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Requirement of the Self-Glucosylating Initiator Proteins Glg1p and Glg2p for Glycogen Accumulation in *Saccharomyces cerevisiae*

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Glycogen, a branched polymer of glucose, is a storage molecule whose accumulation is under rigorous nutritional control in many cells. We report the identification of two *Saccharomyces cerevisiae* genes, *GLG1* and *GLG2*, whose products are implicated in the biogenesis of glycogen. These genes encode self-glucosylating proteins that in vitro can act as primers for the elongation reaction catalyzed by glycogen synthase. Over a region of 258 residues, the Glg proteins have 55% sequence identity to each other and ~33% identity to glycogenin, a mammalian protein postulated to have a role in the initiation of glycogen biosynthesis. Yeast cells defective in either *GLG1* or *GLG2* are similar to the wild type in their ability to accumulate glycogen. Disruption of both genes results in the inability of the cells to synthesize glycogen despite normal levels of glycogen synthase. These results suggest that a self-glucosylating protein is required for glycogen biosynthesis in a eukaryotic cell. The activation state of glycogen synthase in *glg1 glg2* cells is suppressed, suggesting that the Glg proteins may additionally influence the phosphorylation state of glycogen synthase.

The polysaccharide glycogen, a branched polymer of glucose, is widely distributed in nature and is thought to act as a storage molecule, accumulated in times of nutritional sufficiency for later utilization under conditions of deprivation (32). The bulk biosynthesis of glycogen involves chain elongation via the formation of linear α-1,4-glycosidic linkages catalyzed by glycogen synthase (EC 2.4.1.11) and the introduction of branchpoints through α-1,6-glycosidic linkages formed by glycogen branching enzyme (EC 2.4.1.18). From work directed primarily at mammalian systems (see references 41, 43, and 50 for reviews), there is evidence for a distinct initiation step in which a self-glucosylating protein, called glycogenin, grows a covalently attached oligosaccharide primer linked to a unique Tyr residue, Tyr-194 (7, 42). Once the oligosaccharide chain on the glycogenin has been extended sufficiently, glycogen synthase is able to catalyze elongation (7) and, together with the action of the branching enzyme, is thought to form a mature glycogen molecule.

Glycogen accumulation in the yeast *Saccharomyces cerevisiae* has been the subject of considerable study, and yeast counterparts to several of the relevant metabolic enzymes have been identified (for a recent review, see reference 17). *S. cerevisiae* has two genes, *GSY1* and *GSY2*, encoding glycogen synthase proteins that share 45 to 50% sequence identity with mammalian glycogen synthases (14, 15). Both mammalian and yeast glycogen synthases are inactivated by covalent phosphorylation, although full activity can be restored in the presence of the allosteric activator glucose-6-P (17, 41). The −/+ glucose-6-P activity ratio is, therefore, often used as a kinetic indicator of phosphorylation state, a decreased ratio corresponding to increased phosphorylation. The greatest divergence in sequence between the yeast and mammalian enzymes is at the NH_2 and COOH termini, which are implicated in the regulation by covalent phosphorylation (23). A single gene, *GLC3*, encodes a yeast homolog of glycogen branching enzyme (37, 48).

At the outset of this investigation, no yeast equivalent of glycogenin had been reported. None of the various glycogen-deficient mutants of *S. cerevisiae* studied so far (for example, see reference 6) carried defects in genes encoding products similar to glycogenin, and no self-glucosylating proteins with properties similar to glycogenin had been isolated from *S. cerevisiae*. The existence of such proteins in other simple unicellular organisms has been somewhat controversial (see reference 43), although there have been reports of proteins covalently linked to glycogen from *Escherichia coli* (2, 20) and *Neurospora crassa* (20, 45). In a recent study, the NH_2-terminal sequence of a 31-kDa glycogen-associated *N. crassa* protein was reported (20). There have also been descriptions of initiator proteins in plants (19, 29, 33, 34, 43), although this information so far is less detailed than that for mammalian glycogenin. Whether self-glucosylating proteins are commonly involved in polysaccharide biosynthesis, or indeed in other biological processes, remains an interesting but largely unanswered question. Indeed, even in mammals, there is no formal evidence to date that glycogenin in vivo is an obligate requirement for glycogen biosynthesis.

As part of ongoing studies on glycogen metabolism and its regulation in *S. cerevisiae*, we undertook a two-hybrid screen to identify genes encoding proteins that interact with the yeast glycogen synthase Gsy2p. Of the three positive clones identified, one derived from a gene encoding a protein, Glg2p, with several characteristics consistent with its being a self-glucosylating initiator of glycogen biosynthesis. In fact, we found that *S. cerevisiae* contains two such proteins, encoded by the *GLG1* and *GLG2* genes, and that loss of both genes was required to eliminate glycogen accumulation. This result provides the first demonstration that a self-glucosylating protein is required for glycogen biosynthesis in a eukaryotic cell. In addition, the −/+ glucose-6-P activity ratio of glycogen synthase was significantly reduced in *glg1 glg2* cells, suggesting an interplay between Glg proteins and glycogen synthase phosphorylation.

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MATERIALS AND METHODS

Yeast strains and methods. EG328-1A (*MAT*α *trp1 leu2 ura3-52*), which was used as a wild-type strain, has the genetic background of JC302-26B (5) and was obtained from Kelly Tatchell, Louisiana State University. Derived isogenic strains used in our work were DH3 (*MAT*α *trp1 leu2 ura3-52 gsy1*::*LEU2 gsy2*::*URA3*), CC4 (*MAT*α *trp1 leu2 ura3-52 glg1-1*::*LEU2*), CC8 (*MAT*α *trp1 leu2 ura3-52 glg1-2*::*LEU2*), CC6 (*MAT*α *trp1 leu2 ura3-52 glg2*::*URA3*), CC7 (*MAT*α *trp1 leu2 ura3-52 glg1-1*::*LEU2 glg2*::*URA3*), and CC9 (*MAT*α *trp1 leu2 ura3-52 glg1-2*::*LEU2 glg2*::*URA3*). JC482 (*MAT*α *leu2 ura3 his4*) and JC889-14B (*MAT*α *leu2 ura3 can1 his3 trp1 glc3*::*TRP1*) were from John Cannon, University of Missouri. For the two-hybrid screening, strain YPB2 [*MAT*α *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 can1*^{R} *gal4-542 gal80-538 LYS2*::*GAL1-HIS3 URA3*(GAL 17-mers)-*lacZ*] was used (10). Yeast genomic DNA was purified by the method of Hoffman and Winston (24). Routine transformation of *S. cerevisiae* followed the lithium acetate method described by Schiestl and Gietz (38). Cell numbers were determined by counting 100 to 200 cells in a hemocytometer. Other standard methods for yeast genetic analysis were as described previously (35).

