The Human *PICD* Gene Encodes a Cytoplasmic and Peroxisomal NADP⁺-dependent Isocitrate Dehydrogenase*

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The human *PICD* gene was identified by homology probing the data base of expressed sequence tags with the protein sequence of *Saccharomyces cerevisiae* Idp3p, a peroxisomal NADP⁺-dependent isocitrate dehydrogenase. The human *PICD* cDNA contains a 1242-base pair open reading frame, and its deduced protein sequence is 59% identical to yeast Idp3p. Expression of *PICD* partially rescued the fatty acid growth defect of the yeast *idp3Δ* deletion mutant suggesting that PICD is functionally homologous to Idp3p. Kinetic studies on bacterially expressed PICD demonstrated that this enzyme catalyzed the oxidative decarboxylation of isocitrate to 2-oxoglutarate with a specific activity of 22.5 units/mg and that PICD displayed *Km* values of 76 μM for isocitrate and 112 μM for NADP⁺. In subcellular fractionation experiments, we found PICD in both peroxisomes and cytoplasm of human and rat liver cells, with approximately 27% of total PICD protein associated with peroxisomes. The presence of PICD in mammalian peroxisomes suggests a role in the regeneration of NADPH for intraperoxisomal reductions, such as the conversion of 2,4-dienoyl-CoAs to 3-enoyl-CoAs, as well as in peroxisomal reactions that consume 2-oxoglutarate, namely the α-hydroxylation of phytic acid. As for cytoplasmic PICD, the phenotypes of patients with glucose-6-phosphate dehydrogenase deficiency (Luzzatto, L., and Mehta, A. (1995) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) Vol. 3, 7th Ed., pp. 3367–3398, McGraw-Hill (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) Vol. 3, 7th Ed., pp. 3367–3398, McGraw-Hill) suggest that PICD serves a significant role in cytoplasmic NADPH production, particularly under conditions that do not favor the use of the hexose monophosphate shunt (Luzzatto et al.).

Isocitrate dehydrogenases catalyze the nucleotide-dependent oxidative decarboxylation of isocitric acid to 2-oxoglutarate. Mechanistically, these enzymes exist in two distinct subclasses that utilize either NAD⁺ or NADP⁺, respectively, as an electron acceptor. In mammalian cells three highly similar isoforms of NAD⁺-dependent isocitrate dehydrogenase have been thoroughly examined and are localized to the mitochondrial matrix (2) where they catalyze the allosterically regulated rate-limiting step of the tricarboxylic acid cycle (3). Prior studies have also reported the existence of at least two NADP⁺-dependent isocitrate dehydrogenases in mammalian cells, one of which is mitochondrial whereas the other is cytosolic (4). Although the precise metabolic roles of the NADP⁺-utilizing enzymes are not yet clear, they are probably related to the production of NADPH for biosynthetic processes in the cytoplasm and other cellular compartments (5, 6).

It has long been suggested that peroxisomes may contain an NADP⁺-dependent isocitrate dehydrogenase (7), but molecular evidence to support this hypothesis has been provided only recently. Two reports have established that the *Saccharomyces cerevisiae* gene *IDP3* encodes a peroxisomal NADP⁺-dependent isocitrate dehydrogenase (8, 9). Furthermore, the phenotypes of the *idp3Δ* mutant suggest that this enzyme is required to regenerate the NADPH consumed by reductive processes inside the peroxisome (8, 9). Mammalian peroxisomes also contain NADPH-consuming enzymes such as 2,4-dienoyl-CoA reductase (10, 11) and hydroxymethylglutaryl-CoA reductase (12–15) and may also require an intraperoxisomal NADP⁺-dependent isocitrate dehydrogenase. Moreover, such an enzyme has the potential to contribute intraperoxisomal 2-oxoglutarate that is required by peroxisomal enzymes such as phytanoyl-CoA α-hydroxylase (16–18).

