Biochimica et Biophysica Acta 1822 (2012) 1387–1396

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Peroxisomal ABC transporters: Structure, function and role in disease ☆

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ARTICLE INFO

Article history:
Received 1 October 2011
Received in revised form 7 January 2012
Accepted 8 February 2012
Available online 17 February 2012

Keywords:
ABC transporter
Acyl-CoA transport
Adrenoleukodystrophy
Fatty acid β-oxidation
Peroxisome targeting

ABSTRACT

ATP-binding cassette (ABC) transporters belong to one of the largest families of membrane proteins, and are present in almost all living organisms from eubacteria to mammals. They exist on plasma membranes and intracellular compartments such as the mitochondria, peroxisomes, endoplasmic reticulum, Golgi apparatus and lysosomes, and mediate the active transport of a wide variety of substrates in a variety of different cellular processes. These include the transport of amino acids, polysaccharides, peptides, lipids and xenobiotics, including drugs and toxins. Three ABC transporters belonging to subfamily D have been identified in mammalian peroxisomes. The ABC transporters are half-size and assemble mostly as a homodimer after posttranslational transport to peroxisomal membranes. ABCD1/ALDP and ABCD2/ALDRP are suggested to be involved in the transport of very long chain acyl-CoA with differences in substrate specificity, and ABCD3/PMP70 is involved in the transport of long and branched chain acyl-CoA. ABCD1 is known to be responsible for X-linked adrenoleukodystrophy (X-ALD), an inborn error of peroxisomal β-oxidation of very long chain fatty acids. Here, we summarize recent advances and important points in our advancing understanding of how these ABC transporters target and assemble to peroxisomal membranes and perform their functions in physiological and pathological processes, including the neurodegenerative disease, X-ALD. This article is part of a Special Issue entitled: Metabolic Functions and Biogenesis of Peroxisomes in Health and Disease.

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1. Introduction

Peroxisomes are organelles bounded by a single membrane that are present in almost all eukaryotic cells. These organelles are involved in a variety of metabolic processes, including the β-oxidation of fatty acids, especially very long chain fatty acids (VLCFA), and the synthesis of ether phospholipids and bile acids in mammals [1–3]. These metabolic pathways require the transport of metabolites in and out of peroxisomes [4]. Recently it has become clear that the transport of such metabolites is facilitated by at least a few different metabolic transporters. One of the transporter families is the ATP-binding cassette (ABC) transporter.

The ABC transporters comprise a superfamily of membrane-bound proteins found in almost all organisms from eubacteria to mammals. The structure of the ABC proteins is highly conserved, and they catalyze the ATP-dependent transmembrane transport of a wide variety of substrates. The ABC transporters are classified into two groups, ABC importers and ABC exporters [5,6]. The ABC importers are limited to prokaryotes and function as an uptake system for nutrients. The ABC exporters mediate the cellular secretion of toxic compounds and various kinds of lipids. Almost all eukaryotic ABC transporters function as exporters and are localized to plasma membranes as well as the endoplasmic reticulum (ER), Golgi apparatus, lysosomes, peroxisomes and mitochondria. They export various substrates into the extracellular space, and also to the lumen of subcellular organelles or the inner mitochondrial space, which is considered to be an "extracellular space" in eukaryotic cells. The human ABC transporter family currently comprises 49 members, if ABCC13, which lacks the ABC region, is included. They are divided into seven subfamilies based on structural organization and amino acid homology [7,8]. The human ABC proteins are involved in a number of important physiological processes and dysfunction of the ABC transporters is known to cause severe diseases such as Tangier disease (ABCA1), harlequin ichthyosis (ABCA12), cystic fibrosis (ABCC7) and X-linked adrenoleukodystrophy (X-ALD) (ABCD1).

To date, three ABC proteins have been identified in mammalian peroxisomes and classified into "subfamily D". These are adrenoleukodystrophy protein (ALDP/ABCD1), ALDP-related protein (ALDRP/ABCD2) and a 70-kDa peroxisomal membrane protein (PMP70/ABCD3) [9–14]. Dysfunction of ABCD1 is the cause of the human genetic disorder X-ALD, which is characterized by an accumulation of VLCFA because of an impaired peroxisomal β-oxidation of VLCFA [9]. These patients

Abbreviations: ABCD, ATP-binding cassette protein subfamily D; AMN, adrenomyeloneuropathy; CCALD, child cerebral ALD; CNS, central nervous system; DHCA, dihydroxycholestanoic acid; DHA, docosahexaenoic acid; GFP, green fluorescent protein; iPS, induced pluripotent stem; NBD, nucleotide-binding domain; SNP, single nucleotide polymorphism; THCA, trihydroxycholestanoic acid; TMD, transmembrane domain; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; VLCFA, very long chain fatty acids; X-ALD, X-linked adrenoleukodystrophy

☆ This article is part of a Special Issue entitled: Metabolic Functions and Biogenesis of Peroxisomes in Health and Disease.

