcy1 and hence, in all likelihood, on cAMP.

Rgs2 negatively regulates glucose-induced cAMP signalling

Since Rgs2 function apparently requires cAMP control of PKA, we decided to investigate the role of Rgs2 in

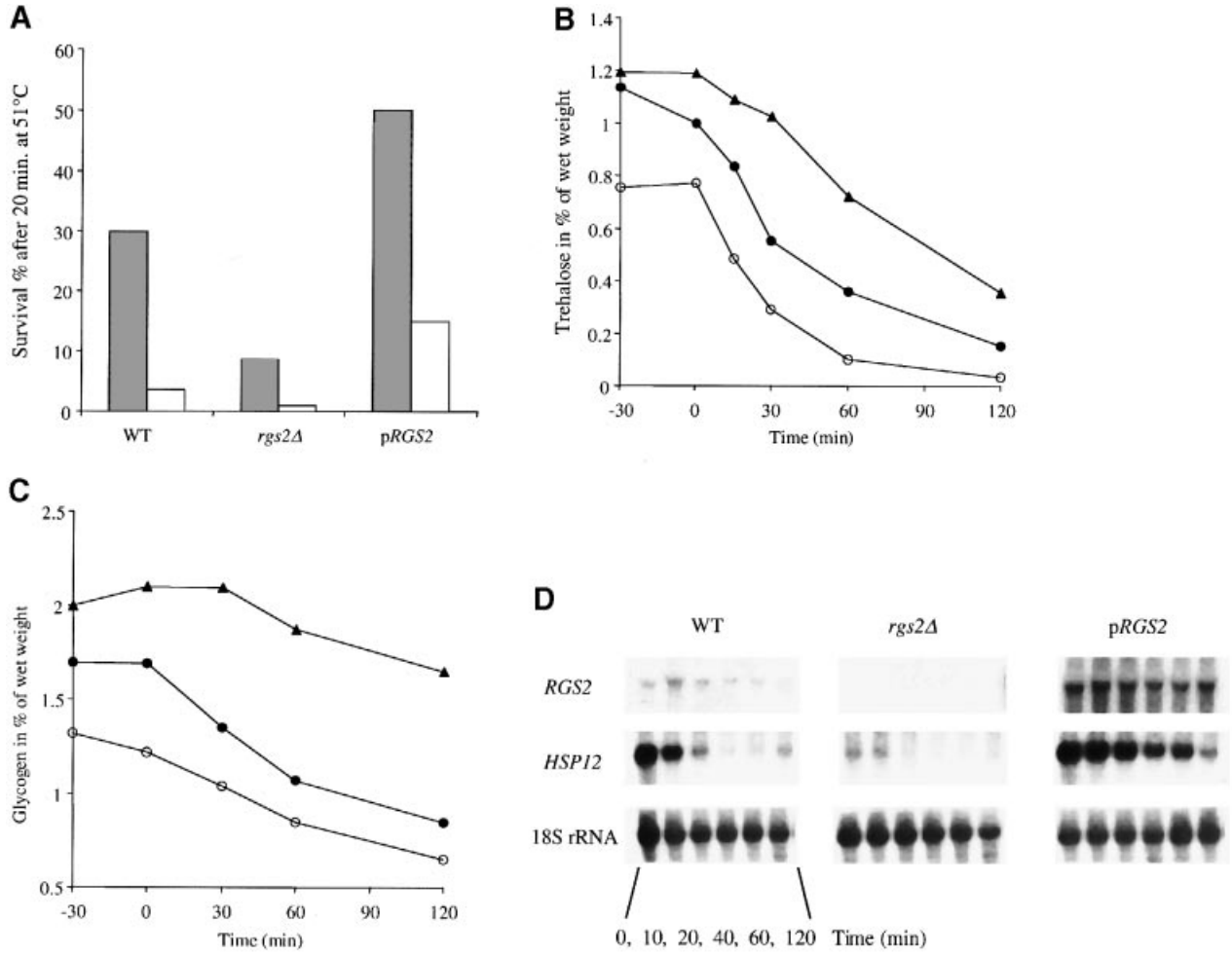

Fig. 2. Glucose-induced loss of heat resistance (**A**), mobilization of trehalose (**B**) and glycogen (**C**), and repression of the STRE-regulated gene, *HSP12* (**D**). Wild-type strain (W303–1A) (●), *rgs2*Δ (MV27) (○) and wild type + YEp*RGS2* (MV29) (▲). For each of the assays, cells were grown to stationary phase on SCD and glucose (100 mM) was added at time zero. In (A), grey bars represent heat resistance before addition of glucose and white bars represent resistance 60 min after addition of glucose.

glucose-induced cAMP signalling directly. Overexpression of *RGS2* suppressed glucose-induced cAMP accumulation to ~50% of the wild-type levels in cells exponentially growing on non-fermentable carbon sources (Figure 4A). Deletion of *RGS2* did not affect the glucose-induced cAMP signal in exponentially growing cells (Figure 4A). This was surprising at first, since deletion of *RGS2* was previously found to cause clearly discernable effects on PKA-dependent phenotypes (see above). However, in an *rgs2*Δ strain grown into stationary phase on non-fermentable carbon sources both the amplitude and the persistence of the glucose-induced cAMP signal were significantly enhanced compared with the wild-type strain (Figure 4B). Moreover, in stationary phase cells over-expressing *RGS2*, the glucose-induced cAMP increase was almost absent. These results indicate that Rgs2 is a negative regulator of cAMP signalling, and that this function is more prominent in stationary phase cells than in exponentially growing cells. It is important to note that for a wild-type strain the glucose-induced cAMP signal is attenuated in stationary phase (Figure 4B) compared with exponential phase (Figure 4A). Apparently, other regulatory mechanisms mask or overcome the negative action of Rgs2 in exponentially growing wild-type cells. One such concealing mechanism could be the strong negative feedback control on cAMP signalling exerted by PKA (Nikawa *et al*., 1987; Mbonyi *et al*., 1990). Therefore, we measured cAMP signalling in exponentially growing cells of a *tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ strain exhibiting low, constitutive (i.e. cAMP-independent) PKA activity (Nikawa *et al*., 1987; see above) and overexpressing or lacking *RGS2*. Because of the low PKA activity and, accordingly, low feedback inhibition, the *tpk1*^{w1} strain accumulated very high levels of cAMP (Figure 4C). Deletion of *RGS2* in this strain caused an even higher cAMP accumulation, whereas overexpression dramatically reduced cAMP levels. These results indicate that under conditions of low negative feedback control, cAMP signalling is very sensitive to negative control by Rgs2. Hence, the stronger negative control on cAMP signalling by Rgs2 in stationary phase cells (exhibiting low PKA activity) compared with exponentially growing cells could be due to reduced feedback inhibition by PKA.

Rgs2 function is dependent on the G-α protein Gpa2

Activation of cAMP accumulation in response to glucose is essentially dependent on the action of Gpa2, a member

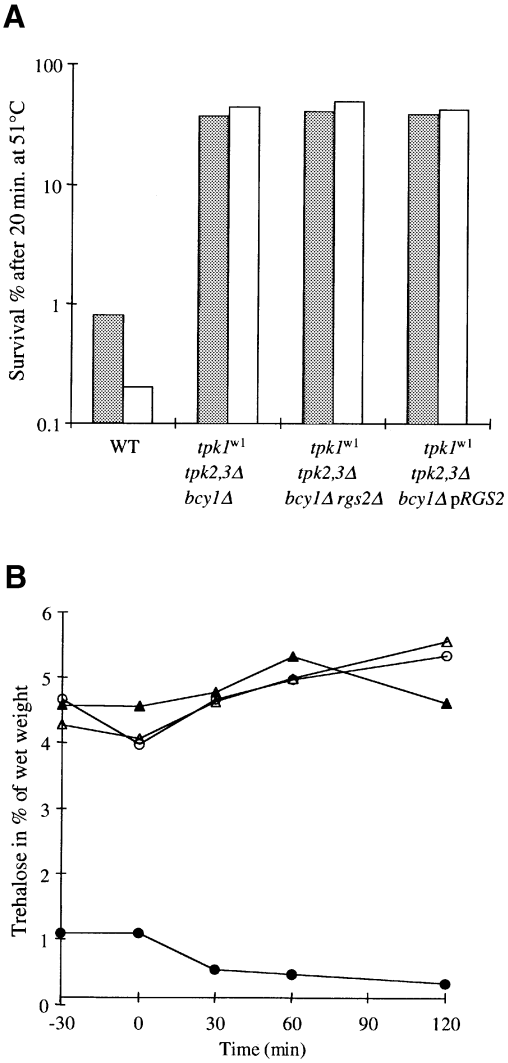

Fig. 3. Glucose-induced loss of heat resistance (**A**) and mobilization of trehalose (**B**) in Tpk-attenuated strains. Wild-type strain (SP1) (●), *tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ (RS13-58A-1) (○), *tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ *rgs2*Δ (MV36) (▲), *tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ + YEp*RGS2* (MV37) (△). Cells were grown to stationary phase on SCD and glucose (100 mM) was added at time zero. In (A), grey bars represent heat resistance before addition of glucose and white bars represent resistance 60 min after addition of glucose; heat resistance is plotted on a logarithmic scale because of the large differences between the wild-type strain and the mutants.

