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YIL042c and *YOR090c* encode the kinase and phosphatase of the *Saccharomyces cerevisiae* pyruvate dehydrogenase complex

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Abstract **In *Saccharomyces cerevisiae* the pyruvate dehydrogenase (PDH) complex is regulated by reversible phosphorylation of its Pda1p subunit. We here provide evidence that Pda1p is phosphorylated by the mitochondrial kinase Yil042cp. Deletion of *YOR090c*, encoding a putative mitochondrial phosphatase, results in a decreased PDH activity, indicating that Yor090cp acts as the corresponding PDH phosphatase. We demonstrate by means of blue native gel electrophoresis and tandem affinity purification that both enzymes are associated with the PDH complex.**

Keywords: Pyruvate dehydrogenase complex; Mitochondria; Phosphoproteins; YIL042c; YOR090c; *S. cerevisiae*

1. Introduction

The role of protein phosphorylation in yeast mitochondria is still poorly understood. In silico analyses predict the presence of putative mitochondrial (mt) proteins that contain either protein kinase or -phosphatase motifs [1]. However, no experimental data on their functional role has been reported so far. In contrast to the growing number of identified phosphorylated proteins in mitochondria of higher eukaryotes only a few mt phosphoproteins are known in the yeast *Saccharomyces cerevisiae*, including subunits of the ATPase complex (Atp1p, Atp2p, [2]) and of the pyruvate dehydrogenase (PDH) complex (Pda1p, [3]). The ~8 MDa PDH multi enzyme complex comprises multiple copies of three enzymes: pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) for the conversion of pyruvate into acetyl-CoA. First evidence for regulation of the yeast PDH complex by phosphorylation in vivo was provided by Uhlinger et al. [3], who showed that the α-subunit (Pda1p) of the yeast enzyme can be phosphorylated by purified heterologous PDH kinase. In contrast to the mammalian PDH complex, which is phosphorylated on three sites of its E1α-subunit [4], Pda1p is solely phosphorylated at a single serine residue. This site correlates with the major inactivation site of the mammalian E1α-subunit. Phosphorylation of Pda1p resulted in the inactivation of the enzyme, whereas the subsequent dephosphorylation by addition of the PDH phosphatase isolated from bovine heart reactivated the enzyme [3].

Authentic Pda1p can be immunoprecipitated in a phosphorylated form from isolated yeast mitochondria [5]. So far the yeast enzymes engaged in these phosphorylation/dephosphorylation mechanisms of PDH regulation have not been detected [3,6].

We screened deletion mutants of putative mt enzymes involved in protein phosphorylation/dephosphorylation, including Δ*yil042c* and Δ*yor090c* strains. The respective ORFs encode proteins that share homology to kinases and phosphatases, respectively, of the PDH complex of other organisms.

Comparison of the mt protein phosphorylation pattern of the Δ*yil042c* deletion strain with that of a wild type strain revealed that Yil042cp is required for phosphorylation of a mt target protein which was isolated by differential immobilized metal ion chromatography and subsequently identified by MALDI-TOF as Pda1p. In addition we observed a decreased PDH activity in cells lacking the putative phosphatase Yor090cp indicating that this protein may act as the corresponding PDH phosphatase in yeast. BN-PAGE analyses indicate that the kinase and the phosphatase are associated with the fully assembled PDH and with PDH subcomplexes. Interestingly, these subcomplexes possess enzymatic activity in strains lacking the PDH kinase, suggesting an additional role of the kinase and/or of the phosphorylation state of Pda1p in PDH complex assembly.

2. Materials and methods

2.1. Strains and media

S. cerevisiae wild type strain BY4741 (Acc. no. Y00000) and deletion strains Δ*pda1* (Acc. no. Y06174), Δ*lat1* (Acc. no. Y07218), Δ*yil042c* (Acc. no. Y01435) and Δ*yor090c* (Acc. no. Y01866) were obtained from Euroscarf. A strain expressing TAP-tagged Pda1p (Pda1p-TAP, Acc. no. YSC1178-7500310) was ordered from Open Biosystems. Yeast media were prepared as described [7].

