Hexokinase PII has a double cytosolic-nuclear localisation in *Saccharomyces cerevisiae*

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Abstract We describe here that the *HXK2* gene product, isoenzyme PII of hexokinase, is located in both the nucleus and the cytoplasm of *Saccharomyces cerevisiae* cells. This conclusion is supported by assays of hexokinase-specific activity in isolated nuclei from wild-type and *hxk1/hxk2* double mutant strains, by immunoblot experiments using anti-Hxk2 antibodies and by observation of the fluorescence distribution of a Hxk2-GFP fusion protein in cells transformed with the *HXK2::gfp* gene.

Key words: *Saccharomyces cerevisiae*; *HXK2*; Nucleus

1. Introduction

In *Saccharomyces cerevisiae*, the phosphorylation of glucose at C6 can be catalysed by three enzymes, namely the isoenzymes PI and PII of hexokinase (encoded by the *HXK1* and *HXK2* genes) and glucokinase (encoded by the *GLK1* gene). Of these, only *HXK2* appears to play a role during in vivo glucose phosphorylation. Thus, Northern analysis has shown that the *HXK2* gene is highly expressed when glucose, fructose or mannose is used as the carbon source. Conversely, the expression of *HXK1* and *GLK1* genes only takes place when the culture medium contains non-fermentable carbon sources or galactose [1]. Recently, this differential expression has been confirmed by exploring the metabolic and genetic control of gene expression on a genomic scale [2].

Genetic analysis of *S. cerevisiae* has led to the identification of several genes necessary for glucose repression and for derepression of enzyme synthesis after depletion of glucose [3]. One of the first genes which act in the glucose repression cascade seems to be *HXK2* [4]. In the absence of this gene the cells become insensitive to glucose repression. It has been proposed that the hexose-phosphorylating activity of hexokinase PII is correlated with glucose repression [5]. However, if the glucokinase gene (*GLK1*) is overexpressed in a *hxk1/hxk2* double-null mutant the strains are still insensitive to glucose repression, even though a threefold increase of phosphorylating activity is achieved [6]. These results indicate that glucose repression is not only associated with the phosphorylating activity of hexokinase PII but they also suggest that the presence of the Hxk2 protein is necessary in the transduction of the glucose repression signal. No further glycolytic steps beyond glucose phosphorylation are necessary for glucose repression [7].

Overall, these results suggest that Hxk2p is a dual enzyme with two major functions: (i) the phosphorylation of glucose at the first step of the glycolytic pathway, and (ii) the triggering of glucose repression, although the actual signal for glucose repression is still unknown.

Because of the interest shown in discovering the mechanism by which Hxk2p controls the expression of glucose-repressible genes, we have studied the cellular localisation of the Hxk2 protein. Thus the aim of this study is to demonstrate a double cytosolic-nuclear localisation of Hxk2p, which may open new possibilities toward explaining its role in glucose repression signalling.

2. Materials and methods

2.1. Strains and plasmids

S. cerevisiae strains DBY1315 (*MATa ura3-52 leu2-3,2-112 lys2-801 gal2*) and DBY2052 (*MATa hxk1::LEU2 hxk2-202 ura3-52 leu2-3,2-112 lys2-801 gal2*) were donated by D. Botstein, and were used in enzymatic assays, immunoblot analysis and as recipients in transformation experiments. Bacterial transformation and large-scale preparation of plasmid DNA were performed in *Escherichia coli* MC1061 [*hsdR mcrB araD139*Δ(*araABC-leu*)*7679*Δ*lacx74 galU galK rpsL thi*].

Plasmids YIp356 [8] and pRS306 [9] are integrative yeast-*E. coli* shuttle vectors.

2.2. Media, growth conditions and enzymatic analysis

Yeasts were grown on 1% yeast extract and 2% peptone supplemented with 2% glucose (YEPD) or 3% ethanol (YEPE). The cells were grown in these media until the optical density at 600 nm reached 1.5 (6.0 mg wet weight/ml). To select for transformants, synthetic medium with yeast nitrogen base, 2% glucose and adequate supplements were used.

