Evidence for involvement of *Saccharomyces cerevisiae* protein kinase C in glucose induction of HXT genes and derepression of SUC2

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Abstract

The *PKC1* gene in the yeast *Saccharomyces cerevisiae* encodes protein kinase C that is known to control a mitogen-activated protein (MAP) kinase cascade consisting of Bck1, Mkk1 and Mkk2, and Mpk1. This cascade affects the cell wall integrity but the phenotype of Pkc1 mutants suggests additional targets which have not yet been identified. We show that a *pkc1*Δ mutant, as opposed to mutants in the MAP kinase cascade, displays two major defects in the control of carbon metabolism. It shows a delay in the initiation of fermentation upon addition of glucose and a defect in derepression of *SUC2* gene after exhaustion of glucose from the medium. After addition of glucose the production of both ethanol and glycerol started very slowly. The *V* \text{max} of glucose transport dropped considerably and Northern blot analysis showed that induction of the HXT1, HXT2 and HXT4 genes was strongly reduced. Growth of the *pkc1*Δ mutant was absent on glycerol and poor on galactose and raffinose. Oxygen uptake was barely present. Derepression of invertase activity and *SUC2* transcription upon transfer of cells from glucose to raffinose was deficient in the *pkc1*Δ mutant as opposed to the wild-type. Our results suggest an involvement of Pkc1p in the control of carbon metabolism which is not shared by the downstream MAP kinase cascade. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Protein kinase C; *Saccharomyces cerevisiae*; Signal transduction

1. Introduction

Yeast cells display several signal transduction pathways containing homologues of the so-called mitogen-activated protein (MAP) kinases found in higher eukaryotes. In general, these pathways comprise a family of threonine/tyrosine-specific protein kinases that mediate consecutive intracellular phosphorylation events. Activation of MAP kinases requires both tyrosine and threonine phosphorylation performed by a ‘dual’ specificity protein kinase called MAP kinase kinase (MAPKK). This MAP kinase activator also requires a tyrosine/threonine phosphorylation for its activation, which is performed by another protein kinase acting upstream of this enzyme, called MAP kinase kinase kinase (MAPKKK) [1]. One of the MAP kinase pathways described in *Saccharomyces cerevisiae* is involved in control of cell wall composition and involves protein kinase C, encoded by the *PKC1* gene [21,31].

Loss of Pkc1 function results in cell lysis due to a deficiency in cell wall biosynthesis. This mutant phenotype was used to identify the downstream components of the Pkc1 pathway. Four genes were discovered that, when over-expressed, suppressed the cell lysis defect. From epis-
tatic analysis and structural relatedness to kinases in other pathways, a model has been proposed for a pathway in which Pkc1 phosphorylates the MAPKKK Bck1, which in turn phosphorylates two redundant MAPKKs, Mkk1 and Mkk2, that phosphorylate the MAP kinase Mpk1. One of the targets of this pathway seems to be a glucan synthase complex that is controlled by MPK1 phosphorylation. Addition of osmotic stabilising agents (sorbitol or NaCl) to the growth medium compensates for the cell wall defect and allows cell proliferation. The cell integrity phenotype is less severe in mutants in the MAP kinase components of the pathway. This suggests that there might be a bifurcation in the pathway at the point of Pkc1 and that this protein kinase controls other important functions in yeast [21]. Up to now such additional targets have not clearly been identified, although hints have been obtained for a role of the Pkc1 and/or related phosphatidylinositol signalling pathways in glucose sensing [5,10,11].

