Glucose-6-P Control of Glycogen Synthase Phosphorylation in Yeast*

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Dongqing Huang, Wayne A. Wilson, and Peter J. Roach‡

From the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5122

Glycogen is a storage form of glucose in a wide variety of organisms and cell types. In the yeast Saccharomyces cerevisiae, glycogen accumulation is normally initiated in response to nutrient limitation, prior to entry into stationary phase (1). Glycogen biosynthesis requires a self-glucosylating initiator protein, glycogenin, glycogen synthase for chain elongation, and branching enzyme to introduce the α-1,6-glycosidic branch points (2). Over the past few years, the yeast genes encoding enzymes responsible for glycogen biosynthesis have been identified, including two glycogen synthase genes, GSY1 and GSY2 (3, 4). GSY2 encodes the major nutritionally regulated form of glycogen synthase (4). One control of GSY2 is by its increased expression at the approach of stationary phase (4). In addition, this protein is regulated by covalent phosphorylation, which decreases its activity when measured in the absence of glucose-6-P (5–7). However, activity is restored even to fully phosphorylated enzyme by the presence of glucose-6-P, so the −/− glucose-6-P activity ratio is frequently used as an index of the activation state of glycogen synthase.

Glycogen synthase can be rate-determining for glycogen synthesis in yeast, and mutation of potential phosphorylation sites in Gsy2p results in overaccumulation of glycogen (7). Mutations in GAC1 and GLC7, which encode type 1 phosphatase subunits, cause a decreased −/+ glucose-6-P activity ratio and concomitantly impaired glycogen deposition (8, 9). Conversely, deletion of PHO85, which encodes a glycogen synthase kinase catalytic subunit, leads to glycogen hyperaccumulation (10, 11).

Another gene that influences glycogen storage is SNF1 (8, 12), which encodes a protein kinase essential for expression of glucose-repressible genes (13). Thus, snf1 mutants grow poorly on an alternative carbon sources (14) and also fail to accumulate glycogen (8, 12). We had previously suggested that the inability of snf1 mutants to synthesize glycogen was caused by hyperphosphorylation, and hence inactivation, of glycogen synthase (15).

In the present study, we searched for second-site suppressors of the glycogen accumulation defect of snf1 cells. One of the resulting suppressor genes was identified as PFK2, which encodes one subunit of the glycolytic enzyme 6-phosphofructo-1-kinase (16). We propose that the glycogen storage phenotype caused by this mutation results from high intracellular glucose-6-P levels and that glucose-6-P acts not only as a direct activator of glycogen synthase but also as an inhibitor of glycogen synthase kinase activity.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Methods—The S. cerevisiae strains used are listed in Table I. Rich medium (YP) contains 1% yeast extract, 2% Bacto-peptone, and 2% concentration of the indicated carbon source. Synthetic medium consists of yeast nitrogen base (6.7 g/liter), lacking the appropriate amino acid, and 2% concentration of the indicated carbon source. Cells carrying a pgil disruption were grown in rich medium with 2% fructose and 0.1% glucose (YPFD). Plasmids were maintained in Escherichia coli strain DH5α. Standard methods for yeast genetic analysis (18) and transformation (19) were used.

Gene Disruptions—For disruption of PFK2, the polymerase chain reaction was used to generate a DNA fragment that contained PFK2 sequences straddling a URA3 marker gene (20). The vector pRS316 (21) was used as the template for the polymerase chain reaction. The resulting polymerase chain reaction product contained the 5′ sequence (+242 to +287, referred to the open reading frame) and 3′ sequence (+2860 to +2851) of PFK2 at each end of a 1.1-kilobase pair sequence containing the URA3 gene. This DNA fragment was then used to transform strains EG328-1A, EG327-1D, and EG335-1C to replace the PFK2 gene and generate strains DH55-0, DH54-0, and DH58-0. Gene disruption in Ura+ transformants was confirmed by polymerase chain reaction. A similar strategy was employed to disrupt PFK1 and PGII1 (Table I). For pgil disruption, Ura+ transformants were selected in synthetic complete media without uracil and containing fructose (2%) and a low concentration of glucose (0.1%) as carbon source.

