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Impaired Very Long-chain Acyl-CoA β-Oxidation in Human X-linked Adrenoleukodystrophy Fibroblasts Is a Direct Consequence of ABCD1 Transporter Dysfunction*

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Background: ABCD1 is a peroxisomal ABC transporter whose dysfunction causes X-linked adrenoleukodystrophy (X-ALD).
Results: β-Oxidation of C26:0 and C22:0 acyl-CoA esters is impaired in X-ALD. ABCD3 accounts for residual β-oxidation activity in X-ALD fibroblasts.
Conclusion: ABCD1 mediates very long-chain acyl-CoA ester β-oxidation without need for additional re-esterification by an acyl-CoA synthetase.
Significance: Our study provides proof of deficient acyl-CoA ester β-oxidation in X-ALD.

X-linked adrenoleukodystrophy (X-ALD), an inherited peroxisomal disorder, is caused by mutations in the *ABCD1* gene encoding the peroxisomal ATP-binding cassette (ABC) transporter ABCD1 (adrenoleukodystrophy protein, ALDP). Biochemically, X-ALD is characterized by an accumulation of very long-chain fatty acids and partially impaired peroxisomal β-oxidation. In this study, we used primary human fibroblasts from X-ALD and Zellweger syndrome patients to investigate the peroxisomal β-oxidation defect. Our results show that the degradation of C26:0-CoA esters is as severely impaired as degradation of unesterified very long-chain fatty acids in X-ALD and is abolished in Zellweger syndrome. Interestingly, the β-oxidation rates for both C26:0-CoA and C22:0-CoA were similarly affected, although C22:0 does not accumulate in patient fibroblasts. Furthermore, we show that the β-oxidation defect in X-ALD is directly caused by ABCD1 dysfunction as blocking ABCD1 function with a specific antibody reduced β-oxidation to levels observed in X-ALD fibroblasts. By quantification of mRNA and protein levels of the peroxisomal ABC transporters and by blocking with specific antibodies, we found that residual β-oxidation activity toward C26:0-CoA in X-ALD fibroblasts is mediated by ABCD3, although the efficacy of ABCD3 appeared to be much lower than that of ABCD1. Finally, using isolated peroxisomes, we show that β-oxidation of C26:0-CoA is independent of additional CoA but requires a cytosolic factor of >10-kDa molecular mass that is resistant to *N*-ethylmaleimide and heat inactivation. In conclusion, our findings in human cells suggest that, in contrast to yeast cells, very long-chain acyl-CoA esters are transported into peroxisomes by ABCD1 independently of additional synthetase activity.

X-linked adrenoleukodystrophy (X-ALD)[2] is the most common peroxisomal disorder with an incidence of 1:16,800 (combined male hemizygotes plus female heterozygotes) (1). Clinically, the disease can manifest very heterogenously with two main phenotypes: the cerebral demyelinating form and adrenomyeloneuropathy (2, 3). In contrast to the huge phenotypic variation observed in X-ALD patients, the accumulation of straight-chain very long-chain fatty acids (VLCFAs; carbon backbone of more than 22 carbon atoms) in tissues and plasma as well as in primary fibroblasts is common to all X-ALD patients and therefore used as diagnostic marker (4, 5).

In search of the molecular defect in X-ALD, different groups independently found the degradation of free (non-esterified) very long-chain fatty acids to be impaired in intact fibroblasts as well as in homogenates and isolated peroxisomes, whereas the degradation of the respective acyl-CoA esters was found to be unaffected. Based on these results, the respective defect was attributed to a very long-chain acyl-CoA synthetase (ACSVL) activity (6, 7).

However, positional cloning identified *ABCD1*, encoding the peroxisomal ATP-binding cassette (ABC) half-transporter ABCD1 (adrenoleukodystrophy protein), as the affected gene (8). Thereupon, it has been suggested that ABCD1 is responsible for the transport of VLCFA into the peroxisome, but the previous findings of defective acyl-CoA synthetase activity remained unexplained. In addition, the molecular mechanisms leading to the clinical manifestations of the disease are still not completely elucidated (3).

Next to ABCD1, the two homologous peroxisomal half-transporters ABCD2 (adrenoleukodystrophy-related protein, ALDRP) (9, 10) and ABCD3 (peroxisomal membrane protein of 70 kDa (PMP70)) (11) have been identified, whereas the fourth

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[2] The abbreviations used are: X-ALD, X-linked adrenoleukodystrophy; ABC, ATP-binding cassette; VLCFA, very long-chain fatty acid; ZWS, Zellweger syndrome; NEM, *N*-ethylmaleimide; ACSVL, very long-chain acyl-CoA synthetase; HB, homogenization buffer; EGFP, enhanced GFP; HPRT, hypoxanthine phosphoribosyltransferase.

member of the ABCD family, ABCD4 (PMP70R), appears to be localized in other subcellular compartments (12, 13). Ectopic overexpression of ABCD2 and to some extent ABCD3 was shown to compensate for ABCD1 dysfunction in fibroblasts from X-ALD patients (14–16). However, the endogenous homologs are obviously not able to rescue the defect in X-ALD. Consequently, either the substrate specificities of the other transporters are too different or their endogenous levels are too low to compensate for the lack of ABCD1.

Different substrate preferences for the three transporters, such as straight-chain saturated fatty acids for ABCD1, unsaturated fatty acids for ABCD2, and branched-chain and/or dicarboxylic acids for ABCD3, have been suggested, but cautious interpretation of the various results is required as they were obtained in different species. Furthermore, it is assumed that the peroxisomal ABC transporters display diverging but overlapping substrate preferences (for a review, see Ref. 17).

Furthermore, the longstanding question of whether the three peroxisomal transporters can form only homodimers or heterodimers as well (18–20) is of relevance for the substrate specificity because it has been shown for some other mammalian ABC transporters, such as TAP1 and TAP2 (21), as well as for the homologous yeast peroxisomal ABC transporters Pxa1p and Pxa2p (22, 23) that two different subunits are needed for proper function. Indeed, ectopic expression of human ABCD1 in the yeast *Saccharomyces cerevisiae* compensates for the lack of both peroxisomal ABC transporter proteins Pxa1p and Pxa2p, showing that ABCD1 can in principle function as a homodimer (24). Based on the fact that in the absence of peroxisomal ABC transporters acyl-CoAs accumulate in yeast, ABCD1 was proposed to accept acyl-CoAs (24). Moreover, recent publications suggested that in yeast and plants (very) long-chain acyl-CoA esters are hydrolyzed by the respective yeast (Pxa1p and Pxa2p) and plant (comatose) ABC transporters during the import into the peroxisome followed by re-esterification of the fatty acids inside of peroxisomes (25, 26). However, with respect to fatty acid metabolism, yeast and plants are fundamentally different from mammals in which β-oxidation of the short-, medium-, and long-chain fatty acids is exerted by mitochondria.

In this work, we addressed the role of a putative ACSVL activity in the β-oxidation of free VLCFAs and their respective acyl-CoA esters. We provide evidence that in human cells ABCD1 directly accepts hexacosanoyl-CoA esters as well as docosanoyl-CoA esters, but in contrast to yeast, no additional activation step is needed. In addition, the contribution of the two other peroxisomal ABC transporters, ABCD2 and ABCD3, to β-oxidation of VLCFAs is targeted in human fibroblasts.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary human fibroblasts were obtained from skin biopsies of male patients affected by X-ALD or ZWS or controls with no metabolic disease and were provided by Dr. Brunhilde Molzer (Medical University of Vienna, Austria) and Dr. Esther Maier (Ludwig-Maximilians-Universität Munich, Germany). The use of these fibroblasts for biochemical characterization was ethically approved to J. B. Fibroblasts were cultivated in RPMI 1640 medium (PAA) supplemented with 10% heat-inactivated fetal bovine serum (PAA), 2 mM L-glutamine (Lonza), 100 units/ml penicillin (Lonza), 100 μg/ml streptomycin (Lonza), and 1 μg/ml Fungizone (Invitrogen) in an atmosphere of 5% CO_2 at 37 °C. HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; PAA) with supplementation as above.

Homogenization of Fibroblasts—Fibroblasts from 10–40 confluent 10-cm-diameter dishes were harvested by trypsinization and washed once with culture medium and three times with homogenization buffer (HB; 250 mM sucrose, 1 mM EDTA (free acid), 0.1% ethanol, 0.1 mM PMSF, Complete protease inhibitor mixture (Roche Applied Science, catalog number 04693132001), 3 mM imidazole, and KOH to pH 7.4) (27).

Cells were homogenized in 3× packed cell volume of homogenization buffer by five strokes with a Teflon-glass tissue grinder (Wheaton, catalog number 358137) followed by centrifugation at 1,000 × *g* for 3 min. The resulting pellet was resuspended in HB, homogenization was repeated twice, the supernatants were combined, and a postmitochondrial supernatant was obtained by centrifugation at 2,000 × *g* for 10 min.

