Glucose-induced inactivation of isocitrate lyase in *Saccharomyces cerevisiae* is mediated by the cAMP-dependent protein kinase catalytic subunits Tpk1 and Tpk2

Isabel Ordiz, Pilar Herrero, Rosaura Rodicio, Fernando Moreno*

Departamento de Bioquímica y Biología Molecular e Instituto Universitario de Biotecnología de Asturias (IUBA), Universidad de Oviedo, 33006 Oviedo, Spain

Received 6 March 1996; revised version received 25 March 1996

Abstract Glucose-induced inactivation of isocitrate lyase (Icl) has been related to protein phosphorylation. Moreover, since rapid reversible inactivation preceded irreversible inactivation of the enzyme, phosphorylation was proposed as the triggering reaction that makes the enzyme accessible to the proteolytic machinery. The protein kinase involved in the process is unknown at the moment. In this work we demonstrate that Tpk1 and Tpk2, the catalytic subunits of cAMP-dependent protein kinase, are involved in the signalling of short-term and long-term inactivation processes of Icl. We also demonstrate that threonine 53 is involved in a regulatory mechanism necessary for short-term reversible inactivation of Icl, probably mediated through its phosphorylation. Other, as yet unidentified, residues are likely to be the target of distinct protein kinases mediating the irreversible long-term inactivation of Icl.

Key words: Yeast; Isocitrate lyase; Glucose inactivation; Protein phosphorylation

1. Introduction

Growth of *S. cerevisiae* on ethanol as sole carbon source requires the glyoxylate pathway that operates as an anaplerotic route for replenishing C_4 compounds to the tricarboxylic acid cycle. Isocitrate lyase (Icl) is a key enzyme of the glyoxylate pathway. However, this enzyme is dispensable when fermentable carbon sources are available and hence, a strict carbon source dependent regulation is observed [1-3]. At least three stages of genetic or biochemical control have been described: (i) Glucose repression of Icl biosynthesis [4]; (ii) Reversible inactivation of the existing enzyme by phosphorylation [5]; (iii) Glucose-induced proteolytic inactivation of the enzyme [6].

The *ICL1* gene has been isolated and its complete coding sequence determined [7,8]. It was also used to study the regulatory mechanisms underlying the transcriptional control of *ICL1* expression [9,10]. Once the Icl protein has been produced on gluconeogenic carbon sources, addition of glucose causes a time-dependent decrease in its activity. The kinetics of the glucose-induced inactivation of the enzyme involves two phases. Within 45 min of glucose addition, the enzyme is reversibly inactivated (short-term inactivation) and apparently protein phosphorylation is implicated in the process [5]. During the following 2–3 h, Icl activity decreases slowly and irreversibly to 25% of the initial activity (long-term inactivation). In this phase, from a loss of protein antigenicity proteolytic degradation of Icl has been deduced [6].

Short-term inactivation of Icl has been related to protein phosphorylation but the protein kinase involved in the process is unknown at present. Two consensus sequences for cAMP-dependent protein kinase (cAPK) phosphorylation were detected at amino acid positions 53 (RRGT) and 343 (KKFT) in Icl [7]; numbers refer to the threonine residues probably phosphorylated. In order to investigate if cAPK is involved in the process, we have used *tpk* mutants to study the glucose-induced short- and long-term inactivation processes. Since rapid reversible inactivation preceded irreversible inactivation, phosphorylation was proposed as the triggering reaction that makes the enzyme accessible to the proteolytic machinery. In order to investigate the role of protein phosphorylation in irreversible inactivation, we exchanged by alanine the threonine residues presumed to be phosphorylated, using site-directed mutagenesis.

Our results showed that Tpk1 and Tpk2, the catalytic subunits of cAPK, are involved in the signalling of short-term and long-term inactivation processes of Icl. Moreover, the Thr53 of Icl is a target residue in short-term inactivation reaction and phosphorylation of other unidentified residues of Icl induces irreversible long-term inactivation of the enzyme.