Plasmids. The plasmids pGBT9-GSY2, pGBT9-GSY2ΔC, and pGBT9-GSY2ΔN, used for two-hybrid analyses, express Gsy2p sequences fused to the DNA binding domain of Gal4p. The plasmid pGBT9-GSY2 was generated by inserting an *Eco*RV-*Bam*HI *GSY2* fragment between the *Eco*RV and *Bam*HI sites of pGBT9 (10) so that the entire Gsy2p coding region (residues 1 to 704) was fused to the Gal4p DNA binding domain. To construct pGBT9-GSY2ΔN, an *Eco*RI-*Sma*I *GSY2* fragment, generated by PCR and containing the COOH terminus of Gsy2p (residues 562 to 704), was inserted between the *Eco*RI and *Sma*I sites of pGBT9. The pGBT9-GSY2ΔC plasmid was formed from pGBT9-GSY2 by deletion of the *Ecl*136II-*Sma*I fragment corresponding to the COOH terminus of Gsy2p. Plasmid pGBT9-GSY2ΔC contains residues 1 to 623 of Gsy2p. The plasmid, pRS314-GLG1, was constructed by ligating a 2.7-kb *Hin*dIII-*Hin*dIII fragment containing *GLG1* and 116 upstream residues into the *Hin*dIII site of pRS314 (40). Similarly, pRS314-GLG2 was generated by cloning a 3.7-kb *Bam*HI-*Hin*dIII fragment containing *GLG2* and 2 kb of 5′ sequence into the *Bam*HI-*Hin*dIII sites of pRS314.

For the expression of mammalian glycogenin in *S. cerevisiae* the coding region of rabbit skeletal muscle was excised from the corresponding cDNA (49) as a *Not*I-*Xba*I fragment and the ends were filled. The plasmid pYcDE2 (21) was cut with *Eco*RI, the ends were filled in, and the fragment bearing glycogenin was inserted by blunt-ended ligation to generate plasmid pYcDE2-GN (constructed by Thomas A. Hardy). Expression of glycogenin in pYcDE2-GN is under the control of the constitutive *ADH1* promoter.

For expression of Glg1p and Glg2p as polyhistidine tagged proteins in *E. coli*, the pET28a(+) vector (Novagen) was used. To make the Glg1p construct, we first used PCR to generate a 196-bp fragment that begins at the start codon and extends to an *Nhe*I site contained within the coding sequence. The 3′ segment of the coding region was independently excised from *GLG1* DNA as a 2,325-bp *Nhe*I-*Hin*dIII fragment. The entire coding sequence was then reconstituted within a 2.5-kb *Nde*I-*Hin*dIII fragment via ligation and inserted into pET28a cut with *Nde*I and *Hin*dIII. The resulting plasmid, pET28-GLG1, encodes a fusion protein containing the NH_2-terminal sequence MGSSHHHHHHSSGLVPRG SH followed by the Glg1p sequence, including the initiator Met residue. The Glg2p construct was created by a similar approach that took advantage of an internal *Afl*III restriction site. Thus, PCR generated a 482-bp *Nde*I-*Afl*III fragment that was combined with a 1,271-bp *Afl*III-*Hin*dIII fragment containing the 3′ segment of the coding sequence. The 1.7-kb *Nde*I-*Hin*dIII fragment containing the *GLG2* coding sequence was ligated into pET28a cut with *Hin*dIII and *Nde*I. The resulting plasmid, pET28-GLG2, encodes the Glg2p sequence with the same NH_2-terminal extension as noted above with respect to Glg1p. In the final plasmid constructs, all segments initially generated by PCR were confirmed by sequencing.

Yeast two-hybrid analysis. The system of Chien et al. (10) was used to search for proteins capable of interacting with Gsy2p sequences, largely following the protocol of Durfee et al. (13). With the plasmid pGBT9-GSY2, a yeast library in the pGAD vector (10) was screened in strain YPB2. From 3.6×10^5 Leu^+ Trp^+ transformants, 26 3-amino-1,2,4-triazole-resistant colonies were positive on a filter β-galactosidase assay (9). These positive colonies were grown to saturation nonselectively in yeast extract-peptone-dextrose liquid medium and plated onto yeast extract-peptone-dextrose plates. Replica plating to SD-Leu and SD-Trp plates identified colonies that had lost one or both plasmids. Colonies that had evicted the pGBT9-GSY2 plasmid but were still positive by the filter β-galactosidase assay were eliminated. The pGAD plasmids were purified from the remaining three colonies and retransformed into *S. cerevisiae* carrying pGBT9-GSY2. All three colonies were confirmed to be positive by β-galactosidase assay. A similar two-hybrid screen with only the COOH terminus of Gsy2p in pGBT9-GSY2ΔN did not result in the identification of any positive clones. At the later stages of the screening, quantitative β-galactosidase solution assays (31) were also performed.

Analysis and sequencing of *GLG1* and *GLG2* genes. A PCR product corresponding to the 1.4-kb insert in pGAD-GLG2 was used to hybridize the Olson-Riles filters, a set of cloned fragments representing most of the yeast genome (30). Hybridizing fragments from both chromosome X and chromosome XI were identified. *GLG2* was found to be located on the left arm of chromosome X in a 3.7-kb *Bam*HI-*Hin*dIII fragment of clone λPM6669 which was subcloned into pRS314. In fact, cross-hybridization of fragments from chromosome XI is almost certainly explained by the fact that our probe also contained a portion of flanking gene, *TIF2* (see Results). The entire *GLG2* coding region as well as 145 bp of upstream sequences was sequenced. *GLG1* is on the right arm of chromosome XI and corresponds to the open reading frame *YKR058w*. The 5′ region of *YKR058w* was sequenced from a 2.7-kb *Hin*dIII-*Hin*dIII fragment of clone λPM6477 subcloned into pRS314.

