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Glucose-induced inactivation of isocitrate lyase in *Saccharomyces cerevisiae* is mediated by an internal decapeptide sequence

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

Departamento de Biología Funcional (Bioquímica) e Instituto Universitario de Biotecnología de Asturias (IUBA), Unidad Asociada al CNB (CSIC), Universidad de Oviedo, 33006-Oviedo, Spain

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Abstract In this work we have investigated the role of specific peptide sequences for glucose-inactivation of the yeast isocitrate lyase. Thus, different fragments of the *ICL1* coding region were fused to the *lacZ* gene of *E. coli* to provide a reporter construction. Determinations of β-galactosidase activities indicated that the decapeptide sequence KTKRNYSARD, located between amino acid residues 37 and 46 of isocitrate lyase, is important for glucose induced proteolytic inactivation. Further experimental evidence was provided by insertion of this sequence into a glucokinase-β-galactosidase fusion protein, which is not sensitive to glucose regulation. The decapeptide inserted conferred glucose inactivation to this construct, confirming that it is both necessary and sufficient as a signal.

Key words: Yeast; Glyoxylate cycle; Glucose inactivation; Proteolytic degradation

1. Introduction

In several yeast strains, addition of glucose to cells adapted to grow in ethanol causes a time-dependent disappearance of enzymes which enable ethanol to be rapidly and efficiently utilized. The proposed physiological role of this glucose inactivation is the regulation of glucose formation [1], since nearly all the enzymes involved are specific for the metabolic reactions providing cells with glucose [2–5].

Growth of *S. cerevisiae* on ethanol as the 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 (ICLase) is the key enzyme of the glyoxylate pathway. This enzyme is dispensable when fermentable carbon sources are available and hence, a strict carbon source dependent regulation is observed. At least three stages of genetic or biochemical control have been described: (i) glucose repression of isocitrate lyase biosynthesis [5]; (ii) reversible inactivation of the existing enzyme by phosphorylation [6]; (iii) glucose induced proteolytic inactivation of the enzyme [7].

The *ICL1* gene has been isolated and its complete coding sequence determined [8,9]. This gene was employed in order to study the transcriptional regulation of its expression. Thus, promoter elements necessary for glucose repression were delimited [10,11]. Once transcription and translation had taken place, addition of glucose to cells growing on a medium with ethanol as the carbon source causes a time-dependent disappearance of ICLase. The kinetics of the glucose induced inactivation of the]

*Corresponding author. Fax: (34) (8) 5103 534.
Email: FMS@dwarfl.quimica.uniovi.es

enzyme involves two phases. Within 45 min of glucose addition, the enzyme is reversibly inactivated and apparently protein phosphorylation is implicated in the process. During the following 2–3 hours, isocitrate lyase activity decreases slowly and irreversibly to 25% of the initial activity. In this phase, from a loss of protein antigenicity, proteolytic degradation of isocitrate lyase has been deduced [7].

In order to investigate if a specialized domain in the Icl protein mediates its susceptibility to glucose induced degradation, several *ICL::lacZ* in frame fusions were constructed. Here we present evidence that the decapeptide KTKRNYSARD, located in the N-terminal region of the protein, is a degradation signal for glucose-triggered degradation of isocitrate lyase and other proteins.

2. Materials and methods

2.1. Strains

S. cerevisiae strain AMW-13C$^+$ (*a trp1(fs) ura3(fs) leu2-3,112 his3-11,15 can1*, where *fs* designates frameshift mutations), was used as a recipient in transformation experiments and as a source of genomic DNA and total RNA. Bacterial transformation and large scale preparation of plasmid DNA were performed in *Escherichia coli* MC1061.

2.2. Media, growth conditions and enzymatic analysis

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 consisting of 0.67% yeast nitrogen base w/o aminoacids 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. Isocitrate lyase was assayed as described in [12]. For preparation of crude extracts, cells were grown on SCD and then transferred to SCE for 8 h to allow for induction of ICLase. β-Galactosidase activity was assayed according to [13]. The protein concentrations were determined according to [14] using bovine serum albumin as the standard. Specific activities are expressed as nmol substrate consumed · min^{-1} · mg $protein^{-1}$ in crude extracts.

