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Glycogen hyperaccumulation in *Saccharomyces cerevisiae ras2* mutant

A biochemical study

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The mechanism by which yeast *ras2* mutant hyperaccumulates glycogen has been investigated. Total glycogen synthase activity was between 2.5 and 1.3 times higher in the *ras2* mutant than in an isogenic strain. In addition, while in the normal strain the glycogen synthase activation state decreased along the exponential phase, in the mutant strain the opposite behaviour was observed: glycogen synthase activation state rose continuously reaching full activation at the beginning of the stationary phase. Glycogen phosphorylase *a* activity was up to 40 times higher in the mutant than in the normal strain. Glucose 6-phosphate and fructose 2,6-bisphosphate levels were slightly more elevated in the mutants. The increase in total glycogen synthase and, particularly, the full activation of this enzyme may explain glycogen hyperaccumulation in the *ras2* mutant even in the presence of elevated levels of glycogen phosphorylase *a*.

cAMP; *ras2* Mutants; Glycogen; Glycogen synthase; Glycogen phosphorylase; Yeast; *Saccharomyces cerevisiae*

1. INTRODUCTION

The yeast *Saccharomyces cerevisae* contains two genes, *RAS1* and *RAS2*, which encode proteins highly homologous to the mammalian RAS proteins [1–3]. Strains lacking *RAS2* sporulate on rich media and hyperaccumulate glycogen [4–6].

In yeast, RAS proteins control adenylate cyclase [4,7,8]. Compared to wild-type strains, adenylate cyclase is significantly depressed in *ras2* mutants and these cells maintain very low levels of cAMF. The main role of cAMP appears to be the activation of cAMP-dependent protein kinases. Phosphorylation of specific substrates by these kinases controls cell metabolism and cell growth [6,9,10].

Glycogen metabolism is controlled by the activity of glycogen synthase and glycogen phosphorylase. Both enzymes are regulated by phosphorylation and dephosphorylation reactions [11–16]. While phosphorylation activates phosphorylase and inactivates synthase, dephosphorylation provokes the opposite effects. Several reports indicate that cAMP-dependent protein kinases are involved in the control of these enzymatic activities in yeast [17,18].

Abbreviations: fructose-2,6,-P_2, fructose 2,6-bisphosphate; glucose 6-P, glucose 6-phosphate; PFK-2, 6-phosphofructo-2-kinase.

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The aim of this work was to study whether changes in the activity of glycogen synthase and glycogen phosphorylase could reflect the decrease in the cAMP-dependent phosphorylation activity, which is a direct consequence of the low cAMP levels present in *ras2* mutants, and whether these changes in activity could, in turn, account for the accumulation of glycogen.

2. MATERIAL AND METHODS

2.1. *Strains*

Saccharomyces cerevisiae 547 (α *leu2 ura3-52 his4-539*) and 546 (α *leu2 ura3-52 his4-539 ras2-530::LEU2*), a *ras2* mutant derived from strain 547 were used for these experiments (both kindly provided by Dr K. Tatchell).

2.2. *Growth conditions*

Yeast cells were grown on an orbital shaker at 200 rpm at 30°C in a medium containing 2% glucose, 1% yeast extract and 1% bactopeptone. The media were inoculated with yeasts grown to the stationary phase in the same medium. Growth was monitored by measuring the turbidity of the culture at 660 nm and samples were started to be collected when absorbance reached 0.5 (about 16 h after inoculation of medium).

2.3. *Preparation of cell-free extracts for enzyme assays*

Samples, all containing equal amounts of cells, were collected during the period of growth of the culture. The volume required was determined by a calibration curve (dry weight vs absorbance).

To obtain cell-free extracts, yeast cells (ca. 30 mg dry weight) were harvested by vacuum filtration, washed with cold distilled water, and suspended in 500 μl of buffer containing 50 mM Tris-HCl (pH 7.0), 25 mM KF, 0.5 mM phenylmethylsulfonyl fluoride and 1 mM β-mercaptoethanol. The suspension was homogenized with 500 mg glass

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beads (0.5 mm diameter) and vigorously shaken for five periods of 1 min with 1 min intervals. During the idle intervals tubes were placed in ice [19]. The resulting homogenate was centrifuged at 1000 × *g* for 15 min at 4°C, and supernatants were used for enzyme assays and protein determination.