Disruption of *GLG1* and *GLG2* genes. Disruption of the *GLG* loci in yeast cells was achieved by one-step homologous recombination (36). PCR was used to generate DNA fragments from primers that contained 45 bp of target gene sequence followed by 21 bp that matched pBluescript sequences straddling the chosen marker gene in an appropriate pRS plasmid (40). Thus, a 934-bp fragment containing the *LEU2* gene was amplified from pRS305 for *GLG1* disruption and a 1,248-bp fragment containing the *URA3* gene was amplified from pRS306 for *GLG2* disruption. The PCR mixture contained, in 100 μl, 100 pmol each of sense and antisense primer (see below), 0.5 μg of template DNA (pRS305 or pRS306), 5 U of *Taq* DNA polymerase (Perkin Elmer), 200 μM each dATP, dCTP, dGTP, and dTTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 1.5 mM $MgCl_2$. Amplification was in a GeneAmp PCR System 9600 (Perkin Elmer) with a single denaturation step at 95°C for 2 min followed by five low stringency cycles (95°C for 30 s, 56°C for 1 min, and 72°C for 8 min), 25 high stringency cycles (95°C for 30 s, 70°C for 8 min), and a final extension for 10 min at 72°C. The PCR products were gel purified with a GeneClean kit (Bio 101) and used to transform yeast cells by the lithium acetate method (38). Transformants were selected for the desired marker, and the disruption was confirmed by restriction mapping and Southern analysis of the genomic DNA. In our experiments, from 10 to 50% of the transformants carried the disruption. In the case of *GLG1*, primer design was initially based on the original *YKR058w* open reading frame so that the sequence deleted corresponded to amino acids 154 to 603. The primers used were ATG TCT AAA CAA ACC ACG TCA CAA GTG GGT GCT ATT GCT GAT ATC AGC AGA TTG TAC TGA GAG TGC (sense) and CTC TAC TGC ATA CTC AAA AAT ATC ATC AGG GAA ACA CCT CTC TAC CAT CTG TGC GGT ATT TCA CAC (antisense). In this way, we generated what we have termed the *glg1-1* allele. Once it was realized that the *GLG1* open reading frame contained additional sequences 5′ to the deletion in *glg1-1*, a second disruption was made by the same technique, this time eliminating the entire coding sequence to generate the *glg1-2* allele. The primers used were GAG GTG CTA TAT ATG TCG ATA AGA AGC AGA GTA TAA GGG TGC CTG AGC AGA TTG TAC TGA GAG TGC (sense) and CTC TAC TGC ATA CTC AAA AAT ATC ATC AGG GAA ACA CCT CTC TAC CAT CTG TGC GGT ATT TCA CAC (antisense). For *GLG2*, the entire coding sequence was deleted with the primers TCA TTT TTT TGA ATA TCT ATC AGA AGT AAG GTT TGT TAC GGA GCT AGC AGA TTG TAC TGA GAG TGC (sense) and TTT CGA AAA AAA AGA AAA AGG TAC GCA AAA TCA ACG CTT TCA CCC CAT CTG TGC GGT ATT TCA CAC (antisense). The single disruptants were generated from a haploid EG328-1A wild-type strain and double disruptants by sequential application of the same technique.

Analysis of glycogen and glycogen synthase in yeast cultures. *S. cerevisiae* was grown in 1-liter cultures of the indicated medium in 2-liter flasks at 30°C from a saturated overnight culture, aliquots were removed at the indicated times, and the cells were harvested by centrifugation and stored at −80°C until analysis. The frozen cell pellets were resuspended in 0.3 ml of a solution of 50 mM Tris-HCl, 1 mM EDTA, 3 mM dithiothreitol, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 5 mM benzamidine, 0.25 μg of leupeptin per ml, and 0.5 μg of aprotinin per ml, pH 7.5. Cells were broken with glass beads and the glycogen levels were determined enzymatically, as described previously (22). Glycogen synthase activity, in the absence or presence of 7.2 mM glucose-6-P, was measured by the method of Thomas et al. (47). A unit of glycogen synthase activity transfers 1 μmol of glucose from UDP-glucose to glycogen per min under conditions of the standard assay.

Purification and analysis of Glg1p and Glg2p. Glg1p and Glg2p were expressed as His-tagged proteins from plasmids pET28-GLG1 or pET28-GLG2, respectively, in *E. coli* BL21/DE3. Typically, cultures of 400 ml were grown at 37°C and induced for 3 h with 0.1 mM isopropyl β-D-thiogalactopyranoside. Cells were collected by centrifugation and suspended in homogenizing buffer (10 ml/g of cells) containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 2 mM benzamidine, 0.5 mM 2-mercaptoethanol, and 0.5% (vol/vol) Triton X-100, pH 7.4. The cells were broken by passage through a French pressure cell at 1,000 lb/in^2. The supernatant, after centrifugation at $10,000 \times g$ for 25 min, was collected and mixed for 1 h at 4°C with Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen). The resin was collected by centrifugation and poured to form a column (~1 ml) that was washed with 10 ml of buffer A (20 mM HEPES, 0.5 M NaCl, 10 mM 2-mercaptoethanol, pH 7.4) plus 2.5 mM histidine, followed by 10 ml of buffer A plus 10 mM histidine. The bound protein was finally eluted with buffer A plus 100 mM histidine. For analysis of self-glucosylation, Glg1p, Glg2p, and glycogenin, each at 40 μg/ml, were incubated for 5 min with 15 μM UDP-[U-^{14}C]glucose (specific activity, 263 Ci/mmol) at 30°C as described previously (7). After incubation, the proteins (0.2 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE) (12% acrylamide), stained with Coomassie blue, and subjected to autoradiography. The efficacies of the proteins as substrates for glycogen synthase were tested by first allowing the proteins to self-glucosylate for 30 min and then adding purified Gsy2p (final concentration, 0.12 mg/ml) for 120 min or mammalian glycogen synthase (final concentration, 0.11 mg/ml) for 45 min together with unlabeled UDP-glucose to a concentration of 27 mM. Samples were analyzed by SDS-PAGE followed by autoradiography.

Sequence analysis and database searching. Routine manipulation of DNA and protein sequences utilized the GeneWorks program (Intelligenetics). Multiple alignments were made with the MACAW program (39). Database searches were run either locally against the yeast protein sequence database maintained by M. Goebl or through the Internet against GenBank databases via the National Center for Biotechnology Information home page.

Other materials and methods. His_6-tagged rabbit muscle glycogenin was prepared as described by Cao et al. (8). Mammalian glycogen synthase was purified from rabbit muscle as described previously (1). His_6-tagged Gsy2p was expressed from a pET28 vector and purified to close to homogeneity in a manner similar to that described for Glg1p and Glg2p (16). Protein was measured by the method of Bradford (4). The SDS-PAGE performed was a modification of the method of Laemmli (25). The method of synthesis of UDP-[U-^{14}C]glucose was modified from that of Tan (46).

Nucleotide sequence accession numbers. The sequences of the *GLG1* and *GLG2* genes are assigned the GenBank accession numbers U25546 and U25436, respectively.

