Mitochondrial Respiratory Mutants in Yeast Inhibit Glycogen Accumulation by Blocking Activation of Glycogen Synthase*

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Control of glycogen synthase activity by protein phosphorylation is important for regulating the synthesis of glycogen. In this report, we describe a regulatory linkage between the ability of yeast cells to respire and activation of glycogen synthase. Strains containing respiration-deficient mutations in genes such as COQ3, required for the synthesis of coenzyme Q, were reduced in their ability to accumulate glycogen in response to limiting glucose. This lowered glycogen accumulation results from inactivation of the rate-determining enzyme, glycogen synthase (Gsy2p). Reduced glycogen synthase activity is coincident with lowered glucose 6-phosphate and ATP levels in the respiration-deficient cells deprived of glucose. Alanine substitutions of three previously characterized phosphorylation sites in Gsy2p, Ser-650, Ser-654, or Thr-667, each suppressed the glycogen defect in cells unable to respire, suggesting that inactivation of this enzyme is mediated by phosphorylation of these residues. Inactivation of glycogen synthase requires the RAS signaling pathway that controls cAMP-dependent protein kinase and is independent of Pho85p previously identified as a Gsy2p kinase. These results suggest that yeast cells unable to shift from a fermentative to a respiratory metabolic regimen block accumulation of glycogen by inactivating Gsy2p through protein phosphorylation.

Expression of many metabolic pathways in yeast is regulated by the availability of nutrients in the growth medium. A well studied example is the coordinate regulation of genes involved in the utilization and storage of sugars (1). Glucose is the preferred carbon source of yeast, and, even when grown aerobically, this sugar is fermented to ethanol and CO₂. Expression of many proteins that are not essential during fermentation is repressed by glucose (1). This repression is released when glucose is depleted from the culture medium, allowing for the utilization of alternative carbon sources requiring respiration. An alternative fate of glucose in yeast is accumulation in the form of the storage carbohydrate glycogen (1–3). Accumulation of glycogen is enhanced in cells limited for any one of several nutrients including nitrogen, phosphorous, sulfur, and carbon. This stored glycogen is degraded during resumption of vegetative growth from stationary phase or during spor germination. Accumulation and degradation of glycogen is controlled by the activities of glycogen synthase and glycogen phosphorylase.

Glycogen synthase in yeast is encoded by two genes, GSY1 and GSY2 (4, 5). GSY2 encodes the major nutritionally regulated form of glycogen synthase and accounts for about 90% of the total enzymatic activity in cells entering stationary phase (5). Two kinds of control ensure that there are appropriate levels of Gsy2p activity in cells responding to nutrient limiting conditions. First, GSY2 transcription is increased as cells deplete glucose from their growth medium (6). Release of this glucose repression is mediated by the cAMP pathway, which, in yeast, is regulated by Ras1p and Ras2p (6–14). A second level of control involves covalent phosphorylation of the carboxyl terminus of Gsy2p, which converts the enzyme to a less active form (15). Glucose 6-phosphate (glucose-6-P) functions as an allosteric activator that can overcome the inactivation of Gsy2p caused by phosphorylation. Thus, the ratio of activity measured in the absence of glucose-6-P to that in the presence of glucose-6-P (-/+ glucose-6-P activity ratio) is often used as a measure of the activation state of the enzyme.

The protein kinases Snf1p and Pho85p have been implicated in the regulation of Gsy2p activity by protein phosphorylation (6, 16–19). Snf1p facilitates expression of many glucose-repressible enzymes and is required for full activity of Gsy2p in cells entering stationary phase (1, 6). Maintenance of Gsy2p activity by Snf1p is thought to be mediated by antagonism of the protein kinase Pho85p (18, 20). Biochemical studies suggest that Pho85p directly phosphorylates Gsy2p at Ser-654 and/or Thr-667 to reduce the enzyme activity. Consistent with these in vitro observations, deletion of PHO85 from cells lowers phosphorylation of Gsy2p as judged by the -/+ glucose-6-P activity ratio. However, Gsy2p continues to be significantly phosphorylated in pho85 mutant cells grown to early stationary phase, suggesting that there is at least one other protein kinase that can reduce glycogen synthase activity by phosphorylation (18, 20). One candidate is cAMP-dependent protein kinase (cAPK), although it is currently uncertain whether this occurs directly or by the function of additional kinases (15, 21).