Hexokinase PII was assayed as described by Moreno et al. [10]. α-Glucosidase was assayed as in [11]. Malate dehydrogenase was assayed as in [12]; NADH dehydrogenase was assayed as in [13] and NADPH-cytochrome P450 reductase was assayed as in [14]. The protein concentrations were determined according to [15], using bovine serum albumin as the standard. Specific activities are expressed as μmol substrate consumed/min/mg protein in crude extracts.

2.3. General DNA techniques

Restriction enzymes and T4 DNA ligase were from Boehringer, Sequenase V2.0 from USB. The dideoxyribonucleotide chain termination procedure was used for DNA sequence analysis [16]. All other DNA manipulations were as previously described [17].

2.4. Construction of yeast strains with HXK2 and HXK2::gfp genes

A DNA fragment containing the complete *HXK2* promoter was isolated from the vector pRS-HXK2 [18] as an 0.88 kbp *Sph*I-*Nco*I fragment and subcloned into a *Sph*I-*Nco*I previously cleaved vector pSP73-HG (this plasmid contains the complete coding region of *HXK2* gene and 254 bp of the 5′ non-coding region in a 2.75 kbp fragment). The resulting plasmid pSP73-HXK2 contains the complete

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HXK2 gene under the control of its own complete promoter in a 3.35 kbp *Sph*I-*Eco*RI fragment. The 3.35 kbp fragment was cloned into YIp356 and the resulting plasmid was named YIp356-HXK2.

A 969 bp *Pst*I-*Bgl*II fragment containing the *gfp* gene was subcloned into the pSP73-HXK2 vector, first cleaved with *Pst*I-*Bgl*II. The resulting plasmid pSP73-HXK2::gfp was used to obtain a *Xho*I-*Bgl*II fragment containing the *HXK2* gene fused in frame with the *gfp* gene. This fragment was also subcloned into pRS306 cleaved with *Xho*I-*Bam*HI rendering plasmid pRS306-HXK2::gfp. All clones used were verified by sequence analysis of fusion points.

Plasmid YIp356-HXK2 was used to transform yeast strain DBY2052 and plasmid pRS306-HXK2::gfp was used to transform yeast strain DBY1315. The plasmids were integrated into the *URA3* locus by digestion with *Stu*I prior to transformation. Single copy integration was confirmed by Southern blot analysis of genomic DNA digested with *Bgl*II and by probing with a 1.1 kbp *Hin*dIII fragment containing the *URA3* gene.

2.5. Preparation of yeast nuclei

Nuclei were prepared by a method based on that of Allen and Douglas [19], with the following modifications. Yeast cells, usually a 300 ml culture, were grown on YEPD medium to mid-log phase. At this time, the cells were harvested or transferred for 6 h to YEPE medium for enzyme induction. Cells were harvested by centrifugation and washed twice in distilled water at room temperature. Cells (1 g wet weight) were resuspended in 10 ml of zymolyase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 M sorbitol and 30 mM DTT) and converted to protoplasts by treatment with 200 U/ml zymolyase. Protoplasts were collected by centrifugation and gently washed twice in zymolyase buffer. The final protoplast pellet was placed in ice, resuspended in 1 ml of cold Ficoll buffer (18% Ficoll-400, 10 mM Tris-HCl, pH 7.5, 20 mM KCl, 5 mM Mg_2Cl, 3 mM DTT, 1 mM EDTA and 1 mM PMSF), and homogenised with 15 strokes of a tight Dounce homogeniser. Complete protoplast lysis was checked in a microscope. The homogenate was centrifuged at $5000\times g$ for 15 min, so that most large cellular debris, unlysed protoplasts and cells were recovered in the pellet. This and all subsequent steps were done at 4°C. The supernatant was centrifuged at $20\,000\times g$ for 20 min. After centrifugation, the supernatant was discarded, and the pellet containing crude nuclei was resuspended and rehomogenised with 10 strokes of the Dounce homogeniser in 10 ml of storage buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol, 100 mM KCl, 1 mM DTT and 1 mM PMSF). The suspension was mixed with an equal volume of 66% Percoll in the same solution and rehomogenised. A Percoll gradient was formed by centrifugation at $18\,000\times g$ for 35 min. A band consisting of clean nuclei as determined by fluorescence microscopy as well as biochemical criteria was collected from near the top of the gradient. The band collected was diluted three times with lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM Mg_2SO_4, 1 mM EDTA, 10 mM potassium acetate, 1 mM DTT and 1 mM PMSF), and centrifuged at $13\,000\times g$ for 10 min. The supernatant was carefully removed by aspiration and the nuclei pellet was either used directly after resuspension in lysis buffer or resuspended in storage buffer and stored at −20°C.