Subcellular Fractionation by OptiPrep Density Gradient Centrifugation—Density gradient centrifugations were carried out as described earlier (27) with some modifications. OptiPrep (Axis-Shield) density gradient solutions were prepared based on the same buffer composition as HB and further diluted with HB. Two milliliters of postmitochondrial supernatant (containing ~4 mg of protein) were layered on top of a prebuilt 10–30% isoosmotic OptiPrep gradient, and a 40% OptiPrep cushion was used. Centrifugation was carried out at 25,000 rpm for 90 min in a Beckman VTi 65.2 vertical rotor (without brake below 3,000 rpm). Gradient fractions were collected from the bottom, and the respective organellar markers were identified by Western blot analysis.

Cytosolic fractions were prepared by centrifugation of fibroblast homogenates at 200,000 × *g*. Separation of cytosolic components was carried out by the use of 10-kDa-cutoff Amicon Ultra Centrifugal filters (Millipore) followed by washing the retentate three times with HB. The first flow-through as well as the retentate, brought to the original volume, was used for the experiments.

Western Blot Analysis—The following primary antibodies were used within the experiments: mouse α-ABCD1 (Euromedex, 1D6), rabbit α-ABCD3 (Thermo Scientific), rabbit α-lactate dehydrogenase (Abcam), mouse α-LAMP2 (Abcam), mouse α-ATPase (Molecular Probes, A21350), and mouse α-GRP78 (BD Transduction Laboratories). Secondary antibodies were goat α-mouse-HRP (Dako) and goat α-rabbit-HRP (Bio-Rad). All procedures were carried out as described previously (28). Detection was carried out using a Chemidoc XRS+ detection system (Bio-Rad), and quantitative analysis was performed using Image Lab 4.0 analysis software.

Cloning and Expression of an ABCD1-ABCD3-EGFP Fusion Protein—A humanized variant of the ABCD3 antibody epitope (NYEFKQITEDTVEFGS; one amino acid exchange relative to rat) was cloned into a SalI site between full-length hABCD1 and EGFP in the EGFP-N2 vector by use of the following oligonucleotides: sense, 5′-TCGAAACTATGAATTCAAACAGATAACAGAAGATACAGTTGAGTTTGGCAG-3′ and antisense,

5′-TCGACTGCCAAACTCAACTGTATCTTCTGTTATCT-GTTTGAATTCATAGTT-3′. The sequence of the insert was verified by sequencing and the plasmid (internal plasmid number 1616) was transfected into HEK 293 cells by electroporation. After 2 days, cells were harvested and homogenized as described above for fibroblasts, and a peroxisome-enriched fraction was obtained by centrifugation at 20,000 × *g*. The pellet was resuspended in radioimmune precipitation assay buffer, and the fusion protein was enriched by the use of an α-GFP antibody (Roche Applied Science, catalog number 11 814 460 001) bound to magnetic beads (Dynal Biotech).

β-Oxidation of 1-^{14}C-Labeled Fatty Acids—1-^{14}C-labeled palmitic acid (C16:0; ARC 0172A), 1-^{14}C-labeled hexacosanoic acid (C26:0; ARC 1253), 1-^{14}C-labeled docosanoyl-coenzyme A (C22:0-CoA; ARC 3066), and 1-^{14}C-labeled hexacosanoyl-coenzyme A (C26:0-CoA; ARC 1818) were obtained from American Radiolabeled Chemicals. Free fatty acids dissolved in ethanol were aliquoted in glass reaction tubes, dried under a stream of nitrogen, and solubilized in α-cyclodextrin by ultrasonication, whereas acyl-CoA esters were soluble in aqueous solution and therefore used directly in the assay.

β-Oxidation of labeled fatty acids/acyl-CoA esters to acetate was carried out similarly as described previously (29). Briefly, unless otherwise indicated, a reaction of 250 μl contained a 4 μM concentration of labeled fatty acid, 2 mg/ml α-cyclodextrin, 30 mM KCl, 8.5 mM ATP, 8.5 mM $MgCl_2$, 1 mM NAD^+, 0.17 mM FAD, 2.5 mM L-carnitine, 0.16 mM CoA, 0.5 mM malate, 0.2 mM EDTA, 1 mM DTT, 250 mM sucrose, 20 mM HEPES, pH 8.0, and 100 μl of postmitochondrial supernatant containing ~1 mg/ml protein. Reactions were started by the addition of protein, incubated for 1 h at 37 °C, and stopped by addition of KOH and saponification at 60 °C for 1 h. After protein precipitation by acidification with $HClO_4$, a Folch partition was carried out, and the amount of [^{14}C]acetate in the aqueous phase was determined by scintillation counting.

Inhibition of β-Oxidation Activity by Specific Antibodies—Inhibition was carried out by preincubation of the protein samples with either an α-ABCD1 (Euromedex, 1D6; 1 μl/reaction) or an α-ABCD3 antibody (Thermo Scientific; 0.5 μl/reaction) for 4 h at 4 °C. Blocking of α-ABCD3 antibody was achieved by preincubation of antibody with 1.25 μg of immunizing peptide (Affinity Bioreagents, PEP-038)/reaction.

RNA Quantification by Quantitative RT-PCR—RNA was obtained from confluent fibroblasts (one 10-cm-diameter dish) by use of the RNeasy Mini kit (Qiagen), and 80 ng of total RNA were reverse transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. ABCD1, ABCD3, and HPRT were detected by the SYBR Green method using SsoFast™ EvaGreen Mix (Bio-Rad), whereas ABCD2 was detected by the TaqMan method using SsoFast™ Probes Supermix (Bio-Rad). Both methods were carried out using the CFX96 Realtime System (Bio-Rad) according to the manufacturer's instructions.

The following primers were used for PCR amplification: ABCD1, 5′-GAGAACATCCCCATCGTC-3′ (forward) and 5′-TGTAGAGCACACCACCGTA-3′ (reverse); ABCD2, 5′-TCCT-ACACAATGTCCATCTCT-3′ (forward), 5′-AGGACATCTTT-CCAGTCCA-3′ (reverse), and fluorescent probe 5′-Cy5-CAAA-GAGAAGGAGGATGGGATGC-BHQ2–3′; ABCD3, 5′-CGGC-TCATCACAAACAGTGA-3′ (forward) and 5′-AGGTGTTCC-ACCAGTTTTCG-3′ (reverse); HPRT, 5′-CCCTGGCGTCGTG-ATTAGT-3′ (forward) and 5′-CAGGTCAGCAAAGAATTTA-TAGCC-3′ (reverse). Standard curves for absolute quantification of cDNA amounts were generated from linearized plasmids of known copy number containing the PCR products of the respective reactions.

Measurement of VLCFA Accumulation in Fibroblasts—Fibroblasts from a confluent 10-cm dish were harvested by trypsinization and washed with PBS. Cell pellets were resuspended in distilled water and sonicated three times for 30 s on ice. Protein concentration was determined by the method of Lowry using bovine serum albumin as standard (30). Extraction of fatty acids and analysis by GC-MS were performed as described previously (31).

Statistical Analysis—All data are shown as mean values, and error bars represent the S.D. of at least three independent cell lines per genotype except for data explicitly given for one specific cell line. Comparison of means was performed by one-way analysis of variance followed by Bonferroni's post hoc test or Student's *t* test if suitable.

RESULTS

β-Oxidation of C26:0-CoA Esters Is Defective in Fibroblasts from X-ALD Patients—Primary human fibroblasts are the most widely used model system to study the β-oxidation defect observed in X-ALD and are generally used either as intact cells or as homogenates. In our studies, we chose homogenization of the cells because this technique provides more possibilities to modify experimental conditions (for example acyl-CoA esters can be used directly) in comparison with intact cells.

To address the question of whether free VLCFAs or very long-chain acyl-CoA esters are accepted by ABCD1 in human cells and to clarify the role of a potential synthetase defect in X-ALD, we compared the relative β-oxidation activity for free C26:0 and the respective acyl-CoA ester as substrates. As widely accepted, we found in our system that β-oxidation of hexacosanoic acid (C26:0) is significantly reduced in X-ALD and absent in ZWS patients (Fig. 1*A*).

Degradation of long-chain fatty acids, such as palmitic acid (C16:0), is commonly used for normalization of VLCFA oxidation rates in X-ALD as this function is mainly exerted by mitochondria that are not primarily affected in X-ALD patients. However, in our approach using homogenates instead of intact cells, mitochondrial activity is underrepresented in comparison with peroxisomal activity because the high levels of ATP present in the assay inhibit mitochondrial function. Indeed, we found a slight but not significant reduction of C16:0 β-oxidation in X-ALD fibroblasts, whereas in homogenates from ZWS fibroblasts, a considerable decrease was observed (Fig. 1*B*).