2. Materials and methods

2.1. Strains, media and enzymatic analysis

S. cerevisiae strains used in this study are listed in Table 1. Rich media were based on 1% yeast extract and 2% peptone (YEP). 2% glucose (D) or 3% ethanol (E), were added as carbon sources. Synthetic media consisted of 0.67% yeast nitrogen base without amino acids, supplemented with amino acids as required (SC) and 2% glucose or 3% ethanol were used to select for transformants of plasmids carrying a *URA3* marker.

2.2. Experimental conditions for glucose-induced inactivation and 'in vivo' ^{32}P-labelling of Icl

For Icl glucose-induced inactivation experiments, cells were grown on YEPD and then transferred to YEPE for 8 h to allow for induction of the enzyme. From this medium the cells were taken at an OD_{600} of 3.0, washed with sterile water and used to inoculate, at the same cell density, a fresh YEPE medium with 3% ethanol as the carbon source. After 60 min incubation with shaking at 30°C, the culture was divided and placed in two flasks, one receiving glucose to a final concentration of 2% and the other an equivalent volume of sterile water. During the glucose inactivation experiment 10-ml samples were withdrawn from the cultures at various times and the cells were harvested by centrifugation at 4000×*g* for 5 min and washed once with distilled water.

For Icl labelling with ^{32}P, the yeast cells were also grown on YEPE until an OD_{600} of 3.0, washed with sterile water and used to inoculate,

*Corresponding author. Fax: (34) (8) 5103534.
E-mail: FMS@dwarf1.quimica.uniovi.es

PII S0014-5793(96)00344-4

at the same cell density, a mineral medium lacking inorganic phosphate with 3% ethanol as the carbon source. After 60 min incubation with shaking at 30°C, [^{32}P]orthophosphoric acid (5 μCi ml^{-1}) was added and the suspension was further incubated for 60 min. For further analysis, the same procedure as described above for Icl glucose-induced inactivation experiments was followed.

Cell-free extracts were prepared by shaking a suspension of 10^9 cells in 1 ml 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 4 mM PMSF and 4 mM sodium fluoride, with glass beads in a vortex mixer for five 1-min intervals alternating with 1-min intervals of cooling in ice. The liquid was carefully removed and centrifuged at 12000×*g* for 15 min. The supernatants were used for enzyme assays, immunoprecipitation experiments and SDS-PAGE.

2.3. Enzymatic and protein analysis

Icl was assayed as described in [11]. The protein concentrations were determined according to [12] using bovine serum albumin as the standard. Specific activities are expressed as the μmoles substrate consumed×min^{-1}×mg protein^{-1} in crude extracts.

2.4. Immunopurification of isocitrate lyase, SDS-PAGE and immunoblotting

Immunopurification of Icl, SDS-PAGE and immunoblotting experiments were performed as described previously [5].

2.5. General DNA techniques

Restriction enzymes and T4 DNA ligase were obtained from Boehringer. Radioactively labelled isotopes were obtained from Amersham International. An *Eco*RI–*Bam*HI and a *Bam*HI–*Hin*dIII fragment from *ICL1* gene were cloned into M13mp18 as a template for mutagenesis using a T7-GEN in vitro mutagenesis kit from United States Biochemicals. The following oligonucleotides were used to introduce point mutations: T53A, 5'GACGCGGGG CATTCCCACCA3'; T343A, 5'AAATTCGCCTCTAAAG3'. All DNA manipulations were carried out as described previously [7,13].

3. Results and discussion

In *S. cerevisiae*, three genes, *TPK1*, *TPK2* and *TPK3*, encode distinct catalytic subunits of cAPK. None of the three *TPK* genes is essential by itself, but at least one of them is necessary and sufficient for viability [14]. The Icl protein has two possible cAMP-dependent protein kinase phosphorylation sites. The sites are located at amino acids 53 and 343 and in both cases threonines are involved.

