Volume 216, number 2, 265-269 FEB 04729 June 1987

Glucose-induced degradation of yeast fructose-1,6-bisphosphatase requires additional triggering events besides protein phosphorylation

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Received 6 February 1987

Glucose addition to yeast cells stimulates a cAMP overshoot with concomitant activation of cAMP-dependent protein kinase, which in turn rapidly phosphorylates fructose-1,6-bisphosphatase. The phosphorylated enzyme subsequently undergoes a slow proteolytic breakdown. Also, it has been proposed that phosphorylation represents the mechanism that initiates proteolysis. Here we present experiments carried out on a yeast mutant defective in adenylate cyclase [(1982) Proc. Natl. Acad. Sci. USA 79, 2355–2359] in which extracellular cAMP triggers full enzyme phosphorylation but a scanty proteolysis, whereas glucose plus cAMP provoke both phosphorylation and complete proteolytic breakdown. Thus, besides a glucose-induced cAMP peak, which results in enzyme phosphorylation, other effects evoked by the sugar are indispensable for its proteolytic degradation.

Fructose-1,6-bisphosphatase; Catabolite inactivation; Protein catabolism; (Yeast)

1. INTRODUCTION

Glucose addition to yeast cells adapted to a sugar-free medium leads to a time-dependent loss of activity of several enzymes [1]. The gluconeogenetic enzyme FBPase has been shown to underly this 'catabolite inactivation' [2], whose proposed regulative roles are prevention of futile cycling and elimination of superfluous protein with subsequent reutilization of its amino acids [3]. Many studies led to elucidation of the degradation mechanism as consisting of the following steps: (i) glucose induced intracellular acidification, with subsequent stimulation of adenylate cyclase [4,5]; (ii) a rapid transient rise of cAMP levels [6,7]; (iii) rapid enzyme phosphorylation [8,9] with concomitant loss of 50–60% of the initial activity [10,11], a process both cAMP-dependent in vivo [12,13] and specifically catalyzed in vitro by cAMP-dependent protein kinase [14,15]; furthermore, this phosphorylation is stimulated by fructose-2,6-bisphosphate [14–16] whose appearance is cAMP-dependent as well, and leads to a quantitative enzyme inhibition with prevention of futile cycling [17,18]; (iv) slow proteolytic breakdown of the phosphorylated form, with an almost complete disappearance of the enzyme protein in 1 h [11]. Also, it has been proposed that enzyme phosphorylation represents the mechanism initiating proteolysis [3,6]. We present here experiments carried out on a yeast mutant defective in adenylate cyclase [19] that confirm the role of phosphorylation as an indispensable prerequisite for protein degradation. However, they also show that other effects evoked by glucose are at the same time re-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

quired for selective proteolysis of the phosphorylated form.

2. MATERIALS AND METHODS

2.1. *Production of antiserum against FBPase*

Commercially available FBPase (66 enzyme units) from *Torula* yeast (Sigma, St. Louis, MO) was desalted by passing it on a PD-10 column (Pharmacia, Uppsala) preequilibrated with 20 mM triethanolamine-HCl, 0.2 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5. The desalted sample was concentrated to 0.35 ml in a Centricon-30 microconcentrator (Amicon, Danvers, MA) and further purified by loading it on an anion Mono Q HR 5/5 exchange column of an FPLC apparatus from Pharmacia, preequilibrated with the aforementioned buffer. Elution was performed with a linear gradient, from 0 to 1 M KCl, in the same buffer at a flow rate of 1 ml/min. The active fractions were immediately pooled, concentrated to 1.2 ml as described above, mixed with an equal volume of complete Freunds adjuvant and injected into a rabbit. Two booster injections were performed 4 and 6 weeks after the first injection, following the same procedure except that 66 and 40 units were respectively used and mixed with incomplete Freund's adjuvant. Removal of contaminating antibodies from the antiserum and subsequent purification of IgG fraction were carried out as described [11].