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doi:10.1016/j.bbadis.2012.02.009

exhibit progressive demyelination in the central nervous system (CNS), adrenal insufficiency and testicular dysfunction. ABCD2 is suggested to be involved in the metabolic transport of VLCFA, since ABCD2 has a high sequence similarity to ABCD1, and the expression of ABCD2 restored VLCFA β-oxidation in X-ALD patient fibroblasts [15]. ABCD3 is suggested to be involved in the metabolic transport of long chain fatty acids, since the overexpression of ABCD3 in CHO cells induced the β-oxidation of palmitic acid [16]. In addition, *Abcd3*(−/−) knockout mice exhibit abnormalities in peroxisomal metabolism of the bile acid intermediates pristanic acid and phytanic acid (Jimenez-Sanchez G et al. Am J Hum Genet 2000, meeting abstract Nr 282), suggesting that ABCD3 is also involved in the transport of bile acid intermediates and branched chain fatty acids. PMP70-related protein (P70R/ABCD4) is also a member of the ABC protein subfamily D [17,18]. Recently, we reported that ABCD4 is localized to ER, not to peroxisomes, but the function of ABCD4 still remains unknown [19].

In this review, we will focus on recent advances in the understanding of how peroxisomal ABC transporters target and assemble to peroxisomal membranes and perform their functions in both physiological and pathological processes. A very recent review about peroxisomal ABC transporters is also helpful in understanding the ABC transporters in health and disease [20].

2. Structure of peroxisomal ABC transporters

Peroxisomal ABC transporters have a predicted structure of an ABC half-transporter with one transmembrane domain (TMD) and one nucleotide-binding domain (NBD). The hydropathy profile of the human peroxisomal ABC transporters predicts that the amino terminal half of the peroxisomal ABC transporters is hydrophobic, with six transmembrane segments, and the COOH-terminal half is hydrophilic, having NBD (Fig. 1) [21]. TMD provides the passageway for the substrate across the membranes and NBD energizes the directional transport of these substrates by cycles of ATP binding and hydrolysis. NBD contains two conserved motifs: Walker A and Walker B,