2.4. *Preparation of cell-free extracts for metabolite determinations*

Cell suspensions were rapidly harvested by vacuum filtration and the yeast pellicle was immediately immersed in liquid nitrogen. Acid extracts were obtained by mixing the frozen cells (about 30 mg dry weight) with 1 ml 10% perchloric acid in a mortar precooled with liquid nitrogen, essentially as described in [20]. For the preparation of alkaline extracts, frozen cells (15 mg dry weight) were suspended in 1 ml cold 54 mM NaOH. The suspension was incubated at 80°C for 15 min. The supernatant obtained by centrifugation at 12 000 rpm in a microfuge for 5 min, was used to measure fructose-2,6-P_2 levels.

2.5. *Enzyme assays*

Total glycogen synthase activity was measured as described in [21]. The assay mixture contained 4.4 mM UDP-[^{14}C]glucose (200 cpm/nmol), 0.66% glycogen, 13 mM EDTA, 25 mM KF, 50 mM Tris-HCl (pH 7.8) and 7,2 mM glucose 6-P.

Active form of glycogen phosphorylase (phosphorylase *a*) was measured following the procedure described in [22]. The assay mixture contained 142 mM KF, 0.66% glycogen, 66.6 mM [^{14}C]glucose 1-P (30 cpm/nmol), 10 mM caffeine and 10 mM EDTA at pH 6.3.

All assays were performed at 30°C. One unit is the amount of enzyme that catalyses the incorporation of 1 μmol of [^{14}C]glucose into glycogen per min under the assay conditions.

2.6. *Metabolites and glycogen determination*

Glycogen was determined enzymatically by the incubation of 100 μl of the acid homogenate (previously neutralized with 5 M K_2CO_3) with 100 μl of 73 U/ml amyloglucosidase (Sigma Chemical Co., USA) in 0.4 M acetate (pH 4.8) at 50°C for 3 h. After the addition of 150 μl of 10% perchloric acid and centrifugation at 22 000 × *g* for 15 min at 4°C, glucose was measured in the supernatant using hexokinase and glucose 6-P dehydrogenase [23].

For measuring glucose 6-P, the acid extract was centrifuged at 22 000 × *g* for 15 min at 4°C. The supernatant was neutralized with 5 M K_2CO_3 and kept in ice for 30 min. Glucose 6-P was measured in the supernatant after centrifugation at 22 000 × *g* by using glucose 6-P dehydrogenase [24]. Fructose-2,6-P_2 was measured in alkaline extracts as in [25]. Auxiliary enzymes were from Boehringer Mannheim (Germany).

2.7. *Other determinations*

Protein was measured in buffered extracts using the Biuret method [26] with bovine serum albumin as a standard.

Glucose in the culture medium was determined, after filtration of the cells, by the hexokinase/glucose 6-P dehydrogenase method [23]. The point where the concentration of glucose in the medium reached 50% of the initial concentration was defined as point 50. This point 50 permits the identification of two parts in the exponential phase, before and after, and also allows a comparison between different experiments.

3. RESULTS

3.1. *Glycogen*

In the wild-type strain, glycogen levels remained low, about 0.5 mg/g during the first part of exponential growth. When the concentration of glucose in the medium was reduced to one half (point 50), glycogen started to accumulate. Values up to 6 mg/g were reached at the beginning of the stationary phase.

Values determined in the *ras2* mutant were much higher. Initially, the glycogen content was about 10 mg/g; it started to rise before point 50 and attained levels around 60 mg/g which are about tenfold higher than in the isogenic strain (Fig. 1).