2.2. *Yeast strains and growth conditions*

The haploid mutant of *Saccharomyces cerevisiae* AM7-11D, deficient in adenylate cyclase activity and cAMP-auxotrophic, was kindly supplied by Professor T. Ishikawa, University of Tokyo. The diploid strain M1 was kindly supplied by Professor H.Holzer, University of Freiburg i.Br. Yeasts were grown for 48 h at 30°C in a low-P_i medium prepared according to Matsumoto et al. [20], in the presence of $^{32}P_i$ (60 μCi/ml) and of [2,5-^{3}H]histidine (20 μCi/ml), both from Amersham (England). For the mutant AM7-11D, 0.5 mM cAMP was also added. At the end of growth, aliquots of AM7-11D cultures were agar-plated in the afore-mentioned medium in the presence or absence of 0.5 mM cAMP. Revertants grown in the absence of cAMP never exceeded 1% of colonies detected in the presence of cyclic nucleotide.

2.3. *Cell collection and incubation with glucose and/or cAMP*

Cell collection and incubation were carried out as reported [13], using 125 mM potassium lactate, pH 4.5, as incubation buffer. Effectors were added to the cell suspension after a 30 min preincubation.

2.4. *Enzyme extraction and specific activity determination*

Cell samples (210 mg wet wt) were collected at the indicated times on cellulose nitrate filters (25 mm diameter, pore size 0.65 μm), washed twice with 5 ml of 0.1 M imidazole-HCl, pH 7.0, resuspended in 0.86 ml of the same buffer and disrupted by vigorous shaking with 1 g glass beads (0.5 mm diameter) for 5 min at 4°C. The homogenates were centrifuged for 20 min at 120000 × g. The supernatants were used for enzyme and protein assay as in [13].

2.5. *Immunoprecipitation and electrophoretic analysis of immunoprecipitates*

Quantitative immunoprecipitation of FBPase from 120000 × g supernatants, SDS electrophoresis of the immunoprecipitates on 12% polyacrylamide slab gels, Coomassie staining and destaining were carried out as described [11]. Each lane was cut into 3-mm slices, which were extracted overnight at 50°C with 0.7 ml NCS tissue solubilizer from Amersham, containing 10% (v/v) water. Then, 5 ml Picofluor 30 from Packard (Downers Grove, IL) was added to each sample and radioactivity determined in a two-channel liquid scintillation counter for separate estimation of ^{3}H and ^{32}P. Immediately after SDS electrophoresis of an immunoprecipitate, FBPase was extracted from the gel by grinding the corresponding slice in a Potter homogenizer in the presence of 1 ml of 15 mM Tris-HCl, pH 7.5. After removal of gel debris by centrifugation, the supernatant was concentrated to about 30 μl and subjected to isoelectric focusing essentially as reported by O'Farrell [21]; the gel rod was cut into 2-mm slices and radioactivity determined. To measure both FBPase ^{3}H radioactivity and protein in the immunoprecipitates, the enzyme band from Coomassie-stained SDS gels was incubated according to Ball [22], which led to quantitative extraction of the dye. Absorbance at 595 nm of the extraction medium provided a reliable protein determination, whereas radioac-

tivity, firmly bound to the gel, was separately quantified.

3. RESULTS

3.1. *Identity and homogeneity of the immunoprecipitated protein*

The protein immunoprecipitated from extracts of the wild-type strain M1 could be unambiguously identified as FBPase on the basis of the following evidence: (i) it was glucose-repressed [2] (not shown); (ii) it was rapidly phosphorylated after glucose addition, and disappeared after a 90 min sugar incubation [8,9] (fig.1A); (iii) it displayed a molecular mass of 40 kDa [15,23] (not shown) and an isoelectric point of about 5.5 [24] (fig.1B). The isoelectric focusing showed, in addition, the substantial homogeneity of the immunoprecipitated protein.