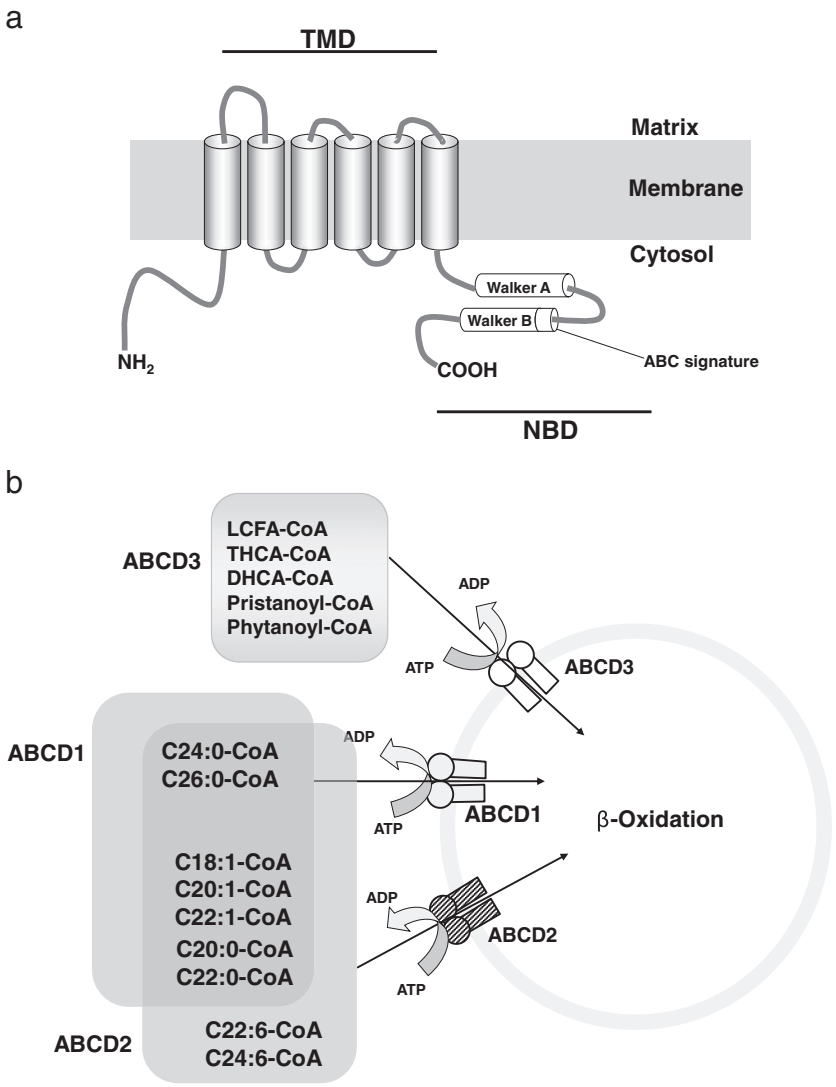

Fig. 1. Hypothesized structure of the peroxisomal ABC half-transporter (a) and the possible function of ABC transporters on peroxisomal membranes (b). ABCD transporters are a half-size ABC transporter with one transmembrane domain (TMD) and one nucleotide-binding domain (NBD). Six transmembrane domains are located in the NH_2-terminal half of the transporter, and Walker A, B and ATP-binding Cassette signature sequence (the ABC signature) are located in the COOH-terminal half of the transporter. In mammals, three ABC proteins (ABCD1, ABCD2 and ABCD3) belonging to subfamily D are known to exist in peroxisomes, and predominately exist as a homodimer. ABCD1 and ABCD2 have overlapping substrate specificities toward saturated and monounsaturated VLCFA-CoAs. However, ABCD1 has higher specificity to C24:0-CoA and C26:0-CoA than ABCD2. In contrast, ABCD2 has higher specificity for C22:0-CoA and C20:0-CoA than ABCD1. ABCD2, but not ABCD1, has an affinity for polyunsaturated VLCFA-CoA. ABCD3 is thought to be involved in the transport of LCFA-CoA, branched chain acyl-CoA, THCA-CoA and DHCA-CoA.

separated by 90–120 amino acids. A third, so-called ABC signature motif is situated upstream of the Walker B sequence [5,7].

Protease treatment of peroxisomes indicated that the NBD of ABCD1 and ABCD3 are exposed to the cytosol [14,21]. In terms of the ATP-binding and hydrolysis activities of ABCD1, Roerig et al. reported that the recombinant NBD of ABCD1 bound and hydrolyzed ATP [22]. Tanaka et al. also detected the ATP binding and hydrolysis activities of native ABCD1 and ABCD3 in rat liver peroxisomes by photoaffinity labeling with 8-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP [23]. Using limited-trypsin digestion, we investigated the effect of ATP-binding and hydrolysis on the conformation of ABCD3. The binding and hydrolysis of ATP were found to induce conformational changes in ABCD3 close to the boundary between the transmembrane and NBD, and the helical domain between Walker A and B motifs [24]. On the other hand, the TMD of ABCD1 is proposed to be involved in substrate-recognition and to form a transport pathway across the peroxisomal membrane. Guimaraes et al. assessed the substrate-induced conformational alterations in ABCD1 with a protease-based assay, and found that long- and very long chain acyl-CoA increased the sensitivity of the NH_2-terminal 44-kDa fragment of ABCD1 to Factor Xa, and this acyl-CoA induced sensitivity was reversed by the presence of ATP-γS [25]. These findings suggest that the NH_2-terminal TMD of ABCD1 is involved in the recognition of these substrates, and undergoes a conformational change upon ATP binding to the COOH-terminal NBD of ABCD1 and ABCD3.

As most of the half-size ABC proteins identified to date dimerize in order to form a functional transporter, it has been suggested that the peroxisomal ABC proteins also need to assemble as homo- or heterodimers on the peroxisomal membranes to form a functional unit. In terms of the quaternary structures of peroxisomal ABC proteins, Liu et al. were the first to show the occurrence of homo- as well as heterodimeric interactions among ABCD1, ABCD2 and ABCD3 by using a yeast two-hybrid system and co-immunoprecipitation experiments [26]. We also showed by means of co-immunoprecipitation studies that ABCD1 forms a stable complex with ABCD3 and certain peroxisomal proteins on rat liver peroxisomal membranes [23]. On the other hand, Guimaraes et al. reported that mouse liver ABCD1 and ABCD3 are a mostly homomeric protein structure, based on sucrose density gradient analysis and immunoprecipitation experiments with digitonin-solubilized mouse liver peroxisomes [27]. Furthermore, FRET microscopy experiments in intact living cells demonstrated that ABCD1 and ABCD3 form a homodimer, although ABCD1 can also form a heterodimer with ABCD3 [28]. These data suggest that ABCD1 and ABCD3 mainly are found as a homodimer in mammalian peroxisomal membranes, although ABCD1 can form a complex with ABCD2 or ABCD3.

Currently, one of the key outstanding questions is how peroxisomal ABC transporters recognize their substrates and import them into the lumen of peroxisomes. Although the X-ray crystallographic structure of peroxisomal ABC transporters has not yet obtained, recently the mouse Abcb1 (Mdr1, P-glycoprotein) was reported at 3.8 Å resolution [29]. The structure of Abcb1 represents a nucleotide-free inward-facing conformation in the plane of the lipid bilayer. The inward facing conformation results in a large internal cavity open to both the cytoplasm and the inner leaflet. The inward-facing conformation represents an initial stage of the transport cycle that is suitable for drug binding. The substrate enters the internal drug-binding pocket through an open portal and the residues in the drug binding pocket interact with the substrate. ATP binds to the NBD, causing a large conformational change, thereby presenting the substrate and drug-binding site(s) to the outer leaflet/extracellular space. Based on these findings and the structure of bacterial multidrug ABC transporters Sav1866 [30] and MsbA [31], peroxisomal ABC transporters might capture acyl-CoA derivatives from cytosolic side and transport them to the lumen of peroxisome through ATP binding and hydrolysis (Fig. 2). The arrangement of TMDs that compose the cavity of acyl-CoA for the substrate, and the linkage between the TMD and NBD, might have a conformation that is different from other ABC transporters in order to transport acyl-CoA derivatives.