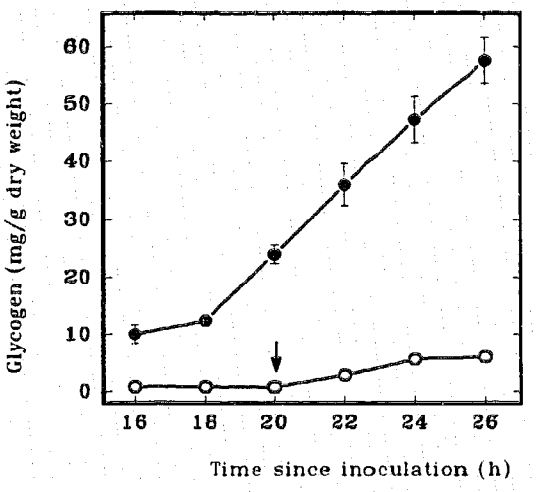

Fig. 1. Glycogen accumulation in wild-type (○) and *ras2* mutant (●) strains during growth of the culture. The arrow indicates point 50: at this moment the concentration of glucose in the medium was decreased to 50% of initial value. The stationary phase began three hours later. Represented values are mean ± SE from 5–7 independent experiments.

3.2. *Glycogen synthase activity*

In the wild-type strain, total glycogen synthase activity rose from 1.7 to 4.4 mU/mg protein during the exponential phase and remained constant along the stationary phase (Fig. 2, Panel A). With regard to the activation state of the enzyme, measured by the (−)glucose 6-P/(+)glucose 6-P activity ratio [21], it decreased continuously along the growth of the culture.

On the other hand, the mutant strain presented an initial total glycogen synthase specific activity of about 4.5 mU/mg protein (2.6 times higher than the wild-type) (Fig. 2, Panel B). Specific activity rose to 6.6 mU/mg

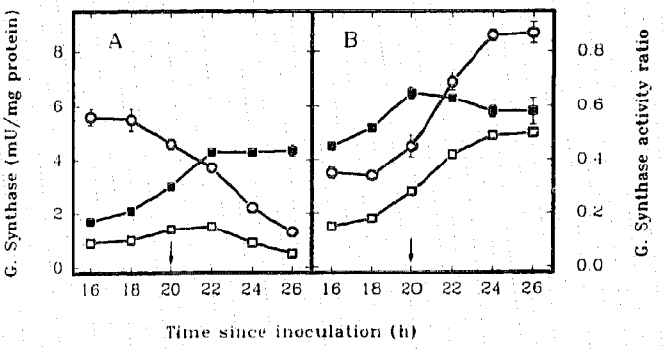

Fig. 2. Glycogen synthase activity in wild-type (panel A) and *ras2* mutant (panel B) strains during the growth of the culture. Total glycogen synthase specific activity ((+)glucose 6-P) (■) and specific activity of the active form ((−)glucose 6-P) (□) were determined. The (−/+)glucose 6-P activity ratio is also represented (○). Values are mean ± SE from 5–7 independent experiments. Arrows indicate point 50.

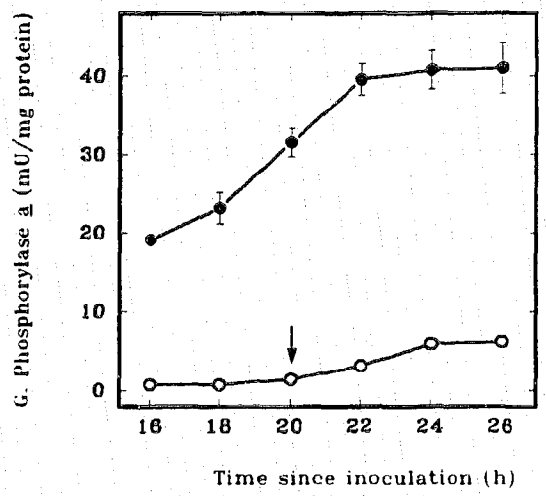

Fig. 3. Glycogen phosphorylase *a* activity in wild-type (○) and *ras2* mutant (●) strains during the growth of the culture. Glycogen phosphorylase *a* activity was measured during the growth culture. Values are mean ± SE from 5–7 independent experiments. Arrows indicate point 50.

protein and then slightly decreased at the beginning of the stationary phase (5.8 mU/mg protein). Opposite to what was observed in the wild-type strain, in the mutant strain the (−)glucose 6-P/(+)glucose 6-P activity ratio remained stable in the first part of the exponential growth, started to rise before point 50 and increased up to values of about 0.85, which were attained in the stationary phase. This is the highest value we have measured in many yeast strains.