2.2. Epitope tagging

Fusion of Pda1p, Yil042cp or Yor090cp with either the cMyc- or HA-tag was achieved by homologous recombination of the corresponding integration cassettes in the chromosomal loci of strain BY4741, deletion strains Δ*yil042c*, Δ*yor090c* or the strain expressing Pda1p-TAP as described [8].

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2.3. Isolation and purification of mitochondria

Yeast cells were grown to early stationary phase in lactate medium. Isolation of mitochondria, purification by two successive sucrose gradient centrifugations and treatment with 1 M NaCl was performed as described [9] in the presence of phosphatase inhibitors (1:100 dilution of phosphatase inhibitor cocktails I + II, Sigma).

2.4. Blue native polyacrylamide gel electrophoresis (BN-PAGE)

The method of BN-PAGE [10] was essentially performed as described [8] except for the use of low salt lysis buffer (5 mM NaCl, 5 mM ε-aminocaproic acid, 50 mM imidazole/HCl, pH 7.0, 1 mM AEBSF, 1× protease inhibitor cocktail (EDTA-free, Roche), phosphatase inhibitor cocktails I + II (Sigma) 1:100) and low salt gel buffer (25 mM imidazole, 50 mM ε-aminocaproic acid, pH 7.0).

2.5. Tandem affinity purification method (TAP)

The TAP-method [11,12] was performed essentially as described [8]. Mt lysate (500 μg) was prepared from strain Pda1p-TAP expressing cMyc-tagged Yil042cp with low salt lysis buffer (500 μl; 1% digitonin in 75 mM Bis–Tris, 50 mM Aminocapronsäure pH 7.0, 1 mM AEBSF, 1× protease inhibitor cocktail (EDTA-free, Roche), phosphatase-inhibitor cocktails I + II (Sigma) 1:100).

2.6. Immobilized metal ion affinity chromatography (IMAC)

The PhosphoPurification kit (Qiagen) was used to enrich phosphorylated proteins according to the manufacturer's instructions.

2.7. SDS–PAGE and Western blot analysis

Protein electrophoresis in the presence of SDS was carried out according to Laemmli [13]. Proteins were transferred onto a PVDF membrane (Millipore), probed with primary antibodies and detected with HRP-conjugated secondary antibodies and the ECL-Plus Kit (Amersham Pharmacia Biotech). Primary antibodies were directed against cMyc (Roche), HA (Roche), Cox2p (Molecular Probes), Aco1p (kind gift of R. Lill, Marburg), Pgk1p (Molecular Probes), and TAP-tag (Open Biosystems). Phosphorylated and total proteins were stained in gel with the ProQ-Diamond and SYPRO Ruby stain, respectively, according to the manufacturer's instructions (Invitrogen).

2.8. Photometric PDH activity assay

The PDH activity in mt lysates was photometrically measured as described [5]. 40 μg mt protein were lysed with 0.25% Triton X-100 and added to the assay buffer (50 mM potassium phosphate buffer, pH 7.4, 1 mM $MgCl_2$, 2 mM pyruvate, 2.6 mM cysteine, 2.5 mM NAD^+ and 2 mM thiamine pyrophosphate). PDH reaction was started by addition of coenzyme A (final concentration 0.13 mM), and the increase of the NADH concentration was followed at 340 nm.

2.9. "In gel" PDH activity assay

A colorimetric assay based on the reduction of nitroblue tetrazolium by NADH was used for determination of PDH activity. BN gels were incubated in buffer containing 50 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.05 mM EDTA, 3 mM pyruvate, 0.3 mM thiamine pyrophosphate, 3 mM NAD^+, 0.75 mM nitroblue tetrazolium (NBT), 0.05 mM phenazine methosulfate and 0.75 mg/ml coenzyme A for 1 h or over night.