3.2. *Effect of extracellular glucose and/or cAMP on FBPase in the yeast mutant AM7-11D*

Incubation of AM7-11D yeast mutant with glucose could not trigger FBPase degradation or phosphorylation (fig.2A,B), because of the absence in this strain of any glucose-stimulated cAMP peak [13]. Also, cAMP addition to a cell suspension did not stimulate any considerable decrease in immunoprecipitated protein, in spite of a significant phosphate incorporation in the protein itself; in contrast, addition of glucose plus cAMP led to an almost complete disappearance of FBPase at the latest incubation time, although phosphate incorporation was somewhat smaller than in the presence of cAMP alone. This shows that protein phosphorylation is not sufficient in itself as a trigger of degradation, but other effectors evoked by glucose are required at the same time. Furthermore, the radioactivity ratio $^{32}P/^{3}H$ showed, at 2 and 4 h incubation with cAMP, maximal and practically identical values (fig.2C). Therefore, we tentatively assumed that, starting

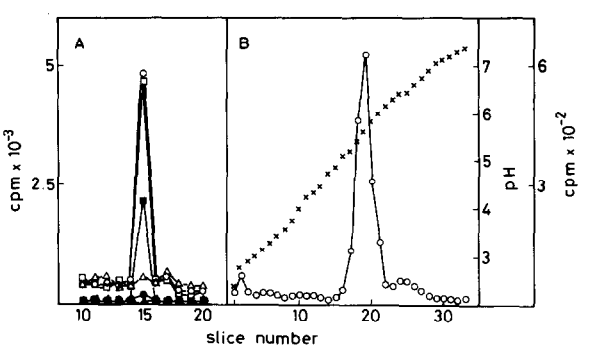

Fig.1. SDS electrophoresis (A) and isoelectric focusing (B) of immunoprecipitates from yeast strain M1 grown to the stationary phase in the presence of [^{3}H]histidine and $^{32}P_i$. Excess antibody was added to extracts from cells incubated 0 min (○,●), 3 min (□,■) and 90 min (△,▲) with glucose. Open symbols, ^{3}H; closed symbols, ^{32}P. After SDS electrophoresis of the immunoprecipitates, the gel was sliced and radioactivity counted in each slice. The radioactive band from a zero-time sample was extracted and further subjected to isoelectric focusing. Again the gel was sliced, and ^{3}H radioactivity and pH values (×) determined in each slice.

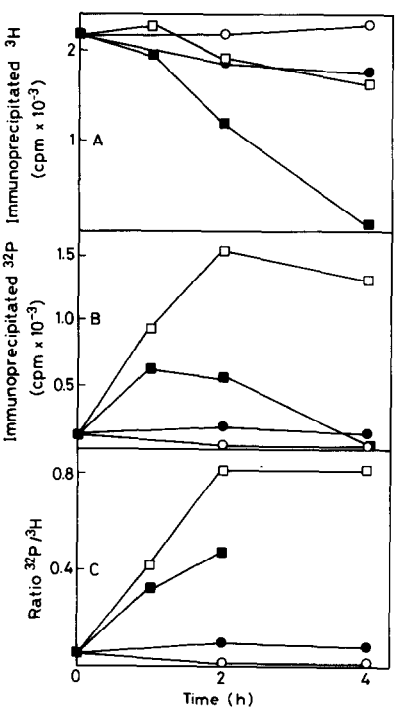

Fig.2. Time course of degradation and phosphorylation of FBPase in AM7-11D yeast mutant. Cells grown in the presence of [^{3}H]histidine and $^{32}P_i$ were incubated in 100 mM potassium lactate, pH 4.5. (○) No addition, (●) 100 mM glucose, (□) 2 mM cAMP, (■) 100 mM glucose plus 2 mM cAMP. At the indicated times, yeast samples were collected and immunoprecipitated ^{3}H (A) and ^{32}P (B) from equal amounts of crude extract protein (2.1 mg) determined. The ratio $^{3}H/^{32}P$ was also calculated (C).

from 2 h of incubation with the cyclic nucleotide, only phosphorylated FBPase is present, which is about 45% active with respect to native enzyme [9–11]. Based on this assumption and on the reported determinations of residual immunoprecipitable protein (fig.1A), we calculated the expected enzyme activity values for each treatment. The substantial concordance between calculated and measured activities (table 1) confirmed our working hypothesis; thus, in spite of complete phosphorylation, a scanty proteolytic degradation of FBPase seems to occur in the absence of glucose. Nevertheless, an alternative interpretation of these data might be that the different time courses of immunoprecipitable protein are not mainly due to different extents of protein degradation, but of protein synthesis. However, this was not supported by experiments carried out with cell preincubation in the presence of cycloheximide, since this effector did not substantially modify the inactivation pattern (not shown). Moreover, in another experiment we preincubated cells grown in the presence of [^{3}H]histidine with 4 mM cold amino acid prior to glucose and/or cAMP addition. Assuming that preincubation prevents radioactive protein synthesis, any difference between time courses of immunoprecipitated enzyme protein detected as Coomassie staining [22] and immunoprecipitated radioactivity should reflect the extent of new protein synthesis during cell incubation. Consistent with the well-known glucose repression of FBPase [2], new protein synthesis was very low in the presence of the sugar (table 2), but also in its absence did not exceed 15% of the initial protein amount, thus showing that its contribution to the different time courses presented in fig.2 was substantially negligible.