2.3. ICL1-lacZ *fusions*

A *Pst*I–*Hin*dIII fragment containing 1000 nucleotides from the 5′ non coding region and 1484 bp of the *ICL1* coding sequence was cloned in frame to *lacZ* into YIp356 [15], Fig. 2A construct 1. Deletions of the coding region were constructed by using available restriction sites in conjunction with polymerase chain reaction, as follows.

A *Pst*I–*Bam*HI fragment containing 1000 nucleotides from the 5′ non coding region and 611 bp of the *ICL1* coding sequence was cloned in frame to *lacZ* into YIp358R (Fig. 2A construct 2). The third construct has been described elsewhere [10], a *Bam*HI–*Hin*dIII fragment containing 1000 nucleotides from the 5′ non coding region and 171 bp of the *ICL1* coding sequence was cloned in frame to *lacZ* into YIp356.

Oligonucleotides 5′-TTTGATCACAAATGGCCA-3′ (primer 1) and 5′-CGCAAGCTTCTCTGGCTGAATAATT-3′ (primer 2) or 5′-CGCAAGCTTCACTCCAACGTGAGTC-3′ (primer 3) were used to generate constructions 4 and 5 with 137 bp and 108 bp of the *ICL1* gene coding region by PCR, respectively. The two fragments were obtained by using 0.1 μg of the primer pair 1 + 2 (construct 4) or the primer pair 1 + 3 (construct 5), 1 μg of construct 3 DNA as template, 2.5 U of *Taq*

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Polymerase (Promega), 0.2 mM dNTPs (Pharmacia) in a total reaction volume of 25 μl in reaction buffer provided by the manufacturer for 30 cycles at 94°C for 30 s, 55°C for 90 s and 72°C for 60 s. The PCR product was isolated from 1% agarose gels. Cut with *Xho*I and *Hin*dIII and ligated into construct 3 digested with the same enzymes.

Finally an *Alw*NI site at 66 bp in the coding region of the *ICL1* gene was used to generate construct 6 of Fig. 2A.

The resulting plasmids were integrated into the *URA3* locus by digestion with *Stu*I prior to transformation of the yeast strain and single copy integration was confirmed by Southern analysis of genomic DNA digested with *Bgl*II and probing with a 1.1 kb *Hin*dIII fragment containing the *URA3* gene.

Restriction enzymes and T4 DNA ligase were obtained from Boehringer, sequenase V2.0 from USB. Radioactively labelled isotopes were obtained from Amersham International. The dideoxyribonucleotide chain termination procedure was used for DNA sequencing analysis [16]. All other DNA manipulations were as previously described [8].

2.4. HXK2-lacZ *fusion*

A *Bam*HI–*Xmn*I fragment from plasmid pRS-HXK2 containing 835 nucleotides from the 5′ non coding region (the *HXK2* promoter sequence appears in the GenBank/EMBL Nucleotide Sequence Databases with the accession no. Z24679) and 39 bp of the *HXK2* coding sequence [17] was cloned in frame to *lacZ* into YIp358 [15], Fig. 2A construct 7.

2.5. GLK1-lacZ *fusion*

A *Sau*3A–*Eco*RI fragment from plasmid pUC-GLK1 containing 881 nucleotides from the 5′ non coding region and 79 bp of the *GLK1* coding sequence [18] was cloned in frame to *LacZ* into YIp358R [15], Fig. 2A construct 8.