3. Results and discussion

3.1. Yil042cp and Yor090cp are homologues of the human PDH-kinases and -phosphatases, respectively

Yil042cp belongs to the pyruvate dehydrogenase kinase/α-ketoglutarate dehydrogenase kinase (PDK/BCKDK) family [14], and shares ~46% homology and ~23% identity with the human PDKs. Interestingly, the yeast protein lacks regions at the extreme C-terminus, which are implicated in binding of the lipoyl-bearing domain (LBD2) of the E2-subunit (dihydrolipoyl acetyltransferase) of PDH (Fig. 1, A–D) [15,16].

Yor090cp (Ptc5p) was classified as a member of the PP2C phosphatase family [17] and possesses an overall homology of ~44% and ~26% identity to human PDH phosphatases (Fig. 2). Unlike other yeast PP2C phosphatases, Yor090cp contains amino- and carboxy-terminal extensions surrounding the catalytic core (aa 181–466). Like other members of this protein family, recombinant Yor090cp is able to dephosphorylate Cdc28p and casein in vitro [17].

3.2. Yil042cp and Yor090cp are mitochondrially localized proteins

A critical aspect in studies of organellar protein phosphorylation is the correct assignment of the enzymes to a specific compartment. Therefore, we re-investigated the intracellular localization of the selected proteins. Highly purified mitochondria were isolated from cells expressing Yil042c-cMyc or Yor090c-3HA by two successive sucrose gradient centrifugation steps and a final salt wash with 1 M NaCl as described by Meisinger et al. [9]. The purity of the obtained fractions was assessed by detection of the cytosolic phosphoglycerate kinase (Pgk1p), the inner mt membrane protein Cox2p (subunit of cytochrome *c* oxidase, COX) and the soluble mt matrix enzyme aconitase (Aco1p).

In line with previous studies [18–21], the majority of Yil042cp and Yor090cp was detected in the mt fractions (Fig. 3). Trace amount of Yil042cp and Yor090cp (not visible in Fig. 3 due to the very low concentration) as well as of Aco1p were also present in the cytoplasmic fractions, likely due to rupture of some mitochondria during preparation.

3.3. Deletion of YIL042c alters the phosphorylation pattern of mt proteins

We next tested the influence of deletions of *YIL042c* and *YOR090c* on the phosphorylation state of mt proteins. Mt proteins from wild type strain BY4741 and from the deletion mutants Δ*yil042c* and Δ*yor090c* were subjected to SDS–PAGE and the gel was subsequently stained with the phospho-specific fluorescent dye Pro-Q Diamond (Fig. 4A) followed by SYPRO-Ruby staining to visualize total proteins (Fig. 4B).

Comparison of the phosphorylation pattern of the strain lacking Yor090cp with that of the wild type does not reveal any obvious differences. In contrast, mitochondria of the Δ*yil042c* strain clearly lack at least one of the stained bands (marked with * in Fig. 4A), indicating a difference in the phosphorylation state of the respective protein. Total protein staining (Fig. 4B) gives no indication that the absence of that band may be due to proteolytical degradation.

3.4. Yil042cp phosphorylates Pda1p, the α-subunit of the PDH complex

Mt lysates of wild type and of Δ*yil042c* strain were subjected to immobilized metal ion affinity chromatography (IMAC) in order to enrich phosphoproteins. After extensive washing steps, bound proteins were eluted, precipitated as described in Section 2, and separated by SDS–PAGE (Fig. 5). Coomassie blue staining revealed two bands of about 36 and 45 kDa (**, * in Fig. 5) that were prominent in the wild type, but hardly detectable in the Δ*yil042c* strain. When analysed by the fluorescent staining procedure for phosphoproteins the upper of the two bands (*) yielded a strong signal in wild type, indicating the presence of phospho-residues (Fig. 5). Whether the