Characterization of yeast pyruvate kinase 1 as a protein kinase A substrate, and specificity of the phosphorylation site sequence in the whole protein

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Pyk1 (pyruvate kinase 1) from *Saccharomyces cerevisiae* was characterized as a substrate for PKA (protein kinase A) from bovine heart and yeast. By designing Pyk1 synthetic peptides containing potential PKA sequence targets (Ser²², Thr⁹⁴ and Thr⁴⁷⁸) we determined that the peptide S22 was a substrate for PKA *in vitro*, with a K_{sp}^* (specificity constant) 10-fold and 3-fold higher than Kemptide for bovine heart and yeast PKA respectively. *In vitro* phosphorylation of the Pyk1 S22A mutant protein was decreased by as much as 90 % when compared with wild-type Pyk1 and the Pyk1 T94A mutant. The K_{sp}^* values for Pyk1 and Pyk1 T94A were the same, indicating that both proteins are phosphorylated at the same site by PKA. Two-dimensional PAGE of Pyk1 and Pyk1 S22A indicates that *in vivo* the S22A mutation prevented the formation of one of the Pyk1 isoforms. We conclude that in yeast the major PKA phosphorylation site of Pyk1 is Ser²².

Phosphorylation of Ser²² leads to a Pyk1 enzyme that is more active in the absence of FBP (fructose 1,6-bisphosphate). The specificity of yeast and mammalian PKA towards the S22 peptide and towards whole Pyk1 protein was measured and compared. The K_{sp}^* for the S22 peptide is higher than that for Pyk1, indicating that the peptide modelled on Pyk1 is a much better substrate than Pyk1, regardless of which tissue was used as the source of PKA. However, the K_m of Pyk1 protein is lower than that of the better substrate, the S22 peptide, indicating that ground-state substrate binding is not the major determinant of substrate specificity for PKA.

Key words: phosphorylation, protein kinase A (PKA), pyruvate kinase 1 (Pyk1), substrate specificity, yeast, kinetics.

INTRODUCTION

The intracellular transduction of signals from the plasma membrane to cellular compartments evokes a variety of physiological responses. Protein phosphorylation is one of the most fundamental mechanisms for signal transduction. The specificity of signal transduction depends upon the ability of each kinase to precisely phosphorylate particular sites on specific substrate proteins. The cAMP-dependent protein kinase [PKA (protein kinase A)] is the most well characterized member of the serine/threonine protein kinase family. Phosphorylation of its protein targets is known to be critical for regulating a multitude of cellular processes including metabolism, gene transcription, ion flux, growth and cell death [1]. The fact that this protein kinase with broad specificity mediates a number of discrete physiological responses following cAMP engagement has raised the question of how specificity is maintained within the cAMP/PKA system. This specificity in phosphorylation is determined by at least two major factors: peptide specificity, and substrate and kinase localization.

It is clear that the cAMP binding to the R (regulatory) subunit of cAMP-dependent protein kinases produces a lower affinity between R and C (catalytic) subunits, but a new concept is emerging, the fact that the PKA holoenzyme is not dissociated by cAMP binding alone [2–4], describing a new role for these substrates. A complete understanding of the activation mechanism of PKA requires the identification and kinetic characterization of particular substrates of this enzyme. Although cAMP-dependent phosphorylation is a highly-studied area there are few comprehensive accounts of the physiological substrates of this enzyme.

These lists form the basis for the determination of the consensus phosphorylation sites for PKA which are Arg-Arg-X-Ser/Thr, Arg/Lys-X-X-Ser/Thr, and Arg/Lys-X-Ser/Thr [5–8]. The effectiveness of protein phosphorylation by protein kinases in general, and PKA in this case, is believed to depend on the primary structure of the protein around the phosphorylation site; synthetic peptides have thus been used to study and define the consensus phosphorylation sequences for PKA [8–9].

Statistical analysis of proteins known to be phosphorylated has shown that many of the phosphorylation sites in the substrate proteins do not correspond exactly with the complete consensus sequence determinant [10]. Loog et al. [11] compared substrate specificity of parent proteins with their short peptide derivatives and demonstrated that alterations made in protein structure had lesser effects than the corresponding alterations made in peptides, whereas the amino acid preferences and the overall specificity pattern remained similar in both cases. Therefore the behaviour of a protein kinase towards its substrate could be different depending on whether the substrate is an entire protein or the peptide derivative.

In *Saccharomyces cerevisiae* PKA is central to an important signal transduction pathway. The regulatory subunit is encoded by the BCY1 gene and the C subunit is encoded by the TPK1, TPK2 and TPK3 genes. Yeast is a good model with which to search for and identify PKA substrates, since its genome is completely known. It is our aim, at present, to understand the role of these substrates in PKA activation.

Several PKA substrates have been described and very recently proteomic approaches that simplify the search for new potential PKA substrates have been described [12]. However, further work

Abbreviations used: C, PKA catalytic subunit C; C_b, PKA catalytic subunit C from bovine heart; DTT, dithiothreitol; K_{sp}^* , specificity constant; FBP, fructose 1,6-bisphosphate; ORF, open reading frame; PEP, phosphoenolpyruvate; PKA, protein kinase A; Pyk1, pyruvate kinase 1; R, PKA regulatory subunit.

