Two Glycogen Synthase Isoforms in Saccharomyces cerevisiae Are Coded by Distinct Genes That Are Differentially Controlled*

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In previous work, we identified a Saccharomyces cerevisiae glycogen synthase gene, GSY1, which codes for an 85-kDa polypeptide present in purified yeast glycogen synthase (Farkas, I., Hardy, T. A., DePaoli-Roach, A. A., and Roach, P. J. (1990) J. Biol. Chem. 265, 20879-20886). We have now cloned another gene, GSY2, which encodes a second S. cerevisiae glycogen synthase. The GSY2 sequence predicts a protein of 704 residues, molecular weight 79,963, with 80% identity to the protein encoded by GSY1. Amino acid sequences obtained from a second polypeptide of 77 kDa present in yeast glycogen synthase preparations matched those predicted by GSY2. GSY1 resides on chromosome VI, and GSY2 is located on chromosome XII. Disruption of the GSY1 gene produced a strain retaining about 85% of wild type glycogen synthase activity at stationary phase, while disruption of the GSY2 gene yielded a strain with only about 10% of wild type enzyme activity. The level of glycogen synthase activity in yeast cells disrupted for GSY1 increased in stationary phase, whereas the activity remained at a constant low level in cells disrupted for GSY2. Disruption of both genes resulted in a viable haploid that totally lacked glycogen synthase activity and was defective in glycogen deposition. In conclusion, yeast expresses two forms of glycogen synthase with activity levels that behave differently in the growth cycle. The GSY2 gene product appears to be the predominant glycogen synthase with activity linked to nutrient depletion.

Glycogen synthase (EC 2.4.1.11) catalyzes the formation of α-1,4-glycosidic bonds in glycogen in a wide variety of species. Mammalian glycogen synthase is regulated by multisite phosphorylation and exists as at least two different isoforms in muscle and liver (1, 2). cDNAs encoding human muscle (3), rabbit muscle (4), and rat liver (5) glycogen synthases have been cloned and the corresponding amino acid sequences deduced. Glycogen synthase in Saccharomyces cerevisiae has not been as extensively characterized. The yeast enzyme is known to undergo reversible phosphorylation (6, 7), and some glycogen-deficient mutants have been shown to have increased activity of cAMP-dependent protein kinase due either to a defective regulatory subunit (8, 9) or to increased CAMP levels (10). Thus, the cAMP pathway is implicated in the regulation of glycogen metabolism. However, glycogen accumulation may also be regulated by mechanisms independent of cAMP (11, 12). Many details of the regulation remain to be established, and no glycogen-deficient mutant with defective glycogen synthase has been characterized so far.

Our yeast glycogen synthase preparations contained two polypeptides of M, 77,000 and 85,000 (13), in agreement with the results of Peng et al. (14) but differing from other reports of a single subunit type (15, 16). We recently cloned a glycogen synthase gene, GSY1, from S. cerevisiae that encodes the 85-kDa polypeptide. Disruption of GSY1 resulted in a viable haploid with significant residual glycogen synthase activity. Also, Southern hybridization of genomic DNA with a GSY1 probe revealed a second fragment hybridizing at low stringency (15). We therefore postulated the existence of a second glycogen synthase gene. Understanding the control of glycogen synthesis in yeast obviously requires characterization of both glycogen synthase genes. Here we report cloning of a second gene, GSY2, and analysis of mutants with one or both genes selectively disrupted.

