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A novel cell model to study the function of the adrenoleukodystrophy-related protein ☆

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Abstract

X-linked adrenoleukodystrophy (X-ALD) is a neurodegenerative disorder due to mutations in the *ABCD1* (*ALD*) gene. ALDRP, the closest homolog of ALDP, has been shown to have partial functional redundancy with ALDP and, when overexpressed, can compensate for the loss-of-function of ALDP. In order to characterize the function of ALDRP and to understand the phenomenon of gene redundancy, we have developed a novel system that allows the controlled expression of the ALDRP-EGFP fusion protein (normal or non-functional mutated ALDRP) using the Tet-On system in H4IIEC3 rat hepatoma cells. The generated stable cell lines express negligible levels of endogenous ALDRP and doxycycline dosage-dependent levels of normal or mutated ALDRP. Importantly, the ALDRP-EGFP protein is targeted correctly to peroxisome and is functional. The obtained cell lines will be an indispensable tool in our further studies aimed at the resolution of the function of ALDRP to characterize its potential substrates in a natural context.

Keywords: Adrenoleukodystrophy; *ABCD2* (*ALDR*); Peroxisome; H4IIEC3; Tet-On; ABC transporters

X-linked adrenoleukodystrophy (X-ALD, OMIM 300100) is the most frequent peroxisomal disorder [1]. This neurodegenerative disease, characterized by the accumulation of very long-chain fatty acids (VLCFA) in plasma and tissues, is due to mutations in the *ABCD1* (ALD) gene located in Xq28 [2]. *ABCD1* encodes a peroxisomal member of the ATP-binding cassette (ABC) family called ALDP, which is thought to participate in the entry of VLCFA into the peroxisome, the unique site of their β-oxidation. ABC transporters form a large family of transmembrane proteins that transport diverse substrates across membranes thanks to binding and hydrolysis of ATP [3,4]. ALDP is a half-transporter, which is supposed to function as a homo- or heterodimer, in association with one of the three other peroxisomal ABC half-transporters: ALDRP (adrenoleukodystrophy-related protein) [5,6], PMP70 (70 kDa-peroxisomal membrane protein) [7], and PMP69 [8]. These transporters are encoded by the *ABCD2* (*ALDR*), *ABCD3*, and *ABCD4* genes, respectively, and their function is still unclear. Overexpression of ALDRP has been demonstrated to compensate for ALDP deficiency in *Abcd1* null mice, thus preventing VLCFA accumulation and the onset of a neurological phenotype [9]. Furthermore, restoration of VLCFA β-oxidation could be obtained in X-ALD human fibroblasts transfected with

☆ *Abbreviations:* ABC, ATP-binding cassette; ACBP, acyl-CoA-binding protein; ALDR, adrenoleukodystrophy-related; EGFP, enhanced green fluorescent protein; FABP, fatty acid binding protein; VLCFA, very long-chain fatty acids; X-ALD, X-linked adrenoleukodystrophy.

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Abcd2 cDNA [6,10–14]. These data suggest a partial overlap in the substrate specificity of the peroxisomal ABC transporters. Even if homodimerization seems to prevail in the liver of rodents [15], heterodimerization cannot be excluded. Peroxisomal ABC transporters have overlapping but distinct expression patterns [5,16]. A mirror expression is often observed between ALDP and ALDRP when specific cell types are analyzed [17]. However, heterodimerization has been demonstrated in vitro [18,19]. Based on the model of transport of pigment precursors in *Drosophila* [20], differences in the relative expression level of each peroxisomal ABC transporter in a single tissue (or single cell type) or under various stimulations could lead to alternative dimerization and consequently to a change in the substrate specificity. ALDRP is the closest homolog of ALDP and its pharmacological induction could represent an alternative therapeutic strategy for X-ALD [11,21]. Whether it is with the aim of better understanding X-ALD or using *Abcd2* as therapeutic target, it is of crucial importance to decipher the exact function of ALDRP and the nature of the partial functional redundancy between ALDP and ALDRP.

Based on the hypothesis that peroxisomal ABC transporters catalyze the transport of specific lipid substrates, it is probable that their access to the substrates strictly depends on the interactions with intracellular fatty acid transporters (fatty acid-binding proteins (FABPs) [22], acyl-CoA-binding proteins (ACBP) [23]), and/or with acyl-CoA synthetases which catalyze an obligatory step before the oxidation reactions. Two synthetases have been found in the peroxisome: VLCS-1, which is constitutively associated with the peroxisomal membrane and is specific for VLCFA [24], and ACS-4, which is transiently associated with the peroxisomal membrane and is specialized in medium or long-chain fatty acid activation [25]. Moreover, dealing with a peroxisomal membrane protein whose membrane insertion depends on a complex mechanism involving at least two peroxins, PEX19 and PEX3, and on the recognition of an internal signal sequence [26,27], we abandoned the idea to use reconstituted lipid vesicles to study the function of ALDRP and chose to create a cell line stably overexpressing a normal or mutated rat ALDRP fused to EGFP in a controlled manner using the Tet-On system [28]. This system allows expression of the reverse tetracycline transactivator (rtTA), which is able to activate a promoter containing a tetracycline response element (TRE) upon binding of doxycycline. This system was shown to yield the highest induced expression levels in comparison with other inducible expression system [29]. The EGFP tag will facilitate the detection of the overexpressed ALDRP and the discrimination between endogenous and exogenous ALDRP. The cell model will allow studying the function of ALDRP in its "natural context" where the putative cytoplasmic and peroxisomal partners, which should be necessary for the function, are present. In this paper, we describe the generation and characterization of the cell clones, and discuss the importance of using this cell model.

