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

Udo Krause-Buchholz[a,*], Uta Gey[a], Jana Wünschmann[a,1], Susanne Becker[b], Gerhard Rödel[a]

[a] *Institut für Genetik, Technische Universität Dresden, 01062 Dresden, Germany*
[b] *Fachbereich für Chemie, Abteilung für Analytische Chemie, Universität Konstanz, 78457 Konstanz, Germany*

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.**
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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-sub-

unit [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].

*Corresponding author. Fax: +49 351 46337725.
E-mail address: ukrause@rcs.urz.tu-dresden.de (U. Krause-Buchholz).

URL: http://www.biologie.tu-dresden.de/genetik/gen-home.html.

[1] Present address: Lehrstuhl für Botanik, Technische Universität München, 85354 Freising, Germany.

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 proteolytic 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
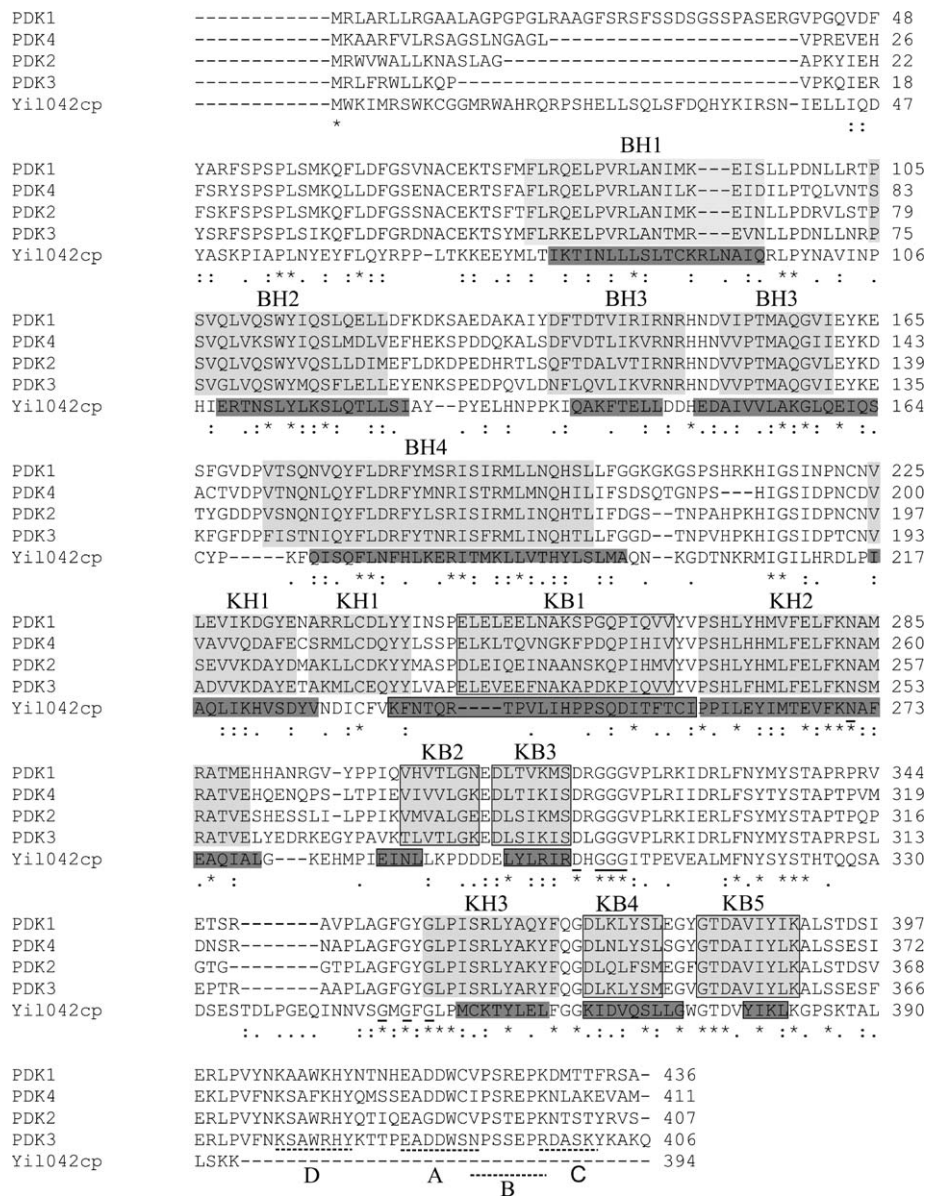
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PDK1      ------------MRLARLLRGAALAGPGPGLRAAGFSRSFSSDSGSSPASERGVPGQVDF 48
PDK4      ------------MKAARFVLRSAGSLNGAGL---------------------VPREVEH 26
PDK2      ------------MRWVWALLKNASLAG------------------------APKYIEH 22
PDK3      ------------MRLFRWLLKQP--------------------------------VPKQIER 18
Yil042cp  ------------MWKIMRSWKCGGMRWAHRQRPSHELLSQLSFDQHYKIRSN-IELLIQD 47
                      *                                      ::
                                        BH1
PDK1      YARFSPSPLSMKQFLDFGSVNACEKTSFMFLRQELPVRLANIMK---EISLLPDNLLRTP 105
PDK4      FSRYSPSPLSMKQLLDFGSENACERTSFAFLRQELPVRLANILK---EIDILPTQLVNTS 83
PDK2      FSKFSPSPLSMKQFLDFGSSNACEKTSFTFLRQELPVRLANIMK---EINLLPDRVLSTP 79
PDK3      YSRFSPSPLSIKQFLDFGRDNACEKTSYMFLRKELPVRLANTMR---EVNLLPDNLLNRP 75