EXPERIMENTAL PROCEDURES

Yeast Strains—Strains YPH52 (ura3-52 lys2-801<sup>med</sup> ade2-101<sup>med</sup> his3-D200 trpl-D1) (31) and YP were used for gene disruption experiments. YP was a diploid obtained by mating YPH52 and YPH54 (a ura3-52 lys2-801<sup>med</sup> ade2-101<sup>med</sup> his3-200 trpl-D1). Strains bearing mutations in the GSY genes were derived from YPH52 as described previously (13) and in the present paper: IF1 (a ura3-52 lys2-801<sup>med</sup> ade2-101<sup>med</sup> his3-D200 trpl-D1 gsy111::URA3) IF2 (a ura3-52 lys2-801<sup>med</sup> ade2-101<sup>med</sup> his3-D200 trpl-D1 gsy121::URA3), IF3 (a ura3-52 lys2-801<sup>med</sup> ade2-101<sup>med</sup> his3-D200 trpl-D1 gsy111::URA3 gsy211::URA3), IF4 (a/a ura3-52 ura3-52 lys2-801<sup>med</sup> trpl-D1 ade2-101<sup>med</sup> his3-D200 his3-D200 trpl-D1 trpl-D1 gsy111::URA3 gsy211::URA3 gsy221::URA3 his3-D200 his3-D200 gsy211::URA3). Growth of Yeast—Yeast were grown in YPD medium containing 1% Bacto-yeast extract (w/v), 2% peptone (Sigma) (w/v), and 2% dextrose (w/v) (18). To monitor the growth curve, 250-ml cultures (YPD) were inoculated with precultures grown to stationary phase and grown at 30 °C with shaking. The initial cell concentration was about 2 × 10<sup>7</sup> cells/ml. Growth was followed by measuring the turbidity of culture at 640 nm. Aliquots producing ~150 μl of pelleted cells were removed at various times for analysis. For determination of water-soluble and water-insoluble glycogen, with or without glutaraldehyde fixation (19), 50-ml cultures (YPD) were inoculated with...
cells from 5-ml saturated cultures and grown for 16 h. Two 20-ml aliquots of each culture were pelleted.

**Cloning of the GSY2 Gene—**Screening of a yeast genomic library constructed in EMBL3a and kindly supplied by Dr. Mike Snyder, Yale University (20), was performed with a GSY1 probe looking for differential hybridization at low versus high stringency. A 2.2-kb SpeI-Ndel fragment of GSY1 containing the coding region of the gene was labeled by random priming (U. S. Biochemical Corp. kit) using α-32PdCTP. Phage were introduced into Escherichia coli LE 392H. Plaques were transferred to duplicate nitrocellulose filters and hybridized with the probe in 6 × SSPE, 10 × Denhardt's solution, 0.1% SDS, sodium pyrophosphate at 58 or 68 °C (1 × SSPE is 0.15 M NaCl, 0.01 M NaH2PO4, 1 mM EDTA, pH 7.4; 1 × Denhardt's solution is 0.2 g/liter Ficol, 0.2 g/liter polyvinyl pyrrolidone, 0.2 g/liter gelatin). The filters were washed with 6 × SSC, 0.1% SDS, 0.05% sodium pyrophosphate at the temperature of hybridization. (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0.) Approximately 30,000 recombinants were screened. Eight clones were selected, which hybridized strongly at 58 °C but more weakly at 68 °C. The eight clones were related to each other as judged by restriction mapping.

**RESULTS**

**Cloning of GSY2—**A yeast genomic library was screened using GSY1 as probe as described under "Experimental Procedures." Eight clones were identified that hybridized strongly at low stringency but much more weakly at high stringency. Because of previous Southern hybridization of genomic DNA, we considered these clones to be candidates for a second gene encoding glycogen synthase. One clone was chosen for sequencing. A 3.8-kb Sall-SalI fragment, which hybridized with the probe, was subcloned into pBluescript KS+ (Stratagene), and a 2,914-base pair segment containing the coding region of the gene was sequenced from both strands.

**DNA and Protein Sequence Analysis—**pBluescript vector containing the subcloned 3.8-kb Sall-SalI fragment of GSY2 inserted in both orientations was digested with NotI. After filling in the 5′ restriction overhang with Klenow, the fragments were cut with EcoRV and nested unidirectional deletions were created by exonuclease III and Mungbean nuclease (Exo III/Mungbean deletion kit, Stratagene). Nucleotide sequence was determined by the dyeoxy chain termination method (21) using a T7 (17-mer) primer. The sequencing projects and sequence alignments were managed using the PCGene program (Intelligenetics). Protein sequence data base searching was achieved using the FASTA algorithm and the data base maintained by Dr. Mark Goebl at Indiana University.

**DNA Hybridization Analysis—**DNA isolated (18) from the wild type strain YPH52 and from mutants containing the gsy1Δ and/or the gsy2Δ mutation was digested with EcoRI and/or EcoRV and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filter was hybridized with the 1.1-kb Sall-EcoRV fragment of GSY2. The fragment was labeled by random priming (U. S. Biochemical Corp. kit) using α-32PdCTP and hybridized at 58 °C in 6 × SSPE, 10 × Denhardt's solution, 0.1% SDS, 0.05% sodium pyrophosphate. Washing was carried out in 6 × SSC, 0.1% SDS, 0.05% sodium pyrophosphate at 60 °C prior to autoradiography.