In contrast to free fatty acids, the situation for VLCFA-CoA ester β-oxidation in humans has remained controversial as there exist conflicting data between human cells that show unaffected β-oxidation of VLCFA-CoA esters in fibroblasts from X-ALD patients (32, 33) and more recent work in yeast suggesting defective VLCFA-CoA β-oxidation in the absence of peroxisomal ABC transporters (25). To resolve this discrep-

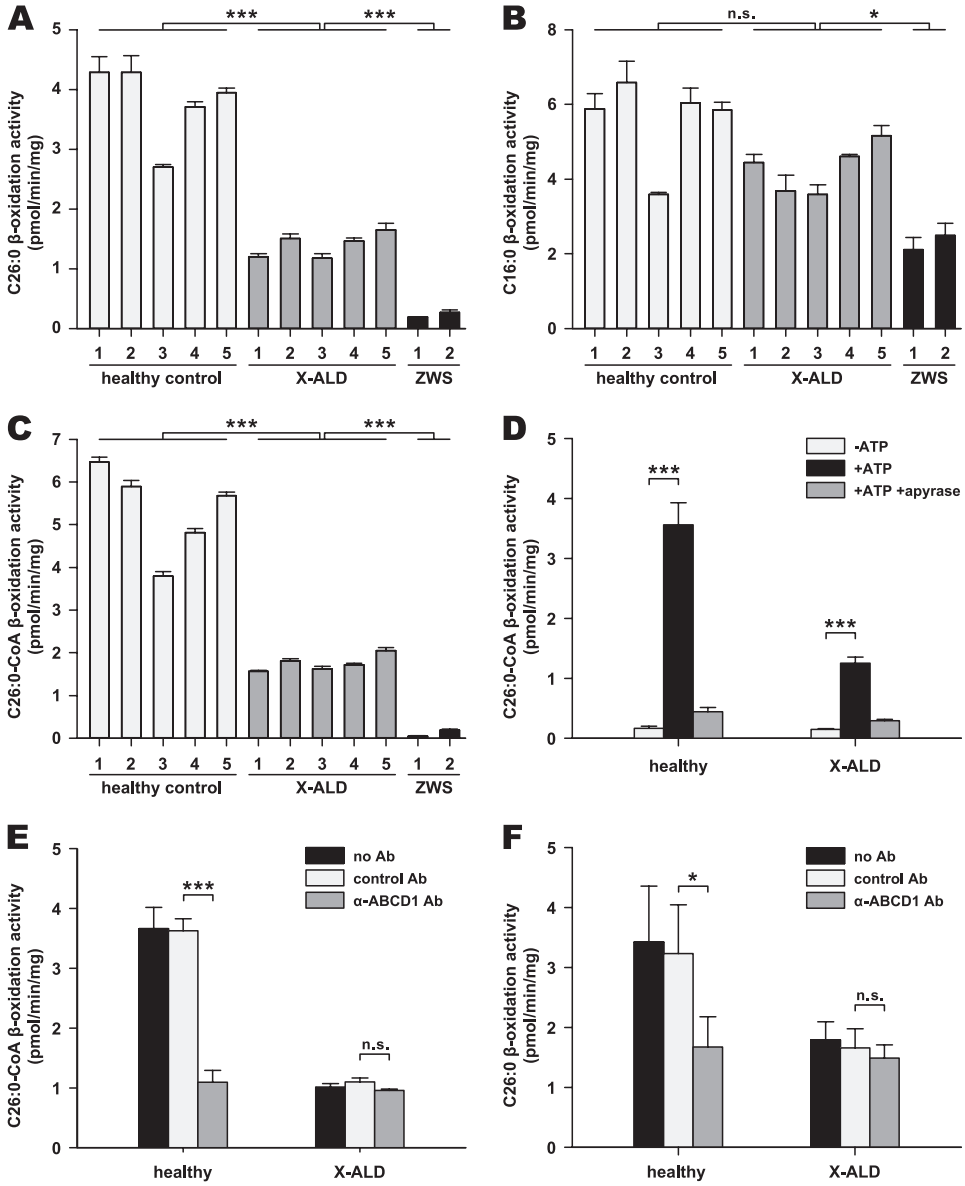

FIGURE 1. **β-Oxidation of free VLCFAs and VLCFA-CoA esters in homogenates from human fibroblasts.** Cultured fibroblasts from healthy controls, X-ALD patients, or ZWS patients were homogenized in isoosmotic buffer, and β-oxidation activities of C26:0 (*A*), C16:0 (*B*), and C26:0-CoA (*C*) were determined in postmitochondrial supernatants. *D*, C26:0-CoA β-oxidation activity of homogenates from healthy and X-ALD fibroblasts was measured in the absence (*light gray*) or presence (*black*) of exogenous ATP and in the presence of ATP after preincubation with apyrase (*dark gray*). *E*, C26:0-CoA β-oxidation activity was measured upon preincubation of homogenates with a specific α-ABCD1 antibody (*Ab*) (*dark gray*), an unspecific antibody (*light gray*), or no addition of antibody (*black*). *F*, C26:0 free fatty acid β-oxidation activity was determined upon antibody inhibition as described in *E*. The rate of β-oxidation is expressed as pmol of labeled acetate released/min/mg of protein of homogenate added. Values given are means ± S.D. (*error bars*) of three measurements in one cell line (*A–C*) or means ± S.D. (*error bars*) of at least three different cell lines (*D–F*) (*, $p < 0.05$; ***, $p < 0.001$; *n.s.*, not significant).

ancy, we carried out β-oxidation of VLCFA-CoA in homogenates of human fibroblasts and for the first time found that the degradation of C26:0-CoA esters in fibroblasts from X-ALD and ZWS patients was decreased to the same extent as degradation of free C26:0. This suggested that the transport of both, free fatty acids and acyl-CoA esters, shares the same molecular defect in X-ALD (Fig. 1*C*).

Very Long-chain Fatty Acids Are Directly Used by ABCD1 as Acyl-coenzyme A Esters—Previous studies showed that the peroxisomal oxidation of free fatty acids depends on ATP, which can be easily explained by the need of a synthetase activity. As this activation step is omitted by the use of C26:0-CoA, the depletion of ATP can be used to test for a different ATP-dependent step, such as substrate transport by ABC transporters. When the β-oxidation activity of acyl-CoA esters was compared in the presence and absence of ATP, we found that β-oxidation in homogenates from controls as well as from X-ALD patients strongly depended on ATP (Fig. 1*D*). Possible indirect effects of nucleotide omittance from the reaction medium were excluded as upon preincubation with apyrase, an ATP-degrading enzyme, in the presence of ATP a similar decrease of β-oxidation activity was found (Fig. 1*D*).

Specific antibodies have been shown previously to be able to block functions carried out by ABC transporters (34). By preincubation with a monoclonal antibody directed against ABCD1 (Euromedex, 1D6) the C26:0-CoA β-oxidation activity of

healthy control samples was decreased to a level surprisingly similar to that in X-ALD patients (Fig. 1*E*). Analogous results were obtained by the use of unesterified C26:0 as substrate for ABCD1 (Fig. 1*F*). This inhibition is specific because a control antibody (α-LAMP2) did not cause an effect and inhibition was dependent on preincubation and concentration of the antibody (data not shown). Similar results, although at higher antibody concentration, were obtained with a different monoclonal antibody (Euromedex, 2B4) (data not shown).

Docosanoic Acid (C22:0) Does Not Accumulate in X-ALD but Is a Substrate for ABCD1—The main biochemical marker used for diagnosis of X-ALD is the accumulation of hexacosanoic acid (C26:0) in plasma and fibroblasts of X-ALD patients, but often the C26:0/C22:0 ratio is given because C22:0 does not accumulate in X-ALD fibroblasts. Based on the structural similarity between C22:0 and C26:0, it can be speculated that C22:0 is as well accepted by ABCD1. To clarify the situation in fibroblasts, we compared accumulation and β-oxidation of C26:0 and C22:0 in fibroblasts from X-ALD and ZWS patients. In both cases, the level of C26:0 was strongly elevated, lignoceric acid (C24:0) showed a moderate increase, and C22:0 remained unaltered (Fig. 2*A*). If the accumulation of VLCFA were a direct consequence of defective β-oxidation, then one would assume that the degradation of C22:0 is unaffected in both diseases. However, it turned out that C22:0-CoA β-oxidation is strongly reduced in fibroblasts from X-ALD patients and essentially absent in ZWS patients, suggesting that β-oxidation of C22:0-CoA is a peroxisomal function and that its transport across the membrane is normally mediated by ABCD1 (Fig. 2*B*). In addition, the observed C22:0-CoA β-oxidation defect in X-ALD was found to be a direct consequence of ABCD1 dysfunction as blocking of ABCD1 function with a specific antibody decreased β-oxidation activity of healthy controls to a level similar to that observed in X-ALD patients (Fig. 2*C*).

Moreover, the apparent β-oxidation rate for C22:0 was ~10-fold higher than for C26:0, suggesting that C22:0 is possibly even a better substrate for ABCD1 than C26:0. Provided that the degradation of C22:0 is defective in fibroblasts derived from X-ALD patients, the observation that C22:0 does not accumulate in X-ALD patients is surprising, and the question of which mechanism prevents the accumulation arises.