Materials and methods

Plasmid constructs. The rat *Abcd2* cDNA cloned in pcDNA3.1/Zeo(+) [6] was amplified to generate *Bam*HI and *Nco*I ends with the following primers: (**ggatcc**ATGATA CACATGCTAAATGCAGC) and (**ccatgg** AT G TGT CCT CTG CAG TTT GC). The *EGFP* cDNA, obtained by *Nco*I–*Not*I digestion of pIRES2-EGFP (Clontech), was fused in-frame to the 3′ end of the rat *Abcd2* cDNA. The fusion did not result in the presence of additional amino acids since the stop codon of *Abcd2* was replaced by the ATG codon of *EGFP*. We expect that the fusion at the C-terminal end does not disrupt the signal of peroxisomal targeting situated in an internal peptide of the N-terminal part of the protein [27,30]. The fused construct was directionally cloned (*Bam*HI/*Not*I) in the pTRE2-Hyg vector (Clontech) for the second step of transfection in the creation of the cell model. Site directed mutagenesis was performed on the ALDRP-EGFP-pTRE2hyg to obtain the mutated construct D207H-ALDRP-EGFP-pTRE2hyg (GAC codon 619 switched to CAC). This mutation in ALDRP, located in a region highly conserved between ALDRP and ALDP, corresponds to the D194H mutation in ALDP, a mutation found in X-ALD patients (see www.x-ald.nl), which disrupts the function without alteration of the peroxisomal localization and probably of the dimerization ability [30]. Both constructs were cloned in the same way in the high expression level constitutive eukaryotic expression vector pcDNA3.1/Zeo (+) (Invitrogen).

Cell culture and transfections. H4IIEC3 rat hepatoma cells were cultured in DMEM/HAM-F12 (1:1) supplemented with 5% FBS at 37 °C in a humidified atmosphere of 5% CO_2. Control fibroblasts and fibroblasts of X-ALD patients were cultured as described previously [31].

To generate H4IIEC3 stably transfected cells, 10^7 cells were resuspended in 400 μL DMEM with 30 μg of the plasmid of interest and electroporated at 230 V and 1800 μF (single pulse) with an EasyjecT Plus Electroporator (Eurogentec). Cells were resuspended into 10 mL of media immediately after electroporation. Cell viability was assayed using trypan blue (Invitrogen) and 200,000 cells were plated in a 6-well plate (NunclonTM). Forty-eight hours after electroporation, the selection agent, G418 (Clontech) or Hygromycin B (Clontech), was added to a final concentration of 400 or 800 μg/mL, respectively. Clones were isolated after 2 weeks, transferred into 24-well plates, and clonally expanded before freezing in 10% DMSO (Sigma) containing media. Stable clones were maintained in the presence of 200 μg/mL G418 (and 400 μg/mL of Hyg) to avoid the loss of the transfected DNA.

Transient transfection of H4IIEC3 cells to validate the ALDRP-EGFP constructs was performed using Exgen500 Reagent (Euromedex, France) following manufacturer's instructions. Ten thousand of H4IIEC3 cells were plated on Lab-Tek chamber slides (pre-coated with gelatine 0.1%). Cells were transiently co-transfected with normal or mutated ALDRP-EGFP-pcDNA3.1/Zeo(+) and the peroxisomal localization vector pDs-Red2-Peroxi (Clontech). After 24 h, cells were washed 3 times with phosphate-buffered saline (PBS, Sigma) and fixed with 2% paraformaldehyde (Sigma) for 15 min. Three washes in PBS were done prior to adding Fluorsave (Merck) between the slide and the cover glass.

Transient transfection of X-ALD fibroblasts with pcDNA3.1/Zeo(+) empty or containing the normal or mutated ALDRP-EGFP cDNA was performed using AMAXA Nucleofector Device and Human Dermal Fibroblast Nucleofector Kit according to the manufacturer's optimized protocol.

Identification of Tet-On and ALDRP-EGFP cell clones. G418-resistant H4IIEC3 cell clones were transiently co-transfected with the reporter plasmid pTRE2hyg-Luc (Clontech) and pCMVβgal (Clontech) using Exgen500 Reagent (Euromedex, France) following manufacturer's instructions. Luciferase activity was measured after incubation with or without doxycycline and corrected for transfection efficiency using β-galactosidase activity as described previously [14]. The clone 13 Tet-On H4IIEC3 displaying the highest inducibility and the lowest background expression was finally selected.

