Adrenoleukodystrophy-related protein can compensate functionally for adrenoleukodystrophy protein deficiency (X-ALD): implications for therapy

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Inherited defects in the peroxisomal ATP-binding cassette (ABC) transporter adrenoleukodystrophy protein (ALDP) lead to the lethal peroxisomal disorder X-linked adrenoleukodystrophy (X-ALD), for which no efficient treatment has been established so far. Three other peroxisomal ABC transporters currently are known: adrenoleukodystrophy-related protein (ALDRP), 70 kDa peroxisomal membrane protein (PMP70) and PMP70-related protein. By using transient and stable overexpression of human cDNAs encoding ALDP and its closest relative ALDRP, we could restore the impaired peroxisomal β-oxidation in fibroblasts of X-ALD patients. The pathognomonic accumulation of very long chain fatty acids could also be prevented by overexpression of ALDRP in immortalized X-ALD cells. Immunofluorescence analysis demonstrated that the functional replacement of ALDP by ALDRP was not due to stabilization of the mutated ALDP itself. Moreover, we were able to restore the peroxisomal β-oxidation defect in the liver of ALDP-deficient mice by stimulation of ALDRP and PMP70 gene expression through a dietary treatment with the peroxisome proliferator fenofibrate. These results suggest that a correction of the biochemical defect in X-ALD could be possible by drug-induced overexpression or ectopic expression of ALDRP.

INTRODUCTION

X-linked adrenoleukodystrophy (X-ALD; McKusick 300100) is a peroxisomal neurodegenerative disease with widely varying clinical phenotypes, ranging from the severe childhood cerebral form with progressive inflammatory demyelination in the brain to the mildest form, Addison-only, characterized by adrenocortical insufficiency as the only clinical symptom (for reviews, see refs 1,2). To date, the only treatment with some benefits for patients with the neurodegenerative variants of the disease appears to be bone marrow transplantation carried out at early stages of the disease. The disorder is characterized by the accumulation of saturated, unbranched very long chain fatty acids (VLCFAs; ≥C22:0) in plasma, tissues and cultured fibroblasts, which is due to impaired VLCFA β-oxidation. The defective step in the peroxisomal β-oxidation is associated with the initial activation of VLCFAs catalyzed by very long chain acyl-CoA synthetase (3–6). However, the ALD gene identified by positional cloning (7) does not encode this enzyme, but a gene product belonging to a family of transmembrane proteins, the ATP-binding cassette (ABC) transporters (8–10). In all patients studied, mutations were found in the ALD gene, and ~70% of patients lack immunoreactive adrenoleukodystrophy protein (ALDP). In cultured fibroblasts from X-ALD patients, β-oxidation can be restored by transfection with normal ALD cDNA (11,12), indicating that the mutant ALDP is indeed the cause of impaired β-oxidation. The exact function of ALDP and its relationship to the acyl-CoA synthetase still remain unclear.

ABC transporters have a wide range of substrates and consist of two hydrophobic transmembrane domains and two hydrophilic nucleotide-binding folds (13). The ALD protein structurally represents a half-transporter, with only one of each of these domains, and presumably has to form a homo- or heterodimer to become a functional unit. Three other mammalian half-ABC transporters, structurally similar to ALDP, have been identified: the ALD-related protein [ALDRP (14)], the 70 kDa peroxisomal membrane protein [PMP70 (15)] and the PMP70-related protein [P70R (16,17)], with 63, 33 and 25% amino acid identity to ALDP, respectively. However, these genes show differing expression patterns (14,18–20).

It was reported recently that transient overexpression of PMP70 and ALDR cDNAs leads to restoration of VLCFA β-oxidation in cultured human X-ALD fibroblasts (21,22). This was unexpected for PMP70, since X-ALD fibroblasts endogenously express PMP70. Apparently, overexpression is required to suppress the β-oxidation defect. In contrast, expression of ALDRP, the closest relative of ALDP, is normally not detectable in human fibroblasts. Here we demonstrate that, as previously shown in transient overexpression (22), ALDRP can also restore peroxisomal β-oxidation efficiently in stably transformed

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X-ALD fibroblasts and, in addition, prevent VLCFA accumulation. Furthermore, VLCFA β-oxidation could be restored in the liver of ALDP-deficient mice after fenofibrate stimulation of ALDR and PMP70 gene expression.