Several signalling pathways are involved in glucose-induced regulatory phenomena in yeast [29]. A major pathway controlling a large number of genes involved in respiration and the utilisation of alternative carbon sources (maltose, galactose, sucrose and raffinose) is the main glucose repression pathway (recent reviews: [7,14]). Major components involved in this pathway are hexokinase 2, protein phosphatase 1 (PP1), the Snf1 protein kinase and the Mig1 transcription factor. Basically, in the presence of high glucose concentrations the Snf1 protein kinase is inactivated and can no longer inhibit the transcriptional repressor Mig1. As a result the genes under control of the pathway are repressed by Mig1. The inactivation of Snf1 is brought about by dephosphorylation which is carried out by PP1. This enzyme is activated by a signal generated from glucose, probably through hexokinase 2. On the other hand, the phosphorylation of Mig1p by Snf1p apparently causes its translocation to the cytoplasm relieving the repression of genes. Glucose also controls the expression of Glucose transporter genes. Two glucose transporter homologues, Snf3p and Rgt2p, have been proposed as putative glucose sensors for control of glucose induction of genuine glucose transporters, respectively by low and high levels of glucose [26,27]. Several glycolytic genes are also induced by glucose and different pathways and mechanisms are apparently involved in this process [3].

In this work, we show that Pkc1 plays a role in carbon metabolism independent of the MAP kinase cascade controlling cell integrity. Pkc1 appears to be involved in controlling the response to glucose during the initiation of fermentative growth and it affects the expression of glucose transporter genes. We also demonstrate that Pkc1p is required for proper derepression upon exhaustion of glucose of the SUC2 gene.

2. Materials and methods

2.1. Strains and growth conditions

The *S. cerevisiae* strains used in this study are shown in Table 1. Yeast cells were grown in medium containing 2% (w/v) peptone and 1% (w/v) yeast extract (YP) supplemented with carbon sources (glucose, galactose, raffinose or fructose) and 1 M sorbitol as indicated. Cellular growth was monitored by measuring the optical density (OD) at 600 nm or by development of colonies on agar plates.

2.2. Ethanol and glycerol determinations

For measurements of glycerol and ethanol production, cells were grown in an orbital incubator at 30°C up to the end of the exponential phase, harvested by centrifugation (approximately 2000 × g) and washed with 25 mM Mes buffer, pH 6.0, containing 1 M sorbitol. The cells were resuspended in 0.5 M Tris buffer pH 7.0 or YP medium both containing 1 M sorbitol and incubated again at 30°C in a shaking waterbath (100 rpm). Each culture was incubated for 20 min before sampling started. At zero time, 100 mM glucose was added.

Ethanol and intra- and extracellular glycerol were determined enzymatically using commercial kits (Boehringer, Mannheim, Germany). At the indicated times, samples containing 40 mg cells (wet weight) were taken. The cells were collected on Whatman GF/C filters (25 mm diameter) by vacuum filtration. In the supernatant, concentrations of ethanol and extracellular glycerol were determined immediately to avoid ethanol evaporation. The filters containing the cells were boiled in tubes with 2 ml 0.5 M Tris, pH 7.0, for 10 min. Part of the extract was centrifuged for

<table>
<thead>
<tr>
<th>Table 1</th>
<th><em>S. cerevisiae</em> strains used in this study</th>
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<tr>
<td>Name</td>
<td>Genotype</td>
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<tr>
<td>1783</td>
<td>Mat a leu2-3,112 ura3-52 trpl-1 his4 can1Δ</td>
</tr>
<tr>
<td>DL253</td>
<td>Mat a leu2-3,112 ura3-52 trpl-1 his4 can1Δ bck1Δ::URA3</td>
</tr>
<tr>
<td>DL376</td>
<td>Mat a leu2-3,112 ura3-52 trpl-1 his4 can1Δ bck1Δ::URA3 bck1Δ::LEU2</td>
</tr>
<tr>
<td>W303</td>
<td>Mat a ade2-1 his3-11.15 leu2-3,112 trpl-1 ura3-1 can1-100 GAL mal SUC2</td>
</tr>
<tr>
<td>YSH813</td>
<td>Mat a bck1Δ::URA3 (isogenic to W303-1A)</td>
</tr>
<tr>
<td>YSH849</td>
<td>Mat a mpk1Δ::TRP1 (isogenic to W303-1A)</td>
</tr>
<tr>
<td>YSH850</td>
<td>Mat a pck1Δ::HIS3 (isogenic to W303-1A)</td>
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3 min at 13000 rpm in a microcentrifuge and the intracellular glycerol was measured in the supernatant.

