Glucose-dependent activation of protein kinase A activity in *Saccharomyces cerevisiae* and phosphorylation of its *TPK1* catalytic subunit

Paula Portela, Silvia Moreno *

Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, Piso 4, Buenos Aires 1428, Argentina

Received 9 August 2005; accepted 9 September 2005

Available online 14 October 2005

Abstract

Protein kinase A (PKA), in yeast, plays a major role in controlling metabolism and gene expression in connection with the available nutrient conditions. We here measure, for the first time, a transient change in the in vivo PKA activity, along a cAMP peak produced by 100 mM glucose addition to glycerol-growing cells as well as a change in the phosphorylation state of its catalytic subunit (Tpk1p) following PKA activation. PKA activity was measured in situ in permeabilized cells, preserving its intracellular localization. Comparison of total PKA activity, measured in situ in permeabilized cells with data obtained from in vitro assays in crude extracts, underscores the inhibitory potency of the regulatory subunit within the cell. Tpk1p phosphorylation was detected through non-denaturing gel electrophoresis. Phosphorylation of Tpk1p increases its specificity constant toward kemptide substrate. The use of mutants of the cAMP pathway showed that phosphorylation depends on the activation of PKA via the G-protein coupled receptor pathway triggered by glucose. The phosphorylation state of Tpk1p was followed during the diauxic shift. Tpk1p phosphorylation is dynamic and reversible: its up-regulation correlates with a fully fermentative metabolism, while its down-regulation with stationary phase or respiratory metabolism. Reversible phosphorylation can thus be considered a new control mechanism possibly pointing to a fine-tuning of PKA activity in response to environmental conditions.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Glucose; PKA (protein kinase A); cAMP signalling; Phosphorylation; Yeast; In situ assay

1. Introduction

In the unicellular eukaryote, *Saccharomyces cerevisiae*, cAMP-dependent protein kinase (PKA) controls a multitude of processes. Its activation stimulates glycolysis, ensures rapid progression through the cell cycle, triggers the mobilization of trehalose and glycogen, represses stress-related and gluconeogenic genes and induces growth-related genes [1]. The PKA regulatory subunit (R) is encoded by the *BCY1* gene, and the partially redundant *TPK1, TPK2* and *TPK3* genes encode the catalytic subunits (C).

Glucose is a potent activator of cAMP synthesis through a G-protein coupled receptor (GPCR) system in combination with a still elusive glucose-phosphorylation-dependent system [2]. It has been recently described that the receptor Gpr1 directly senses sugars, and that sugars can effectively bind GPCRs with a low affinity in a binding pocket formed by the transmembrane domains [3].

PKA is responsive to fermentable carbon sources; the addition of glucose or sucrose to cells growing on a non-fermentable carbon source results in a rapid, transient accumulation of cAMP through activation of adenylate cyclase, followed by hydrolysis of cAMP by the low-affinity phosphodiesterase, Pde1p, as part of the feedback inhibition by activated PKA [4]. The glucose-induced cAMP signal seems to be required for the rapid transition from respiratory or basal stationary phase metabolism to fermentation [5,6].

Although changes in PKA-dependent phenotypes in response to glucose have been well studied, there has been no report on activation of PKA during stimulation of the cAMP signal. The measurement of PKA activity in the complex...
cellular scene requires methods that reflect the behaviour of PKA in its natural context within the cell, particularly now as the importance of the spatial and temporal aspects of signal transduction as well as the interaction of PKA with anchoring proteins (AKAPs) and substrates are becoming better appreciated [7–9]. In S. cerevisiae, the endogenous in situ PKA activity (measured using permeabilized cells in the absence of exogenously added cAMP) has been shown to correlate with the activity (measured using permeabilized cells in the absence of proteases (AKAPs) and substrates are becoming better appreciated [7–9]. In S. cerevisiae, the endogenous in situ PKA activity (measured using permeabilized cells in the absence of exogenously added cAMP) has been shown to correlate with the activity (measured using permeabilized cells in the absence of proteolytic activity). In the AGC family, the PKA has been extensively studied. Phosphorylation of mammalian catalytic subunit has been reported to occur at specific sites. The phosphorylation of some amino acids has been demonstrated to influence the assembly of an active enzyme and/or some of its kinetic properties [13,14]. However, little is known about the in vivo regulation of this phosphorylation, specifically about the effector-dependent change in catalytic subunit phosphorylation.

The present study was undertaken to investigate the in vivo activation of PKA during the transition from fermentative metabolism. Through the measurement of PKA activity in situ, in permeabilized cells in the presence of cAMP, we could evaluate its in vivo activation and make evident that the inhibition of C activity by R subunit within the cell can not be easily relieved by exogenous cAMP addition. We also show that the reconfiguration of metabolism implicates a transient increase of PKA activity with a change in the phosphorylation state of Tpk1p, dependent on PKA activation triggered by glucose, through the GPCR pathway. Preliminary studies indicate that phosphorylation increases the specificity constant of Tpk1p. Finally, we show that the phosphorylation degree of Tpk1p in cells grown in different carbon sources and during the diauxic shift correlates positively with the presence of a fermentable carbon source.

2. Materials and methods

2.1. Yeast strains, growth media and growth conditions

The yeast strains, genotype and genetic nomenclature used in this study are listed in Table 1. Strains were grown on rich medium containing 2% bactopeptone, 1% yeast extract and 2% glycerol (YPGly), 2% potassium acetate (YPA), 2% galactose (YPGal) or 2% glucose (YPG). Carbon source-depressed cells used for the experiments were grown on YPGly until exponential phase ($D_{600}$ 0.8). Cells were collected by centrifugation at 4 °C, washed once with ice-cold 25 mM MES-KOH pH 6 and incubated for 15 min in fresh YPGly, before glucose (100 mM final concentration) was added. Aliquots were taken at different times and processed according to each determination (see below). The zero time point always corresponds to samples taken immediately before glucose addition.

2.2. Standard PKA assay

The assay was started by mixing the different amounts of PKA coming from different sources (permeabilized cells, crude extracts or partially purified samples) with assay mixture to give a final volume of 70 μl containing 50 mM Tris–HCl pH 7, 0.1 mM EGTA, 0.1 mM EDTA, 15 mM MgCl2, 10 mM 2-mercaptoethanol, 0.1 mM [γ-32P] ATP (Perkin-Elmer Life Science, used at 700–1200 dpm/pmol), 200 μM kemptide and 10 μM cAMP (when added). After incubation at 30 °C for the times indicated in each determination, aliquots were processed according to the phosphocellulose paper (Whatman) method [15]. PKA activity is expressed in units (U), defined as the amount of enzyme catalyzing the incorporation of 1 pmol of phosphate to substrate per minute at 30 °C [pmol min⁻¹].