Hygromycin-resistant cell clones were selected by flow cytometry analysis after treatment with doxycycline and fixation in 4% paraformaldehyde. Responsive clones were incubated in the presence of 2 μg/ml

doxycycline for 12 h and sterile-sorted in several rounds by fluorescence-activated cell sorting (FACS) to select the cells with the highest expression of the EGFP fusion protein. For flow cytometry experiments, the cells were grown in 24-well culture plates, treated with doxycycline, and fixed in 4% paraformaldehyde. Ten thousand cells were counted each time in a single experiment. Flow cytometry analysis and cell sorting were performed using an Epics Elite ESP flow cytometer (Beckman Coulter). An air-cooled argon laser operating at 20 mW was used for excitation at 488 nm. Forward scatter (FCS) at linear amplification was used as the threshold parameter to reduce electronic and small particle noise. Signal fluorescence from EGFP was collected using DL 500 nm and bandpass 525 nm set filters. Sorting was carried out through a 100 μm nozzle at a flow rate of no more than 800 cells/s. Flow cytometry results were analyzed using WinMDI software.

Immunoprecipitation and Western blotting. Cells were grown in 25 cm^2 culture flasks to 80% confluence, washed two times with PBS, and incubated on ice with the lysis buffer (1% Triton X-100, 100 mM NaCl, 10 mM EDTA, and 100 mM Tris–HCl, pH 8, supplemented with 1% PMSF, complete protease inhibitor cocktail (Roche)) for 30 min. Nuclei were removed by centrifugation for 10 min at 4 °C at 1000*g*. Supernatants from each flask were used in immunoprecipitation with agarose-conjugated anti-GFP beads (MBL) according to the manufacturer's instructions. Proteins were resolved on a 7.5% SDS–PAGE. After electrophoresis, the proteins were transferred onto a PVDF membrane, probed with a 1/1000 diluted mouse anti-GFP antibody (Roche; 0.4 mg/ml) and consequently with 1/2000 diluted rabbit anti-mouse antibody coupled to HRP (Santa Cruz Biotechnology, USA), and revealed by ECL (Santa Cruz Biotechnology, USA).

Confocal microscopy. Lab-Tek slides containing ALDRP-EGFP cell clones or transiently transfected H4IIEC3 were allowed to dry in the dark for 12 h before observation using a TCS SP2 AOBS confocal laser microscope (Leica Microsystemes SA) and a 40× oil-immersion objective. EGFP fluorescence and DsRed fluorescence were detected at 520 and 572 nm after excitation at 488 and 543 nm, respectively. Images were recorded from a cross-section through the cells and analyzed with the Leica Confocal software.

In vitro translation. In vitro translation was performed using the TnT rabbit reticulocyte T7 expression Kit (Promega) in the presence of L[^{35}S]methionine (Amersham) according to the manufacturer's instructions. One microgram of the ALDRP, ALDRP-EGFP, or D207H-ALDRP-EGFP pcDNA3.1/Zeo(+) constructs was used as template in a final volume of 50 μl. The in vitro translation products were resolved by 10% polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by autoradiography.

Northern blot analysis and quantitative real-time RT-PCR. Total RNA was extracted from H4IIEC3 cells as described previously [14]. For Q-RT-PCR, they were extracted from transfected H4IIEC3 cells treated (or not) with doxycycline using RNeasy Kit (Qiagen).

The *Abcd1-4* cDNA probes, similar in length and in G + C%, were generated by PCR from rat cDNA using the following primers : 5′-TGAC CAAGCCACTCCTGG-3′ and 5′-CACATATTTCATGAGGAACTG-3′ for *Abcd1*, 5′-GTGTATGCCACTGCTAAAGT-3′ and 5′-TCAGCTC CAGAGGCCAGT-3′ for *Abcd2*, 5′-ATGGCGGCCTTCAGCAAG-3′ and 5′-CCAGAGAGATAAGAGGCATG-3′ for *Abcd3*, and 5′-TGAG CTGGAGGAAGGACC-3′ and 5′-CATTCATCTGAATCTGCAT-3′ for *Abcd4*. Probes were labeled by random priming using the Hexalabel Kit (Fermentas) with [α-^{32}P]dATP (Amersham) and purified on G50 Sephadex micro-columns. Membranes containing 20 μg/lane of total RNA were hybridized in Church buffer. To allow comparison, specific activity of each probe was adjusted; hybridization and autoradiography were performed in the same conditions.

Quantitative analysis of *Abcd2* expression, normalized to the phosphoriboprotein *36B4*, was performed using the SYBR Green I real-time PCR technology using an iQ real-time PCR detection system (Bio-Rad). The primers 5′-CAGCGTCCACCTCTACCACATAG-3′ (rat *Abcd2* sense), 5′-CGTCCAGCAATGCGTACTTCG-3′ (rat *Abcd2* antisense), 5′-GGCATCACCACTAAAATCTCC-3′ (*36B4* sense), and 5′-GCTCC CACCTTGTCTCCA-3′ (*36B4* antisense) were chosen using the Beacon Designer Software (Bio-Rad). Two microgram of total RNA was treated with RQ1 DNase (Promega) and reverse transcribed using M-MLV Reverse transcriptase (Promega) and 250 ng of random hexanucleotides following the standard protocol. PCRs were carried out in duplicate in a final volume of 25 μl containing 12.5 μl of SYBR Green qPCR Mastermix (Eurogentec), 1 mM EDTA, 300 nM of sense and antisense primers and 1 μl cDNA. The amplification was conducted as follows: *Taq* DNA polymerase activation for one cycle of 10 min at 95 °C, then 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s followed by a melting curve analysis to control the absence of non-specific products. For each transcript, the amplification efficiency was determined by the slope of the standard curve generated from 2-fold serial dilutions of cDNA. Relative quantification of transcripts in comparison with the *36B4* reference gene was calculated using the Pfaffl mathematical model which takes the differences in efficiency into account [32].