Genetic Analysis of ssg1—Since diploids homozygous for snf1 do not sporulate, we crossed an ssg1 snf1:: URA3 mutant to an isogenic wild-type strain DH66 (Ura+). Tetrad analysis revealed segregation ratios of 2:2:3:1 and 4:0:0 for glycogen accumulation, with most tetrads 3:1:1, indicating that mutation in a single gene was responsible for the
phenotype. The sporation also yielded tetratypes consisting of one Ura− colony with normal glycogen (SSG1 SNF1), one Ura− colony defective for glycogen (SSG1 snf1), and one Ura− colony with hyperaccumulation of glycogen (ssg1 SNF1). For spores segregating 2:2:2:2, the two glycogen-positive clones always hyperaccumulated and were Ura−, while the two glycogen-negative clones were Ura+. These results suggest that, besides restoring glycogen in snf1 cells, ssg1 caused hyperaccumulation of glycogen in a wild-type background. We also tested whether ssg1 would overcome the glycogen deficit in glc7-1 cells, which are defective in the type 1 protein phosphatase catalytic subunit (8). When an ssg1 strain (DH59-13) was crossed with EG327-1D (glc7-1), the resulting tetrads exhibited a 3:1 segregation ratio for positive glycogen storage, suggesting that the ssg1 mutation suppresses the glc7-1 glycogen-deficient phenotype. Multiple independent ssg1 glc7-1 double mutant strains were isolated and found to accumulate an intermediate level of glycogen. Thus, the ssg1 mutation partially suppressed the glycogen phenotype of glc7-1.

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The spores from a cross of a strain (DH5-0) with hyperaccumulation of glycogen, ssg1-1, were electroporated with the Gsy2p expression vector pEF2-PFK2 and plated on 1% glucose (normal glycogen) and 4% glucose. The frequency of the strain with normal glycogen was about twice that in a normal extract of wild-type cells. The amount of added Gsy2p gives a level of activity measured in the absence of glucose-6-P. The amount of added Gsy2p gives a level of activity measured in the absence of glucose-6-P activity ratio. In the reaction, 20 µl of purified Gsy2p (0.76 mg/ml) 50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, was mixed with 300 µl of yeast lysate (adjusted to ~4 mg of protein/ml) prepared as described above. The reaction was started by the addition of 80 µl of 25 mM ATP to 750 mM Tris-HCl, 150 mM MgCl2, pH 7.4, together with any other additions. After incubation at 30 °C for different times, a portion (40 µl) was removed, mixed with 10 µl of 0.3 M EDTA, 0.4 M NaF to terminate the reaction, and loaded onto a spin column (Sephadex G25, 1.5 ml volume). After centrifugation at 1000 rpm in an IEC Clinical Centrifuge for 3 min, the pass-through was collected, mixed with 50 µl of the same homogenization buffer described above but with 100 mM NaF, and assayed for glycogen synthase activity. In some experiments, cell extracts were treated with Sephadex G25 columns to remove endogenous glucose-6-P prior to initiating the protein kinase reaction. In control experiments in which we measured glucose-6-P levels, we found that the spin column removed over 95% of the glucose-6-P. To measure glycogen synthase kinase activity in this assay, we had to use gsy2 strains so that the endogenous glycogen synthase activity, which is reduced to about one-tenth of wild type, does not interfere significantly with that of the added Gsy2p. The amount of added Gsy2p gives a level of activity about twice that in a normal extract of wild-type cells.

In the second assay for glycogen synthase kinase activity, the direct phosphorylation of purified Gsy2p added to cell extracts was determined by analyzing the incorporation of [32P] into Gsy2p from γ-[32P]ATP. Yeast cells were grown and collected as described above. The cells were resuspended in a homogenization buffer containing 50 mM Tris-HCl, 1 mM EDTA, 3 mM dithiothreitol, 50 mM NaF, 1 mM phenylmethylsulfon fluoride, 0.1 mM Nα-p-tosyl-l-lysine chloromethyl ketone, 5 mM benzamidine, 0.25 µg/ml leupeptin, and 0.5 µg/ml aprotinin, pH 7.4. The cells were broken with glass beads as described previously (7).

