Volume 279, number 2, 341–345 FEBS 09441 February 1991

ADONIS 001457939100150B

Saccharomyces cerevisiae gene SIT4 is involved in the control of glycogen metabolism

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Received 12 December 1990; revised version received 7 January 1991

The gene SIT4 of *S. cerevisiae*, which codes for a protein structurally related to the catalytic subunit of mammalian protein phosphatase 2A, was disrupted in vitro. Analysis of glycogen synthase activity ratio in mutant haploid cells indicated that the enzyme was less active than in wild-type cells. On the contrary, glycogen phosphorylase *a* activity was much higher. The activation of glycogen synthase observed in wild-type cells after incubation with lithium ions was not detected in mutant cells. These results suggest that the product of gene SIT4, a putative protein phosphatase, could be involved in the control of glycogen metabolism in yeast cells.

Protein phosphatase; Glycogen synthase; Glycogen phosphorylase; Gene disruption; *S. cerevisiae*

1. INTRODUCTION

Covalent modification of proteins by phosphorylation/dephosphorylation reactions is known to be a major system for controlling many biological processes, including metabolic pathways. Glycogen synthase and glycogen phosphorylase, the key enzymes in glycogen metabolism, are under control of protein kinase and protein phosphatase activities in eukaryotic cells. Phosphorylation causes the inactivation of glycogen synthase and the activation of glycogen phosphorylase whereas dephosphorylation reverses these effects [1–3]. In mammalian cells dephosphorylation of phosphoserine and phosphothreonine residues is catalyzed by serine/threonine protein phosphatases. These enzymes have been divided into two groups: type 1 and 2. Type 1 enzymes are inhibited by nanomolar concentrations of protein inhibitors (inhibitor-1 and -2) but type 2 phosphatases are much less sensitive. Type 2 enzymes can be grouped into 3 categories: type 2A (active in the absence of divalent cations), type 2B (calcium dependent) and type 2C (magnesium dependent) [4–6]. In mammalian cells, type 1 and 2A phosphatases appear to be responsible for the dephosphorylation of glycogen synthase and phosphorylase [7].

In yeast cells glycogen is the most important energy reserve [8] and, as in higher eukaryotic cells, interconversion between phosphorylated and dephosphorylated forms of glycogen synthase and phosphorylase is believed to be the main regulatory mechanism in glycogen metabolism [9]. It is generally accepted that phosphorylation of glycogen synthase and phosphorylase is controlled by the levels of cAMP through activation of the cAMP-dependent protein kinase, although cAMP-independent mechanisms could be important in the regulation of glycogen metabolism in yeast [10]. Despite that little work has been done on yeast protein phosphatases (see [11] for review), it is known that there exist very remarkable similarities between yeast and mammalian protein phosphatases in terms of requirements for divalent cations and sensitivity to mammalian protein inhibitors and to okadaic acid [12]. Recently, yeast genes encoding proteins showing a very high identity with the catalytic subunit of mammalian protein phosphatase 1 (DIS2S1) and protein phosphatase 2A (SIT4) have been isolated and sequenced [13–15]. The gene SIT4 has been found to be involved in the regulation of transcription of a certain number of genes and is 57% identical (71% if conservative substitutions are considered) to mammalian protein phosphatase 2A. Since phosphatase 2A has been shown to be involved in the control of glycogen metabolism we considered it interesting to test the possible role of the SIT4 gene product in this metabolic pathway. In the present paper we show that the gene SIT4 is involved in the control of the activity of glycogen-metabolizing enzymes.

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2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes were from Boehringer Mannheim. *Thermus aquaticus* DNA polymerase (Taq polymerase) was from Perkin-Elmer/Cetus. Oligonucleotide primers were synthesized on an Milligen 7500 DNA synthesizer.

2.2. *Strains and media*

S. cerevisiae M5 (MATa/MATα, homozygous for leu2-3/112 ura 3-52 trp1), generously provided by Dr I. Schaaff-Gerstenslager and Dr F. Zimmermann, was used in gene disruption experiments. *E. coli* NM522 was used to amplify plasmid constructions. Yeast strain was grown at 30°C in YPD medium or, in the case of those harboring plasmids, in SD synthetic complete minimal medium [16] lacking tryptophan (unless otherwise stated). The bacterial strain was grown in LB medium containing 50 μg/ml ampicillin, when needed, for plasmid selection.

2.3. *Genetic methods and recombinant DNA techniques*

Bacterial cells were transformed by the method of Chung et al. [17]. Yeast cells were transformed after preparation of spheroplasts as described in [16]. Gene disruption was carried out by the one-step gene replacement method [18]. Tetrad analysis and scoring of markers were carried out by standard methods.