3. Targeting of peroxisomal ABC transporters

Recent studies have demonstrated that peroxisomes originate from ER as pre-mature peroxisomes and mature as the result of post-translational transport of peroxisomal membrane and matrix proteins. The peroxisomes then undergo growth and division [32–34]. According to certain very recent findings, the biogenesis of the peroxisomal membranes is different in yeast and mammals. In yeast, it is suggested that a set of peroxisomal membrane proteins such as Pex3p, Pex13p, Pex14p and Pex15p is transported to the ER and then bud as premature peroxisomes [35,36]. However, in mammalian

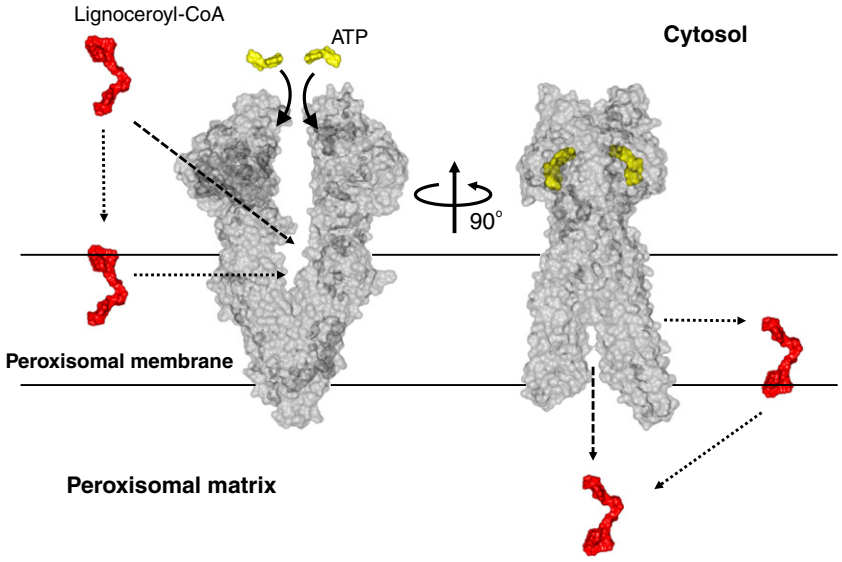

Fig. 2. A hypothetical model of substrate transport by the peroxisomal ABC transporter. Substrate (lignoceroyl-CoA) enters putative substrate-binding pocket of the ABC transporter from cytosol and/or leaflet of the lipid bilayer facing cytosol. ATP binds to the NBD, causing a large conformational change of the ABC transporter. The substrate is released into the lumen of peroxisome and/or the leaflet facing lumen of peroxisome. This model is drawn up on the base of structures of p-gp, Sav1866 and MsbA deposited in Protein Data Bank [29–31]. A possible conformation of lignoceroyl-CoA in aqueous solution is also made up based on energy minimization calculation, and in lipid bilayer based on NMR study [112]. The molecular-graphics software, CCP4mg was used to generate this model [113].

cells, peroxisomal membrane proteins are posttranslationally induced to become either premature or mature peroxisomes through the steps of maturation and division.

At least two peroxin proteins, Pex3p and Pex19p, have central functions in the generation of the peroxisome membrane structure [37]. In the cycle of peroxisome formation, Pex3p and Pex19p play a role in the insertion of newly synthesized peroxisomal membrane proteins into preexisting peroxisomes (Fig. 3). Pex19p is a hydrophilic and acidic protein which functions as both an acceptor and chaperone, thereby preventing the aggregation and degradation of peroxisomal membrane proteins [38,39]. We showed that the *in vitro* translation of ABCD3 in the presence of purified Pex19p resulted in soluble ABCD3 that co-immunoprecipitated with Pex19p. However, in the absence of Pex19p, ABCD3 formed aggregates during translation. Pex19p complexed with peroxisomal membrane proteins associates with its docking factor Pex3p on peroxisomal membranes, and peroxisomal membrane proteins are unloaded on the membranes (Fig. 3) [40,41].

Concerning the trafficking of the newly synthesized peroxisomal ABC transporters, ABCD3 has been characterized in some detail [39,42–44]. We investigated the targeting signal of ABCD3 (AA.1–659) to peroxisomes using various NH_2-terminal or COOH-terminal truncated ABCD3 fused to green fluorescent protein (GFP) [43]. The COOH-terminally truncated ABCD3 (AA.1–144)-GFP, composed of TMD1 and TMD2 of ABCD3, and GFP-ABCD3 (AA.263–375), composed of TMD5 and TMD6, were found to be localized to peroxisomes. Through further analysis of the targeting signal of ABCD3 by site-directed mutagenesis, we suggest that ABCD3 is recognized and binds to Pex19p at the NH_2-terminal hydrophobic motif constituted by Leu^{21}-Leu^{22}-Leu^{23} and the region of TMD5-TMD6. The ABCD3-Pex19p complex is transported to peroxisomes by the peroxisomal membrane protein targeting signals (mPTSs) located in the NH_2-terminal 124-amino acid region and the region of AA.263–375 (the hydrophobicity of Ile^{70}-Leu^{71} and Ile^{307}-Leu^{308} might be essential). Finally, ABCD3 is inserted into the peroxisomal membranes through the putative proteinaceous components on the peroxisomal membranes. In this process, at least two TMDs (TMD1 and TMD2) are required for proper insertion [43].