RESULTS

Expression of ALDP and ALDRP leads to restoration of VLCFA β-oxidation in X-ALD fibroblasts

Normally, fibroblasts do not express ALDR protein or mRNA. To investigate the influence of expression of ALDP and ALDRP on β-oxidation in an ALDP/ALDRP-deficient system, we used SV40-transformed skin fibroblasts from an X-ALD patient with the missense mutation A626T, which lack the punctate immuno-reactive staining pattern characteristic for ALDP (23). Expression plasmids were constructed containing the human ALD and ALDR cDNA, respectively, under control of the cytomegalovirus (CMV) promoter. Since the subcellular localization of ALDRP has only been shown for the mouse protein (24), a haemagglutinin (HA) tag was added to the C-terminus of one ALDRP expression construct. Indirect immunofluorescence demonstrated a peroxisomal localization of the ALDR-HA protein in transfected fibroblasts (data not shown), indicating that the ALDR construct is expressed and localized correctly. As controls for unspecific stimulation of peroxisomal β-oxidation, the expression vector alone (pcDNA3.1), the vector expressing chloramphenicol acetyltransferase (pcDNA3.1/CAT), and pEGFP-C3, a vector expressing green fluorescent protein (GFP) under the control of the CMV promoter, were used. GFP was selected as it should not influence β-oxidation, and transfection efficiency can be established easily. The mean transfection efficiency was estimated to be ∼20% (15–33%; data not shown) for the constructs expressing ALDP, ALDRP-HA and GFP.

The ratio of β-oxidation of lignoceric acid (C24:0) versus palmitic acid (C16:0) was determined in extracts from transfected normal and X-ALD fibroblasts, showing that β-oxidation of VLCFAs was decreased in ALDP-deficient fibroblasts to ∼25% of the control (Fig. 1). Expression of wild-type ALD protein in X-ALD fibroblasts led to restoration of β-oxidation to about the same value as in the healthy control fibroblasts, in good agreement with previous data (11,12). The differences between X-ALD fibroblasts and healthy controls or X-ALD fibroblasts transfected with normal ALD were statistically significant (multiple range test at the 95% confidence level). In contrast, measurement of β-oxidation in cells transfected with vector (Fig. 1), vector expressing CAT or pEGFP-C3 (data not shown) all showed a slight, but statistically not significant, increase relative to the value in X-ALD fibroblasts. To investigate the influence of ALDRP expression on VLCFA β-oxidation, X-ALD fibroblasts were also transiently transfected with the ALDR construct, leading to a similar increase in β-oxidation as that in expression of ALDP (Fig. 1) and, thus, to a specific activity, which was not significantly different from the β-oxidation in healthy control cells. Transfection with the ALDR-HA construct also increased β-oxidation, although to a slightly lower extent than the unmodified ALDRP (75% of ALDRP; data not shown), which might be due to a negative influence of the HA tag on the functionality of the protein.

Normalization of VLCFA content by stable expression of ALDRP in X-ALD fibroblasts

X-ALD fibroblasts accumulate ∼10-fold amounts of C26:0 fatty acid in comparison with healthy controls. To investigate whether this accumulation can be suppressed by expression of ALDRP, we generated fibroblasts stably expressing ALDRP. RNA was isolated from individual clones for northern blot analysis, and strong expression of ALDR could be shown in a stable clone, whereas no ALDR mRNA expression was detectable in control or in X-ALD fibroblasts (Fig. 2a). Neither the absence of ALDP (Fig. 2a, X-ALD) nor the overexpression of ALDR (Fig. 2a, X-ALD + ALDR transfected) appeared to have any influence on the level of PMP70 mRNA.