RESULTS

Identification of the *GLG1* and *GLG2* genes. In a search for proteins involved in yeast glycogen biosynthesis, we employed a two-hybrid screen to identify genes whose products are capable of interacting with glycogen synthase. We chose Gsy2p, the predominant yeast glycogen synthase, to fuse to the Gal4p DNA binding domain for the screening of a library expressed from the pGAD vector, as described in Materials and Methods. Three positive clones were identified. One positive clone, now named pGAD-GLG2, expressed the 47 COOH-terminal amino acids of a protein encoded by what we ultimately designated *GLG2* (for glycogenin-like gene). The characterizations of the other two positive clones, derived from genes *PIG1* and *PIG2* (for protein interacting with Gsy2p), will be described elsewhere; Pig1p and Pig2p have limited sequence identity with Gac1p (18). The partial *GLG2* sequence at the time had no match in GenBank, and we went on to identify and sequence the entire *GLG2* gene, which was located on chromosome X (Fig. 1). The sequence of this gene has recently appeared independently in GenBank as SCX731_18 (accession no. X87371), predicting a protein sequence identical to what we obtained for Glg2p. While the *GLG2* gene was being analyzed, the sequence of yeast chromosome XI (12) became available and we found that an open reading frame on chromosome XI, corresponding to *YKR058w*, had similarity both to mammalian glycogenin and to the Glg2p sequence. We propose renaming *YKR058w* as *GLG1*. As published, *YKR058w* would encode a protein significantly truncated at the NH_2 terminus compared with that of Glg2p. After independently determining the sequence of the 5′ region of Glg1p, we found two differences (a one-base insertion and a one-base deletion) from the published *YKR058w′* sequence. These changes would NH_2 terminally extend the coding region for Glg1p, with a sequence highly homologous to the corresponding region of Glg2p (Fig. 1). The *GLG1* and *GLG2* genes are located immediately upstream of the highly conserved *TIF1* and *TIF2* genes, respectively (27), suggesting the occurrence of a gene duplication event involving multiple genes. This conserved juxtaposition with respect to the *TIF* genes also explains why our initial probe, which contained both *GLG2* and *TIF2* sequences, hybridized strongly with fragments from both chromosome X and chromosome XI; the DNA sequences corresponding to the coding regions of the *TIF* genes are >99% identical.

GLG1 and *GLG2* encode proteins of 618 and 380 residues, respectively, that have 55% sequence identity over their NH_2-terminal 258 amino acids (Fig. 1). In the COOH termini, there are two small segments of sequence similarity. One section, with 13 of 19 identities between the two proteins, lies in the region of Glg2p identified in the two-hybrid screen and hence in the region likely to be responsible for interaction with glycogen synthase. The sequences of Glg1p and Glg2p are 33 and 34% identical, respectively, to that of rabbit muscle glycogenin in the NH_2-terminal region (49), and each contains a Tyr residue in correspondence with Tyr-194, the unique site of carbohydrate attachment in the mammalian protein (7, 42).

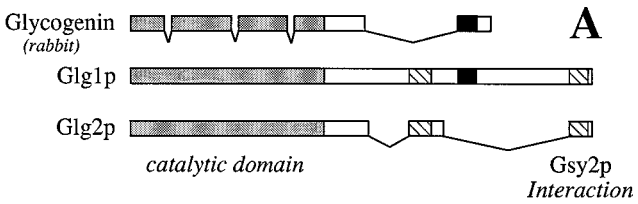

FIG. 1. Comparison of the structures of Glg1p, Glg2p, and rabbit muscle glycogenin. (A) Diagram indicating the disposition of conserved regions referred to Glg1p, which is the longest of the three proteins. Regions of similarity among all three proteins, between Glg1p and Glg2p, and between Glg1p and glycogenin are indicated by shaded, hatched, and black boxes, respectively. (B) Alignment of primary sequences of all three proteins. The alignment was made with the MACAW program (39). Regions where two or more residues are identical are boxed. The arrowheads point to a series of conserved hydrophobic residues present every seventh residue as in leucine zipper motifs. GN, rabbit muscle glycogenin.

Glg2p interactions with glycogen synthase. Interaction between full-length Gsy2p, expressed from the pGBT9-GSY2 plasmid, and the two-hybrid positive clone encoded by pGAD-GLG2 was monitored by qualitative filter β-galactosidase assays during screening. After isolation and characterization of the pGAD-GLG2 plasmid, solution β-galactosidase assays were also performed. In addition, other constructs of Gsy2p were tested by this assay. Protein lacking the COOH-terminal 81 residues of Gsy2p, encoded by pGBT9-GSY2ΔC, tested positively for interaction with Glg2p, whereas the COOH terminus alone, encoded by pGBT9-GSY2ΔN, gave no signal above background (Fig. 2). The latter result is consistent with a failure to identify any consistently positive clones in a screen of the library with pGBT9-GSY2ΔN. Western hybridization (immunoblotting) with antibodies raised against a synthetic peptide based on COOH-terminal Gsy2p sequences detected protein expressed from pGBT9-GSY2 and pGBT9-GSY2ΔN (data not shown). Therefore, the failure of the COOH terminus alone to test positive for interaction with Glg2p was presumably not for lack of expressed Gsy2p fusion protein. We conclude that the COOH terminus of Gsy2p is not essential for the interaction with Glg2p.

Glycogen metabolism in strains disrupted in the *GLG1* and/or *GLG2* genes. In order to establish whether *GLG1* and *GLG2* influence glycogen storage, the genes were disrupted by homologous recombination, as described in Materials and Methods. For *GLG1*, we generated two disruptants, the first one based on the original *YKR058w* sequence, in which the sequence encoding residues 154 to 603 was removed (*glg1-1* allele), and a later one based on our final Glg1p sequence, in which the entire coding sequence was removed (*glg1-2* allele). In *glg2* cells, the entire *GLG2* coding region was excised. Disruption of either *GLG1* or *GLG2* led to little change in glycogen accumulation compared with wild-type levels, as determined by iodine staining of colonies (data not shown) or by enzymatic measurement of glycogen levels (Fig. 3). In the case