Yil042cp  YASKPIAPLNYEYFLQYRPP-LTKKEEYMLTIKTINLLLSLTCKRLNAIQRLPYNAVINP 106
          ::  . :**. : :*::          ::  .: :   : : :*:    :  . :  . 
              BH2                              BH3        BH3
PDK1      SVQLVQSWYIQSLQELLDFKDKSAEDAKAIYDFTDTVIRIRNRHNDVIPTMAQGVIEYKE 165
PDK4      SVQLVKSWYIQSLMDLVEFHEKSPDDQKALSDFVDTLIKVRNHHNVVPTMAQGIIEYKD 143
PDK2      SVQLVQSWYVQSLLDIMEFLDKDPEDHRTLSQFTDALVTIRNRHNDVVPTMAQGVLEYKD 139
PDK3      SVGLVQSWYMQSFLELLEYENKSPEDPQVLDNFLQVLIKVRNRHNDVVPTMAQGVIEYKE 135
Yil042cp  HIERTNSLYLKSLQTLLSIAY--PYELHNPPKIQAKFTELLDDHEDAIVVLAKGLQEIQS 164
           :  .:* *::*:  ::.   . : .:   .  : : *.:.: .:*:*: * :.
                                   BH4
PDK1      SFGVDPVTSQNVQYFLDRFYMSRISIRMLLNQHSLLFGGKGKGSPSHRKHIGSINPNCNV 225
PDK4      ACTVDPVTNQNLQYFLDRFYMNRISTRMLMNQHILIFSDSQTGNPS---HIGSIDPNCDV 200
PDK2      TYGDDPVSNQNIQYFLDRFYLSRISIRMLINQHTLIFDGS--TNPAHPKHIGSIDPNCNV 197
PDK3      KFGFDPFISTNIQYFLDRFYISRISFRMLINQHTLLFGGD--TNPVHPKHIGSIDPTCNV 193
Yil042cp  CYP-----KFQISQFLNFHLKERITMKLLVTHYLSLMAQN--KGDTNKRMIGILHRDLPT 217
          . : .. **:  .  .**:::*:.:: ::  .   .    ** :.   :
              KH1       KH1                KB1               KH2
PDK1      LEVIKDGYENARRLCDLYYINSPELELEELNAKSPGQPIQVVYVPSHLYHMVFELFKNAM 285
PDK4      VAVVQDAFECSRMLCDQYYLSSPELKLTQVNGKFPDQPIHIVYVPSHLYHMLFELFKNAM 260
PDK2      SEVVKDAYDMAKLLCDKYYMASPDLEIQEINAANSKQPIHMVYVPSHLYHMLFELFKNAM 257
PDK3      ADVVKDAYETAKMLCEQYYLVAPELEVEEFNAKAPDKPIQVVYVPSHLFHMLFELFKNSM 253
Yil042cp  AQLIKHVSDYVNDICFVKFNTQR----TPVLIHPPSQDITFTCIPPILEYIMTEVFKNAF 273
          :::.  :   . :*   .   .   . : * .. :*. * ::: *:*** ::
                                KB2      KB3
PDK1      RATMEHHANRGV-YPPIQVHVTLGNEDLTVKMSDRGGGVPLRKIDRLFNYMYSTAPRPRV 344
PDK4      RATVEHQENQPS-LTPIEVIVVLGKEDLTIKISDRGGGVPLRIIDRLFSYTYSTAPTPVM 319
PDK2      RATVESHESSLI-LPPIKVMVALGEEDLSIKMSDRGGGVPLRKIERLFSYMYSTAPTQP 316