In order to investigate if cAMP-dependent protein kinase is involved in the glucose-induced phosphorylation of Icl, we have used double disruption *tpk* mutants [15].

Icl activity was followed in cell-free extracts of wild type and mutants at different intervals after glucose addition. A typical result of glucose-induced inactivation of Icl in *S. cerevisiae* is shown in Fig. 1. The yeast cells were grown on YEPE medium to derepress Icl and then shifted to fresh medium with glucose, which leads to repression of Icl synthesis and triggers inactivation and degradation of the enzyme. Another sample of derepressed cells was shifted to fresh medium containing ethanol as a control. As evident from Fig. 1, a rapid decrease of about 55% in the specific Icl activity can be observed for the wild-type strain during the first 60 min after glucose addition. This is followed by a slower decrease in enzymatic activity to almost 25% of the initial activity after 4 h. In contrast, no significant inactivation of Icl activity was observed when cells continued to grow on ethanol.

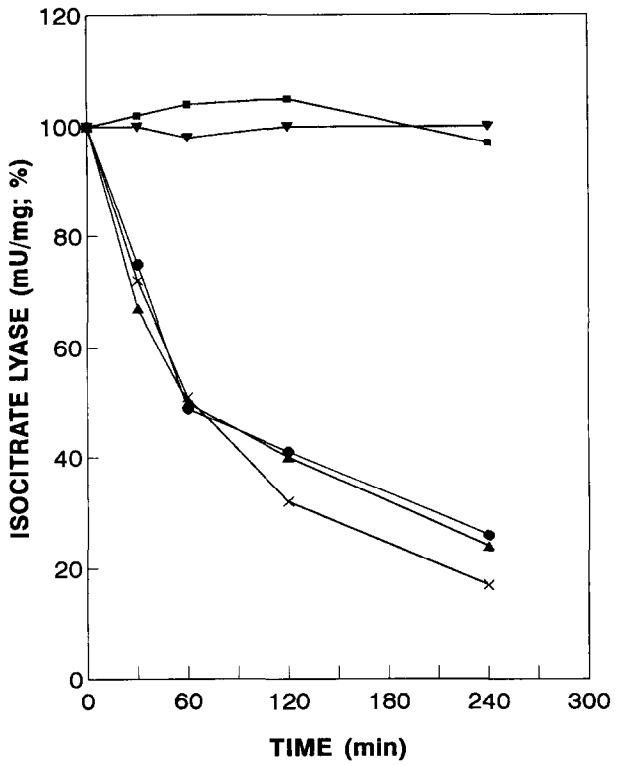

Fig. 1. Pattern of isocitrate lyase activities in *tpk* mutants during glucose inactivation. *S. cerevisiae* strain W303.1A and the mutant strains MB23 (*TPK1*), MB13 (*TPK2*) and MB12 (*TPK3*) [functional genes indicated in parentheses], were grown and derepressed as indicated in section 2. Inactivation was initiated by the addition of glucose at zero time. Cell-free extracts were prepared from aliquots taken at the various times indicated as described in section 2. Symbols: Isocitrate lyase activity in W303.1A cells growing on YEPE treated with 2% glucose (▲); in W303.1A cells grown on YEPE (▼); in MB23 cells growing on YEPE treated with 2% glucose (●); in MB13 cells growing on YEPE treated with 2% glucose (×); and in MB12 cells growing on YEPE treated with 2% glucose (■). Initial activities were: 80 mU/mg (▲,▼,●); 74 mU/mg (×) and 42 mU/mg (■).