Total genomic yeast DNA was isolated as described in [19]. DNA probes were labeled by the random hexanucleotide priming method as described in [20]. Restriction enzyme digestions, phosphatase alkaline treatment, DNA ligations and other standard molecular biology techniques were performed essentially as described in [21].

2.4. *Amplification and cloning of SIT4 gene*

The SIT4 gene was amplified and cloned from total genomic DNA using PCR techniques. For that purpose two oligonucleotides were synthesised (SIT4A, GGATCCGGATCCATCTTTCTGCGGG-TAAT and SIT4B, GGATCCGGATCCAAACCGTGGGAGGT-GAC). Their sequence was based on the sequence of the gene reported by Arndt et al. [14]. Both oligonucleotides contained repeated *Bam*HI recognition sequences at their 5' end. Total genomic DNA (0.2 μg) from *S. cerevisiae* X2180 was amplified in a mixture containing 10 mM Tris-HCl, pH 8.4, 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.1 mg/ml gelatin, 0.2 mM each dNTP, 0.5 μM each oligonucleotide and 2.5 units of Taq polymerase. Annealing was performed at 55°C for 2 min and extension at 72°C for 4 min. After 30 cycles the mixture was incubated 4 additional minutes at 72°C. The product was analyzed by agarose gel electrophoresis and presented a single amplification product with an estimated size analogous to the expected (1.72 kb). The DNA was eluted from the gel, digested with *Bam*HI and cloned into the *Bam*HI site of plasmid Bluescript SK(-) (Stratagene) to give plasmid pJA1. Identification of the cloned DNA as gene SIT4 was performed by restriction analysis and partial DNA sequencing.

2.5. *Preparation of yeast extracts*

For glycogen synthase and phosphorylase activity measurements, yeast cells (150 mg wet weight) were harvested by filtration under vacuum in 0.45 μm nitrocellulose filters (Millipore), washed with cold water and immediately resuspended in 0.5 ml of cold buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM KF, 5 mM EDTA, 2 mM EGTA, 0.6 M sucrose, 0.5 mM phenylmethylsulphonyl fluoride, 0.5 mM benzamidine and 2 mM dithiothreitol. An equal volume of acid-washed glass beads were added and homogenization was carried out by vigorous vortexing (five 60 s pulses with 60 s intervals at 4°C). Samples were centrifuged at 4°C (3000 × *g*, 5 min) and supernatants immediately used for enzyme activity measurement and protein concentration determinations.

2.6. *Glycogen synthase and phosphorylase assays*

Glycogen synthase activity was determined by the incorporation of [^{14}C]UDPG into glycogen as described in [22]. The state of activation of glycogen synthase is expressed as the ratio between the activity measured in the absence of exogenous glucose 6-phosphate (*a* form) and the activity measured in the presence of 6.6 mM glucose-6P (total activity). Glycogen phosphorylase *a* was measured as radioactive glucose incorporated into glycogen from [^{14}C]glucose-1P essentially as described in [23].

2.7. *Other determinations*

Glucose levels in the medium were measured using a Gluco-quant kit from Boehringer-Mannheim, adapted for a Cobas Bio-autoanalyzer (Roche). Protein concentration was determined by the Biuret method as described in [24] using bovine serum albumin as standard.

3. RESULTS

3.1. *Disruption of gene SIT4*

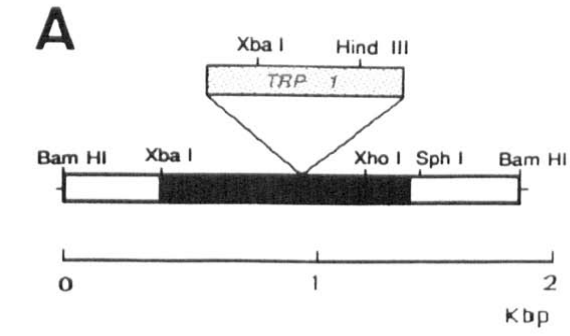
The plasmid YRp7 [25] was digested with *Eco*RI and the 1.45 kbp DNA fragment containing the TRP1 gene isolated. This fragment was ligated into the *Eco*RI site of plasmid Bluescript SK(-) and the resulting construction digested with *Bgl*II and *Bam*HI. Then, the 0.85 kbp containing the TRP1 coding region but lacking the ARS sequences was gel purified.