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Table 1 Description of yeast strains and Pyk1 wild-type and mutant proteins used in the present study

Strain	Genotype	Genes
KT 1115*	<i>Mata leu2 ura3 his3 pep4Δ</i>	1115
Pyk1-5†	<i>Mata pyk1-5 ade1 leu1 met14 ura3 ade6 CUP(r)</i>	<i>pyk1-5</i>
Pyk1	<i>Mata pyk1-5 ade1 leu1 met14 ura3 ade6 CUP(r) + pYK101</i>	<i>pyk1-5 + Pyk1</i>
Pyk1(S22A)	<i>Mata pyk1-5 ade1 leu1 met14 ura3 ade6 CUP(r) + pYK101-S22A</i>	<i>pyk1-5 + Pyk1-S22A</i>
Pyk1(T94A)	<i>Mata pyk1-5 ade1 leu1 met14 ura3 ade6 CUP(r) + pYK101-S22A</i>	<i>pyk1-5 + Pyk1-T94A</i>
S13-3A‡	<i>Mata his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2</i>	<i>TPK1-bcy1Δ</i>
JT20454§	<i>Mata his3 leu2 ura3 trp1 tpk1::ade8 tpk2::HIS3 tpk3::TRP1 msn2::LEU2 msn4::HIS3</i>	<i>JT20454</i>
JT20454(WT)	<i>Mata his3 leu2 ura3 trp1 tpk1::ade8 tpk2::HIS3 tpk3::TRP1 msn2::LEU2 msn4::TRP1 + pPYK101</i>	<i>JT20454 + Pyk1</i>
JT20454(S22A)	<i>Mat a his3 leu2 ura3 trp1 tpk1::ade8 tpk2::HIS3 tpk3::TRP1 msn2::LEU2 msn4::TRP1 + pPYK101-S22A</i>	<i>JT20454 + Pyk S22A</i>
JT20454(T94A)	<i>Mat a his3 leu2 ura3 trp1 tpk1::ade8 tpk2::HIS3 tpk3::TRP1 msn2::LEU2 msn4::TRP1 + pPYK101-S22A</i>	<i>JT20454 + Pyk T94A</i>
YJL 164C	<i>Mata his3 leu2 met15 ura3 TPK1TAP</i>	<i>TPK1-TAP</i>

These strains have been previously described in the literature: * [26], † [27], ‡ [28], § [provided by J. Thevelein, Catholic University of Leuven, Leuven, Belgium].

is needed to demonstrate that the candidate proteins identified are indeed PKA substrates, and to identify the target sequences.

Yeast Pyk1 (pyruvate kinase 1) catalyses the irreversible conversion of PEP (phosphoenolpyruvate) to pyruvate, the final step in glycolysis. We have previously demonstrated that it is a substrate of yeast PKA, both *in vitro* and *in vivo* [13]. In the present study we have characterized Pyk1 as a substrate of PKA and identified the target site for Pyk1 phosphorylation by yeast PKA. The phosphorylated serine residue is located within a consensus sequence site, RRTS. We have analysed, through the comparison of the specificity constants, whether the behaviour of a yeast PKA substrate is the same when it is a whole protein or a short peptide derivative. The peptide modelled on Pyk1 is a much better substrate than Pyk1 itself. However, the K_m of the parent Pyk1 protein is lower than that of the peptide.

EXPERIMENTAL

Materials

All chemicals were of analytical grade. Yeast growth medium supplies were from Difco Laboratories and Merck. The rabbit anti-(goat IgG)-horse radish peroxidase conjugated antibody was from Santa Cruz Biotechnology Inc., Chemiluminescence Luminol Reagent, IPG buffer, Immobiline DryStrip Gels, IgG Sepharose 4B and calmodulin Sepharose 4B columns were from Amersham Biosciences. [γ -³²P]ATP was from New England Nuclear. Phosphocellulose paper (P81) was from Whatman. C_b (PKA catalytic subunit C from bovine heart), goat anti-(rabbit IgG)-horse radish peroxidase conjugated antibody, PEP, NADH, ATP, ADP, FBP (fructose 1,6-bisphosphate) and lactate dehydrogenase were from Sigma Chemical Co. The polyclonal anti-Pyk1 (rabbit muscle) antibody was from Rockland. EDTA-free protease inhibitor cocktail was from Roche Diagnostics GmbH. The peptides LRRTS-IIGT (S22), IRTGTTTNDR (T94) and LKKGDTYVSI (T478) were synthesized and purified commercially by Bio-Synthesis, Inc. AcTEV protease was from Invitrogen Life Technology.

Yeast strains and plasmids

The strains, genotype and genetic nomenclature used in this study are listed in Table 1. The yeast strain *Pyk1-5* (generously provided by T. Nowak, Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN, U.S.A.) harbours a mutation that abolishes Pyk1 expression, and cannot grow on glucose as the sole carbon source. The strain instead harbours a plasmid pPYK101 for Pyk1 expression under its own promoter. Strains Pyk1(S22A) and Pyk1(T94A) are *Pyk1-5* strains which

contain pPYK1-S22A (where p is plasmid) and pPYK1-T94A expressing Pyk1 S22A and Pyk1 T94A mutants respectively, under the Pyk1 promoter. Strains JT20454(S22A), JT20454(T94A) and JT20454(wild-type) are JT20454 strains which contain pPYK1-S22A, pPYK1-T94A and pPYK1-wild-type expressing Pyk1 S22A, Pyk1 T94A mutants and Pyk1 wild-type proteins respectively, under a Pyk1 promoter. Yeast strains were transformed by the lithium acetate method [14]. The strain *TPK1 bcy1Δ* was used as source of Tpk1 enzyme.

Growth media

Yeast media were prepared as described [15]. Strains were grown on rich medium containing 2 % bactopectone, 1 % yeast extract and 2 % galactose (YPgal), or plus 2 % glucose (YPG), or plus 2 % glycerol/ethanol (YPgly/Et). Synthetic medium containing 0.67 % yeast nitrogen base without amino acids, 2 % glucose, plus the necessary additions to fulfill auxotrophic requirements were used to maintain the SD (selectable plasmids). Solid medium contained 2 % agar.

Site-directed mutagenesis of Pyk1

The single-site mutants of yeast Pyk1 in which Ser²² and Thr⁹⁴ were replaced with an alanine residue were obtained using conventional PCR-based methods, using complementary mutagenic oligonucleotide primers and a pPYK101 construct as the template.