In this report we present the identification of *PICD*, a human homolog of *S. cerevisiae* IDP3. Activity analysis and kinetic characterization of a bacterially expressed form of PICD revealed that this protein is an NADP⁺-dependent isocitrate dehydrogenase, and subcellular fractionation experiments demonstrated that PICD is localized to both peroxisomes and the cytoplasm in both human and rat cells. We discuss the potential metabolic roles of both peroxisomal and cytoplasmic PICD.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The human cDNA clone (GenBank™ accession number AA313574) for the candidate peroxisomal NADP⁺-dependent isocitrate dehydrogenase was obtained from American Type Culture Collection (Manassas, VA) and was sequenced in its entirety. Two forms of the *PICD* open reading frame (ORF) were amplified by PCR using the cDNA clone as a template. The complete ORF was amplified using the primers 5'-AAAGTCGACAAATGTCGAGCTGAGATTTGGTGAACGCTAGTGG-3' and 5'-AAACGCGGCCGCTTAAAGGTTGGTGGCAGCTTATGTTG-3'. A form of *PICD* lacking the final three codons of the ORF (*PICDΔKL*) was amplified using the primer 5'-GAAAGGGGGCGCTTAAGGACTGAAGTTGATCCTC-3' in conjunction with the first primer above. Both sets of oligonucleotides append SalI and NotI sites (underlined sequences) at the 5’- and 3’-ends of the *PICD* ORF. All PCR reactions were performed with a low error rate mixture of polymerases (Expand, Roche Molecular Biochemicals). The PCR product from each reaction was digested with SalI and NotI and cloned between the SalI and NotI sites of pMBP (19). The sequence of each form of the *PICD* ORF in pMBP was confirmed by automated fluorescent sequencing, and the

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‡ The abbreviations used are: ORF, open reading frame; PCR, polymerase chain reaction; MBP, maltose-binding protein.
resulting plasmids were denoted pMBP-PICD and pMBP-PICDΔAKL. The SacI-NorI fragment of pMBP-PICD was excised and transferred to the oleic acid-inducible yeast expression vector pOE19 (19), as well as the T7-based expression vector, pT7 (20).

**Strains, Media, and Growth Curves**—All bacterial manipulations were performed with the *Escherichia coli* strain DH10B (21). The yeast strains BY4733 (22), BY4733, idp3Δ::HIS3 (23), and BY4733, pex8Δ::HIS3 (23) have been described. Methods for yeast transformations, routine culture of yeast strains, and growth curves have been described (19).

**Production of Recombinant PICD**—The plasmids pMBP-PICD and pMBP-PICDΔAKL are designed to express the PICD and PICDΔAKL proteins, respectively, in fusion with *E. coli* maltose-binding protein (MBP). Induction of protein expression, cell growth and lysis, and amylose-affinity chromatography methods were as described in Geisbrecht et al. (19). Following elution of the MBP-PICD fusion protein, the fractions containing highly purified MBP-PICD (>95% pure by SDS-polyacrylamide gel electrophoresis) were pooled, precipitated with 0.4 g/ml (NH₄)₂SO₄, aliquoted, and stored at -70 °C until use. Recombinant MBP-PICDΔAKL and MBP were expressed and purified according to a similar procedure.

The vector pT7-PICD was used as a template for all *in vitro* translocations of PICD from a T7-coupled rabbit reticulocyte lysate system (TrT, Promega; Madison, WI). Reactions were carried out at 37 °C according to the manufacturer’s suggestions.

**Generation of Antibodies and Immunoblotting**—Bacterially expressed MBP-PICDΔAKL was used to elicit the production of polyclonal anti-PICD antibodies in New Zealand White rabbits. Rabbits were purchased from, maintained at, and immunized according to the standard protocols of Cocalico Biologicals, Inc. (Reamstown, PA). Crude serum was obtained from the supplier. The specificity of the crude sera for PICD was confirmed as follows. Samples containing either total human skin fibroblast protein (40 µg per lane, prepared as described (24)), *in vitro* synthesized PICD, or rabbit reticulocyte lysate alone were separated by SDS-polyacrylamide gel electrophoresis and transferred to membranes. Immune sera were used at a 1:2500 dilution, and immunoblotting was performed as described in Crane et al. (25). Following chemiluminescent detection (data not shown), anti-PICD immunoreactivity was present as one band of approximately 46 kDa in lanes corresponding to the fibroblast cellular protein and *in vitro* synthesized PICD but not in the control lane. Thus, the immune sera was judged to be specific for PICD.

**Analytical Procedures**—Isocitrate dehydrogenase activity was monitored spectrophotometrically at 340 nm as described by Loftas et al. (26), with the exception that assays were performed at 20 °C. One unit of NADP⁺-dependent isocitrate dehydrogenase activity is defined as the amount of enzyme catalyzing the oxidative decarboxylation of 1.0 µmol of α-isocitrate to 2-oxoglutarate in 1 min under standard assay conditions (26). A molar extinction coefficient (ε₄12) of 6220 M⁻¹ cm⁻¹ was assumed for NADPH, and this figure was used in the calculation of all reaction rates. Assays for catalase (a peroxisomal marker) (27, 28), succinate dehydrogenase (a mitochondrial marker) (29), NADPH-cytochrome c reductase (an endoplasmic reticulum marker) (30), and lactate dehydrogenase (a cytosolic marker) (31) have been described. Total protein concentration was determined using the Bradford method (Bio-Rad) with bovine serum albumin as a reference. Curve fitting was performed with GraFit data analysis software. Quantitation of film exposure was performed by densitometry using MacBas software (version 2.0).

