Glycogen synthesis in the absence of glycogenin in the yeast *Saccharomyces cerevisiae*

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Received 9 May 2005; revised 27 May 2005; accepted 7 June 2005

Available online 20 June 2005

Edited by Horst Feldmann

Abstract In eukaryotic cells, glycogenin is a self-glucosylating protein that primes glycogen synthesis. In yeast, the loss of function of *GLG1* and *GLG2*, which encode glycogenin, normally leads to the inability of cells to synthesize glycogen. In this report, we show that a small fraction of colonies from *glg1glg2* mutants can switch on glycogen synthesis to levels comparable to wild-type strain. The occurrence of glycogen positive *glg1glg2* colonies is strongly enhanced by the presence of a hyperactive glycogen synthase and increased even more upon deletion of *TPS1*. In all cases, this phenotype is reversible, indicating the stochastic nature of this synthesis, which is furthermore illustrated by colour-sectoring of colonies upon iodine-staining. Altogether, these data suggest that glycogen synthesis in the absence of glycogenin relies on a combination of several factors, including an activated glycogen synthase and as yet unknown alternative primers whose synthesis and/or distribution may be controlled by *TPS1* or under epigenetic silencing.

Keywords: Glycogenin; Glycogen; Trehalose; Glycogen synthase; *GSY2*; *TPS1*

1. Introduction

Glycogen is a polymer of glucosyl units linked by α-1,4-bonds with α-1,6-branches. It can accumulate to account for up to 10–15% of the cell dry mass in yeast under conditions of growth restriction, upon specific physicochemical stresses and at the end of growth on a glucose-limited medium [1]. In eucaryotic cells, the biogenesis of glycogen is initiated by glycogenin, a self-autoglucosylating protein that produces, from UDP-glucose, a short oligosaccharide covalently linked to a tyrosine residue of this initiator protein. Once the oligosaccharide chain has been extended sufficiently (6–10 glucose residues), glycogen synthase catalyzes the elongation and, together with the action of a branching enzyme, generates a mature glycogen molecule of very high molecular mass [2,3]. In the yeast *Saccharomyces cerevisiae*, *GLG1* and *GLG2* encode glycogenin-like proteins that are 55% identical to each other and 33% identical to the rabbit muscle glycogenin [4]. Disruption of either gene causes no defect in glycogen accumulation, but deletion of both genes was shown to abolish glycogen synthesis. The same result was recently obtained by disrupting the *gnn* gene that encodes the *Neurospora crassa* glycogenin [5]. Taken together, these genetic data are the strongest proof to date that a protein primer is necessary for glycogen biogenesis in eucaryotic cells.

Until now, no glycogenin-like protein has been found in bacteria [6], which suggests that the initiation of this polymer must occur in a different way. Ugalde et al. [7] recently showed that the de novo synthesis of glycogen in *Agrobacterium tumefaciens* is initiated directly on glycogen synthase, which catalyzes both the autoglucosylation and the elongation process. Also, in mammalian tissues, it was shown that alkylglucosides and aromatic glucosides can serve as artificial acceptors for the transfer of glucosyl unit from UDP-glucose by glycogen synthase yielding alkylmaltooligosaccharide products, which can be further elongated into α-(1,4) glucosyl chains by the same enzyme [8]. Whether such oligosaccharide acceptors exist in vivo is still an open question. There was also a report of the existence of a manganese sulfate-dependent glucose transfer to glycoproteins that is catalyzed by a non-glucose 6-phosphate-activated glycogen synthase [9], but the role of this process in the early stage of glycogen biogenesis is still obscure. Together, these data raise the question of whether glycogen biogenesis in eukaryotic cells could still occur in the absence of glycogenin. In this report, we show that glycogen synthesis can take place in glycogenin-defective strains of *S. cerevisiae*, and discuss possible mechanisms underlying this process.