For the S22A mutation four primers were used: BS47: 5'-CA-CCCAGACATCGGGCTTC-3'; BA47: 5'-GAACACCGTTCCTTGACACCGA-3'; BS70: 5'-GGGTCCAGAAATCAGAACTGGT-GCCACCACCAACGATGTTGACTACC-3' BA70: 5'-ACCA-GTTCTGATTTCTGGACCC-3'. The codon TCC(Ser²²) was replaced by GCC(Ala²²). The first rounds of PCR were performed with the primer pairs BS47 with BA70, and BA47 with BS70. Two PCR fragments were purified. Using the two previous PCR products as a template a second round of PCR was performed using BS47 and BA47 as primers. The 1300 bp PCR product was purified and cut with BglII and XbaI restriction enzymes to generate a 510 bp fragment. The fragment was cloned into the pPYK101 vector which had been cut with the same enzymes. Clones were isolated and positive clones were sequenced using BS47 as the primer. For the T94A mutation four primers were used: BS47: 5'-CACCCAGACATCGGGCTTC-3'; BA47: 5'-GAACACCGTTCCTTGACACCGA-3' BS48: 5'-GGTCTGACTTGAGAAGAACCGCCATCATTGGTACCATCGGTC-3'; BA48: 5'-CGGTTCTTCTCAAGTCAGAACCCAG-3'. The codon ACC(Tyr⁹⁴) was replaced by GCC(Ala⁹⁴). The first rounds of PCR were performed with the primer pairs BS47 with BA48, and

BA47 with BS48. Two PCR fragments were purified. Using the two previous PCR products as the templates and using BS47 and BA47 as primers, we performed the second round of PCR. The 1300 bp PCR product was purified and cut with BglII and XbaI restriction enzymes to generate a 510 bp fragment. The fragment was cloned into the pPYK101 vector which had been digested with the same enzymes. Clones were isolated and positive clones were sequenced using BS47 as the primer.

Standard PKA assay

PKA subunit C activity was determined by assay of phosphotransferase activity with Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), S22, T94 or T478 peptides as the substrate. The assay was initiated by mixing different amounts of PKA sources (either C_b , crude extracts of *TPK1 bcy1Δ* as source of Tpk1, or purified TPK1-BCY1 holoenzyme) with 15 mM Tris/HCl, (pH 7.5), 0.1 mM EGTA, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, cocktail phosphatase inhibitors [10 mM NaF, 10 mM $Na_4P_2O_7 \cdot 10H_2O$, 0.1 mM Na_3VO_4 , 0.1 mM $(NH_4)_6Mo_7O_{24}$] and EDTA-free protease inhibitor (buffer A); 15 mM $MgCl_2$, 0.1 mM $[\gamma\text{-}^{32}P]ATP$ (700 d.p.m./pmol) plus the concentration of different substrates indicated in each experiment (see Figures 1–3) and 10 μM cAMP, when added. After 15 min at 30°C, aliquots were processed according to the phosphocellulose paper method [16]. PKA assays were linear with time and protein concentration. PKA activity is expressed in pmol of phosphate incorporated into substrate/min, at 30°C. C_b was reconstituted in 50 mM DTT (dithiotreitol) and the activity is expressed in units as defined commercially. TPK1 units from purified holoenzyme were defined as the amount of enzyme which transfers 1 pmol of phosphate to Kemptide/min, at 30°C.

Pyk1 expression and purification

Pyk1 was purified from the *S.cerevisiae* strain JT20454 containing pPYK101 or pPYK101-S22A or pPYK101-T94A vectors, using a previously described method [17]. These yeast strains over-express Pyk1, Pyk1(S22A) or Pyk1(T94A) forms of Pyk1 when grown in 2% glucose media. Briefly, the semi-purification of the enzyme involved two chromatography steps, DEAE-cellulose, and phosphocellulose and ammonium sulphate precipitation. The precipitated protein was stored at –20°C. The steps and quality of the purification were analysed by SDS/PAGE, Western blotting and pyruvate kinase activity assay.

PKA purification

The yeast PKA holoenzyme (TPK1-BCY1) was purified from a yeast TAP (tandem affinity purification)-fusion strain that contains the ORF (open reading frame) YIL033 of TPK1 which is TAP tagged (Open Biosystems). Tpk1-TAP was purified using the standard TAP procedure [18] using two specific affinity purification/elution steps. The TAP tag consists of two Protein A IgG-binding domains and a calmodulin-binding peptide separated by a TEV (tobacco etch virus) protease cleavage site which allows for purification using IgG Sepharose 4B and calmodulin Sepharose 4B columns respectively.

In vitro phosphorylation of Pyk1

The *in vitro* phosphorylation assays were performed in a final volume of 40 μl using crude extracts of the *TPK bcy1Δ* strain, C_b , or TPK1-BCY1 as PKA sources. The assay was started by mixing the substrates Pyk1, Pyk S22A or Pyk1 T94A (substrate amounts are indicated in Figures 1–3) with 0.1 mM $[\gamma\text{-}^{32}P]ATP$ (1000–1300 d.p.m./pmol) in buffer A. The reaction time was 15 min, at 30°C. The incorporation of phosphate into Pyk1

was determined by scintillation counting of phosphorylated enzyme excised from dried SDS/PAGE gels. Alternatively, SDS/PAGE gels were dried and subjected to digital image analysis (Bio-Imaging Analyzer Bas-1800II and Image Gauge 3.12, FUJIFILM). The total amount of PKA catalytic activity in the crude extract preparations and the purified TPK1 holoenzyme was determined under the standard PKA assay conditions.

PKA activity in permeabilized cells

Culture samples ($1\text{--}2 \times 10^8$ cells) from a *1115* yeast strain were cultured in YPD [1% yeast extract (w/v), 1% peptone (w/v) and 2% dextrose (w/v)] medium. The cells were collected by centrifugation at 4°C. The pelleted cells were washed once with ice-cold buffer A, suspended in 0.5 ml of buffer A, and mixed with 0.075 ml of toluene/ethanol (1:4, v/v) and vortex-mixed for 5 min. The cells were immediately pelleted, washed three times 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 $0.5\text{--}1.5 \times 10^6$ permeabilized cells in a final 70 μl volume of the standard PKA assay mixture for 3 min at 30°C. The PKA assays were linear with time, and cell number.

