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A role for the human peroxisomal half-transporter ABCD3 in the oxidation of dicarboxylic acids

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ABSTRACT

Peroxisomes play a major role in human cellular lipid metabolism, including fatty acid β-oxidation. Free fatty acids (FFAs) can enter peroxisomes through passive diffusion or by means of ATP binding cassette (ABC) transporters, including *Hs*ABCD1 (ALDP, adrenoleukodystrophy protein), *Hs*ABCD2 (ALDRP) and *Hs*ABCD3 (PMP70). The physiological functions of the different peroxisomal half-ABCD transporters have not been fully determined yet, but there are clear indications that both *Hs*ABCD1 and *Hs*ABCD2 are required for the breakdown of fatty acids in peroxisomes. Here we report that the phenotype of the *pxa1/pxa2Δ* yeast mutant, i.e. impaired oxidation of oleic acid, cannot only be partially rescued by *Hs*ABCD1, *Hs*ABCD2, but also by *Hs*ABCD3, which indicates that each peroxisomal half-transporter can function as homodimer. Fatty acid oxidation measurements using various fatty acids revealed that although the substrate specificities of *Hs*ABCD1, *Hs*ABCD2 and *Hs*ABCD3 are overlapping, they have distinctive preferences. Indeed, most hydrophobic C24:0 and C26:0 fatty acids are preferentially transported by *Hs*ABCD1, C22:0 and C22:6 by *Hs*ABCD2 and most hydrophilic substrates like long-chain unsaturated-, long branched-chain- and long-chain dicarboxylic fatty acids by *Hs*ABCD3. All these fatty acids are most likely transported as CoA esters. We postulate a role for human ABCD3 in the oxidation of dicarboxylic acids and a role in buffering fatty acids that are overflowing from the mitochondrial β-oxidation system.

1. Introduction

In humans, peroxisomes harbor a variety of different enzymes involved in a range of different metabolic functions, including α and β-oxidation of fatty acids. Based on biochemical work in rodents and clinical data of patients [1], it is now clear that a variety of substrates are chain-shortened by β-oxidation in peroxisomes, either by one (bile intermediates, some poly unsaturated fatty acids (PUFAs)) or more cycles such as very long-chain fatty acids (VLCFA), terpenoid acids like pristanic acid, PUFAs, eicosanoids, epoxy fatty acids, xenobiotics, phytanic acid and various ϖ-oxidized metabolites derived from saturated long chain fatty acids, leukotriene, PUFA, and likely phytanic acid [2].

Substrates for peroxisomal β-oxidation, can enter the organelle through passive diffusion [3] or by active transport mediated by different ATP-binding cassette (ABC) transporters [4,5], belonging to subclass D. Two models for peroxisomal fatty acid transport are described in literature ([6–9]). In one model, the ABC transporters deliver esterified fatty acids directly to the peroxisomal matrix while in parallel free fatty acids may enter the peroxisomal matrix via passive diffusion after which they are subjected to esterification by different acyl-CoA synthetases, such as Faa2p and Fat1p. According to the other model, the ABC transporters hydrolyze the CoA esters prior to their entry into the peroxisomes after which the latter are re-esterified by Faa2p or other peroxisomal synthetases, which are dependent on the presence of Pxa1/Pxa2p heterodimer.

The ABCD subfamily contains four half-transporters of which three are localized in peroxisomes, including *Hs*ABCD1 (ALDP, adrenoleukodystrophy protein), *Hs*ABCD2 (ALDRP, adrenoleukodystrophy-related protein) and *Hs*ABCD3 (PMP70, peroxisomal membrane protein) [10–13]. The fourth member *Hs*ABCD4 (PMP70R, PMP70-related protein) has long thought to be also peroxisomal, but recently appears to be localized in other subcellular compartments [14,17]. To be functional, the peroxisomal half transporters have to dimerize. Most experimental data suggest that *Hs*ABCD1, *Hs*ABCD2 and *Hs*ABCD3 function as homodimers [15], but a heterodimeric structure has also been suggested [16].