ABCD2 Gene Expression in Human Fibroblasts Is Very Low—As the residual peroxisomal β-oxidation activity in X-ALD fibroblasts depends on ATP, the remaining homologous ABC transporters are candidates to account for this activity. We first compared the mRNA levels of the three peroxisomal ABC transporter genes *ABCD1*, *ABCD2*, and *ABCD3* (Fig. 3*A*). The mRNA levels of all three genes in X-ALD as well as in ZWS fibroblasts were comparable with the levels of healthy controls (except for one X-ALD case with clearly lower ABCD1 mRNA amount). When comparing the absolute amounts, the ABCD2 mRNA level was ~300-fold lower than that of ABCD1. In contrast, expression of ABCD3 was found to be ~4-fold higher than that of ABCD1 (Fig. 3*D*). This suggests that the relative amount of ABCD2 is so low that a functional contribution to the observed ATP-dependent β-oxidation activity in X-ALD fibroblasts can be excluded. Thus, ABCD3 becomes the major candidate for this function.

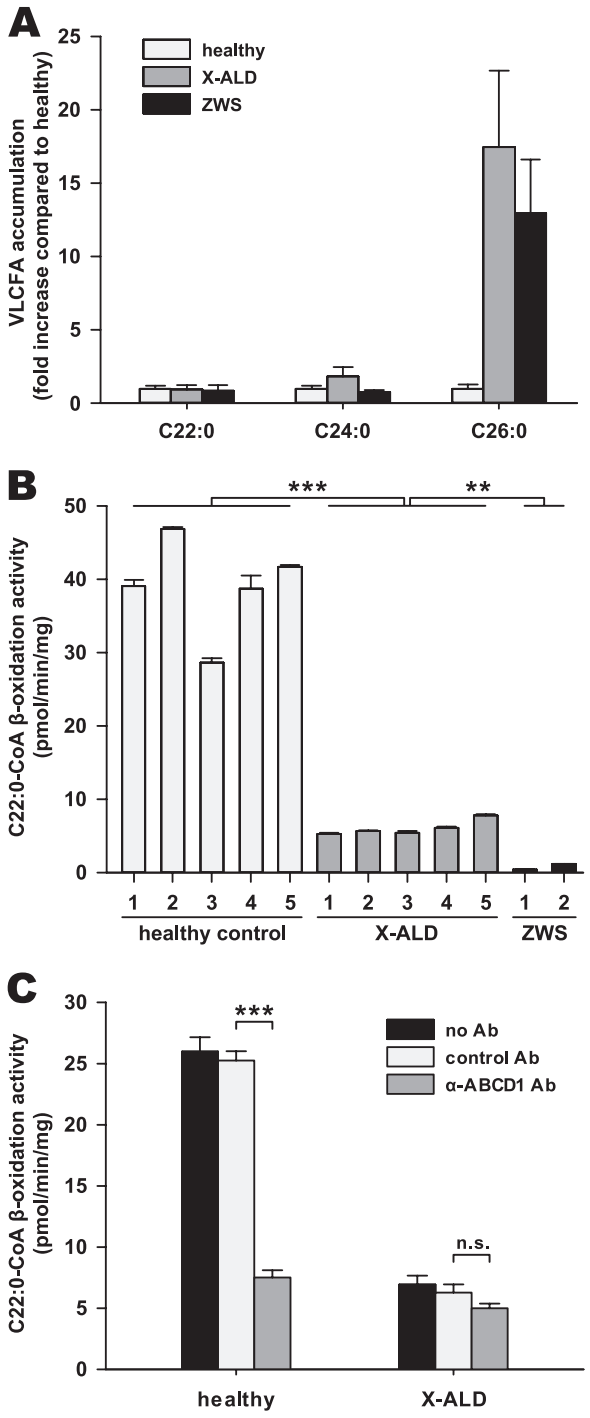

FIGURE 2. **β-Oxidation of C22:0-CoA is defective in X-ALD but does not lead to accumulation of C22:0.** *A*, fibroblasts were grown to confluence, and after extraction and hydrolysis, the levels of C22:0, C24:0, and C26:0 fatty acids were measured by GC-MS and are depicted as -fold increase over healthy control values. Values represent data normalized to the healthy controls and are given as means ± S.D. (*error bars*) of at least three different cell lines. *B*, β-oxidation activities of C22:0-CoA in fibroblast homogenates were determined similarly as described in Fig. 1. *C*, C22:0-CoA β-oxidation was determined upon inhibition of ABCD1 function by preincubation with an α-ABCD1 antibody (*Ab*) (*dark gray*), an unspecific antibody (*light gray*), or no addition of antibody (*black*). Values represent means ± S.D. (*error bars*) of three measurements (**, $p < 0.01$; ***, $p < 0.001$; *n.s.*, not significant).

We next confirmed the relative abundance of ABCD1 and ABCD3 protein levels by direct comparison of protein levels by Western blot analysis (Fig. 3*B*). In contrast to ABCD3, which