In strains with *TPK1* or *TPK2* as the sole active *TPK* genes,

Table 1. Yeast strains

Strain	Genotype	Reference
W303-1A	*MATa ade2-1 his3-11,15 leu2-3,112 trp 1-1 ura 3-1 can1-100 TPK1 TPK2 TPK3*	[18]
MB23	isogenic to W303-1A, except for *TPK1 tpk2::HIS3 tpk3::URA3*	[15]
MB13	isogenic to W303-1A, except for *tpk1::LEU2 TPK2 tpk3::URA3*	[15]
MB12	isogenic to W303-1A, except for *Tpk1::LEU2 tpk2::HIS3 TPK3*	[15]
FMY401	*MATα trp1(fs) ura3(fs) leu2-3,112 his3-11,15 icl1::LEU2*	this work
FMY490[a]	isogenic to FMY401, with Yep352-*ICL1*	this work
FMY491[a]	isogenic to FMY401, with Yep352-*ICL1(T53A)*	this work
FMY492[a]	isogenic to FMY401, with Yep352-*ICL1(T343A)*	this work
FMY492[a]	isogenic to FMY401, with Yep352-*ICL1(T53A, T343A)*	this work

[a] All these strains were transformed with the multicopy plasmid YEp352 that carries the *ICL1* gene or the indicated point mutation in the *ICL1* gene.

we determined inactivation patterns closely resembling that of the wild-type strain (Fig. 1). In the strain carrying only *TPK3* in an active form, no inactivation at all was observed during the 4 h of incubation with glucose. Thus, the strain lacking *TPK1* and *TPK2* is unable to inactivate isocitrate lyase in response to glucose addition. Because this strain shows nearly undetectable phosphorylating activity and *TPK3* expression is not detectable at the mRNA level [15], this experiment strongly suggests that the glucose-induced inactivation depends on the function of one active cAPK. Similar results were obtained for fructose 1,6-bisphosphatase by Mazón et al. [15]. However, this does not seem to constitute a general function of the Tpks in the inactivation induced by glucose as the sugar transporter maltose permease seems to be inactivated independent of the three *TPK* genes [16].

In order to investigate whether the *tpk1 tpk2* double mutant strain is capable of phosphorylating the Icl subunits, immunoprecipitation experiments of in vivo ^{32}P-labelled Icl were carried out using cell-free extracts obtained from the three double *tpk* mutants (Fig. 2). Because no phosphorylation could be observed in the strain containing *TPK3* as the sole intact *TPK* gene, these experiments lead us to the conclusion that the products of *TPK1* and *TPK2* are indeed involved in the glucose-induced phosphorylation of Icl.

As stated above, two consensus sites for cAPK phosphorylation are present in the Icl at threonines 53 and 343. In order to elucidate if both or either one of these sites are involved in the phosphorylation process, they were exchanged