2.3. Determination of metabolite levels and invertase and SNF1 activities

Cell extraction for determination of metabolites was performed with the cold methanol rapid-quenching method [12]. The metabolites were determined essentially as described by Bergmeyer [2]. Measurement of specific activity of invertase activity was measured as described by Goldstein and Lampen [15] with the modification introduced by Celenza and Carlson [8], except that the assay was carried out at pH 5.1 and 37°C. Snf1 kinase activity induced by Celenza and Carlson [8], except that the assay was carried out at pH 5.1 and 37°C. Snf1 kinase activity was measured as described by Bergmeyer [2]. Measurement of specific activity of invertase activity was measured as described by Goldstein and Lampen [15] with the modification introduced by Celenza and Carlson [8], except that the assay was carried out at pH 5.1 and 37°C. Snf1 kinase activity induced by Celenza and Carlson [8], except that the assay was carried out at pH 5.1 and 37°C. Snf1 kinase activity was measured as described by Bergmeyer [2]. Measurement of specific activity of invertase activity was measured as described by Goldstein and Lampen [15] with the modification introduced by Celenza and Carlson [8], except that the assay was carried out at pH 5.1 and 37°C. Snf1 kinase activity was measured as described by Bergmeyer [2].

2.4. Glucose transport

The initial uptake rate of glucose was measured with \( ^{3}H \)-glucose (Amersham) in 40 \( \mu l \) of a cellular suspension containing around 2 mg (dry weight) of biomass in 50 mM Tris-citrate buffer, pH 5.0, plus 1 M sorbitol. At zero time, 10 \( \mu l \) of a water solution of labelled glucose (concentration range 0.2–75 mM; specific activity 30 Bq mmol\(^{-1}\)) was added. After 5 s, the uptake was arrested by addition of 5 ml of ice-cold water, also containing 1 M sorbitol. The suspension was filtered on Whatman GF/C filters (25 mm diameter) which were washed immediately with ice-cold 0.5 M glucose and the radioactivity was counted by liquid scintillation. Controls were prepared by adding 5 ml of ice-cold water prior to the addition of the labelled solution.

2.5. Determination of glucose consumption and protein content

Glucose consumption was measured with the classical glucose oxidase/peroxidase method. Protein was determined according to Lowry et al. [22].

2.6. Measurement of respiratory activity

Oxygen consumption was monitored using a Clark oxygen electrode. The cells were grown in YP medium supplemented with 2% (w/v) glucose and 1 M sorbitol, harvested at the end of the exponential phase and washed with a cold solution of 1 M sorbitol. The cells were resuspended in 50 mM phosphate buffer, pH 6.0, containing 1 M sorbitol and incubated at 30°C. The biomass concentration in the assay was 5 mg ml\(^{-1}\) (wet weight), and the cell suspension was bubbled with air during 5 min prior to the assay. To a final volume of 4 ml of cell suspension a pulse of 50 mM glucose was added and the oxygen consumption was continuously recorded.

2.7. RNA isolation and Northern blot analysis

For a shift from growth on glucose to growth on raffinose, yeast cells were grown in YP glucose (2%, w/v) plus 1 M sorbitol at 30°C up to the early exponential phase. The cells were washed quickly by centrifugation with 1 M sorbitol. The pellet was resuspended in YP raffinose (4%, w/v) and rapidly mixed. Samples of 20 ml were taken at time intervals, immediately cooled to 0°C by mixing with ice-cold water, and cells were recovered by centrifugation at 4°C and stored at −20°C.