2.3. In situ PKA activity

Culture samples (1–2 x 10⁸ cells) obtained at different times before and after addition of glucose were immediately cooled by addition of ice-cold water. The cells were collected by centrifugation at 4 °C. Sedimented cells were washed once with ice-cold water and suspended in 0.5 ml 40 mM Tris–HCl pH 7, 0.3 mM EGTA, 0.5 mM EDTA, 5 mM β-glycerophosphate, 10 mM 2-mercaptoethanol, complete EDTA-free protease inhibitor cocktail (Roche) (Buffer A), mixed with 0.075 ml toluene/ethanol (1:4, v/v) and vortexed for 5 min. The cells were immediately pelleted, washed once and resuspended in the same buffer. Permeabilized cells were used within 30 min of preparation. The in situ PKA activity was measured by incubation of 0.5–1.5 x 10⁶ permeabilized cells in a final volume of 70 μl for 3 min at 30 °C in the standard PKA assay mixture. Alternatively, the permeabilized cells were preincubated at 4 °C

<table>
<thead>
<tr>
<th>Table 1: List of strains and nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>KT1115</td>
</tr>
<tr>
<td>SP1</td>
</tr>
<tr>
<td>W303</td>
</tr>
<tr>
<td>S7-7 A</td>
</tr>
<tr>
<td>S13-3 A</td>
</tr>
<tr>
<td>KT1449</td>
</tr>
<tr>
<td>LK41</td>
</tr>
<tr>
<td>RS13-58A-1</td>
</tr>
<tr>
<td>35,2, m2/4</td>
</tr>
</tbody>
</table>
with an assay mixture containing the same compounds, as described before, except kemptide. After 10 min, the reaction was started by kemptide addition. The PKA assays were linear with time and cell number.

2.4. Crude extract preparation and partial purification of PKA

Samples were taken as for in situ PKA activity determination. Sedimented cells were lysed by disruption with glass beads at 4 °C in 0.5 ml buffer A. Crude extracts were either used directly for enzymatic assay or submitted to partial purification as described previously [16]. Protein was determined by the Bradford assay with Bovine serum albumin as standard.

2.5. Determination of PKA subunit activities

The determination of regulatory subunit activity was based on the measurement of its binding capacity for \(^{3}H\)cAMP (DuPont New England Nuclear). Aliquots of partially purified samples (10–50 μg) were processed as described [16]. Total catalytic subunit activity was determined in aliquots of partially purified samples or crude extract under the standard assay condition, in the presence of cAMP. The assay was started by mixing the samples containing different amounts of protein (0.2–0.4 μg) with assay mixture. After 10 min at 30 °C, aliquots were processed as described in the standard PKA assay. PKA assays were linear with time and protein concentration.

2.6. Determination of cAMP levels

A cAMP assay kit (Amersham) based on the competition of cAMP with \(^{3}H\)cAMP for the regulatory subunit of cAMP-dependent protein kinase was used for the measurement of total cAMP. Aliquots (approximately 70 mg wet weight) from glucose-induced derepressed cell cultures were removed at different times and processed as described [17]. Results are expressed as pmol (g ww)\(^{-1}\). Effective concentration of the 10 μM cAMP added to the in situ PKA assay after the 3 min incubation was estimated by a standard phosphodiesterase assay [18] under identical incubation conditions as those for in the in situ PKA assay. The cAMP hydrolysis was followed by addition of \(^{3}H\)cAMP as tracer, to a final specific activity of 460 dpm/pmol.

2.7. SDS-PAGE electrophoresis

Samples of the partially purified preparations of PKA (20–30 μg per lane) or aliquots of exponentially growing cells (0.25 ml) disrupted with glass beads were separated by SDS-PAGE 10% T Laemmli system. Blots were probed with α-TPKI, α-BCY1, α-P-CREB (S133) (Santa Cruz Biotechnology) and α-Msn2 antibodies (kindly provided by F. Estruch, Universitat de Valencia, Spain). The blots were developed with Chemiluminescence Luminol reagent (Santa Cruz Biotechnology), and immunoreactive bands were visualized by autoradiography and analyzed by digital imaging.

2.8. Native gel electrophoresis

Cells were grown to exponential phase on glycerol and 100 mM glucose was added at time zero. Samples were harvested and suspended in 0.2 ml cold extraction buffer A. The pelleted cells were disrupted in buffer A with glass beads, and the lysate was clarified and mixed with native sample buffer (0.015 M Tris–HCl pH 9, 5% glycerol, 0.0125% Bromophenol Blue). In control experiments, buffer A was prepared in the presence of a complete phosphatase inhibitor cocktail containing, besides 5 mM β-glycerol phosphate, 0.3 mM EGTA and 0.5 mM EDTA the following phosphatase inhibitors: 10 mM NaF, 10 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate and 0.1 mM ammonium molybdate. No differences in the ratio of high and low phosphorylated isoforms of Tpk1p during the glucose stimulus was observed using this aggressive phosphatase inhibitor cocktail when compared with buffer A. Gels were prepared essentially as described for SDS-PAGE but SDS was omitted and the upper buffer was prepared with Tris–HCl 0.15 M pH 9. The native molecular mass of Tpk1p isoforms and Bcy1p dimer were determined by PAGE as described [19], followed by Western blot as described above. This method involves electrophoresis under non-denaturing and non-reducing conditions in a series of gels with various concentrations of polyacrylamide. The standard proteins were Trypsin Inhibitor (Sigma), Carbonic Anhydrase B (Sigma) and Bovine serum albumin Fraction V (Gibco BRL). The immunoreactive bands were visualized by autoradiography and analyzed by digital imaging. The densitometric scanning of the bands are shown on top of each lane in Figs. 3–6. They are intended to show the relative abundance of the isoforms within the lane. Grey and black arrowheads indicate, respectively, the lower and higher mobility Tpk1p isoforms.

2.9. Alkaline phosphatase treatment of catalytic subunit

Alkaline phosphatase was used to dephosphorylate Tpk1p proteins present in crude extracts from SP1WT, TPK1 BCY1, TPK1 bcy1 Δ and tpk1\(^{+}\) strains. To analyze the Tpk1p isoforms on native PAGE upon phosphatase treatment, samples containing 80 μg of total protein were incubated for 15 min at 37 °C in a total volume of 40 μl with 0.1 M Tris–HCl pH 9.5, 50 mM MgCl\(_2\), 0.1 M NaCl and 100 units of alkaline phosphatase (Sigma). The reaction was stopped by addition of phosphatase inhibitors. Samples treated with or without phosphatase were separated by native gel electrophoresis and the membrane blotted with α-TPK1.

2.10. Determination of kinetic parameters of Tpk1p isoforms

The \(K_m\) and \(V_{max}\) for Tpk1p isoforms were determined in extracts containing Tpk1p with different degrees of phosphorylation, submitted or not to dephosphorylation conditions. Crude extracts from TPK1 bcy1 Δ (350 μg) or TPK1 BCY1 (100 μg) glycerol-grown cells were harvested before or shortly after glucose addition (as indicated) and incubated with alkaline phosphatase-biotinimidocaproyl (Sigma); alkaline phosphatase
was magnetically separated, as described previously [20]. The supernatant was used as source of dephosphorylated isoform of Tpk1p for kinetic parameter determinations. Mock treated crude extract was used as source of phosphorylated isoforms of Tpk1p protein. The kinase activity was measured as described under standard PKA assay but using variable kemptide concentration. Kinetic data were analyzed according to the Michaelis–Menten equation, in order to estimate the kinetic constants $K_m$ and $V_{\text{max}}$.