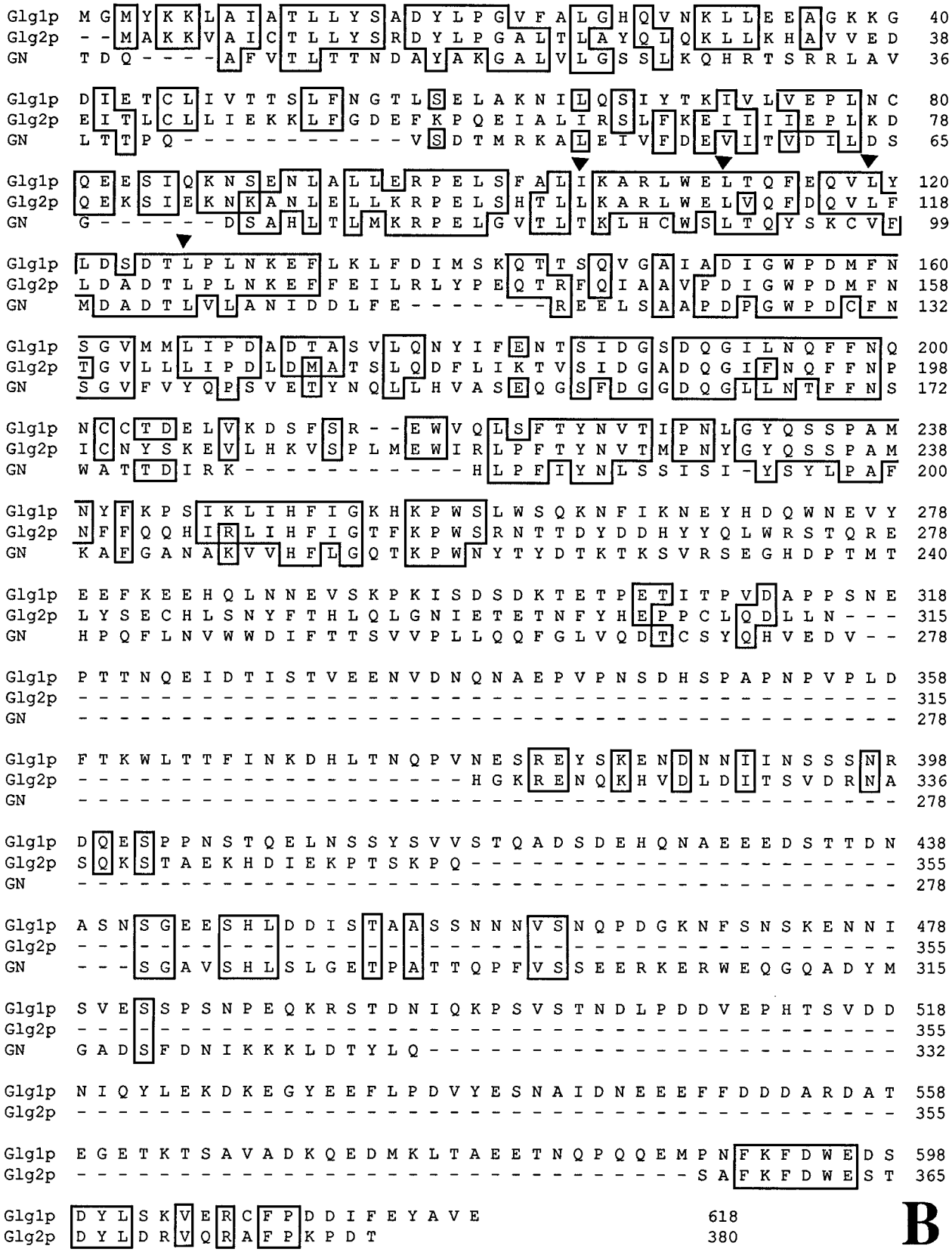

FIG. 1—*Continued.*

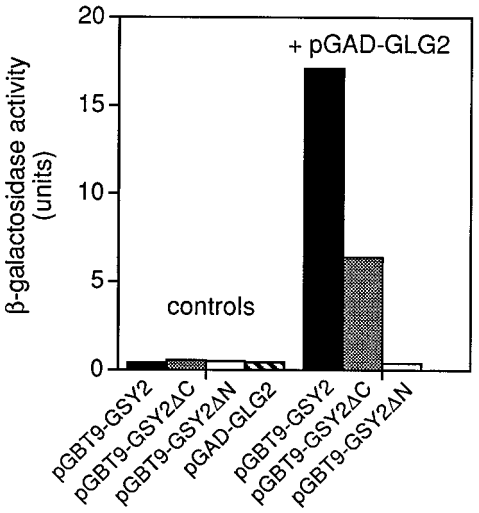

FIG. 2. Two-hybrid analysis of Glg2p interaction with Gsy2p. The clone pGAD-GLG2 was tested in conjunction with plasmids encoding full-length (pGBT9-GSY2), COOH-terminally truncated (pGBT9-GSY2ΔC) or NH_2-terminally truncated (pGBT9-GSY2ΔN) Gsy2p. Interaction was estimated by measurement of β-galactosidase activity. As controls, yeast cells expressing the indicated single plasmids were analyzed.

of *GLG1* disruptions, there was no significant difference in behavior between the *glg1-1* and *glg1-2* alleles. Loss of both *GLG* genes correlated with a severe deficit in glycogen storage (Fig. 3). This result was the same irrespective of which *glg1* allele was present, arguing that the presence of only the 5′ end of *GLG1* was insufficient for function. The glycogen deficiency was comparable to that observed in strains lacking glycogen synthase (15) (Fig. 3). The deficit in glycogen stores in the *glg1 glg2* strain could be overcome by extrachromosomal expression of either Glg1p or Glg2p from a low-copy-number plasmid (Fig. 3). Furthermore, expression of rabbit muscle glycogenin (pYcDE2-GN) caused a substantial restoration of glycogen levels, indicating functional complementation by the mammalian protein.

Glycogen and glycogen synthase levels were monitored as a function of growth in strains defective at the *GLG* loci (Fig. 4). Loss of *GLG* gene function had no significant effect on growth beyond normal variability in liquid cultures. Consistent with the results noted above, *glg1* or *glg2* strains accumulated glycogen upon approach of stationary phase. This deposition of glycogen was absent, however, in *glg1 glg2* cells. The amount of glycogen synthase, as reflected by the total glycogen synthase activity measured in the presence of the allosteric activator glucose-6-P, was not seriously affected by mutation at the *GLG* genes and increased through logarithmic growth and into the stationary phase (Fig. 4C). This was true also for the *glg1 glg2* strain. Therefore, lack of glycogen synthesis was not due to the absence of glycogen synthase. Besides control at the level of expression, yeast glycogen synthase is thought to be regulated by covalent phosphorylation (see reference 17). The best-studied example of this is Gsy2p, which is postulated to undergo phosphorylation at three COOH-terminal sites, which decreases the −/+ glucose-6-P activity ratio (23). In wild-type cells, the −/+ glucose-6-P activity ratio increased slightly with growth and then decreased by about 50% over the period during which glycogen was accumulating; however, the activity ratio did not fall below 0.15 (Fig. 4D). In the *glg1 glg2* strain, the glycogen synthase activity ratio was always lower than that in controls. The most significant difference was a severe fall in activity ratio, almost reaching zero, over the period during which the wild-type strain was synthesizing glycogen. Since the *glg1 glg2* cells were the only ones in the experiment to lack glycogen, we were concerned that the absence of glycogen somehow affected the measurements of glycogen synthase activity ratio. Thus, we tested whether simply adding back glycogen to the homogenizing buffer would result in a higher activity ratio for glycogen synthase. There was no significant effect, arguing against posthomogenization effects of glycogen on the analysis (data not shown). To determine whether the absence of glycogen inside the cell influenced glycogen synthase activity, we took advantage of the fact that *glc3* cells, defective in the branching enzyme, are unable to accumulate glycogen (37, 48). Both the level of glycogen synthase total activity and the activity ratio were somewhat elevated in the *glc3* strain compared with those of a control, even though glycogen accumulation was severely impaired (Table 1). We conclude, therefore, that the absence of glycogen alone did not result in a suppressed glycogen synthase activity ratio. Thus, the results described above suggest a novel connection between the presence of the Glg proteins and the control of glycogen synthase via phosphorylation.

Properties of Glg1p and Glg2p proteins. Glg1p and Glg2p were expressed in *E. coli* as fusion proteins with NH_2-terminal polyhistidine tracts and purified by nickel-chelate chromatography (Fig. 5). When analyzed by SDS-PAGE, Glg2p ran as an almost homogeneous species of 50 kDa, which is close to the expected mass of the fusion protein. Similar analysis of Glg1p revealed several species, the largest having an apparent M_r of 117,000. Most likely, Glg1p is susceptible to proteolysis. Since purification utilized the NH_2-terminal tag, such proteolysis presumably must have occurred towards the COOH terminus.