β-Oxidation analysis and VLCFA analysis. Fatty acid β-oxidation in cultured fibroblasts expressing ALDRP-EGFP or not was determined as described [33]. Assays contained 100 μg of protein along with 50 μM [1-^{14}C]cerotic acid (C26:0). Duplicate or triplicate reactions were measured for each sample and activity was expressed as pmol of radiolabeled water-soluble products plus CO_2 as produced in one hour per milligram of protein.

Results and discussion

Choice of the cell type

The major aim of this study was to provide a novel tool to study the function of ALDRP. Peroxisomal ABC transporters have been hypothesized to function as lipid importers. The idea behind this work was to reconstitute a functional system to check whether some specific lipids can be transported by ALDRP. Because this translocation is probably strictly dependent on the presence of other cellular proteins in charge of the cellular transport of fatty acids (cytoplasmic ACBP, FABP) and the proteins in charge of their specific activation (ACS), we chose to generate a cell model overexpressing a normal or a mutated ALDRP-EGFP fusion protein under the tight control of doxycycline. First, it was necessary to choose a suitable cell type in which ALDRP would be virtually absent, but should be completely functional if it was present. Liver is considered as the main organ for peroxisomal lipid metabolism. ALDRP expression has been demonstrated to be induced in vivo in the liver of rodents upon treatment with peroxisome proliferators (PPs) [14] suggesting its active participation in peroxisomal lipid metabolism. We then chose the rat hepatoma H4IIEC3 cell line, which presents the advantages of expressing all the peroxisomal β-oxidation enzymes and responding to classical peroxisome proliferators (PPs) [34], suggesting that all the functional parameters of peroxisomal lipid metabolism are present. As shown by Northern blotting, H4IIEC3 cells do not express demonstrable levels of ALDRP, while the three other peroxisomal ABC transporters are present (Fig. 1). Thus, this cell line offers a good reference with regard to the cells expressing the ALDRP-fusion proteins under the control of a doxycycline treatment.

Establishment of the Tet-On H4IIEC3 cell line

The first step of the strategy consisted in the generation of a stable transfectant cell line expressing rtTA. H4IIEC3

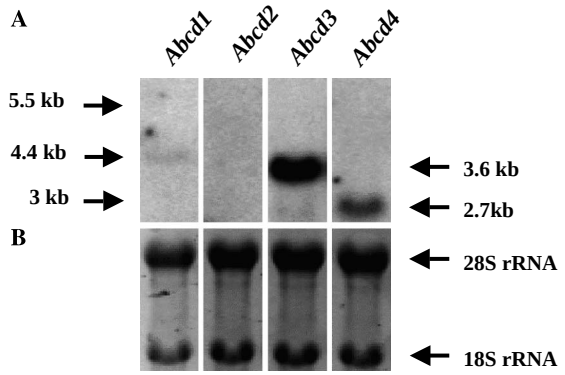

Fig. 1. Northern blot analysis of the *Abcd* subfamily mRNAs in H4IIEC3 cells. (A) Total RNA (20 μg/lane) from H4IIEC3 cells was probed using cDNA probes, similar in length, (G + C) content, and [^{32}P]cpm, under the same conditions of hybridization and autoradiography. The size of the transcripts is indicated (4.4 kb for *Abcd1*, 3 and 5.5 kb for *Abcd2*, 3.3 kb for *Abcd3*, and 2.7 kb for *Abcd4*). (B) Ethidium bromide-stained gel prior to blotting showing rRNA was used as an index of loading.

cells were electroporated with the pTet-On plasmid (Clontech) and then clonally selected in the presence of 400 μg/ml G418. G418-resistant cells, whose growth and morphological aspect were normal, were transiently transfected with the reporter plasmid pTRE2hyg-Luc and cultured in the absence or presence of 1 μg/mL doxycycline. After 48 h, cells were lysed and luciferase activity was assessed and normalized to the transfection efficiency. One of the clones (clone 13), showing the strongest induction of the luciferase activity in response to doxycycline and the lowest leakiness (activity in the absence of doxycycline), was selected and further studied. A dose-dependent induction was observed for doxycycline concentrations ranging between 0.1 and 2 μg/mL (Fig. 2A). Using 1 μg/mL doxycycline, the maximum increase in the luciferase activity (16-fold) was reached after 9 h of treatment (Fig. 2B).