We report here that there is a regulatory linkage between the ability of yeast cells to respire and the synthesis of glycogen. Strains containing respiration-deficient mutations in genes such as COQ3, required for the synthesis of coenzyme Q, were reduced in their ability to accumulate glycogen in response to depletion of glucose in the culture medium. Our results strongly suggest that this lowered glycogen accumulation is the direct result of hyperphosphorylation of Gsy2p, leading to a block in the activation of this rate-determining enzyme. This inactivation of Gsy2p requires the Ras2p signaling pathway that controls cAPK and is independent of Pho85p kinase.

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1 The abbreviations used are: glucose-6-P, glucose 6-phosphate; cAPK, cAMP-dependent protein kinase; kb, kilobase pair(s).
### MATERIALS AND METHODS

**Yeast Strains and Plasmds—**Strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1. The COQ3 deletions in strains RY52, RY53, and RY71 were constructed by replacing the entire COQ3 coding sequence with *LEU2* or *TRP1*. The *COQ3-URA3* disruptions were introduced into strains JC452 and EG328–1A by transforming a 3.3-kb *Bgl*II restriction fragment derived from plasmid pY9 and selecting for ura3 prototrophy. GSY2, GSY2-S654A, GSY2-S660A, GSY2-S685A, GSY2-T663A, and GSY2-T673A were introduced into strain RY10. A 2.6-kb *BanHI* restriction fragment encoding the COQ3 gene was removed from pRY7 and inserted into pRS315, generating pRY10, and this plasmid was shown to restore glycogen accumulation in strain RY10. The gap repair method was used to obtain the COQ3 sequences disrupted by the *URA3*-marked transposon from strain RY10 (27). A 5.5-kb *NruI* and *NsiI* DNA fragment was removed from plasmid pRY7, and the remaining portion of the plasmid DNA was introduced into strain RY10. After selecting for *Leu*+ transformants, a plasmid designated pRY9 was rescued from the RY10 strain derivative.

**Measurement of Glycogen—**Glycogen accumulation was measured qualitatively by inverting agar plates containing yeast cells over iodine crystals (28, 29). Yeasts develop a brown color proportional to their levels of glycogen. Glycogen-deficient strains are yellow. A quantitative measure of glycogen levels was determined as described previously (15). Glycogen was digested with α-amylase and amyloglucosidase, and released glucose was measured as described (30) and reported as mg of glucose released per 100 mg of total protein.

**Measurement of Glycogen Synthase—**Glycogen synthase was assayed as described by Hardy and Roach (15). Where indicated, extracts were first passed over a Sephadex G25 spin column to remove low molecular weight compounds (31). A unit of glycogen synthase activity is defined as the amount of enzyme that catalyzes 1 μmol of glucose from UDP-glucose to glycogen/min/mg of total protein. Total activity of glycogen synthase is measured in the presence of 7.2 mM glucose 6-phosphate. The *−/−* glucose-6-P activity ratio is defined as the glycogen synthase activity measured in the absence of glucose-6-P divided by the activity measured in the presence of glucose-6-P. Each reported measurement was the average of three independent experiments.

**Assay for Glucose 6-Phosphate, ATP, ADP, AMP, cAMP, and cAPK—**Cells were grown to the indicated phase in YPD medium and collected by filtration, followed by rapid freezing in liquid nitrogen. Determination of glucose-6-P was carried out as described (32). To measure ATP, ADP, and AMP, cell extracts were prepared as described for the glucose 6-P assay. ATP was measured using hexokinase (33). ADP and AMP levels were quantitated using pyruvate kinase and myokinase as described by M. Goebl (23). ATP and ADP are expressed in pmol per mg of protein.

### RESULTS

**Mutants Defective for Respiration Reduce Glycogen Accumulation—**As part of a screen for genes required for glycogen
Deficiency was the result of a biosynthetic pathway (24). We confirmed that the glycogen alcalizes the fourth and rate-limiting step in the coenzyme Q dihydroxy-5-hexaprenylbenzoate methyltransferase that catalyzes a brown color after iodine staining (Fig. 1). We added a 2.6-kb sresively smaller DNA sequences were tested for complementation, and the entire coding sequence of the gene. The coq3 gene is strain JC482, coq3 is RY33, and coq3 + pCOQ3 is RY33 containing plasmid pRY10 encoding COQ3.

