The Hexokinase Isoenzyme PII of *Saccharomyces cerevisiae* is a Protein Kinase

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The *HXK2* gene product has an important role in controlling carbon catabolite repression in *Saccharomyces cerevisiae*. We have raised specific antibodies against the hexokinase PII protein and have demonstrated that it is a 58 kDa phosphoprotein with protein kinase activity. The predicted amino acid sequence of the *HXK2* gene product has significant homology to the conserved catalytic domain of mammalian and yeast protein kinases. Protein kinase activity was located in a different domain of the protein from the hexose-phosphorylating activity. The hexokinase PII protein level remained unchanged in P2T22D mutant cells (*hvk1 HXK2 glk1*) growing in a complex medium with glucose. The protein kinase activity of hexokinase PII is regulated by the glucose concentration of the culture medium. Exit from the carbon catabolite repression phase and entry into the derepression phase may be controlled, in part, by modulation of the 58 kDa protein kinase activity by changes in cyclic AMP concentration.

INTRODUCTION

Carbon catabolite repression, or glucose repression, is an important, global regulatory system in both prokaryotic and eukaryotic cells. Studies on the yeast *Saccharomyces cerevisiae* have indicated that the regulatory mechanisms affecting carbon catabolite repression in eukaryotes are different from those in bacteria. The evidence suggests that glucose repression does not appear to involve low levels of cAMP, a key metabolite in catabolite control of gene expression in *Escherichia coli* (Matsumoto *et al.*, 1983; Eraso & Gancedo, 1984).

To identify the different elements implicated in the mechanism of catabolite repression in yeast, a large number of mutants have been described (Entian, 1986; Gancedo & Gancedo, 1986). Although the gene products and their biochemical functions are unknown in most cases, some biochemical knowledge exists from the study of *snf1* (Celenza & Carlson, 1986) and *hex1* (Zimmermann & Scheel, 1977) mutants.

The *SNF1* function is required for expression of glucose-repressible genes and the gene encodes a protein kinase (Celenza & Carlson, 1986). The following genetic evidence suggests the *SSN6* gene product as a possible target of *SNF1* activity. The defect in *SUC2* expression in *snf1* mutants can be suppressed by mutations at the *SSN6* locus (Carlson *et al.*, 1984). Both *ssn6* mutants and *snf1* double mutants show high-level, constitutive (glucose-insensitive) synthesis of secreted invertase. This suggests that *SSN6* is a negative regulator of *SUC2* and that *SNF1* prevents the repressive effect of *SSN6* (Sarokin & Carlson, 1984).

hex1 mutants have a mutation in the structural gene for hexokinase PII (Entian & Mecke, 1982) and are mainly affected in the repression of enzymes of disaccharide hydrolysis and the galactose pathway (Michels *et al.*, 1983). The essential role of hexokinase PII in triggering glucose repression seems to be established (Fröhlich *et al.*, 1984), but the molecular mechanism is unknown.

There is evidence that the proteolysis of hexokinase PII is not the triggering signal of carbon catabolite derepression in *S. cerevisiae* (Fernández *et al.*, 1987). On the other hand, it has been

suggested that xylose-induced phosphorylation of hexokinase PII *in vivo* may produce a change in the enzyme conformation which could provide the triggering signal to activate an additional activity (Fernández *et al.*, 1986, 1987). Recently it has been reported that hexokinase PII can undergo autophosphorylation. The rate of autophosphorylation and the final extent of ^{32}P incorporation are stimulated by the presence of xylose in the assay mixture (Fernández *et al.*, 1988). These results suggest that hexokinase PII could have a protein kinase activity which could catalyse the phosphorylation of an inactive derepressing signal to an active one. In this paper, we report that in addition to the hexose-phosphorylating activity, hexokinase PII also presents a protein kinase activity regulated by glucose and cAMP.

METHODS

Materials. These were all described recently (Fernández *et al.*, 1988). In addition, [^{35}S]sulphate (carrier free) was from New England Nuclear.

Strains and growth conditions. *Saccharomyces cerevisiae* G-517 (CECT 1317) was used as a wild-type strain. The mutant strains P2T22D (*MAT α ade1 hxx1 glk1*), lacking hexokinase PI and glucokinase, and D.308.3 (*MAT α hxx1 hxx2 glk1 ade1 trp1 his2 met14*), lacking all three glucose-phosphorylating enzymes, were obtained from the Yeast Genetic Stock Center (University of California, Berkeley, USA). The mutant strain *hex1 (MAT α his4 ade1 hex1-18 MAL2-8C SUC3)* was kindly provided by K.-D. Entian (University of Tübingen, FRG).

