The Saccharomyces cerevisiae PCD1 Gene Encodes a Peroxisomal Nudix Hydrolase Active toward Coenzyme A and Its Derivatives*

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The PCD1 nudix hydrolase gene of Saccharomyces cerevisiae has been cloned and the Pcd1p protein characterized as a diphosphatase (pyrophosphatase) with specificity for coenzyme A and CoA derivatives. Oxidized CoA disulfide is preferred over CoA as a substrate with $k_m$ and $k_{cat}$ values of 24 $\mu$M and 5.0 s$^{-1}$, respectively, compared with values for CoA of 280 $\mu$M and 4.6 s$^{-1}$ respectively. The products of CoA hydrolysis were 3′-phosphoadenosine 5′-monophosphate and 4′-phosphopantetheine. F− ions inhibited the activity with an $IC_{50}$ of 22 $\mu$M. The sequence of Pcd1p contains a potential PTS2 peroxisomal targeting signal. When fused to the N terminus of yeast-enhanced green fluorescent protein, Pcd1p was shown to locate to peroxisomes by confocal microscopy. It was also shown to co-localize with peroxisomal thiolase by immunofluorescence microscopy. N-terminal sequence analysis of the expressed protein revealed the loss of 7 or 8 amino acids, suggesting processing of the proposed PTS2 signal after import. The function of Pcd1p may be to remove potentially toxic oxidized CoA disulfide from peroxisomes in order to maintain the capacity for $\beta$-oxidation of fatty acids.

The nudix$^1$ hydrolases are members of a phylogenetically widespread enzyme family that hydrolyze predominantly the diphosphate (pyrophosphate) linkage in a variety of nucleoside triphosphates, dinucleoside polyphosphates, nucleotide sugars, and related compounds having the general structure of a nuclide diphosphate linked to another moiety, X (1, 2). They all possess the nudix box sequence signature motif $GX_{2,3}EX_{10,12}(UA$)-XRE(UA$)X_{1,2}$XEGXGU (where U is a hydrophobic amino acid), formerly known as the MutT motif (1, 3). The functions proposed for members of this protein family are to cleanse the cell of potentially deleterious endogenous nucleotide metabolites and to modulate the accumulation of metabolic intermediates by diverting them into alternative pathways in response to biochemical need (1).

Genome sequencing studies show that the number of nudix hydrolases varies from 0 in Mycoplasma genitalium (4) to 24 in Deinococcus radiodurans (5), whereas cDNA sequencing reveals at least 15 family members in mammalian cells. The budding yeast Saccharomyces cerevisiae has genes for five nudix hydrolases. YSA1 (ORF YBR111C) encodes a 26-kDa ADP-sugar diphosphatase$^2$ (1), NPY1 (ORF YGL067W) encodes a 43.5-kDa NADH diphosphatase$^3$ (6), PSU1 (DCP2, ORF YNL118C) encodes a 109-kDa protein with an N-terminal nudix hydrolase domain whose enzymic activity is as yet undefined but which may be involved in both transcriptional activation (7) and mRNA decapping (8), while DDP1 (ORF YOR163W) encodes a 21.5-kDa enzyme that is a member of a unique subgroup of nudix hydrolases that can hydrolyze both diadenosine polyphosphates and non-nudix diphosphoinositol polyphosphate substrates (9, 10). Here we show that the fifth S. cerevisiae nudix hydrolase gene, PCD1 (ORF YLR151C) on chromosome XII, encodes a protein with an entirely new enzyme activity: a peroxisomal coenzyme A diphosphatase, Pcd1p, that cleaves 3′-phosphoadenosine 5′-monophosphate (3′,5′-ADP) from coenzyme A and CoA derivatives. Oxidized CoA disulfide (CoASSCoA) is preferred to CoA as a substrate. Pcd1p also appears to be the second documented S. cerevisiae protein to have an N-terminal PTS2 peroxisomal targeting signal.