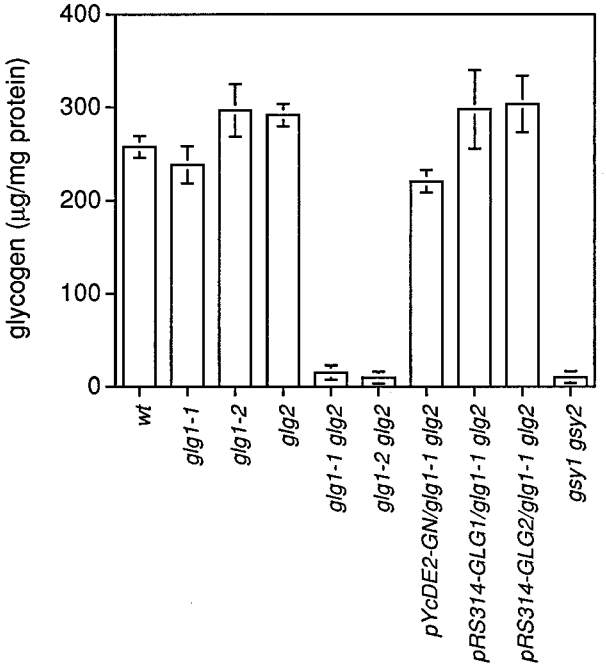

FIG. 3. Requirement of *GLG* genes for glycogen accumulation in *S. cerevisae.* Yeast strains with the indicated genetypes were grown for 2 days on SD medium to a density of 1×10^8 to 2×10^8 cells per ml, and glycogen accumulation was determined enzymatically as described in Materials and Methods. The relevant genotype of each yeast strain as well as that of any transforming plasmid is indicated. Shown are the averages and standard deviations (bars) of three or four independent experiments. wt, wild type.

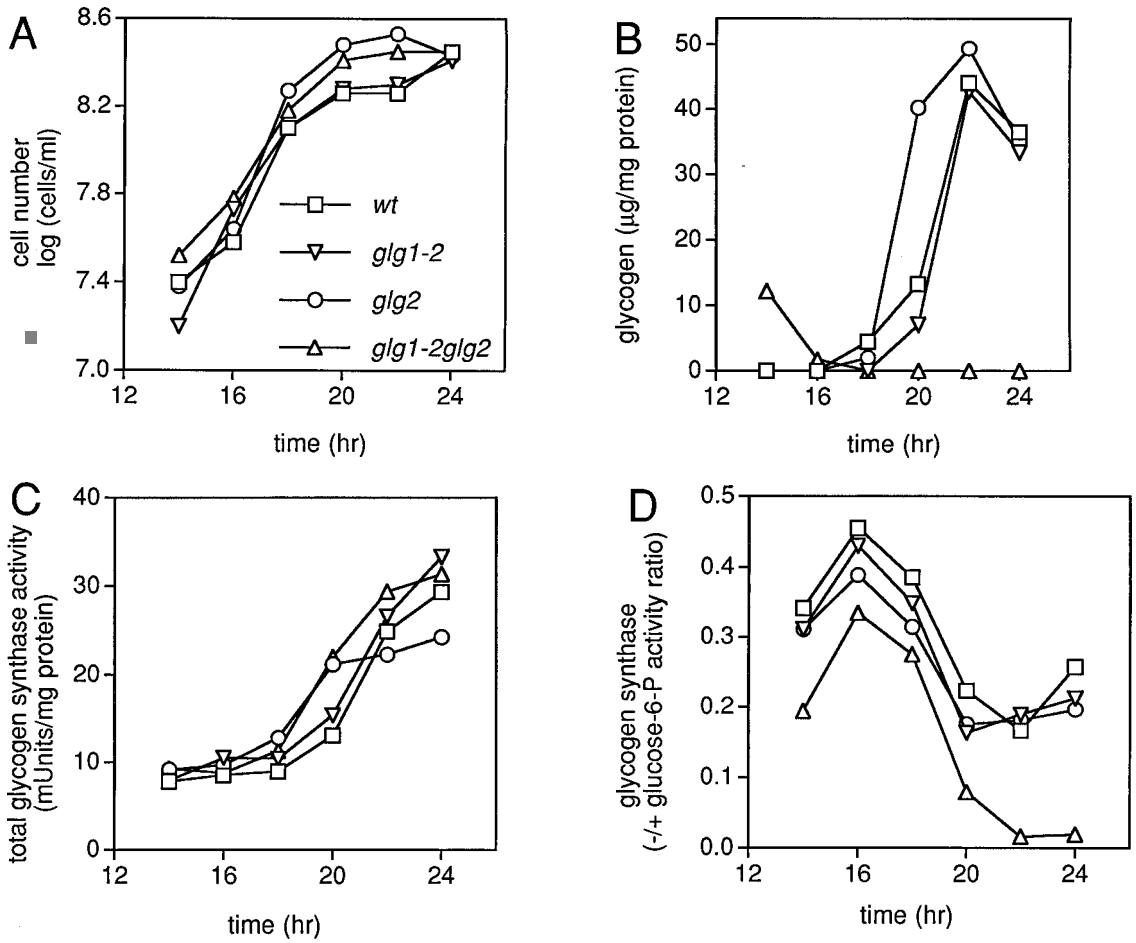

FIG. 4. Effects of *GLG* genes on glycogen and glycogen synthase as a function of growth. Liquid cultures of wild-type (wt), *glg1-2*, *glg2*, or *glg1-2 glg2 S. cerevisiae* were grown, as described in Materials and Methods. The cell number (A), glycogen accumulation (B), total glycogen synthase activity, i.e., that measured in the presence of glucose-6-P (C), and glycogen synthase −/+ glucose-6-P activity ratio (D) were measured at the indicated times. Note that the absolute levels of glycogen accumulation are lower than those reported in Fig. 3, reflecting growth in rich medium as opposed to that on the selective media needed for the experiments of Fig. 3. The detectable glycogen level seen at 14 h in the *glg1-2 glg2* cells was not reproducible. Symbols for all panels are defined in panel A.