The physiological function of the different human half-ABC transporters has not been established unambiguously yet, although there are strong indications that at least *Hs*ABCD1 and *Hs*ABCD2 are involved in the breakdown of very long chain fatty acids in peroxisomes [17]. This is concluded from the fact that in patients suffering from X-linked adrenoleukodystrophy due to mutations in *HsABCD1*, very-long-chain fatty acid oxidation is impaired. This results in elevated plasma and tissue

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levels of C24:0, and especially C26:0. Similar fatty acid abnormalities have been found in *Abcd1* −/− mice. At present, no defect of human ABCD2 has been reported. However, *Abcd2* −/− mice also show fatty acid abnormalities, especially at the level of mono- and polyunsaturated fatty acids [12]. Interestingly, over expression of *Hs*ABCD2 or *Hs*ABCD3 restores VLCFA oxidation in X-ALD fibroblasts, indicating that *Hs*ABCD1, *Hs*ABCD2 and *Hs*ABCD3 have overlapping substrate specificities [18]. On the other hand the levels of other peroxisomal β-oxidation substrates, such as leukotrienes, terpenoid acids, dicarboxylic acids or PUFAs are normal in X-ALD patients [2], which could be caused by a different substrate specificity of the three peroxisomal ABC transporters.

Recently we demonstrated that HsABCD1 is involved in the transport of saturated, monounsaturated and polyunsaturated VLCFA-CoA esters across the peroxisomal membrane by expressing ABCD1 in a *Saccharomyces cerevisiae pxa1/pxa2* deletion strain[15], which lacks the two yeast half ABC transporters. This enabled us to study the substrate specificity of each transporter without interference of the other two. In addition, the level of C24-CoA and C26:0-CoA in X-ALD fibroblasts was reported to become elevated when X-ALD fibroblasts were incubated with C24:0 [19]. These findings strongly suggests that ABCD1 and most likely ABCD2[19,20] are involved in the uptake of a wide range of VLCFA-CoA esters into peroxisomes [17,21].

In this paper we have now also characterized the substrate specificity of human ABCD3 using the same expression system and an extended substrate panel. Because we extended our panel of substrates, we compared the substrate specificity of *Hs*ABCD3 with that of *Hs*ABCD1 and *Hs*ABCD2. We found that *Hs*ABCD3 can function as a homodimer as reported before for *Hs*ABCD1 and *Hs*ABCD2. Furthermore, we show that *Hs*ABCD1, *Hs*ABCD2 and *Hs*ABCD3 have overlapping substrate specificities but that *Hs*ABCD3 has a role in the transport of dicarboxylic acids.

2. Materials and methods

2.1. Yeast strains and culture conditions

The wild-type strain (WT) used in this study was *S. cerevisiae* BJ1991 (Matα, *leu2*, *trp1*, *ura3*-251, prb1-1122, *pep4*-3, *gal2*). The deletion strains used in this study are: *fox1Δ*, (or *pox1Δ*; peroxisomal acyl-CoA oxidase), *pxa1Δ* and *pxa2Δ* (peroxisomal ABC transporters 1 and 2) and *faa2Δ* (peroxisomal acyl-CoA synthetase). The mutants were constructed from BJ1991 as described previously [8,15,22]. Double and triple mutants used in this study are *pxa1/pxa2Δ*, *pxa1/pxa2/faa2Δ* deletion strains.

Yeast transformants containing the expression plasmids p*ABCD1* (human ALDP), p*ABCD2* (human ALDRP) or p*ABCD3* (human PMP70) were selected and grown in minimal medium containing 6.7 g/L yeast nitrogen base without amino acids (YNB-WO), supplemented with 3 g/L glucose and amino acids (20 mg/L) if required. For the induction of peroxisome proliferation, cells were shifted to YPO medium containing 5 g/L potassium phosphate buffer pH 6.0, 3 g/L yeast extract, 5 g/L peptone, 1.2 g/L oleate and 2 g/L Tween-80 and supplemented with 20g/L glycerol when indicated. Prior to shifting to these media, the cells were grown in minimal 3 g/L glucose medium for at least 24 h.