The targeting of ABCD1 to peroxisomes has also been characterized. Rottensteiner et al. found a Pex19p binding site consisting of a 15-amino-acid conserved consensus motif, which consists of a cluster of basic and possibly hydrophobic amino acids: [F(F/L)X(R/Q/K)(L/F)(L/I/K)XLLKIL(F/I/V)P] that has been identified in the amino-terminal close to the first TMD [45]. Deletion of the Pex19p binding site in ABCD1 (amino acid residues 71–84) abolished peroxisomal targeting. A similar sequence exists at AA.59–73 and 82–96 of ABCD3, but the affinity of these regions was found to be very weak compared to that of ABCD1 [46]. Nonetheless, these sequences are located in the region adjacent to the TMD1 of ABCD1 and 3, and may have an important role in the targeting of the proteins to peroxisomes together with TMD1 and TMD2. In ABCD2, a potential Pex19p binding site was also identified as ABCD1 [46]. It corresponds to AA.84–97, which are localized in the proximity of the putative TMD1. Unfortunately, no experimental data are available as yet to support the functionality of this putative Pex19p binding site.

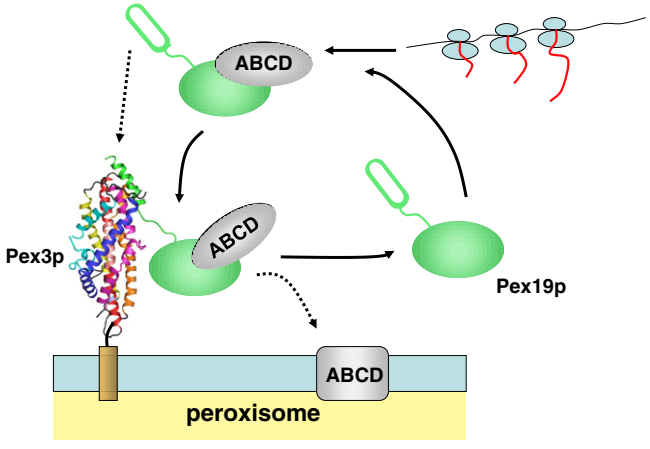

Fig. 3. Targeting of the peroxisomal ABC transporter to the peroxisomal membrane. The peroxisomal ABC transporter translated on free ribosomes is selectively captured by Pex19p in the cytosol. The interaction becomes more stable by the TMDs located downstream of the NH_2-terminal Pex19p binding motif. As a result, the peroxisomal ABC transporter keeps soluble and proper conformation in the cytosol. Then, the peroxisomal ABC transporter-Pex19p complex binds to Pex3p through the interaction of the NH_2-terminal Pex3p binding motif of Pex19p, consisting of Leu18, Leu21, Leu22, Phe29, and Trp104 in the α2-helix of Pex3p. Finally, the peroxisomal ABC transporter is inserted into the peroxisomal membrane and Pex19p shuttles back to the cytosol to initiate another import cycle. However, the mechanisms by which Pex19p unloads the ABC transporter and shuttles back have not yet elucidated.

ABCD4 is a member of the ABC transporter subfamily D. We demonstrated in CHO cells that transiently expressed ABCD4 in fusion with HA (ABCD4-HA) was localized to the ER, not peroxisomes and endogenous ABCD4 in rat liver was detected in microsomal fraction by subcellular fractionation [19]. These results suggest that ABCD4 might be located to the ER. The ABC subfamily D transporters destined for peroxisomes have an NH_2-terminal hydrophilic region that binds to the Pex19p adjacent to TMD1, as mentioned above. However, only ABCD4 lacks the region and is translated with only the NH_2-terminal hydrophobic TMD1. Therefore, it seems likely that the hydrophobicity of the NH_2-terminal region determines the subcellular localization of the ABC subfamily D transporters.

4. Function and regulation of peroxisomal ABC transporters

4.1. ABCD1

X-ALD is caused by mutations in the *ABCD1* gene that result in a defect in the ABCD1 protein and the accumulation of VLCFA and/or VLCFA-CoA. Therefore, it is thought that ABCD1 functions as a transporter of VLCFA across the peroxisomal membrane. This is supported by the result that transfection of *ABCD1* cDNA into X-ALD skin fibroblasts restored the VLCFA β-oxidation, and consequently, the VLCFA content returned to normal in the fibroblasts [47,48]. Recently, van Roermund et al. have further demonstrated that ABCD1 is involved in the transport of saturated, monounsaturated and polyunsaturated VLCFA-CoA (such as C18:0-, C22:0-, C24:0-, C26:0-, C18:1- and C24:6-CoA) across the peroxisomal membranes by expressing human ABCD1 in a *PXA1/PXA2* knockout yeast mutant [49,50]. In fact, the level of C24:0-CoA and C26:0-CoA in X-ALD fibroblasts was reported to become elevated when X-ALD fibroblasts were incubated with C24:0 [51]. These findings strongly suggest that ABCD1 is involved in the uptake of a wide range of VLCFA-CoA forms into peroxisomes.