**EXPERIMENTAL PROCEDURES**

**Materials**

*S. cerevisiae* strain INVSc1 (MATa, his3-D1, leu2, trp1-289, ura3-52) was from Invitrogen. All nucleotides and nucleotide derivatives were from Sigma. Calf intestinal alkaline phosphatase, yeast inorganic pyrophosphatase, and restriction enzymes were from Roche Molecular Biochemicals. The *Escherichia coli* expression vector, pET 17b (+) was from Novagen, and the pPGY1 yeast centromere vector was a gift from L.D. Barnes. Rhodamine B hexyl ester was from Molecular Probes. Rhodamine-conjugated goat anti-rabbit IgG was from Santa Cruz Biotechnology. The yeast-enhanced green fluorescent protein (yEGFP) fusion vectors pUG35 and pUG36 were a gift from J. H. Hegemann. A rabbit polyclonal antibody to yeast 3-oxoacyl-CoA thiolase (Fox3p) was kindly donated by W.-H Kunau.

**Methods**

Cloning of PCD1 from Genomic DNA—The PCD1 coding region was amplified from genomic DNA using the polymerase chain reaction and the 36-mer oligonucleotide forward and reverse primers d(AGAAAA-GAAGTCATGATATTAAGTCAGAGGAGGATG) and d(ATCTCTCTC-GAGTTTTCTAGGGAACCCGTTATACCC). The synthesized primers provided an EcoRI restriction site at the start of the amplified ORF and a XhoI site at the end. After amplification with Pfu DNA polymerase (Stratagene), the DNA was recovered by phenol/chloroform extraction, followed by ethanol precipitation. The Pfu DNA polymerase was used because it was found to produce more correct products than either the Taq or Pwo polymerases. The amplified DNA was digested with EcoRI and XhoI and cloned into the EcoRI and XhoI restriction sites of the yeast centromeric vector pPGY1. The resulting plasmids were transformed into yeast INVSc1. Yeast colonies were screened for the presence of the PCD1 gene using EcoRI and HindIII digestion. 

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‡ The abbreviations used are: nudix, nucleoside diphosphate linked to another moiety, X; 3′,5′-ADP, 3′-phosphoadenosine 5′-monophosphate; CoASSCoA, coenzyme A disulfide; CoASSP, mixed disulfide of coenzyme A and 4′-phosphopantetheine; DTT, dithiothreitol; yEGFP, yeast-enhanced green fluorescent protein; ORF, open reading frame; SC-Ura, synthetic complete medium without uracil; PCR, polymerase chain reaction.

§ The term “diphosphatase” is used here instead of “pyrophosphatase” in line with the expected recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature.

¶ S. R. Abdel Raheim and A. G. McLennan, unpublished observations.
digested with EcoRI and XhoI, and the gel-purified restriction fragment ligated between the EcoRI and XhoI sites of both pET17b(+) and pPGY1. The resulting pET1751C construct (from pET17b) yielded an N-terminal fusion of the T7 tag sequence and Pcd1p under the control of the T7 promoter while the pPGY151C construct (from pPGY1) generated an ATG for downstream expression of Pcd1p, a galactosidase inducible promoter, and yielded native Pcd1p when expressed in yeast. Both plasmids were used to transform E. coli XL1-Blue cells for propagation.

Expression of Pcd1p in E. coli and Preparation of Antibody—E. coli strain BL21(DE3) was transformed with pET151C. A single colony was picked from an LB agar plate containing 60 \(\mu\)g/ml ampicillin and inoculated into 10 ml of LB medium containing 60 \(\mu\)g/ml ampicillin. After overnight growth, the cells were transferred to 1 liter LB medium containing 60 \(\mu\)g/ml ampicillin and grown to an \(A_{600}\) of 0.6. Isopropropyl-1-thio-\(\beta\)-galactopyranosidase was added to 0.4 m am and the cells induced for 4 h. The induced cells (4.2 g) were harvested, washed, and resuspended in 50 ml of sonication buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1 m NaCl). The cell suspension was sonicated and the inclusion bodies recovered by centrifugation at 10,000 \(\times\) g for 20 min. After washing by resuspension in sonication buffer containing 2.5 \(\mu\)l urea, the inclusion bodies were dispersed in 27 ml of 25 mM Tris-HCl, pH 8.0, 5 mM, 10 mM dithiothreitol (DTT) and the extract centrifuged at 100,000 \(\times\) g for 1 h. The supernatant was applied to Mono Q HR 5/5 anion exchange column (Amersham Pharmacia Biotech) and eluted with a linear gradient from 0 to 0.5 m NaCl in Buffer A (25 mM Tris-HCl, pH 8.0, 6 mM urea) and the protein eluted with a linear gradient from 0 to 0.5 m NaCl in Buffer A. Homogeneous Pcd1p eluted at 0.27 m NaCl. Fractions containing the protein were dialyzed extensively against phosphate-buffered saline and used to generate a rabbit anti-Pcd1p polyclonal antiserum by standard procedures.