3.3. *Glycogen phosphorylase activity*

Active glycogen phosphorylase (phosphorylase *a*) in the wild-type strain was very low at the beginning, about 0.5 mU/mg protein; it increased along the second part of the exponential growth and reached values of 7.5 mU/mg protein in the stationary phase (Fig. 3).

In the mutant strain, glycogen phosphorylase *a* activity was very high. Initial values were already about 19 mU/mg protein and constantly increased along the growth of the culture, attaining 40 mU/mg protein in the stationary phase.

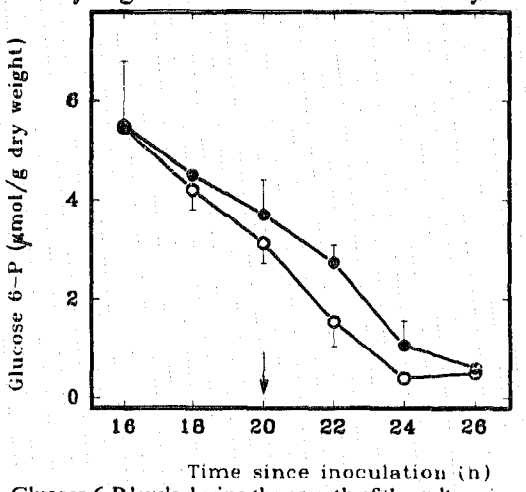

Fig. 4. Glucose 6-P levels during the growth of the culture in wild-type (○) and *ras2* mutant (●) strains. Values are mean ± SE from three independent experiments. Arrows indicate point 50

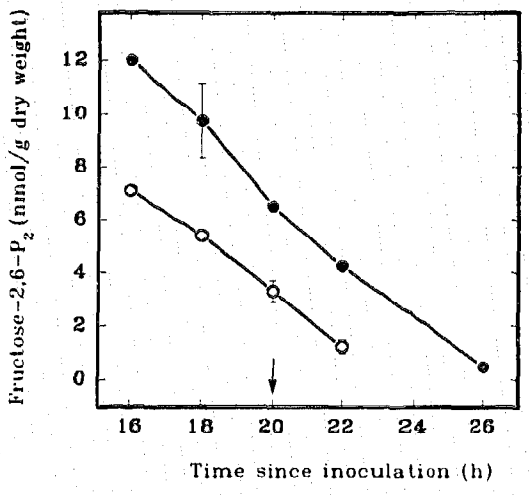

Fig. 5. Fructose-2,6-P_2 levels during growth in wild-type (○) and *ras2* mutant (●) strains. Values are mean ± SE from three independent experiments. Arrows indicate point 50.

3.4. *Glucose 6-P*

Glucose 6-P levels were slightly higher in the mutant than in the wild-type strain (Fig. 4). In both cases, values progressively decreased about 10 times along the growth of the culture. Initially, glucose 6-P levels were around 5 μmol/g and, at the beginning of the stationary phase, they had decreased to values around 0.5 μmol/g in both strains.

3.5. *Fructose-2,6-P_2*

Fructose-2,6-P_2 levels in the mutant were higher (about two-fold) than those determined in the wild-type strain (Fig. 5). In both cases, values constantly decreased along the cell growth reaching very low values (around 0.5 nmol/g) in the stationary phase.

4. DISCUSSION

There is ample evidence showing that cAMP plays an important role in the regulation of carbohydrate metabolism in yeast [10,27,28]. Direct evidence of the importance of cAMP stems from mutants which have disorders in the production of cAMP. These mutants present alterations in glycogen accumulation. *RAS2* proteins in yeast are involved in the production of cAMP [4,8,29]. Therefore, *ras2* mutants maintain very low levels of cAMP and this results in the hyperaccumulation of glycogen [5].