In parallel, plasmid pJA1 was digested with *Bgl*II in order to linearize the molecule by cutting specifically at the single *Bgl*II site located in the coding region of SIT4. The linear molecule was then dephosphorylated by incubation with alkaline phosphatase and ligated with the 0.85 kbp TRP1 fragment. Therefore, the resulting plasmid, pJA2, contains a copy of the SIT4 gene carrying a 0.85 kbp disruption at position 476 of the coding region (Fig. 1A).

A 2.57 kbp fragment containing the disrupted SIT4 gene was isolated from pJA2 by digestion with BAM HI and used to transform diploid M5 cells. Transformants were selected by their ability to grow in minimal medium lacking tryptophan. Transformants were sporulated and the phenotype of the mutants analyzed by dissection of the resulting tetrads. Analysis of 12 tetrads indicated that two spores per tetrad showed the Trp + phenotype and that it was consistently associated to a slow growing phenotype (Fig. 1B). The presence of a copy of the disrupted gene in the yeast genome was confirmed by Southern blot analysis using both SIT4 and TRP1 probes (data not shown).

3.2. *Analysis of glycogen synthase and phosphorylase activities*

Several haploids carrying the SIT4::TRP1 disruption were selected for glycogen synthase and phosphorylase studies. For that purpose, cells were grown in YPD medium and aliquots were taken at different times. Extracts were immediately prepared and glycogen synthase activity ratio and glycogen phosphorylase *a* activity were measured. As shown in Fig. 2A, wild-type haploid cells presented a very high glycogen synthase activity ratio during the exponential phase. When cells reached stationary phase and glucose in the medium was almost exhausted, the glycogen synthase activity ratio dropped suddenly. However, activity ratio in the mutant haploid cells remained low even during the exponential phase. Total synthase activity was not significantly different in wild-type and mutant cells. In order to verify that this difference was a result of the



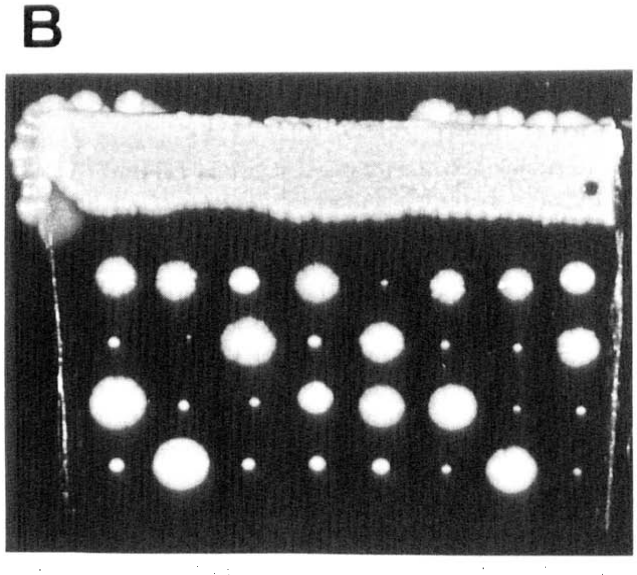


Fig. 1. Disruption of gene SIT4. (A) Graphic description of the disrupted gene. A 0.85 kbp fragment corresponding to *S. cerevisiae* TRP1 gene was ligated into the unique *Bgl*II site in the coding region of gene SIT4 (indicated as a black box). (B) Tetrad analysis of a diploid Trp+ strain obtained by transformation of M5 cells with the 2.57 kbp *Bam*H1-*Bam*H1 fragment from the construction shown in (A). Transformants were sporulated and asci dissected. Slow growing colonies were Trp+ in 23 out of 24 cases.

disruption of SIT4, we constructed a multicopy plasmid containing a wild-type copy of SIT4 by ligating the 1.72 kbp SIT4 fragment into the *Bam*HI site of the shuttle plasmid YEplac195 [26]. Mutant haploid cells were then transformed and glycogen synthase activity ratio measured. As shown in Fig. 2A, the activity ratio in transformed cells was almost identical to wild-type cells.

Glycogen phosphorylase *a* activity was very low in both exponential and stationary phases in wild-type cells. However, a dramatic increase in activity was detected in mutant cells, reaching the highest value at stationary phase (Fig. 2B). When mutant cells carrying extrachromosomal copies of SIT4 were grown, glycogen phosphorylase *a* activity was reduced, although it was still higher than in wild-type cells.