Expression of Pcd1p in S. cerevisiae and Purification—S. cerevisiae strain INVScI was transformed with pPGY151C. A single colony was picked from an SC-Ura (synthetic complete medium without uracil) agar plate and inoculated into 40 ml of SC-Ura medium supplemented with 5% glucose. After 36 h the cells were harvested by centrifugation and resuspended in 4 liters of SC-Ura + 5% glucose and grown for another 24 h. The cells were again recovered by centrifugation, resuspended in 4 liters of SC-Ura + 2% galactose, 1% raffinose, and grown for 24 h to fully induce expression of Pcd1p.

The induced cells (26.8 g) were harvested, washed, and resuspended in 50 ml of breakage buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 50 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 \(\mu\)m trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, 1 mM benzamidine). The cells were disrupted in a French pressure cell and the cell extract recovered by centrifugation at 100,000 \(\times\) g for 4 h. The extract was then dialyzed against Buffer B (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 10 mM 2-mercaptoethanol) before application at 1.5 ml/min to a 25 \(\times\) 250-mm column of DEAE-Sephacel (Amersham Pharmacia Biotech). After removal of unbound protein, a 450-ml linear gradient from 0 to 0.5 m NaCl in Buffer B was applied. Fractions containing Pcd1p were identified by immunoblotting and pooled (67 ml).

Solid (NH_4)_2SO_4 was added to the pooled fraction to a final concentration of 1 m and the sample was loaded at 1.5 ml/min on to a 15 \(\times\) 50-mm column of Phenyl-Sepharose CL-4B (Amersham Pharmacia Biotech) previously equilibrated in Buffer C (50 mM Tris-HCl, pH 7.5, 5 m 2-mercaptoethanol) containing 1 m (NH_4)_2SO_4. After removal of unbound protein, a 100-ml linear gradient (0 to 2.5 m) (NH_4)_2SO_4 in Buffer C was applied. Fractions containing Pcd1p were identified by immunoblotting and pooled (52 ml) before dialysis against 10 m sodium phosphate, pH 6.8, 10 \(\mu\)m CaCl_2.

The dialysate was applied at 1 ml/min to a 100 \(\times\) 7.8-mm Bio-Gel HPHT column (Bio-Rad) and the protein eluted with a 30-ml linear gradient from 10 to 350 m sodium phosphate, pH 6.8, containing 10 \(\mu\)m CaCl_2. Homogeneous Pcd1p eluted at 300 m sodium phosphate, and fractions containing the pure protein were dialyzed extensively against 25 mM Tris-HCl, pH 7.5, 50 m NaCl prior to assay.