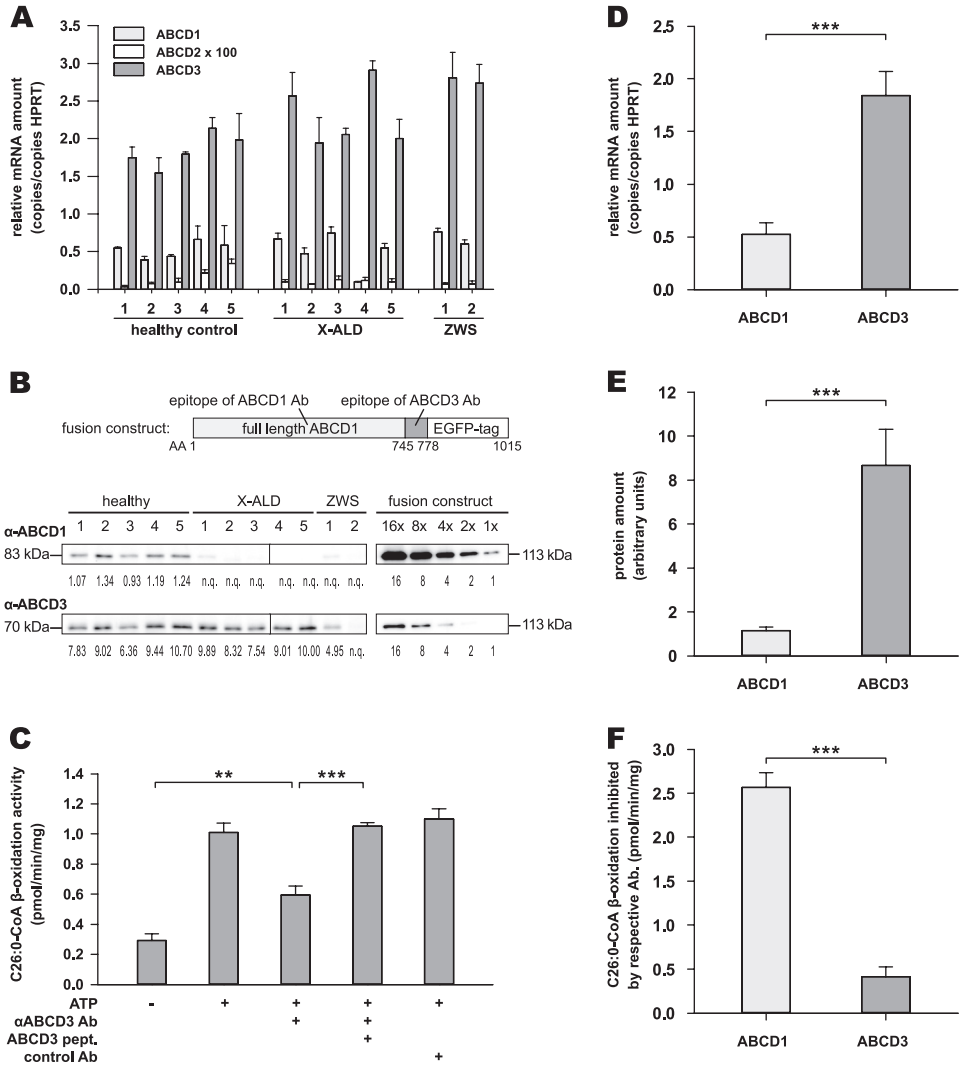

FIGURE 3. **The abundance of the three peroxisomal ABC transporters in human fibroblasts and the relative contribution of ABCD3 to C26:0-CoA degradation.** *A*, mRNA was isolated from cultured human fibroblasts of healthy controls, X-ALD patients, and ZWS patients. The amount of mRNA encoding ABCD1, ABCD2, and ABCD3 was quantified by quantitative RT-PCR and is presented as relative copy number normalized to the housekeeping gene HPRT (means ± S.D. (*error bars*) of three independent RNA preparations). Note that ABCD2 is virtually absent as a different scale was used for ABCD2. *B*, the relative abundance of ABCD1 and ABCD3 at the protein level was determined by comparing the immunoblot signals of ABCD1 and ABCD3 with a fusion protein containing the epitopes recognized by the respective antibodies. The fusion construct consisted of full-length ABCD1 (as the exact epitope of the antibody is not known), the humanized epitope of the α-ABCD3 antibody (*Ab*), and an EGFP tag. Equal amounts of fibroblast homogenates were separated by SDS-PAGE and subjected to Western blot analysis, and the signals of either ABCD1 or ABCD3 were compared with serial dilutions of fusion protein applied in parallel lanes. The indicated values below each panel represent the quantification (arbitrary units) relative to the fusion protein (*n.q.*, not quantified). *C*, the fraction of C26:0-CoA β-oxidation activity mediated by ABCD3 was obtained by measuring C26:0-CoA β-oxidation in homogenates from X-ALD fibroblasts and determining the effects upon addition of ATP (*lane 2*) or preincubation with either α-ABCD3 antibody (*lane 3*) or α-ABCD3 antibody blocked by an excess of the immunizing peptide (*pept*) (*lane 4*). Values represent means ± S.D. (*error bars*) of specific activity of at least three independent cell lines. *D* and *E*, summary of the ABCD1 and ABCD3 mRNA (*D*) and protein (*E*) levels in healthy fibroblasts. Values represent means ± S.D. (*error bars*) of five healthy controls. *F*, the relative contribution of ABCD1 and ABCD3 to C26:0-CoA β-oxidation activity was estimated by determination of the inhibitory effect exerted by α-ABCD1 or α-ABCD3 antibodies. The values shown represent the decrease obtained by inhibition with antibodies as determined in Fig. 1*E* for ABCD1 (in healthy control fibroblasts) or Fig. 3 *C* for ABCD3 (**, $p < 0.01$; ***, $p < 0.001$). *AA*, amino acids.

was present at a similar level in all patient fibroblasts (except one ZWS patient), ABCD1 expression varied from notably decreased (patients 1–3) to below the level of detection (patients 4 and 5) in X-ALD cases.

For a direct comparison of the cellular amounts of ABCD1 and ABCD3, a fusion construct that encoded the epitopes recognized by α-ABCD1 and α-ABCD3 antibodies was generated. Therefore, a peptide sequence encoding the domain of human ABCD3 that is recognized by the α-ABCD3 antibody was fused together with EGFP to the C terminus of full-length human ABCD1 (Fig. 3*B*, *top* element), and this reference protein was used as an internal standard. Quantification of the Western blot signals estimated about 7.5 times higher protein levels of ABCD3 compared with ABCD1 in healthy fibroblasts (Fig. 3*E*).

ABCD3 Can Accept Straight-chain Very Long-chain Fatty Acids but with Lower Efficacy than ABCD1—From the fact that ABCD2 was (nearly) absent and ABCD3 expression was higher than the expression of ABCD1, we supposed that ABCD3 could account for the remaining peroxisomal β-oxidation activity in X-ALD fibroblasts. Because of the lack of fibroblasts with dysfunctional ABCD3, again an antibody was used to interfere with ABCD3 function. In contrast to a control antibody, the

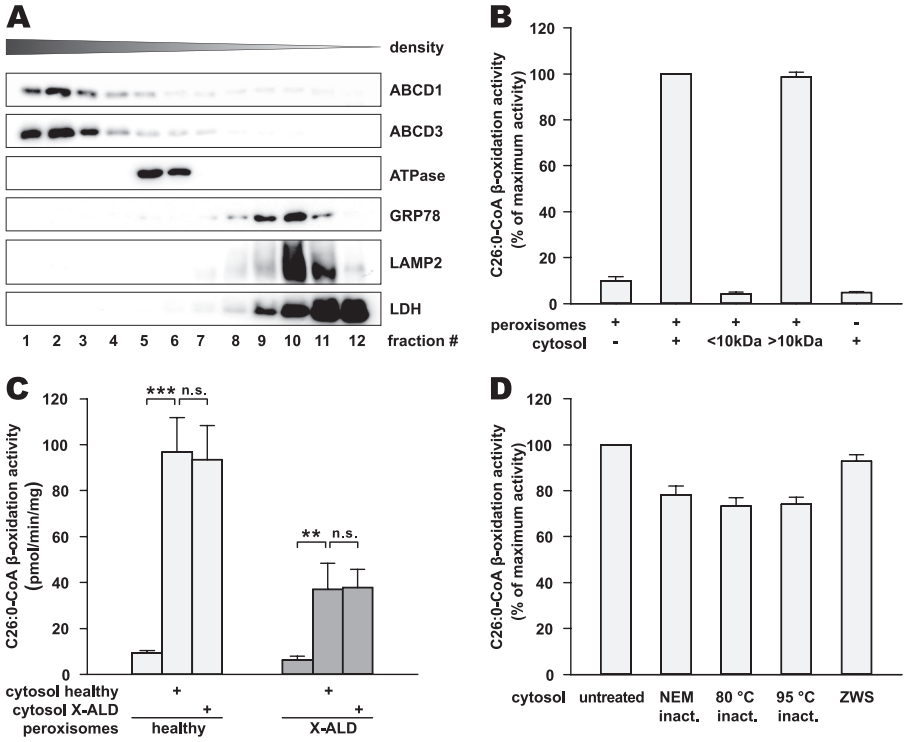

FIGURE 4. **A cytosolic factor is needed for functional β-oxidation of VLCFA in purified peroxisomes.** *A*, fibroblast homogenates were subfractionated by OptiPrep density gradient centrifugation. The distribution of the different organelles in the gradient is shown by Western blot analysis using the respective marker enzymes ABCD1 and ABCD3 (peroxisomes), ATPase (mitochondria), GRP78 (endoplasmic reticulum), LAMP2 (lysosomes), and lactate dehydrogenase (*LDH*) (cytosol). *B*, C26:0-CoA β-oxidation in the peroxisomal fraction (pool of fractions without detectable levels of other marker proteins) of healthy controls in the presence and absence of a cytosolic fraction (supernatant of 200,000 × *g*). Separation of the cytosolic fraction into low (<10-kDa) and high (>10-kDa) molecular mass molecules was carried out by ultrafiltration with a cutoff size of 10 kDa. *C*, peroxisomes and cytosol of either X-ALD or healthy fibroblasts were combined crosswise, and C26:0-CoA β-oxidation rates were determined. *D*, C26:0 β-oxidation was determined in peroxisomal fractions derived from healthy fibroblasts supplemented with cytosolic fractions inactivated (*inact.*) either by NEM or heat treatment for 10 min as indicated. Cytosol derived from ZWS fibroblasts was used to exclude the possibility of leakage of peroxisomal enzymes into the cytosol during homogenization. β-Oxidation rates in the presence of cytosol were taken as reference (100%) for each individual cell line. Values represent means ± S.D. (*error bars*) when comparing at least three different cell lines (**, $p < 0.01$; ***, $p < 0.001$; *n.s.*, not significant).

α-ABCD3 antibody decreased the remaining β-oxidation activity of C26:0-CoA in X-ALD fibroblasts by ~50% (Fig. 3*C*). Although similar inhibitory effects of α-ABCD3 antibody were observed in healthy controls, results are presented for X-ALD fibroblasts because in this genotype involvement of possible ABCD1-ABCD3 heterodimers can be excluded. Moreover, a direct effect of the antibody was confirmed as the inhibition depended on preincubation of the homogenate with the antibody, and the inhibitory effect could be antagonized by blocking of the antibody with the respective immunizing peptide (Fig. 3*C*). However, in contrast to the higher amount of protein, the contribution of ABCD3-dependent C26:0-CoA β-oxidation was estimated to be 6-fold lower than the contribution of ABCD1-dependent β-oxidation activity (Fig. 3*F*).

A Cytosolic Factor Is Needed for VLCFA β-Oxidation in Isolated Peroxisomes—To exclude that additional non-peroxisomal factors modulate the defect in X-ALD fibroblasts, we aimed at determining β-oxidation rates in isolated peroxisomes. Purified peroxisomes were prepared by OptiPrep density gradient fractionation (modified from Ref. 27). As shown by Western blot analysis, peroxisomes (marker proteins, ABCD1 and ABCD3) were well separated from mitochondria (ATPase), endoplasmic reticulum (GRP78), lysosomes (LAMP2), and the cytosol (lactate dehydrogenase) (Fig. 4*A*). Unexpectedly, purified peroxisomes alone exhibited only low C26:0-CoA β-oxidation activity, but when a cytosolic fraction (200,000 × *g* supernatant of homogenates) was added back, activity was restored (Fig. 4*B*). In contrast, the cytosolic fraction showed no β-oxidation activity by itself. For discrimination of small (*e.g.* cofactors) and high molecular mass factors (like proteins or lipid complexes), the cytosol was separated by ultrafiltration through filter devices with a molecular cutoff of 10 kDa. The responsible factor was located in the retentate fraction, suggesting a macromolecule (>10kDa) to be responsible for activity recovery (Fig. 4*B*). This allowed us to compare the β-oxidation activity of the peroxisomal fractions from healthy and X-ALD patient fibroblasts and to test for possible additional effects mediated by cytosolic factors derived from either healthy or X-ALD fibroblasts. Upon crosswise combination of the factors, we found that the β-oxidation defect was clearly mediated by the peroxisomal and not by cytosolic fractions, which boosted the activities to the same extent regardless of genotype (Fig. 4*C*). The fact that a cytosolic factor from ZWS fibroblasts could substitute as well excluded the possibility that some part of peroxisomes leaked into the cytosol during homogenization. Furthermore, treatment of the cytosol with *N*-ethylmaleimide (NEM) or heat (80 or 95 °C) before complementation showed only a modest effect, suggesting that the putative cytosolic factor is not an enzymatic activity (such as for example a synthetase) (Fig. 4*D*).