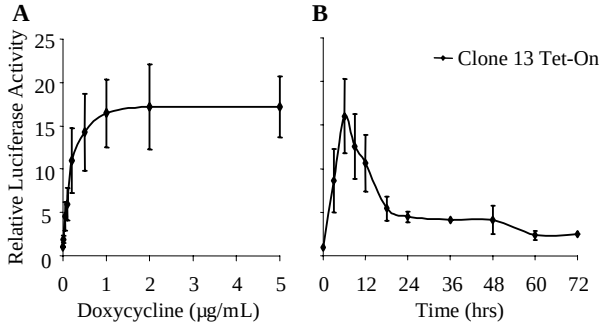

Fig. 2. Characterization of a positive Tet-On clone. The clone 13 was transfected with pTRE2hyg-Luc and incubated for 9 h with various doses of doxycycline (0, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 μg/mL) (A) or incubated for various times (0, 3, 6, 9, 12, 18, 24, 36, 48, 60, and 72 h) with 1 μg/mL doxycycline (B). Luciferase activity was corrected for transfection efficiency by measuring β-galactosidase activity. Results are presented as fold induction ± SE in relation to luciferase activity of untreated cells.

Validation of the constructs

The second step of the strategy consisted in obtaining a stable transfectant cell line from the Tet-On H4IIEC3 clone 13 expressing a normal or a mutated ALDRP fused to EGFP. The constructs were generated (as described under Materials and methods) in two versions: pTRE2-Hyg for the transfection experiment and in pcDNA3.1/Zeo(+) for validation experiments. We first checked by in vitro translation whether the ALDRP and ALDRP-EGFP fusion proteins were correctly synthesized. A band at 110 kDa, which corresponds to the combined sizes of ALDRP (83.3 kDa), and EGFP (27.4 kDa), was observed for both constructs (Fig. 3A). On the autoradiogram, a band with a lower molecular weight was present in all the lanes (59 kDa for ALDRP and 87 kDa for the fusion proteins). This might be an in vitro artifact corresponding to an internal translation initiation site at position 643 that eliminates the first 214 amino acids.

Since the fusion proteins are correctly expressed in vitro, we then tested whether the EGFP tag did not alter the peroxisomal targeting of the fusion proteins. H4IIEC3 cells were transiently co-transfected with the ALDRP-EGFP constructs and with the subcellular localization vector pDS-Red2-Peroxi. Confocal microscopic analysis demonstrated colocalization of the red and green signals indicating that the ALDRP-EGFP fusion proteins are correctly targeted to the peroxisome (Fig. 3B). This result was confirmed by immunofluorescence showing the colocalization of the fusion proteins with endogenous peroxisomal proteins (PMP70 and PMP22) (data not shown). It is noteworthy that in a few cases, we observed an increase in the labeling intensity and a decrease in the number of spots suggesting aggregation events (data not shown).

Previously, it has been demonstrated that transfection of X-ALD fibroblasts with *Abcd2* results in partial complementation of the ALDP defect: the restoration of VLCFA β-oxidation and decrease of the VLCFA content [6,10–14]. To investigate the functional impact of the EGFP tag on the capability of ALDRP to complement ALDP function, we transfected X-ALD fibroblasts with the normal ALDRP-EGFP constructs. As expected, the C26:0 β-oxidation level in X-ALD fibroblasts was found to be strongly reduced in relation to control fibroblasts [33]. Transient transfection with the ALDRP-EGFP resulted in a 1.9-fold increase of C26:0 β-oxidation while no effect was observed in the vector-transfected cells (Fig. 4). If transfection efficiency is taken into account (no more than 40% of cells were expressing ALDRP-EGFP), we can conclude that C26:0 β-oxidation level is normalized in transfected cells and therefore that the EGFP tag does not alter the functionality of ALDRP.

Identification of double transfectant clones

The H4IIEC3 Tet-on cell clone (clone 13) was transfected by electroporation either with ALDRP-EGFP-pTRE2hyg