Determination of Pyk activity

The Pyk activity was determined in 50 mM imidazole buffer (pH 7.0), containing 62 mM $MgCl_2$ and 100 mM KCl. Under standard conditions, 1.5 mM ADP, 1.5 mM FBP as activator, and various concentrations of PEP (0.5–30 mM in the absence of FBP and 0.1–10 mM in its presence). The reaction was coupled to NADH oxidation by the addition of 1 unit/ml lactate dehydrogenase and 0.22 mM NADH. The time-course of the reaction was monitored at 30°C by measuring the decrease in absorbance at 340 nm.

For the determination of kinetic parameters, the Pyk preparation used was partially purified from crude extracts by precipitation with 60% $(NH_4)_2SO_4$. Pellets were stored at –20°C and desalted through a Sephadex G-25 column in imidazole buffer immediately before the assay. Pyk activity is expressed in units: 1 enzyme unit causes the transphosphorylation of 1 μmol of phosphate from PEP to ADP/min, at 37°C, under standard assay conditions.

Protein determination and analysis of kinetic data

Protein concentration was determined by the Bradford assay [18a] with BSA as the standard. The quantification of purified Pyk1 for all the phosphorylation assays was made by SDS/PAGE using BSA as the standard. Kinetic analysis of PKA activity was performed using the Hill equation [18b]. The K_m and V_{max} kinetic values were determined by the Michaelis–Menten equation.

Two-dimensional PAGE and protein identification

The *S. cerevisiae* *Pyk1-5* strain containing pPYK101, pPYK101-S22A or pPYK101-T94A mutant vectors was cultured for 16 h at 30°C. Cultures of 50 ml were centrifuged at 4°C. Pelleted cells were washed once with ice-cold buffer A and suspended in 1 ml of buffer A and an equal volume of glass beads (425–600 μm). Disruption of the cells was achieved by vortex-mixing the sample for 3 min at 4°C. The supernatant was collected and its protein concentration was determined. The sample was mixed with an equal volume of 20% (v/v) TCA in acetone with 20 mM DTT and incubated for 45 min at –20°C before centrifugation for 15 min at 15 000 g at 4°C. The pellet was washed with cold acetone/20 mM DTT, incubated for 1 h at –20°C, centrifuged, dried and finally solubilized in lysis solution (50 μl /mg dried

Figure 1 Phosphorylation of Pyk1 peptides *in vitro* and *in situ*

Peptide phosphorylation was assayed using 2 units of C_b (A) or 1 μ g of crude extract from a *TPK1 bcy1Δ* strain (B) and 0.1 mM [γ - 32 P]ATP. The *in situ* phosphorylation of exogenous peptides was carried out using permeabilized cells from a 1115 strain (C); 0.5 – 1×10^6 permeabilized cells were used for the assay with 0.2 mM [γ - 32 P]ATP in the presence or absence of cAMP. In each case the data shown is representative of three independent experiments. Table (D) shows the peptides that were designed to include the putative phosphorylation sites. Possible phosphorylation sites are indicated in bold and the possible consensus sequences recognized by PKA are underlined.

pellet) for 2–4 h. The lysis solution contained 8 M urea, 2% (w/v) Nonidet P40, 20 mM DTT, Bromophenol Blue and 0.5% IPG buffer. The samples were used immediately or stored at -80°C until further use. Protein samples (50 μ g in 125 μ l IPG buffer) were transferred on to IPG strips (pH 3–10, 7 cm, Amersham Biosciences) for a period of 15 h for rehydration. Isoelectric focusing was carried out at 20°C using the Multiphor II system (Amersham Biosciences) at 500 V for 2 h, 1000 V for 2 h and 8000 V for 2 h. The strips were equilibrated in equilibration buffer [6 M urea, 20 mM DTT, 50 mM Tris/HCl (pH 8.8), 30% glycerol, 2% SDS and Bromophenol Blue] for a period of 15 min, and subjected to two-dimensional SDS/PAGE. Proteins were blotted onto nitrocellulose and Pyk1 protein was detected with an anti-Pyk antibody.

Reproducibility of results

All experiments were repeated at least three times with independent transformants, cultures and enzymatic preparations. The results showed consistent trends, i.e. different extracts and transformants gave highly reproducible results. In all cases, results from representative experiments are shown. Values in all Figures are the means $\pm$ S.D. for $n = 3$ experiments.

In silico analysis

The putative PKA phosphorylation sites in the Pyk1 protein were determined by analysing its amino acid sequence using two programs for the prediction of phosphorylation sites: NetPhos2.0 (www.cbs.dtu.dk/services/NetPhos/) and Scan site (scansite.mit.edu/dbsearch.one.phtml). The Scan site predicted only one PKA phosphorylation site and NetPhos yielded multiple serine and threonine phosphorylation sites, from which we chose those that

had the highest score for possible consensus PKA phosphorylation sites.

The phosphorylation site surface exposure predictions for Pyk1 were estimated from the de PHDAcc solvent accessibility prediction algorithm (www.predictprotein.org).

RESULTS

Pyk1 synthetic peptides containing a PKA consensus phosphorylation motif assayed as substrates for PKA

We have previously demonstrated that yeast Pyk1 is phosphorylated by yeast PKA both *in vivo* and *in vitro* [13]. In order to identify the PKA phosphorylation site in the protein we analysed its primary sequence for consensus PKA phosphorylation motifs using prediction programs, as described in the Experimental section. The protein has three potential phosphorylation sites (Ser²², Thr⁹⁴ and Thr⁴⁷⁸) within a PKA sequence motif. Three peptides LRTSIIGT (S22), IRTGTTINDR (T94) and LKKGDTYVSI (T478) containing the potential phosphorylation sites (in bold) were synthesized based on the deduced protein sequence of Pyk1 (Figure 1D). The T94 peptide was designed with an additional positively charged residue so that the peptide would be retained in the phosphocellulose paper used for the measurement of PKA activity. The ability of these peptides to serve as substrates for PKA was examined and compared with that of Kemptide by assaying the degree of phosphorylation of each one using different sources of PKA.