2. Materials and methods

2.1. Yeast strains, plasmids and growth conditions

Construction of strains from EG3218-1A and CEN.PK113-1A background was described previously [10,11]. Unless otherwise stated, yeast strains were grown at 30 °C in a synthetic minimal medium containing 2% (w/v) galactose (YNGal) or glucose (YNGlu), 0.17% (w/v) yeast nitrogen base without amino acids and ammonium, 0.5% (w/v)

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0014-5793/$30.00
doi:10.1016/j.febslet.2005.06.007

ammonium sulfate, supplemented with the appropriate auxotrophic requirements. The same medium with only 0.02% (w/v) ammonium sulfate was prepared for nitrogen limitation experiments. Agar was added at 2% (w/v) for solid media. The *tps1* strains cannot grow on glucose. Therefore, for rigorous comparisons, galactose was routinely used. This carbon source further leads to enhanced glycogen deposition in yeast cells as compared to glucose, and makes easier identification and counting with better contrast between strains that do and do not accumulate glycogen.

Plasmids YEp356 and pYADE4 were used as 2μ control vectors carrying *URA3* and *TRP1* markers, respectively. Plasmids carrying full length *GSY2* and mutated variants in the COOH-terminal have been described previously [12,13]. These constructs will be referred in this study to as pGSY2 (pYcDE2-GSY2; 2μ, *TRP1*, *GSY2* CDS under the *ADH1* promoter), pGSY2*-CEN (pRS314-GSY2 S650A/S654A; CEN/ARS, *TRP1*, own promoter), pGSY2Δ643-2μ (pYcDE2-GSY2Δ643; 2μ, *TRP1*, *GSY2* CDS under *ADH1* promoter). The multicopy vector carrying the full length *GAC1* gene is referred to as pGAC1-1 (pST93; 2μ, *URA3* [14]) or pGAC1-2 (same as pGAC1-1 with *TRP1* as marker; unpublished).

2.2. Biochemical and analytical procedures

Yeast samples (50 OD_{600} units) were filtered through nitrocellulose membranes. The cells were rapidly scraped, frozen in liquid nitrogen and stored at −80 °C until use. Preparation of extracts and assay of glycogen synthase were carried out as described by François et al. [15] in the presence of 0.25 mM UDP [U–^{14}C] glucose. To estimate the active and total form, the assay was done in the absence and in the presence of 5 mM galactose-6-P instead of glucose-6-P because galactose-6-P can act as a glycogen synthase activator with a $K_a \cong 0.5$ mM (François, unpublished), and this avoided isotopic dilution of UDP [U–^{14}C] glucose due to the presence of active galactose-1-phosphate uridyl transferase and UDP-galactose epimerase in crude extract of galactose-grown cells.

2.3. Determination of glycogen and metabolites

Qualitative assessment of glycogen content was carried out by the iodine-staining method of Chester [16] following the modification of Enjalbert et al. [17]. Quantitative assays of glycogen and trehalose levels were performed according to Parrou and François [18]. Collection of yeast cells for extraction of intracellular metabolites and their measurement were carried out as in [19,20].

2.4. Isolation of glycogen and determination of its structure by proton nuclear magnetic resonance spectrometry (^{1}H NMR)

Glycogen was purified from wild-type and mutant strains grown on YNGal (i.e., at $OD_{600} \approx 15$). Briefly, about 0.5–1 g cells (dry mass) were disrupted in 20 ml of 50 mM sodium β-glycerophosphate buffer, pH 7.6, containing 2 mM EDTA, 2 mM EGTA and a protease inhibitor cocktail (Roche, 1836170; 1 capsule for 10 ml of buffer) with 0.5 g glass beads (0.5 mm diameter) by vigorous vortex mixing, 6 times for 15 s, with 15 s intervals on ice. The supernatant was collected by a 10 min centrifugation at 3000 × *g*, 4 °C. A second centrifugation at 15000 × *g*, 4 °C for 45 min was followed by a third ultracentrifugation of the latter supernatant at 100000 × *g*, 4 °C for 1 h 20 min. The pellet, which contained glycogen, was washed with 10 ml of extraction buffer, resuspended in 1 ml of the same buffer, and ethanol (to a final 66% v/v) was added. The glycogen pellet was collected by centrifugation (5 min at 10000 × *g* in a microfuge), rinsed with 66% cold ethanol. After removal of excess of ethanol by incubation at room temperature, the purified glycogen (about 20 mg) was resuspended in a minimal volume of 50 mM ammonium bicarbonate, pH 7.6, in the presence of 0.02 mg/ml of trypsin. The suspension was incubated at 37 °C for 5 h, then the same amount of trypsin was added and the solution was incubated for another 5 h. After digestion, samples were dialyzed overnight at 4 °C against MilliQ water (SpectraPor Membrane MWCO: 6-8000 Spectrum) and lyophilized. The samples were then analysed by Nuclear Magnetic Resonance. ^{1}H NMR analyses were performed on a Bruker AMX-500 spectrometer at 500.13 MHz using a 5 mm BBI probe at 343 °C in D_2O. COSY experiments were performed using the Bruker pulse field gradient program cosygpmf, with 1.5 s recycle delay and 0.52 s acquisition time. A sine-bell apodization function was applied before Fourier transformation.