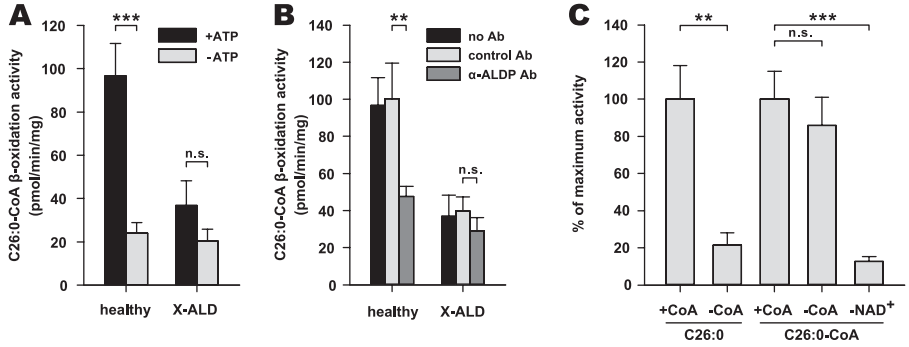

FIGURE 5. **Degradation of C26:0-CoA is independent of an additional synthetase activity.** Peroxisomal fractions derived from either healthy or X-ALD fibroblasts were supplemented with NEM-inactivated cytosolic fractions lacking cofactors of less than 10 kDa, and C26:0-CoA β-oxidation activity was determined in the presence (*black*) or absence (*gray*) of ATP (*A*) and upon preincubation with either an α-ABCD1 antibody (*Ab*) (*dark gray*), an unspecific antibody (*light gray*), or no addition of antibody (*black*) (*B*). *C*, a peroxisomal fraction derived from healthy fibroblasts was supplemented with an NEM-inactivated cytosolic fraction lacking cofactors of less than 10 kDa, and β-oxidation activities of free C26:0 fatty acids or the respective C26:0-CoA esters were determined in the presence or absence of CoA. Dependence on NAD^+ was used as a control for the permeability of the peroxisomal membrane for cofactors. β-Oxidation rates in the presence of all cofactors were set as 100% for C26:0 and C26:0-CoA, respectively. Values represent means ± S.D. (*error bars*) of at least three different cell lines (**, $p < 0.01$; ***, $p < 0.001$; *n.s.*, not significant).

Hexacosanoyl-CoA Degradation (C26:0) Is Independent of Additional ACSVL Activity—In contrast to whole cell homogenates in which a variety of metabolic processes and cofactors could potentially influence β-oxidation results, isolated peroxisomes in combination with an NEM-inactivated cytosolic fraction provide an excellent opportunity to investigate peroxisomal processes in the absence of mitochondria and other non-peroxisomal enzymatic activities. Similar to homogenates, β-oxidation activity for C26:0-CoA of isolated peroxisomes from fibroblasts of healthy and X-ALD patients was found to depend on ATP (Fig. 5*A*). This suggests that either an ABC transporter, a pathway including a thioesterase and following reactivation step, or both of these pathways could be involved in the degradation of C26:0-CoA. Furthermore, the direct involvement of the ABC transporter ABCD1 was supported by the use of the α-ABCD1 antibody, which reduced the C26:0-CoA β-oxidation activity of control peroxisomes to approximately the level seen in peroxisomes isolated from X-ALD fibroblasts (Fig. 5*B*).

In light of recent work in yeast that suggests an additional intraperoxisomal activation step after the import of fatty acids into peroxisomes, it was of particular interest to clarify the role of a possible intraperoxisomal reactivation step in human fibroblasts. As no suitable inhibitor for ACSVL activity was available and ATP could not be omitted because of its contribution to transport, the dependence on additional free CoA was used as measure of the need for a synthetase. In contrast to the degradation of free C26:0 that as expected required CoA for activation, the β-oxidation of C26:0-CoA was independent of exogenously added CoA (Fig. 5*C*). In addition, β-oxidation was found to strictly depend on additional NAD^+ (Fig. 5*C*), demonstrating that the peroxisomal membrane is permeable to cofactors under the assay conditions used. Therefore, a dedicated CoA pool within peroxisomes can be excluded. Finally, endogenous supply of CoA from the added cytosol can be excluded based on the lack of low molecular mass components that had been removed beforehand from the added cytosolic fraction by ultrafiltration.

DISCUSSION

Here, we show for the first time in primary fibroblasts from human patients that not only is degradation of free very long-chain fatty acids impaired in X-ALD but also that of very long-chain acyl-CoA esters. Similar conclusions were recently drawn from heterologous expression of the human ABCD transporters in yeast (25). However, the question of why conflicting results were obtained by previous studies in human fibroblasts (32, 33) is raised. The fact that no defects in acyl-CoA β-oxidation were found in fibroblasts from X-ALD patients before could be due to different experimental conditions used in previous work. Examples include the lack of osmoprotection during homogenization, leading to disintegration of peroxisomes (33); the use of KCN, which is intended to inhibit mitochondria but probably affects ABC transporters as well (32); the omission of ATP (35); or different methods for solubilization of fatty acids.

To exclude a non-peroxisomal factor that could contribute to β-oxidation differences, such as competing enzymatic activities from other organelles or endogenous cofactors, we established a modified isolation and assay procedure. This allowed us to determine β-oxidation from isolated peroxisomes with a high recovery rate of β-oxidation activity and a higher specific activity compared with descriptions in previous reports (35). We found that freshly isolated peroxisomes showed only very low β-oxidation activity, but when a cytosolic fraction lacking other membranous structures, such as mitochondria or microsomes, was supplemented, full activity was recovered. Judging by the size of the cytosolic factor, a protein, like for example fatty acid-binding protein, could be accommodated.

In addition, the β-oxidation defect caused by mutations in the *ABCD1* gene is reflected by blocking ABCD1 function in isolated peroxisomes with a specific antibody that exerts its inhibitory action in the course of the assay. The similar extent of these effects proves a direct contribution of ABCD1 to peroxisomal β-oxidation and excludes possible secondary mechanisms, such as long term effects of ABCD1 absence or a different leakiness of peroxisomes during isolation.

It was suggested from yeast experiments that human ABC transporters are capable of mediating C22:0 transport into peroxisomes (36). However, in humans, in contrast to yeast, β-oxidation of non-VLCFAs is carried out in mitochondria, implying differences between yeast and man especially as no accumulation of C22:0 can be found in X-ALD patients. Indeed, the finding that C22:0 β-oxidation is defective in X-ALD and absent in ZWS suggests that mitochondria are not involved in C22:0 degradation and that ABCD1 is responsible for the import of C22:0 into peroxisomes. Thus, different mechanisms, such as fatty acid elongation and ω-oxidation, must be considered as the explanation for the lack of C22:0 accumulation in X-ALD fibroblasts as well as in patients (3, 37, 38).

Before cloning of the gene responsible for X-ALD, a deficiency of an ACSVL was suspected to be the primary defect in X-ALD. This assumption was primarily based on the finding that the degradation of free VLCFAs was impaired in contrast to degradation of the respective acyl-CoA esters, which appeared to be unaffected (32, 33). Using our model, we found a clear difference in C26:0-CoA β-oxidation between healthy and X-ALD fibroblasts, rendering the main argument for an involvement of an ACSVL activity unconvincing.

Interestingly, recent work in yeast suggests that acyl-CoAs are hydrolyzed and again re-esterified in the peroxisomal lumen during the transport of acyl-CoAs by the respective yeast (25) and plant (26) ABC transporters, and a similar pathway was proposed for mammalian peroxisomes. However, in mammals, the situation for peroxisomal β-oxidation might be different because in contrast to yeast and plants the β-oxidation of non-VLCFAs is located in mitochondria instead of peroxisomes. In addition, the question of whether any of the mammalian ACSVLs is located in the peroxisomal lumen or only adhering to the outside of peroxisomal membranes is still a matter of debate (39–42). In particular for ACSVL5 (SLC27A4; FATP4), which was found to account for the majority of ACSVL activity in fibroblasts, accepted peroxisomal targeting signals are missing as well as clear indications for intraperoxisomal localization of the protein (40, 43).