For chromosomal analysis of GSY1 and GSY2, chromosomes from yeast strain AB 1050 were separated by contour-clamped homogeneous electric field electrophoresis (22) (CHEF-DRII System, Bio-Rad) on a 0.5 M Tris-C1, 1 M NaCl, 10 mM sodium fluoride, 0.01 M NaH2P04, 1 mM EDTA, pH 7.5, plate for 45 h at 1,700 g for 2 min. To separate chromosomes V and VI, the plates were treated with 0.15 M NaCl for 30 min at room temperature. The chromosomes were then run on a 3.9 M urea-10% PAGE gel, stained with ethidium bromide, and separated by pulsed field electrophoresis and transferred to nitrocellulose. Fourteen of the 16 chromosomes were clearly separated, with chromosomes V and VIII running as poorly resolved doublets (not shown). A 2.3-kb SpeI-Ndel fragment of GSY1 hybridized to chromosome VI. The identity of chromosome VI was confirmed by its hybridization to a fragment of CDC4, which is known to reside in chromosome VI (30). A 2.3-kb EcoRI-Sall fragment of GSY2 hybridized to the same chromosome as a RNA probe. Therefore, GSY2 is located on chromosome XII, which is known to contain rDNA sequences (30).

**Disruption of GSY2—**For disruption of the GSY2 gene, pRS303 vector (31) containing the HIS3 marker gene was used. An EcoRV-Apal fragment bearing the 5′ region of GSY2 was introduced into pRS303 followed by insertion of the 3′ Sall-EcoRV fragment of the gene into the corresponding sites of the modified plasmid. The resulting pRS303-GSY2Δ1 construct (Fig. 4) contained a unique EcoRV site. Digestion of the plasmid DNA with EcoRV and Apal released a 3-kb (filled bar) fragment containing the coding region of GSY2 and a 1-kb fragment (open bar) corresponding to the 3′ untranslated region. When this construct was transformed into yeast strain AB 1050, tetrad analysis was performed by standard methods of yeast genetics (18).

![FIG. 1. Partial restriction map of the GSY2 gene and sequencing strategy](image-url)
the plasmid with EcoRV resulted in a linear DNA in which most of the coding sequence was replaced by pRS303 sequences, including the HIS3 marker. This linearized DNA was used for one-step gene replacement (32). Haploid wild type (YPH52) and gsy1A1 mutant yeast cells were transformed with the linearized pRS303-GSY2Al DNA, using the LiAc method (33). Selection was made for His+ transformants. Southern hybridization was used to confirm that GSY2 was replaced by the disrupted gene (Fig. 5). DNA from wild type and from haploid mutant strains were digested with appropriate restriction enzymes and probed with a 1.1-kb SacI-EcoRV fragment containing a segment of the coding region of GSY2. DNA isolated from wild type and gsy1A1 mutant cells contained a 3.5-kb EcoRV-EcoRV fragment (Fig. 5, tracks 1 and 3), as predicted from the restriction map of GSY2. Similarly, the 2.6-kb hybridizing fragments in the EcoRV-EcoRI digests of wild type (Fig. 5, track 2) and gsy1A1 mutant (Fig. 5, track 4) strains were indicative of the presence of GSY2. In the gsy2A1 mutant and in the double mutant, these fragments were absent, and a 6.2-kb fragment appeared (Fig. 5, tracks 5-8), as expected from the strategy for the disruption of GSY2. Thus, haploid yeast strains were generated in which either or both of the glycogen synthase genes were defective.