The cells were grown in flasks containing 300 ml YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose] supplemented with 30 mg adenine l^{-1} , on a rotatory shaker at 28 °C. Strain D.308.3 was grown in flasks with 300 ml medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 3% (v/v) glycerol and 3% (v/v) ethanol, supplemented with 30 mg adenine l^{-1} , on a rotatory shaker at 28 °C. Growth was followed by determination of the optical density at 600 nm.

For labelling with [^{35}S]sulphate, *S. cerevisiae* G-517 cells were grown to a density of $6 \times 10^6 \text{ ml}^{-1}$ in YPD medium, collected by centrifugation, washed once in mineral medium (MM) (Kitazume *et al.*, 1962) lacking inorganic sulphate, and resuspended at the same density in sulphate-free MM with 2% (w/v) glucose as carbon source. After incubation at 30 °C for 20 min, [^{35}S]sulphate was added to a concentration of 20 $\mu\text{Ci ml}^{-1}$ ($4 \times 10^7 \text{ d.p.m. ml}^{-1}$), and the cells were labelled for 1 h.

The cells were harvested from cultures, washed twice with distilled water and resuspended in the extraction buffer [10 mM-potassium phosphate, pH 7.0, 1 mM-EDTA, 4 mM-phenylmethylsulphonyl fluoride (PMSF) and 4 mM-sodium fluoride] for further treatment. PMSF and sodium fluoride were included as proteinase and phosphatase inhibitors respectively.

Purification of hexokinase PII. Hexokinase PII was purified to homogeneity, as described by Fernández *et al.* (1986), using a four-step procedure: cell-free extract preparation, hydroxyapatite chromatography, chromatofocusing and DEAE-Sephadex A-50 chromatography.

Preparation of antibodies. Antisera against homogeneously prepared hexokinase PII were raised by injecting New Zealand White rabbits with 0.1 mg protein at one-week intervals as described by Fernández *et al.* (1987). Control sera were obtained from the same rabbits prior to the first injection of antigen and stored frozen at -70 °C. The titres of antisera were determined by Ouchterlony double diffusion analysis (Ouchterlony & Nilsson, 1978) of serially diluted samples against purified enzyme. Maximal titre was attained about 5 weeks after the first injection.

Double diffusion analyses have demonstrated that hexokinase PII, hexokinase PI and glucokinase are not immunologically related. The hexokinase PI and glucokinase partially purified proteins did not react with the antiserum to hexokinase PII (Fernández *et al.*, 1987).

Extraction of proteins, immunoprecipitation and Western blotting. Cells were collected by centrifugation and cell-free extracts prepared by vortexing in extraction buffer with glass beads (five times for 1 min with 1 min intervals of cooling in ice). Hexokinase PII was specifically immunoprecipitated from crude extracts with antiserum and protein A-Sepharose as described by Anderson & Blobel (1983). Anti-hexokinase PII serum (25 μl) was added to 200 μl of cell-free extract containing 0.6 mg protein and then incubated at 4 °C for 60 min. A suspension of protein A-Sepharose CL-4B (200 μl , 5 mg per tube) was added, and incubated with shaking at 4 °C for 30 min. The Sepharose beads were pelleted by a 2 min centrifugation and the supernatant discarded. The beads were washed four times (1 ml per wash in 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 100 mM-NaCl, 0.1% (w/v) sodium azide, 10 mM-potassium phosphate buffer, pH 7.5, with vortexing at each wash. Western blotting was performed as described previously (Fernández *et al.*, 1987).

Analytical methods. Protein kinase activity of hexokinase PII was determined using immune complexes or purified hexokinase PII as sources of enzyme. Immune complexes were washed twice with assay buffer (40 mM-HEPES/NaOH, pH 7.4, 10 mM-MgCl₂, 0.5 mM-DTT). The protein A-Sepharose beads were then resuspended in assay buffer (40 μl) containing 20 μM -ATP, casein or histone (1 mg ml^{-1}), and 3 μCi of [γ - ^{32}P]ATP (6×10^6

d.p.m.) and incubated at 30 °C for 15 min. The reaction was terminated and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were run and analysed as described previously (Fernández *et al.*, 1988). Glucose-phosphorylating activity of hexokinase PII was assayed as previously described (Fernández *et al.*, 1985).