Pcd1p-GFP Fusion Constructs and Subcellular Localization—Expression plasmids encoding C-terminal and N-terminal fusions of Pcd1p to yEGFP (11) were constructed by amplification of the PCD1 coding region from genomic DNA using the polymerase chain reaction with either PCD1-yEGFP or yEGFP primers described above to give PCR product C, or the 3-mer forward and reverse primers (dAAGAAAAGATTCATGATAATTACGAGGAGGAT) and (dCAGTTTTCGAGGACACCGGCGCAGTCCACG) to give product N. After amplification with Pfu DNA polymerase, both DNA products were recovered by phenol/chloroform extraction and digested with EcoRI and XhoI. The digested and gel-purified PCR product C was ligated between the EcoRI and XhoI sites of pEGFP-C1 (Clontech) and transformed into E. coli XL1-Blue cells for propagation. Protein expression and localization were analyzed by confocal microscopy (12) using the Zeiss LSM 510 confocal microscope with a 1.4 numerical aperture objective. Enzyme Assays and Protein Identification—Potential substrates were serendipitously identified by high performance anion-exchange chromatography. Reaction mixtures (100 ml) containing 50 m 1, 3-bis[tris(hydroxymethyl)methylamino]propane-HCl, pH 7.0, 5 m MgCl_2, 0.5 m substrate, and 0.125 m Pcd1p were incubated for up to 10 min at 37 °C and assayed by measuring the released P_i by co-incubation of nucleotides with Pcd1p and either inorganic pyrophosphatase or alkaline phosphatase as described previously (9, 13). Reaction products generated by Pcd1p were identified by high performance anion-exchange chromatography. Reaction mixtures (100 ml) containing 50 m 1, 3-bis(tris(hydroxymethyl)methylamino)propane-HCl, pH 7.0, 5 m MgCl_2, 0.5 m substrate, and 0.125 m Pcd1p were incubated for up to 10 min at 37 °C and assayed by monitoring the released P_i. Peaks were identified with the aid of standards and quantitated by area integration for kinetic analysis.

Other Methods—Immunoblotting was performed as described previously (9). Protein concentrations were estimated by the Coomassie Blue binding method (15). RESULTS

Cloning, Expression, and Purification of Pcd1p

The intronless PCD1 gene contains an open reading frame, YLR151C, that potentially encodes a 39,758-Da protein containing a putative pyridoxal binding domain (Fig. 1A). Initial and various attempts to express soluble Pcd1p to a high level in E. coli were unsuccessful, with all the recombinant protein being found in inclusion bodies. Resolubilization of this material was considered undesirable. It was therefore decided to use the protein isolated from inclusion bodies to raise a polyclonal rabbit antibody that could then be used in an immunoassay for the purification of the overexpressed protein.
from yeast. The YLR151C ORF was amplified by PCR from genomic DNA and the PCR product inserted into the yeast centromere vector, pPGY1. The resulting plasmid, pPGY151C, was transformed into S. cerevisiae strain INVSc1 and expression of the insert induced by growth on galactose. By following the major immunoreactive species on Western blots of chromatographic fractions, the protein product of PCD1 was purified to homogeneity from extracts of the induced cells. The purified protein had a molecular mass of 38 kDa according to SDS-PAGE (Fig. 1B). Throughout the purification, the immunoblots revealed more clearly than the gels that the overexpressed Pcd1p actually comprised two species of very similar size (Fig. 1C). N-terminal sequencing of the two bands excised from these blots showed the upper and lower species to have the sequences MLSSKQLI and LSSKQLI, respectively, suggesting that Pcd1p may have undergone some N-terminal processing with the loss of either 7 or 8 amino acids (Fig. 1A). N-terminal sequencing of the two bands excised from these blots showed the upper and lower species to have the sequences MLSSKQLI and LSSKQLI, respectively, suggesting that Pcd1p may have undergone some N-terminal processing with the loss of either 7 or 8 amino acids (Fig. 1A).