Figure 1 shows the results of using C_b as a source of PKA (Figure 1A), a crude extract of a *TPK1 bcy1Δ* strain (Figure 1B), or permeabilized 1115 yeast cells (Figure 1C). In all three cases

it was observed that only the S22 peptide, containing the Ser²² residue, was a substrate for PKA *in vitro*. The *in situ* assay (Figure 1C) showed that phosphorylation of the S22 peptide was cAMP-dependent. None of the other two peptides proved to be a substrate for PKA in this assay. The kinetic parameters $V_{\max}$ and K_m for the S22 peptide as compared with those for Kemptide, calculated from Figures 1(A) and 1(B) and further summarized in Figure 6, indicate that the S22 peptide is a better substrate for both enzymes, suggesting that Ser²² is a potential phosphorylation site for PKA in the parent Pyk1 protein.

Effect of Pyk1 S22A and T94A mutations on PKA phosphorylation *in vitro*

Taking into account that T94 and T478 peptides were not phosphorylated by PKA (and C_b or Tpk1) we studied the *in vitro* PKA phosphorylation of Pyk1 wild-type protein and two mutant proteins in which the Ser²² and Thr⁹⁴ residues were mutated to alanine. The Thr⁹⁴ mutation was chosen as representative of the two non-phosphorylatable sites. Mutations were introduced by site-directed mutagenesis into pPYK101, a high-copy number yeast expression vector expressing Pyk1 under its own promoter, to yield pPYK101-S22A and pPYK101-T94A. To overexpress and semi-purify the wild-type and Pyk1 mutant proteins we transformed the corresponding plasmids into JT20454, a strain devoid of the three TPK genes (TPK1, TPK2 and TPK3). The rationale behind this approach was to avoid the co-purification of Tpk proteins with Pyk1, since we have previously shown [13] that Pyk1 co-immunoprecipitated with the PKA holoenzyme. In this way we could study the phosphorylation of Pyk1 *in vitro* by the exogenous addition of different sources of the PKA subunit C. The purification procedure resulted in a semi-purified preparation of Pyk1 wild-type or mutant proteins as shown by SDS/PAGE. The identity of Pyk1 proteins was confirmed by Western blot analysis using anti-pyk1 antibodies (results not shown). Different aliquots of the purified preparations were submitted to phosphorylation conditions, using [γ -³²P]ATP and either C_b (Figure 2) or a crude extract containing Tpk1 (Figure 3) as the source of enzyme. The samples were subjected to SDS/PAGE and autoradiographed.

Figure 2(A) shows the dependence of Pyk1, Pyk1 S22A and Pyk1 T94A phosphorylation by C_b on enzyme concentration. The extent of phosphorylation was decreased by greater than 90% in Pyk1 S22A as compared with Pyk1 or Pyk1 T94A and it was almost equal in these two latter proteins. These results indicate that Ser²² is the major Pyk1 phosphorylation site for PKA under the conditions analysed. At maximum phosphorylation rates, under these experimental conditions, C_b catalysed the incorporation of 0.6 ± 0.2 and 0.5 ± 0.1 mol of phosphate/mol of Pyk1 and Pyk1 T94A respectively, demonstrating the incorporation of one mol of phosphate/mol of protein in both cases.

We determined the specificity constants for the two proteins Pyk1 and Pyk1 T94A (Figure 2B). We define ' K_{sp}^* ' as a relative parameter that gives a measure of the substrate phosphorylation efficiency that allows us to determine whether Pyk1 and Pyk1 T94A exhibit similar behaviour as substrates [K_{sp}^* was calculated as $V_{\max}$ (expressed relative to C subunits used in the assay, i.e. $\text{pmol} \cdot \text{min}^{-1} \cdot \text{U}^{-1}) / K_m (\mu\text{M}^{-1})$]. The K_{sp}^* values were calculated from the initial-rate versus substrate concentration plots. The K_{sp}^* was the same for Pyk1 and Pyk1 T94A (0.24 and $0.27 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{U}^{-1} \cdot \mu\text{M}^{-1}$ respectively) indicating that both proteins had a similar behaviour as substrates for PKA. Almost no phosphorylation could be observed in Pyk1 S22A.

Figure 3 shows the phosphorylation of Pyk1 by a *TPK1 bcy1* Δ crude extract. Even though the phosphorylation efficiency was low, as expected for a crude extract as a source of enzyme, it

Figure 2 Kinetics of the *in vitro* phosphorylation of Pyk1 and peptide mutants using C_b

Pyk1, Pyk1 S22A and Pyk1 T94A (1–2 μg) were incubated with the indicated amounts of C_b and 0.1 mM [γ -³²P]ATP for 15 min at 30°C (A). In (B) 2.5 units/40 μl of C_b and 0.1 mM [γ -³²P]ATP were incubated with the indicated concentrations of Pyk1, Pyk1 S22A and Pyk1 T94A. After incubation the samples were subjected to SDS/PAGE and digital imaging. The amount of incorporation of phosphate into the substrate was determined by scintillation counting of the phosphorylated protein band excised from SDS/PAGE gels.

was very clear that the lack of phosphorylation of Pyk1 S22A and that the phosphorylation of wild-type Pyk1 was dependent upon enzyme (Figure 3A) and substrate concentrations (Figure 3B).