As no feasible inhibitor for ACSVL activity was available, we targeted this question by testing β-oxidation of C26:0-CoA for CoA dependence. By using purified peroxisomes in combination with NEM-inactivated high molecular mass cytosolic factors, non-peroxisomal ACSVL activities as well as influences of endogenously present CoA were excluded. Indeed, β-oxidation of acyl-CoA was found to be independent of additional CoA, whereas β-oxidation of free fatty acids relied on additional free CoA, suggesting that no additional synthetase either inside or outside of the peroxisome is needed for β-oxidation of very long-chain acyl-CoA esters in humans. The possible presence of a particular peroxisomal CoA pool seems unlikely because in contrast to the substrates of β-oxidation, which are most likely present in the assay medium as larger complexes with their respective binding proteins, cofactors such as CoA or NAD^+ can reach the peroxisomal lumen under assay conditions as peroxisomal membranes are considered permeable (44–47).

For the treatment of X-ALD patients, pharmacological induction of the remaining peroxisomal ABC transporters, especially ABCD2, has been proposed (14–16, 48, 49). However, the remaining endogenous transporters are obviously not able to compensate for defective ABCD1 most likely because of different expression levels of the three ABC transporters in different tissues (50–52).

We provide evidence that ABCD2 is virtually absent in human fibroblasts and therefore most probably does not contribute to the peroxisomal β-oxidation in this cell type. In combination with a relatively high ABCD1 to ABCD3 ratio, this finding explains why fibroblasts show a much more pronounced deficit in VLCFA β-oxidation activity when compared with for example tissues from Abcd1 knock-out mice (53).

In line with these expression results, we provide evidence that endogenous ABCD3, not ABCD2 as suspected previously, contributes to the remaining degradation of VLCFAs in human fibroblasts. It seems likely that this activity is mediated by ABCD3 homodimers and not by ABCD1-ABCD3 heterodimers as ABCD3-dependent activity was present even in fibroblasts without detectable ABCD1 protein. Nonetheless, it remains to be resolved whether VLCFAs can be directly used by ABCD3 or only after modification by other pathways, such as for example ω-oxidation.

In conclusion, with a novel assay system that allows the use of highly purified peroxisomes, we found that β-oxidation of very long chain acyl-CoA esters directly depends on ABCD1 without the need for additional re-esterification by a synthetase. Furthermore, we provide the first description of a C22:0-CoA β-oxidation defect in X-ALD fibroblasts. Finally, we demonstrated that ABCD3 is responsible for most of the residual VLCFA β-oxidation in X-ALD fibroblasts.

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REFERENCES

1. Bezman, L., Moser, A. B., Raymond, G. V., Rinaldo, P., Watkins, P. A., Smith, K. D., Kass, N. E., and Moser, H. W. (2001) Adrenoleukodystrophy: incidence, new mutation rate, and results of extended family screening. *Ann. Neurol.* **49,** 512–517
2. Ferrer, I., Aubourg, P., and Pujol, A. (2010) General aspects and neuropathology of X-linked adrenoleukodystrophy. *Brain Pathol.* **20,** 817–830
3. Kemp, S., Berger, J., and Aubourg, P. (2012) X-linked adrenoleukodystrophy: clinical, metabolic, genetic and pathophysiological aspects. *Biochim. Biophys. Acta* **1822,** 1465–1474
4. Moser, A. B., and Moser, H. W. (1999) The prenatal diagnosis of X-linked adrenoleukodystrophy. *Prenat. Diagn.* **19,** 46–48
5. Schutgens, R. B., Bouman, I. W., Nijenhuis, A. A., Wanders, R. J., and Frumau, M. E. (1993) Profiles of very-long-chain fatty acids in plasma, fibroblasts, and blood cells in Zellweger syndrome, X-linked adrenoleukodystrophy, and rhizomelic chondrodysplasia punctata. *Clin. Chem.* **39,** 1632–1637
6. Wanders, R. J., van Roermund, C. W., van Wijland, M. J., Schutgens, R. B., Schram, A. W., Tager, J. M., van den Bosch, H., and Schalkwijk, C. (1988) X-linked adrenoleukodystrophy: identification of the primary defect at the level of a deficient peroxisomal very long chain fatty acyl-CoA synthetase using a newly developed method for the isolation of peroxisomes from skin fibroblasts. *J. Inherit. Metab. Dis.* **11,** 173–177
7. Singh, H., Derwas, N., and Poulos, A. (1987) Beta-oxidation of very-long-chain fatty acids and their coenzyme A derivatives by human skin fibro-