Table 1

Comparison between measured FBPase activities in AM7-11D yeast mutant incubated with glucose and/or cAMP, and values expected on the assumption that after 1 h of cAMP incubation only phosphorylated enzyme occurs

Treatment		FBPase activity[a]	
		Expected	Measured
No addition	2 h	100	101
	4 h	105	109
2 mM cAMP	1 h	78	85
	2 h	41	46
	4 h	35	31
0.1 M glucose	2 h	82	83
	4 h	80	76
0.1 M glucose + 2 mM cAMP	1 h	73	77
	2 h	39	32
	4 h	–	b.d.[d]

[a] Values are expressed as percentage of zero times
[b] Below detection

Table 2

Determination of ^{3}H radioactivity and enzyme protein in FBPase immunoprecipitates from AM7-11D yeast mutant[a]

Treatment		Radioactivity[b]	Protein[b]	Difference
No addition	2 h	91	96	5
	4 h	90	102	12
2 mM cAMP	2 h	79	85	6
	4 h	62	77	15
0.1 M glucose	2 h	56	62	6
	4 h	75	80	5
0.1 M glucose + 2 mM cAMP	2 h	56	62	6
	3 h	26	29	3
	4 h	b.d.[c]	b.d.[c]	–

[a] Cells were grown in the presence of [^{3}H]histidine and preincubated with 4 mM cold amino acid prior to addition of glucose and/or cAMP
[b] Values are expressed as percentage of zero times
[c] Below detection

4. DISCUSSION

The present data show that incubation with cAMP of the adenylate cyclase defective yeast mutant, triggers FBPase phosphorylation, although more slowly than does glucose in wild-type strains; this difference is probably due to a slow cyclic nucleotide permeation. However, the inactivation pattern here presented fully confirms that, as previously proposed [3,6], FBPase phosphorylation is indispensable for protein degradation, but shows, in addition, that other effectors evoked by glucose are required to trigger the process. This is

supported by the fact that cyclic nucleotide alone is not able to stimulate a significant enzyme degradation, which instead occurs in the presence of glucose plus cAMP.

Thus the question raised by these findings is how glucose stimulates degradation of phosphorylated FBPase. One might account for the glucose-induced stimulation of the proteolytic process in terms of its possible energy dependence. However, this can be ruled out by the fact that the time course of FBPase inactivation is not detectably affected by sulfite (not shown), an effector which drastically lowers intracellular ATP levels [25]. Furthermore, previous work showed that ATP levels in the AM7-11D mutant incubated with cAMP were not very different for the first 2 h from those detected on incubation with cAMP plus glucose [13].

On the other hand, reports of other groups have demonstrated that the inactivation depends on the accumulation of glucose metabolites not beyond hexose monophosphates, quite probably via the stimulation of long-term proteolytic degradation [26,27]. Thus, our finding that no such degradation occurred to a significant extent in the absence of the sugar is in agreement with these reports.

No definite models have been yet proposed for the mechanism of FBPase proteolysis; however, Funaguma et al. [28] have recently shown that no long-term inactivation is detectable in a pleiotropic mutant devoid of many intravacuolar proteolytic activities. Glucose metabolites might therefore stimulate internalization of phosphorylated enzyme into vacuoles and/or its proteolysis by intravacuolar proteinases.

ACKNOWLEDGEMENTS

Thanks are due to Professor Tatsuo Ishikawa (Institute of Applied Microbiology, University of Tokyo, Japan) for his generous gift of the yeast mutant AM7-11D. This work was supported by grants from the Italian Ministero della Pubblica Istruzione, DPR 382/80, art. 65.

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