2.11. Reproducibility of results

All the experiments were repeated several times (indicated in each figure) with independent culture and enzymatic preparations. The in vivo PKA activity+cAMP and densitometric analysis of relative abundance of isoforms of Tpk1p were analyzed using T test dependent samples. The ratio of higher to lower phosphorylated isoforms, derived from the densitometric analysis of the bands from the native gels are included in the legend to each corresponding figure. The effect of phosphatase treatment on catalytic efficiency was analyzed using ANOVA–Tukey HSD test.

3. Results

3.1. In situ PKA activity during glucose-induced signalling

Changes in the activity of cAMP-PKA signal transduction pathway are usually monitored through phenotypic or biochemical changes that are assumed to be mediated by in vivo PKA activation. In order to directly evaluate the in vivo activation of PKA we undertook the measurement of in situ PKA activity during a physiological and transient increase in cAMP levels, and established a correlation with the phosphorylation state of a bona fide endogenous PKA substrate.

From our own previous results [10], we knew that although measurement of PKA activity in vitro in crude extracts gives a good estimate of total PKA activity in the cell (when assayed +cAMP), it is not a useful tool when trying to monitor the level of in vivo activation of PKA. The principal reason is that the intracellular concentrations of its regulatory (R) and catalytic (C) subunits as well as the one of cAMP are not preserved due to the dilution conditions of the extract preparation and further enzymatic assay. On the contrary, the in situ assay, in which PKA activity is measured in permeabilized cells using kemptide as an exogenous substrate (+/−cAMP) allows to control the ionic and metabolic environment of the assay, while preserving the in vivo protein concentration, some of the in vivo intracellular compartments and the endogenous relationships between protein kinase, protein phosphatases, anchoring proteins and substrates. Therefore, the method of choice of this work was the in situ assay.

As a first step, we measured the cAMP levels upon addition of glucose 100 mM to cells growing exponentially on glycerol, to verify the activation of the cAMP pathway under our experimental conditions. Three different wild type background strains (1115WT, SPIWT and W303WT) were used for these experiments. In every case, cAMP accumulation reached maximum values at 1−2 min post-glucose addition (Fig. 1A and data not shown).

The measurement of in vivo activation of PKA during glucose-induced cAMP signalling was determined, as a first approach, by the in situ assay, in the absence of exogenously added cAMP. The endogenous kinase activity of 1115WT strain displayed a consistently reproducible fluctuation with maximum values at 2.5−3 min post-glucose addition, showing a temporal correlation with the cAMP peak (Fig. 1B left, −cAMP curve). This result was highly reproducible and indicates there was an activation of PKA in response to the in vivo increase in cAMP. However, in SPIWT (Fig. 1B right, −cAMP curve) and W303WT strains (data not shown), the endogenous PKA activity was systematically below the detection level of the assay; therefore no change could be detected during the 10−15 min post-glucose addition. The difference is probably due to the fact that C subunit is an unstable protein [21,22] and that the 1115WT strain has a deletion in the PEP4 gene, which decreases proteolysis in general. We therefore foresee that in situ PKA activity measured in the absence of exogenous cAMP, will not be the suitable parameter to measure if one wants to follow in vivo PKA activation in all kind of strains.

The in situ kinase activity was also measured in the presence of 10 μM cAMP added exogenously to the kinase assay. Samples of cells from the three wild type strains were taken at different times after glucose addition. The results (Fig. 1B, +cAMP curve and data not shown for W303WT) show that an increase in cAMP in vivo was correlated with a higher activation of PKA by the exogenously added cAMP. During the design of this experiment we were aware that this in situ assay (+cAMP) did not detect the total PKA activity in the cell, and that the levels of in situ specific PKA activity were 100−200 fold lower than those estimated from in vitro assays (+cAMP) in crude extracts of the same cells [10] (For examples of these differences using SPIWT and TPK1 BCY1 strains, see Table 2). This peak of PKA activity (+cAMP) could not be observed in vitro, when PKA was measured in crude extracts (+/−cAMP) along the 10 min after glucose addition, which yielded a constant value (data not shown).

However, before hypothesizing on the meaning of the results of Fig. 1B (+cAMP), we needed to discard some possible artifactual reasons. The first one was that, since the exogenous 10 μM cAMP was added simultaneously with kemptide and ATP/Mg$^{2+}$ to the protein kinase assay mixture, and the incubation time is of only 3 min, no time for efficient cAMP binding was being left before assaying the catalytic phosphorylating activity; therefore, if under this condition one intends to measure not only endogenously activated PKA, but also PKA activated by the cAMP added to the assay, this incubation conditions might result inefficient and distort the results. The second one comes from the knowledge that addition of glucose causes a transient drop in the ATP level, coinciding with a rapid build-up of sugar phosphate [17] and that this difference in ATP concentration could be reflected in a different degree of occupancy of PKA with intracellularly prebound non-
labeled ATP. To exclude these two possibilities and to facilitate the PKA activation process we measured the in situ kinase activity in two steps: permeabilized cells from $1115\text{WT}$ strain were preincubated with assay mixture containing [$\gamma$-32P]ATP, Mg$^{2+}$ and 10 $\mu$M cAMP for 10 min at 4 °C; during this time cAMP could bind to its sites and the ATP pool could be equilibrated. In the second step, kemptide was added and its phosphorylation measured after 3 min at 30 °C. The activity detected, although higher, still shows the same behaviour for PKA activation (Fig. 1 panel B, right, +cAMP prein curve).

A last artifact that could somehow explain the results was that the effective levels of the exogenously added 10 $\mu$M cAMP would not remain constant during the 3 min assay, due to differential degradation by phosphodiesterases during the time...
course. However, by adding [3H]cAMP as a tracer to the same incubation mixture used for the in situ PKA assay, we could measure, using the classical phosphodiesterase assay, that not more than 1% of the 10μM cAMP was degraded during the 3 min of the PKA assay.