The predicted M_r for the full-length fusion protein is only 72,000, indicating that Glg1p has abnormal electrophoretic mobility. Treatment of the protein with α-amylase caused only a small increase in mobility (data not shown), suggesting that the aberrant behavior was not due to attachment of large amounts of glucose in glycosidic linkages. Glucose transfer catalyzed by mammalian glycogenin effectively utilizes UDP-glucose as the glucosyl donor (43, 50). When incubated with UDP-glucose and Mn^{2+}, both Glg1p and Glg2p were capable of self-glucosylation (Fig. 5). As a positive control, the self-glucosylation of mammalian glycogenin is also shown. In the case of Glg1p, multiple glucosylated species were observed, essentially in correspondence with the polypeptides seen after Coomassie blue staining. The smallest self-glucosylated Glg1p species was ~35 kDa. To test whether the glucosylated proteins could serve as substrates for elongation by glycogen synthase, purified glycogen synthase, either yeast or mammalian, was added after an initial self-glucosylation stage (Fig. 6). Together with the glycogen synthase, a high concentration of unlabeled UDP-glucose was also added. The resulting reduction in UDP-glucose-specific activity in the elongation reaction means that the label is essentially tracking the initial glucose residues introduced at the self-glucosylation stage, and hence, the glycogenin or Glg protein. When Gsy2p was added, species with significantly reduced electrophoretic mobilities were observed. Virtually all of the 50-kDa Glg2p polypeptide was converted to very low mobility forms. With Glg1p, only the largest, 117-kDa species appeared to participate in the elongation reaction, suggesting that loss of the extreme COOH terminus reduced its ability to serve as a glycogen synthase substrate. Mammalian glycogenin was, by comparison, a poor substrate for elongation by Gsy2p. Rabbit muscle glycogenin,

TABLE 1. Glycogen synthase and glycogen in cells lacking branching enzyme[a]

Relevant genotype (strain)	Glycogen (μg/mg of protein)	Glycogen synthase	
		Total activity (mU/mg)	Activity ratio
Wild type (JC482)	49.3	27.6	0.19
glc3 (JC889-14B)	0.2	35.9	0.25

[a] Cells were grown on yeast extract-peptone-dextrose in liquid culture to a density of ~10^8 cells per ml. Glycogen and glycogen synthase were determined as described in Materials and Methods.

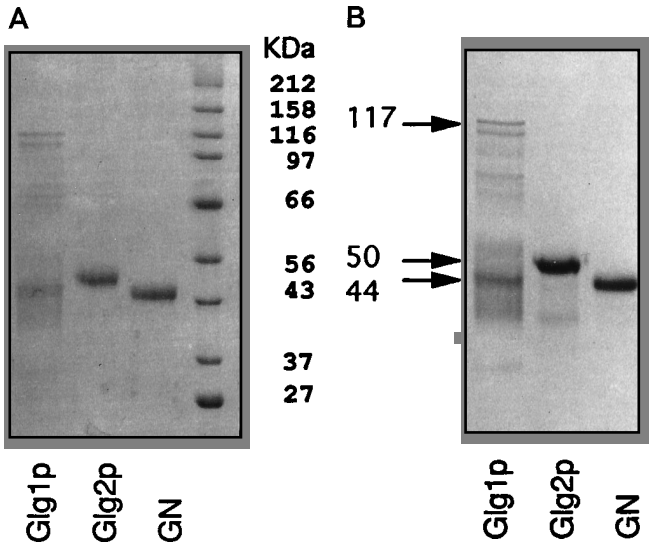

FIG. 5. Self-glucosylation of Glg1p, Glg2p, and glycogenin (GN). The purified proteins were incubated for 5 min with UDP-[U-^{14}C]glucose as described in Materials and Methods. Samples (0.2 μg) were separated by SDS-PAGE (12% acrylamide) and stained with Coomassie blue (A) or subjected to autoradiography (B). The numbers show the molecular masses of standards (A) or the apparent masses (in kilodaltons) of Glg1p, Glg2p, and glycogenin (B).

Glg1p, and Glg2p were all substrates for the mammalian glycogen synthase, and the reductions in polypeptide mobility are seen in Fig. 6B. Rabbit muscle glycogenin was the preferred substrate, as is more clearly seen at earlier times of reaction (data not shown). Interestingly, the mammalian glycogen synthase seemed not to discriminate among the full-length Glg1p and the degraded forms of the protein, almost all of which were subject to elongation.

DISCUSSION

The outcome of this investigation is to define two *S. cerevisiae* genes as encoding self-glucosylating proteins with roles in glycogen metabolism. These proteins appear to be yeast homologs of the mammalian protein glycogenin, which has been postulated to act in the initiation of glycogen biosynthesis in mammals. This conclusion is based on (i) sequence similarities among Glg1p, Glg2p, and glycogenin, (ii) the ability of all three proteins to self-glucosylate, with UDP-glucose as the glucosyl donor, (iii) the ability of all three proteins to serve as substrates for elongation by glycogen synthase, and (iv) the loss of glycogen accumulation in strains lacking Glg1p and Glg2p together with the ability of rabbit glycogenin to complement this defect. The existence of two distinct genes, each sufficient to sustain glycogen synthesis, can explain why the *GLG* genes were not previously identified by the screening of mutants defective in glycogen (6).

TABLE 2. Some properties of Glg1p, Glg2p, and glycogenin

Protein	Chromosome no.	No. of residues	Mol wt[a]	pI[a]	% Acidic residues (D + E)	% Asn
Glg1p	XI	618	69,836	3.95	18.4	9.31
Glg2p	X	380	44,495	5.60	13.9	4.48
Glycogenin (rabbit)		332	37,224	4.84	12.0	3.38

[a] Molecular weights and isoelectric points were predicted from primary structure with the GeneWorks 2.4 program (Intelligenetics).

Glg1p and Glg2p, like mammalian glycogenins, are acidic proteins (Table 2) that are also relatively hydrophilic. In Glg1p, 18.4% of the residues are Asp or Glu. Glg1p differs from Glg2p in being significantly longer (Fig. 1) as well as containing an unusually high proportion, 9.3%, of Asn residues. The two proteins each have a conserved NH_2-terminal region, stretching through residue 258, that, accounting for inserts, aligns with mammalian glycogenins and presumably contains the glucosyl transferase domain. Though contained in this region of overall similarity, the unique site of glucosylation of rabbit muscle glycogenin, Tyr-194, is in a stretch of poor local homology. Nonetheless, a Tyr common to both Glg1p and Glg2p, Tyr-232, can be aligned with Tyr-194. Interestingly, a fragment of Glg1p as small as 35 kDa was glucosylated in