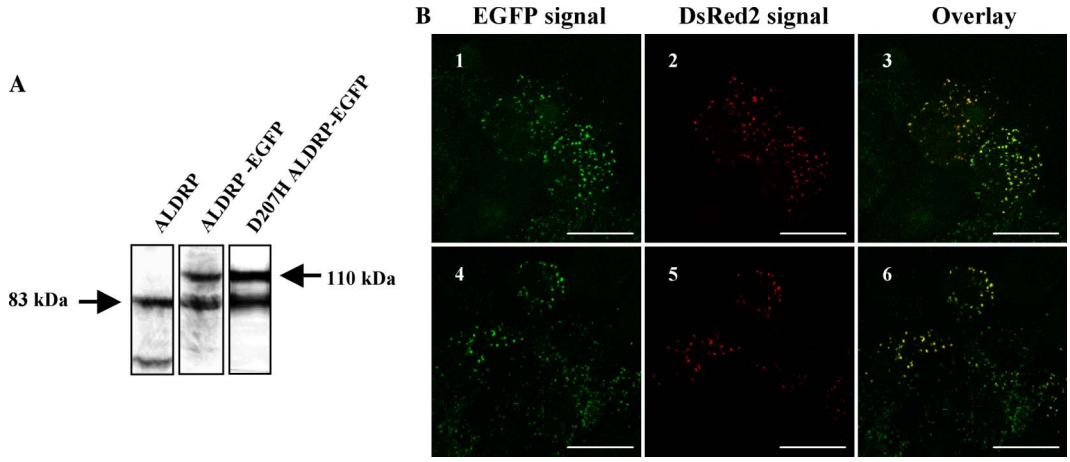

Fig. 3. Analysis of the expression and of the subcellular localization of the normal and mutated (D207H) ALDRP-EGFP. (A) In vitro translation of the ALDRP, ALDRP-EGFP, or D207H-ALDRP-EGFP pcDNA3.1/Zeo(+) constructs using L[^{35}S]methionine. After SDS–PAGE, the proteins were transferred onto a PVDF membrane and visualized by autoradiography. (B) Confocal microscopy analysis showing colocalization between EGFP and DsRed2 signals in H4IIEC3 cells transiently co-transfected with normal (1, 2, and 3) or mutated (4, 5, and 6) ALDRP-EGFP-pcDNA3.1/Zeo(+) and the peroxisomal localization vector pDsRed2-Peroxi. The bar represents 20 μm.

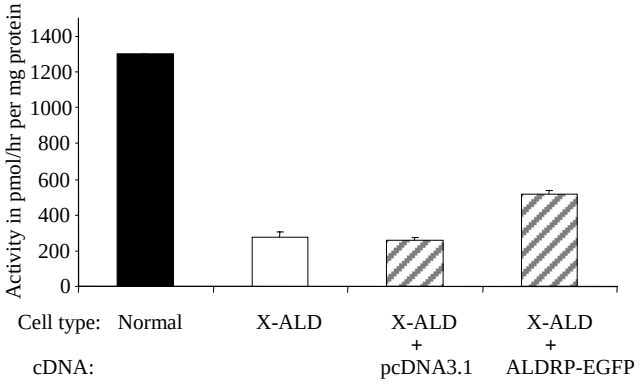

Fig. 4. ALDRP-EGFP complementation of C26:0 β-oxidation. Human X-ALD fibroblasts transformed with SV40 T antigen were transfected with the expression vector pcDNA3.1/Zeo(+) alone or containing the ALDRP-EGFP cDNA (hatched bars). The transfection efficiency estimated by immunofluorescence was about 40%. Triplicate measurements were done for each transfection ($n = 10$).

or with D207H-ALDRP-EGFP-pTRE2hyg. After 15 days of selection with 800 μg/ml Hygromycin, about 30 clones for each construct were isolated and amplified before analysis for their ability to express the fusion protein in response to doxycycline. The most responsive clones expressing normal (clone 28) or mutated (clone 19) ALDRP-EGFP were selected. Successive flow cytometry analyses spanning several weeks demonstrated a progressive decrease in doxycycline response, suggesting the presence of a phenomenon of epigenetic inhibition (data not shown). This repression mechanism is frequently encountered within Tet-regulated stable transfectant systems [35]. Although clonal, the cell population showed a weak percentage of responsive cells as observed by confocal microscopy. The percentage of responsive cells was considerably increased (up to 50%) after three consecutive sorting experiments following doxycycline treatment (data not shown). Both clones were then frozen and further studied. Immunoprecipitation followed by a Western blot analysis of the cell clones with the GFP specific antibody demonstrated a strong increase in the expression of the fusion proteins after doxycycline treatment (Fig. 5A). A faint signal was observed in untreated cells showing that even in the absence of doxycycline, there is a weak leakiness of the system. This phenomenon already observed in cells transiently transfected with pTRE2hyg-Luc (see above) is probably associated with traces of antibiotics in the FBS. The use of "Tet system approved" FBS may improve the induction window by reducing the background expression. The Western blot results were supported by flow cytometric analysis showing the increase in EGFP fluorescence of the double-transfected cells upon doxycycline induction (Fig. 5B). Finally, confocal microscopic analysis demonstrated that, whereas untreated cells did not show any peroxisomal labeling, a strong punctuate labeling reminiscent of peroxisomes was observed in the cells treated with 2 μg/ml doxycycline for 24 h (Fig. 5C). Taken together, the results indicate that the clones 28 and 19 express ALDRP-EGFP and D207H-ALDRP-EGFP, respectively, at a high level in the peroxisome upon doxycycline treatment.

To quantify the expression of the fusion proteins and to evaluate the level of the induction, quantitative RT-PCR analysis was performed on the two cell clones cultivated for different times in the absence or in the presence of varying doses of doxycycline. Dose-dependent induction was observed for both cell clones (Fig. 6A). Maximum level of induction (9-fold for the ALDRP-EGFP mRNA and 15-fold for D207H-ALDRP-EGFP mRNA) was reached with 2 μg/ml doxycycline. The time course of induction was also evaluated (Fig. 6B). Expression of the fusion proteins mRNA was gradually enhanced and reached maximum after 12 h. As expected, no induction was observed in the H4IIEC3 control cells under doxycycline treatment (data not shown).