ABCD1 gene expression is reportedly affected by the cholesterol level in human THP-1 cells and primary macrophages [52]. In *Abcd1*-deficient mice, the plasma cholesterol level was higher than that in wild-type mice and was not increased by cholesterol-feeding whereas the level in cholesterol-fed wild-type mice was increased [53]. These findings suggest a link between ABCD1 function and cellular cholesterol metabolism. However, the mechanism by which cholesterol affects *Abcd1* gene expression has not yet been elucidated.

4.2. ABCD2

It is generally accepted that ABCD2 has a certain functional redundancy with ABCD1 [54]. However, the differences in the expression patterns and phenotypes of *Abcd1*-deficient and *Abcd2*-deficient mice suggest that there are certain other specific roles for ABCD2 in lipid metabolism. This is supported by the report that ABCD2 complements the germination phenotype of an *Arabidopsis* cts-1 mutant that lacks the peroxisomal ABCD transporter CTS/AtABCD1, but ABCD1 does not [55].

The β-oxidation activity of C24:6n−3, an immediate precursor of docosahexaenoic acid (DHA), was reduced in *Abcd2*-deficient mice brain. In addition, ABCD2 expression is highly sensitive to dietary polyunsaturated fatty acids (PUFA), suggesting that Abcd2 is involved in the transport of PUFA in relation to DHA metabolism [50,56,57]. This is supported by experiments using yeast expression system in which ABCD1 and/or ABCD2 was expressed in a *pxa1*/*pxa2* knockout yeast mutant. van Roermund et al. have shown that ABCD2 has overlapping substrate specificities with ABCD1 toward saturated fatty acids and monounsaturated fatty acids such as C24:0-CoA, C26:0-CoA, C20:1-CoA and C22:1-CoA [50], although ABCD2 has a higher specificity for C22:0-CoA and C20:0-CoA than ABCD1. In contrast, ABCD2 has an affinity for PUFA such as C22:6-CoA and C24:6-CoA, but ABCD1 does not. These results are good agreement with the results from ABCD2-overexpressing H4IIEC3 cells [57].

It has been suggested that ABCD2 might be more important than ABCD1 in the control of oxidative stress, because ABCD2 is involved in the metabolism of monounsaturated VLCFAs (C26:1) which might be related to oxidative damage to proteins [58]. These results are consistent with the report by Lu et al. that in *Abcd2*-deficient mice, the oxidative stress in adrenal cells was greater than that in *Abcd1*-deficient mice [59]. Taken together, ABCD2 appears to have a central role in the metabolism of mono- and polyunsaturated VLCFA rather than saturated VLCFA, and may be involved in the regulation of oxidative stress and the synthesis of DHA.

ABCD2 gene expression is reported to be regulated by nuclear factors such as the peroxisome proliferator-activated receptor (PPARα), retinoid X receptor (RXR), thyroid hormone receptor (TRβ) and the sterol regulatory element (SRE) binding proteins (SREBP1a, SERBP1c and SREBP2). The SRE located in the *ABCD2* promoter overlaps with a direct repeat separated by 4 nucleotides (DR-4), suggesting cross talk between SREBPs and liver X receptor α (LXRα) or TRβ, which are known to dimerize with RXRα [60]. Weinhofer et al. demonstrated that depression of the cholesterol content results in a decrease in the C26:0 level, and the expression of the *ABCD2* gene is up-regulated *via* the activation of the SREBPs [52,61]. In addition, the *ABCD2* promoter contains a functional thyroid hormone response element (TRE). Recently, it was shown that the ligand-activated thyroid hormone receptors TRα and TRβ stimulate or derepress, respectively, the SREBP1-dependent induction of the *ABCD2* promoter [60]. These thyroid hormone receptors bind the SRE/DR-4 motif. Therefore, novel tissue-specific ligands for TRα, TRβ or other DR-4 binding factors that interact with SREBP1, might enhance *ABCD2* expression.