2.6. Electrophoretic analysis, immunoblotting and antibodies

Electrophoresis of proteins (SDS-PAGE) was performed on 10% polyacrylamide gels using the buffer system described in [20]. Western transfer of proteins to a nitrocellulose membrane was carried out as described in [21]. Hxk2 protein was detected by sequential incubation with crude polyclonal antibody (1:1500 dilution) and goat-peroxidase-coupled anti-rabbit IgG (1:3000 dilution).

Specific anti-Hxk2 serum was raised in rabbits by sequential immunisation with a purified fraction of hexokinase PII. Polyclonal antibodies against fructose 1,6-bisphosphatase (Fbp1p) and phosphoenolpyruvate carboxykinase (Pck1p) were donated by M. Rose and antibodies against Msn2, a zinc finger protein required for transcriptional induction through the stress-response element [22], were donated by F. Estruch.

3. Results

3.1. Hexokinase activity and immunoblotting

In the glucose repression cascade model, Hxk2p has always been placed as one of the first steps (for a review see [3,23]). However, approaches to test this hypothesis have not so far given any positive result. Early immunolocalisation studies in eukaryotic cells suggested that the homologous protein to Hxk2p could also be found in the nucleus associated with its outer-side membrane or within it [24–26]. However, these results aroused considerable criticism due to the lack of proper subcellular markers to exclude cross-contamination.

To clarify this point, we made a subcellular fractionation and analysed the pure nuclear extracts. Yeast nuclei were isolated from cells of a wild-type strain (DBY1315), a *hxk1*/*hxk2* double mutant strain (DBY2052) and a DBY2052 transformed strain containing either YIp356 or YIp356-HXK2 plasmids (see Section 2). Cells were grown in YEPD at repressing conditions. As is shown in Table 1, about 14% of total hexokinase activity was found in the nuclear fraction of a wild-type strain, whereas no hexokinase activity was detected in isolated nuclei of a *hxk1*/*hxk2* double mutant strain. Furthermore, the wild-type phenotype was restored after transformation of a *hxk1*/*hxk2* double mutant strain with the YIp356-HXK2 plasmid. To rule out cross-contamination in our subcellular fractionation we assayed several subcellular enzymatic markers. As determined by quantitative enzymatic assays the nuclear fraction had less than 0.4% cross-contamination of the cytosolic and mitochondrial enzyme malate dehydrogenase and less than 0.1% of either the mitochondrial enzyme NADH dehydrogenase or the microsomal enzyme NADPH cytochrome P450 reductase.

These results were confirmed by immunoblot analysis using a polyclonal antibody against Hxk2p. As cytosolic markers we used Fbp1p and Pck1p, two gluconeogenic enzymes of a high expression level. Msn2p, a transcriptional factor involved in

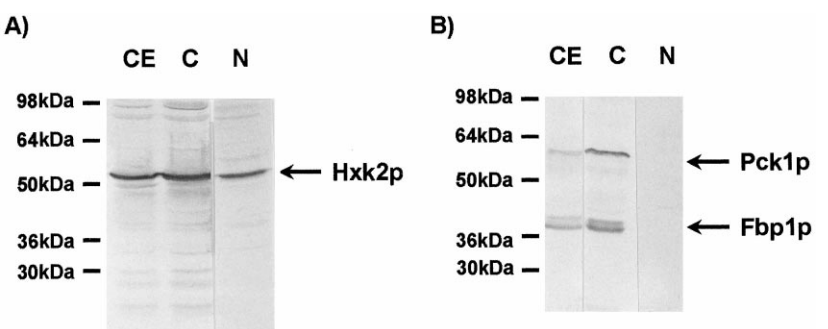

Fig. 1. Immunoblot analysis of the subcellular fractionation. A wild-type strain (DBY1315) was grown on YEPD to the mid-log phase and derepressed for 6 h with 3% ethanol. Protein samples from crude extract (CE), cytosol (C) and nuclear (N) fractions were separated by SDS-PAGE, electroblotted, and immunodetected with Hxk2p (A), and Pck1 and Fbp1 (B) antisera.