Having discarded the possible artifacts, we propose that the peak of PKA activity detected in the presence of exogenous cAMP, along the glucose pulse, is very probably the consequence of a differential activation state acquired by the holoenzyme molecules after being in contact with the endogenously produced cAMP, triggered by the addition of glucose to derepressed cells. These “primed” molecules are thus sensitive to further activation by the exogenously added 10μM cAMP. This peak is observed in the three wild type strains tested. To investigate the consequence glucose activation of PKA had on the levels of its subunits, we analyzed the total amount of R and C by means of two methods: determination of their corresponding activities assayed in vitro in crude extracts and Western blot. To obtain a direct relationship between expression level and activity of catalytic subunit, this experiment was carried out in a strain lacking TPK2 and TPK3 genes, since we can only follow Tpk1p with the available antibodies. The TPK1 BCY1 strain showed the same correlation between total cAMP levels and in situ activation of PKA plus cAMP (Fig. 2 panel A and B) demonstrated with the wild type strains (see Fig. 1). Fig. 2C shows that, after the peak of cAMP, the total levels of holoenzyme subunits remained constant. We can therefore infer that the decrease in kinase activity observed in the in situ assay plus exogenous cAMP, after 5–10 min post-glucose addition is the consequence of the shift of the holoenzyme equilibrium toward the associated state promoted by the reduction of intracellular cAMP level and not due to a decrease in the C subunit levels.

As an independent and indirect way of detecting the in vivo activation of PKA, we were interested in following the phosphorylation of a direct target of PKA. One of these targets is the transcription factor Msn2, responsible for the expression of stress genes, such as HSP12 and SSA3. After glucose addition to stationary phase cells, the expression of stress genes is rapidly turned-off, due to the relocalization of Msn2 to cytoplasm, in a PKA-mediated process [23,24]. It has been recently demonstrated that the main nuclear localization signal (NLS) of Msn2p is directly modified by PKA in vitro and in vivo, thereby influencing its overall intracellular distribution [25]. We therefore chose to follow the phosphorylation status of

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic nomenclature</th>
<th>In vitro specific activity&lt;sup&gt;a&lt;/sup&gt; (U/μg protein)</th>
<th>In situ specific activity&lt;sup&gt;b&lt;/sup&gt; (U/μg protein)</th>
<th>Ratio&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>SP1WT</td>
<td>0.80</td>
<td>0.0080</td>
<td>100</td>
</tr>
<tr>
<td>S7-7A</td>
<td>TPK1 BCY1</td>
<td>0.45</td>
<td>0.0025</td>
<td>0.005</td>
</tr>
<tr>
<td>S13-3A</td>
<td>TPK1 bcy1Δ</td>
<td>0.066</td>
<td>0.019</td>
<td>3.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> In vitro specific PKA activity in crude extracts was experimentally measured in presence of 10μM cAMP as described under Materials and methods (these values did not change during the 10 min after glucose addition).

<sup>b</sup> In situ specific PKA activity was experimentally measured in permeabilized cells at Time 0 (before glucose addition) and at Time max (time of maximal response after addition of glucose) in the presence of 10μM cAMP; data were expressed as U/μg protein assuming that 1 cell contains 1.6×10<sup>7</sup>μg of protein.

<sup>c</sup> Ratio between the in vitro and the in situ PKA specific activities.

![Fig. 2](image-url)
Msn2p after glucose addition to glycerol-growing 1115WT and SP1WT cells, by means of Western blot with anti-P-CREB antibody [25]. Fig. 1C shows that upon glucose addition, and in both wild type strains there is a clear increase in Msn2 phosphorylation (evaluated by the ratio of the densitometry of phosphorylated Msn2p to total Msn2p) that begins to be detected at 3 min, delayed in time with the increase in cAMP levels. This result is compatible with a phosphorylation of this substrate by the PKA activated in vivo following the increase in cAMP triggered by glucose.

Summarizing, the temporal correlation between cAMP levels, in situ PKA activity (+cAMP) and in vivo phosphorylation of a PKA target (Fig. 1), indicate that the endogenous activation of PKA triggered by the glucose-stimulated cAMP peak can be monitored by the in situ assay of PKA activity in the presence of exogenously added 10 μM cAMP. This assay monitors the activation phase of PKA as well as its shut-down, while substrate phosphorylation, besides sometimes indirect, is delayed and does not monitor the shut-down phase.

### 3.2. Phosphorylation state of Tpk1p during the transition from respiratory to fermentative metabolism

The activity of protein kinases, as an enzyme group, is controlled by several mechanisms including additional subunits or domains that may function in response to second messengers, subcellular localization, phosphorylation and dephosphorylation by kinases and phosphatases [11]. Two-dimensional gel electrophoretic analysis of Tpk1p indicated the presence of several isoforms [26], suggesting that the catalytic subunit contains several points of post-translational modification.

The results of Figs. 1 and 2 indicate that PKA presents a differential activation state throughout the cAMP peak triggered in response to glucose. We investigated a possible change on the phosphorylation state of the catalytic subunit upon addition of...
glucose. SDS-PAGE Western blot analysis, using anti-TPK1, of crude extracts from glycerol-growing cells exposed to glucose (Fig. 2C, left panel for TPK1 BCY1 and data not shown for SP1WT) did not reveal either at 0 or 10 min, the presence of Tpk1p isoforms with different mobility. However, native polyacrylamide gel electrophoresis followed by Western blot analysis of crude extracts from wild type strains with different backgrounds (Fig. 3) revealed the presence of two principal Tpk1p isoforms. The estimated molecular mass for the two bands was 50 kDa. No bands were detected on native Western blot analysis of crude extract from TPK2Δtpk1ΔΔtpk3Δ BCY1 strain, used as control (data not shown). These results indicate that the isoforms detected correspond to monomers of Tpk1p. We do not claim that these monomers of C correspond to the free in vivo C subunit. In fact, the total amount of Tpk1p isoforms, quantitated by phosphoimager technology, evaluated during the 15 min glucose stimulus revealed that the amount of catalytic subunit detected in these gels did not change. We think that the free C arises from a spontaneous dissociation of the holoenzyme due to the dilution in pH 9 buffer, to which the extract has to be submitted for the gel electrophoresis. Upon alkaline phosphatase treatment of crude extract from glycerol-grown cells, a change in the migration pattern was observed (Fig. 3B), resulting in a decrease in mobility of the faster-migrating isoforms of Tpk1p. Note that the reduction in mobility of the faster-migrating isoforms was accompanied by an increase in the intensity of the immunoreactive band that could correspond to the sum of the two isoforms detected without phosphatase treatment (compare Fig. 3B lane −, + Ppase). This result indicates that the modification in the migration pattern is due to phosphorylation on Tpk1p. The mobility of the slowermigrating isoform and of the immunoreactive band after alkaline phosphatase treatment was the same, indicating that either this isoform is still phosphorylated, although phosphatase-resistant, or that it corresponds to the unphosphorylated isoform of Tpk1p.

The evaluation of the phosphorylation status of Tpk1p was followed after glucose addition to glycerol-growing cells. It can be observed that in the three wild type strains (Fig. 3 A,D and not shown for W303WT) as well as in the TPK1 BCY1 strain (Fig. 3C), the ratio of phosphorylated isoforms of Tpk1p shifted from a higher abundance of the slower mobility band at time 0 to a higher abundance of the higher mobility isoform at 10–15 min. The ratio of higher to lower phosphorylated isoforms was obtained from densitometric analysis of the three wild type strains and TPK1 BCY1 strain; these values oscillated between 0.6–0.8 at time 0 (before glucose addition) to 1.1–1.4 at time 10 min post stimulus, being this difference statistically significant (for details see legend to Fig. 3). No change in the phosphorylation state of Tpk1p was observed when cells from the gpr1Δ strain, in which the membrane sugar receptor was deleted, were exposed to 100 mM glucose (Fig. 3E).