Isolation of total yeast RNA was performed essentially as described by De Winde and Grivell [13], with minor modifications. Total RNA was separated on 1% (w/v) agarose in 50 mM boric acid, 1 mM sodium citrate, 5 mM NaOH, pH 7.5, containing 1% (w/v) formaldehyde. Subsequently RNA was blotted onto Hybond-N membranes in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and hybridised with gene-specific probes as described by De Winde and Grivell [13]. Probe fragments were radioactively labelled using the High Prime labelling kit (Boehringer). Hybridised Northern blots were evaluated and quantified using phosphorimager technology (Fuji, BAS-1000; Software, PCBASE2.0). In all cases equal amounts of RNA were loaded. Gene-specific probes were obtained by PCR. A 1.6-kb BamH I-KpnI fragment from pAct1 was used as a specific probe for the actin gene (constitutive endogenous control). For each time the relative expression of a given gene was estimated by dividing the intensity of the band (detected in the phosphorimager) by the correspondent actin band multiplied by 100.

2.8. Incorporation of [2-\textsuperscript{14}C]uracil

Total RNA synthesis activity was measured by following incorporation of [2-\textsuperscript{14}C]uracil with an adaptation of the method described by Van Laere et al. [30]. All the steps in the assay were performed in the presence of 1 M sorbitol. The cells were grown on YP raffinose (2%, w/v), harvested at the end of the exponential phase, washed twice by centrifugation (approximately 2000×g) and resuspended in 5 ml of YP medium. After addition of a solution of [2-\textsuperscript{14}C]uracil (specific activity 2.3 GBq mmol\(^{-1}\)), the cells were incubated at 30°C in a shaking waterbath. At time zero, 4% (w/v) glucose (final concentration) was added and samples of 150 \( \mu l \) (containing \( 5 \times 10^7 \) cells) were taken at time intervals, quickly (30 s) spun down in a microcentrifuge and washed twice with 1 M sorbitol. The final pellets were resuspended in a liquid scintillation cocktail and radioactivity was counted.

2.9. Reproducibility of results

All experiments were performed at least three times with consistent results. Representative results are shown.
3. Results

3.1. Slower initiation of growth and fermentation in the \textit{pkc1} mutant

Yeast cells with a deletion of the \textit{PKC1} gene initiated growth on glucose more slowly than the wild-type strain and the \textit{bck1} mutant. Initiation of glucose consumption and ethanol production were also delayed (Fig. 1). Moreover, the \textit{pkc1} mutant did not exhibit the typical diauxic growth observed in the other strains. Once the glucose was exhausted growth on ethanol was very poor. When respiration was blocked with antimycin the wild-type strain and the \textit{bck1} mutant did not show an increase in OD in the second growth phase. For the \textit{pkc1} mutant there was practically no difference between the conditions with and without antimycin (Fig. 1). The ethanol measurements showed that the \textit{pkc1} mutant consumed ethanol, albeit at a lower rate than the other strains. Apparently it used ethanol much less efficiently than the other strains (Fig. 1). Oxygen consumption measurements confirmed that respiration of the \textit{pkc1} mutant was severely reduced compared to that of the wild-type strain and the \textit{bck1} mutant. This was observed in two different genetic backgrounds (Table 2). Oxygen consumption in the \textit{bck1} strain was also reduced 20–60\% compared to that of the wild-type under the same conditions, but the \textit{pkc1} mutant showed a much greater reduction. Oxygen uptake was barely present in this strain.

We also checked whether the delay in glucose consumption and fermentation were a consequence of the growth defect. For this purpose we measured the initiation of...
ethanol and glycerol accumulation in the medium and the accumulation of intracellular glycerol immediately after addition of glucose to cells incubated in Mes buffer or YP medium (Fig. 2). The cells were grown to late exponential phase in the presence of 1 M sorbitol, washed and reincubated in the presence of 1 M sorbitol. The cells were then incubated at 30°C in Mes buffer, pH 6.0, (open symbols) or in YP medium (closed symbols), both containing 1 M sorbitol. After 20 min 100 mM glucose was added. A: Ethanol concentration in the medium; B: extracellular glycerol concentration; C: intracellular glycerol concentration.