Properties of the Protein

Substrates—Nucleotides were tested as potential substrates for Pcd1p using a spectrophotometric assay. No activity was found with the following compounds: (deoxy)nucleoside 5'- monophosphates, nucleoside 5'-di- or monophosphates, diadenosine polyphosphates, nucleoside 5'-diphosphosugars, cytidine 5'-diphosphoalcohols, NAD+, NADH, or FAD. However, substantial activity was found with CoA and some CoA derivatives (Table I). HPLC analysis of the products of CoA hydrolysis showed that the enzyme was a CoA diphosphatase, cleaving the diphosphate linkage in CoA to give 3',5'-ADP and the dimer of 4',5'-phosphopantetheine (Fig. 2A). Pcd1p is the first nudix hydrolase to be described with such a substrate specificity. The Km and kcat for CoA were 280 μM and 4.6 s⁻¹, respectively, while the corresponding values for oxidized CoA disulfide (CoASSCoA) were 24 μM and 5.0 s⁻¹, respectively. Thus, the enzyme has a 13-fold higher kcat/Km ratio for CoASSCoA compared with CoA. These kinetic parameters were calculated by non-linear regression analysis of the data in Fig. 3A. The reciprocal plots in Fig. 3B clearly show that the enzyme follows Michaelis-Menten kinetics with these two substrates. The initial products of CoASSCoA hydrolysis were 3',5'-ADP and what is presumed to be CoASSP, the mixed disulfide of CoA and 4',5'-phosphopantetheine, i.e. CoASSCoA lacking a single 3',5'-ADP moiety (Fig. 2B). Significant accumulation of this product was observed with time before it too was degraded, presumably to 3',5'-ADP and the dimer of 4',5'-phosphopantetheine, suggesting that CoASSP is not as efficient a substrate as CoASSCoA. When measured at a single fixed substrate concentration, moderate activity was also obtained with several short chain acyl-CoA esters while 3'-dephospho-CoA was a very poor substrate (Table I). Thus, the 3' phosphate on the adenosine moiety appears to be important for substrate recognition. Attempts to demonstrate enzyme activity in crude yeast extracts proved impossible due to 3' dephosphorylation of the CoA substrates. The mixed CoA-glutathione disulfide, which may exist in vivo but which is more usually found as an extraction artifact (16, 17), was also a relatively poor substrate. Therefore, recognition of CoASSCoA as a good substrate must involve more than just the disulfide bond.

Pcd1p was optimally active at pH 7.0 with 5 mM Mg²⁺ ions. Mn²⁺ at 300 μM supported 83% of the activity observed with 5 mM Mg²⁺ ions. Like all other nudix hydrolases tested, Pcd1p was very sensitive to inhibition by fluoride ions with an IC₅₀ of 22 μM using CoASSCoA as substrate (data not shown).

Subcellular Localization—A likely subcellular location for an enzyme with the properties described would be the mitochondria or peroxisomes, as these contain the major cellular CoA pools. The latter organelle is the sole site of fatty acid β-oxidation in yeast. The N-terminal 30–40 amino acids of Pcd1p are rich in Lys, Arg, Ser, and Thr, suggesting they may comprise a mitochondrial targeting signal (Fig. 1A) (18). The PSORT algorithm suggests a possible mitochondrial location (19) while a hydrophobic transmembrane segment following the potential leader sequence that could anchor the imported protein in the inner mitochondrial membrane is predicted by the TMpred (20) and TMHMM (21) algorithms (Fig. 1A). The sequence of Pcd1p does not contain a typical C-terminal tripeptide peroxisomal targeting signal (PTS1) (22); however, the sequence RRMLSSKQL in the N-terminal region (Fig. 1A) is a close match to the PTS2 N-terminal peroxisomal matrix tar-

Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (mg of Pcd1p for 10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoASSCoA</td>
<td>100.0</td>
</tr>
<tr>
<td>CoA</td>
<td>63.9</td>
</tr>
<tr>
<td>Succinyl-CoA</td>
<td>20.5</td>
</tr>
<tr>
<td>3-Hydroxymethylglutaryl-CoA</td>
<td>16.3</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>14.3</td>
</tr>
<tr>
<td>CoA-glutathione disulfide</td>
<td>6.1</td>
</tr>
<tr>
<td>3'-Diphospho-CoA</td>
<td>0.8</td>
</tr>
</tbody>
</table>

FIG. 2. Identification of reaction products from hydrolysis of CoA (A) and CoASSCoA (B). Assays containing 0.5 mM substrate were incubated with or without 0.125 μg of Pcd1p for 10 min then applied to a 1-ml Resource-Q anion exchange column as described under “Experimental Procedures.” Positions of standards are indicated. — — —, without enzyme; - - -, with enzyme; gradient (---).
geting signal consensus (R/K)(L/V/I) (22, 23). Thus, Pcd1p could be either mitochondrial or peroxisomal. The similarity in peroxisomal and mitochondrial N-terminal targeting signals has been noted before (24).