β-Oxidation was determined in control, X-ALD and stably transfected fibroblasts. VLCFA β-oxidation was increased more strongly in the ALDRP-expressing clone than in the transiently transfected fibroblasts, leading to a value ∼2-fold higher than in healthy control fibroblasts (Fig. 2b). This elevated level of lignoceric acid β-oxidation probably reflects that now each single cell is overexpressing ALDR protein, whereas in the transiently transfected cells an average of only 20% of the fibroblasts displayed ALDRP expression.

After 2 months of growth, the VLCFA content of X-ALD cells stably expressing ALDRP was determined by gas chromatography. VLCFA levels were now normalized (Fig. 2c), indicating that overexpression of ALDRP is capable of suppressing the accumulation of VLCFAs in X-ALD fibroblasts. The gas chromatographic profiles of the normal and the ALDRP-restored X-ALD fibroblasts displayed no major differences, providing additional evidence for similar substrate specificities for ALDP and ALDRP.

Restoration of β-oxidation is not due to stabilization of ALDP in ALDR-expressing fibroblasts

In yeast, only two peroxisomal half-ABC transporters, Pxa1p and Pxa2p, are known (25,26). These form a heterodimer, and the absence of Pxa2p leads to destabilization of Pxa1p (27). Thus, a possible mechanism for restoration of β-oxidation by ALDR
Restoration of peroxisomal $\beta$-oxidation in the liver of fenofibrate-treated mice

Dietary treatment with the peroxisome proliferator fenofibrate leads to up-regulation of ALDR and PMP70 gene expression in the liver of rodents (28), and both PMP70 (21) and ALDRP (22) are able to restore $\beta$-oxidation upon overexpression in X-ALD fibroblasts. Since ALDP-deficient mice show VLCFA accumulation in many tissues, including the liver (29), it is possible to investigate whether fenofibrate treatment can restore $\beta$-oxidation also in vivo. Wild-type and ALDP-deficient mice (30) were fed a diet supplemented with 0.3% fenofibrate. After 7 days of treatment, the livers of control and fenofibrate-treated mice were dissected, RNA and protein were isolated for northern and western blot analyses, respectively, and the rate of lignoceric acid $\beta$-oxidation was determined. Northern blot analysis of ALDR and PMP70 mRNA expression showed that fenofibrate stimulated expression of both genes in the liver of wild-type as well as ALDP-deficient mice (Fig. 3a). The increase in mRNA was ~4-fold for PMP70 and 10-fold for ALDR. Western blot analysis showed a comparable (4-fold) increase in PMP70 expression in ALDP-deficient and control mice (data not shown), similar to that shown in the liver of fenofibrate-treated rats (28).

In the liver of the ALDP-deficient mice on a regular diet, lignoceric acid $\beta$-oxidation was reduced to ~50% of the value in wild-type mice (Fig. 3b). Whereas fenofibrate treatment had no significant effect on the rate of C24:0 $\beta$-oxidation in the liver of wild-type mice, it resulted in an increase in the ALDP-deficient mice, such that no statistically significant difference remained between wild-type and ALDP-deficient mice (post hoc Tukey-test at a significance level of 0.05). These results suggest that overexpression of ALDR and/or PMP70 can restore peroxisomal $\beta$-oxidation also in the liver of ALDP-deficient mice.

**DISCUSSION**

The high degree of amino acid identity between the closest relatives ALDRP and ALDP already suggested functional similarity, and here we could show that expression of ALDRP in X-ALD fibroblasts lacking detectable ALD protein leads to restoration of $\beta$-oxidation and—in clones stably overexpressing ALDRP—to normalization of the VLCFA concentration. In contrast to a recently published study (22) reporting a normalized peroxisomal $\beta$-oxidation activity in transiently ALDR-transfected X-ALD fibroblasts only after correcting for transfection efficiency, we achieved a level similar to that of the healthy control without correcting for transfection efficiency in the transient study. The apparently more efficient restoration in our study may be due to differences in the ALDR expression constructs or transfection procedure. As expected, the $\beta$-oxidation rate was even higher (twice the control) in our stable ALDR-expressing clone. This suggests that, at least in normal fibroblasts, ALDP may be a rate-limiting factor for VLCFA $\beta$-oxidation.