We have previously demonstrated by *in vitro* assay using crude extracts as a source of PKA that the phosphorylation of Pyk1 is PKA dependent [13]. The *in vitro* assays were performed using crude extracts from a wild-type strain (*1115*) as a source of

Figure 3 Kinetics of the *in vitro* phosphorylation of Pyk1 and its mutants using TPK1

In (A), Pyk1 and Pyk1 S22A (1–2 μg) were incubated with the indicated amounts of TPK1 *bcy1* Δ crude extracts. In (B), 2 μg of Pyk1 and Pyk1 S22A in 40 μl of crude extracts from a TPK1 *bcy1* Δ strain and 0.1 mM [γ - ^{32}P]ATP were incubated with the indicated concentrations of Pyk1 or mutants proteins. After incubation the samples were subjected to SDS/PAGE and digital imaging. The amount of incorporation of phosphate into the substrate was determined by scintillation counting of the phosphorylated protein band excised from SDS/PAGE gels.

PKA, which contained normal levels of holoenzyme, and from a strain in which the TPK2 and TPK3 genes have been deleted thus producing an attenuated form of TPK1 (*tpk1*^{wl}). A clear dependence on PKA activity was evident, because there was no phosphorylation of Pyk1 using extracts from the PKA attenuated strain (*tpk*^{wl}) [13]. This result, together with those shown in Figure 3, clearly indicate that Ser²² is the residue in Pyk1 that is phosphorylated by Tpk1.

Figure 4 Two-dimensional PAGE

An aliquot of protein (50 μg) from each extract prepared from the *Pyk1-5* strain containing pPYK101, pPYK101-S22A or pPYK101-T94A and from strain JT20454 (PKA null) containing pPYK101, were separated on two-dimensional polyacrylamide gels. Proteins were then transferred to membranes and revealed by an anti-Pyk antibody. The panels on the right show the Red Ponceau staining of the same membranes as a loading control before the Western blot.

In vivo phosphorylation analysis by two-dimensional PAGE

In a previous study we have demonstrated the *in vivo* phosphorylation of Pyk1 by metabolically labelling yeast cells from *1115* and *tpk1*^{wl} strains with ^{32}P . The results indicated that Pyk1 phosphorylation was PKA-dependent, since there was no radio-labelled Pyk1 phosphoprotein in the *tpk1*^{wl}-radiolabelled strain.

In order to demonstrate that the Ser²² residue is phosphorylated *in vivo*, we analysed the effect of the S22A mutation on the phosphorylation of Pyk1 by two-dimensional PAGE. Extracts from the *Pyk1-5* strain (defective in endogenous Pyk1 expression, but otherwise containing the three TPK genes) transformed with pPYK101, pPYK101-S22A or pPYK101-T94A were analysed by two-dimensional PAGE and Western blotting using an anti-Pyk1 antibody (Figure 4). In extracts of Pyk1 overexpressing cells, we observed four spots which migrated to approx. 54 kDa and had a pI of approx. 7.5. The presence of several spots for Pyk1 is in agreement with results published previously [19]. The migration pattern of Pyk1 S22A differed from that of Pyk1 and Pyk1 T94A in that there was a loss of one of the spots, present both in Pyk1 and Pyk1 T94A, towards the acidic pH side of the gel (Figure 4). This result indicates that the S22A mutation prevented the formation of a Pyk1 low pI isoform. In order to confirm this result, we analysed the Pyk1 isoforms, through two-dimensional PAGE, using extracts from a yeast strain null for the three Tpk's (strain JT20454), and overexpressing Pyk1. These results (Figure 4) showed that the same fourth spot, with the lowest pI, disappears from extracts of this strain, suggesting that Ser²² was not phosphorylated in the absence of Tpk's. We concluded that the phosphorylation of the Ser²² residue *in vivo* is PKA dependent.

Figure 5 Effect of phosphorylation on Pyk1 activity

(A) Pyk1 activity was measured as a function of PEP in the absence (upper panel) or presence of 1.5 mM of FBP (lower panel) in Pyk1 and Pyk1 S22A samples. In (B) the Table shows the kinetic parameters derived from the experiments in (A).

Effect of phosphorylation on Pyk1 activity

Strain *Pyk1-5*, as already mentioned, is a mutant strain lacking Pyk1, which neither grows on or ferments sugars as a sole carbon source, but grows on glycerol or pyruvate. The expression of Pyk1 by pPYK101 restores the capacity for growth on glucose to the strain. Strain *Pyk1-5*, transformed with either pPYK101-S22A or pPYK101-T94A also grew in glucose, indicating that the mutation did not significantly affect Pyk1 activity. By analysis of the growth rate in solid or liquid medium at 30 °C or 37 °C (results not shown) we determined that there was no difference between *Pyk1-5* strains expressing either wild-type or mutant Pyk1 protein. Finally, by Western blot analysis it was determined that there was no significant difference in the expression level of the three Pyk1 proteins.

Previously, we have clearly shown [13] a correlation between PKA activity and Pyk1 activity in the absence of FBP which was independent from a change in the amount of Pyk1 protein. These experiments were carried out by measuring Pyk1 activity in crude extracts from yeast strains which had different levels of PKA activity.

In order to evaluate whether phosphorylation of Ser²² has an effect on Pyk1 activity, in the present study we analysed the effect of this mutation on enzyme activity. We measured the kinetic behaviour of semi-purified Pyk1 (wild-type and Pyk1 S22A) towards PEP in the presence or absence of FBP (Figure 5A, upper and lower panels). The semi-purified preparations of Pyk1 were obtained from the same extracts for which the two-dimensional gels were performed and are shown in Figure 4. The kinetic parameters derived from the Hill plot of the curves shown in Figure 5(A) are given in Figure 5(B). In the presence of the allosteric activator FBP, the $K_{0.5}$ for PEP, as well as the k_{cat} for the reaction for the two proteins were quite similar. The $K_{0.5}$ for PEP and n_H (Hill coefficient) values for the Pyk1 activity measured in the absence of the allosteric activator, FBP, differed between Pyk1 and Pyk1 S22A. There was a decrease in co-operativity displayed by Pyk1 S22A, but the most remarkable result is the increase in the $K_{0.5}$ for PEP (Figures 5A and 5B). Taken together, the enzyme parameters derived from the measurements of Pyk1 and Pyk1 S22A activity, which cannot be phosphorylated by PKA, suggest that Ser²² phosphorylation of Pyk1 by PKA modulates its activity and results in a more active enzyme.