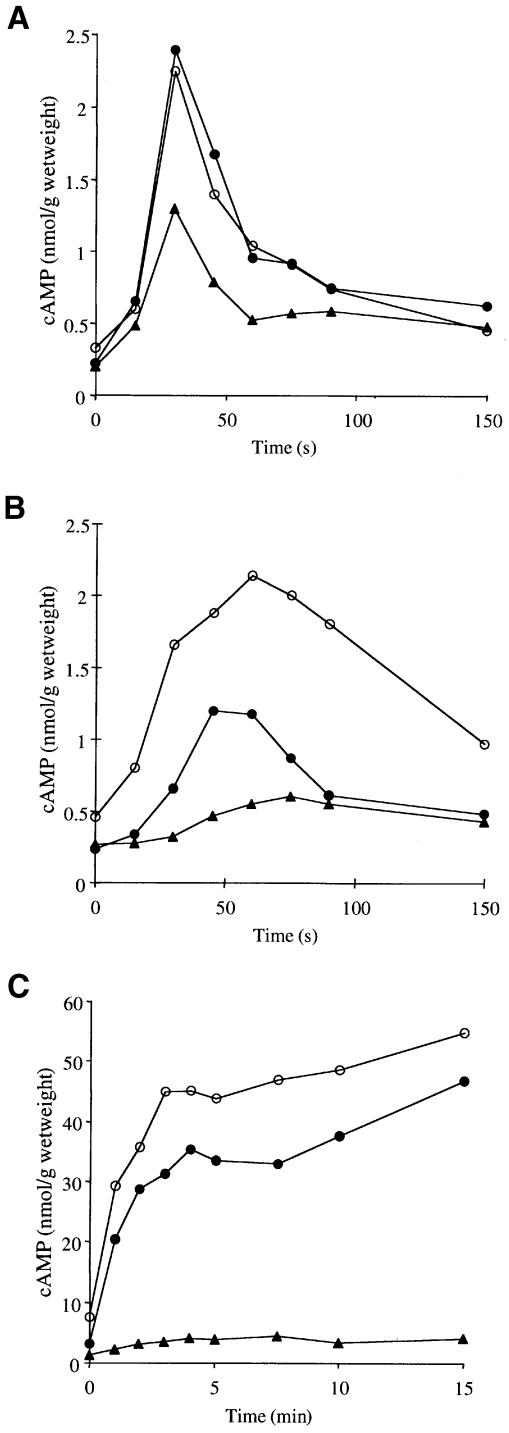

Fig. 4. Glucose-induced cAMP signalling in cells exponentially growing on SCEGd (**A**) or in cells grown into stationary phase on SCEGd (**B**). Wild-type strain (W303–1A) (●), *rgs2*Δ (MV27) (○) and wild type + YEp*RGS2 (*MV29) (▲). (**C**) cAMP content as a function of time after addition of glucose to cells of Tpk-attenuated strains exponentially growing on SCEGd. *tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ (RS13-58A-1) (●), *tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ *rgs2*Δ (MV36) (○), *tpk1*^{w1} *tpk2*Δ *tpk3*Δ *bcy1*Δ + YEp*RGS2* (MV37) (▲). In each case 100 mM glucose was added at time zero.

of the conserved family of α subunits of heterotrimeric G proteins (Colombo *et al*., 1998). We therefore tested whether Rgs2 would be a negative regulator of Gpa2. The heat stress sensitivity (Figure 5A) and low trehalose (Figure 5B) and glycogen (Figure 5C) content, in a strain lacking Rgs2 (see above), were completely suppressed when *GPA2* was also deleted. We have shown previously that double deletion of *GPA2* and *SCH9* severely reduces growth, while deletion of *GPA2* alone has no detectable effect on the growth rate (Colombo *et al*., 1998; Kraakman *et al*., 1999). Overexpression of *RGS2* in an *sch9*Δ strain mimicked the severe growth defect of a *gpa2*Δ *sch9*Δ strain, while *RGS2* overexpression in a wild-type background had no effect on growth (results not shown). These results clearly indicate that the function of Rgs2 is fully dependent on the presence of Gpa2. We have shown recently that Gpa2-mediated activation of cAMP signalling requires the presence of the cognate GPCR homologue Gpr1 (Kraakman *et al*., 1999). A *gpr1*Δ strain is intrinsically more thermotolerant and has higher trehalose and

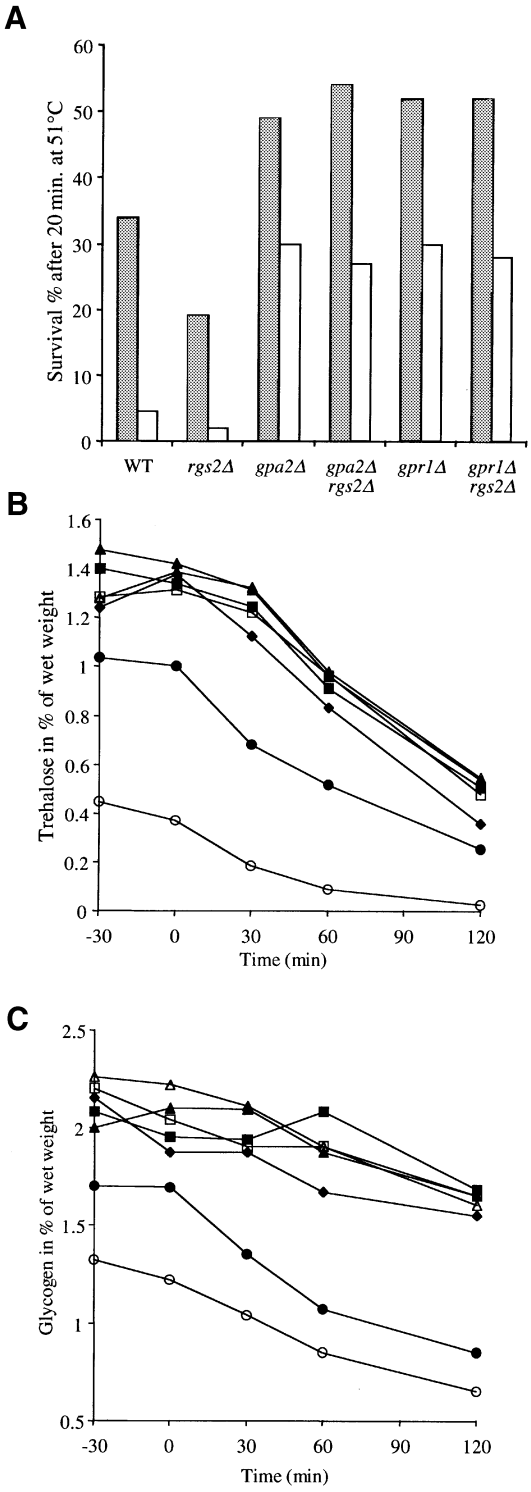

Fig. 5. Glucose-induced loss of heat resistance (**A**), mobilization of trehalose (**B**) and glycogen (**C**). Wild-type strain (W303–1A) (●), *rgs2*Δ (MV27) (○), wild type + YEp*RGS2* (MV29) (▲), *gpa2*Δ (JW8106) (△), *gpa2*Δ *rgs2*Δ (MV30) (■), *gpr1*Δ (LK5) (□), *gpr1*Δ *rgs2*Δ (MV31) (◆). For each of the assays, cells were grown to stationary phase on SCD, and glucose (100 mM) was added at time zero. In (A), grey bars represent heat resistance before addition of glucose and white bars represent resistance 60 min after addition of glucose.

glycogen levels than a wild-type strain both before and after initiation of fermentation. These characteristics were not affected by deletion of *RGS2* (Figure 5A, B and C). Overexpression of *RGS2* in either the *gpr1*Δ or the *gpa2*Δ background did not have any additional effects on the low PKA phenotypes of these strains (results not shown).