Comparison of the kinetics of the transient in vivo activation of PKA by glucose with the appearance of endogenously phosphorylated Tpk1p isoforms suggests that activation of PKA, via the GPCR pathway, was followed by an increase in phosphorylated species of Tpk1p.

3.3. Analysis of Tpk1p isoforms and in situ PKA activity in PKA mutants

The results of Fig. 3 suggest that the accumulation of phosphorylated isoforms of Tpk1p is a consequence of the in vivo activation of PKA by glucose. To test this hypothesis we studied whether there was a correlation between the phosphorylation state of Tpk1p and PKA activity in strains with low, intermediate and high constitutive PKA activity.

Yeast tpklw1 mutant cells show greatly reduced activity of PKA. In this strain, two of the TPK genes are deleted, whereas the third TPK gene is partially inactivated with no detectable PKA activity either in crude extracts or in permeabilized cells [20,27]. Addition of glucose to derepressed cells of this strain results in a huge, long lasting increase in cAMP levels [28]. Native gel Western blot analysis of extracts from tpklw1 cells growing on glycerol showed two isoforms of Tpk1p (Fig. 4, lane 0 min), with a high predominance of the slow migrating isoform. Alkaline phosphatase treatment prior to Western blot analysis of crude extracts obtained at 0 min resulted in the disappearance of the small amount of higher mobility band, indicating the phosphorylated nature of this tpklw1 isoform (Fig. 4 bottom panel). The important difference displayed by this strain is that the profile of isoforms does not change upon glucose addition, and remains with the same relative abundance.

![Image](365x339 to 548x373)

![Image](366x608)

Fig. 4. The change in the phosphorylation state of Tpk1p is dependent on PKA activity. The weak PKA activity strain tpklw1 was grown until exponential phase on YP Gly. Glucose (100 mM) was added at time zero. Samples were collected at different times. Equal amounts of protein from crude extracts were separated by native gel electrophoresis and Tpk1p isoforms were developed using anti-TPK1 antibody. The densitometric ratio of higher to lower phosphorylated isoforms was constant (0.33±0.12, n:3) throughout the 30 min. A native gel with samples of tpklw1 extract, with and without alkaline phosphatase treatment is shown in the bottom. The first two lanes contain the same amount of sample, while lane 3 was loaded with twice the amount of the untreated sample for better visualization of the low amount of higher mobility band. As a mobility control, samples from SPIWT extracts were loaded in the same gels. Grey and black arrows indicate, respectively, the lower and higher mobility Tpk1p isoforms.
of the slower-migrating isofrom up to 30 min (Fig. 4). These results indicate that in the presence of very low levels of PKA activity, the phosphorylation state of Tpk1p is shifted toward the lower phosphorylation isofroms and no change is observed during the glucose stimulus. The slight difference in mobility displayed by the mutant Tpk1p when compared to its wild type counterpart might be attributed to a difference in protein conformation resulting from the change of Leu217, in an hydrophobic pocket, to an hydrophobic Ser217. The presence of a minor proportion of phosphorylated isofroms in tpk1Δ could indicate that this strain contains a minimal kinase activity, undetectable in kinase assays. Cells with a deletion of the three TPK genes are not viable, suggesting that the weak mutants could present certain kinase activity. In accordance with the behaviour of the tpk1w1 strain, the cdc35Δ pde2Δ msn2/4Δ strain, in which almost no kinase activity is predicted due to the lack of adenylate cyclase, revealed only the presence of the slow migrating Tpk1p isofrom (data not shown).

As an example of a mutant with constitutively high PKA activity, we used a TPK1 bcy1Δ strain. It has been demonstrated that these kind of strains have very low levels of cAMP, and a strongly reduced glucose-induced increase in cAMP [29]. As shown in Fig. 5A, in the absence of the PKA regulatory subunit, the in situ PKA activity of strain TPK1 bcy1Δ remained almost constant after glucose addition. The phosphorylation state of Tpk1p showed the same pattern during the 10 min shift of the experiment; this pattern consisted of several bands with higher mobility (Fig. 5B shows a representative example) than the isofroms of the SPT1WT strain, shown for comparison. Phosphatase treatment of the samples shifted the bands upward, toward one band of lower mobility (Fig. 7A), indicating that the isofroms arose from a differential degree of phosphorylation. These results indicate that a strain with unregulated kinase activity presents hyperphosphorylated isofroms of Tpk1p. Table 2 shows that in the case of this strain, specific PKA activity measured either in situ or in vitro (+cAMP) is almost the same and the in situ specific activity (+cAMP) is 7-fold higher than the one measured for TPK1 BCY1 permeabilized cells in the presence of cAMP (0.019 vs. 0.0025 U/mg protein). This result was expected for a strain with unbridled PKA activity.

As an example of a strain with constitutively intermediate PKA activity we chose to use the 16 strain which contains the mutated bcy1-16 allele with a complete deletion of cAMP-binding domain B of the R subunit, and displays intermediate PKA activated phenotypes [10]. We analyzed the behaviour of this strain grown in glycerol in response to glucose addition, measuring cAMP levels, in situ PKA activity (−/+exogenous cAMP addition) and phosphorylation status of Tpk1p (Fig. 5C, D, E). As expected for a strain with intermediate PKA activity and therefore, intermediate feedback inhibition, the bcy1-16 strain accumulated a certain level of cAMP but showed a highly reduced glucose-induced cAMP accumulation (Fig. 5C). This indicates a strong inhibitory effect of high PKA activity in the bcy1-16 mutant on the cAMP response. The in situ PKA activity with and without 10 μM cAMP was almost constant through glucose stimulation, as expected for the very low cAMP signal (Fig. 5D). Native gel Western blot analysis of crude extract from bcy1-16 revealed three Tpk1p isofroms, without change in the relative abundance of the isofroms (Fig. 5E).

These results show there is a direct correlation between the basal in vivo level of PKA activity (either genetically predicted or as detected in the in situ assay without exogenously added cAMP) of the strains used: tpk1w1<TPK1 BCY1<16<TPK1 bcy1Δ and the phosphorylation state of Tpk1p. Even among the wild type strains, a difference can be observed in the phosphorylation state of Tpk1p in the 1115WT, which has a higher basal PKA activity, both in the higher abundance of the more phosphorylated isofrom, as well as in the appearance of a third, although minor, more phosphorylated isofrom (Fig. 3). A higher PKA activity is therefore corresponded with a higher phosphorylation state of Tpk1p. The presence of several isofroms indicates that Tpk1 protein is capable of being multi-phosphorylated.