Fig. 2. Initiation of ethanol and glycerol accumulation. The wild-type strain (1783; ■, ○), bck1Δ mutant (DL253; ▲, ▼) and pkc1Δ mutant (DL376; ●, ▴) were grown in YP glucose medium plus 1 M sorbitol up to the end of the exponential phase, harvested and washed with 1 M sorbitol. The cells were then incubated at 30°C in Mes buffer, pH 6.0, (open symbols) or in YP medium (closed symbols), both containing 1 M sorbitol. After 20 min 100 mM glucose was added. A: Ethanol concentration in the medium; B: extracellular glycerol concentration; C: intracellular glycerol concentration.

Fig. 3. Glucose transport capacity. The $V_{\text{max}}$ of glucose transport was measured at 0, 15, 60 and 120 min after addition of glucose. The cells were pre-grown in YP glucose medium plus 1 M sorbitol up to the end of the exponential phase, harvested and resuspended in YP medium plus 1 M sorbitol. At time zero 100 mM glucose was added. Two different genetic backgrounds were used: W303-1A, wild-type, and isogenic YSH850, pkc1Δ strain; 1783, wild-type, and DL376, isogenic pkc1Δ strain.

It was observed that only in cells grown on galactose the production of glycerol and ethanol was comparable to that observed in the wild-type (data not shown). On the other hand we performed experiments of cellular growth, glucose consumption, ethanol and glycerol production using strains from a second genetic background (W303) and all results obtained were essentially the same as those observed in Figs. 1 and 2. Thus, these observations suggest that the defects are direct consequences of the pkc1Δ mutation and they can be attributed neither to an indirect consequence of a delay in growth initiation nor to the fact that the strains used were from different genetic backgrounds. The hypothesis that loss of viability of cells at the end of the exponential phase could interfere with the results was also considered. This possibility was discarded since the ability to form colonies in this phase was similar in the mutant pkc1Δ and in the wild-type strain (data not shown).
3.2. Deficient induction of HXT glucose carrier genes in the \textit{pkc1} mutant

The $V_{\text{max}}$ of glucose transport was measured at different time points after addition of 2% glucose to cells incubated in YP medium containing 1 M sorbitol. The \textit{pkc1}$\Delta$ mutant showed a clear reduction in the $V_{\text{max}}$ of glucose transport as a function of time of incubation of the cells in the glucose-containing medium (Fig. 3). This was observed in two genetic backgrounds and suggested that the \textit{pkc1}$\Delta$ mutant was affected in glucose control of HXT gene expression. The $K_m$ of glucose transport was about 2–8 mM and was the same in all strains (results not shown), suggesting that low- and high-affinity carriers were affected by the \textit{pkc1}$\Delta$ mutation in a similar way.

Subsequently, we performed Northern blot analysis of the expression of three major glucose carriers, \textit{HXT1}, \textit{HXT2} and \textit{HXT4}, as a function of time after addition of 4% (w/v) glucose to cells of the wild-type, \textit{bck1}$\Delta$ and \textit{pkc1}$\Delta$ strains pre-grown on raffinose. As shown in Fig. 4, induction of the three genes was strongly reduced in the \textit{pkc1}$\Delta$ mutant. The similar reduction for the three genes is consistent with the absence of an effect on the $K_m$ of glucose uptake in the \textit{pkc1}$\Delta$ mutant (data not shown).