4.3. ABCD3

ABCD3 is one of the most abundant peroxisomal membrane proteins, at least in hepatocytes, and has been reported to have a capacity to transport various fatty acids. Overexpression of ABCD3 stimulates C16:0 β-oxidation activity in CHO cells, but inhibits C24:0 β-oxidation activity [16]. In addition, no decrease in the C24:0 β-oxidation activity was observed by the silencing ABCD3 in ABCD1-knockdown U87 cells (manuscript in preparation), suggesting that the contribution of ABCD3 to peroxisomal VLCFA β-oxidation might be negligible. It has been reported in *Abcd3*-deficient mice that the bile acid precursors trihydroxycholestanoic acid (THCA) and dihydroxycholestanoic acid (DHCA), as well as pristanic acid, were accumulated in plasma, and that the β-oxidation of phytanic and pristanic acid, plant-derived fatty acids in *Abcd3*-deficient mouse fibroblasts, was decreased to 50% of control (Jimenez-Sanchez G et al. Am J Hum Genet 2000, meeting abstract Nr 282). These results suggest that ABCD3 might function in the transport of LCFA-CoA, THCA-CoA, DHCA-CoA and branched chain acyl-CoA. This is consistent with the fact that hepatic peroxisomes potently express ABCD3 and are essential for bile acid synthesis. In the mouse liver, ABCD3 expression is known to be induced by treatment with fibrates, such as ciprofibrate and fenofibrate, and plasticizers *via* PPARα.

4.4. ABCD4

As mentioned above, we recently reported that ABCD4 is not a peroxisomal membrane protein but an ER-resident protein, even though human ABCD4 shares 46% homology with human ABCD1 [19]. It is therefore speculated that ABCD4 functions as a transporter of certain lipid molecules essential for lipid metabolism in the ER. However no information on such a function has yet been reported.

5. Role of ABCD1 in CNS and pathogenesis in X-ALD

As judged from the phenotype observed in *Abcd1*- or *Abcd2*-deficient mice as well as X-ALD patients, both ABCD1 and ABCD2 seem to be involved in the maintenance of axonal integrity and/or myelination. In the CNS, ABCD1 is expressed in endothelial cells, astrocytes and microglia, and also in subpopulations of oligodendrocytes located in the corpus callosum, internal capsules and anterior commissure, but not in neurons in the adult mouse or human brain [62]. In contrast, ABCD2 is expressed in all mouse CNS cells except for endothelial cells, and the expression pattern mirrors the ABCD1 distribution [63]. Since no disease associated with the mutation of ABCD2 has been reported, we focus our attention on ABCD1 and X-ALD in the following subsections.

5.1. VLCFA metabolism in X-ALD

Mutations of the *ABCD1* gene [9] result in X-ALD with a birth incidence of 1:17,000 [64]. The affected patients exhibit progressive demyelination in the CNS as pathological characteristics [65]. Dysfunction of ABCD1 results in increased levels of saturated (C24:0 and C26:0) and monounsaturated (26:1) VLCFAs in plasma and tissues of patients with X-ALD, and reduced VLCFA β-oxidation in peroxisomes [66]. Increased cellular VLCFA-CoAs are thought to be further elongated by an ER-resided enzyme called elongation of very long chain fatty acids-1 (ELOVL1), which catalyzes the synthesis of both saturated (C26:0) and mono-unsaturated VLCFA (C26:1) [51]. The abnormal accumulation of VLCFA is the most likely culprit behind both the initiation and progression of the disease [67]. Asheuer et al. found that increase of C26:0 in normal appearing white matter in child cerebral ALD (CCALD) was higher than adrenomyeloneuropathy (AMN) [68]. Recently, Jang et al. established induced pluripotent stem (iPS) cells from X-ALD patients and demonstrated that oligodendrocytes differentiated from X-ALD induced-iPS cells displayed an accumulation of C26:0 [69]. Interestingly, C26:0 accumulation was reported to be much higher in child cerebral ALD (CCALD) oligodendrocytes than AMN oligodendrocytes, although the study involved a single CCALD and a single AMN line.

5.2. The pathogenesis of X-ALD

The mechanism by which the accumulation of VLCFAs in the brain causes neurodegeneration, especially demyelination, remains obscure. However, the linkage of VLCFA accumulation with the immunoresponse is thought to be important. It has been demonstrated that VLCFA accumulation subsequently leads to a neuroinflammatory response, with the production of proinflammatory cytokines by activated astrocytes and microglial cells together with demyelination and a loss of oligodendrocytes [70,71]. Hein et al. have shown a direct toxic effect of VLCFA on primary neurons and glial cells, especially oligodendrocytes, in rat hippocampus culture, *via* Ca^{2+} deregulation and mitochondrial dysfunction, probably due to an incorporation of VLCFA into the mitochondrial inner membrane [72]. The presence of less mature myelin in both weaning and postweaning rats treated with C26:0 [73], suggests that an increase of VLCFA may lead to myelin instability, followed by an inflammatory or immune-mediated process, and therefore contribute, at least in part, to the loss of oligodendrocytes observed

in the X-ALD brains. The complex lipids of VLCFAs or VLCFA-phospholipids may comprise an antigen recognized by the CD1 pathway, which would be a plausible trigger of inflammatory demyelination [74].