Glyogen synthase was assayed by the method of Thomas et al. (24) as described by Hardy et al. (15) except that the extracts were first passed over a Sephadex G25 spin column to remove low molecular weight compounds as described below. A unit of activity is defined as the amount of enzyme that catalyzes the transfer of 1 pmol of glucose from UDP-glucose to glycogen per min under conditions of the standard assay. The total activity of glycogen synthase is that measured in the presence of 7.2 mM glucose 6-phosphate. The ~1/glucose-6-P activity ratio is defined as the activity measured in the absence of glucose-6-P divided by that measured in its presence. Each measurement was the average of duplicate assays.

Glyogen synthase kinase activity in extracts was measured by two procedures. In one, the inactivation of added, purified recombinant Gsy2p protein was followed with time, monitoring the decrease in the ~1/glucose-6-P activity ratio. In the reaction, 20 µl of purified Gsy2p (0.76 mg/ml) 50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 20% (v/v) glycerol was mixed with 300 µl of yeast lysate (adjusted to ~4 mg of protein/ml) prepared as described above. The reaction was started by the addition of 80 µl of 25 mM ATP to 750 mM Tris-HCl, 150 mM MgCl2, pH 7.4, together with any other additions. After incubation at 30 °C for different times, a portion (40 µl) was removed, mixed with 10 µl of 0.3 M EDTA, 0.4 M NaF to terminate the reaction, and loaded onto a spin column (Sephadex G25, 1.5 ml volume). After centrifugation at 1000 rpm in an IEC Clinical Centrifuge for 3 min, the pass-through was collected, mixed with 50 µl of the same homogenization buffer described above but with 100 mM NaF, and assayed for glycogen synthase activity. In some experiments, cell extracts were treated with Sephadex G25 columns to remove endogenous glucose-6-P prior to initiating the protein kinase reaction. In control experiments in which we measured glucose-6-P levels, we found that the spin column removed over 95% of the glucose-6-P. To measure glycogen synthase kinase activity in this assay, we had to use gsy2 strains so that the endogenous glycogen synthase activity, which is reduced to about one-tenth of wild type, does not interfere significantly with that of the added Gsy2p. The amount of added Gsy2p gives a level of activity about twice that in a normal extract of wild-type cells.

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TABLE I

| Strain      | Genotype                                      | Source  |
|-------------|-----------------------------------------------|---------|
| EG328-1A    | MATa trp1 leu2 ura3-52                         | K. Tatchell |
| EG327-1D    | MATa trp1 leu2 ura3-52 glc7-1                 | Ref. 17 |
| EG353-1C    | MATa trp1 leu2 ura3-52 snf1::URA3            | K. Tatchell |
| F243        | MATa thr4                                      | R. C. Wek |
| DH2         | MATa trp1 leu2 ura3-52 ssg2::URA3             | Ref. 17 |
| DH5         | MATa trp1 leu2 ura3-52 snf1::LEU2             | This study |
| DH22        | MATa trp1 ura3-52 thr4 snf1::URA3            | This study |
| DH53        | MATa trp1 leu2 ura3-52 ssg1-1                 | This study |
| DH35-64     | MATa ura3-52 thr4 pho85::URA3                 | This study |
| DH51        | MATa trp1 ura3-52 thr4 glc7-1                 | This study |
| DH54-0      | MATa trp1 leu2 ura3-52 glc7-1 pfk2::URA3      | This study |
| DH54-102    | MATa trp1 leu2 ura3-52 glc7-1 ssg1-1          | This study |
| DH55-0      | MATa trp1 leu2 ura3-52 pfk2::URA3             | This study |
| DH56-0      | MATa trp1 ura3-52 pfk1::URA3                  | This study |
| DH57-0      | MATa trp1 ura3-52 glc7-1 pfk1::URA3           | This study |
| DH58-0      | MATa trp1 leu2 ura3-52 snf1::LEU2 pfk2::URA3  | This study |
| DH59-13     | MATa trp1 ura3-52 thr4 ssg1-1                 | This study |
| DH60        | MATa trp1 leu2 ura3-52 pfk1-1::URA3          | This study |
| DH61-0      | MATa trp1 leu2 ura3-52 snf1::LEU2 pfk1::URA3  | This study |
| DH62-60     | MATa trp1 ura3-52 glc7-1 pgl1::URA3            | This study |
| DH63-0      | MATa trp1 ura3-52 pg1::URA3                    | This study |
| DH64-42     | MATa trp1 leu2 ura3-52 gsy2::URA3 pfk2::URA3  | This study |
| DH65        | MATa thr4                                      | This study |
| DH66        | MATa ura3 thr4                                 | This study |