By indirect immunofluorescence, it could be excluded that the effect of ALDRP leading to restoration of $\beta$-oxidation in X-ALD fibroblasts was due to stabilization of mutant ALDP. These data strongly indicate that ALDRP itself is able to replace ALDP functionally.

To evaluate whether overexpression of peroxisomal ABC proteins other than ALDP could correct $\beta$-oxidation also in vivo, expression of ALDR and PMP70 was stimulated in the liver of...
The peroxisome proliferator fenofibrate, a frequently used hypolipidemic drug without severe side effects, recently has been shown to stimulate ALDR and PMP70 expression in the liver of rats (28). Treatment of wild-type and ALDP-deficient mice with fenofibrate also results in strong stimulation of ALDR and PMP70 gene expression in the liver. This overexpression did not exhibit any significant effect on β-oxidation in the wild-type mice. In the liver of ALDP-deficient mice, VLCFA β-oxidation was normalized after fenofibrate treatment. These results indicate that ALDR and/or PMP70 can take over the function of deficient ALDP in X-ALD. In addition to ALDP and ALDRP stimulation, fenofibrate may exert pleiotropic effects on other enzymes, including very long-chain acyl-CoA synthetase, that could also affect β-oxidation of VLCFAs. Redundant function of ALDP and ALDRP is suggested as there currently is no proof that the accumulation of VLCFAs could be the reason why fenofibrate is not effective in the brain could be a lack of the particular PPAR subtype necessary for activation in brain. However, it has been shown that PPARα and β are ubiquitously expressed in rat brain (32,33), and fenofibrate presumably activates PPARα (34). Therefore, it appears more likely that fenofibrate would, in principle, have an effect in brain, but is not able to cross the blood–brain barrier. In this case, it might be possible to find some way to mediate penetration of fenofibrate or a similarly acting substance into the brain. Several drugs, such as 4-phenylbutyrate (22), lovastatin and sodium phenylacetate (35), or compounds increasing the cAMP level (36), have been shown to normalize VLCFAs in skin fibroblasts of X-ALD patients. In the case of 4-phenylbutyrate, this seems to be accomplished by a stimulation of ALDRP expression in addition to peroxisome proliferation (22), whereas for the other drugs the mechanisms underlying the functional rescue require more detailed investigation. It can be anticipated that pharmaceutical correction of the biochemical defect in X-ALD can be achieved via a single drug (4-phenylbutyrate, lovastatin or fenofibrate) or via a combination of different drugs. However, it is necessary to evaluate further whether the correction of the VLCFA metabolism will influence the neurodegenerative course of the disease, as there currently is no proof that the accumulation of VLCFAs per se is the cause of the neurological symptoms.

**MATERIALS AND METHODS**

**Cell lines and cell culture**

SV40-transformed skin fibroblasts from an X-ALD patient lacking detectable ALD protein were kindly provided by Dr K.D. Smith and Dr L.T. Branteman (Baltimore, MD; patient no. 11 with mutation A626T in ref. 23). Control fibroblasts were obtained from skin biopsies of probands without metabolic diseases and were transformed using the plasmids pSVBam and pE6.6 (37) containing the entire SV40 genome and activated Ha-RAS, respectively, in pBR322. Cells were maintained in RPMI 1640 medium (Biowhittaker, Walkersville, MD) supplemented with 2 mM L-glutamine (Biowhittaker), 10% (v/v)
inactivated fetal calf serum (Serva, Heidelberg, Germany) and 50 U/ml penicillin/streptomycin (BioWhittaker).