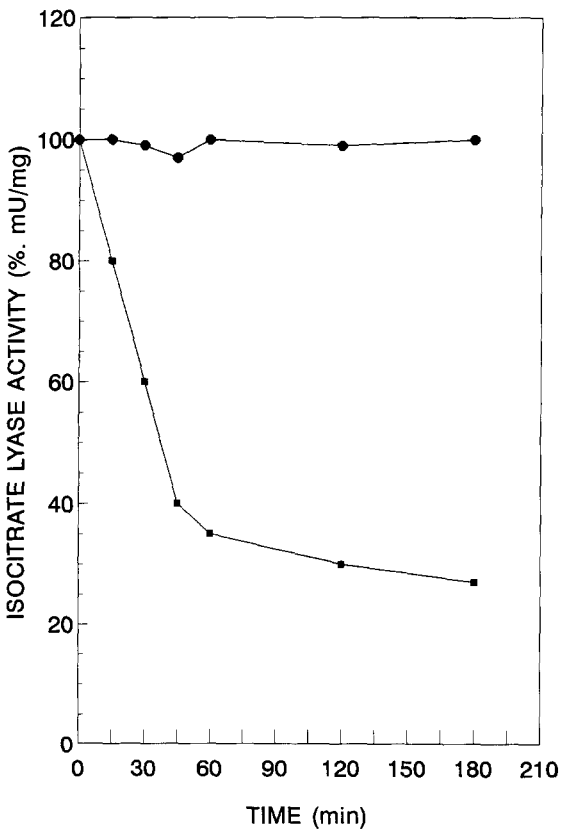

Fig. 1. Isocitrate lyase activity pattern during glucose inactivation. *S. cerevisiae* strain AMW13C$^+$ was grown and derepressed as indicated in section 2. Inactivation was initiated by the addition of glucose at zero time. Cell-free extracts were prepared from aliquots taken at the various times indicated as described in section 2. Symbols: (■) isocitrate lyase activity in cells growing on SCE treated with 100 mM glucose; (●) isocitrate lyase activity in cells grown on SCE. 100% activity corresponds to a specific activity of 50 mU/mg.

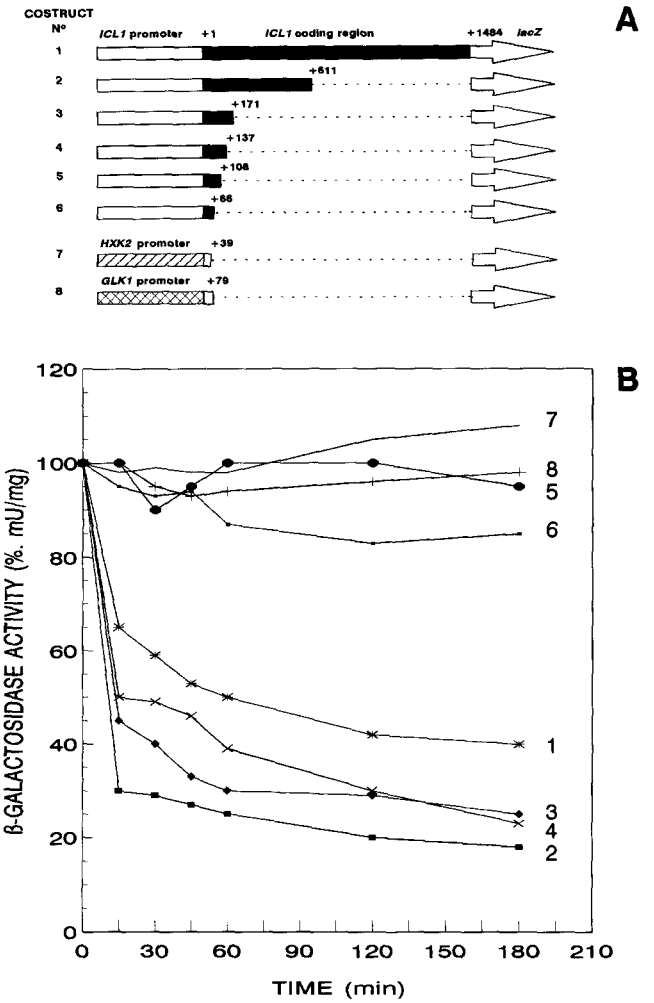

Fig. 2. Glucose induced inactivation of Icl-β-galactosidase fusion proteins. (A) The length of the *ICL1* fragment in each fusion is shown. Control constructs using the *HXK2* gene promoter and 39 bp of the coding region or the *GLK1* gene promoter and 79 bp of the coding region were obtained by fusion with *lacZ* gene. (B) Different fragments of Icl protein were fused with β-galactosidase and the glucose induced inactivation of the fusion protein was followed as described in Fig. 1. 100% activity corresponds to a β-galactosidase specific activity between 400 and 600 mU/mg. The number of the construction used is given next to each curve.