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EG328-1A is the wild type of this study. EG327-1D and EG353-1C are isogenic to EG328-1A. They were all kindly provided by Dr. Kelly Tatchell. DH65 and DH66 are the progeny of five and four backcrosses, respectively, to the wild type strain EG328-1A from F243. The DH strains are all related to EG328-1A.
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hydrochloride, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-ethylmaleimide, and 1 mM β-mercaptoethanol. Cell extracts were prepared using glass beads and, in some cases, were passed over a Sephadex G25 spin column as described above. Yeast extract (5 μl, diluted to ~2.5 mg of protein/ml with homogenization buffer) was combined with 2.5 μg of His6Gsy2p and 100 mM oxadiazol in a final volume of 20 μl. In some experiments, glucose-6-phosphate was also added from an aqueous stock solution. The reaction was initiated by the addition of 5 μl of [γ-32P]ATP mix (1 mM ATP, 25 mM MgCl₂, ~1200 cpm/pmol). After incubation at 30 °C for 15 min, 25 μl of a 1:1 slurry of nickel-nitrotriacetic acid-agarose was collected by centrifugation, and the pellet was washed four times with 500 μl of wash buffer. Bound His6Gsy2p was eluted using 25 μl of wash buffer with the imidazole concentration increased to 500 mM. The eluted material was analyzed by polyacrylamide gel electrophoresis in the presence of SDS (25) and autoradiography.

Measurement of Glucose-6-P Levels—Determination of glucose-6-P was a variation of the method of Lang and Michal (26) as described by Wilson (27). Cells were harvested by rapid filtration, resuspended in perchloric acid (5%), and broken with glass beads. The acid extracts were collected and centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was extracted twice to remove perchloric acid with 10% hydrochloric, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-ethylmaleimide, and 1 mM β-mercaptoethanol. Cell extracts were prepared using glass beads and, in some cases, were passed over a Sephadex G25 spin column as described above. Yeast extract (5 μl, diluted to ~2.5 mg of protein/ml with homogenization buffer) was combined with 2.5 μg of His6Gsy2p and 100 mM oxadiazol in a final volume of 20 μl. In some experiments, glucose-6-phosphate was also added from an aqueous stock solution. The reaction was initiated by the addition of 5 μl of [γ-32P]ATP mix (1 mM ATP, 25 mM MgCl₂, ~1200 cpm/pmol). After incubation at 30 °C for 15 min, 25 μl of a 1:1 slurry of nickel-nitrotriacetic acid-agarose was collected by centrifugation, and the pellet was washed four times with 500 μl of wash buffer. Bound His6Gsy2p was eluted using 25 μl of wash buffer with the imidazole concentration increased to 500 mM. The eluted material was analyzed by polyacrylamide gel electrophoresis in the presence of SDS (25) and autoradiography.