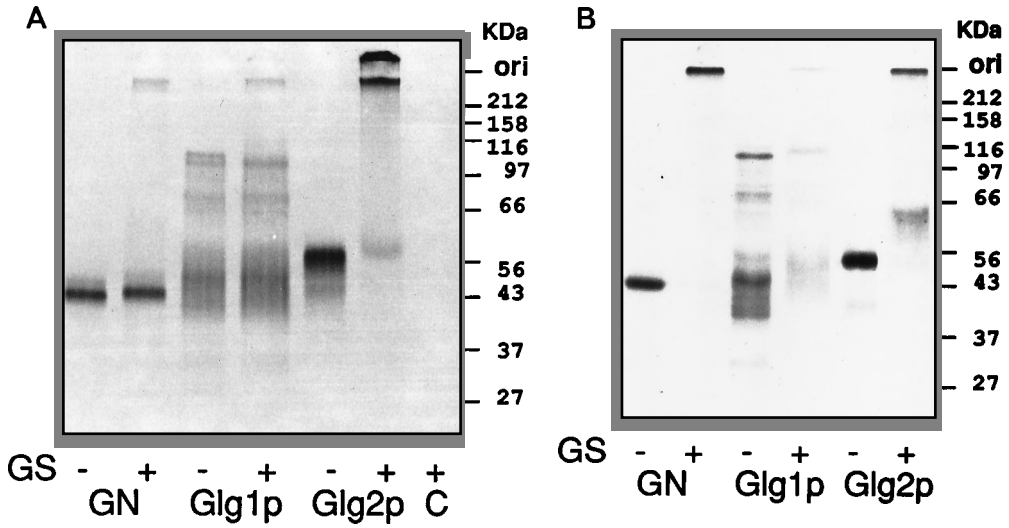

FIG. 6. Ability of Glg1p, Glg2p, and glycogenin (GN) to serve as substrates for yeast or mammalian glycogen synthase (GS). Samples of the indicated proteins were first allowed to self-glucosylate prior to the addition of purified Gsy2p (A) or mammalian glycogen synthase (B), as indicated by the plus sign. At the same time, an excess of unlabeled UDP-glucose was added and the incubation continued for another 120 min. A minus sign indicates the addition of only buffer and UDP-glucose. Samples (0.2 μg) were analyzed by SDS-PAGE followed by autoradiography. Track C is a control containing only Gsy2p. The numbers show the molecular masses and migration of protein standards.

self-glucosylation experiments, and on the assumption that the cleavage was COOH terminal, at least one site of glucose attachment would then lie in the NH_2-terminal portion of, say, ~300 residues. However, it remains to be seen whether Tyr-232 is in fact modified in the yeast proteins. Within the catalytic region, four Leu or Ile residues in the Glg proteins and three Leu or Val residues in mammalian glycogenins are disposed in what might be a minimal leucine zipper motif (26). No such role in determining protein-protein interactions has been established for either glycogenin or the Glg proteins, although it has been proposed that glycogenin can dimerize (8, 44). Outside the presumed catalytic domain, there are two small segments of sequence similarity between Glg1p and Glg2p (Fig. 1). One of these, at the extreme COOH terminus, appears to be involved in interaction with glycogen synthase, as discussed below. The significance of the large insert in Glg1p is unknown, but it does contain a short region of similarity to rabbit but not human glycogenin.

The yeast Glg proteins and rabbit glycogenin can be elongated by glycogen synthase from either species, consistent with the observation that rabbit glycogenin could restore glycogen accumulation to *glg1 glg2* cells. We conclude that basal elongation by glycogen synthase can occur irrespective of the origin of the glycogenin, perhaps simply by interaction with the growing carbohydrate chain. However, the results would argue that more specific determinants exist. For example, the COOH termini of the Glg proteins appear to confer enhanced interaction between the initiator protein and the elongating activity. In mammalian glycogenin, such determinants have yet to be identified. Does the glycogen synthase also contribute specificity? We have shown that the COOH terminus of Glg2p interacts with the NH_2-terminal 623 residues of Gsy2p but, other than this, we have little idea of the glycogen synthase sequences involved. Does this interaction involve more than the residues strictly involved in transglucosylation? Such questions raise the possibility that the interactions between glycogen synthase and Glg proteins serve other roles than simply allowing glucosyl transfer, as discussed below.

It is intriguing that *S. cerevisiae* contains two genes for the self-glucosylating initiator proteins as well as glycogen synthase. One can ask whether this complexity has a significance, such as allowing for a specific pairing between the Gsy and Glg proteins. Loss of either *GLG* gene on its own resulted in no apparent defect in glycogen accumulation. Were one of the Glg proteins strictly paired with Gsy2p, the dominant nutritionally controlled glycogen synthase, one might have expected a partial but definite reduction in the ability to synthesize glycogen, similar to that seen in a *gsy2* strain (15). One explanation is that Glg functions are overlapping, at least as regards glycogen storage, and disruption of both *GLG* genes is required in order to eliminate the normal nutritionally induced accumulation of glycogen. This observation would fit with the role postulated for mammalian glycogenin from biochemical studies, the absence of the self-glucosylated initiator proteins physically disabling the elongation process. In fact, the observed lack of glycogen in *glg1 glg2* cells would provide the first evidence of a requirement for self-glucosylating initiator proteins in the biosynthesis of glycogen in eukaryotes; no formal proof of an obligate requirement has been possible so far in studies of the mammalian proteins. However, the results of the present investigation could imply a more complex role for the Glg proteins. There is evidence for an interplay between the Glg proteins and the phosphorylation state of glycogen synthase, which presumably would entail the activation of phosphatase(s) and/or the suppression of protein kinase(s). Thus, we cannot exclude the possibility that the lack of glycogen accumulation in *glg1 glg2* cells is explained solely by the low level of activity of glycogen synthase. Such was true in *snf1* strains in which we found that the inability to accumulate glycogen could be explained by maintenance of the glycogen synthase in an inactivated state (22). This control of glycogen synthase phosphorylation could be indirect, for example, as a consequence of the lack of glycogen. Alternatively, the Glg proteins might have multiple functions, both priming glycogen biosynthesis as well as exerting control over the phosphorylation of glycogen synthase. Many different molecular mechanisms could be hypothesized for such regulation. However, a model involving physical interaction, consistent with the two-hybrid experiments with Gsy2p and Glg2p, is attractive. Perhaps the Glg proteins have a role in assembling or organizing the proteins involved in glycogen biosynthesis, along the lines of the scaffolding function proposed for Ste5p in the control of the mating factor protein kinase cascade (11, 28).

As noted in the introduction, relatively few self-glucosylating proteins have been identified to date and the generality of their involvement in polysaccharide biosynthesis is not yet clear. The NH_2-terminal 10-residue sequence reported for the *N. crassa* glycogen-associated protein (20) does not match any Glg1p, Glg2p, or glycogenin sequence but does line up effectively (seven of nine identities) with an expressed sequence tag from *Arabidopsis thaliana* (accession no. Z26549) that likely encodes a light-induced protein precursor. GenBank searches identified several proteins with similarity to glycogenin and the Glg proteins. The clearest examples were expressed sequence tags from *Caenorhabditis briggsae* which together define two closely related but distinct amino acid sequences (accession no. R04793 [105 residues] and accession no. R03881 and R05077 [merged together, 137 residues]). These sequences have 50 to 57% identity to rabbit glycogenin and 26 to 35% identity to the Glg proteins. GenBank searches also identify a *Rhodobacter sphaeroides* gene that is putatively involved in sulfolipid biosynthesis, a pathway that interestingly utilizes UDP sugars (3), as well as a rice protein induced by water stress (accession no. D26537). It is therefore possible that self-glycosylating proteins are more widespread in nature than is currently appreciated, and in pathogens, their involvement in the formation of polysaccharides essential for survival, like cell wall constituents, could provide useful new loci for chemotherapeutic intervention.

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