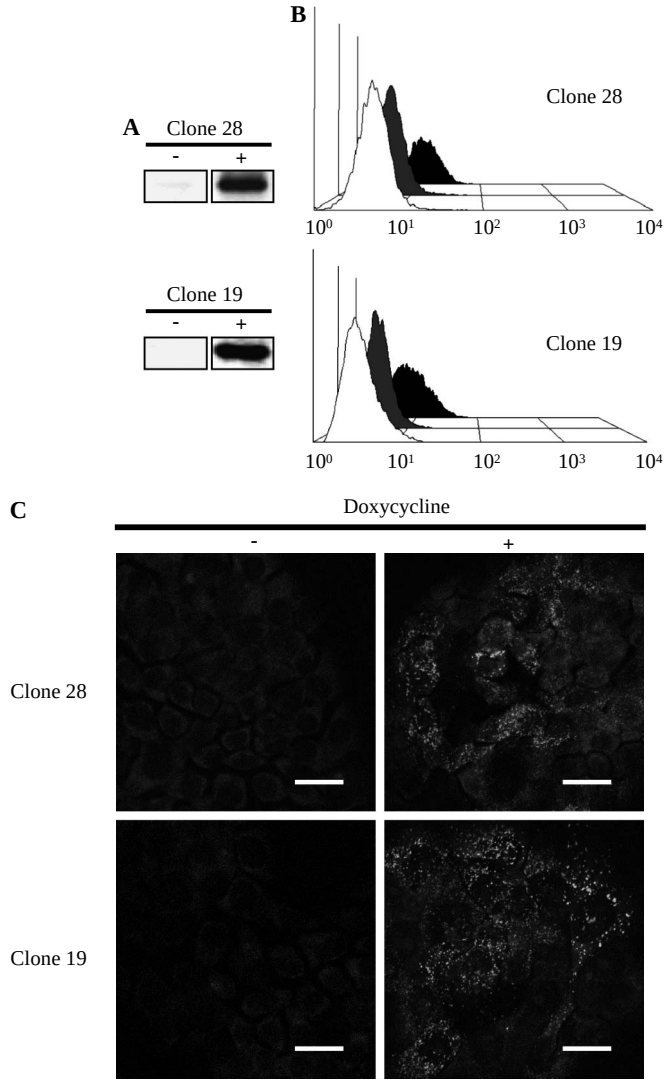

Fig. 5. Characterization of the H4IIEC3-TetOn cell clones expressing a normal (clone 28) or mutated (D207H, clone 19) ALDRP-EGFP fusion protein upon doxycycline treatment. (A) Western blot analysis of the H4IIEC3-TetOn ALDRP-EGFP cell clones: "+," induced with doxycycline (2 μg/ml 24 h); "−," no doxycycline. Cytoplasmic cellular extracts of the cells were used in immunoprecipitation using anti-GFP beads as described under Materials and methods. The proteins were resolved on a 7% SDS–PAGE and transferred onto PVDF membrane, probed with an anti-GFP antibody, and visualized by ECL. (B) Flow cytometry analysis of normal or mutated ALDRP-EGFP-expressing cell clones. Ten thousand cells were counted after culturing in the absence (filled gray) or in the presence (filled black) of doxycycline (2 μg/ml) for 24 h. The open black histogram represents untransfected H4IIEC3 cells. *Y* axis—relative cell number, *X* axis—EGFP fluorescence intensity. (C) Expression of the fusion proteins in the cell clones upon doxycycline treatment as visualized by confocal microscopy: Cells were incubated for 24 h with normal medium (minus) or with doxycycline-supplemented medium (2 μg/ml) and analyzed by confocal microscopy for EGFP fluorescence. The typical peroxisomal staining pattern can be observed only in induced cells. The bar represents 20 μm.

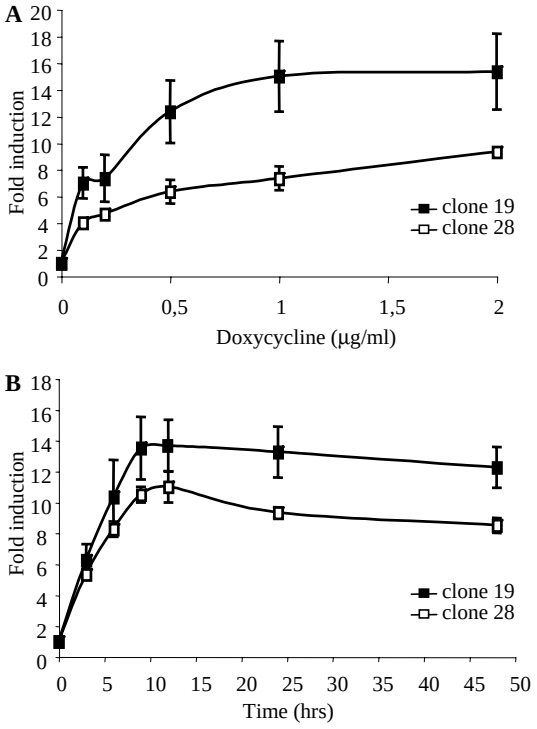

Fig. 6. Quantification of the relative expression levels of mRNA of the fusion proteins by quantitative real-time RT-PCR with primers against *Abcd2* in the H4IIEC3-TetOn cell clones expressing normal ALDRP-EGFP protein—clone 28 (open rectangles) and mutated (D207H) ALDRP-EGFP protein—clone 19 (filled rectangles). (A) Cells were incubated for 12 h with various doses of doxycycline (0.1, 0.2, 0.5, 1, and 2 μg/mL); or (B) incubated for various times (3, 6, 9, 12, 24, and 48 h) with 2 μg/mL doxycycline. Results are presented as fold induction in relation to *Abcd2* mRNA expression of untreated cells. Expression levels were normalized to *36B4* (see Materials and methods). Data are mean values, and standard errors are calculated for 3–4 reactions per group. The H4IIEC3 non-transfected cells show virtually no expression of *Abcd2* mRNA. The experiments in (A) and (B) have been performed independently.