Phenotype of Yeast Cells Defective in Glycogen Synthase Genes—Disruption of GSY2 in both wild type (YPH52) and in gsy1A1 haploid strains resulted in viable mutants. Therefore, GSY2 itself is not an essential gene nor is the presence of at least one of the two glycogen synthase genes an obligate requirement for viability. No obvious morphological difference between wild type and mutant cells was apparent. To determine the specific effects of the loss of GSY1 and GSY2 functions, wild type and mutant strains were grown under the same conditions (YPD) and monitored for A600, glycogen synthase activity, and glycogen content during the approach to stationary phase (Fig. 6). No significant difference in the doubling time during exponential growth was observed, and growth profiles of wild type and mutant strains were similar. Glycogen synthase activity increased and glycogen accumulated in haploid wild type and gsy1A1 mutant yeast approaching stationary phase, although the levels of glycogen synthase and glycogen were somewhat lower in the mutant without functional GSY1. In the gsy2A1 cells, however, the level of glycogen synthase remained at a constant low level throughout, about 10% that of wild type cells in stationary phase. A small increase in glycogen content was observed, but the increase was significantly lower than in wild type cells entering the stationary phase. The strain defective for both GSY1 and GSY2, no glycogen synthase activity was detected at any stage in the growth cycle, and very little or no glycogen accumulated. The experiment was also performed using a homozygous gsy1A1 gsy2A1 diploid strain (described below) with similar results as the gsy1A1 gsy2A1 haploid (data not shown). The phenotype for glycogen accumulation was also demonstrable using iodine staining to detect glycogen (Fig. 7).

Gunja-Smith et al. (19) had suggested that the water-soluble and -insoluble glycogen fractions obtained after treating cells with hot alkali represented two different pools of glycogen. They proposed that insoluble glycogen was associated with a component of the cell wall (19). An obvious possibility was that the two glycogen synthases defined above might be correlated with the synthesis of "soluble" or "insoluble" glycogen pools. To test this hypothesis, we determined the content of glycogen 2-4 days we consistently saw a slightly higher intensity of stain in the gsy2A1 mutant as compared with wild type. Thus, maintenance of glycogen deposits over prolonged periods is correlated with the disruption of GSY1, a seemingly paradoxical result unless the expression of GSY1 was somehow linked to glycogen degradation.
centration of soluble and insoluble glycogen in gly1Δ and gly2Δ mutant yeast cells from cultures grown to stationary phase, using procedures based on those of Gunja-Smith et al. (19) (Table 1). To avoid overestimation of soluble glycogen due to enzymatic hydrolysis during cell homogenization, cells were pretreated with glutaraldehyde in one set of determinations. In gly1Δ mutant cells, the concentration of total and insoluble glycogen was only slightly lower than in wild type cells. In gly2Δ mutant cells, however, the concentration of both the soluble and insoluble glycogen was significantly lower than in YPH52 cells. Glycogen detection without glutaraldehyde fixation gave a higher estimate of the proportion of soluble glycogen. However, there was no abolition of either the soluble or insoluble pools of glycogen associated with the disruption of either of the glycogen synthase genes.

To test whether gly1Δ and gly2Δ are required for sporulation, we generated a homozygous gsy2A1 gsy2A2 gsy1A1 gsy1A2 strain. Mutant yeast strains were digested with EcoRV (tracks 1, 2, 3, 4, or 5) or with EcoRI (tracks 6, 7, 8) and analyzed as described under "Experimental Procedures." The filter was hybridized with a 1.1-kb SacI-EcoRV fragment of gSYa1 or gSYa2.

Fig. 3. Comparison of the primary structures of the two S. cerevisiae glycogen synthases with those of glycogen synthases from rabbit muscle and rat liver. Identities with the sequence encoded by gSY1 are denoted by solid dots; gaps are shown as dashes. Numbering of the rabbit muscle and rat liver sequences includes the initiator methionine, whereas numbering of the muscle and yeast sequences were identical to the parental YP strain were then sporulated in McClary's medium (1% potassium acetate, 0.1% glucose). Tetrads were dissected and scored for uracil and histidine prototrophy and analyzed as described under "Experimental Procedures." The ascis from the mutant and wild type diploids showed a similar capacity to form colonies (data not shown). Glycogen can be converted to trehalose (34, 35), which has been reported to accumulate in yeast cells upon exposure to both the soluble and insoluble glycogen.

The parent pRS303-GSY2Δ plasmid was linearized at the unique EcoRV site. Restriction sites are abbreviated as in the legend to Fig. 1.
heat (36). One hypothesis is that glycogen reserves might influence trehalose availability and hence the ability of the cell to withstand heat shock. Therefore, we compared the thermostolerance of wild type and glycogen-deficient cells exposed for 30 min to 55 and 50 °C on YPD plates or in YPD liquid cultures, respectively (results not shown).