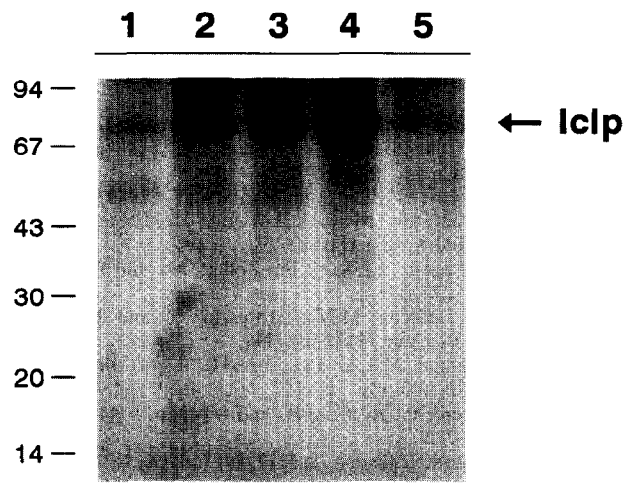

Fig. 2. Phosphorylation of isocitrate lyase in *tpk* mutants during glucose inactivation. Icl from wild-type and *tpk* mutant strains was labelled with ^{32}P in vivo during glucose inactivation as described in section 2. Cell-free extracts obtained from the different strains, were treated with antibodies against Icl, immunoprecipitates were subjected to SDS-PAGE and the gel exposed to autoradiography. Lanes 1 and 2, specific immunoprecipitates of phosphorylated Icl from W303.1A strain after 0 and 90 min of glucose addition, respectively. Lane 3, specific immunoprecipitate of phosphorylated Icl from MB23 (*TPK1*) 90 min after glucose addition. Lane 4, specific immunoprecipitate of phosphorylated Icl from MB13 (*TPK2*) 90 min after glucose addition. Lane 5, specific immunoprecipitate of phosphorylated Icl from MB12 (*TPK3*) 90 min after glucose addition. At time zero the three *tpk* mutant strains showed an identical strength of the Icl signal to the one shown in lane 1 for the wild-type strain (data not shown). The arrow points to the 75 kDa phosphorylated protein corresponding in size to the isocitrate lyase subunit.

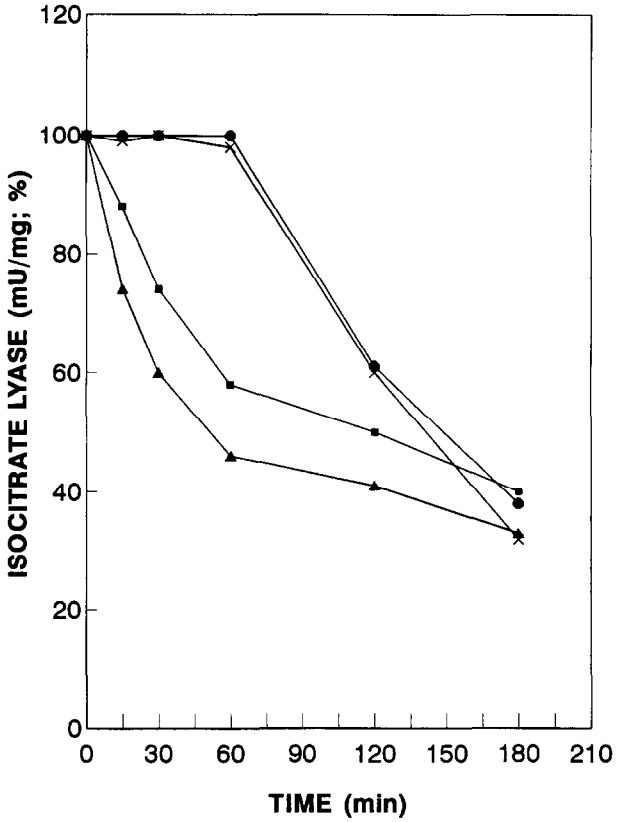

Fig. 3. Pattern of isocitrate lyase activities in *ICL1* mutants during glucose inactivation. The glucose-induced inactivation of isocitrate lyase was followed as described in the legend to Fig. 1. Symbols: Cells transformed with YEp352 carrying the wild-type *ICL1* gene (▲); the *ICL1* (T53A) mutant gene (●); the *ICL1* (T343A) mutant gene (■) and the *ICL1* (T53A/T343A) double mutant gene (×). Initial activities were: 0.98 U/mg (▲); 1.8 U/mg (●); 2.9 U/mg (■) and 4.0 U/mg (×).

to alanines by in vitro mutagenesis. Therefore, we obtained three different *ICL1* mutants: T53A, T343A and the double mutant T53A/T343A. The *icl1* null mutant strain FMY401 was transformed with the multicopy plasmid YEp352 carrying either the wild-type copy of the *ICL1* gene (strain: FMY490) or the genes with the point mutations (strains: FMY491, FMY492 and FMY493; Table 1).

When glucose was added to derepressed transformants carrying the T53A mutation or the double mutation, no inactivation was observed within the first 60 min. In contrast, the wild-type control and the strain with the T343A mutation lost about 55% and 45% of their specific isocitrate lyase activities, respectively (Fig. 3). Thus, the short-term rapid reversible inactivation is eliminated when threonine 53 is replaced by an alanine. Yet specific isocitrate lyase activity of the T53A mutant strain started to decrease 60 min after the addition of glucose and reached about 35% of the initial value within 3 h. This indicates that the long-term inactivation (i.e. proteolytic degradation) is still functioning in this mutant.