Gpa2^{val132} abrogates negative control by Rgs2

If Rgs2 indeed functions as an RGS-type negative regulator, it would enhance the intrinsic GTPase activity of its cognate G-α protein Gpa2. The dominant gain-of-function mutation, Gly132Val in Gpa2, locks this G protein in its activated, GTP-bound conformation (Lorenz and Heitman, 1997; Scheffzek *et al*., 1997; Kraakman *et al*., 1999). This constitutively activated *GPA2^{val132}* allele causes phenotypic characteristics indicative of high PKA activity, such as sensitivity to heat stress and low trehalose levels (Figure 6A and B). Neither overexpression nor deletion of *RGS2* affected these high PKA activity phenotypes (Figure 6A and B), indicating that negative control by Rgs2 is abrogated by *GPA2^{val132}*. In a strain with constitutively active Gpa2^{val132}, addition of glucose still triggers a cAMP signal, because only after addition of glucose is the second requirement for glucose activation of cAMP synthesis, i.e. sugar phosphorylation, fulfilled (Kraakman *et al*., 1999; F.Rolland, J.H.de Winde, V.Wancke, K.Lemaire, P.Ma, E.Boles, M.Vanoni, J.M.Thevelein and J.Winderickx, manuscript submitted). However, in contrast to the wild type, neither overexpression nor deletion of *RGS2* significantly affected cAMP signalling in a *GPA2^{val132}* strain (Figure 6C), again indicating that Rgs2 function is irrelevant in the presence of the constitutive G-α mutation. These results are fully consistent with a role for Rgs2 as GTPase activator of Gpa2.

Rgs2 physically interacts with Gpa2

Several mammalian RGS proteins (Watson *et al*., 1996; Druey and Kehrl, 1997; Natochin *et al*., 1997) and yeast Sst2 (Apanovitch *et al*., 1998) have been shown to bind with low affinity to both the inactive GDP-bound form and the active GTP-bound form of their cognate G-α proteins. However, these RGS proteins exhibit high affinity for the G-α protein in the transition state for GTP hydrolysis, which can *in vitro* be induced by binding of GDP and AlF_4^- on the G protein (Chabre *et al*., 1990; Berman *et al*., 1996b; Mittal *et al*., 1996; Tesmer *et al*., 1997). We have set up an *in vitro* binding system by coupling GST–Rgs2 fusion protein to glutathione–Sepharose beads. These beads were incubated with yeast extracts containing HA-tagged Gpa2. Figure 7 shows that GST–Rgs2 strongly and specifically bound HA-tagged Gpa2, after pre-incubation with GDP/AlF_4^-, and only weakly interacted with GDP-bound HA-Gpa2.

We have also detected a specific interaction between Gpa2 and both full-length Rgs2 or its isolated RGS domain (amino acids 35–166) using the yeast two-hybrid system (Fields and Song, 1989; James *et al*., 1996). However, this two-hybrid interaction was relatively weak and could only be detected with Gpa2 fused to the DNA binding domain and Rgs2 fused to the activating domain of Gal4 (results not shown; see Materials and methods). This poor interaction might be due to the specific requirement of the transition state for GTP hydrolysis in order to establish a strong interaction between the RGS protein and its cognate G-α protein.

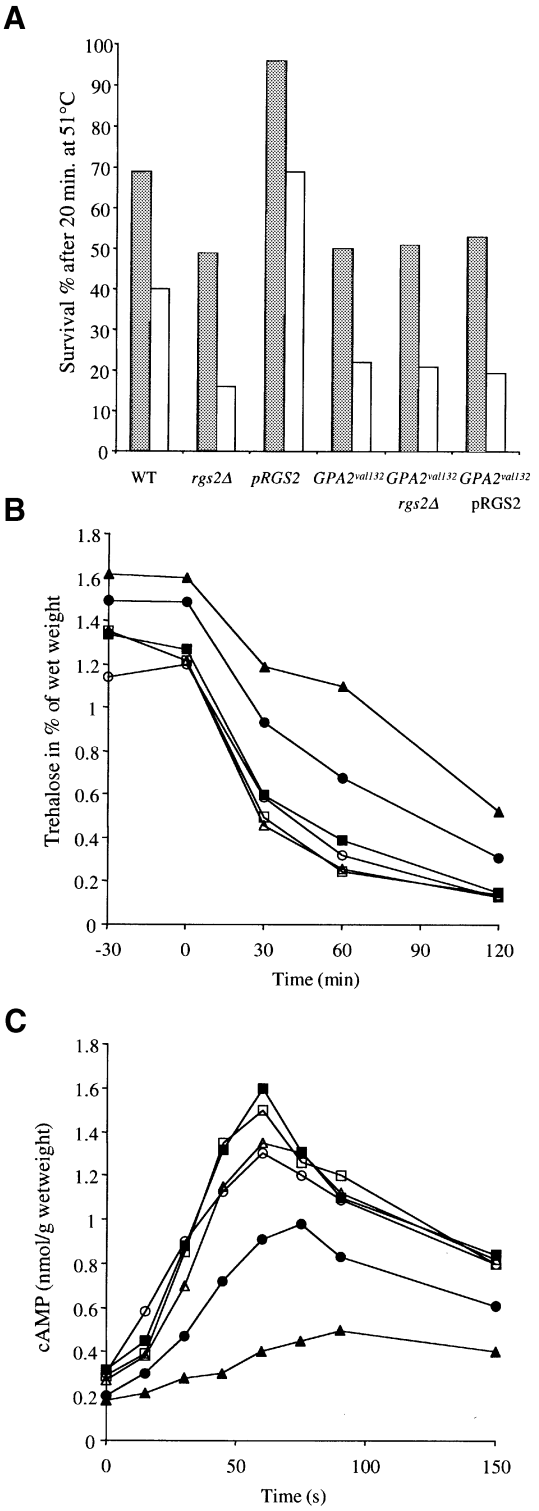

Fig. 6. Glucose-induced loss of heat resistance (**A**), mobilization of trehalose (**B**) and glucose-induced cAMP signalling (**C**). Wild-type strain (W303–1A) (●), *rgs2*Δ (MV27) (○), wild type + YEp*RGS2* (MV29) (▲), *GPA2*^{val132} (PM735) (△), *GPA2*^{val132} *rgs2*Δ (MV32) (■), *GPA2*^{val132} + YEp*RGS2* (MV27) (□). For (A) and (B), cells were grown on SCD; for (C), cells were grown on SCEGd into stationary phase. Glucose (100 mM) was added at time zero. In (A), grey bars represent heat resistance before addition of glucose and white bars represent resistance 60 min after addition of glucose.

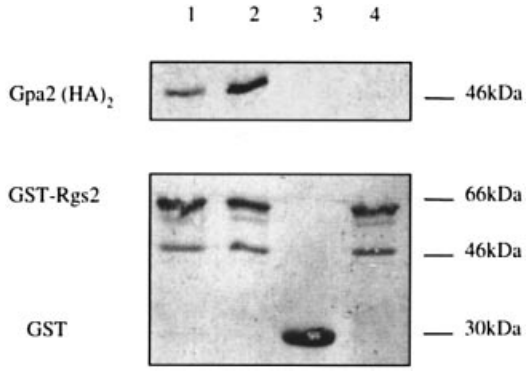

Fig. 7. *In vitro* binding of HA-Gpa2 to GST–Rgs2. The upper panel shows anti-HA detection, the lower panel shows anti-GST detection of the same amount of boiled precipitates after gel separation. Lane 1, GST–Rgs2 incubated with Gpa2(HA)$_2$–GDP; lane 2, GST–Rgs2 and Gpa2(HA)$_2$–GDP/AlF$_4^-$; lane 3, GST and Gpa2(HA)$_2$–GDP/AlF$_4^-$; lane 4, GST–Rgs2 and a control yeast extract with GDP/AlF$_4^-$.

Rgs2 accelerates hydrolysis of GTP on Gpa2 in vitro

The phenotypes for Rgs2 described above, and the direct interaction between Rgs2 and Gpa2, strongly argue for a typical RGS function of Rgs2 on Gpa2; thus, Rgs2 would be expected to stimulate hydrolysis of GTP on Gpa2. To investigate this possibility directly, we measured the effect of Rgs2 on turnover of GTP on Gpa2 *in vitro*. For this purpose we purified both Gpa2 and Rgs2 proteins using a His$_6$ tag at the N-terminus. The tagged proteins were expressed in *Escherichia coli* and purified by means of Ni-NTA affinity chromatography (see Materials and methods). We determined both the effect of Rgs2 on the steady-state GTP turnover on Gpa2 and on the single GTP turnover. Steady-state hydrolysis of GTP on a G-α protein is determined by two steps: the binding of GTP and the subsequent hydrolysis of GTP to GDP and free phosphate. We followed the release of free phosphate during incubation of [γ-^{32}P]GTP with Gpa2 in the absence and presence of Rgs2. As shown in Figure 8A, a clear stimulating effect of Rgs2 on steady-state hydrolysis on Gpa2 was observed. We also determined whether Rgs2 stimulates hydrolysis of GTP using a single GTP turnover assay. Gpa2 was loaded in the absence of Mg^{2+} with [γ-^{32}P]GTP and then Mg^{2+} and an excess of cold GTP was added to initiate hydrolysis and prevent further hydrolysis of unbound radiolabelled GTP. The results of such a single turnover experiment are presented in Figure 8B. Rgs2 drastically accelerates the hydrolysis of GTP on Gpa2. Quantification of this acceleration is difficult since even at 4°C Rgs2-stimulated hydrolysis of GTP was already complete at the first time point (15 s). These results provide a molecular mechanism for the negative regulation of Rgs2 on Gpa2 indicated by the phenotypic effects observed *in vivo*.