Potential use of the obtained cell clones

In summary, we have generated two cell clones expressing the normal (clone 28) or mutated (clone 19) ALDRP-EGFP fusion proteins under the control of doxycycline. The normal fusion protein has been demonstrated to be fully functional since the biochemical defect in X-ALD fibroblasts is restored by transfection of the construct. Thus, incubation of the cell clones with radiolabeled lipids, measurement of the β-oxidation level, and of the peroxisomal import of these lipids after purification of the peroxisomes and subsequent lipid analysis might shed light on the function of ALDRP. The peroxisomal import of labeled lipids and β-oxidation activity will be compared in both cell clones and in control cells upon varying doses of doxycycline. The inducibility of the expression will permit us to directly correlate the lipid results to the expression level of the ALDRP-EGFP fusion protein. Moreover, by allowing changes in the relative abundance of ALDRP-EGFP towards the other peroxisomal ABC transporters, which should consequently modify the possible dimerization states, the system may lead to relevant observations concerning the substrate specificity. Candidate lipid substrates will be chosen using the following criteria: the lipids must be known to be imported into peroxisomes, must be structurally related to the

putative substrates of ALDP (saturated or mono-unsaturated VLCFA), and must be abundant in the tissues where ALDRP is highly expressed (brain, adrenals, and adipose tissue). For instance, we plan to test in priority docosahexaenoic acid (DHA, C22:6 n-3) and its immediate precursor, tetracosahexaenoic acid (C24:6 n-3). DHA is a major fatty acid in the brain and is β-oxidized preferentially in the peroxisome [36]. Moreover, the last step of its biosynthesis results from the peroxisomal import of tetracosahexaenoic acid either as free acid, CoA ester, or in some other form, and of its subsequent β-oxidation [37]. Knowledge of the biochemical features of the *Abcd2* transgenic mice [38] or the *Abcd2*−/− mice [38] should also help in the choice of candidate substrates for this functional study of ALDRP. Obviously, the interpretation of these models with regard to the function of ALDRP will be made delicate by the existence of mechanisms of compensation associated to the presence of the other peroxisomal ABC transporters, in particular ALDP. However, it will be interesting to look carefully at the transport and oxidation of cerotic acid (C26:0) since cerotic acid accumulation (the phenotype of ALDP deficiency) has clearly been observed in dorsal root ganglions of *Abcd2*−/− mice [38].

The cell clones expressing the normal and mutated ALDRP-EGFP protein will also serve to analyze the cross-talk between ALDRP and endogenous peroxisomal ABC transporters, particularly ALDP. The D207H mutation in ALDRP is similar to the D194H mutation in ALDP that has been previously studied by stable transfection and has been shown to disrupt function without suppressing the capacity of dimerization and of import in the peroxisomal membrane [30]. Based on the fact that ALDP and ALDRP are closely related both functionally and structurally, we suppose that the expression of this mutated ALDRP should induce a trans-dominant negative effect on the function of ALDP if ALDRP-ALDP heterodimerization occurs. Thus, the cell model may serve not only to study the function of ALDRP but also of ALDP.

Data on other ABC transporters indicate that it is probable that the peroxisomal transporters interact with other proteins (fatty acid transporters, activators or enzymes of the peroxisomal matrix) in order to optimize the transport of fatty acids. For instance, the transport of antigenic peptides by the ABC transporter heterodimer Tap1/Tap2 is made particularly effective due to the participation of intermediate proteins (Tapasin, Calreticulin, and ERp57) which connect the transporter and the major histocompatibility complex by physical interaction [39]. As already shown in the case of the mitochondrial ABC transporter, ABCB10 [40], intracellular cross-linking carried out in the obtained cell clone under doxycycline treatment followed by proteomic analysis should lead to differential identification of proteins interacting with ALDRP. The inducibility of expression would provide an efficient way to study whether these putative interactions are linked to the relative quantity of ALDRP in the cell. This study can shed light on the dimerization question and might allow identification of proteins involved in peroxisomal biogenesis or lipid metabolism, which will help to understand the exact role of ALDRP in the peroxisomal membrane.

In summary, we have generated a novel cell model, which holds great promises with respect to the identification of the physiological function of ALDRP. Furthermore, it may also give clues about the function of the other peroxisomal ABC transporters, particularly of ALDP, the protein deficient in X-ALD, and to address the question of selectivity of fatty acid transport in the peroxisomal oxidation pathway.

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