**Plasmids**

The entire coding regions of the human ALD (7) and ALDR (16) cDNAs, respectively, were EcoRI-inserted into the eukaryotic expression vector pcDNA3.1 (+) (Invitrogen, Groningen, The Netherlands). An HA tag was added to the 3' end of the ALDR cDNA by PCR using an upstream oligonucleotide primer corresponding to the translational start (nucleotide 136 of the ALDR cDNA; EMBL accession no. AJ000327) and the following downstream primer: 5'-GCTCTAGATTAAGGCGCTAGTC- TGAGCGCTGATGAGGTTAAGATGTCTCATCTTCAATT- AAATG-3'. The HA-tag-introducing PCR primer consists of a part complementary to the 3’ end of the ALDR-coding sequence followed by the nucleotides introducing the nine amino acids of the HA epitope (YPYDVPDYA), two stop codons and a recognition site for the restriction enzyme XbaI. The PCR product was ligated into the ALDR expression vector and the resulting construct was sequenced. As controls, the vector alone, pcDNA3.1(+)/CAT (Invitrogen), and an expression vector for GFP, pEGFP-C3 (Clontech, Palo Alto, CA), were used.

**Transfection**

SV40-transformed fibroblasts were transfected with plasmid DNA by electroporation. About 5 x 10^6 cells were suspended in 0.5 ml of normal growth medium. 20 µg of plasmid DNA was added, and the suspension was subjected to an electric pulse of 200 V at a capacitance of 950 µF using the Gene Pulser II and Capacitance Extender PLUS (Bio-Rad, Hercules, CA). For transient transfection, electroporated cells were grown for 4 days prior to analysis.

To obtain stable cell lines, transfected cells were subjected to selection by G418 (Serva) at 200 µg/ml medium starting 2 days after transfection, and individual clones were isolated and expanded after 2 weeks of selection.

**Identification of transfected clones by PCR**

After 4 weeks of selection, the transfected cells were isolated for identification of clones with stably integrated ALDR cDNA. The cells were grown to confluency in 24-well plates, washed twice with phosphate-buffered saline (PBS; BioWhittaker) and incubated in lysis buffer (10 mM Tris, pH 7.7, 10 mM EDTA, 10 mM NaCl, 0.5% sarcosyl, 1 mg/ml proteinase K) at 60°C overnight. DNA was precipitated with a mixture of NaCl and ethanol (150 µl of 5 M NaCl in 10 ml of cold ethanol), resuspended in TE buffer (10 mM Tris, 1 mM EDTA) and used for PCR. The oligonucleotide primers for amplification of ALDR cDNA were Oli 210 (nucleotides 177–199 of EMBl accession no. AJ000327) and Oli 214 (nucleotides 195–199 of EMBl accession no. AJ000327), leading to amplification of an 1818 bp fragment only in clones containing the integrated ALDR cDNA constructs. The endogenous ALDR gene contains introns between these two primers and, therefore, the genomic fragment is too large for amplification under the conditions used.

**Antibodies and immunofluorescence detection**

Indirect immunofluorescence studies of fibroblasts were performed as described elsewhere (38). Aliquots of the transfected cells were plated on glass coverslips immediately after transfection and analyzed by immunofluorescence 4 days later with the following commercially available antibodies: mouse monoclonal anti-ALDP antibody (ALD-1D6, dilution 1:500; Euromedex, Souffelweyersheim, France), mouse monoclonal anti-HA antibody (1:40; Boehringer Mannheim, Mannheim, Germany), sheep anti-human catalase antibody (1:100; The Binding Site, Birmingham, UK), biotinylated anti-mouse antibody (1:200; Amersham, Little Chalfont, UK), FluoroLink Cy2 Avidin (1:50; Amersham) and rhodamine-conjugated anti-sheep antibody (1:100; Accurate Chemical and Scientific, Westbury, NY). GFP was visualized by direct exposure to blue light. Co-localization with catalase was confirmed by simultaneous incubation with anti-catalase and anti-ALDP or anti-HA antibodies and comparison of the staining pattern. Transfection efficiency was determined by counting immunostained and non-stained cells in 10 visual fields at 400x magnification.

**β-Oxidation assay**

The rate of β-oxidation in fibroblasts and freshly dissected or frozen mouse liver was determined in duplicate as described elsewhere (39). In separate reactions, 1–2 x 10^5 d.p.m. of [1-14C]glycerol (acid C24:0) and [1-14C]palmitic acid (C16:0) (both obtained from American Radiolabeled Chemicals, St Louis, MO) were brought to 5 nmol with the respective unlabeled fatty acid and added to fibroblast and mouse liver extracts. After 1 h incubation at 37°C, the amount of degraded fatty acids was determined by measuring the release of water-soluble radioactivity. The β-oxidation activity was calculated as nmol/h/mg protein, and the C24:0/C16:0 ratio was determined.