Sst2 and Rgs2 are specific RGS proteins for Gpa1 and Gpa2, respectively

The previous results have shown that yeast Rgs2 is a GTPase-activating protein (GAP) for Gpa2. Hence, these proteins constitute a second cognate RGS–G-α protein pair in *S.cerevisiae*. The first pair characterized in yeast consists of Sst2 and Gpa1 (Dohlman *et al.*, 1996; Apanovitch *et al.*, 1998). We have tested the possibility that Sst2 and Rgs2, despite some different structural

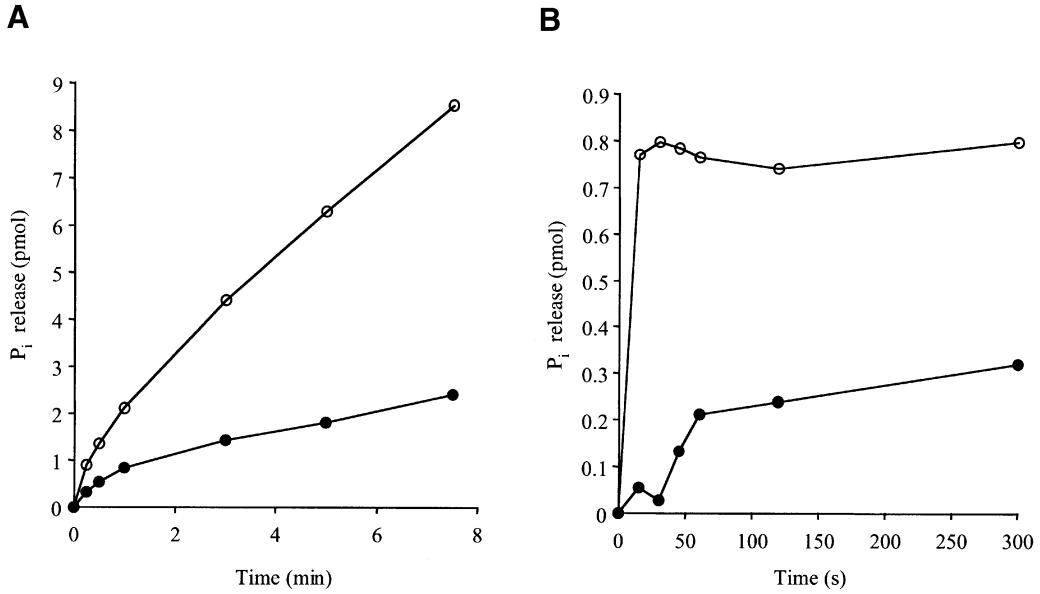

Fig. 8. GTP turnover on Gpa2. (**A**) Steady-state GTP turnover (at 4°C) in the presence of boiled (●) or native (○) Rgs2. (**B**) Single GTP turnover (at 4°C). GTP hydrolysis was initiated 12 min after the start of GTP loading on Gpa2 by addition of $MgCl_2$ and cold GTP in the presence of either boiled (●) or native (○) Rgs2 (time zero is set at the initiation of GTP hydrolysis). The amount of GTP hydrolysed into free phosphate at time zero (~4 pmol) is subtracted from the phosphate subsequently released.

characteristics (Figure 1B), might have (partially) overlapping functions. Deletion of *SST2* in a wild-type or *rgs2*Δ background did not affect trehalose levels before or after addition of glucose to stationary phase cells (Figure 9A). In addition, overexpression of *SST2* could not suppress the low trehalose levels of an *rgs2*Δ strain (Figure 9A). Also, glucose-induced cAMP signalling in stationary phase (Figure 9B) or exponentially growing cells (results not shown) was not affected by deletion or overexpression of Sst2 in a wild-type or an *rgs2*Δ strain. Furthermore, overexpression and deletion of *RGS2* in an *sst2*Δ strain did not significantly affect the higher sensitivity to pheromone-induced growth arrest of the *sst2*Δ strain (Dohlman *et al*., 1995), as determined in an α-factor halo assay (Figure 9C). Thus, the two RGS proteins in *S.cerevisiae*, Rgs2 and Sst2 appear to exert specific, non-overlapping control functions on the G-α proteins Gpa2 and Gpa1, respectively.

Functional importance of conserved residues in Rgs2

The highly conserved RGS domain, consisting of ~125 amino acids, has been shown to contact the G-α interface directly and be sufficient for GAP activity (De Vries *et al*., 1995; Tesmer *et al*., 1997). Several fully conserved residues within the helical core domain of mammalian RGS4 interact with the switch regions of its cognate G-α protein, thereby stabilizing the GTPase cycle transition state (Tesmer *et al*., 1997). Mutation of a conserved Asn or Leu residue within the RGS domain has been reported to affect binding and GAP activity (Druey and Kehrl, 1997; Natochin *et al*., 1998). We have mutated the corresponding residues in yeast Rgs2, yielding mutations Asn63Ser and Leu144His (indicated by * in Figure 1A). Whereas overexpression of wild-type Rgs2 counteracts both glucose-induced loss of heat stress resistance and mobilization of trehalose (see also above, Figure 2), $Rgs2^{Ser63}$ (partially) and $Rgs2^{His144}$ (fully) lacked this effect (Figure 10A and B). Consistently, $Rgs2^{Ser63}$ (partially) and $Rgs2^{His144}$ (fully) lacked the ability to suppress glucose-induced cAMP signalling when overexpressed (Figure 10C). These phenotypes are largely in agreement with results for the corresponding mutations in mammalian RGS4, where the Leu159Phe mutation had a more stringent effect than the Asn88Ser mutation (Druey and Kehrl, 1997).

Mammalian RGS proteins can fulfil the function of yeast Rgs2

To investigate further the extent of functional evolutionary conservation between mammalian RGS proteins and yeast Rgs2, we overexpressed mouse RGS2, RGS5 and RGS16 (Chen *et al*., 1997) in an *rgs2*Δ strain, and measured trehalose accumulation and glucose-induced activation of cAMP synthesis. The cells were grown on galactose to ensure efficient expression of the mouse cDNAs from the *GAL1* promoter fusion constructs (see Materials and methods). Both RGS5 and RGS16 mimicked yeast Rgs2 in enhancing trehalose accumulation when overexpressed both before and after addition of glucose to galactose-grown cells (Figure 11A). Moreover, overexpression of both RGS5 and RGS16 caused almost complete suppression of glucose-induced cAMP accumulation (Figure 11B). In contrast, mouse RGS2 could not replace yeast Rgs2 in enhancing trehalose content (Figure 11A) and had only a minor inhibiting effect on cAMP signalling (Figure 11B). Since the *sst2*Δ mutation was only rescued by mouse RGS16 (Chen *et al*., 1997), these results further support the previous notion that yeast Rgs2 and Sst2 exert specific and separate functions. In addition, we checked complementation of the *rgs2*Δ phenotype by human RGS1, RGS2, RGS3, RGS3T and rat RGS4 (Druey *et al*., 1996). Of these RGS proteins only RGS4 could complement the low trehalose levels of the *rgs2*Δ strain (results not shown),