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RESULTS

Mutations in a Phosphofructokinase Gene Suppress the Glycogen-deficient Phenotype of snf1 Mutants—Cells defective in the Snf1p protein kinase fail to accumulate glycogen, a deficit that we attributed to hyperphosphorylation of glycogen synthase (15). Several second site suppressors of this glycogen phenotype, designated ssg1-5 (suppressor of snf1 for glycogen phenotype), were identified, and the wild-type allele of one, ssg3, was found to encode Pgo85p, a protein kinase catalytic subunit (11). Another mutant allele, ssg1, also restored the wild-type glycogen synthase activity ratio to snf1 cells, suggesting that a ssg1 snf1 strain could have reduced glycogen synthase activity. Also, the presence of the ssg1 allele in a wild-type background caused glycogen hyperaccumulation (Fig. 1). Cloning ssg1 by complementation was complicated by the fact that transformation of the ssg1 snf1 cells was extremely difficult, and we could not obtain sufficient numbers of transformants for effective library screening. Since ssg1 also complemented the glycogen defect in glc7-1 cells, which have an impaired type 1 phosphatase catalytic subunit (8), we screened for loss of glycogen accumulation in an ssg1 glc7-1 strain (DH5241-102) after transformation with a yeast genomic library (22). We isolated a plasmid that complemented the ssg1 phenotype and identified the responsible gene as PFK2, which encodes the β-subunit of phosphofructokinase-1-kinase (16). As described under “Experimental Procedures,” we confirmed by genetic methods that the relevant phenotype was indeed linked to the PFK2 locus.

Deletion of PFK2 Causes the Same Phenotype as the ssg1 Mutation—Strains harboring deletions of PFK2 were found to hyperaccumulate glycogen in a wild-type background, and glycogen storage was restored to glc7-1 and snf1 cells (Fig. 1). Deletion of PFK2 has been reported to increase glucose-6-P levels (28), and we therefore measured glucose-6-P in ssg1 and several other relevant strains. In wild-type cells, glucose-6-P decreases from about 2 nmol/mg, dry weight, during logarithmic growth to less than half of that level before the onset of stationary phase. Elevated levels of glucose-6-P were observed in ssg1, pfk2, ssg1 glc7-1, and pfk2 glc7-1 cells (Table II). The double mutants ssg1 snf1 and pfk2 snf1 contained less glucose-6-P than pfk2 cells but still more than wild type. In a snf1 mutant, the glucose-6-P concentration was significantly lower than that in wild-type cells, especially at late logarithmic phase (Table II).

Since glucose-6-P is a potent activator of glycogen synthase, an obvious hypothesis is that enhanced glycogen storage by pfk2 mutants is caused by this activation. In our initial analysis of glycogen synthase activity in extracts from ssg1 snf1 cells, we observed that the −/+ glucose-6-P activity ratio was significantly elevated as compared with snf1 cells (11). However, not knowing that glucose-6-P levels might be increased, we had taken no special measures in the earlier study to remove small molecules from the extract. Thus, there could have been carry-over of endogenous glucose-6-P from the cell extract into the glycogen synthase assay. For the assay in the absence of added glucose-6-P, this could cause increased activity and an erroneously high −/+ glucose-6-P activity ratio. Therefore, cell extracts were treated with a Sephadex G25 spin column to remove endogenous glucose-6-P prior to the assay. The −/+ glucose-6-P activity ratio in pfk2 mutants was still over twice that of wild-type cells (Fig. 2). In pfk2 snf1 cells, the activity ratio was restored to wild-type levels, whereas deletion of PFK2 in a glc7-1 background only partially restored the activity (Fig. 2). This result indicates that the intrinsic activity, and by inference the phosphorylation state, of glycogen synthase was affected in these mutants.