2.2. Construction of HsABCD1-3-expression plasmids

The full length *Hs*ABCD1 and *Hs*ABCD2 ORF was cloned into a low copy (ARS/CEN4) yeast expression vector (pIJl30) downstream of the oleate-inducible CTA1 promoter, described by van Roermund et al. [15,20]. The coding region of *HsABCD3* was optimized according to the preferred codon usage of *S. cerevisiae* using algorithms that replace rare codons, and cope with problematic mRNA structure, and various *cis*-elements in transcription and translation (GenScript, Piscataway, NJ). This optimized ORF of *HsABCD3* (Supplementary Fig. 1) was cloned into pIJl30 [23].

2.3. Subcellular fractionation and Nycodenz gradients

Subcellular fractionation was performed as described by Van der Leij et al. [24]. Organellar pellets were layered on top of a 150 to 350 g/L Nycodenz gradient (12 ml), with a cushion of 1.0 ml of 500 g/L Nycodenz solution. All Nycodenz solutions contained 5 mM MES (morpholinoethanesulfonic acid, pH 6.0), 1 mM EDTA, 1 mM KCl, and 85 g/L sucrose. The sealed tubes were centrifuged for 2.5 h in a vertical rotor (MSE 8 × 35) at 19,000 rpm at 4 °C. After centrifugation, gradients were aliquoted in 8 fractions, which were assayed for activity of various marker enzymes as described below. In addition, 150 μL aliquots were taken from the individual fractions derived each from Nycodenz gradient, to which 150 μL of Laemmli sample buffer was added followed by analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Western blotting

Yeast extracts were subjected to SDS-PAGE on 10% acrylamide gels. *Hs*ABCD3 was visualized using a polyclonal antibody (1:1000) following the manufacturer's instructions (Euromedex, Souffelweyersheim, France).

2.5. Enzyme assays

β-Oxidation assays in intact cells were performed as described previously by Van Roermund et al. [25] with some modifications. Cells were grown overnight in media containing oleate/glycerol to induce fatty acid β-oxidation. The β-oxidation capacity was measured in 50 mM MES (pH = 6.0) supplemented with 10 μM of [1-^{14}C]-fatty acids. Subsequently, [^{14}C]−CO_2 was trapped in 2 M NaOH and used to quantify the rate of fatty acid oxidation. [1-^{14}C]-fatty acids were purchased from American Radiolabeled Chemicals (ARC).

The activity of the peroxisomal marker 3-hydroxyacyl-CoA dehydrogenase was measured on a Cobas-Fara centrifugal analyzer by monitoring the acetoacetyl-CoA-dependent rate of NADH consumption at 340 nm [26]. Protein concentrations were determined by the bicinchoninic acid method described by Smith et al. [27].

3. Results

3.1. Expression of human HsABCD3 in S. cerevisiae

Previous work in *S. cerevisiae* has shown that Pxa1p and Pxa2p, in cooperation with at least two acyl-CoA synthetases, transport acyl-CoA esters across the peroxisomal membrane [8]. We showed that the *Hs*ABCD1 and *Hs*ABCD2 can functionally complement a *pxa1/pxa2Δ* deletion strain, suggesting that these human orthologues are functional copies of Pxa1p and Pxa2p.

Initial attempts to express *Hs*ABCD3 in the *pxa1/pxa2Δ* deletion strain were unsuccessful. Immunoblot analysis of yeast cells transformed with *Hs*ABCD3 revealed that the human protein was not expressed, indicating problems at the translational level (not shown). It is known that the codon usage preference among organisms differs markedly, which may lead to low heterogeneous protein expression. Analysis of the ORF of *Hs*ABCD3 using Graphical Codon Usage Analyser (http://gcua.schoedl.de/) indeed revealed several regions that potentially prevent proper translation. To improve the expression, we therefore synthesized a codon optimized ORF of *Hs*ABCD3 and expressed this in yeast. The results in Fig. 1A show that the optimized *HsABCD3* ORF was not only properly expressed in the *pxa1/pxa2* double mutant but also predominantly present in the crude organellar pellet (P) with very little cross-reactive material in the high-speed supernatant