3. Results

3.1. Yeast can synthesize glycogen in the absence of glycogenin

A very simple method to evaluate glycogen accumulation in yeast is to spot yeast cultures on agar plates and then to check whether these patches stain brown upon exposure to iodine vapour [16,17]. As indicated in Fig. 1A, the patch of the *glg1glg2* mutant remained yellow whereas the wild-type strain was brown. However, when a culture of *glg1glg2* cells was spread on YNgal agar plates to generate isolated colonies, we surprisingly found that 2–3% of these colonies were brown after iodine staining (Fig. 1B, left panel). The colour of the colonies, but not the frequency of their apparition, was considerably enhanced when the medium was nitrogen-limited (0.02% ammonium sulfate instead of 0.5%; Fig. 1B, right panel), a condition known to favour glycogen deposition [17,21]. Moreover, this phenotypic trait was associated neither with nature of the carbon source (identical results with glucose, data not shown), nor with the genetic background of the strain since similar results were obtained with the CC9 strain [4], a *glg1glgl2* mutant generated in a different genetic context (data not shown). Under this condition, 100% of the isolated colonies from wild-type strains turned brown when exposed to iodine vapour (data not shown).

To verify that the brownish colouration of *glg1glg2* mutant colonies was due to glycogen, and not to other molecules such as lipids that are known to interfere somehow with the iodine staining [22], we performed two types of experiments. In the first, brown colonies of *glg1glg2* mutant were cultivated in

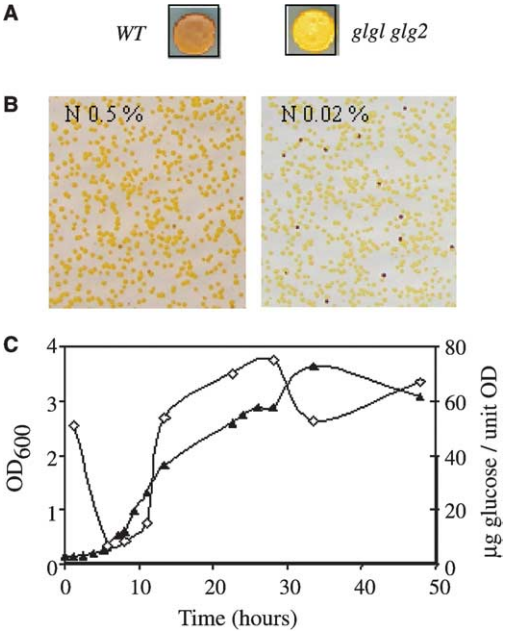

Fig. 1. (A, B) Iodine staining of a wild-type and glycogenin-defective strains. The wild-type CENPK113-1A and corresponding *glg1glg2* mutant were pre-cultured in liquid YNGal and 10 μl were spotted on a YNgal plate (A) or spread to obtain isolated colonies on galactose plates with high (left panel) or low ammonium sulfate (right panel). (C) Enzymatic determination of glycogen during growth of 'iodine-positive' glycogenin-defective colonies. An overnight pre-culture of iodine-positive colonies from a *glg1glg2* mutant in YNGal medium limited for ammonium sulfate was inoculated in 100 ml of the same medium for glycogen determination. Symbols: (▲) OD_{600}, (◇) glycogen levels.