**Subcellular Fractionation**—Preparation of a post-nuclear supernatant from HepG2 cells and fractionation of this post-nuclear supernatant by ultracentrifugation on a 15–40% (w/v) linear Nycodenz gradient has been described (32). Following ultracentrifugation, fractions (750 µl) were drawn from the bottom of the gradient and assayed for a peroxisomal marker enzyme catalase, for a mitochondrial marker enzyme succinate dehydrogenase, and for an endoplasmic reticulum marker enzyme NADPH-cytochrome c reductase. The proteins present in each fraction were then precipitated by adding trichloroacetic acid to 15% and incubating on ice for 10 min, and the precipitate was collected by centrifugation at 15,000 × g for 5 min. The precipitated samples were analyzed for polyacrylamide gel electrophoresis as described by Doidt et al. (32). Generation and fractionation of a post-nuclear supernatant from rat liver was performed as described (33), using a 15–42% (w/v) linear Nycodenz density gradient.

**Selective Peptidemization Experiments**—Human hepatocellular carcinoma cells (HepG2) were grown to confluency on two 10-cm dishes. These cells were harvested with trypsin and washed with an isotonic sucrose buffer as described previously (32). The washed cells were divided into 6 aliquots, pelleted by centrifugation at 1,500 × g for 5 min, and placed on ice. The cells in individual aliquots were gently resuspended in isotonic sucrose buffer containing 0, 50, 100, 250, and 500 µg/ml digitonin, and the remaining aliquot was resuspended in the same buffer containing 500 µg/ml digitonin with 1% Triton X-100. The cell suspensions were incubated on ice for 10 min and then centrifuged at 15,000 × g to yield a pellet and supernatant. The solubile proteins present in the supernatant were precipitated by adding trichloroacetic acid as described above, and the samples were prepared for SDS-polyacrylamide gel electrophoresis and immunoblotting as described (32).

**RESULTS**

**Identification of a Human Homolog of *S. cerevisiae* Idp3p—** IDP3 encodes the peroxisomal NADP⁺-dependent isocitrate dehydrogenase of *S. cerevisiae* (8, 9). We used the BLAST algorithm to scan the human data base of expressed sequence tags (dbEST) for candidate human homologs of yeast Idp3p. Successive rounds of prioritized BLAST screening identified a human colon carcinoma cDNA (clone AA313574) whose insert had the potential of encoding a protein with a high degree of similarity to the N-terminal sequence of Idp3p. This cDNA was sequenced in its entirety and was found to contain a 1242-base pair ORF (Fig. 1). The initial ATG codon of this ORF has a near-consensus match for high efficiency translation initiation (34, 35) and is preceded by three in-frame stop codons in the 5'-untranslated region at positions −38, −41, and −135 relative to the A of the putative initiator codon. The deduced product of this cDNA is 59% identical to Idp3p (Fig. 2). Idp3p and PICD were more similar to one another than to any other proteins in these species, indicating that this cDNA (PICD) encoded the human homolog of yeast Idp3p.

Idp3p plays a critical role in regenerating the NADPH equivalents necessary to complete the β-oxidation of unsaturated fatty acids (8, 9). Oleic acid (C18:1ω9) is such a fatty acid, and yeast strains lacking the IDP3 gene (idp3Δ) display a reduced growth rate on oleic acid (9). In order to test whether or not the PICD cDNA encoded a functional homolog of Idp3p, we examined the ability of PICD expression to complement the oleate growth defect of an idp3Δ yeast strain (BY4733, idp3Δ::HIS3) (23). Growth rates were determined in oleic acid medium for the wild-type strain BY4733 (22), the idp3Δ derivative of BY4733 containing the PICD cDNA on an episomal plasmid, the idp3Δ derivative of BY4733, and the pex8A derivative of BY4733 (BY4733, pex8Δ::HIS3) (pex mutants of *S. cerevisiae* are unable to grow on fatty acids (36)). We observed that expression of PICD restored growth of the idp3Δ strain to approximately 75% of wild-type or about twice the growth observed for the idp3Δ strain alone (Fig. 3).