Glucose was determined by a glucose oxidase–peroxidase coupled assay. Protein was determined by the Lowry method with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Immunological and biochemical evidence for the identification of the hexokinase PII as a protein kinase

Immunoprecipitation from [³⁵S]sulphate-labelled *S. cerevisiae* protein extracts followed by gel electrophoresis showed a single radioactive band in the immune complex, with an apparent molecular mass of 58 kDa (data not shown). This band was not present when preimmune serum was used for immunoprecipitation or when the antibody was preincubated with a slight excess of unlabelled enzyme prior to addition to the labelled crude extract. This suggests that the antibodies elicited in the rabbit by immunization with pure hexokinase PII were specific for the enzyme.

To establish whether hexokinase PII has protein kinase activity immune complexes containing the 58 kDa protein were prepared and transfer of phosphate from [γ -³²P]ATP to either caseins or histone was assayed. Protein kinase activity was present in the immune complexes (Fig. 1, lanes 1–3). This activity was absent when preimmune serum was used (Fig. 1, lane 6), suggesting that the 58 kDa protein has protein kinase activity.

Previous work in our laboratory showed that a single protein band with an apparent molecular mass of 58 kDa, which corresponded to hexokinase PII, was obtained by using a four-step purification procedure followed by gel electrophoresis (Fernández *et al.*, 1986). A protein kinase activity was present in the purified hexokinase PII preparation (Fig. 1, lanes 4–5).

We have demonstrated that immune complexes prepared using antisera raised against purified hexokinase PII from *S. cerevisiae* contain a protein kinase activity that is able to transfer phosphate from [γ -³²P]ATP to histone III-S, α -casein or β -casein. The simplest interpretation of these data is that the hexokinase PII (58 kDa) is a protein kinase, although we cannot formally eliminate the possibility that the 58 kDa protein coprecipitates with a protein kinase. The interpretation that hexokinase PII is itself a protein kinase is, however, supported by the observation that the purified hexokinase PII also has protein kinase activity. This interpretation is also supported by the fact that hexokinase PII is autophosphorylated and that the mechanism of hexokinase PII autophosphorylation is intramolecular in nature (Fernández *et al.*, 1988).

Sequence homology between hexokinase PII and several protein kinases

To gain insight into the possible function as protein kinase of the *HXK2* gene product, we compared the predicted amino acid sequence of hexokinase PII (Fröhlich *et al.*, 1985; Stachelek *et al.*, 1986) with the sequences of three serine/threonine protein kinases and two tyrosine protein kinases. In all cases, a cluster of glycines within the sequence Gly-X-Gly-X-X-Gly lies 15–28 residues to the amino terminal side of the lysine implicated in binding ATP (Kamps *et al.*, 1984; Bennett & Kennedy, 1987). Within the hexokinase PII sequence, Lys-252 is in a position that corresponds to the ATP-binding residue in other protein kinases; the sequence Gly-X-Gly-X-X-Gly begins at residue 232 (Fig. 2). We can conclude that the deduced amino acid sequence of hexokinase PII contains a region of high homology to a number of other protein kinases (Fig. 2). Amino acid sequences outside the putative catalytic domain are not homologous among the various protein kinases and are probably important for functions that are specific to particular kinases (Parker *et al.*, 1986; Knopf *et al.*, 1986).

Fig. 1. Protein kinase activity of hexokinase PII. Immunoprecipitates containing a 58 kDa protein were prepared from proteins extracted from wild-type cells and assayed for their ability to transfer [32 P]phosphate from [γ - 32 P]ATP to the following proteins: histone III-S (lane 1); α -casein (lane 2); β -casein (lane 3). Hexokinase PII purified to homogeneity was assayed for its ability to transfer [32 P]phosphate from [γ - 32 P]ATP to α -casein (lane 4) or whole casein (lane 5). Lane 6 shows the absence of α -casein phosphorylation by an immunoprecipitate obtained with preimmune serum. After 15 min incubation at 30 °C, the products of the reaction were analysed by SDS-PAGE. After staining, the gel was dried and fluorographed for 2 d at -70 °C using X-ray film.