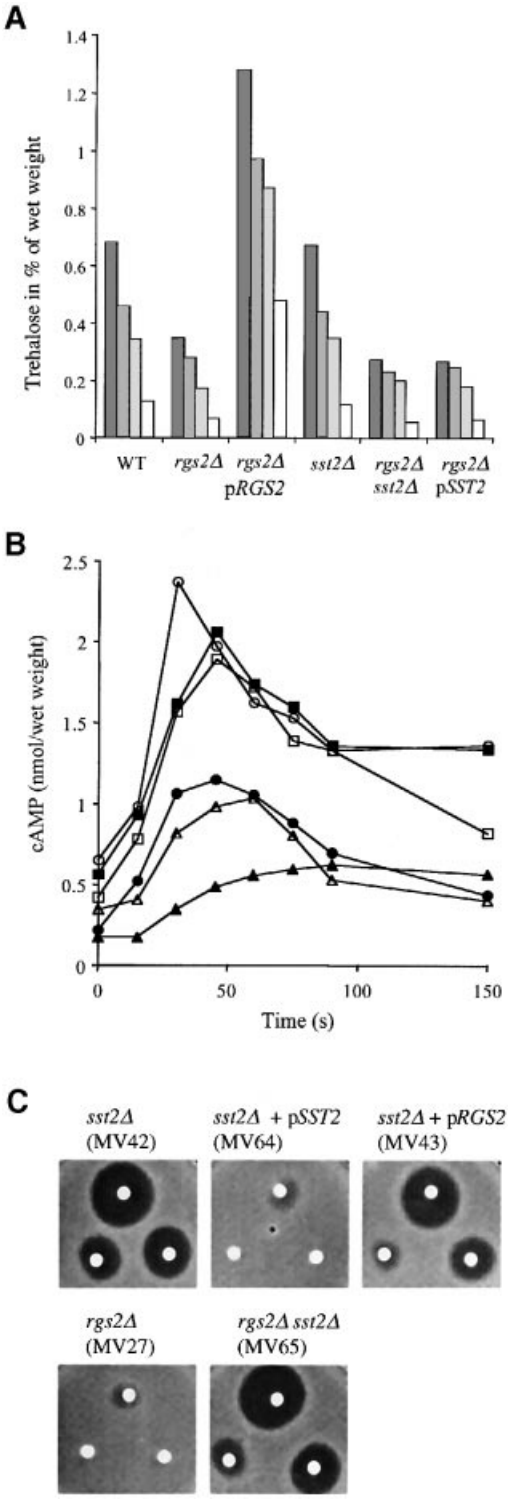

Fig. 9. Rgs2 and Sst2 fulfil distinct functions. (**A**) Glucose-induced mobilization of trehalose in cells grown into stationary phase on SCD. Dark grey bars represent trehalose levels before addition of glucose, lighter shade bars represent trehalose levels 30, 60 and 120 min after addition of 100 mM glucose. (**B**) Glucose-induced cAMP signalling in cells grown into stationary phase on SCEGd. Wild-type strain (W303–1A) (●), *rgs2*Δ (MV27) (○), *rgs2*Δ + YEp*RGS2* (MV67) (▲), *sst2*Δ (MV42) (△), *sst2*Δ *rgs2*Δ (MV65) (■), *rgs2*Δ + YEp*SST2* (MV66) (□). Glucose (100 mM) was added at time zero. (**C**) α-factor induced growth inhibition as determined by the halo assay. Top disk, 1000 pmol; right, 300 pmol; and left, 100 pmol α-factor. The plates were incubated for 2 days at 30°C.

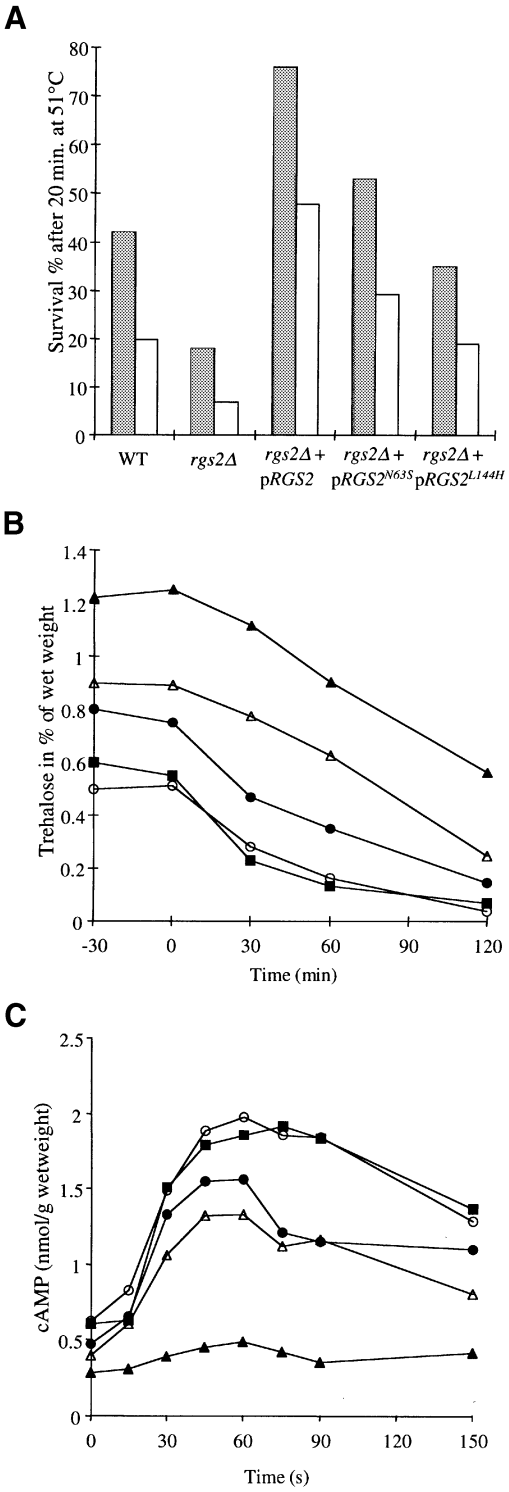

Fig. 10. Glucose-induced loss of heat resistance (**A**), mobilization of trehalose (**B**) and glucose-induced cAMP signalling (**C**). Wild-type strain (W303–1A) (●), *rgs2*Δ (MV27) (○), *rgs2*Δ + YEp*RGS2* (MV67) (▲), *rgs2*Δ + YEp*RGS2*^{N63S} (△), *rgs2*Δ + YEp*RGS2*^{L144H} (■). For (A) and (B), cells were grown into stationary phase on SCD; for (C), cells were grown on SCEGd into stationary phase. Glucose (100 mM) was added at time zero. In (A), grey bars represent heat resistance before addition of glucose and white bars represent resistance 60 min after addition of glucose.

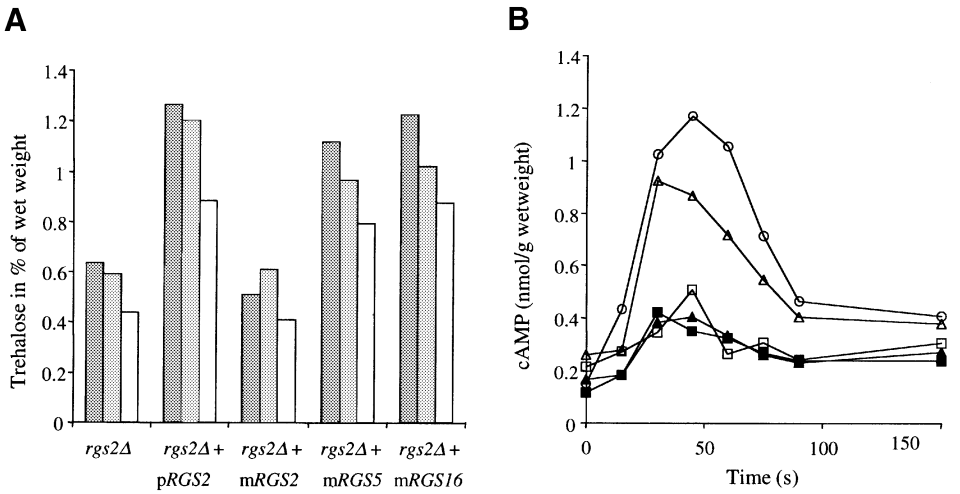

Fig. 11. Glucose-induced mobilization of trehalose (**A**) and glucose-induced cAMP signalling (**B**). *rgs2*Δ (MV27) (○), *rgs2*Δ + YEpyeast*RGS2* (MV67) (▲), *rgs2*Δ + pMW29mouseRGS2 (△), *rgs2*Δ + pMW29mouseRGS5 (■), *rgs2*Δ + pMW29mouseRGS16 (□). For (A), cells were grown into stationary phase on SCD; for (B), cells were grown on SCEGd into stationary phase. Glucose (100 mM) was added at time zero.

while RGS1, RGS3T and RGS4 were reported to complement the *sst2*Δ phenotype (Druey *et al*., 1996).

Discussion

Rgs2 downregulates the cAMP-PKA pathway

We have previously been able to identify positively acting components essential for glucose activation of the cAMP pathway by screening for mutants deficient in the loss of thermotolerance after addition of glucose to stationary phase cells, i.e. the putative glucose receptor Gpr1 (Kraakman *et al*., 1999) and adenylate cyclase (P.Van Dijck, P.Ma, M.Versele, M.-F.Gorwa, S.Colombo, K.Lemaire, A.Loïez and J.M.Thevelein, manuscript in preparation). In the present paper we describe the isolation of a negative regulator of this pathway in a screen for multi-copy suppressors of the glucose-induced loss of heat resistance in stationary phase cells. The unknown open reading frame (ORF) *YOR107w* was isolated in this screen. The presence of an RGS domain at the N-terminus of this gene suggested a role in negatively regulating the G-α protein involved in glucose-induced cAMP signalling, Gpa2 (Colombo *et al*., 1998). We therefore renamed the gene *RGS2*. The effects of deletion and overexpression of *RGS2* on several targets of PKA (trehalose and glycogen levels, glucose-induced repression of STRE-regulated genes and heat resistance) are consistent with a negative role of Rgs2 on the cAMP-PKA pathway.