3. Results and discussion

A typical result of glucose induced inactivation of ICLase in *S. cerevisiae* can be seen in Fig. 1. The yeast cells were grown in YPE medium to derepress ICLase. Derepressed cells were shifted to glucose fresh medium which leads to repression of ICLase synthesis and triggers inactivation and degradation of the enzyme, or to ethanol fresh medium as a control [6]. As shown in Fig. 1, a rapid decrease of almost 60% of ICLase activity is observed during the first 45 min after glucose addition, followed by a slow disappearance of ICLase activity, to almost 25% of the initial activity after 3 h. In contrast, no significant inactivation of ICLase activity was observed when ethanol was used as the sole carbon source.

To investigate the sequences responsible for ICLase proteolytic degradation several *ICL1::lacZ* fusions were con-

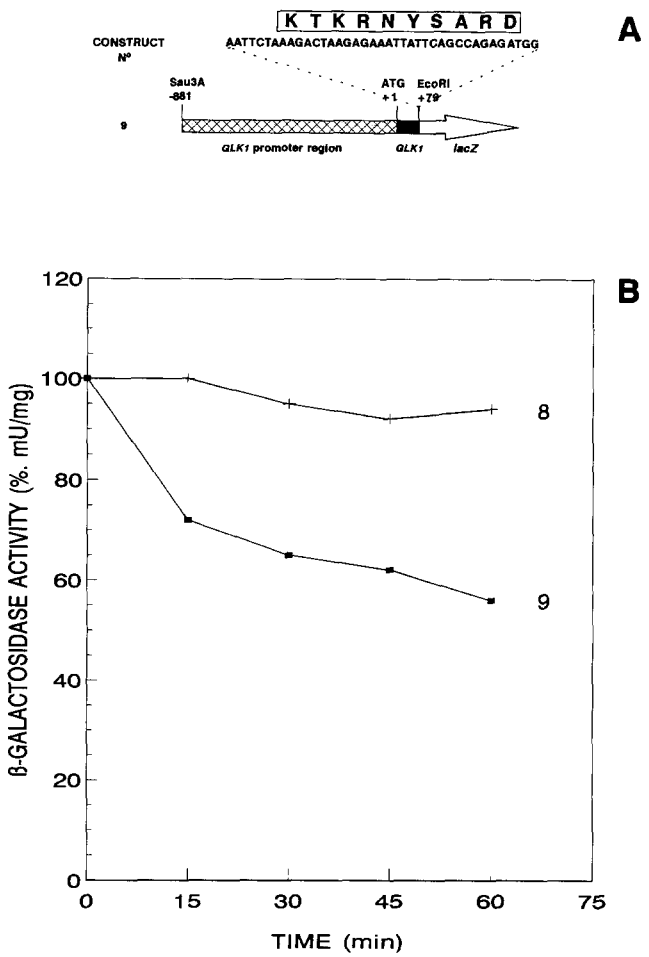

Fig. 3. Icl-decapeptide induced inactivation of Glk-β-galactosidase fusion protein. (A) Scheme of the experimental procedure followed to insert the Icl-decapeptide, KTKRNYSARD into a Glk-β-galactosidase fusion protein. A 30 bp DNA fragment containing *Eco*RI ends was subcloned at the +79 bp *Eco*RI site of the *GLK1-lacZ*. (B) The glucose induced inactivation of the fused protein was followed as described in Fig. 1. Yeast cells were grown on YPE and treated with 100 mM glucose at zero time. 100% activity corresponds to a β-galactosidase specific activity between 250 and 400 mU/mg.