<i>HXK2</i> (228-260)	G	V	I	F	G	T	G	V	N	G	A	F	Y	D	V	C	S	D	I	E		K	L	Q	G	K	L	S	D	D	I	P	P	S		
<i>cGPK</i> (362-397)	I	D	T	L	G	V	G	G	F	G	R	V	E	L	V	Q	L	K	S	E	E	S	K	T	F	A	M	K	I	L	K	K	R	H	I	V
<i>cAPK</i> (45-79)	I	K	T	L	G	T	G	S	F	G	R	V	M	L	V	K	H	M	E	T	G	N	H	Y	A	M	K	I	L	D	K	Q	K	V	V	
<i>SNF1</i> (59-92)	K	T	L	G	E	G	S	F	G	K	V	K	L	A	Y	H	T	T	T	G	Q	K	V	A	L	K	I	I	N	K	K	V	L	A		
<i>src</i> (270-303)	E	A	K	L	G	Q	G	C	F	G	E	V	W	M	G	T	W	N	D	T	T		R	V	A	I	K	T	L	K	P	G	T	M	S	
<i>fps</i> (924-958)	G	E	R	I	G	R	G	N	F	G	E	V	F	S	G	R	L	R	A	D	N	T		P	V	A	V	K	S	C	R	E	T	L	P	

Fig. 2. Related amino acid sequences of protein kinases. Alignment of amino acid sequences surrounding the ATP-binding region of three serine/threonine protein kinases - cGMP-dependent protein kinase (cGPK), cAMP-dependent protein kinase (cAPK), yeast *SNF1* gene product (Hunter & Cooper, 1985; Celenza & Carlson, 1986) - and two tyrosine protein kinases - pp60^{v-src} (*src*), P140^{src} (*fps*) (Hunter & Cooper, 1985) with the hexokinase PII (*HXK2*). Amino acids homologous to lysine 252 and the Gly-X-Gly-X-X-Gly sequence of hexokinase PII are boxed. Amino acid abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Fig. 3

Fig. 4

Fig. 3. Protein kinase activity of hexokinase PII extracted from several yeast mutant strains. Immunoprecipitates containing a 58 kDa protein were prepared. After extensive washing, they were assayed for their ability to transfer [γ - 32 P]phosphate from [γ - 32 P]ATP to histone III-S. The reaction products were analysed by SDS-PAGE. After staining, the gel was dried and fluorographed for 2 d at -70°C using X-ray film. Lanes 1 and 4, immunoprecipitates obtained from the wild-type strain G-517 (with preimmune serum in lane 1); 2, immunoprecipitate obtained from mutant *hex1*; lane 3, immunoprecipitate obtained from mutant D.308.3.

Fig. 4. Phosphorylation of histone by hexokinase PII. Immunopurified hexokinase PII from the wild-type strain G-517 was used to assay phosphate transfer from [γ - 32 P]ATP to histone III-S. At different times the reaction products were analysed by SDS-PAGE and autoradiography. The radioactive protein band of 36 kDa was quantified by integrating the area of the peak obtained by scanning for absorbance at 558 nm. Typical results are shown.

Hexokinase-PII-dependent protein kinase activity in different mutant strains

To confirm that the kinase activity in the immune complex was associated with the hexokinase PII protein, two mutant strains were examined to check if mutations in the hexokinase PII structural gene could also affect the protein kinase activity.

Immunoprecipitates were prepared from the wild-type strain G-517 and the mutant strains *hex1* and D.308.3 (*hxx1 hxx2 gkl1*). The cells were harvested when the glucose concentration reached 70% of the original value. The 58 kDa protein kinase from the wild-type strain (Fig. 3, lane 4) catalysed phosphate transfer from [γ - 32 P]ATP to histone III-S. This activity was absent

when the immunoprecipitation reaction was performed with preimmune serum (Fig. 3, lane 1). The 58 kDa phosphoprotein corresponds to hexokinase PII which was autophosphorylated during the reaction (Fernández *et al.*, 1988). The protein kinase extracted by immunoprecipitation from *hex1* (lane 2) and D.308.3 (lane 3) was almost undetectable. This experiment demonstrated that the protein kinase activity in immune complexes prepared from the wild-type strain, using antibody against hexokinase PII, was severely impaired in the *hex1* mutant, with less than 4% of the wild-type activity, and was decreased in the triple mutant D.308.3 (*hxx1 hxx2 glk1*) to 10% of the wild-type activity. Western blotting of the proteins in the immune complexes showed that approximately equal amounts of 58 kDa protein were precipitated from the cells of the strains tested (data not shown). Combining the present data, we suggest that the 58 kDa immunoprecipitated protein kinase activity was associated with the *HXX2* gene product.