Inactivation of Gsy2p in Respiration-deficient Cells

Inactivation of glycogen synthase in the coq3::URA3 strain suggests that increased phosphorylation of Gsy2p lowers the activity of this biosynthetic enzyme, thus leading to reduced levels of glycogen. Hardy and Roach (15) showed that deletion of the carboxyl terminus of Gsy2p blocked in vivo phosphorylation of glycogen synthase and increased the activity ratio. We addressed whether the expression of GSY2::Δ463, deleted for 61 carboxyl-terminal residues, could restore glycogen accumulation in a coq3 strain (Fig. 3). When GSY2::Δ463 was expressed from the GSY2 promoter or the heterologous ADC1 promoter, which is not subject to glucose repression, we found accumulation of glycogen in the coq3::URA3 strain. In contrast, we found reduced levels of glycogen in coq3::URA3 cells as measured by iodine staining when we similarly expressed a full-length version of Gsy2p. Consistent with earlier observations (15), we also found that expression of GSY2::Δ463 in coq3::URA3 cells using the GSY2 promoter resulted in an elevated activity ratio compared with the full-length GSY2 (0.55 versus 0.09). These results support the idea that reduced activation of glycogen synthase in the respiration-deficient strains results from increased phosphorylation of residues in the carboxyl terminus of Gsy2p. The total activity of glycogen synthase was similar in the coq3::URA3 and COQ3 cells (Table II), indicating that there was no significant difference in GSY2 expression. Furthermore, these experiments indicate that the coq3 cells contain sufficient glucose and energy stores to synthesize glycogen provided an activated form of Gsy2p is present.

GSY2p Residues Ser-650, Ser-654, and Thr-667 Are Required to Block Activation of Glycogen Synthase in coq3 Mutant Cells—Previously it was found that three carboxyl-terminal residues in Gsy2p, Ser-650, Ser-654, and Thr-667, were probable regulatory phosphorylation sites (15). Alanine substitutions at each of these sites resulted in an increased glycogen synthase activity ratio and elevated glycogen accumulation in cells grown to late exponential phase. To determine whether these residues were required for inactivation of glycogen synthase in respiration-deficient cells, we compared glycogen accumulation in a coq3::LEU2 strain expressing different GSY2 alleles encoding carboxyl-terminal residue substitutions or deletions (Fig. 4). Each GSY2 allele was expressed from the TRPI-marked low copy number plasmid pRS314 in isogenic strains DH2 (gsy2::URA3) and RY53 (gsy2::URA3 coq3::LEU2). As judged by iodine staining, each of the GSY2 alleles complemented the gsy2 defect in DH2 cells, indicating that each Gsy2p mutant protein was functionally expressed (data not shown). Consistent with our earlier observation, expression of GSY2::Δ463 suppressed the glycogen deficiency in the coq3::LEU2 mutant (Fig. 4B). By comparison, no glycogen accumulation was found in coq3::LEU2 cells expressing the shorter deletion GST2::Δ691, and low, but detectable levels, of glycogen were found in GST2::Δ656 cells. These results suggest that the sequences between residues 643 and 691 directly participate in the inactivation of Gsy2p in response to a failure to respire, with sequences carboxyl-terminal to residue 691 performing no regulatory role.

GSY2 alleles containing alanine substituted for each individual serine or threonine between residues 650 and 704 were next assessed for glycogen content in the coq3::LEU2 strain.
Iodine indicates cells grown on SD medium and stained with iodine for 2 days at 30 °C on agar plates containing glucose (SD), and on glycerol containing plates (YPG). Iodine staining was darker brown than cells expressing mutant Gsy2p containing alanine substitutions at Ser-650, Ser-654, or Thr-667, is required for full inactivation of Gsy2p in response to respiration deficiency. Together, these results suggest that phosphorylation of three residues, Ser-650, Ser-654, and Thr-667, is required for full inactivation of Gsy2p in response to respiration deficiency.

Inactivation of Glycogen Synthase in Respiration-deficient Cells

Glycogen levels were also reduced in strain RY53 (coq3::URA3) expressing Gsy2p deleted at the carboxyl terminus can accumulate glycogen. Strain RY53 (gsy2::URA3 coq3::LEU2) expressing Gsy2p or Gsy2Δ643 from the ADCl or GSY2 promoters was grown for 2 days at 30 °C on agar plates containing SD medium. In the Iodine panel cells are exposed to iodine vapor to assess glycogen content. Vector indicates strain RY53 that contains only parent plasmid pYCDE2 or pRS314 and does not express GSY2p.

Fig. 3. Respiration-deficient cells expressing Gsy2p deleted at the carboxyl terminus can accumulate glycogen. Strain RY53 (gsy2::URA3 coq3::LEU2) expressing Gsy2p or GSY2Δ643 from the ADCl or GSY2 promoters was grown for 2 days at 30 °C on agar plates containing SD medium. In the Iodine panel cells are exposed to iodine vapor to assess glycogen content. Vector indicates strain RY53 that contains only parent plasmid pYCDE2 or pRS314 and does not express GSY2p.