PKA activity is regulated by the cAMP pathway through a complex network of upstream regulatory components including the Ras system, the Gpr1–Gpa2 system and also the glucose phosphorylation enzymes (Beullens *et al*., 1988; F.Rolland, J.H.de Winde, V.Wancke, K.Lemaire, P.Ma, E.Boles, M.Vanoni, J.M.Thevelein and J.Winderickx, manuscript submitted). The PKA targets are also controlled by cAMP-independent mechanisms such as the so-called FGM pathway involving the protein kinase Sch9 (Crauwels *et al*., 1997). Since deletion of the regulatory subunit of PKA (Bcy1) suppressed the effects of Rgs2, cAMP control of PKA activity is essential for the action of Rgs2.

Rgs2 negatively regulates glucose-induced cAMP signalling

In yeast, addition of glucose to glucose-deprived cells results in a transient cAMP increase. In cells growing exponentially on non-fermentable carbon sources, a relatively small effect of overexpression of *RGS2* and no effect at all of the deletion of *RGS2* on glucose-induced cAMP signalling was observed. However, in stationary phase cells both deletion and overexpression of *RGS2* resulted in clear effects on glucose-induced cAMP signalling. This supports that Rgs2 affects the PKA targets through control of the cAMP level, and emphasizes the importance of Rgs2 control on glucose-induced cAMP signalling in stationary phase cells. In a strain with low constutitive PKA activity, the effect of deletion and overexpression of *RGS2* was very strong, which was also the case when the cells were exponentially growing on non-fermentable carbon sources. This suggests that in a wild-type background, PKA-mediated feedback may mask control by Rgs2 in cells exponentially growing on non-fermentable carbon sources. Previously, the low-affinity cAMP phosphodiesterase, Pde1, was also shown to be involved in downregulation of cAMP signalling (Ma *et al*., 1999). Hence, it seems that multiple layers of negative control regulate cAMP signalling and that one of these layers consists of the Rgs2 control.

Rgs2 downregulates cAMP signalling via Gpa2

The PKA phenotypes associated with overexpression and deletion of *RGS2* are completely dependent on the presence of Gpa2. Moreover, the constitutively active *GPA2*^{val132} allele blocks the effects of Rgs2 both at the level of the PKA phenotypes and cAMP signalling. This allele is expected to render the G-α protein insentive to stimulation of its intrinsic GTPase activity (Scheffzek *et al*., 1998). Therefore, these data strongly argue for Rgs2 acting as a GAP on Gpa2 in *vivo*. This is further supported by the fact that alleles in which one of two strictly conserved residues in the RGS domain was mutated were either partially or completely unable to complement the *rgs2*Δ phenotypes. As will be discussed below, the observation

that RGS proteins from higher eukaryotes complement the *rgs2*Δ phenotype provides an additional argument for the *in vivo* RGS function of Rgs2.

We obtained more direct evidence that Rgs2 acts on Gpa2 by using an *in vitro* binding assay. Inactive, GDP-bound Gpa2 was found to bind weakly to Rgs2. However, strong binding was induced when the transition state for GTP hydrolysis of Gpa2 is mimicked *in vitro* by addition of GDP, Mg^{2+} and AlF_4^-. This dependency on the transition state is typical for the interaction between G-α proteins and RGS proteins (De Vries *et al*., 1996; Watson *et al*., 1996; Natochin *et al*., 1997; Apanovitch *et al*., 1998). It has been proposed that the mechanism by which an RGS protein stimulates GTP hydrolysis on the G-α protein consists of stabilizing the transition state of the G-α protein during GTP hydrolysis (Tesmer *et al*., 1997). Thus, Rgs2 binds to Gpa2 in a typical RGS–G-α manner. In addition, we demonstrated that Rgs2 enhances the steady-state GTP turnover on Gpa2. For several other RGS proteins, no effect on the steady-state hydrolysis was found. Only in single turnover assays was a clear stimulating effect observed (e.g. Sst2, GAIP and RGS4; Berman *et al*., 1996a; Apanovitch *et al*., 1998). These authors have argued that the GTP turnover in steady-state conditions is determined by the rate of GDP dissociation on the G-α proteins. This GDP dissociation is not regulated by the RGS proteins but rather by the β–γ dimer, which stabilizes the GDP-bound G-α protein. On the other hand, retinal RGS (RGS-r) has been shown to stimulate steady-state hydrolysis of the transducin α subunit (Chen *et al*. 1996). In addition, the authors showed that GTP binding or GDP dissociation was not stimulated by RGS-r, leading to the conclusion that the enhanced steady-state hydrolysis by RGS-r was caused by stimulation of the GTP hydrolysis on transducin α. This was confirmed later by single GTP turnover assays (Natochin *et al*., 1997). With such a single turnover assay we have directly shown that Rgs2 also stimulates GTP hydrolysis on Gpa2, providing a straightforward mechanism for the negative regulation of Rgs2 on Gpa2 observed *in vivo*.

Gpa2 and Rgs2 constitute a second and specific RGS–G-α protein pair in yeast

The first RGS–G-α protein pair characterized in yeast consisted of Sst2 and Gpa1 (Dohlman *et al*., 1996; Apanovitch *et al*., 1998). Activation of Gpa1 releases the Ste4/Ste18 β–γ dimer and activates the mating pheromone response cascade, leading to a G_1-specific block in cell cycle progression (Dohlman *et al*., 1998). GTP hydrolysis causes reassociation with the β–γ dimer and desensitization to mating pheromone. Accordingly, the *sst2* mutant was isolated as a mutant hypersensitive to pheromone because of its deficiency in desensitization (Dietzel and Kurjan, 1987). Sst2 is the founding member of the RGS protein family (Koelle, 1997); however, Sst2 differs relatively strongly from the other members of the RGS family. The RGS domain present in Sst2 for instance, contains two gaps, which are absent in other RGS proteins (Figure 1B). In contrast with Sst2, Rgs2 is a relatively small protein, containing a more typical RGS domain. The C-terminus does not show any significant homology to other known proteins or protein domains. No functional redundancy between Sst2 and Rgs2 was found, neither in an Sst2-related assay nor in an Rgs2-related phenotype (Figure 9). This high specificity of the two RGS–G-αprotein pairs in yeast is remarkable in view of the difficulty in assigning specific RGS proteins to specific G-α proteins in mammalian cells (Berman *et al*., 1996a; Watson *et al*., 1996). Investigation of the molecular basis for this specificity in the yeast RGS–G-α systems could provide clues to unravel the much more complicated situation in higher eukaryotes.

The observation that the *sst2*Δ phenotype (which is commonly used to determine *in vivo* RGS activity of a putative RGS protein; Druey *et al*., 1996; Watson *et al*., 1996; Chen *et al*., 1997) and the *rgs2*Δ phenotype are complemented to some extent by different mammalian RGS proteins also points to a distinct function of both RGS proteins. For RGS5 this is the first time that *in vivo* RGS activity could be demonstrated, since it did not complement the *sst2*Δ mutant (Chen *et al*., 1996) and it also did not show any activity in other *in vivo* assays (Zhang *et al*., 1999). The RGS4, RGS5 and RGS16 proteins have been put into a subgroup of the RGS protein family based on the observation that their extreme N-termini are very similar (Srinivasa *et al*., 1998). This N-terminus is essential to target these RGS proteins to the membrane (Srinivasa *et al*., 1998; Druey *et al*., 1999). Other RGS proteins (like RGS3; Dulin *et al*., 1999) are targeted to the membrane via their cognate G-α protein. We have observed that Rgs2 is present in membrane fractions and that this membrane localization is independent of the presence of Gpa2; the distribution of HA-tagged Rgs2 between the membrane and cytosolic fraction was identical in a wild-type strain and a *gpa2*Δ strain (results not shown). Therefore, we speculate that full complementation of the *rgs2*Δ strain by RGS4, RGS5 and RGS16 could be aided by their membrane targeting mechanism, based on their similar N-terminus and independent of the G-α protein.

Another difference between the Sst2–Gpa1 protein pair and Rgs2–Gpa2, is that Gpa1 is an inhibitory G-α protein (Dohlman *et al*., 1998), while all available data indicate that Gpa2 functions as a stimulating G-α protein (Lorenz and Heitman, 1997; Colombo *et al*., 1998; Kraakman *et al*., 1999). No mammalian RGS proteins characterized so far stimulate GTP hydrolysis on G-α s proteins (Berman *et al*., 1996a; De Vries and Farquhar, 1999). For mammalian G-α proteins, a single amino acid has been shown to be responsible for the difference in interaction between G-α i or q and G-α s proteins with RGS proteins (Ser202 in G-α i or q, while the corresponding residue in G-α s is an aspartate; Natochin and Artemyev, 1998a,b). When Gpa2 is aligned with mammalian G-α proteins the residue corresponding to Ser202 is also a serine (Ser302 in Gpa2). Since Gpa2 is able to interact with Rgs2, this is in agreement with the proposed role for the serine residue in the RGS–G-α interaction. However, it makes Gpa2 stand out as the first G-α s protein regulated by an RGS protein. It might be an exception or it could indicate that the relationship between G-α i character and RGS interaction observed up to now was only fortuitous.