Interestingly, the \textit{bck1}$\Delta$ mutant showed a more pronounced induction as compared to the wild-type. These results should be analysed with caution since, at first view, and especially in relation to the expression of the \textit{HXT2} and \textit{HXT4} genes, the addition of 4% glucose seems to trigger an increase in the level of expression of such genes. These findings are apparently in strong contradiction to the model currently accepted indicating that both \textit{HXT2} and \textit{HXT4} genes are induced by raffinose (or low glucose concentrations) and repressed by high glucose concentrations. In our opinion, this could be related to differences between the experimental conditions used here and those used to study the regulation of the expression of HXT genes and also due to cross-hybridisation between HXT genes that present a high level of similarity (about 60–85% identity) [26,27]. The most important conclusion that we can draw from our results is that in a \textit{pkc1}$\Delta$ mutant the delay in glucose consumption and fermentation seems to be related to a clear incapacity to sustain an
efficient glucose uptake, most probably due to an atypical and low rate of HXT genes expression.

We checked whether the pkc1 mutant displayed a general defect in the stimulation of transcription upon feeding with glucose. However, measurement of the incorporation of radioactive uracil indicated a similar stimulation by glucose for the wild-type, bck1Δ, mpk1Δ and pkc1Δ strain (Fig. 5).

The defective initiation of fermentation in the pkc1Δ mutant might have been due to a reduction in glycolytic flux because of the lowered glucose transport. This might be reflected in reduced levels of glycolytic intermediates after addition of glucose. However, we could not detect a significant difference in the levels of glucose-6-phosphate, fructose-1,6-bisphosphate, ATP, NAD⁺ and phosphoenolpyruvate (results not shown).

3.3. The pkc1Δ mutant is deficient in derepression

The growth measurements shown in Fig. 1 indicated that the pkc1Δ mutant grew very poorly on ethanol, which might have been due to a defect in derepression of the genes controlled by the main glucose repression pathway. As shown in Fig. 6, growth of the pkc1Δ mutant was also very poor on glycerol and affected on galactose and raffinose as compared to the wild-type. These growth defects were not present in the bck1Δ and mpk1Δ mutants.

We further examined a possible derepression defect by measuring invertase activity on media with different carbon sources. As shown in Fig. 7 wild-type cells growing on galactose or raffinose displayed much higher invertase activity than cells growing on glucose or fructose. This was also true for cells of the mpk1Δ and bck1Δ mutants. Derepression of invertase activity upon transfer of the cells from glucose to raffinose medium was clearly deficient in the pkc1Δ mutant as compared to the three other strains (Fig. 7). Northern blot analysis showed that the SUC2 gene, encoding invertase, was less efficiently derepressed in the pkc1Δ strain than in the wild-type upon transfer of the cells from glucose to raffinose medium (Fig. 8).

3.4. Increase of the Snf1 kinase activity in the pkc1Δ mutant depends on the growth phase of the cells transferred to derepression conditions

Since Snf1p is known to be involved in the derepression process we investigated subsequently whether the pkc1Δ mutant might be affected in the Snf1 kinase activity. Transferring wild-type yeast cells pre-grown on glucose to derepression conditions (media low in glucose or raffinose) caused a sharp increase in Snf1 kinase activity, measured in cell extracts. This increase was independent of the growth phase of the cells transferred to derepression conditions. In cells pre-grown on glucose up to the early exponential phase or up to the mid-exponential phase, the Snf1 activity varied, respectively, from 8.43 to 42.2 and from 3.02 to 16.9 nmol Pi min⁻¹ (mg protein)⁻¹ when

![Fig. 6. Growth on agar plates with different carbon sources.](image)

![Fig. 7. Invertase activity under repressive and non-repressive growth conditions.](image)

![Fig. 8. Northern blot analysis of derepression of SUC2.](image)
the cells were transferred to low glucose. On the other hand, in \( pck1 \Delta \) mutant cells pre-grown on glucose up to the mid-exponential growth phase such an increase in the Snf1 activity was not observed \((1.05-1.69\text{ nmol Pi min}^{-1}\text{ (mg protein)}^{-1})\) when the cells were transferred to a low glucose concentration. However, for cells pre-grown on glucose up to the early exponential phase the Snf1 activity increased from 12.8 to 72 nmol Pi min\(^{-1}\) (mg protein\(^{-1}\)) when the cells were transferred to a low glucose concentration. These results suggest that the repressive phenotype observed in \( pck1 \Delta \) strains cannot be related to a defect in the activation of Snf1p. Thus, the involvement of the Pkc1p in the derepression process is most probably exerted by an alternative pathway.