Fig. 2. Glycogen does not accumulate in different respiration-deficient strains. Yeast strains were grown for 2 days at 30 °C on agar plates containing glucose (SD), and on glycerol containing plates (YPG). Glycogen synthase activity is blocked in coq3 mutant cells compared with isogenic wild-type strain EG328–1A. The activity ratio of glycogen synthase increased in the coq3 strain from lag phase to the second growth phase. In the coq3::URA3 strain grown to lag phase, the activity ratio was slightly reduced compared with wild-type cells. However, 14 h later when COQ3 cells were in the second growth phase, the respiration-deficient mutant did not resume growth and displayed an even greater reduction in the glycogen synthase activity ratio (Fig. 5B). The activation state of glycogen synthase in the coq3::URA3 mutant was 9 times lower than that measured in wild-type cells.

We also monitored the levels of glucose-6-P and ATP in the coq3::URA3 and COQ3 strains during each of the growth phases (Fig. 5, C and D). As wild-type COQ3 cells became deprived of glucose and entered the lag phase, the level of glucose-6-P was reduced by 50%, and this level was maintained as the cells entered the second growth phase. The ATP levels in the COQ3 strain were also modestly reduced during this transition from fermentative growth to oxidative phosphorylation. Coincident with this reduction, ADP levels were increased about 2-fold. By comparison, the glucose-6-P levels were reduced over 6-fold when coq3::URA3 cells entered the lag phase. The failure of the coq3::URA3 strain to shift to a respiratory regimen was accompanied by a dramatic lowering of the ATP levels.

Table II

| Relevant genotype | Glycogen synthase activity^a |
|------------------|-----------------------------|
|                  | Glycogen levels^b | Total activity (+G-6-P) | Activated (-G-6-P) | Glycogen synthase activity ratio^c |
| COQ3             | 120 ± 11           | 34 ± 0.2                 | 23 ± 0.8           | 0.69 ± 0.02                           |
| coq3::URA3       | 20 ± 1.4           | 41 ± 2.4                 | 3.2 ± 0.5          | 0.08 ± 0.01                           |

^a Relevant genotypes of strain JC482 (COQ3) and RY33 (coq3::URA3) are indicated. Glycogen accumulation was also reduced in strain RY52 (coq3::TRP1) compared with isogenic wild-type strain EG328–1A.

^b Glycogen was measured as mg glucose released/100 mg of total protein. Results presented are from three independent experiments.

^c Glycogen synthase was measured as milliliters/mg of total protein in the presence or absence of glucose 6-phosphate (G-6-P).

^d Ratio of glycogen synthase activity in the absence of glucose-6-P to that measured in the presence of the sugar phosphate.

Wild-type

| Strain | SD | Iodine | YPG |
|--------|----|--------|-----|
| coq3   |    |        |     |
| coq4   |    |        |     |
| coq6   |    |        |     |
| coq8   |    |        |     |
| mip1   |    |        |     |

Wild-type

| Strain | SD | Iodine | YPG |
|--------|----|--------|-----|
| atp2   |    |        |     |
| cor1   |    |        |     |
| coq3   |    |        |     |

Vector

P_{ADC1} - GSY2

P_{ADC1} - GSY2Δ643

P_{GSY2} - GSY2

P_{GSY2} - GSY2Δ643

Iodine

Increased over 5-fold from late exponential to lag phase and that this enzyme level was retained in the second growth phase (Fig. 5). As noted earlier, the levels of total activity were similar in the coq3::URA3 and COQ3 strains (Table II and Fig. 5A). The activity ratio of glycogen synthase increased in the COQ3 strain from lag phase to the second growth phase. In the coq3::URA3 strain grown to lag phase, the activity ratio was slightly reduced compared with wild-type cells. However, 14 h later when COQ3 cells were in the second growth phase, the respiration-deficient mutant did not resume growth and displayed an even greater reduction in the glycogen synthase activity ratio (Fig. 5B). The activation state of glycogen synthase in the coq3::URA3 mutant was 9 times lower than that measured in wild-type cells.