This result also suggests that hexokinase PII is a protein kinase whose activity is located in a different domain of the protein from the hexose-phosphorylating activity. In mutants *hex1* and D.308.3 the structural gene of hexokinase PII carried two different mutations and the residual protein kinase activity was different. These results indicate that hexokinase PII is a bifunctional enzyme having a hexose-phosphorylating and a protein kinase activity. These two activities could be located in different domains of the protein and, therefore, be affected to a different extent by the two mutations. The existence of two domains in the hexokinase PII protein was demonstrated previously by results obtained with hexokinase PII structural gene mutants *hex1^r*. The *hex1^r* mutation caused defects in carbon catabolite repression, but did not affect normal hexose-phosphorylating activity (Entian & Fröhlich, 1984).

Time course of protein (histone III-S) phosphorylation by immunoprecipitated hexokinase PII

S. cerevisiae G-517 was harvested at an OD₆₀₀ of 0.9, when the glucose concentration had reached 70% of the original value. The 58 kDa protein kinase activity extracted by immunoprecipitation was used to assay phosphate transfer from [γ -³²P]ATP to histone III-S (Fig. 4). Within 2 min, phosphorylation of histone III-S reached 50% of the maximum. Maximal phosphorylation was reached after about 15 min.

Changes in protein kinase and hexose-phosphorylating specific activities of hexokinase PII during cell growth

The level of protein kinase activity of hexokinase PII in yeast cultures growing under different nutritional conditions is a subject of interest. Hexokinase PII was immunopurified from cultures of mutant P2T22D as described in Methods. Hexokinase PII immunopurified from fermentative yeast cultures (28 mM-glucose), showed a high histone-phosphorylating specific activity (Fig. 5). Hexokinase PII from cultures growing on 1 mM-glucose showed 30% of maximum histone-phosphorylating specific activity. The glucose-phosphorylating specific activity remained constant during the time of the experiment. The level of 58 kDa protein per cell, as detected by Western blotting analysis, did not change significantly. These results indicate that the protein kinase activity of the hexokinase PII is regulated by the glucose level in the culture. The specific activity of protein kinase decreased when the predominant metabolic conditions were gluconeogenic and carbon catabolite repression was relieved.

Effect of cAMP and other nucleotides on the protein kinase activity of hexokinase PII

The protein kinase activity of hexokinase PII was stimulated *in vitro* by physiological concentrations of cAMP: 1.25 μ M-cAMP elicited a 3.5-fold stimulation (Fig. 6). A similar extent of stimulation was also observed with 2 μ M-dibutyryl cAMP but not with the same concentration of dibutyryl cGMP, GDP, GTP or AMP (data not shown). François *et al.* (1984) showed that addition of glucose to a suspension of *S. cerevisiae* caused a transient increase in the concentration of cAMP; thus the effect of glucose level on the protein kinase activity of hexokinase PII could be mediated by the intracellular cAMP concentration.

Fig. 5

Fig. 6

Fig. 5. Effect of different nutritional conditions on the protein kinase activity of hexokinase PII. The mutant strain P2T22D was grown as indicated in Methods and the cells were harvested at different times. Glucose concentration was determined in the supernatant (●). After washing with distilled water the cells were resuspended in extraction buffer, extracts were prepared as indicated in Methods, and glucose-phosphorylating activity was determined in the extracts (Δ). Immunoprecipitates containing the 58 kDa protein were also prepared and assayed for their ability to transfer [32 P]phosphate from [γ - 32 P]ATP to histone III-S (▲). The reaction products were analysed by SDS-PAGE and autoradiography. The radioactive protein band of 36 kDa was quantified as indicated in the legend to Fig. 4. Typical results are shown.

Fig. 6. Dependence of protein kinase activity of hexokinase PII on the concentration of cAMP. The wild-type strain G-517 was grown as indicated in Methods and the cells were harvested at an OD_{600} of 0.9, when the glucose concentration had reached 70% of the original value. The protein kinase activity of the 58 kDa protein prepared by immunoprecipitation was assayed in the presence of the indicated concentrations of cAMP. Typical results are shown.

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