In conclusion, the change in the phosphorylation pattern of Tpk1p after glucose addition to cells grown in glycerol was completely dependent on the activation of PKA. The absence of either PKA activity or cAMP signalling abolished a change in the phosphorylation status of Tpk1p after glucose addition.

3.4. Analysis of the phosphorylation state of Tpk1 protein during respiratory and fermentative metabolisms

Until now we have shown that the Tpk1p phosphorylation state gradually changes toward higher phosphorylated isofroms during the transition from respiratory to fermentative metabolism, when glucose is added to glycerol-growing cells. We decided to investigate the phosphorylation pattern of Tpk1p during the growth on different carbon sources. In cells grown on acetate and glycerol, the major Tpk1p isofrom detected was the slower-migrating isofrom (Fig. 6A, lanes Ac and Gly). Cells grown on a poor fermentable carbon source, such as galactose, show a relative abundance of isofroms similar to extracts from cells growing on non-fermentable sources (Fig. 6, lane Gal). In contrast, cells grown on glucose show equal proportion of slower- and faster-migrating isofroms (Fig. 6A, lane Glu). Together with the observation of the difference in the relative abundance of Tpk1p isofroms under different carbon sources we observed an increase in Tpk1p levels during growth on non-fermentable carbon sources (Fig. 6B). In cells grown on acetate and glycerol, an increase in Bcy1p expression and slower-migrating isofroms of Bcy1p were also detected (Fig. 6B). Increased expression, phosphorylation and change in localization of Bcy1p have already been reported to occur in cells grown to stationary phase or non-fermentable carbon source [30].

Considering the direct relationship between carbon source and phosphorylation state of Tpk1p, we studied the behaviour of Tpk1p phosphorylation during the diauxic shift of an aerobic culture grown on glucose (Fig. 6C). The diauxic shift involves a transition from an oxido-reductive (fermentation) to a purely oxidative (respiration) metabolism. Inoculation of yeast into a medium rich in sugar is followed by rapid growth fueled by fermentation, with the production of ethanol. When the
fermentable sugar is exhausted, the yeast cells turn to ethanol as carbon source for aerobic growth. Throughout logarithmic growth, we observed an accumulation of lower mobility isoforms and an increase in the Tpk1p level (Fig. 6C), as cells enter the diauxic shift.

These results taken as a whole, suggest that the expression of TPK1 and BCY1 genes increase concertedly once glucose levels begin to decrease. These data confirm those of a published report [31], and are in accordance with data obtained from gene expression studies at genomic scale performed during diauxic shift [32].

From the evaluation of PKA-dependent phenotypes, it is currently accepted that both respiratory metabolism as well as growth to stationary phase are dependent on the down-
Fig. 6. The level of expression and phosphorylation state of Tpk1p is carbon source dependent. Equal amount of crude extract samples (20–30 μg) from cells growing exponentially on YP medium supplemented with acetate (Ac), glycerol (Gly), galactose (Gal) or glucose (Glu) were submitted to native gel electrophoresis (panel A) and SDS-PAGE (panel B). Tpk1p was visualized using anti-TPK1 antibody. Panel A bottom shows higher densitometric exposures of the same lanes. The denatured gel was stripped and further developed with anti-BCY1 antibody. A Coomassie Brilliant Blue stained gel is included as a loading control. Densitometric ratio between higher to lower phosphorylated isoforms for Ac:0.3±0.1 (n=3); Gly:0.4 ±0.2 (n=3); Gal:0.6±0.1 (n=3); Glu:1±0.1 (n=3). Panel C shows the glucose concentration (open symbols) as function of cell growth (closed symbols) during diauxic shift of the W303WT (circles) and gpr1Δ (triangles) strains. Phosphorylation state of Tpk1p from samples selected along the diauxic shift (indicated by numbers and arrows on C, top panel), detected by native gel electrophoresis and Western blot with anti-TPK1 is shown in C, bottom panel. Panel D (left panel for W303WT and right panel for gpr1Δ) shows a Western blot of samples from W303WT (3, 5 and 6, the amount of samples 3 and 5 was increased for better visualization) and gpr1Δ (2, 3, 4 and 5). Densitometric ratio between higher to lower phosphorylated isoforms were the following: W303WT strain: T3:1; T5:0.5; T6:0.4; gpr1Δ strain: T2:0.5; T3:0.6; T4:0.4; T5:0.6.
regulation of PKA activity [1]. In fact, our own previous results
[10] indicate that the endogenous PKA activity measured in the
1115WT strain is three-fold lower in cells from stationary phase
as compared to cells in logarithmic growth. It remains to be
explained how PKA activity is down-regulated, when para-
doxically both R and C subunits increase their levels during
derpressed metabolic conditions.

A GPCR system has been proposed to act as a high glucose-
sensing system for control of the cAMP pathway by glucose
availability and Gpr1p is the receptor protein responsible for the
detection of glucose. Considering the close connection between
phosphorylation state of Tpk1p and the presence of glucose in
the medium, we studied the behaviour of Tpk1p-phospho-
rylation throughout logarithmic growth focusing on the period
when the extracellular glucose levels gradually begin to
decrease from 100 to 80 mM and then to almost undetectable
levels (Fig. 6D, samples 3, 5 and 6, respectively). As the
Figure concentration decreased from 100 to 80 mM (Fig. 6D
left panel, samples 3 and 5, respectively), the ratio of
phosphorylated species of Tpk1p changed from equal abun-
dance of low and high mobility isoforms to higher abundance of
less phosphorylated species, to finally end with an even higher
ratio of lower mobility species when glucose was exhausted
(Fig. 6D left panel, sample 6). This change implies that the more
phosphorylated states of Tpk1p depend on high glucose levels.
Strains lacking the glucose sensor Gpr1p, showed a high

![Figure A](image1)

**Fig. 7.** Phosphorylated Tpk1p isoforms display higher catalytic efficiency toward kemptide. Tpk1p isoforms activities were measured as a function of kemptide concentration. (A) Crude extract from TPK1 bcy1Δ strain was treated or not with alkaline phosphatase previous to the kinase activity assay, varying kemptide concentration (left panel). The kinetic parameters were: □ − Ppase: $K_m$ (mM): 0.066 ± 0.009; $V_{max}$: 0.095 ± 0.035; ■ + Ppase: $K_m$ (mM): 0.13 ± 0.01; $V_{max}$: 0.10 ± 0.03
(n:3). The specificity constant $K_m / V_{max}$ values (right panel) and a native PAGE followed by Western blot of isoforms of Tpk1p used in the kinetic analysis are shown. A lane with an aliquot from crude extract from SP1WT strain is shown for mobility comparison. Dark grey arrows indicate hyperphosphorylated Tpk1p isoforms. B) Samples of cultures from TPK1 BCY1 strain were taken at time 0 (previous glucose addition) and 5 min post-glucose addition. Crude extracts from time 0 and time 5
min with or without alkaline phosphatase treatment, were used for the kinase assay, varying kemptide (left panel). The kinetic parameters obtained were: ● time 0: $K_m$ (mM): 0.163 ± 0.069; $V_{max}$: 0.50 ± 0.15 (n:3); ○ time 5 − PPase: $K_m$ (mM): 0.084 ± 0.029; $V_{max}$: 0.49 ± 0.16 (n:3). ▲ time 5 + PPase: $K_m$ (mM): 0.195 ± 0.007; $V_{max}$:
0.42 ± 0.02 (n:3). The specificity constant ($K_m / V_{max}$) values (right panel) and a native PAGE followed by Western blot of isoforms of Tpk1p used in the kinetics
analysis are shown. (* time 5 − Ppase significantly differs from time 5 + Ppase and time 0; p:0.001).
portion of the lower phosphorylated species during the logarithmic phase of growth on glucose (Fig. 6D, right panel). This is a strong, although preliminary, evidence indicating that the change in phosphorylation of the C subunit observed during the diauxic shift depends on the GPCR system.