We also monitored the levels of glucose-6-P and ATP in the coq3::URA3 and COQ3 strains during each of the growth phases (Fig. 5, C and D). As wild-type COQ3 cells became deprived of glucose and entered the lag phase, the level of glucose-6-P was reduced by 50%, and this level was maintained as the cells entered the second growth phase. The ATP levels in the COQ3 strain were also modestly reduced during this transition from fermentative growth to oxidative phosphorylation. Coincident with this reduction, ADP levels were increased about 2-fold. By comparison, the glucose-6-P levels were reduced over 6-fold when coq3::URA3 cells entered the lag phase. The failure of the coq3::URA3 strain to shift to a respiratory regimen was accompanied by a dramatic lowering of the ATP levels.
concentration and increased ADP levels in the cell. At a later time, when wild-type cells entered the second growth phase, ATP levels in the respiration-deficient mutant continued to be low and AMP began to accumulate.

Since glucose-6-P is a potent activator of glycogen synthase, it was possible that the observed increase in the glycogen synthase activity ratio in the COQ3 strain compared with respiration-defective cells was due to endogenous glucose-6-P from the cell lysate being carried over into the glycogen synthase assay. We therefore treated cell lysates with a Sephadex G25 spin column to remove endogenous glucose-6-P prior to the assay. The 1/6 glucose-6-P activity ratios were unchanged from that reported in Fig. 5B. Thus, the intrinsic activity, and by inference the phosphorylation state, of glycogen synthase is altered in the coq3::URA3 mutant.

Reduction of Glycogen Accumulation in Respiration-deficient Cells Is Independent of the Glycogen Synthase Kinase Pho85p—Pho85p is a cyclin-dependent protein kinase that directly phosphorylates Gsy2p to reduce glycogen synthase activity (18). Two approaches were used to address whether inactivation of glycogen synthase in a coq3 strain is mediated by Pho85p. In the first experiment, we mated strain RY75 (MATα trp1 ura3–52 coq3::TRP1) with RY26 (MATα trp1 ura3–52 pho85::URA3). The resulting diploid strain was sporulated, and 21 tetrads were analyzed. Of 17 Trp+ Ura+ spores identified, all displayed the glycogen-deficient phenotype (Glc−) as judged by iodine staining and found them all to display a Glc− phenotype, indicating that PHO85 function is not required to block glycogen accumulation in coq3 cells.

To confirm whether Pho85p kinase functions to inactivate glycogen synthase in respiration-deficient cells, we deleted PHO85 and COQ3 independently or in combination in strain EG328–1A (Fig. 6). As previously reported, both the pho85::URA3 and coq3::TRP1 mutants were unable to grow in media containing glycerol (19). However, pho85::URA3 cells were able to grow poorly in ethanol-containing media, indicating that the respiratory pathway is at least in part functional in the absence of this kinase. Consistent with our earlier analysis,

**Fig. 4.** Alanine substitutions of Gsy2p residues Ser-650, Ser-654, and Thr-667 suppress the glycogen deficiency in coq3 mutant cells. A, sequence of the carboxy terminus of Gsy2p from amino acid residue 640 to the terminal residue 704. Altered serine and threonine residues are indicated in larger letters, and underlined residues are those that individually suppress the glycogen defect in coq3 mutants. Sequences deleted in alleles, GSY2-Δ643, GSY2-Δ656, and GSY2-Δ691 are indicated. B, strain RY53 (gsy2::URA3 coq3::LEU2) expressing the indicated GSY2 alleles was grown for 2 days at 30 °C on SD agar plates and exposed to iodine vapor to determine glycogen accumulation. Vector indicates strain RY53 containing only pRS314 used to express each GSY2 allele.

**Fig. 5.** Inactivation of glycogen synthase in coq3 mutants cells is accompanied by reductions in glucose-6-P and ATP levels. Strain JC482 encoding wild-type COQ3 (open bars) was grown to the indicated phase and assayed for glycogen synthase activity or the indicated metabolite levels. Cultures of coq3::URA3 strain RY33 (solid bars) were synchronized with JC482, and cells were harvested and analyzed at the identical time points as this COQ3 strain. The coq3::URA3 mutant does not resume the second growth phase because of the inability to respire, and the results listed under this phase are from the same time point when the COQ3 cells were in the second growth. Units for total glycogen synthase (GS) activity and glucose-6-P (G-6-P), ATP, ADP, and AMP levels are indicated on the right side of each panel. Glycogen synthase activity ratio is the activity measured in the absence of glucose-6-P divided by that measured in the presence of glucose-6-P. ND, not determined.
glycogen levels were similarly reduced in both the coq3 and pho85 coq3 mutant strains. We conclude that inactivation of glycogen synthase in respiration-deficient cells is independent of the protein kinase Pho85p.