structed (Fig. 2A). This approach assumes that the machinery that inactivates ICLase is also able to inactivate other proteins if they carry the degradation sequences. The fusion proteins constructed contain ICLase fragments of different lengths in conjunction with β-galactosidase, as a reporter enzyme (Fig. 2A, constructs 1–6). Constructs 7 and 8 (Fig. 2A) were used as control fusion proteins which are not susceptible to glucose induced proteolytic degradation. Construct 7 contains the *HXK2* promoter and 39 bp of the *HXK2* coding region in frame with the *lacZ* gene. The encoded fusion protein is synthesized in response to the presence of glucose in the culture medium in accordance with the regulatory properties of *HXK2* promoter [20]. Construct 8 carries the *GLK1* promoter and 79 bp of the *GLK1* coding region in frame with *lacZ* gene. The synthesis of the encoded fusion protein is repressed by the presence of glucose in the culture medium [20]. Yeast was transformed with the different constructs and the transformant yeast strains were used to analyse the glucose induced inactivation kinetics of β-galactosidase (Fig. 2B). When the fusion protein contained as few as 45 N-terminal aminoacids from ICLase, β- galactosidase activity was inactivated in a glucose dependent manner and faster than the native ICLase (Fig. 1). These results show a better susceptibility to glucose inactivation in the fusion proteins and thus faster degradation than the native ICLase. When the fusion protein included only 36 or 22 N-terminal aminoacids from ICLase, β-galactosidase activity was not further affected by the addition of glucose. The stability of the fusion proteins encoded by constructs 5 and 6 or the control fusion proteins 7 and 8, indicate that β-galactosidase itself is not destabilised in glucose containing media. Thus, the fragment between +137 and +108, encoding the decapeptide KTKRNYSARD (amino acids 37 to 46 of isocitrate lyase), appears to be essential for degradation.

To confirm the importance of this region as a signal for glucose induced degradation, it was placed into a heterologous test system (construct 9). To this end, a 30 bp DNA fragment, containing the sequence of the putative autonomous degradation signal decapeptide, was subcloned in a plasmid containing an in frame fusion of part of the *GLK1* gene and the *lacZ* gene (Fig. 3A). As can be seen in Fig. 3B, the presence of the isocitrate lyase degradation signal decapeptide in an otherwise stable Glk-lacZ fusion protein, increases protein degradation in response to the presence of glucose in the culture medium. A nearly 45% decrease in β-galactosidase activity is observed during the first 60 min after glucose addition. This experimental model takes advantage of using the *GLK1* gene promoter. The *GLK1* promoter controls gene expression in a manner similar to the *ICL1* promoter [10,20]. When exponentially growing cells in YPE medium were placed in fresh YPD medium, this resulted in a loss of *GLK1* mRNA with levels declining to about 5% within 60 min following the shift of the medium (results not shown).

Our results demonstrate that glucose induces inactivation of proteins (ICLase and β-galactosidase) that contain the decapeptide KTKRNYSARD. When this sequence is removed the protein is stabilized in glucose containing media. On the other hand, insertion of this decapeptide in a glucose-stable Glk-lacZ fusion protein induces protein degradation in response to the presence of glucose in the medium. Considering all these results together, we suggest that in the N-terminal region of the ICLase there is a decapeptide that acts as a degradation signal. This signal shows no similarity to the destruction box described for the ubiquitin-dependent proteolysis of cyclins [21], and it is not present in the segments of MATα2 repressor that confer instability on β-galactosidase [22].

Previous results in relation to what determines the susceptibility of *S. cerevisiae* fructose 1,6-bisphosphatase to glucose inactivation show that there are several regions in the fructose 1,6-bisphosphatase capable of destabilizing the protein [23], but no specific sequence of the protein implicated in the enzyme destruction was identified. Regions with similar sequences to the ICLase decapeptide signal are not found in FBPase [24] and PCKase [25], two other gluconeogenic enzymes regulated by glucose inactivation in yeast.

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