Figure 6 Comparison of the kinetic constants of Pyk1 protein with the S22 peptide

Different amounts (up to 40 μ M) of Pyk1 were incubated with C_b (2 units/40 μ l) (A) and with purified TPK1–BCY1 holoenzyme (B) and 0.1 mM [γ - 32 P]ATP with cAMP (10 μ M) for 15 min at 30 °C. The K_m and V_{max} values for the S22 peptide and Kemptide in (A) were calculated from data in Figure 1(A), whereas those in (B) were estimated from experiments carried out (results not shown) with purified TPK1–BCY1 holoenzyme.

Comparison of the Pyk1 protein and S22 peptide kinetic constants

Up until now the specificity of protein kinases for their substrates has been determined from studies performed with short peptides. Loog et al. [11] addressed this question by comparing the specificity of a mammalian PKA (from bovine heart) in reaction with a series of mutant protein substrates and synthetic peptides derived from L-type pyruvate kinase. In that study the authors demonstrated that amino acid preferences and the specificity pattern remained similar in both cases but alterations made in the parent protein sequence had lesser effects than the corresponding alterations to peptides. It has been reported that yeast and mammalian PKAs recognize similar specific features in peptide substrates [20]. The sequence that includes the only phosphorylation site that we have defined in yeast Pyk1 is conserved in all the pyruvate kinases of fungal origin sequenced at present, but not in pyruvate kinase isoforms from higher eukaryotes. To analyse whether the specificity of yeast PKA for its substrate is the same when it is a whole protein or a short peptide derivative, the kinetic parameters for the parent Pyk1 protein and the S22 peptide, which includes the PKA phosphorylation site in this protein, were further compared using C_b and a purified preparation of TPK1–BCY1 holoenzyme, obtained as described in the Experimental section, as a source of kinase.

Figure 6 shows the hyperbolic plots for the phosphorylation rate versus the substrate concentration using C_b (Figure 6A) or

purified TPK1–BCY1 holoenzyme (Figure 6B). The V_{max} and K_m values were calculated from these two plots. These values and those obtained using the S22 peptide are summarized in the Tables shown in Figure 6(A) and 6(B).

The S22 peptide is a better substrate than the Pyk1 protein using either C_b or TPK1 holoenzyme as the kinase source. The difference in the K_{sp}^* value is the result of changes in the K_m and the V_{max} values. The K_m of the Pyk1 protein is lower than that of the S22 peptide, thus indicating a higher affinity of the parent protein for both kinases. On the other hand, the V_{max} value of the S22 peptide is higher than that of Pyk1, indicating slower enzymatic catalysis for the whole protein. The catalytic efficiencies of PKA, using either C_b or TPK1 as the source, obtained using these substrates indicates that the peptide is a better substrate than the Pyk1 protein, but only as a consequence of the increase in its V_{max} value. Given that both peptides differ by only four amino acids it is noteworthy that the S22 peptide is a better substrate than Kemptide derived from L-type pyruvate kinase, for both C_b (K_{sp}^* 3-fold higher) and for yeast PKA (K_{sp}^* 10-fold higher).

DISCUSSION

In the present study we characterized Pyk1 as a PKA substrate by identifying its phosphorylation site, studying the effect of phosphorylation on Pyk1 activity. We have also compared the

behaviour of the Pyk1 protein and of the small peptide derivative that contains the phosphorylated Pyk1 amino acid, as substrates of PKA.

The identification of the PKA target site in Pyk1 was addressed in order to gain information about the consensus and specificity of the target sequences for PKA.

The peptide LRRTSIIGT that contains the PKA sequence motif including the Ser²² residue was the only one that behaved as a peptide substrate, among the three that were assayed *in vitro*, for PKA, using both C_b and yeast PKA. *In situ* assays of permeabilized cells confirmed this result and showed the dependence of the phosphorylation with cAMP. These data provide support for the hypothesis that Ser²² in Pyk1 might be the target for PKA phosphorylation. A Pyk1 protein with an S22A mutation was constructed and used to confirm this hypothesis. The extent of phosphorylation of the Pyk1 S22A mutant was decreased by as much as 90 % as compared with Pyk1 and Pyk1 T94A (other putative phosphorylation site). The kinetic parameters for the phosphorylation reaction were determined by assaying the rate of phosphorylation of Pyk1 by C_b and TPK1. The K_{sp}^* for Pyk1 and Pyk1 T94A proteins, which is a measure of the efficiency of phosphorylation, were the same for both proteins. Taken together these results indicate that both Pyk1 or Pyk1 T94A are phosphorylated at the same site by PKA.

By two-dimensional PAGE of Pyk1 and Pyk1 S22A we showed that the S22A mutation prevented the formation of one of the Pyk1 isoforms. Taking into account that we have previously demonstrated [13] that the *in vivo* phosphorylation of Pyk1 in yeast is PKA-dependent, together with the results for the Pyk1 mutant proteins both *in vitro* and *in vivo* obtained in the present study, we conclude that in yeast the major PKA phosphorylation site is Ser²².

The sequence surrounding residue Ser²² is a consensus sequence for PKA phosphorylation. It has already been reported that the most prominent specificity determinants that contribute to the recognition of substrates by PKA is the positioning of arginine at the P-6, P-3 and P-2 positions. Most physiological substrates have either a P-3 and P-2 arginine combination, as occurs in the case of the Pyk1 PKA phosphorylation site [21–23].