Mutants in cAPK Pathway Suppress Glycogen Defect in Respiration-deficient Cells—cAPK plays an important role in the regulation of glycogen synthase in response to starvation (6–13). The cAMP levels in yeast are controlled by adenylate cyclase, whose activity is modulated by Ras1p and Ras2p, GTP-binding proteins that are homologs of the mammalian Ras oncogene. To address whether reduction in the activity of cAPK can suppress the glycogen defect in coq3 cells, we introduced coq3::URA3 into strain LRA24 containing a temperature-sensitive mutant allele of CDC25 that mediates the guanine nucleotide exchange for Ras1p and Ras2p (35–37). The cdc25–10 allele suppressed the glycogen defect associated with the coq3::URA3 mutant when grown at the semipermissive temperature, 30 °C, with the cdc25–10 coq3::URA3 cells accumulating glycogen similar to wild-type COQ3 cells as judged by iodine staining (Fig. 7).

To determine whether the cdc25–10 suppression of the glycogen deficiency in coq3::URA3 mutant cells was linked to glycogen synthase, we measured the $\frac{-/+}{+/}$ glucose-6-P activity ratio of this enzyme. While combining cdc25–10 with coq3::URA3 had little impact on the total activity of glycogen synthase, the glycogen synthase activity ratio was elevated about 10-fold compared with the coq3 strain (Fig. 8). These results suggest that reduced activity of cAPK can suppress the glycogen deficiency found in coq3 mutant strains by lowering the inhibitory phosphorylation of Gsy2p. To further test this premise, coq3::URA3 was introduced into strain LRA86 containing the cdc35–13 allele. cAMP is synthesized by the CDC35 (CYR1)-encoded adenylate cyclase (38, 39), and the cdc35–13 allele is temperature-sensitive with a partially defective phenotype (40). We found that the resulting strain, RY135 (coq3::URA3 cdc35–13), accumulated glycogen as judged by iodine staining and exhibited an elevated glycogen synthase activity ratio as compared with the isogenic coq3::URA3 strain RY33 (0.39 versus 0.16).

Our genetic analyses indicates the RAS-cAMP pathway is required for inactivation of Gsy2p in respiration-deficient cells. In the simplest model, increased cAPK activity during respiration deficiency would result in hyperphosphorylation of Gsy2p, inactivating its enzymatic function. However, given that three different residues, Ser-650, Ser-654, and Thr-667, facilitate the block in Gsy2p function, we presume that one or more additional protein kinases are required for full inactivation of Gsy2p. In this combinatorial model, the activities of only a portion of the individual protein kinases and phosphatases that modulate Gsy2p function may be altered by the respiration-deficient conditions, while others may remain relatively unchanged. To directly address whether the cAMP pathway is induced under the respiration-deficient conditions, we measured cAMP and cAPK activity in our strains. Consistent with earlier studies (41, 42), we found cAMP levels were reduced about 50%, from 14 to 6 pmol/A600, as wild-type cells made the transition from exponential growth to lag phase and the lower levels were retained in the second growth phase. Previously, it was reported that there was a modest reduction in the activity of adenylate cyclase in cdc25–10 cells grown at the permissive temperature (40). However, we found no reproducible difference in cAMP levels among wild-type, coq3::URA3, cdc25–10, and coq3::URA3 cdc25–10 cells grown in YPD medium at the semipermissive temperature, 30 °C, in which our glycogen studies were carried out. Given that cAMP changes can be transitory, we cannot rule out that there is a short term difference between these cells. Measurements of cAPK activity indi-
Inactivation of Gsy2p in Respiration-deficient Cells

Coordinate Control of Glycogen Accumulation and Respiratory Function—As glucose is depleted from the media, yeast cells begin to accumulate glycogen and shift to a respiratory pathway. During this metabolic transition, there is increased expression of Gsy2p along with many genes required for respiration (1, 25) (Fig. 5A). Subsequent to elevated Gsy2p levels, this enzyme is dephosphorylated, leading to its activation and the accumulation of glycogen (Fig. 5B) (15, 18). In cells unable to respire, such as coq3 mutants, depletion of glucose from the medium is accompanied by a dramatic reduction in glucose-6-P and ATP levels (Fig. 5). Under these conditions, respiration-deficient cells do not transfer their limited energy reserves into the synthesis of glycogen. This metabolic decision is due to inactivation of Gsy2p by protein phosphorylation involving the cAMP-pathway (Figs. 7 and 8).