Effect of Intracellular Glucose-6-P on Glycogen Accumulation—Since pfk2 mutants have elevated glucose-6-P, we explored the possibility that the high level of this metabolite caused the glycogen hyperaccumulation. Mutation of two other genes, PFK1 and PGII, is also known to affect glucose-6-P levels, and we asked whether such mutations also influenced glycogen accumulation. Mutants defective in PFK1, which encodes the α subunit of phosphofructokinase, contain elevated glucose-6-P but less than in pfk2 strains (Ref. 28; see also Table II) and in general have a less severe phenotype than pfk2
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The glucose-6-P level is normalized to that in wild-type cells during logarithmic growth. The absolute value of glucose-6-P in wild-type cells under these conditions was 2 nmol/mg, dry weight, of cells.

| Strain Mutation | Glycogen synthase activity ratio |
|-----------------|----------------------------------|
| EG328-1A Wild type | 100 |
| EG327-1D glc7-1 | 59 |
| EG353-1C snf1 | 12 |
| DH56-0 pfk1 | 194 |
| DH55-0 pfk2 | 351 |
| DH59-13 ssg1-1 | 298 |
| DH57-0 glc7-1 pfk1 | 254 |
| DH61-0 snf1 pfk1 | 113 |
| DH54-0 glc7-1 pfk2 | 341 |
| DH54-102 glc7-1 ssg1-1 | 394 |
| DH58-0 snf1 pfk2 | 186 |
| DH23 snf1 ssg1-1 | 258 |
| DH63-0 pgi1c | 61 |

a Glucose-6-P was measured in yeast cell extracts as described under "Experimental Procedures." Cells were harvested during either logarithmic growth (~10^8 cells/ml) or late logarithmic phase (~8 × 10^7 cells/ml).

b ND, not determined.

c Strains were grown in rich medium containing 2% fructose and 0.1% glucose.

Glucose-6-P Inhibits Glycogen Synthase Kinase Activity—If glycogen synthase phosphorylation is decreased, this must be due to inhibition of a protein kinase, activation of a phosphatase, or both. To test whether glycogen synthase kinase activity was affected in a pfk2 strain, we first constructed a pfk2 gsy2 double mutant, so that the endogenous glycogen synthase activity was significantly reduced (4). Extracts from gsy2 pfk2 and gsy2 mutants were then compared for Gsy2p kinase activity. In one assay, the ability to inactivate added recombinant Gsy2p protein was followed by monitoring the decrease in the -/+/+ glucose-6-P activity ratio with time (Fig. 3A). The glycogen synthase total activity was essentially unchanged over the period of incubation (data not shown). The endogenous glycogen synthase activity in gsy2 cells was only ~5% of that due to the added recombinant enzyme and therefore did not make a major contribution to the measured activity ratio. Glycogen synthase kinase activity was readily detected in unfiltered extracts from gsy2 cells but was markedly reduced in extracts from a pfk2 gsy2 mutant (Fig. 3A). In these experiments, 50 mM NaF was present to inhibit phosphatase activity. In other experiments, identical results were obtained when 0.1 µM okadaic acid instead of NaF was present during the protein kinase incubation (data not shown). A similar result was obtained by analyzing the direct phosphorylation of Gsy2p (Fig. 3B). Using either assay, however, we found that the difference in glycogen synthase kinase activity observed with unfiltered extracts was eliminated by treatment with Sephadex G25, consistent with a low molecular weight compound having been removed (Fig. 3).

The effect of glucose-6-P on glycogen synthase kinase activity was therefore tested directly. Filtered extracts from gsy2 cells were used as a source of protein kinase activity. With the Gsy2p inactivation assay, a concentration-dependent inhibition of the glycogen synthase kinase activity was observed, to about 25% of the uninhibited level. Half-maximal inhibition was seen at ~2 mM (Fig. 4A). This value is within the range of physiological glucose-6-P concentrations (Table II). Similar results were observed by direct analysis of phosphate incorporation into Gsy2p (Fig. 4B). To test whether the inhibitory effect on glycogen synthase kinase activity was specific for glucose-6-P, some related metabolites were also tested for their inhibition of this kinase activity. As compared with glucose-6-P, fructose-1,6-P_2, and fructose-2,6-P_2 showed little inhibitory effect, while fructose-6-P caused a moderate inhibition of the kinase activity (Fig. 5).