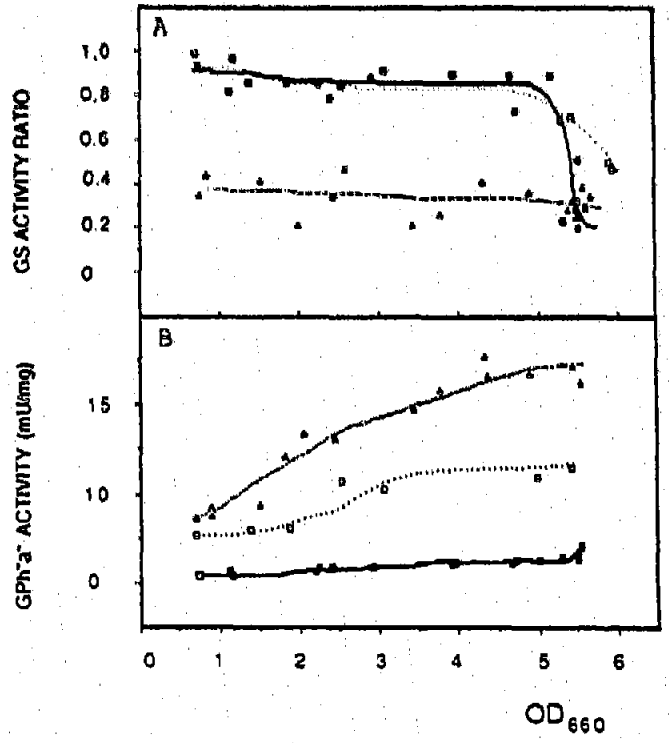


Fig. 2. Effects of disruption of gene SIT4 on glycogen synthase and phosphorylase activities. Cells were grown in YPD medium and aliquots collected at different periods during growth. Extracts were prepared as described and glycogen synthase activity ratio (-glucose-6-P/+glucose-6-P) (panel A) or glycogen phosphorylase *a* (panel B) measured. (■—■) wild-type haploid strain; (△▪▪△) SIT4::TRP1 haploid strain; (□▪▪□) SIT4::TRP1 strain harboring a multicopy plasmid containing wild-type SIT4 gene. Data from 3-6 independent experiments is presented.

3.3. *Effect of lithium ions on glycogen synthase activity ratio*

Since lithium ions are able to cause the activation of glycogen synthase in different cell types, we decided to

Table 1

Effect of lithium ions on glycogen synthase activity in wild-type and mutant yeast cells

	Glycogen synthase activity (mU/mg)		
	−glucose-6-P	+glucose-6-P	−/+glucose-6-P ratio
Wild-type			
+20 mM NaCl	0.88 ± 0.13	1.70 ± 0.12	0.51 ± 0.06
+20 mM LiCl	1.55 ± 0.32	2.06 ± 0.29	0.73 ± 0.09
SIT4::TRP1 mutant			
+20 mM NaCl	0.92 ± 0.18	2.20 ± 0.10	0.42 ± 0.05
+20 mM LiCl	1.00 ± 0.20	2.12 ± 0.22	0.47 ± 0.06

Cultures in stationary phase were made with 20 mM LiCl and further incubated for 30 min at 30°C with shaking. NaCl (20 mM) was added to control cultures. Cell extracts were prepared as described and assayed for glycogen synthase activity in the presence and in the absence of glucose-6-phosphate. Results are mean ± SE from at least 4 independent experiments.

test whether or not the ion could activate glycogen synthase in yeast cells carrying a disruption in the gene SIT4. Lithium was capable of activating the enzyme when added at a concentration of 20 mM to wild-type cells in stationary phase. The effect was already detected after 15 min of incubation and, after 45 min, glycogen synthase activity ratio was still higher than in control cells (data not shown). However, the ion failed to activate glycogen synthase in mutant cells at the different incubation times tested (Table I).

4. DISCUSSION

Glycogen phosphorylase *a* activity was quite low during exponential growth in haploid cells derived from strain M5, as has been described for other yeast strains [27,28]. Disruption of gene SIT4 resulted in a remarkable increase (about 10-fold) in phosphorylase *a* activity and the increase was partially abolished in mutant cells containing extrachromosomal copies of the original gene. It is worth noting that it has been reported that a very strong increase in glycogen phosphorylase mRNA levels occurs when the glucose in the medium is decreased to values of about 50 mM (that is, about half of its initial concentration) [28]. The increase in glycogen phosphorylase transcripts correlates very well with the increase in total phosphorylase activity reported by François et al. [27], although the enzyme was essentially in the inactive form. These results are consistent with the idea that the product of gene SIT4 controls glycogen phosphorylase activity in yeast cells. Since phosphorylase phosphatase activity has been described in yeast [29], a possible hypothesis would be that the SIT4 gene product could dephosphorylate and inactivate yeast phosphorylase. Therefore, in wild-type cells, glycogen phosphorylase would be induced at the late exponential phase, but the enzyme would be mostly inactive as a result of balanced kinase and phosphatase activities. The lack of the putative phosphatase encoded by the gene SIT4 would upset such a balance and increase the amount of the phosphorylated, more active, form of glycogen phosphorylase.