**PICD Encodes an NADP⁺-dependent Isocitrate Dehydrogenase**—The fact that PICD expression was able to complement the oleate growth defect of the idp3Δ strain suggested that the PICD protein, like Idp3p, was an NADP⁺-dependent isocitrate dehydrogenase. To test this directly, we expressed a recombinant form of PICD in fusion with *E. coli* maltose-binding protein (MBP), purified this protein by amylose affinity chromatography, and assayed the purified protein for NADP⁺-dependent isocitrate dehydrogenase activity. The purified MBP-PICD protein catalyzed the oxidative decarboxylation of isocitric acid with a specific activity of 22.5 ± 2.3 units/mg PICD, whereas purified MBP alone was devoid of detectable isocitrate dehydrogenase activity (Table 1). In addition, we were unable to detect isocitrate dehydrogenase activity with purified MBP-PICD when NADP⁺ was replaced with NAD⁺ (Table 1). To examine the kinetics of the PICD catalyzed reaction in more detail, we measured the initial velocities of the enzymatic reaction at various isocitrate and NADP⁺ concentrations and used nonlinear least squares analysis to deter-
mine both $K_M$ constants of the enzyme (data not shown). The $K_M$ constants for the purified enzyme were found to be 76 $\mu$M for isocitrate and 112 $\mu$M for NADP$^1$. These values are in the range of those reported for a bacterially expressed form of Idp3p (9). Interestingly for PICD, the $K_M$ constant observed with respect to NADP$^1$ is higher than for isocitrate, a unique property that has not been observed for the peroxisomal NADP$^1$-dependent isocitrate dehydrogenases of yeasts (9, 37).

The PICD Protein Is Bimodally Distributed between Peroxisomes and the Cytosol—

The above data demonstrated that the human PICD gene encodes a protein highly similar to $S$. cerevisiae Idp3p that can partially complement the yeast idp3Δ mutant and that PICD has NADP$^1$-dependent isocitrate dehydrogenase activity. The presence of the type 1 peroxisomal targeting signal sequence (PTS-1), Ala-Lys-Leu-COOH, at the C terminus of PICD suggested that this enzyme might be targeted to peroxisomes in human cells (38). Rabbit polyclonal antibodies were generated against a recombinant form of PICD lacking its three amino acids (to avoid generating antibodies to the PTS-1 (39)) and were used to examine PICD distribution in subcellular fractionation experiments. Human hepatocellular carcinoma (HepG2) cells were homogenized, and a post-nuclear supernatant was prepared by differential centrifugation. This post-nuclear supernatant was fractionated further by ultracentrifugation on a linear, 15–40% Nycodenz density gradient. Equal portions of each fraction were assayed for peroxisomal, mitochondrial, and endoplasmic reticulum marker enzyme activities (Fig. 4A) and for PICD by Western blot (Fig. 4B). PICD immunoreactivity (approximate mass 46 kDa) was found to be bimodally distributed across the gradient. Significant amounts of PICD were found to colocalize with the peroxisomal marker enzyme catalase, suggesting that PICD is a resident peroxisomal enzyme. However, we observed that the majority of PICD was present in cytosolic fractions at the top of the gradient. Based on the amount of catalase present at the top of the gradient, the levels of PICD in these fractions were

![Fig. 1. Structure of the human PICD cDNA and its deduced product.](image-url)

The nucleotide sequence of the PICD cDNA is presented, together with the amino acid sequence of its deduced protein product. Positions are shown to the right, and the nucleotide sequence is numbered from the first nucleotide of the PICD ORF. The deduced protein is predicted to terminate in the PTS-1 (Ala-Lys-Leu-COOH, underlined). Three in-frame, upstream stop codons in the 5′-untranslated region are also underlined.
disproportionate to the amount expected from peroxisome rupture during homogenization and centrifugation. Thus PICD appeared to be present in the cytoplasm as well as peroxisomes.

We next tested whether PICD from any other mammals shared a similar bimodal distribution. The anti-PICD antibodies detected a protein of the appropriate molecular mass in rat liver homogenates (data not shown), and we found that rat PICD was also present in both peroxisomes and the cytoplasm (Fig. 5, A and B). Comparative densitometry of the anti-PICD immunoreactivity in peroxisome-containing fractions (numbers 1–8) with the non-peroxisomal fractions (numbers 9–16) revealed that approximately 27% of total cellular PICD was peroxisome-associated. We compared this ratio to that of a

### Table I

| Nucleotide | MBP-PICD (units/mg) | MBP |
|------------|----------------------|-----|
| NADP⁺      | 22.5 ± 2.3           | 0   |
| NAD⁺       | 0                    | NA* |

* NA, not applicable.
cytoplasmic marker enzyme activity, lactate dehydrogenase (Fig. 5C), to determine if the peroxisome-associated PICD was due to contamination of the peroxisome-containing fractions with cytoplasmic proteins. The very low level of lactate dehydrogenase activity in the peroxisomal fractions (2% total) confirmed that the peroxisomal PICD was not due to cytoplasmic contamination and instead supported the hypothesis that PICD is a bimodally distributed protein.