PDK3      RATVELYEDRKEGYPAVKTLVTLGKEDLSIKISDLGGGVPLRKIDRLFNYMYSTAPRPSL 313
Yil042cp  EAQIAIG---KEHMPIEINLLKPDDDELYLRIRDHGGGITPEVEALMFNYSYSTHTQQSA 330
          .* :        . : ..::* ::: *   ... * :.:. . :*.* *** .
                                  KH3        KB4        KB5
PDK1      ETSR-------AVPLAGFGYGLPISRLYAQYFQGDLKLYSLEGYGTDAVIYIKALSTDSI 397
PDK4      DNSR-------NAPLAGFGYGLPISRLYAKYFQGDLNLYSLSGYGTDAIIYLKALSSESI 372
PDK2      GTG--------GTPLAGFGYGLPISRLYAKYFQGDLQLFSMEGFGTDAVIYLKALSTDSV 368
PDK3      EPTR-------AAPLAGFGYGLPISRLYARYFQGDLKLYSMEGVGTDAVIYLKALSSESF 366
Yil042cp  DSESTDLPGEQINNVSGMFGFGLPMCKTYLELFGGKIDVQSLLGWGTDVYIKLKGPSKTAL 390
          :: . ....      ::*:*:***.: * . * *.:.: *: * ***. * :*. *. ::
PDK1      ERLPVYNKAAWKHYNTNHEADDWCVPSREPKDMTTFRSA- 436
PDK4      EKLPVFNKSAFKHYQMSSEADDWCIPSREPKNLAKEVAM- 411
PDK2      ERLPVYNKSAWRHYQTIQEAGDWCVPSTEPKNTSTYRVS- 407
PDK3      ERLPVFNKSAWRHYKTTPEADDWSNPSSEPRDASKYKAKQ 406
Yil042cp  LSKK------------------------------------ 394
                D           A        C
                                B
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Fig. 1. Sequence alignment of Yil042cp and human PDH kinases PDK1-4 [32] Identical (∗), partially conserved (:) and homologous amino acids (·) are indicated below the sequences. The human kinases comprise two domains (B- and K-domain) with either helical (light grey boxes; BH1-4, KH1-3) or β-sheet structure (framed boxes KB1-5) [33]. The predicted secondary structure of Yil042cp [34] (highlighted in dark grey) corresponds to that of the human kinases, suggesting structural conservation. Amino acids essential for nucleotide binding [33] are underlined (solid line). Regions (A–D) implicated in binding of the lipoyl-bearing domain (LBD2) of the E2-subunit of PDH [15] are marked by dashed lines.