In addition to the effect of the mutation on phosphorylase activity, the activation state of glycogen synthase was greatly reduced suggesting a role of SIT4 in the control of the activity of this enzyme. This effect was a result of a decrease in the active form and not of changes in the total amount of the enzyme. However, the enzyme was never fully inactive in the absence of its allosteric activator glucose-6-P. This result suggests the idea that protein phosphatases able to control glycogen synthase activity, other than the SIT4 gene product, could exist in yeast cells. The existence of more than one glycogen synthase phosphatase activity in yeast has been previously postulated on the basis of chromatographic analysis of yeast extracts [30]. In fact, type 1 phosphatase activity, which is also involved in the control of glycogen metabolism in mammals, has been described in yeast [12]. Recently, two genes structurally very related to the catalytic subunit of mammalian protein phosphatase 1 have been isolated from *S. pombe* and shown to play an important role in chromosome dysjunction in mitosis [13]. The same authors reported the sequence of the *S. cerevisiae* homologue gene, DIS2S1. Such a gene product could also be involved in glycogen metabolism in yeast. In this regard, a very recent report [31] describes a strain defective for glycogen accumulation, 22R1(glc7), which shows a glycogen synthase activity ratio lower than in wild-type cells. Interestingly, the gene apparently responsible for such a defect (GLC7) appears to be identical to DIS2S1. Experiments carried out in our laboratory showed that glycogen phosphorylase was dramatically activated in diploid cells carrying a disrupted allele of DIS2S1. In addition, DIS2S1 was found to perform an essential role in the cell, since its disruption was lethal (Clotet et al., unpublished observations). Therefore, although the product of gene SIT4 is involved in the control of glycogen synthase activity, most probably it is not the only phosphatase responsible for such control.

Lithium ions have been shown in the past to cause the activation of glycogen synthase in mammalian cells [32–34] and, very recently, in yeast [31]. In our hands, glycogen synthase was also activated when cells were exposed to lithium ions. Since the M5 strain shows a very high glycogen synthase activity ratio during exponential growth, we tested the effect of lithium in stationary phase cultures. Lithium chloride was added directly to the cultures, instead of collecting the cells and resuspending them in fresh YEPD [31], because resuspension of the cells in medium containing high amounts of glucose results by itself in a remarkable activation of glycogen synthase (data not shown). In fact, the ability of glucose to activate glycogen synthase when the sugar is added to the medium has been reported previously [27]. In any case, the fact that lithium ions failed to activate glycogen synthase in the mutants suggests that the SIT4 product could be either a target for lithium or an intermediate step in the mechanism of action of the ion.

It is important to note that our data, although suggesting a role for the SIT4 protein in the control of the activity of glycogen synthase and phosphorylase, do not prove that it is a synthase and phosphorylase phosphatase. The possibility of an indirect mechanism should always be considered. One course, it is very suggestive, the fact that SIT4 was originally described as structurally related to mammalian phosphatase 2A, which has been shown to be involved in the control of glycogen metabolism. However, the molecular cloning of two cDNAs whose predicted proteins are even closer to SIT4 than phosphatase 2A is, has been very recently reported. One of them, termed PPX [35,36] was cloned from a rabbit liver library and shows 61% identity to SIT4 (74% when conservative substitutions are con-

sidered). The second is a cDNA from *Drosophila* [36] comprising almost nearly the coding region of a protein 63% identical to SIT4 (74%, considering conservative changes). Those novel phosphatases have not been biologically characterized and, therefore, their functional roles remain unknown.

Acknowledgements: We thank Drs F. Zimmermann and I. Schaaff-Gerstenschläger (Institut für Mikrobiologie, Darmstadt) for their valuable help in tetrad analysis and C. Costa, A. Casamayor, J. Cerominas and M. Camps for their technical help in enzyme measurements. We gratefully acknowledge Prof. C. Gancedo for critical discussion of the manuscript. This work was supported by Grant PB89/0313 from the DGICYT (Spain) and by an 'Acción Integrada Hispano-Alemana', no. 78A. J.C. is the recipient of a fellowship from the 'Conselleria d'Ensenyament de la Generalitat de Catalunya'.

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