4. Discussion

4.1. The \( pck1 \Delta \) mutant displays a defect in the initiation of fermentation

The \( pck1 \Delta \) mutant displayed two discernible major defects in carbon metabolism: a defect in the initiation of fermentation and a defect in derepression upon exhaustion of glucose in the medium. When yeast cells are provided with glucose a variety of regulatory mechanisms are activated, aimed at the rapid and optimal initiation of fermentation. This results in a rapid production of ethanol which allows the yeast cells to outcompete other microorganisms in their environment. We noticed that cells of the \( pck1 \Delta \) mutant showed a conspicuous delay in the start-up of fermentation, as measured by the increase in the ethanol level in the medium. This delay could have been a consequence of a growth defect due to the \( pck1 \Delta \) mutation. However, measurement of ethanol production during the first 2 h of fermentation indicated that the ethanol production defect was manifest from the very beginning of fermentation. This was evidenced by a very slow accumulation of ethanol during the first 2 h. Also the accumulation of glycerol in the medium and the increase in intracellular glycerol were very slow during this period. We considered a possible deficiency in glucose transport as a plausible cause and found that the \( V_{\text{max}} \) of glucose transport rapidly dropped in the \( pck1 \Delta \) mutant after addition of glucose but not in the wild-type strain. The kinetics of glucose transport are determined by contributions from different glucose transporters and glucose itself causes a strong redistribution in the expression of the \( HXT \) genes encoding the glucose transporters [19,20,27]. Since the \( K_m \) of glucose transport was not different between the wild-type and the \( pck1 \Delta \) mutant, more than one carrier was probably affected. Northern blot analysis indeed showed that the induction of \( HXT1, HXT2 \) and \( HXT4 \) after glucose addition was aberrant in the \( pck1 \Delta \) strain. This was not due to a general defect in the initiation of transcription since uracil incorporation after addition of glucose was not significantly different in the wild-type, \( bck1 \Delta, mpk1 \Delta \) and \( pck1 \Delta \) strain. Induction of the \( HXT \) genes is controlled by an elaborate sensing system in which two non-transporting glucose carrier homologues, Snf3 and Rgt2, play a major role [27]. The transcription factor, Rgt1, has also been identified but neither its expression nor its DNA-binding activity are regulated by glucose [28]. Hence, it seems most likely that Rgt1 is regulated posttranslationally and that this could affect its nuclear localisation and/or its transcriptional repression or activation abilities. All these components constitute possible targets for the \( PKC1 \) gene product. Our results demonstrate that the \( pck1 \Delta \) defect in the initiation of fermentation is not shared by mutants in the downstream MAP kinase cascade. Hence, the partial fermentation defect of the \( pck1 \Delta \) mutant might contribute to the more stringent phenotype of this strain compared to that of mutants in the downstream MAP kinase cascade.

4.2. The \( pck1 \Delta \) mutant displays a defect in derepression upon glucose exhaustion in the medium

The diauxic growth curve of the \( pck1 \Delta \) mutant on glucose medium indicated poor growth in the second growth phase on the ethanol produced during fermentation. This observation was confirmed by addition of antimycin which produced only little further inhibition of growth, and by incubation of the \( pck1 \Delta \) mutant with other carbon substrates. The latter revealed a general defect, respectively a weakness to grow on non- and poorly fermented carbon sources. This can have many causes but subsequent work revealed a defect in the derepression of invertase both at the level of enzyme activity and of the expression of \( SUC2 \).

The Snf1 protein kinase plays a major role in glucose repression and derepression [7,14]. Moreover, it has been demonstrated that Snf1p is a central component of highly conserved protein kinase cascades present in most eukaryotic organisms. Like for the mammalian homologues, in yeast there is a good correlation between increasing AMP/ATP ratio and activation of the Snf1p under conditions of glucose limitation. For this reason, these kinases are denominated AMP-activated protein kinases (AMPKs).