Glucose-6-P and Other Phenotypes of snf1 Cells—In snf1 mutants, the glucose-6-P level is low relative to wild-type cells, especially at late logarithmic phase (Table II), but this is not responsible for glucose repression defects, since a snf1 pfk2 double mutant does not grow well on carbon sources other than glucose (data not shown). Also, invertase activity in the snf1 pfk2 double mutant was not induced by low glucose (data not shown). Likewise, low glucose-6-P does not account for sporulation defects, since diploids homozygous for both snf1 and pfk2 alleles still do not sporulate (data not shown).
DISCUSSION

Starting with a glycogen-deficient snf1 strain in which the lack of glycogen appeared to be due to hyperphosphorylated glycogen synthase (15), we sought second site suppressor mutations that restored glycogen accumulation (11). This screen already identified PHO85 as encoding a glycogen synthase kinase catalytic subunit, and we now report that another mutant, ssg1, carries an allele of PFK2. Interestingly, a genetic defect in human muscle phosphofructokinase also results in overaccumulation of glycogen (type VII glycogen storage disease; Ref. 30). The most important outcome of the present study is to refocus attention on the role of metabolites, specifically glucose-6-P, in regulating glycogen synthase. Such a role for glucose-6-P has been acknowledged for years, based mainly on biochemical analyses (for recent reviews, see Refs. 31 and 32). What is novel in the current work is, first, that the connection should be generated by a genetic screen and, second, to suggest that mechanistically glucose-6-P may inhibit glycogen synthase kinase activity.

Mutations in PFK1 and PFK2 were already known to impair glycolysis and cause elevation in the levels of metabolites such as glucose-6-P (23). We have additionally shown that PFK2
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**FIG. 6.** Correlation of glycogen accumulation and glucose-6-P levels in various yeast strains. Glycogen levels (see Fig. 1) and relative glucose-6-P (see Table II) for several of the strains studied are plotted against one another. Strains containing the glc7-1 allele are indicated by filled circles. The other strains (open circles) include those with pfk1, pfk2, and snf1 mutations.

mutations also cause glycogen hyperaccumulation. Mutation of PFK1 gives a less severe phenotype but can suppress the glycogen defect in snf1 cells. We also confirmed the previous observation that loss of phosphoglucomutase results in excess glycogen synthesis (29). Grown on fructose with trace amounts of glucose, pgi1 mutants accumulate large amounts of glucose-6-P, UDP-glucose, and glycogen (29). If we exclude strains containing glc7-1 mutations, there is good correlation between glycogen accumulation and the glucose-6-P level at late logarithmic phase (Fig. 6). Mutations in PGI1 provide a temporal correlation in that elevated glycogen levels are seen only during logarithmic growth, when glucose-6-P is high, whereas at late logarithmic or early stationary phase, when glucose-6-P has returned to wild-type levels, glycogen too is normal. We propose that the glucose-6-P concentration is one of the major positive signals for glycogen synthesis in yeast. However, not all defects in glycogen storage are overcome simply by elevating glucose-6-P, since neither pfk1 nor pfk2 mutations completely correct glycogen storage in a glc7-1 background, meaning that sensitivity to glucose-6-P levels is greater in snf1 mutants.

Does the low glucose-6-P level observed in snf1 cells account for their inability to accumulate glycogen? From the discussion of the preceding paragraph, this may be partly so. However, based on our results, we cannot exclude the possibility that other defects of snf1 strains contribute to the impaired glycogen synthesis. For example, two of the five ssg snf1 mutant strains (ssg2 and ssg3) retained the low glucose-6-P level associated with the parent snf1 strain, and the other three (ssg1, ssg4, and ssg5) had the sugar phosphate at wild-type levels or higher (data not shown). Mutation in PFK1 also falls into the latter category. Thus, suppressors of the glycogen defect of snf1 cells are of two types, those that elevate glucose-6-P and those that do not. Note that mutation of PHO85 (ssg3) was among those mutations that did not increase glucose-6-P in snf1 cells; therefore, Pho85p either acts downstream of changes in glucose-6-P concentration or else is involved in glucose-6-P-independent control of glycogen synthase phosphorylation.