FIG. 6. Changes in the levels of glycogen synthase and glycogen during growth of haploid wild type (YPH52) and mutant yeast strains disrupted for one or two glycogen synthase genes. YPD medium was inoculated with precultures as described under "Experimental Procedures." Panel A, absorbance at 640; panel B, glycogen levels; panel C, total glycogen synthase activity.

FIG. 7. Glycogen staining of wild type and mutant yeasts disrupted in glycogen synthase genes. Yeast strains were patched onto a YPD plate, incubated at 30 °C for 24 h, and exposed to iodine vapor. 1, YPH52; 2, gsy1Δ (IF1); 3, gsy2Δ (IF2); 4, gsy1Δ gsy2Δ haploid (IF3); 5, YP; 6, homozygous gsy1Δ gsy2Δ diploid (IF4).
mammalian glycogen synthases (3–5). Furthermore, the COOH-terminal region has a notable net negative charge, again in common with all the other glycogen synthases for which sequences are known (3–5). The COOH terminus of glycogen synthase-2 is very similar to that of glycogen synthase-1 and is the part of the molecule that differs most from the mammalian enzymes. This region is implicated in the regulation of the enzyme by phosphorylation and may contain sites for phosphorylation by cAMP-dependent protein kinase (13). In the glycogen synthase-1 sequence, three potential COOH-terminal sites for this protein kinase were predicted (13) based on a minimal recognition sequence of R/K-X-Y-X-S. Interestingly, the presence of an extra residue in the glycogen synthase-2 sequence would destroy one of these potential sites (Ser-660 of glycogen synthase-2) leaving as candidates Ser-650 and Ser-662. Biochemical and genetic experiments to define the exact phosphorylation sites are under way.

Though many yeast mutants with aberrant glycogen accumulation have been identified, none of these has, to our knowledge, involved defective glycogen synthase. The presence of two quite similar genes, of course, can account for this failure. Having cloned both the GSY1 and GSY2 genes, we were in a position to examine the viability and phenotypes of mutant yeast defective in one or both genes. Yeast lacking functional versions of both genes were viable and yet lacked detectable glycogen synthase activity, even at stationary phase. Glycogen content was very low (Table I). Since glycogen was determined from the assay of glucose released by hydrolysis, the small values measured may be due to the lack of specificity for glycogen of amyloglucosidase. The absence of measurable glycogen synthase activity in the double mutant argues that, at least under the growth conditions used, no other gene for glycogen synthase was expressed. We consider it probable that yeast contains only two genes encoding glycogen synthase, although one cannot rigorously exclude the possibility that another gene might be functional under other unstressed circumstances. In any event, our results with the double mutants demonstrated that loss of glycogen synthase activity and defective glycogen accumulation were not correlated with any major impairment in viability, ability to sporulate or recover from sporulation, or survival and rate of growth after heat shock. We conclude that glycogen is not essential for S. cerevisiae under the limited number of conditions tested. However, the ability of the organism to accumulate glycogen and to do so in a carefully regulated manner makes it difficult to view glycogen deposition as a redundant process. Future work will seek conditions in which defective glycogen accumulation correlates with impaired performance of the cells.

Our studies of strains selectively disrupted in one of the GSY genes permit us to speculate on differences in the regulation of the two genes. Yeast containing only the GSY1 gene express a low but constant level of glycogen synthase activity throughout the growth cycle and are severely restricted in their ability to accumulate glycogen upon entry into stationary phase. Though our analyses to date have been only of enzyme activity, it appears as if the GSY1 gene is expressed constitutively. In yeast that express only glycogen synthase-2, glycogen deposition is only slightly less than in the wild type, and large increases in glycogen synthase activity are observed prior to the onset of stationary phase, as with the wild type. Thus, GSY2 encodes the predominant glycogen synthase, in keeping with the relative proportions of 77- and 85-kDa polypeptides in the purified enzyme samples. Furthermore, we hypothesize that expression of GSY2 is controlled by environmental signals such as nutrient deprivation. Why yeast contain glycogen synthase genes under different controls remains unexplained.

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