The introduction of cdc25 or cdc35 mutant alleles in coq3 cells led to modest reductions in cAPK activity accompanied with an increased glycogen synthase activity ratio. This indicates that the cAMP-mediated pathway is required for the inhibition of glycogen accumulation in coq3 cells. However, we did not find any significant difference in the levels of cAMP or cAPK activity between wild-type and respiration-deficient cells, suggesting that cAPK works in conjunction with other regulatory pathways. Three different phosphorylation sites in Gsy2p are thought to mediate inactivation of this enzyme, and only Ser-650 shares a minimal resemblance with the substrate recognition sequence, RRX5, required for yeast cAPK (34). Regulatory sites Ser-654 and Thr-667 contain the flanking sequences (S/T)PXDXL recognized by cyclin-dependent kinases, including Pho85p (18) (Fig. 4A). Our genetic studies clearly show that Pho85p does not participate in the inactivation of Gsy2p in respiration-deficient cells (Fig. 6), suggesting that one or more additional protein kinases facilitate the block in Gsy2p function in coq3 cells. The activities of these additional protein kinase and/or protein phosphatases may be modulated by the metabolic changes occurring during the respiration-deficient conditions. A final cautionary note is that during the extended time course we measured cAMP levels, we cannot rule out the possibility that a short term change in its levels occurred in the respiration-deficient cells exiting exponential growth. As an illustration of this point, Francois et al. (43) found that the addition of dinitrophenol to yeast cells, which disrupts coupling of electron transport and ATP synthesis in mitochondria, sharply increased the concentration of cAMP concomitant with a reduction in the activity of glycogen synthase. Only 10 min after the addition of the uncoupling agent, cAMP in the cells returned to its original levels.

Phosphorylation of Glycogen Synthase Is Regulated by Multiple Pathways—Several different physiological conditions have been described that stimulate or inactivate glycogen synthase. First, glycogen synthesis in yeast cells is increased in response to limitation for carbon, nitrogen, phosphorus, or sulfur (1–3). In response to glucose deprivation, signaling pathways involving cAPK and the Snf1p and Pho85p kinases control the activation state of Gsy2p through phosphorylation of three regulatory residues, Ser-650, Ser-654, and Thr-667. Although it is currently unknown whether these same kinases and phosphorylation sites participate in Gsy2p activation in response to other nutrient limitations, it is presumed that there is some regulatory overlap.

While starvation for different nutrients increases the synthesis of glycogen, there are two physiological conditions that inhibit glycogen synthase activity. First, Gsy2p is inactivated in respiration-deficient cells. This inhibition also involves cAPK and the Gsy2p regulatory sites, Ser-650, Ser-654, and...
Thr-667, but is independent of Pho85p. A second condition observed to inhibit glycogen synthase is treatment of MATa cells with the mating pheromone α-factor. The α-factor leads to G1 arrest of MATa cells and blocks accumulation of glycogen by inactivation of glycogen synthase (44). Results from genetic studies demonstrated that inhibition of glycogen synthase is inactivation of glycogen synthase (44). Reduced glycogen accumulation in response to mating pheromone was independent of capK. Currently it is not known whether Pho85p kinase mediates α-factor inhibition of glycogen accumulation in MATa cells.