**Gas chromatographic analysis of VLCFAs**

For VLCFA determination, a confluent 75 cm² flask of each cell type was harvested by trypsinization. The cell pellet was dispersed in 0.5–1.0 ml of distilled water, sonicated for 30 s at 70 W efficiency, and the protein content determined. As an internal standard, 2.5 µg of C23:0 fatty acid in 50 µl was added with 5 ml of chloroform/methanol (1:1) to 0.5 ml of fibroblast homogenate (500 µg–2 mg of protein) and kept at room temperature for 1 h. The protein precipitate was removed by centrifugation and Folch partition was performed (40). Fatty acid methyl esters were prepared according to ‘procedure 1’ for plasma samples (41), and analyzed by gas chromatography as previously described (30).

**Fenofibrate treatment of mice**

Three-month-old ALDP-deficient male mice and wild-type littermates on a C57BL/6×129 hybrid background (30) were fed a normal diet supplemented with 0.3% fenofibrate for 1 week. Pellets were soaked with fenofibrate (Sigma, St Louis, MO) dissolved in acetonitrile, which was allowed to evaporate overnight at room temperature. Fenofibrate-treated and control groups of wild-type and ALDP-deficient mice each consisted of three animals.
Northern blot analysis

Frozen mouse liver was homogenized by grinding in liquid nitrogen, and total cellular RNA was isolated using TriPure Isolation Reagent (Boehringer Mannheim) according to the manufacturer’s instructions. From fresh fibroblasts, total RNA was isolated with RNeasy Mini kit (Qiagen, Hilden, Germany) as described by the manufacturer. The poly(A)^+ RNA fraction was oligo(dT)-selected from 120 µg of total RNA samples using oligo(dT)-cellulose (Boehringer Mannheim) or Dynabeads (Dynal, Oslo, Norway). Poly(A)^+ RNA and a size marker (RNA ladder; Gibco BRL, Paisley, UK) were fractionated on 1.2% agarose–formaldehyde gels (42) and transferred to Biodyne B nylon membranes (Pall, Pullman, UK). Probes were radioactively labeled by random priming using [α-32P]dCTP (Amersham). As a control of equal loading and transfer, the blots were probed with the mouse cDNA of the ubiquitously expressed β-actin mRNA. For mouse liver, a 1049 bp ALDR cDNA fragment (nucleotides 31–1089 of EMBL accession no. Z48570) was used to detect ALDR mRNA. As an mPMP70-specific probe, a 697 bp cDNA fragment (nucleotides 1015–1711 of GenBank accession no. M11182) was used. RNA from human fibroblasts was probed with an 1818 bp hALDR cDNA fragment (nucleotides 172–1989 of EMBL accession no. AJ000327) and a fibroblasts was probed with an 1818 bp hALDR cDNA fragment (nucleotides 172–1989 of EMBL accession no. AJ000327) and a

Western blot analysis

Fresh or frozen mouse liver was homogenized and total membranes were isolated by sodium carbonate precipitation and subsequent high speed centrifugation (43). Gel electrophoresis and western blot analysis of the membrane protein fraction was performed with the PhastSystem (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. The membranes were incubated with rabbit anti-human PMP70 antibody kindly provided by Dr J. Gärtner (Düsseldorf), with biotinylated anti-rabbit antibody (1:400; Amersham) and with avidin–peroxidase (1:400; Sigma). For visualization, ECL detection reagents and Hyperfilm (Amersham) were used as described by the manufacturer.

ABBREVIATIONS

ABC, ATP-binding cassette; ALDP, adrenoleukodystrophy protein; ALDRP, adrenoleukodystrophy-related protein; HA, hae-magglutinin; PMP70, 70 kDa peroxisomal membrane protein; VLCFA, very long-chain fatty acid; X-ALD, X-linked adrenoleukodystrophy.

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