Conclusions

In this work, we have shown that glucose activation of the cAMP pathway in yeast is controlled by a novel

Table I. Yeast strains used in this study

Strain	Relevant genotype	Source/reference
YSH315	*MAT*α *leu2-3/112 ura3-52 trp1-92 SUC GAL tps1::TRP1 hxk2::LEU*	Hohmann *et al*. (1993)
MV26	YSH315 *hsp104::ura3*- (FOA selected)	this study
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)
MV27	W303–1A *rgs2::URA3*	this study
MV28	W303–1A *rgs2::loxP-kanMX-loxP*	this study
MV29	W303–1A + YEplac195-*RGS2*	this study
JW8106	W303–1A *gpa2::LEU2*	Kraakman *et al*. (1999)
MV30	W303–1A *gpa2::LEU2 rgs2::URA3*	this study
LK5	W303–1A *gpr1::LEU2*	Kraakman *et al*. (1999)
MV31	W303–1A *gpr1::LEU2 rgs2::URA3*	this study
PM735	W303–1A $GPA2^{val132}$ (*LEU2)*	Kraakman *et al*. (1999)
MV32	W303–1A $GPA2^{val132}$ (*LEU2) rgs2::URA3*	this study
MV33	W303–1A $GPA2^{val132}$ (*LEU2)* + YEplac195-*RGS2*	this study
MV42	W303–1A *sst2::loxP-kanMX-loxP*	this study
MV43	W303–1A *sst2::loxP-kanMX-loxP* + YEplac195-*RGS2*	this study
MV64	W303–1A *sst2::loxP-kanMX-loxP* + pYX012-*SST2*	this study
MV65	W303–1A *sst2::loxP-kanMX-loxP rgs2::URA3*	this study
MV66	W303–1A *rgs2::loxP-kanMX-loxP* + pYX012-*SST2*	this study
MV67	W303–1A *rgs2::loxP-kanMX-loxP* + YEplac195-*RGS2*	this study
LC101	W303–1A *TPI1-GPA2*$(HA)_2$ (*URA3)*	this study
SP1	*MAT***a** *his4 leu2 ura3 trp1 ade8 can1*	Nikawa *et al*. (1987)
RS13-58A-1	SP1 $tpk1^{w1}$ *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*	Nikawa *et al*. (1987)
MV36	SP1 $tpk1^{w1}$ *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 rgs2::URA3*	this study
MV37	SP1 $tpk1^{w1}$ *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2* + YEplac195-*RGS2*	this study
pJ69-4A	*MAT***a** *leu2-3,112 ura3-52 trp1-901 his3-200 gal4*Δ *gal80*Δ *GAL2-ADE2 lys2::GAL1-HIS3 met2::GAL7*-LacZ	James *et al*. (1996)

member of the RGS protein family called Rgs2. It acts as a stimulator of the GTPase activity of Gpa2. Hence, yeast appears to have two similar GPCR systems, one regulated by the RGS–G-α protein pair Sst2–Gpa1 for sensing pheromones, and a second one with Rgs2–Gpa2 for sensing glucose. The function of the two RGS proteins is specific and is complemented to a certain extent by different mammalian RGS proteins.

Materials and methods

Strains and growth conditions

Yeast strains used in this study are listed in Table I. Yeast cells were grown in minimal media containing 0.67% w/v yeast nitrogen base without amino acids supplemented with synthetic 'drop-out' amino acid/nucleotide mixture as required and with 25 mg/l additional adenine, and supplemented with 2% w/v glucose (SCD) or galactose (SCGal), or with 2% w/v glycerol, 2% w/v ethanol and 0.1% w/v glucose (SCEGd). Solid media contained 1.5% w/v Bacto-agar in addition. In all experiments care was taken to use strains with identical auxotrophies in order to avoid any marker effects on growth (whenever necessary empty plasmids were introduced). The higher overall levels of both heat resistance and trehalose in Figure 6 compared with the other experiments is due to a difference in growth medium; this experiment was done in SCD lacking both leucine and uracil, while the other experiments were done in SCD lacking uracil only. *Escherichia coli* strain TOP10 (Invitrogen) was used for cloning, and BL21(DE3) (Pharmacia) was used to express the GST–Rgs2 fusion protein and the His-tagged proteins (Gpa2 and Rgs2).

Plasmids and constructs

A genomic fragment containing *HSP104* was amplified by PCR using 5′ and 3′ primers with a *Bam*HI and *Pst*I site, respectively, and cloned into pUC19 to create pUC*HSP104*. A *Bgl*II–*Bcl*I internal fragment was replaced by a *Bam*HI–*Bam*HI fragment from YDpU (Berben *et al*., 1991) to create pUC*hsp104::URA3*. *YOR107w/RGS2* was isolated (see below) from a multi-copy genomic DNA library in vector pFL44L, originally constructed by Dr F.Lacroute (Bonneaud *et al*., 1991). *RGS2* was subcloned from the genomic fragment by digestion with *Nsi*I and *Sph*I, and ligation into the *Pst*I and *Sph*I sites of pUC19, creating pUC*RGS2*. Via digestion with *Bam*HI and *Sph*I, *RGS2* with its own promoter region was transferred from pUC*RGS2* to YEplac195 (Gietz and Sugino, 1988), yielding YEp*RGS2*. Two deletion constructs were made from pUC*RGS2*: a 1.5kb internal *Sna*BI–*Sna*BI fragment was removed from pUC19-*RGS2* and replaced by either a *Sma*I–*Sma*I fragment from pJJ242 containing *URA3* (Jones and Prakash, 1990), yielding pUC*rgs2*::*URA3*, or an *Eco*RV–*Pvu*II fragment from pUG6 (Guldener *et al*., 1996), giving pUC*rgs2::loxP-kanMX-loxP*. Mutations in *RGS2 (*the N88S and the L144H replacements) were introduced using the Quick Change Mutagenesis kit (Stratagene) on pUC19*RGS2*. Mutant *RGS2* genes were subcloned in YEplac195 as described above. Both constructs were sequenced (using the Thermosequenase kit, Amersham) to verify correct introduction of the mutations. The complete coding region of *GPA2* was amplified by PCR using 5′ and 3′ primers containing *Eco*RI and *Sma*I sites, respectively. The amplified gene was cloned in pYX012 (R&D systems) behind a *TPI1* promotor, putting a C-terminal HA-epitope tag in-frame with *GPA2*. A second HA-epitope tag was added by PCR using a 3′ primer containing an additional HA-tag sequence. The final pYX*GPA2*-HA_2 construct was sequenced at both junctions. The construct was linearized with *Pst*I for integration at the *URA3* genomic locus. Expression of Gpa2$(HA)_2$ was verified by Western blot analysis using anti-HA antibodies (CF10A, Boehringer Mannheim). For the GST–Rgs2 fusion we used pGEX-4T-1 (Pharmacia). The coding sequence of *RGS2* was amplified with primers containing an *Eco*RI and a *Xho*I site and cloned into these sites of the Multiple Cloning Site of pGEX-4T-1. The constructs were sequenced at both junctions and expression of fusion protein was verified by Western blot analysis using anti-GST antibodies (Pharmacia). For the yeast two-hybrid constructs, the complete ORF of *RGS2* and the RGS domain (amino acids 35–166) were amplified by PCR with primers containing a *Bam*HI and *Pst*I site (5′ and 3′, respectively) and cloned (in-frame) into the corresponding sites of the two-hybrid fusion vectors pGBT9 and pGAD424 (Clontech). The two-hybrid constructs for Gpa2 have been described previously (Kraakman *et al*., 1999). The ORFs from Gpa2 and Rgs2 were cloned in the pRSTA vector (Invitrogen) to create N-terminal His_6-tagged proteins. For *RGS2*, the ORF (without start codon) was amplified with primers containing the restriction sites *Bam*HI and *Xho*I, and the digested PCR product was cloned in the corresponding sites of pRSTA. For *GPA2*, the ORF (without start codon) was amplified with primers containing the restriction sites *Bam*HI and *Eco*RI, and the digested PCR product was cloned in the corresponding sites of pRSTA. Both constructs were sequencened at the cloning junctions and proper expression was checked by immunoblotting using the anti-Express antibody (Invitrogen). *SST2* was deleted by the one-step PCR-mediated KAN^r disruption method (Guldener *et al*., 1996). The complete ORF of *SST2* was replaced by

the *loxP-kanMX-loxP* cassette. The overexpresssion construct of *SST2* was constructed by cloning the amplified ORF (containing *Eco*RI and *Xho*I sites at the 5′ and 3′ ends, respectively) into the integrative plasmid pYX012 (R&D systems), by means of these sites, behind the strong constutitive *TPI1* promotor. The construct was linearized with *Pst*I for integration at the *URA3* genomic locus, and functional expression is shown by complementation of the *sst2*Δ strain (Figure 9C). Centromeric URA3-based plasmids containing the *GAL1* promoter pMW29, pMW29-mouseRGS2, pMW29-mouseRGS5 and pMW29-mouseRGS16 were a gift of Dr S.-C.Ling (Chen *et al*., 1997). Plasmids pBM743, pBM743-huRGS1, pBM743-huRGS2, pBM743-huRGS3, pBM743-huRGS3T and pVT102U-ratRGS4 were a gift of Dr J.Kehrl (Bethesda, MA) (Druey *et al*., 1996).