Glucose-6-P levels appear not to affect other snf1 phenotypes, since high glucose-6-P levels in pfk2 snf1 double mutants did not restore growth on alternate carbon sources, induction of invertase, or the ability to sporulate. Also, the other ssg snf1 mutants with elevated glucose-6-P did not grow on alternate carbon sources (not shown).

Why snf1 cells have reduced glucose-6-P is not known with certainty. One possible explanation, at least for the more substantial glucose-6-P deficit seen during late logarithmic phase in these cells, is the impairment of gluconeogenesis that occurs in snf1 mutants. Expression of the genes encoding the gluconeogenic enzymes fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase is repressed by glucose (33). Derepression occurs as glucose is depleted from the medium and requires the activity of the Snf1p protein kinase (34). Thus, in wild-type cells, as glucose is depleted, gluconeogenesis can feed the glucose-6-P pool, whereas snf1 mutants would lack such a route for glucose-6-P production. Mitochondria are required for gluconeogenesis and, interestingly, Yang and Wek1 have found that a number of mutations affecting mitochondrial function also cause aberrant glycogen phenotypes, implying that mitochondria may be needed for proper glycogen synthesis. This finding is consistent with the old observation that petite strains are often impaired for glycogen storage (35).

Mechanistically, how does the glucose-6-P concentration regulate glycogen metabolism? First, glucose-6-P is a precursor of UDP-glucose, and hence increased availability of UDP-glucose could increase glycogen accumulation by mass action. Indeed, Corominas et al. (29) had ascribed the elevated glycogen levels in pgi1 mutants to this mechanism. While increased UDP-glucose levels may contribute to increasing the flux to glycogen in such instances, where both glucose-6-P and UDP-glucose are severely elevated, we would suggest that glucose-6-P also has a regulatory function. For example, elevated glucose-6-P levels in pfk glc7-1 mutants did not cause hyperaccumulation of glycogen, suggesting that more than mass action is involved in overaccumulating the polysaccharide. Second, glucose-6-P is a potent activator of glycogen synthase that can, as noted earlier, override the inactivating effects of phosphorylation. Thus, the instantaneous glucose-6-P concentration and the phosphorylation state combine to determine the activity of glycogen synthase (36). Third, glucose-6-P can control glycogen synthase by regulating the phosphorylation state of the enzyme. Using crude extracts of yeast, François and Hars (37) had proposed that glucose-6-P stimulated the activation of endogenous glycogen synthase by activation of a protein phosphatase. Here, we provide evidence that glucose-6-P inhibits a major glycogen synthase kinase activity present in yeast cells at concentrations that are physiologically relevant and that are certainly exceeded in some of the mutant strains examined in this study. Note that these two proposals are not mutually exclusive. Indeed, our work provides indirect support for the idea that glucose-6-P also affects glycogen synthase phosphatase. Genetically, glc7-1 is downstream of elevated glucose-6-P, since a glc7-1 mutation is epistatic to a pfk mutation as regards glycogen levels. Also, the fact that elevated glucose-6-P was less effective in suppressing a glc7-1 than a snf1 mutation would be consistent with the presence of a glucose-6-P-activated phosphatase. The snf1 mutants, possessing both protein kinase and phosphatase, might thus be more sensitive to increased glucose-6-P than glc7-1 cells containing only a glucose-6-P inhibitable kinase.

The mechanism by which glucose-6-P affects glycogen synthase kinase activity is not known and is the subject of ongoing study. First, we do not know whether the sugar phosphate acts by binding to the glycogen synthase or to the modifying enzyme(s). As regards protein kinases, at least two glycogen synthase kinases exist in yeast, one containing Pho85p as catalytic subunit. Future efforts will need to address the molecular basis by which glucose-6-P affects glycogen synthase phosphorylation.

1 R. Yang and R. C. Wek, unpublished results.
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