weak signal corresponding to the lower band (∗∗) reflects a phospho-specific staining is doubtful. Unspecific binding of the dye due to the high abundance of the protein cannot be excluded. MALDI-TOF analysis identified the excised protein bands as Pda1p (∗) and Pdb1p (∗∗), the E1α- and E1β-subunits of PDH.

The data provide strong evidence that Yil042cp is involved in phosphorylation of Pda1p. Therefore, we propose "Pkp1p" (for protein kinase of PDH) as the standard name for the protein encoded by *YIL042c*. Copurification of Pdb1p along with Pda1p is likely due to the physical interaction of both proteins in a heterotetrameric complex [22] that is resistant to the detergent and salt concentrations used for cell lysis.

Our results are in line with previous finding that purified PDH complex of yeast is phosphorylated on Pda1p [3].

3.5. PDH activity is decreased in cells lacking the putative protein phosphatase Yor090cp

We next tested whether the absence of either Yil042cp or Yor090cp has an effect on the enzymatic activity of PDH. To this end, mitochondria of the respective null mutants and of the wild type were isolated and subjected to the photometric assay of PDH activity as described in Section 2. To document the specificity of this assay, we included the Δpda1 strain (Fig. 6).

As expected, cells lacking the intact PDH complex due to the deletion of *PDA1* convert only negligible amounts of NAD$^+$ to NADH, clearly demonstrating the specificity of the assay. Interestingly, the activities of the wild type and the Δyil042c strain were almost identical. Obviously, absence of the kinase in Δyil042c does not alter the PDH activity compared to wild

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PDP1        MPAPTQLFFPLIRNCELSRIYGTACYCHHKHLCCSSSYIPQSRLRYTPHPAYATFCRPKE  60
PDP2        MSSTVSYWILNSTRNSIATLQGGRR--LYSRYVSNRNKLKWRLFSRVPPTLNSSPCGG--  56
Yor090cp    MSPLTRTVAIKKTVKVLSKCQSGRE--YTQKFLQRAYSTSHANSTYYSRTKLFISSHSKA  58
            *..              ::        .         .:        .   .   .    .

PDP1        NWWQYTQGRRYASTPQK---FYLTPPQVNSILKANEYSFKVPEFDGKNVSSILGFDSNQL  117
PDP2        --FTLCKAYRHTSTEEDDFHLQLSPEQINEVLRAGETTHKILDLESRVPNSVLRFESNQL  114
Yor090cp    LNIALLSGSLLLTYSYYSPKKILSLDTINGIKDYSTNTSGNINMPSPNPKGTETQKSQRS  118
                           ..      .      *:   .:   .:    ::     ..    .*::

PDP1        PANAPIEDRRSAATCLQTRGMLLGVFDGHAG----CACSQAVSERLFYYIAVSLLPHETL  173
PDP2        AANSPVEDRRGVASCLQTNGLMFGIFDGHGG----HACAQAVSERLFYYVAVSLMSHQTL  170
Yor090cp    QNDQSVLILNDSKIEAKLHDREESHFVNRGTGIFRYDVAQLPSNHPIEDDHVEQIITIPI  178
              :  .:   ..      :  .. . * .:. .     :*  *:: :    *. :   .:

PDP1        LEIEN-AVESGRALLPILQWHKHP--NDYFSKEASKLYFNSLRTYWQELIDLNTGESTDI  230
PDP2        EHMEG-AMESMKPLLPILHWLKHP--GDSIYKDVTSVHLDHLRVYWQELLDLHMEMG--L  225
Yor090cp    ESEDGKSIEKDLYFFGIFDGHGGPFTSEKLSKDLVRYVAYQLGQVYDQNKTVFHSDP-NQ  237
            :..::*.       .::  ::   *.     * :.: :  *:        *     :::       .

PDP1        DVKEALINAFKRLDNDISLEAQVGDPNS--FLNYLVLRVAFSGATACVAHVDGVD--LHV  286
PDP2        SIEEALMYSFQRLDSDISLEIQAPLEDE--VTRNLSLQVAFSGATACMAHVDGIH--LHV  281
Yor090cp    LIDSAISKGFLKLDNDLVIESFRKLFQDPNNTNIANTLPAISGSCALLSLYNSTNSILKV  297
             :..*:   .*  :**.*: :*        :... .      *:**: * ::  :. .:   *:*

PDP1        ANTGDSRAMLGVQEEDGSWSAVTLSNDHNAQNERELERLKLEHPKSEAKSVVKQDRLLGL  346
PDP2        ANAGDCRAILGVQEDNGMWSCLPLTRDHNAWNQAELSRLKREHPESEDRTIIMEDRLLGV  341
Yor090cp    AVTGDSRALICGLDNEGNWTVKSLSTDQTGDNLDEVRRIRKEHP--GEPNVIRNGRILGS  355
            * :**.**::    :::* *:  .*: *:..  *   *: *:: ***      .:: :.*:**

PDP1        LMPFRAFGDVKFKWSIDLQKRVIESGPDQLNDNEYTKFIPPNYHTPPYLTAEPEVTYHRL  406
PDP2        LIPCRAFGDVQLKWSKELQRSILERGFNTEALNIY-QFTPPHYYTPPYLTAEPEVTYHRL  400
Yor090cp    LQPSRAFGDYRYKIKEVDGKPLSDLP---EVAKLYFRREPRDFKTPPYVTAEPVITSAKI  412
            * * ***** : *  .     : : :      : * :  * .: ****:**** :*  ::

PDP1        RPQDKFLVLATDGLWETMHRQDVVRIVGEYLTGMHHQQPIAVGGYKVTLGQMHGLLTERR  466
PDP2        RPQDKFLVLASDGLWDMLSNEDVVRLVVGHLAEADWHK-TDLAQRPANLGLMQSLLLQR-  458
Yor090cp    GENTKFMVMGSDGLFELLTNEEIASLVIRWMDKN--MNLAPVKAEPGKLPKVIDVSEDKE  470
              : **:*:.:***:: : .:::. :*     :       :       :      .*  .:  ::