The overall specific isocitrate lyase activities in the strains transformed with the multicopy plasmids differed broadly (see legend to Fig. 3). Therefore similar experiments were carried out with the *icl1* null mutant strain transformed with derivatives of the centromeric plasmid pRS316, carrying the same *ICL1* mutant alleles as described above (data not shown). The

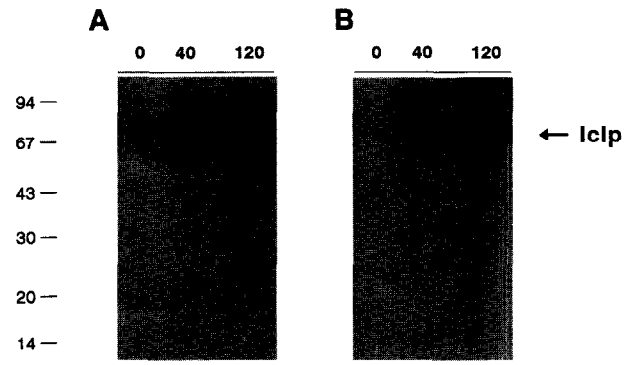

Fig. 4. Glucose-induced phosphorylation of wild-type (A) and mutant (B) Icl. The *icl1* null mutant strain, FMY401 (Table 1) was transformed with the multicopy plasmid YEp352 carrying the *ICL1* gene (A) or the *ICL1* gene carrying the point mutation T53A (B). Transformed yeast cells were incubated in low-phosphate mineral medium in the presence of [^{32}P]orthophosphoric acid. Samples were taken at 0, 40 and 120 min after glucose addition and extracted as described in section 2. Isocitrate lyase was immunoprecipitated with polyclonal antiserum and the immunoprecipitates were subjected to SDS-PAGE followed by autoradiography on X-ray film. The results in B are representative for all three *ICL1* mutants; identical results were obtained with FMY401 cells transformed with the plasmid YEp352 carrying the *ICL1* gene with the point mutations T343A or the double mutation T53A/T343A. The arrow points to the 75 kDa phosphorylated protein corresponding in size to the isocitrate lyase subunit.

results obtained confirmed that threonine 53 is involved in the regulatory mechanism necessary for short-term reversible inactivation of Icl. In addition, the catalytic activity of the enzyme was not affected by the point mutation introduced as the specific activities in the mutants did not differ significantly from those of the wild-type strain.

Immunoprecipitation experiments of cell-free extracts from T53A, T343A or the T53A/T343A mutant labelled in vivo with ^{32}P-orthophosphoric acid did not show any difference with the wild-type strain (Fig. 4). Thus, other residues than threonines 53 or 343 must serve as target sites for phosphorylation. Since there are no consensus sequences for cAPK phosphorylation other than those of threonines 53 and 343, this finding suggests that other protein kinases that are in turn regulated by the *TPK* gene products are involved in Icl modification.

From the results described above, we conclude that threonine 53 appears to be involved in a regulatory mechanism necessary for short-term reversible inactivation of Icl. Most likely the effect is mediated through its phosphorylation. However, from our results it is not possible to definitively establish if the requirement of *TPK*s for long-term inactivation of Icl is due to their influence on the phosphorylation of other residues than threonines 53 or 343, or is due to some kind of effect on the regulatory system controlling the proteolytic machinery involved in the process.

On the other hand, one cannot rule out the possibility that a modification of some amino acid residues, other than T53, could trigger inactivation more indirectly. Thus, a conformational change could be induced in a region adjacent to an internal decapeptide located between amino acid residues 37 and 46, which has been previously described as mediating glucose inactivation of isocitrate lyase [17].

Acknowledgements: We would like to thank Jürgen Heinisch for helpful comments and critical reading of the manuscript and María Jesús Mazón for kindly providing the *tpk* mutant strains. This work was supported by Grant PB94-0091-C02-02 from DGICYT (Spain).

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