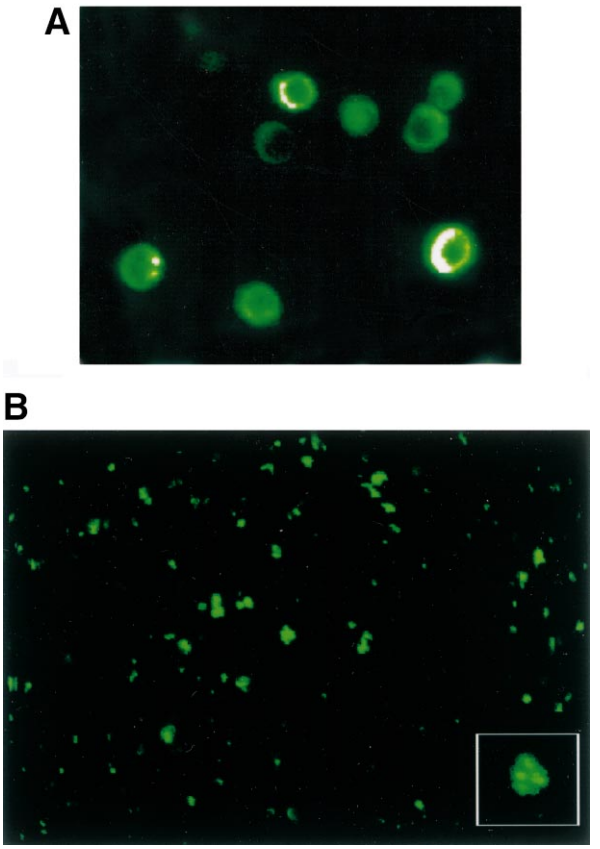

Fig. 2. Detection of Hxk2-GFP fusion protein in living yeast cells. Yeast strain DBY1315 was transformed with the integrative expression plasmid pRS306-HXK2::gfp. The resulting single copy transformed strain was grown exponentially in YEPD liquid medium and cell-associated fluorescence was analysed in whole cells transformed with the pRS306-HXK2::gfp plasmid (A) by confocal microscopy. The fluorescence associated with the nuclei isolated from cells transformed with the pRS306-HXK2::gfp plasmid (B) was also analysed by confocal microscopy. In the insert of B a 10-fold magnification of one isolated nucleus is shown. Confocal images of Hxk2-GFP fusion protein expression were obtained on a Bio-Rad MRC 600 inverted laser confocal microscope using a standard fluorescein isothiocyanate filter providing excitation at 490 nm and emission at 527 nm. The images files were processed using a computer-based graphic system (COMOS) where they were arranged and annotated. A, ×150; B, ×1000.

the stress response, served as nuclear marker. Wild-type cells grown in glucose were shifted to ethanol-containing medium for 6 h. This time was considered sufficient to induce the synthesis of Pck1 and Fbp1 proteins, but not long enough to affect the level of Hxk2p (unpublished results). Both Hxk2p (Fig. 1A) and the gluconeogenic enzymes (Fig. 1B) were detected in the crude extract and in the cytosolic fraction. However, Hxk2p was also present in the nuclear fraction, whereas the gluconeogenic enzymes were undetectable. Furthermore, Msn2p was found in the nuclear fraction, but not in the cytosol as expected.

3.2. Fluorescence distribution of a Hxk2-GFP fusion protein

To confirm the nuclear localisation of Hxk2p, we made a fusion of the green fluorescent protein (GFP) to the C-terminal end of hexokinase PII. The fusion was placed under the control of the *HXK2* gene promoter, thus the expression of the fluorescent fusion protein was regulated in a *HXK2*-specific manner: it increased when glucose was used as the carbon source and decreased on ethanol culture medium (data not shown). Laser scanning confocal microscopy (Fig. 2) demonstrated that Hxk2-GFP fusion protein was uniformly distributed in the cell except for the vacuole which was virtually free of green fluorescence (Fig. 2A). The fluorescence was also observed in the nuclear region, confirming the double cytosolic-nuclear localisation of the fusion (Fig. 2A). Furthermore, nuclei purified by Percoll gradients showed a clear associated fluorescence, which was distributed irregularly (Fig. 2B, insert).