We determined the K_{sp}^* for the S22 peptide which contains the Ser²² residue phosphorylated by PKA. The K_{sp}^* for peptide S22 is higher than that for Kemptide using either C_b or TPK1 as the source of PKA, although the difference in the behaviour of both peptides was greater than when using the yeast enzyme (10-fold versus 3-fold). Kemptide sequence, LRRASLG, differs from the S22 peptide, LRRTSIIGT, in the residues indicated in bold and is three residues longer. These differences make the S22 peptide a better substrate for both kinases, C_b or TPK1, that were assayed. The importance of a bulky and hydrophobic residue in the +1 position has been described as one of the determinants of the specificity of PKA for synthetic peptides [10]. In the S22 peptide there are two hydrophobic residues in the +1 and +2 positions and in Kemptide only one in the +1 position; perhaps this additional residue results in a better structure of the peptide substrate around this position and ultimately results in a more effective substrate. It has previously been demonstrated using the peptides derived from the alcohol dehydrogenase regulator 1 from yeast, a PKA substrate [20], as a model, that differences in peptide length and composition, as occurs in the case of the S22 peptide and Kemptide, also differentially affect their efficiency as substrates of mammalian and yeast PKAs.

The sequence surrounding the Ser²² position in Pyk1 is conserved in the yeast isoform Pyk2 at Ser²⁴, and is conveniently located at the interface of the catalytic domain A and the regulatory domain C of the protein, according to its crystal structure [24].

The sequence is also conserved in all of the pyruvate kinases of fungal origin that have been sequenced up until now, but not in pyruvate kinase isoforms from higher eukaryotes. We have demonstrated previously [13] that Pyk2 is also a PKA substrate, with preliminary results suggesting that it is a better substrate than Pyk1. It remains to be confirmed whether Ser²⁴ is the target site for PKA phosphorylation. It will be very interesting to study why Pyk2 appears to be a better substrate than Pyk1 if both have the same PKA phosphorylation site.

The K_{sp}^* for the S22 peptide is higher when compared with the K_{sp}^* for the Pyk1 protein, indicating that the peptide modelled on Pyk1 is a much better substrate than Pyk1 regardless of which protein kinase was used as the PKA source. It has been assumed that natural substrates are not always better than the corresponding peptide derivatives that are used as models, because even when sharing the same substrate determinants, the phosphorylation site structure in the protein substrate is likely to be more constrained [25]. Although our results indicate that the Pyk1 protein is a less efficient substrate than the S22 peptide and Kemptide, it has a higher affinity for PKA than these peptides.

We also addressed the physiological relevance of Pyk1 phosphorylation by PKA and we have demonstrated that phosphorylation of Ser²² leads to a Pyk1 enzyme that is more active in the absence of FBP, due to a decrease in its K_m towards PEP. We had already suggested this effect in a previous report [13] in which we correlated PKA activity with Pyk1 phosphorylation and Pyk1 activity. It is already known that the activation of PKA stimulates glycolysis through the phosphorylation and activation of 6-phosphofructo-2-kinase, with a consequent increase in FBP levels, a strong allosteric activator of 6-phosphofructo-2-kinase, one of the key enzymes in regulating glycolysis [27]. We now suggest that PKA can also activate this metabolic pathway through the phosphorylation of another key enzyme, Pyk1. The addition of glucose to yeast cells growing on non-fermentable carbon sources, produces a transient increase in cAMP levels and activation of the PKA pathway. Under these conditions, metabolism is shifted from respiration to fermentation. We speculate that phosphorylation and activation of Pyk1 by PKA might be important during this transition, when the FBP concentration is still low before the full glycolytic flux is established.

The specificity of protein kinases is essentially determined by the primary sequence of the phosphorylation site but also, as described above, the protein structure around the residue to be phosphorylated, which appears to be important for the recognition of secondary and tertiary structural elements in the protein target. Kreegipuu et al. [7] in their statistical study have proposed that protein kinases can phosphorylate sites of rather different structure and have suggested that the specificity of these proteins should not be presented only in terms of consensus sequences. In the present study we have demonstrated that a very highly conserved primary sequence in its structural context is less effective as a substrate than this sequence isolated in a peptide. The solvent accessibility predictions of the phosphorylation site for Pyk1 (PHDacc method) determined that the Ser²² is a buried residue. Predictions for the localization of the phosphorylated residue in a good number of proteins kinase targets indicates that although 70 % are predicted to be on the surface of the protein, 25 % are predicted to be buried [10]. We can therefore predict that in Pyk1 the PKA phosphorylation site is not significantly exposed, resulting in a less effective substrate.

Experimental observations have lead to the hypothesis that conformational adjustments of the enzyme, the substrate, or both are important aspects in enzymatic catalysis. The enzymes are conformationally dynamic. One hypothesis that has emerged is that conformational adjustments are used to distort the substrate,

the enzyme or both in order to bring the system towards the transition-state structure. One model used to explain the conformational flexibility and the optimization of active site-transition state complementarity is that in which forces existing between the enzyme and the substrate are used directly to induce strain in the substrate molecule, distorting it towards the transition state structure in order to facilitate the reaction.

The structural architecture of the enzyme active site further dictates the substrate specificity for the reaction. A structural complementarity exists between the enzyme active-site and the substrate in its transition state configuration [26]. Other observations that suggest a need to invoke conformational distortion is the manifestation of substrate specificity in the k_{cat} rather than in the K_{m} values. The K_{m} values of various substrates can be quite similar but their k_{cat} values may vary greatly; a good example of this comes from studies of the hydrolysis of synthetic peptides by the enzyme pepsin [28].

In the present study we determined that the K_{m} of the less effective substrate, the Pyk1 protein, is lower than that of the better substrate, the S22 peptide, indicating that ground-state substrate-binding is not the major determinant of substrate specificity for PKA. We suggest that the substrate specificity is determined by the transition state structure and that this structure is differentially attained if the substrate is either a peptide or a protein which includes the same phosphorylation residue.

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