Selection of multi-copy suppressors of the glucose-induced loss of heat stress resistance

Plasmid DNA (50 μg) from the multi-copy genomic library in pFL44L was transformed into MV26. The total number of transformants was estimated to exceed 30 000. Transformants were pooled in selective liquid medium. A large aliquot was grown into stationary phase on SCD. Cells were washed and resuspended in SC (no carbon source) and incubated at 30°C for 1 h. Then glucose was added to a final concentration of 2% and cells were again incubated at 30°C for 1 h. The culture was then heat shocked at 56°C for 30 min by mixing it with 1 l of preheated medium. After the heat stress treatment, surviving transformants were enriched by growing the complete culture again to stationary phase. The complete selection procedure was repeated six times. Restriction analysis of plasmids isolated from transformants surviving the last round confirmed that a thorough selection had taken place compared with the starting pool of library transformants (six different restriction patterns on a total of 61 plasmids analysed). These six plasmids were then retransformed to MV26 to confirm the heat resistant phenotype and subsequently the inserts were sequenced. One of those plasmids contained both the *YOR106w*/*VAM3* and *YOR107w* genes. The plasmid containing this insert was found five times on a total of 61 plasmids. Both genes were subcloned and *YOR107w* was shown to be responsible for the heat resistant phenotype.

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 75 mg (wet weight) per ml at 30°C in 25 mM Mes buffer (pH 6) for 10 min before addition of 100 mM glucose. Samples containing 75 mg of cells were quenched in 10 ml of 60% methanol at –40°C at the time points indicated, and extracts were prepared and cAMP was determined as described previously (Colombo *et al*., 1998). For trehalose and glycogen determination, cells were collected by filtration, washed once with cold water, weighed and frozen in liquid nitrogen. Trehalose and glycogen determinations have also been described previously (Colombo *et al*., 1998). Concentrations are expressed as trehalose or glycogen weights per wet weight of cells.

RNA extraction and Northern blot analysis

Culture samples for Northern blot experiments were immediately cooled 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, and 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 *RGS2*, *HSP12*, *SSA3* and 18S rRNA. Northern blots were analysed using phosphoimager technology (Fuji, BAS-1000; software, PCBAS 2.0).

Determination of heat shock resistance

For determination of heat shock resistance, samples were taken from the culture at the time points indicated and heated for 20 min at 51°C (as indicated). After cooling, aliquots were diluted and spread on nutrient plates. Colonies were counted after 3 days of growth at 30°C.

In vitro protein binding assay

Freshly transformed BL21(DE3) cells (Pharmacia) containing the GST–Rgs2 fusion protein or GST alone (see constructs and plasmids), were grown to an OD_{600} of 1.5. Isopropyl-β-D-galactopyranoside (IPTG, 0.3 mM final concentration) was then added to induce expression. After 2 h the cells were cooled on ice and harvested. Protein extracts were prepared by incubating the cells in buffer A (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA) containing the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and pepstatin A, and with 0.2 mg/ml lysozyme for 5 min at room temperature. Five freeze/thaw cycles were then applied to complete the cell lysis. After clearing the extracts (10 min at 8000 r.p.m.), 20 μl of glutathione–Sepharose beads (Pharmacia) were added to 0.5 ml of extract to precipitate GST–Rgs2 or GST alone. The precipitates were washed three times with buffer A. Crude extracts from yeast strain LC101 (containing *GPA2*-HA_2) and a control strain without the HA-tagged Gpa2 were prepared by vigorous vortexing of the cells in buffer A with 0.4% Nonidet P-40 and glass beads. Cleared extracts (10 min, 8000 r.p.m.) were incubated with GDP (10 μM) alone or with GDP (10 μM), $AlCl_3$ (30 μM) and NaF (10 mM) for 30 min at room temperature. These extracts were then added to the GST precipitates and incubated for 1 h at 4°C with moderate shaking. After precipitation, the extracts were washed three times with buffer A containing 0.2% Nonidet-P40. To 20 μl of precipitate, 40 μl of SDS-loading buffer was added and 10 μl was used for SDS–PAGE.

Two-hybrid analysis

Constructs for yeast two-hybrid analysis are described above. Protein–protein interactions were monitored in strain pJ69-4A by serial dilution on selective media lacking histidine or adenine.

GTP turnover assays

At the N-terminus of both Gpa2 and Rgs2 a His_6 tag was added by cloning the respective ORFs in the pRSTA vector supplied by Invitrogen (see Plasmids and constructs). Vectors containing His-tagged Gpa2 and Rgs2 were transformed into *E.coli* strain BL21(DE3) and grown at 37°C (Gpa2) or 30°C (Rgs2) to an OD_{600} of 0.5. Then IPTG was added (0.2 mM final concentration) to the cultures and they were incubated for an additional 4 h. Extracts were prepared by freezing the cells rapidly in liquid nitrogen, followed by thawing in lysis buffer (50 mM Tris–HCl pH 8.0, 2 mM DTT, 20 μM GDP, 1 mM $MgCl_2$) containing 0.2 mg/ml lysozyme for 30 min. Extracts were cleared for 10 min at 13 000 r.p.m. and the supernatant was applied on a Ni-NTA column (Qiagen). The proteins were washed and eluted under native conditions as described by the manufacturer (washing with lysis buffer containing 10 mM imidazole and elution with 250 mM imidazole). The proteins were concentrated and the buffer was exchanged for the storage buffers by the use of Vivaspin columns (Vivascience). The storage buffer for Gpa2 was 50 mM Na–HEPES pH 7.6, 2 mM DTT, 10 μM GDP, 1 mM EDTA; RGS storage buffer was 50 mM Na–HEPES pH 8.0, 2 mM DTT, 1 mM EDTA. Purity of proteins was estimated with Coomassie-stained SDS–PAGE (Gpa2 was ~90% pure, Rgs2 was ~75% pure); approximate protein concentrations were determined using the Bradford method (Bio-Rad) with thyroglobuline as a standard. Both the steady-state GTP hydrolysis and the single turnover assay have been performed as described by Apanovitch *et al*. (1998) with some modifications. Briefly, for steady-state GTP hydrolysis, purified Gpa2 (250 nM) and boiled (15 min) or native Rgs2 (1 μM) were mixed on ice with 2× GTP buffer (50 mM HEPES pH 8.0, 2 mM DTT, 1 mM EDTA, 2 μM GTP, 10 mM $MgCl_2$) supplemented with [γ-^{32}P]GTP (~7500 c.p.m./pmol, 2 μM final concentration). Samples were taken at the time points indicated and mixed with a 5% (w/v) charcoal solution (Norit A, Aldrich) in 50 mM NaH_2PO_4 pH 2.3 on ice, and processed to determine release of free phosphate after centrifugation for 5 min at 13 000 r.p.m. and counting 200 μl of the supernatant in a scintillation counter. For the single GTP turnover measurements, the GTP binding reaction was carried out as indicated above except that no Mg^{2+} was added to the 2× GTP buffer and 5 mM EDTA was added. The GTP hydrolysis reaction was initiated by addition of $MgCl_2$ (15 mM final concentration), in the presence of an excess of cold GTP (150 μM final concentration) in 2× GTP buffer containing either native or boiled Rgs2 (1 μM). The P_i release was determined as described above.

α-factor halo assay

A halo assay to measure response to and recovery from pheromone-induced growth arrest was performed as described by Druey *et al*. (1996). Briefly, overnight cultures were diluted 20× in a final volume of 2 ml of selective medium and mixed with 2 ml of 1.5% molten agar (50°C). The mixture was rapidly spread onto a plate containing the same selective medium. Sterile filter disks were laid on top of the nascent lawn and different concentrations of α-factor were applied on the disks.

Reproducibility of the results

All experiments were repeated at least twice with independent transformants and cultures. The results always showed consistent trends, i.e.

differences between strains and mutants were highly reproducible. In all cases, results from representative experiments are shown.

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