PDP1        TKMSSVFE-DQNAAT-------------HLIRHAVGNNEFGTVDHERLSKMLSLPEELAR  512
PDP2        -KASGLHEADQNAAT-------------RLIRHAIGNNEYGEMEAERLAAMLTLPEDLAR  504
Yor090cp    AQRPAFRYKDNNSSSPSGSNPEYLIEDKNVATHLIRNALSAGGRKEYVSALVSIPSPMSR  530
            ::  ...     *:*:::::.:...    ....:    *  : *  .    *  :: :::*. .*.  ::*

PDP1        MYRDDITIIVVQFNSHVVGAYQNQE----------------  537
PDP2        MYRDDITVTVVYFNSESIGAYYKGG----------------  529
Yor090cp    RYRDDLTVTVAFFGDSGTPSIVSNATSIVMNPEATTKPKPRL  572
            ****:*: *. *..    :   . ::    ...::....
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Fig. 2. Sequence comparison of Yor090cp and the human PDH phosphatases PDP1 and PDP2 [32]. Identical (∗), partially conserved (:) and homologous amino acids (·) are indicated below the sequences.
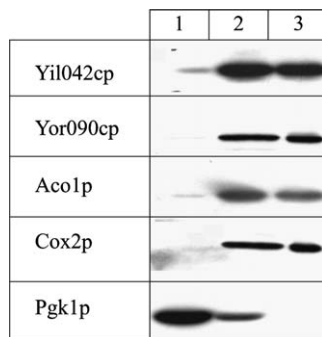

Fig. 3. Mitochondrial localization of Yil042cp and Yor090cp. 50 μg of cytoplasmic (lane 1) or mt proteins of strains expressing either Yil042cp-cMyc or Yor090cp-HA before (lane 2) and after (lane 3) purification by two successive steps of sucrose gradient centrifugation were analysed by Western blot.
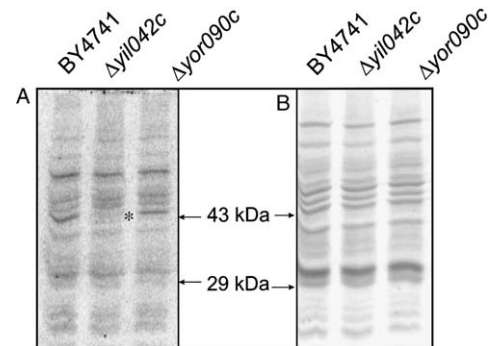

Fig. 4. Comparison of the pattern of phosphorylated mt proteins. Phosphorylated (A) and total (B) mt proteins of wild type and strains lacking Yil042cp or Yor090cp were detected using fluorescence dyes as described in Section 2. The phosphorylated protein band absent in Δyil042c is indicated by an asterisk.

type. This indicates that: (i) PDH is active in its non-phosphorylated state as it is in human cells [22,23]; and (ii) that the Pda1p subunit of wild type cells grown on lactate as a carbon source seems to exist predominantly in its dephosphorylated state. The relatively high fluctuation of PDH activity of BY4741 in the replicate measurements may reflect differences
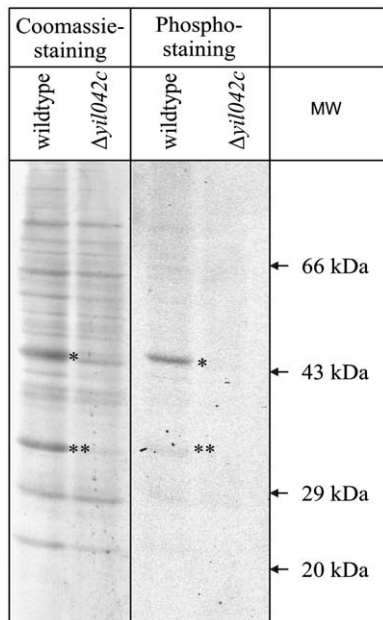
Fig. 5. Coomassie blue staining (left) and phosphoprotein specific staining (right) of IMAC-enriched mt proteins from wild type and Δyil042c. Proteins that lack in the deletion mutant were identified by MALDI as Pda1p (*) and Pdb1p (**).
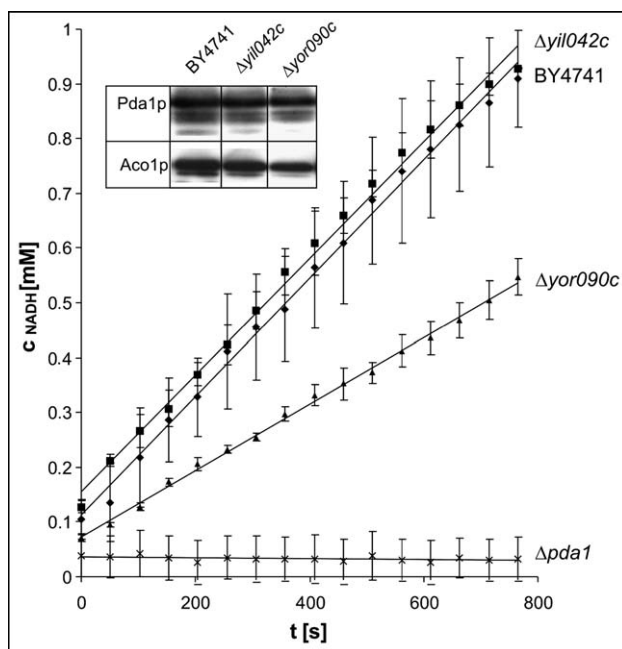