4. Discussion

Elucidation of the mechanism involved in the control of glucose repression by hexokinase PII is crucial to understand this main regulatory system of glucose metabolism. The finding that glucose repression was correlated with the sugar-phosphorylation activity of Hxk2p led initially to the idea that hexokinase PII could be placed in one of the first steps of the glucose repression pathway, controlling glucose influx into glycolysis [5]. The existence of a putative glucose-sensing complex, of which Hxk2p could form a part, has also been suggested [23]. However, recent work has shown that early glucose repression of invertase does not require a specific sugar kinase, and that Hxk2p is only necessary for the long-term glucose response [27,28]. The correlation between glucose phosphorylation activity of Hxk2p and glucose repression also seems less likely at present. Recent work with several mutant alleles of *HXK2* shows that glucose repression is not relieved linearly with decreasing kinase activity, indicating that sugar kinase activity and sugar signalling are mediated at least in part through separated domains of Hxk2p [29]. Glucose repression is also not affected in a *tps2* mutant under conditions in which very high levels of trehalose 6-phosphate, an inhibitor of hexokinase PII activity [30], have been accumulated [31].

Recently, it was reported that yeast galactokinase (Gal1p) has a dual function as an enzyme involved in galactose phosphorylation and as a transcriptional regulator. In the presence

Table 1
Specific hexokinase activity in protoplasts and nuclei from different yeast strains

Strain	Plasmid	Hexokinase (mU/mg protein)	
		Lysed protoplasts	Lysed nuclei
DBY1315	–	1570	221
DBY2052	–	n.d.	n.d.
DBY2052	YIp356	n.d.	n.d.
DBY2052	YIp356-HXK2	1585	225

Cells were grown in YEPD, harvested and fractionated as described in Section 2. The hexose phosphorylating activity was measured using fructose as substrate. n.d., not detectable.

of galactose and ATP, Gal1p activates Gal4p by direct binding to the Gal4p inhibitor Gal80p [32].

Our results obtained by measuring hexokinase activity and by detecting Hxk2 protein with specific antibodies in isolated nuclei clearly indicate a double cytosolic-nuclear localisation of a fraction of the Hxk2 protein. This was further confirmed by expressing a Hxk2-GFP fusion protein in yeast ruling out a possible cross-contamination during subcellular fractionation. Therefore, in a similar way as Gal1p, Hxk2p could participate in the transduction of the glucose repression signal by interacting with transcriptional factors related to this regulatory mechanism.

Which proteins are affected by Hxk2p is still unknown, but possible candidates could be transcriptional factors such as Mig1p, Tup1p or Ssn6p, or other elements of the glucose repression cascade, such as the Hex2p/Glc7p complex or the Snf1p kinase complex. The nuclear localisation of Hxk2p shown in this work does not necessarily indicate that this putative interaction has to take place in the nucleus. Recently, it was shown that Mig1p may move from the nucleus to the cytosol in response to carbon source [33]. In such a way, one might think that hexokinase PII could interact with some proteins outside the nucleus and then be internalised.

Once the nuclear localisation of hexokinase PII had been probed we analysed the deduced amino acid sequence of Hxk2 protein for the presence of signal sequences described previously as nuclear targeting signals. All the nuclear localisation sequences that have been characterised in yeast to date contain a number of basic residues, but they do not conform to the consensus bipartite sequence proposed previously [34]. Comparison of amino acid sequences of other known or presumed yeast nuclear proteins with the amino-terminal 16 residues of Hxk2p reveals a sequence that might be important for nuclear targeting, Lys^{8}-Pro-Gln-Ala-Arg^{12}. A similar sequence of two positively charged amino acids flanking three residues, one of which is proline, is present in several other yeast nuclear proteins [34]. This sequence is not present in any yeast cytoplasmic proteins currently known.

Experiments to further investigate the nuclear targeting ability of this putative nuclear localisation sequence in hexokinase PII are now in progress.

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