In order to determine the true subcellular location of Pcd1p, yeast cells were transformed with expression vectors encoding Pcd1p fused to either the C terminus (pyEGFP-PCD1) or the N terminus (pPCD1-yEGFP) of yeast-enhanced green fluorescent protein (yEGFP) (11) and then examined by confocal microscopy. Cells transformed with pyEGFP-PCD1 showed a diffuse cytoplasmic fluorescence with no clear subcellular localization (Fig. 4, A and B) while cells transformed with pPCD1-yEGFP showed a clear punctate fluorescence characteristic of peroxisomes (Fig. 4C). The same cells stained with the mitochondrial-specific dye rhodamine B hexyl ester revealed a quite distinct pattern of mitochondrial staining (Fig. 4D). Superimposition of the latter two images showed only limited coincidence of green and red emissions due to physical overlap of some organelles (Fig. 4E). The structural integrity of the cells was apparent under bright field conditions (Fig. 4F). The peroxisomal location of Pcd1p in cells transformed with pPCD1-yEGFP (Fig. 4G) was confirmed by indirect immunofluorescence microscopy using an antibody to 3-oxoacyl-CoA thiolase (Fox3p), a known peroxisomal enzyme (12, 25), and a rhodamine-conjugated second antibody (Fig. 4H). Both signals were clearly coincident (Fig. 4I). These results show that the N-terminal sequence of Pcd1p directs the enzyme to peroxisomes, most probably via the PTS2-like sequence RRMLSSKQL, but not to the mitochondria. Thus, Pcd1p is only the second protein identified in S. cerevisiae to be imported into peroxisomes by virtue of a PTS2 signal, the first being Fox3p (26). Interestingly, purified Fox3p has been reported to lack its 5 N-terminal amino acids (25) while purified Pcd1p appears to have lost 7 or 8 amino acids from its predicted sequence. This would lend support to the suggestion that the PTS2 signal in yeast may undergo specific proteolytic processing after import into the peroxisomes (25).

DISCUSSION

In addition to the nudix box, the sequence of Pcd1p contains a second, contiguous signature motif to the N-terminal side identified in the PROSITE data bank as UPF0035 (Fig. 5). This motif has the sequence LLTXRXGG (Fox3p) and 34% sequence identity with each other and 26% and 20% sequence identity with Pcd1p, respectively. The Nudt7 gene product is also a peroxisomal CoA diphosphatase with a C-terminal tripeptide targeting signal, 2

\[ \text{Fox3p: } M_{30}L_{11}T_{11}S_{11}K_{11}L_{11}I_{11} \]

\[ \text{Pcd1p: } M_{30}L_{11}S_{11}R_{11}M_{11}L_{11}S_{11}K_{11}Q_{11}L_{11}I_{11}E_{11} \]

\[ \text{Structure 1} \]

FIG. 3. Michaelis-Menten (A) and Lineweaver-Burk (B) plots for the hydrolysis of CoA (●) and CoASSCoA (○) by Pcd1p. Enzyme assays were performed and product peak areas quantified by ion-exchange chromatography as described under “Experimental Procedures.”

FIG. 4. Subcellular localization of Pcd1p by fluorescence confocal microscopy. A, yEGFP fluorescence of cells transformed with pyEGFP-PCD1; B, as panel A but superimposed on a bright field picture; C, yEGFP fluorescence of cells transformed with pPCD1-yEGFP; D, fluorescence of same cells as in panel C stained with rhodamine B hexyl ester; E, superimposition of panels C and D; F, bright field picture of cells in panels C–E; G, yEGFP fluorescence of cells transformed with pPCD1-yEGFP; H, fluorescence of cells in panel G incubated with an antibody to peroxisomal Fox3p and a rhodamine-conjugated second antibody; I, superimposition of panels G and H.