Fig. 6. PDH activity of wild type and the deletion strains Δyil042c and Δyor090c. PDH activity was measured as described in Section 2 (average of three replicates, standard deviation marked by bars). Inset: Steady state concentrations of Pda1p-HA were analyzed by Western blot of 50 μg mt protein.

in the phosphorylation state due to the activity of phosphatase(s) and kinase(s) present in the lysate.

The PDH activity of cells from strain Δyor090c was about 50% of wild type activity, suggesting that Pda1p is effectively phosphorylated in the absence of this phosphatase and par-

tially inactivated by the respective PDH kinase. This finding is in line with previous studies demonstrating a reduced PDH activity due to phosphorylation in a variety of eukaryotic organisms [24,25]. To exclude the possibility that variations in the steady state concentration of Pda1p could account for the observed differences in PDH activity, mitochondria of wild type, Δyor090c and Δyil042c strains bearing HA-tagged versions of Pda1p were analyzed by Western blot (Fig. 6, inset). In none of the strains the steady state level of Pda1p was affected. Taken together, our data suggest that Yor090cp acts as regulating PDH phosphatase by dephosphorylating Pda1p.

In mammalian cells the enzyme is completely inactivated by phosphorylation by a concerted and tissue-specific action of four kinase isoforms [26,27]. There are several possible explanations for our observation that the yeast enzyme activity decreases to only about 50%. (i) Despite of the presence of the Yil042cp in the Δyor090c strain not all Pda1p molecules associated with the complex are phosphorylated, perhaps due to regulation of the expression and/or activity of the kinase. (ii) Even the complete phosphorylation of Pda1p may not result in an entirely inactivated enzyme. This, however, is in contradiction to the finding that complete inactivation of the isolated enzyme is observed upon addition of recombinant PDH kinase [3] and of ATP to isolated mitochondria [5]. (iii) There might exist one or more additional phosphatase(s) with overlapping specificity as it is the case in the human system [28]. Inspection of the S. cerevisiae genome sequences revealed no protein that is homologous to Yor090cp. However, three proteins (Ycr079wp, Yhr076wp and Yjl005wp) of the mt proteome [18,21] share the sequence motif of type 2C protein phosphatases and are candidates for substituting Yor090cp function.

Under in vitro conditions Yor090cp exhibits Mg^{2+}/Mn^{2+}-dependent casein- and very weak Cdc28p-phosphatase activity [17], leading to the proposed name Ptc5p (for type 2C Ser/Thr phosphatase). Although we cannot completely exclude the possibility that a small portion of the protein may be localized outside of mitochondria, its preferential mt localization and its effect on PDH activity strongly argues against a function in the cytosol or the nucleus. Therefore we propose to rename this protein as Ppp1p (for protein phosphatase of PDH).