blasts. *Arch. Biochem. Biophys.* **254,** 526–533
8. Mosser, J., Douar, A. M., Sarde, C. O., Kioschis, P., Feil, R., Moser, H., Poustka, A. M., Mandel, J. L., and Aubourg, P. (1993) Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature* **361,** 726–730
9. Holzinger, A., Kammerer, S., Berger, J., and Roscher, A. A. (1997) cDNA cloning and mRNA expression of the human adrenoleukodystrophy related protein (ALDRP), a peroxisomal ABC transporter. *Biochem. Biophys. Res. Commun.* **239,** 261–264
10. Lombard-Platet, G., Savary, S., Sarde, C. O., Mandel, J. L., and Chimini, G. (1996) A close relative of the adrenoleukodystrophy (ALD) gene codes for a peroxisomal protein with a specific expression pattern. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 1265–1269
11. Kamijo, K., Taketani, S., Yokota, S., Osumi, T., and Hashimoto, T. (1990) The 70-kDa peroxisomal membrane protein is a member of the Mdr (P-glycoprotein)-related ATP-binding protein superfamily. *J. Biol. Chem.* **265,** 4534–4540
12. Coelho, D., Kim, J. C., Miousse, I. R., Fung, S., du Moulin, M., Buers, I., Suormala, T., Burda, P., Frapolli, M., Stucki, M., Nürnberg, P., Thiele, H., Robenek, H., Höhne, W., Longo, N., Pasquali, M., Mengel, E., Watkins, D., Shoubridge, E. A., Majewski, J., Rosenblatt, D. S., Fowler, B., Rutsch, F., and Baumgartner, M. R. (2012) Mutations in ABCD4 cause a new inborn error of vitamin B12 metabolism. *Nat. Genet.* **44,** 1152–1155
13. Kashiwayama, Y., Seki, M., Yasui, A., Murasaki, Y., Morita, M., Yamashita, Y., Sakaguchi, M., Tanaka, Y., and Imanaka, T. (2009) 70-kDa peroxisomal membrane protein related protein (P70R/ABCD4) localizes to endoplasmic reticulum not peroxisomes, and NH_2-terminal hydrophobic property determines the subcellular localization of ABC subfamily D proteins. *Exp. Cell Res.* **315,** 190–205
14. Netik, A., Forss-Petter, S., Holzinger, A., Molzer, B., Unterrainer, G., and Berger, J. (1999) Adrenoleukodystrophy-related protein can compensate functionally for adrenoleukodystrophy protein deficiency (X-ALD): implications for therapy. *Hum. Mol. Genet.* **8,** 907–913
15. Kemp, S., Wei, H. M., Lu, J. F., Braiterman, L. T., McGuinness, M. C., Moser, A. B., Watkins, P. A., and Smith, K. D. (1998) Gene redundancy and pharmacological gene therapy: implications for X-linked adrenoleukodystrophy. *Nat. Med.* **4,** 1261–1268
16. Braiterman, L. T., Zheng, S., Watkins, P. A., Geraghty, M. T., Johnson, G., McGuinness, M. C., Moser, A. B., and Smith, K. D. (1998) Suppression of peroxisomal membrane protein defects by peroxisomal ATP binding cassette (ABC) proteins. *Hum. Mol. Genet.* **7,** 239–247
17. Morita, M., and Imanaka, T. (2012) Peroxisomal ABC transporters: structure, function and role in disease. *Biochim. Biophys. Acta* **1822,** 1387–1396
18. Liu, L. X., Janvier, K., Berteaux-Lecellier, V., Cartier, N., Benarous, R., and Aubourg, P. (1999) Homo- and heterodimerization of peroxisomal ATP-binding cassette half-transporters. *J. Biol. Chem.* **274,** 32738–32743
19. Hillebrand, M., Verrier, S. E., Ohlenbusch, A., Schäfer, A., Söling, H. D., Wouters, F. S., and Gärtner, J. (2007) Live cell FRET microscopy: homo- and heterodimerization of two human peroxisomal ABC transporters, the adrenoleukodystrophy protein (ALDP, ABCD1) and PMP70 (ABCD3). *J. Biol. Chem.* **282,** 26997–27005
20. Guimarães, C. P., Domingues, P., Aubourg, P., Fouquet, F., Pujol, A., Jimenez-Sanchez, G., Sá-Miranda, C., and Azevedo, J. E. (2004) Mouse liver PMP70 and ALDP: homomeric interactions prevail *in vivo*. *Biochim. Biophys. Acta* **1689,** 235–243
21. Hinz, A., and Tampé, R. (2012) ABC transporters and immunity: mechanism of self-defense. *Biochemistry* **51,** 4981–4989
22. Shani, N., Sapag, A., Watkins, P. A., and Valle, D. (1996) An *S. cerevisiae* peroxisomal transporter, orthologous to the human adrenoleukodystrophy protein, appears to be a heterodimer of two half ABC transporters: Pxa1p and Pxa2p. *Ann. N.Y. Acad. Sci.* **804,** 770–772
23. Hettema, E. H., van Roermund, C. W., Distel, B., van den Berg, M., Vilela, C., Rodrigues-Pousada, C., Wanders, R. J., and Tabak, H. F. (1996) The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*. *EMBO J.* **15,** 3813–3822
24. van Roermund, C. W., Visser, W. F., Ijlst, L., van Cruchten, A., Boek, M., Kulik, W., Waterham, H. R., and Wanders, R. J. (2008) The human peroxisomal ABC half transporter ALDP functions as a homodimer and accepts acyl-CoA esters. *FASEB J.* **22,** 4201–4208
25. van Roermund, C. W., Ijlst, L., Majczak, W., Waterham, H. R., Folkerts, H., Wanders, R. J., and Hellingwerf, K. J. (2012) Peroxisomal fatty acid uptake mechanism in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **287,** 20144–20153
26. De Marcos Lousa, C., van Roermund, C. W., Postis, V. L., Dietrich, D., Kerr, I. D., Wanders, R. J., Baldwin, S. A., Baker, A., and Theodoulou, F. L. (2013) Intrinsic acyl-CoA thioesterase activity of a peroxisomal ATP binding cassette transporter is required for transport and metabolism of fatty acids. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 1279–1284
27. Sandhir, R., Khan, M., Chahal, A., and Singh, I. (1998) Localization of nervonic acid β-oxidation in human and rodent peroxisomes: impaired oxidation in Zellweger syndrome and X-linked adrenoleukodystrophy. *J. Lipid Res.* **39,** 2161–2171
28. Kunze, M., Neuberger, G., Maurer-Stroh, S., Ma, J., Eck, T., Braverman, N., Schmid, J. A., Eisenhaber, F., and Berger, J. (2011) Structural requirements for interaction of peroxisomal targeting signal 2 and its receptor PEX7. *J. Biol. Chem.* **286,** 45048–45062
29. Watkins, P. A., Ferrell, E. V., Jr., Pedersen, J. I., and Hoefler, G. (1991) Peroxisomal fatty acid β-oxidation in HepG2 cells. *Arch. Biochem. Biophys.* **289,** 329–336
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275
31. Unterberger, U., Regelsberger, G., Sundt, R., Bernheimer, H., and Voigtländer, T. (2007) Diagnosis of X-linked adrenoleukodystrophy in blood leukocytes. *Clin. Biochem.* **40,** 1037–1044
32. Wanders, R. J., van Roermund, C. W., van Wijland, M. J., Nijenhuis, A. A., Tromp, A., Schutgens, R. B., Brouwer-Kelder, E. M., Schram, A. W., Tager, J. M., and van den Bosch, H. (1987) X-linked adrenoleukodystrophy-defective peroxisomal oxidation of very long-chain fatty-acids but not of very long-chain fatty acyl-coA esters. *Clin. Chim. Acta* **165,** 321–329
33. Hashmi, M., Stanley, W., and Singh, I. (1986) Lignoceroyl-CoASH ligase: enzyme defect in fatty acid β-oxidation system in X-linked childhood adrenoleukodystrophy. *FEBS Lett.* **196,** 247–250
34. Hipfner, D. R., Mao, Q., Qiu, W., Leslie, E. M., Gao, M., Deeley, R. G., and Cole, S. P. (1999) Monoclonal antibodies that inhibit the transport function of the 190-kDa multidrug resistance protein, MRP. Localization of their epitopes to the nucleotide-binding domains of the protein. *J. Biol. Chem.* **274,** 15420–15426
35. Lazo, O., Contreras, M., Hashmi, M., Stanley, W., Irazu, C., and Singh, I. (1988) Peroxisomal lignoceroyl-CoA ligase deficiency in childhood adrenoleukodystrophy and adrenomyeloneuropathy. *Proc. Natl. Acad. Sci. U.S.A.* **85,** 7647–7651
36. van Roermund, C. W., Visser, W. F., Ijlst, L., Waterham, H. R., and Wanders, R. J. (2011) Differential substrate specificities of human ABCD1 and ABCD2 in peroxisomal fatty acid β-oxidation. *Biochim. Biophys. Acta* **1811,** 148–152
37. Ofman, R., Dijkstra, I. M., van Roermund, C. W., Burger, N., Turkenburg, M., van Cruchten, A., van Engen, C. E., Wanders, R. J., and Kemp, S. (2010) The role of ELOVL1 in very long-chain fatty acid homeostasis and X-linked adrenoleukodystrophy. *EMBO Mol. Med.* **2,** 90–97
38. Sanders, R. J., Ofman, R., Valianpour, F., Kemp, S., and Wanders, R. J. (2005) Evidence for two enzymatic pathways for ω-oxidation of docosanoic acid in rat liver microsomes. *J. Lipid Res.* **46,** 1001–1008
39. Steinberg, S. J., Kemp, S., Braiterman, L. T., and Watkins, P. A. (1999) Role of very-long-chain acyl-coenzyme A synthetase in X-linked adrenoleukodystrophy. *Ann. Neurol.* **46,** 409–412
40. Watkins, P. A., and Ellis, J. M. (2012) Peroxisomal acyl-CoA synthetases. *Biochim. Biophys. Acta* **1822,** 1411–1420
41. Smith, B. T., Sengupta, T. K., and Singh, I. (2000) Intraperoxisomal localization of very-long-chain fatty acyl-CoA synthetase: implication in X-adrenoleukodystrophy. *Exp. Cell Res.* **254,** 309–320
42. Watkins, P. A. (2008) Very-long-chain acyl-CoA synthetases. *J. Biol. Chem.* **283,** 1773–1777
43. Jia, Z., Moulson, C. L., Pei, Z., Miner, J. H., and Watkins, P. A. (2007) Fatty acid transport protein 4 is the principal very long chain fatty acyl-CoA synthetase in skin fibroblasts. *J. Biol. Chem.* **282,** 20573–20583

44. Antonenkov, V. D., and Hiltunen, J. K. (2012) Transfer of metabolites across the peroxisomal membrane. *Biochim. Biophys. Acta* **1822,** 1374–1386
45. Agrimi, G., Russo, A., Scarcia, P., and Palmieri, F. (2012) The human gene SLC25A17 encodes a peroxisomal transporter of coenzyme A, FAD and NAD^+. *Biochem. J.* **443,** 241–247
46. Visser, W. F., van Roermund, C. W., Ijlst, L., Waterham, H. R., and Wanders, R. J. (2007) Metabolite transport across the peroxisomal membrane. *Biochem. J.* **401,** 365–375
47. Antonenkov, V. D., Rokka, A., Sormunen, R. T., Benz, R., and Hiltunen, J. K. (2005) Solute traffic across mammalian peroxisomal membrane—single channel conductance monitoring reveals pore-forming activities in peroxisomes. *Cell. Mol. Life Sci.* **62,** 2886–2895
48. Berger, J., Pujol, A., Aubourg, P., and Forss-Petter, S. (2010) Current and future pharmacological treatment strategies in X-linked adrenoleukodystrophy. *Brain Pathol.* **20,** 845–856
49. Flavigny, E., Sanhaj, A., Aubourg, P., and Cartier, N. (1999) Retroviral-mediated adrenoleukodystrophy-related gene transfer corrects very long chain fatty acid metabolism in adrenoleukodystrophy fibroblasts: implications for therapy. *FEBS Lett.* **448,** 261–264
50. Berger, J., Albet, S., Bentejac, M., Netik, A., Holzinger, A., Roscher, A. A., Bugaut, M., and Forss-Petter, S. (1999) The four murine peroxisomal ABC-transporter genes differ in constitutive, inducible and developmental expression. *Eur. J. Biochem.* **265,** 719–727
51. Langmann, T., Mauerer, R., Zahn, A., Moehle, C., Probst, M., Stremmel, W., and Schmitz, G. (2003) Real-time reverse transcription-PCR expression profiling of the complete human ATP-binding cassette transporter superfamily in various tissues. *Clin. chem.* **49,** 230–238
52. Troffer-Charlier, N., Doerflinger, N., Metzger, E., Fouquet, F., Mandel, J. L., and Aubourg, P. (1998) Mirror expression of adrenoleukodystrophy and adrenoleukodystrophy related genes in mouse tissues and human cell lines. *Eur. J. Cell Biol.* **75,** 254–264
53. McGuinness, M. C., Lu, J. F., Zhang, H. P., Dong, G. X., Heinzer, A. K., Watkins, P. A., Powers, J., and Smith, K. D. (2003) Role of ALDP (ABCD1) and mitochondria in X-linked adrenoleukodystrophy. *Mol. Cell. Biol.* **23,** 744–753