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REFERENCES
1. Johnston, M., and Carlson, M. (1992) in The Molecular and Cellular Biology of the Yeast Saccharomyces (Broach, J. R., Pringle, J. R., and Jones, E. W., eds) Vol. 2, pp. 193–292, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Francois, J.-M., Blazquez, J., Arino, J., and Gancedo, C. (1997) in Yeast Sugar Metabolism: Biochemistry, Genetics, Biotechnology (Zimmermann, F. K., ed) pp. 285–311, Technomics, Lancaster, PA
3. Lillie, S. H., and Pringle, J. R. (1980) J. Bacteriol. 143, 1384–1394
4. Farkas, I., Hardy, T. A., and Roach, P. J. (1990) J. Biol. Chem. 265, 20879–20886
5. Farkas, I., Harday, T. A., Goebl, M. G., and Roach, P. J. (1991) J. Biol. Chem. 266, 15602–15607
6. Hardy, T. A., Huang, D., and Roach, P. J. (1991) J. Biol. Chem. 269, 27909–27913
7. Toda, T., Uno, I., Ishikawa, T., Powers, S., Katoaka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985) Cell 40, 27–36
8. Tatchell, K., Robinson, L. C., and Breitenbach, M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3785–3789
9. Broek, D., Samiy, N., Pasano, O., Fujiyama, A., Tamanoi, F., Northup, J., and Wigler, M. (1985) Cell 41, 763–769
10. Cannon, J. F., and Tatchell, K. (1987) Mol. Cell. Biol. 7, 2651–2663
11. Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987) Cell 50, 277–287
12. Deschenes, R. J., and Broach, J. R. (1989) Adv. Cancer Res. 54, 79–138
13. Gibbs, J. B., and Marshall, M. S. (1989) Microbiol. Rev. 53, 171–185
14. Cameron, S., Levin, S., Zoller, M., and Wigler, M. (1988) Cell 33, 555–566
15. Hardy, T. A., and Roach, P. J. (1993) J. Biol. Chem. 268, 23799–23805
16. Thompson-Jeager, S., Francois, J., Gaughran, J. P., and Tatchell, K. (1991) Genetics 129, 697–706
17. Cannon, J. F., Pringle, J. R., Fiechter, A., and Khalil, M. (1994) Genetics 136, 485–503
18. Huang, D., Farkas, I., and Roach, P. J. (1990) Mol. Cell. Biol. 10, 4357–4365
19. Timblin, H. K., Tatchell, K., and Bergman, L. W. (1990) Genetics 153, 57–66
20. Huang, D., Moffat, J., Wilson, W. A., Moore, L., Cheng, C., Roach, P. J., and Andrews, B. (1998) Mol. Cell. Biol. 18, 3289–3299
21. Peng, Z. Y., Trumbly, R. J., and Reimann, E. M. (1990) J. Biol. Chem. 265, 13571–13577
22. Hadfield, C., Cashmore, A. M., and Meacock, P. A. (1986) Gene (Amst.) 45, 149–158
23. Kaiser, C., Michaelis, S., and Mitchell, A. (1994) Methods in Yeast Genetics, pp. 207–217, Cold Spring Harbor Laboratory Press, Plainview, NY
24. Clarke, C. F., Williams, W., and Teruya, J. H. (1991) J. Biol. Chem. 266, 16636–16644
25. Tzagoloff, A., and Dieckmann, C. L. (1990) Microbiol. Rev. 54, 211–225
26. Chun, K. T., and Goebl, M. G. (1991) Methods in Enzymol. 194, 281–301
27. Rothstein, R. (1991) in Methods Enzymol., pp. 39–50
28. Adams, H. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) pp. 539–543 and 573–577, Verlag Chemie Weinheim Academic Press, Inc., New York
29. Huang, D., Wilson, W. A., and Roach, P. J. (1997) J. Biol. Chem. 272, 22495–22501
30. Wilson, W. A., Hawley, S. A., and Hardie, G. D. (1996) Curr. Biol. 6, 1426–1434
31. Estabrook, R. W., Williamson, J. R., Frenkel, R., and Maitra, P. K. (1967) Methods Enzymol. 10, 474–482
32. Deris, C. L., Kemp, B. E., and Zoller, M. J. (1996) J. Biol. Chem. 266, 17932–17935
33. Robinson, L. C., Gibbs, J. B., Marshall, M. S., Sigal, I. S., and Tatchell, K. (1987) Science 235, 1218–1221
34. Brooks, D., Toda, T., Micault, L., Levin, L., Birnmeier, C., Zoller, M., Powers, S., and Wigler, M. (1987) Cell 48, 789–799
35. Jones, S., Vignais, M. L., and Broach, J. R. (1991) Mol. Cell. Biol. 11, 2641–2646
36. Matsumoto, K., Uno, I., and Ishikawa, T. (1984) J. Bacteriol. 157, 277–282
37. Kataoka, T., Broek, D., and Wigler, M. (1985) Cell 43, 493–505
38. Pettiet, A., Hilger, F., and Tatchell, K. (1990) Genetics 124, 797–806
39. Russell, M., Bradshaw-Rouse, J., Markwardt, D., and Heideman, W. (1993) Mol. Biol. Cell 4, 757–765
40. Ma, P., Goncalves, T., Maretzek, A., Dias, M. C. L., and Thevelein, J. M. (1997) Mol. Cell. Biol. 17, 493–505
41. Papp, G., Gondre, T., Maretzek, A., Dias, M. C. L., and Thevelein, J. M. (1997) Microbiology 143, 3451–3459
42. Francois, J., Villanueva, M. E., and Hers, H.-G. (1988) Eur. J. Biochem. 174, 551–559
43. Francois, J., Higgins, D. L., Chang, F., and Tatchell, K. (1991) J. Biol. Chem. 266, 6174–6180
44. Do, T. Q., Schultz, J. R., and Clarke, C. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7534–7539
45. Tzagoloff, A., Wu, M., and Crivellone, M. (1986) J. Biol. Chem. 261, 17163–17169