3.6. Yil042cp and Yor090cp are associated with the PDH complex

Human PDH kinases and -phosphatases are associated with the enzyme complex. The kinase is tightly bound via the covalently attached lipoyl-tail of the E2-component [29], whereas the phosphatase is loosely attached to the complex [23]. Both the kinase and the phosphatase were copurified with the PDH complex by biochemical isolation procedures [23]. In contrast, attempts to identify the corresponding enzymes in isolated yeast PDH complexes failed [3,6]. We investigated the molecular organization of Yil042cp and Yor090cp with respect to their association with the PDH complex by BN-PAGE. To maintain the salt labile PDH complex [30], mitochondria of wild type, Δyor090c and Δyil042c strains expressing the HA-tagged version of Pda1p were lysed by the mild detergent digitonin in low salt buffer. Protein complexes were separated in a 3–13% acrylamide gel with a 3% stacking gel by BN-PAGE and subsequently subjected to a second dimension under denaturing conditions (Fig. 7A). Native
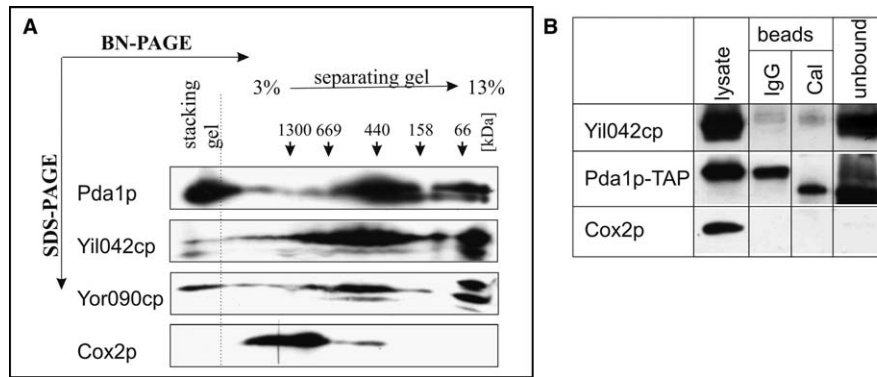
Fig. 7. Molecular organization of Yil042cp and Yor090cp. (A) Mt complexes of strains expressing tagged versions of Pda1p, Yil042cp and Yor090cp were separated in a 3–13% BN-PAA gel. Complex composition was studied by Western blot after separation in a 12% SDS–PAGE. (B) In the TAP purification 500 μg mt proteins of a strain coexpressing Pda1-TAP and Yil042c-cMyc were lysed and subjected to the two consecutive affinity steps (for details see text).

separation conditions were confirmed by detection of Cox2p in the molecular weight (MW) region between 650 and 1300 kDa. These signals represent supramolecular complexes of COX with one or two copies of complex III [31]. In addition COX complexes with an even higher molecular mass of more than 1300 kDa were detectable under the low salt conditions used. Pda1p was detected to a large extent as part of a huge complex residing within the 3% stacking gel, possibly reflecting its association with the 8 MDa PDH complex. This complex can enter 3% polyacrylamide gels [30], but further migration of the complex is prevented by the smaller poresize of the separating gel. In addition, Pda1p accumulates in the lower MW-range of about 400 kDa. The signal in the MW-range smaller than 66 kDa is likely to correspond to the monomeric form of Pda1p. Interestingly, both the kinase and the phosphatase and Pda1p showed a similar separation profile with the majority of the proteins in the 400 kDa MW-range (Fig. 7A). This may hint at a preferential association of Yil042cp and Yor090cp with PDH subcomplexes. Faint signals within the stacking gel may result from the portion of the kinase or phosphatase molecules that are associated with the fully assembled PDH complex.

Further support for an interaction of the kinase with the PDH complex in vivo was obtained by tandem affinity purification (TAP, [12]) with lysates of a strain coexpressing a TAP-tagged version of Pda1p and Yil042cp-cMyc. The successful

affinity purification was monitored by detection of Pda1p-TAP with antibodies directed against the calmodulin-binding moiety. Full-length Pda1p-TAP was detected in the lysate (Fig. 7B, lane 1, 1/50 of total) and on the IgG-beads (Fig. 7B, lane 2, 1/10 of total). The ~16 kDa shift of the signal on calmodulin-beads (Fig. 7B, lane 3, quantitatively applied) documents the efficient cleavage of the TAP-tag by the TEV-protease. A large portion of the protein was found in the fraction of unbound material (Fig. 7B, lane 4, quantitatively applied) indicating less efficient binding to calmodulin beads. Co-precipitation of a small portion of Yil042cp on both matrices hints at a weak or transient interaction with Pda1p or its associated complex. The observation that the mt membrane protein Cox2p is not bound by the beads, documents the specificity of Yil042cp binding.