Although the simplest explanation for these data is that PICD is only poorly imported into peroxisomes and that most PICD is cytoplasmic, some studies have described peroxisomal proteins that are preferentially lost from the organelle during homogenization and/or fractionation (40). Therefore, in an alternative approach to determine whether PICD is truly predominantly cytoplasmic, we permeabilized HepG2 cells with increasing amounts of digitonin and followed the amount of PICD released as compared with the release of a peroxisomal marker protein catalase (41). At low concentrations of digitonin, most PICD was released from the cells, as expected for a predominantly cytoplasmic protein (Fig. 6). In contrast, release of catalase was observed only when all cellular membranes were permeabilized with 1% Triton X-100. Thus, the cytoplasmic localization of PICD suggested by the subcellular fractionation experiments appeared to reflect bona fide cytoplasmic enzyme instead of peroxisomal PICD that had been released by damage to the organelle.

DISCUSSION

Recent genetic and biochemical studies revealed the presence of an NADPH-dependent isocitrate dehydrogenase, Idp3p, in peroxisomes of S. cerevisiae. These studies also established that Idp3p is essential for the activity of peroxisomal 2,4-dienoyl-CoA reductase, an NADPH-dependent enzyme, and thus that Idp3p is required for producing intraperoxisomal NADPH. To determine whether mammalian peroxisomes also require intraperoxisomal enzymes for the regeneration of enzyme cofactors, we searched for human homologs of yeast IDP3, and we identified a single candidate gene, PICD. We observed that human PICD can rescue the phenotypes of the yeast idp3Δ mutant and that PICD encodes a peroxisomal protein with intrinsic NADP+ -dependent isocitrate dehydrogenase activity, thereby providing strong evidence that PICD is the human homolog of yeast IDP3.

Mammalian peroxisomes contain multiple NADPH-dependent enzymes, including 2,4-dienoyl-CoA reductase (10, 11), hydroxymethylglutaryl-CoA reductase (12–15), and acyl-CoA reductase (42). Furthermore, they contain at least one enzyme, phytanoyl-CoA a-hydroxylase, that requires 2-oxoglutarate as a cosubstrate (16–18). However, the source of the intraperoxisomal NADPH and 2-oxoglutarate that are required by these enzymes has not previously been identified. The presence of PICD in human peroxisomes suggests that this enzyme is responsible for generating these compounds within the organelle. Furthermore, it suggests that mutations in PICD may result in defects in multiple peroxisomal metabolic pathways, namely the oxidation of unsaturated fatty acids and the degradation of phytanic acid. This combination of phenotypes is
highly likely to result in human disease given that defects in phytic acid degradation alone are sufficient to cause Refsum disease (18, 43). Yet to be determined is the source of intraperoxisomal isocitrate, the PICD substrate, as well as the mechanism(s) by which peroxisomes take up the biochemical quantities of NADP⁺ and/or NADPH required to initiate a metabolically relevant NADP⁺/NADPH cycle within the organelle.

In addition to resolving the intraperoxisomal source of NADPH and 2-oxoglutarate, the detection of PICD in human peroxisomes has significant implications for peroxisome permeability. Prior studies of mammalian peroxisomes have suggested that they contain a porin-like protein and are permeable to virtually all small molecules, including sugars as large as sucrose (44). This model implies that the substrates and products of peroxisomal enzymatic reactions can diffuse freely across the peroxisome membrane and, furthermore, that there is no need for peroxisomal metabolite transporters and antiporters, such as those present in the inner mitochondrial membrane. However, this model is based primarily on *in vitro* data using purified peroxisomes that are subject to the caveat that the peroxisomal isocitrate, the PICD substrate, as well as the NADP⁺, NAD⁺, CoA, acetyl-CoA, etc. (45). Since a peroxisome with a freely permeable membrane should not require an intraperoxisomal NADPH-regenerating enzymatic activity *a priori*, the mere identification of PICD supports the hypothesis that mammalian peroxisomes also have an impermeable membrane. This hypothesis predicts the existence of a selective permeabilization experiments.

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