These results indicate a carbon source-dependent phosphorylation state of Tpk1p, since the presence of a fermentable carbon source, such as glucose, produces an accumulation of phosphorylated isoforms.

3.5. Effect of phosphorylation on catalytic kinase activity

To analyze whether glucose-induced phosphorylation changes on Tpk1p had a consequence on catalytic kinase activity, we examined the effect of alkaline phosphatase treatment on Tpk1p activity. As a first approach, a crude extract from a \( \text{TPK1 \ bcy1} \Delta \) strain carrying hyperphosphorylated isoforms (see Fig. 5B) was used as catalytic subunit source. The alkaline phosphatase used could be removed from the reaction with magnetic beads to avoid interference in the ulterior catalytic kinase activity. \( \text{TPK1} \) activity was measured in the hyper-phosphorylated or in the dephosphorylated enzymatic preparation using different concentrations of kemptide (Fig. 7A, left panel). Kinetic parameters are shown in the right panel of Fig. 7A. The affinity between kemptide and Tpk1 protein, as measured by \( K_m \) value, decreased significantly after phosphatase treatment. The \( V_{\text{max}}/K_m \) parameter is the most critical parameter in determining the specificity of an enzyme for a substrate. When comparing both differentially phosphorylated isoforms of Tpk1p, it can be seen that the phosphatase treatment of Tpk1p decreases the \( V_{\text{max}}/K_m \). These results suggest that phosphorylated isoforms of Tpk1p have more reactivity toward the substrate, mainly due to an increase in the affinity of kemptide for the phosphorylated isoforms.

In order to analyze whether a similar change in kinetic parameters was also observed under the physiological conditions where a change in the relative abundance of phosphorylated isoforms of Tpk1p took place, we used crude extracts obtained from the \( \text{TPK1 \ BCY1} \) cells grown in glycerol, harvested at time 0 (before glucose addition) and at time 5 min after glucose addition (Fig. 7B, left panel). The right panel of Fig. 7 shows the change in the measured specificity constants (\( V_{\text{max}}/K_m \)). It can be observed that the increase in the abundance of more phosphorylated isoforms of Tpk1p, triggered by glucose addition, is accompanied by a higher specificity constant of the enzyme mixture, principally attributed to an increase in the affinity for kemptide. The alkaline phosphatase treatment of the 5 min time point post-glucose (time 5 +Ppase), had as a consequence a decrease in the specificity constant, corroborating thus the positive effect phosphorylation has on the enzyme reactivity toward substrate. It is remarkable that, even though the enzyme samples used in Fig. 7A and B are both heterogenous and different regarding the relative abundance and phosphorylation state of the Tpk1p isoforms (shown on the lower right panel of each Figure), the same qualitative effect on catalytic activity is observed.

4. Discussion

4.1. In situ PKA activation in glucose-stimulated cells

Here we describe the dynamic in vivo activation of PKA during the transient peak of cAMP that is produced after 100 mM glucose addition to non repressed cells. The method of choice was to measure PKA activity in situ using permeabilized cells, in the absence or presence of exogenously added cAMP. The in situ activity, measured in the absence of exogenous cAMP had been proven to be very suitable when applied to cells with a differential, but constant, degree of activation of PKA, relatively independent of cAMP levels [10]. However, in this case, when the increase in cAMP is transient, activation of PKA could only be followed in one of the wild type strains (WT1115) when using this approach, since in the rest of the strains activity was below the detection level.

To our surprise, when performing the in situ PKA assay in the presence of 10 \( \mu \text{M cAMP} \), a peak of activity following the dynamic of the cAMP peak was observed, when glucose was added to cells growing on glycerol. Although we knew from our own previous results [10] that the maximum in situ PKA activity measured plus cAMP was much lower than the one measured in crude extracts in vitro plus 10 \( \mu \text{M cAMP} \), we did not understand the reason for this difference. We obtained a clue when we compared the numerical values of PKA specific activity obtained with the two strains \( \text{TPK1 \ BCY1} \) and \( \text{TPK1 \ bcy1} \Delta \) (Table 2). While in \( \text{TPK1 \ BCY1} \), the in vitro PKA specific activity measured in crude extracts, plus cAMP, was around 180-fold higher than the one measured in situ, in the \( \text{TPK1 \ bcy1} \Delta \) strain, specific PKA activity was the same no matter whether it was assayed in crude extracts or in permeabilized cells. The main difference between these two strains is the presence or absence of the Bcy1p. The fact that one can measure so less activity in permeabilized cells than in crude extracts, when adding 10 \( \mu \text{M cAMP} \) exogenously, can be interpreted as being the consequence of the difficulty in overcoming the inhibition of catalytic activity by the R subunit within the cell. At the light of this analysis we now interpret and hypothesize that the transient peak of activity observed with the in situ assay plus cAMP is the result of the extra-activation potency this exogenously added cAMP has on PKA holoenzyme molecules that have somehow been “primed” by the in vivo increase of cAMP within the cell. However, in the point of maximum activation of the curve, we still measure 40–90 fold less activity in situ than in crude extracts (Table 2), indicating that not all the holoenzyme molecules were “primed” by the in vivo increase in cAMP levels.

One possibility we can envisage to interpret these results is the following. These “primed” holoenzyme molecules correspond to those that have had access to the cAMP produced endogenously evoked by glucose addition, and have their two cAMP binding sites occupied. During the permeabilization procedure, free cAMP is lost from the cell (our own unpublished results), as well as cAMP from one binding site, probably site B, since it is this site the one that is freely accessible to cAMP binding in the holoenzyme. The loss of
cAMP from one site makes the other site (probably site A) inactive (although with cAMP still bound). The addition of exogenously added 10 μM cAMP to the in situ PKA assay, reverts the cAMP binding to site B, and activates the holoenzyme. If this explanation would turn to be true, then it might also be an explanation for the initial steps of reversal of PKA activation once cAMP levels are decreased after the signal is shut-off. This proposal is somehow backed by the PKA activation once cAMP levels are decreased after the holoenzyme. If this explanation would turn to be true, then it reverts the cAMP binding to site A, and activates the exogenously added 10 μM cAMP to the in situ PKA assay, which is around 2–3 fold less than in the wild type strain $SP^1_{WT}$, indicating that Tpk2p and Tpk3p (to a lower extent) contribute to the activity in the wild type strain, as already reported [34]. The regulation of PKA activity, measured in situ, is the same in the wild type strain, where there is a contribution in activity of the three Tpks and in the $TPK1\ BCY1$ strain, where only Tpk1p is the active PKA isoform. We can therefore conclude that with the in situ assay plus cAMP we can also follow, at least, the in vivo activation of Tpk2p.