3.7. Molecular organization of PDH is influenced by Yil042cp

The role of the kinase and phosphatase on PDH activity is well documented [3,5,6]. In contrast, no data on their influence on the molecular organisation and/or assembly of the complex are available. To investigate this issue, we compared the BN-SDS–PAGE profiles of Pda1p of wild type strain BY4741 and strains lacking either the kinase or phosphatase. As shown in Fig. 8A, loss of phosphatase function (Δyor090c) did not alter the separation profile for Pda1p, as the signals almost completely overlap with those of the wild type. However, deletion
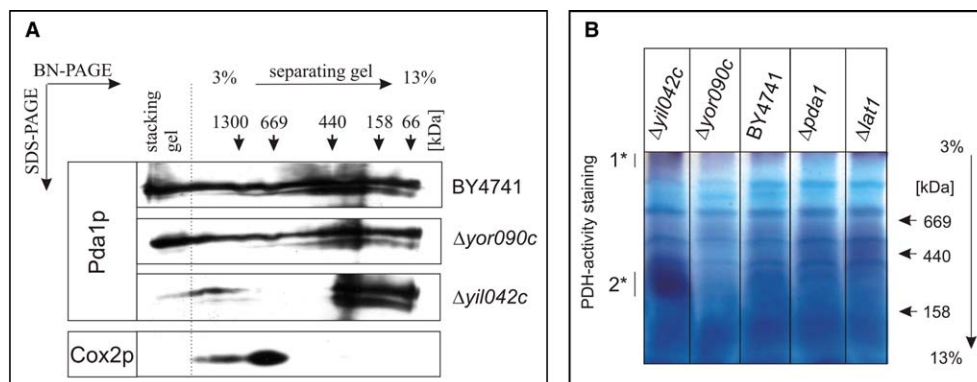

Fig. 8. PDH activity and complex association of Pda1p in wild type and the deletion strains Δyil042c and Δyor090c. Mt complexes were separated by either BN-SDS–PAGE (A) or BN-PAGE and subsequently subjected to PDH *in gel* activity staining (B). Immunodetection was carried out with HA- and Cox2p antibodies.

of the kinase resulted in a preferential accumulation of Pda1p in the MW-range between 300 and 400 kDa. To test whether these smaller complexes possess PDH activity, we performed a newly developed in gel-staining assay for PDH activity after native separation (Fig. 8B). In addition to the above described strains we analysed strains devoid of an active PDH complex due to deletion of PDA1 or LAT1 (the E2 component of the PDH complex). Activity staining revealed a purple band on top of the separating gel in all strains. This may be in part due to activity of the fully assembled PDH complex (Fig. 8B, 1*) that resides in the stacking gel as outlined before. However, other enzyme complexes that are able to reduce NBT may accumulate at the interface between stacking and separating gel and could account for the staining in that region. In contrast, gel staining of PDH activity revealed a second active complex of about 300–400 kDa that is specific for Δyil042c (Fig. 8B, 2*). Since the activity is measured on the basis of the final of the three PDH catalyzed reaction steps, one can assume that at least one copy of each of the three components of PDH are assembled in that subcomplex. Calculated from the MW of E1, E2 and E3 and their known quarternary structure (E1: 2 copies of each of α- and β-subunit; E2: homotrimer; E3: homodimer) this would yield a MW of 446 kDa, which fits to the observed MW-range of the subcomplex in BN-gels. These subcomplexes are also present in the wild type and Δyor090c as shown in Fig. 8A, but they are enzymatically inactive. We assume that these subcomplexes are efficiently phosphorylated by the associated Yil042cp (cf. Fig. 7A) resulting in a complete inactivation. The observed accumulation of PDH subcomplexes in strains devoid of Yil042cp (Fig. 8A) may hint at an additional role of the kinase in complex assembly. In that context, Yil042cp could assist the formation of the fully assembled complex as a structural component and/or due to regulation of Pda1p phosphorylation.

In summary, we provide evidence that the activity of the PDH complex in yeast is regulated by the activities of the PDH kinase Yil042cp and PDH phosphatase Yor090cp, respectively. Both enzymes are associated with the fully assembled PDH complex and with PDH subcomplexes. An additional role of the kinase in complex assembly remains to be elucidated. Further studies will address the quantification of Pda1p phosphorylation and its correlation with PDH activity and/or assembly.

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