In yeast cells, after addition of glucose, Snf1 is dephosphorylated by the Glc7/Reg1 protein phosphatase which causes its inactivation. As a result the Mig1 transcriptional repressor is no longer phosphorylated by Snf1 and becomes active. Upon exhaustion of glucose or transfer to a non-fermentable carbon source Snf1 kinase activity rapidly increases. But, in spite of the fact that in mammalian systems the AMPKs are regulated through a phosphorylation mechanism by an upstream kinase – referred to as AMPKK [17,32], a Snf1-activating factor was not identified up to now. This reasoning led us to imagine that Pkc1p would be the kinase involved in this regulation. Nevertheless, our results suggest that Pkc1p is not the
protein kinase that phosphorylates Snf1. Future work will have to elucidate by which mechanism Pkc1 directly or indirectly controls the derepression process in yeast cells.

As was the case for the partial defect of the pkc1Δ mutant in the initiation of fermentation, the derepression defect was also specific for this strain and not observed in the MAP kinase mutants. Hence, the derepression defect might also contribute to some extent to the more stringent phenotype of the pkc1Δ mutant as opposed to that of the MAP kinase mutants. Up until now, no clear role of MAP kinase pathways in nutrient sensing or in the control of carbon metabolism has been observed in yeast. The specific involvement of Pkc1 in activation of the derepression process raises the question as to what upstream factor controls Pkc1 activity. So far, Pkc1 has been located in a cell integrity pathway which is believed to sense the composition and/or strength of the cell wall. On the other hand, it is well known that yeast cells growing on non-fermentable carbon sources have a different cell wall composition compared to cells growing on glucose, as evidenced by a higher resistance to cell wall-lytic enzymes. Up to now, this property has only been related to the activity of the cAMP-PKA pathway [4]. Possibly, upon derepression Pkc1p is activated by a hitherto undefined mechanism and activates two branches of the PKC MAP kinase pathway: one of them would control, in some way, a mechanism leading to rapid derepression of genes involved in respiration, gluconeogenesis and the utilisation of alternative carbon sources, and the other would be the MAP kinase pathway leading to adaptation of the composition of the cell wall.

4.3. Does Pkc1 have a central role in control of yeast metabolism and growth?

The Pkc1 pathway is not only essential for the maintenance of cellular integrity through its control of the expression of genes that encode enzymes involved in cell wall construction [18]. It also has links to other signal transduction pathways and/or cellular processes such as the mating MAP kinase pathway [6] and the components of the cell division system [24]. In addition, it is also involved in bud emergence [16]. Moreover, this pathway seems to be required for polarised cell growth and actin organisation [25]. A dual control was already suggested of the temperature-induced expression of yeast FKS2 by protein kinase C and by calcineurin, a Ca2+/calmodulin-dependent protein phosphatase [34]. On the other hand, one of the two complex forms of RNA polymerase II seems to function downstream of the PKC MAP kinase pathway cascade to regulate the expression of a subset of yeast genes [9].

Our present results add another level of complexity to the function of Pkc1p. In contrast to the other components of the pathway, this enzyme seems to be involved in the control of carbon metabolism. Of course, the possibility cannot be ruled out of occurrence of pleiotropic and less specific effects at the origin of the severe growth defects and/or that pkc1Δ cells would be sicker than the other mutants for some more trivial reasons, such as insufficient ATP or redox imbalance. However, both the results from the experiments on the incorporation of [2-14C]uracil and those related to the measurement of the intracellular levels of such compounds indicate that this is not the case.

Taken together, our findings suggest that Pkc1p might play a central role in the control of growth and metabolism in yeast by linking different signal transduction pathways to each other. Experiments to investigate in more detail such interactions are currently being performed.

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