Glycogen synthase and glycogen phosphorylase, the two key enzymes in the control of biosynthesis and degradation of glycogen, respectively, are regulated by phosphorylation and dephosphorylation mechanisms [11–16]. There is also evidence that, in yeast, the activity

of these two enzymes is regulated by a mechanism involving phosphorylation by a cAMP-dependent protein kinase [17,18]. Therefore, it seemed logical to assume that in *ras2* mutants the activity of these enzymes would be altered in such a way that would explain the observed hyperaccumulation of glycogen.

Our results confirm that *ras2* mutants hyperaccumulate glycogen [5]. At the beginning of the stationary phase, the mutant strain had glycogen levels ten times higher than its isogenic strain. In the normal strain, glycogen accumulation started when the concentration of glucose in the medium had decreased by one half. This had been observed previously in other strains [30,31]. It is worth noting that in the *ras2* mutant glycogen initiated its accumulation earlier, before cells had consumed 50% of initial glucose. This suggests a possible alteration in the glucose signalling mechanism in this mutant.

Major differences in the activity of glycogen metabolizing enzymes between these two strains have been observed. Theoretically, glycogen phosphorylase should have been inactivated in a strain with a failure in the adenylate cyclase activity, and therefore, with low cAMP levels and with a low degree of protein phosphorylation. However, the active form of the glycogen degrading enzyme was between 5 and 40 times higher in the *ras2* mutant than in the isogenic strain. This was highly unexpected since phosphorylase is not supposed to be very active in a strain that hyperaccumulates glycogen. In this context it is worth noting that a similar situation has been observed recently in mutants carrying a disruption in the *SIT4* gene, which in comparison with isogenic strains also present higher phosphorylase *a* activity [32] and hyperaccumulate glycogen (Clotet, J., unpublished results). Point mutations in the *SIT4* gene also induce hyperaccumulation of glycogen [33].

On the other hand, total glycogen synthase activity was between 2.5 and 1.3 times higher in the mutant than in the wild-type strain. Moreover, when the activation state of glycogen synthase is taken into account, a completely different pattern is observed. In the normal strain, glycogen synthase activity ratio, which was high at the beginning and steadily decreased along the exponential phase. In the mutant strain we observed the opposite behaviour: glycogen activity ratio rose continuously, reaching values close to 0.85 at the end of the stationary phase. This is a remarkably high value and corresponds to a fully activated enzyme. This finding can be perfectly explained by the decrease in cAMP-dependent phosphorylation activity consequence of the *ras2* mutation. The observed increase in the total amount of glycogen synthase and especially in its activation state may explain the hyperaccumulation of glycogen observed in the *ras2* mutants. However, since glycogen phosphorylase is found to be highly active in the mutant strain we have to postulate that this enzyme ought to be inhibited in vivo, so that a futile cycle does not operate. An obvious candidate for such inhibition is glucose 6-P, since this compound is able to inhibit glycogen phosphorylase, in addition to activating glycogen synthase.

Fructose-2,6-P_2 concentrations were higher (about double) in *ras2* mutants than in wild-type cells. This is not in accordance with the accepted activation of PFK-2 by a cAMP-dependent protein kinase in yeast cells [34]. Nevertheless, we have to mention that in other cases discrepancies between cAMP and PFK-2 activity have been observed [35], suggesting that determinations other than cAMP-dependent protein kinase are able to regulate PFK-2 [35–37].

Two general conclusions might be derived from our results: (1) cAMP may be involved in the expression of glycogen synthase and glycogen phosphorylase in yeast cells, since the specific activity of these two enzymes is concomitantly elevated in *ras2* mutants; however, only in the case of glycogen synthase the activation state of the enzyme is in accordance to the low cAMP levels characteristic of *ras2* mutants; and (2) glycogen synthesis in yeast may occur in the presence of elevated levels of phosphorylase *a* under conditions where glycogen synthase is also highly active. The unique behaviour of glycogen synthase in *ras2* mutants, of which the activation state raises along with the culture attaining full activation, may explain the hyperaccumulation of glycogen in these mutants.

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