FIG. 5. Subcellular localization of Pcd1p by fluorescence confocal microscopy. A, yEGFP fluorescence of cells transformed with pyEGFP-PCD1; B, as panel A but superimposed on a bright field picture; C, yEGFP fluorescence of cells transformed with pPCD1-yEGFP; D, fluorescence of same cells as in panel C stained with rhodamine B hexyl ester; E, superimposition of panels C and D; F, bright field picture of cells in panels C–E; G, yEGFP fluorescence of cells transformed with pPCD1-yEGFP; H, fluorescence of cells in panel G incubated with an antibody to peroxisomal Fox3p and a rhodamine-conjugated second antibody; I, superimposition of panels G and H.

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SKL. Therefore, the UPF0035 motif may be a determinant of CoA substrate specificity among the nudix hydrolases. Since it overlaps with the predicted transmembrane segment, the latter may not be genuine. An additional sequence feature is the substitution of the usual glutamate residue in the nudix box (marked with + in Fig. 5) by either aspartate or glutamine and the inclusion of an extra amino acid in several cases between this residue and the invariant alanine (marked with † in Fig. 5). Thus, the consensus nudix box in this family of potential CoA diphosphatases is GX\_DX\_AXREXXEEXGU.

Pcd1p represents a new class of nudix hydrolase and a new class of enzyme. The existence of such an activity has been inferred from the results described above, but it has not been isolated. In E. coli, presumably in other cells, regulation of the concentration of CoA includes turnover to form 3′,5′-ADP and 4′-phosphopantetheine, the latter being formed directly or by transfer to and removal from acyl carrier protein (27). The former route would require a CoA diphosphatase. CoA diphosphatase has also been proposed as an activity associated with the 400-kDa CoA synthesizing protein complex from S. cerevisiae in which it forms part of an alternative pathway for CoA biosynthesis that differs from the principal route of 3′-dephospho-CoA and CoA synthesis by this complex (28). This CoA/4′-phosphopantetheine cycle also includes hydrolysis of CoA to 3′,5′-ADP and 4′-phosphopantetheine, which then reacts with ATP to give 3′-dephospho-CoA and then CoA. At the moment we do not know if Pcd1p is responsible for this activity. A recent comprehensive two-hybrid analysis of protein-protein interactions in S. cerevisiae revealed no interacting partners for Pcd1p (29). However, stable interactions requiring three or more partners would not have been detected.

Alternatively, the high activity of Pcd1p toward oxidized CoA disulfide and its peroxisomal location suggest a function that may be more in keeping with the proposal that a major role of the nudix hydrolases is the elimination of toxic nucleotides. Oxidative stress generates increased levels of several of the substrates for nudix hydrolases, e.g. 8-oxo-dGTP for the MutT protein (30), diadenosine tetraphosphate (ApA) for ApA hydrolase (31, 32), and ADP-ribose for ADP-sugar diphosphatases (13). Many of the oxidative reactions in peroxisomes generate hydrogen peroxide and the resultant oxidizing environment would be expected to increase the CoA disulfide/CoA ratio (cf. the oxidized glutathione/glutathione ratio). Indeed, some organisms such as Staphylococcus aureus use a thiol/disulfide redox system based on CoA, CoA disulfide, and a CoA disulfide reductase instead of the more common glutathione system to maintain a reducing environment (33). In the probable absence of a specific CoA disulfide reductase to regenerate CoA within the yeast peroxisomes (33), accumulation of CoA disulfide could lead to a reduction in the ability to oxidize fatty acids, hence the need for Pcd1p. Since the S. cerevisiae NPT1 NADH diphosphatase is also peroxisomal,2 both enzymes may participate in the maintenance and protection of essential nucleotide pools for β-oxidation. Although preliminary experiments with a yeast strain disrupted for PCD1 have failed to show any substantial deficiency in growth on oleic acid,3 more detailed investigations are underway to determine the precise function of Pcd1p and the consequences of PCD1 disruption.

In conclusion, a nudix hydrolase with a previously undescribed enzyme activity has been characterized in yeast. It is the first nudix hydrolase to be shown to be peroxisomal and only the second protein known in S. cerevisiae to be targeted by a PTS2 signal sequence. It will be of interest to determine if a deficiency in the human orthologue is associated with impaired peroxisomal function and disease.

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S. cerevisiae Coenzyme A Diphosphatase

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