The fact that PKA can not be fully activated in situ by addition of exogenous cAMP to permeabilized cells suggests that in order to have an "activating" effect, cAMP has to be generated endogenously, in accordance with what has been proposed to occur in metazoan cells, where compartmentalization of the signal transduction pathway is necessary in order to have an adequate response to the signal [7,35].

Even if the interpretation of the mechanism of change of P. Portela, S. Moreno / Cellular Signalling 18 (2006) 1072–1086 activity of PKA in permeabilized cells in response to exogenous cAMP is still speculative, we believe that the in situ measurement of PKA activity observed plus cAMP is really useful to monitor the in vivo transient activation of PKA.

4.2. Dynamic changes in the phosphorylation state of Tpk1p is carbon source-dependent

In this work we present evidence demonstrating, for the first time, a modification in the phosphorylation state of the catalytic subunit of PKA in response to a physiological signal. In this case, the phosphorylation state of the Tpk1p from S. cerevisiae changes toward more phosphorylated isoforms during the metabolic transition from respiratory to fermentative metabolism. The change in phosphorylation of Tpk1p occurs after the cAMP peak produced by glucose addition, and depends on the activation of PKA triggered by this increase in cAMP. The difference in timing between the “priming” of PKA, as detected in situ plus cAMP, and the increase in phosphorylated isoforms of Tpk1 are not compatible with phosphorylation of C subunit being one reason for the “priming” effect, described above.

The PKA kinase can therefore be PKA itself, or a PKA-regulated protein kinase. Even though none of the three Tpks show a conventional PKA consensus motif RRXS, it has already been demonstrated that this motif represents only one-half of all physiological PKA phosphorylation sites [36]. The less-frequent phosphorylation of degenerate/variant consensus sequences in physiological substrates makes it difficult to predict likely phosphorylation sites solely on the basis of the substrate's primary structure.

Two-dimensional gel electrophoretic analysis of full-length Tpk1p indicates the presence of several phosphorylation sites, mainly serines, on the enzyme [26]. However, except for Thr-241, the rest of the phosphorylated aminoacids have not been yet identified. Thr-241 has been demonstrated to be stably phosphorylated since it was identified as phospho-Thr-241 in Tpk1p crystals [37]. This aminoacid is analogous to Thr-197 of mammalian C subunits [38], and to Thr-356 in the C subunit from Schizosaccharomyces pombe [39]. Phosphorylation of this residue is critical, as this phosphate in the activation loop is essential for coordinating the active conformation, for optimal enzymatic activity and for adequate inhibition by the regulatory subunit [13,26,38,39]. In mammalian C, phospho Thr-197, is resistant to phosphatase hydrolysis in vitro [40], at least as isolated from the reductive environment of the cytoplasm [41]. It is possible therefore, that the Tpk1p isoform shown in this work to display the lowest mobility on native PAGE, corresponds to alkaline phosphatase-resistant phospho-Thr-241. The phosphorylation changes shown by Tpk1p in response to glucose very probably occur on other aminoacid(s), which we are at present on the way of characterizing.

As demonstrated by the results of the kinetic experiments shown in Fig. 7, performed with samples with different abundance of phosphorylated isoforms of Tpk1p, the more phosphorylated isoforms present an increased specificity constant ($V_{max}/K_m$) toward kemptide. This preliminary result suggests that phosphorylation of Tpk1p produces a positive effect on the efficiency of the catalytic activity of PKA toward its substrates.

The state of phosphorylation of Tpk1p is dynamic. On one hand, during the transition from respiratory to fermentative metabolism (Fig. 1), there is a change in the proportion of the phosphorylated isoforms, from less phosphorylated to more phosphorylated after glucose addition. On the other hand, during the diauxic shift (Fig. 6) the change is the opposite; the decrease in the extracellular glucose concentration is followed by an increase in the proportion of less phosphorylated isoforms. Experiments of diauxic shift or of glucose addition to glycerol-grown cells, performed with WT and gpr1Δ strains, indicate that glucose sensing through GPCR is necessary for the change in the phosphorylation state of Tpk1p. The GPCR system has been postulated to have an important role in the stimulation of cAMP synthesis during the transition from respiratory to fermentative growth, and a minor role in the
control of cAMP pathway in cells growing on glucose [6,29]. However, an explanation to the effect a gpr1 deletion has on several PKA-dependent targets, might be found in the positive influence GPCR may have, through the sensing of high extracellular glucose levels, on C subunit phosphorylation and therefore on its activity.

The physiological relevance of the reversible phosphorylation state of PKA, adjustable to the presence/absence of glucose, could be the fine-tuning of PKA activity, up regulating it through phosphorylation when it is necessary to sustain the full fermentative capacity of the cells, or down-regulating it, through dephosphorylation, when PKA needs to be shut-down, like in stationary phase or respiratory metabolism. We therefore introduce here a new control mechanism of PKA activity through reversible phosphorylation; this mechanism could act in addition or concertedly with the already known regulatory mechanisms: intracellular cAMP levels, inhibition by R subunit, and subcellular compartmentalization.

In conclusion, the main objective of this work was to follow the activation of PKA during a physiological peak of cAMP. During the accomplishment of this objective we have discovered that: 1) in vivo activation of PKA can be monitored by measuring in situ kemptide phosphorylating activity in permeabilized cells in the presence of exogenously added cAMP; 2) R subunit inhibition of C subunit, within the permeabilized cell, can not be completely relieved by exogenous cAMP; 2) R subunit inhibition of C subunit, within the permeabilized cell, can not be completely relieved by exogenous cAMP addition; 3) concomitant and following the cAMP peak, the Tpk1p changes its phosphorylation state toward more phosphorylated isoforms, in a process that is dependent on cAMP and on PKA activation through the GPCR pathway; 4) phosphorylation of Tpk1p increases its specific activity toward kemptide.

Acknowledgements

We thank S. Rossi for critical revision of the manuscript, P. Valacco for help with written english, and JM Thevelein with help in the provision of several strains. This work was supported by grants from Universidad de Buenos Aires (UBA), CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), ANPCYT (Agencia Nacional de Promoción Científica y Tecnológica) and Fundación Antorchas. Paula Portela had a fellowship from UBA and from Fundación Antorchas.

References