In addition, there is increasing evidence that oxidative stress contributes to the pathogenesis of the cerebral inflammatory phenotype based on *in vivo* and *in vitro* studies. Powers et al. found convincing evidence of oxidative stress and oxidative damage from lipid peroxidation, as well as nitrosylated proteins, in the post-mortem brain tissue of four X-ALD patients with the cerebral inflammatory phenotype [75]. In Abcd1(−/−) mice, oxidative damage in the spinal cord was observed at as early as 3.5 months of age, more than 1 year before the neuropathological signs typically appear [76–78]. Furthermore, incubation of human fibroblasts in the presence of C26:0 resulted in an increase in the intracellular reactive oxygen species (ROS) level and the activation of enzymatic antioxidant defenses, indicating a role for VLCFA in the generation of ROS. In the CNS, a decreased plasmalogen level due to the dysfunction of ABCD1 might be involved in the vulnerability to oxidative stress because plasmalogen is known to possess antioxidant activity [79,80]. Taken together, the dysfunction of ABCD1 may be correlated with a defective antioxidant response, and oxidative damage may thus be linked to both the initiation and the progression of the demyelination.

However, initiation and progression of the X-ALD pathologies cannot be accounted for only by the accumulation of VLCFA. In addition to the VLCFA metabolic abnormality, modifier genes and/or environmental factors have been implicated in triggering inflammatory demyelination [81,82]. Recently, head trauma was reported to initiate the onset of neurodegeneration [83]. At present, identification of the genetic, epigenetic and environmental factors which trigger this neurodegeneration remains a central issue.

5.3. Role of ABCD1 in glial cells and association with X-ALD

It is plausible that a dysfunction of ABCD1 in glial cells causes the demyelination. Oligodendrocytes are myelin-producing cells, and astrocytes and microglia are known to be important for the support of axonal growth and myelination. In contrast, the neuron-specific peroxisome-knockout mouse model, *NEX-Pex5*, did not exhibit any metabolic abnormality or axonal damage, suggesting that ABCD transporters in neurons are not involved in such neuropathogenesis [84].

5.3.1. Oligodendrocytes

The dysfunction of ABCD1 in oligodendrocytes unambiguously plays a crucial role in the disease progression of X-ALD. Is ABCD1 involved in either axonal integrity or myelination? A study using iPS cells suggested that ABCD1 is not required for oligodendrocytic maturation, because ABCD1-deficient iPS cells normally differentiate into mature oligodendrocytes [69]. Furthermore, a recent study showed that the lack of functional peroxisomes in oligodendrocytes resulted in the degradation of myelinated axons, but did not affect oligodendrocyte survival [84,85]. Indeed, myelination itself is conspicuously normal during brain development in oligodendrocyte-specific peroxisome-deficient mice and X-ALD patients. It has been thought that the support of axonal survival by oligodendrocytes is essential for axonal integrity [86,87]. Therefore, ABCD1 dysfunction in oligodendrocytes might exert an effect on axonal survival but not myelination.

5.3.2. Astrocytes

Astrocytes have important roles in both remyelination and the maintenance of myelin structure [88]. In astrocyte-specific peroxisome-deficient mice, the accumulation of VLCFA and decrease of plasmalogen in the brain were observed, although a definitive pathogenetic phenotype was not identified. Interestingly, in these mice, the VLCFA accumulation was detected in myelin [84]. This result suggests a metabolic interaction between astrocytes and oligodendrocytes. Astrocytes are known to synthesize various lipids, including cholesterol and DHA, and to supply these lipids to other neural cells for myelination and synaptogenesis [89,90]. Fatty acid β-oxidation may thus provide acetyl-CoA for de novo fatty acid and cholesterol synthesis in the brain. It is thus postulated that disruption of lipid metabolism in *ABCD1*-deficient astrocytes might affect the oligodendrocytic function and further accelerate the demyelination which occurs in X-ALD.

5.3.3. Microglia

Hematopoietic stem cell transplantation exhibits remarkable efficacy in halting cerebral demyelination in X-ALD, and this effect is likely due to the functional replacement of brain microglia by bone marrow-derived cells [91]. This result suggests that the dysfunction of ABCD1 in microglia contributes to the demyelination in X-ALD. When X-ALD fibroblasts were co-cultured with N9 cells, a mouse microglial cell line, the VLCFA level in X-ALD fibroblasts was reduced by a cell-mediated process [92]. Therefore, ABCD1 in microglia might be important for the maintenance of VLCFA metabolism in the CNS and the suppression of the onset or progression of demyelination. In fact, microglial apoptosis in perilesional white matter represents an early pathogenic change in CCALD [93].

Taken together, in addition to the dysfunction of ABCD1 in oligodendrocytes, a defect in the metabolic support from astrocytes and microglia to oligodendrocytes and probably neurons is suggested to be involved in the demyelination in X-ALD.

6. Peroxisomal ABC transporters in other organisms

A variety of genetic and biochemical studies on ABC transporter subfamily D from other organisms has been reported. To date, two ABCD transporters have been described in *Saccharomyces cerevisiae* (Pxa1p and Pxa2p), two in *Podospora anserina* (pABC1 and pABC2), three in *Dictyostelium discoideum* (ABCD.1, ABCD.2 and ABCD.3), five in *Caenorhabditis elegans* (pmp1, pmp2, pmp3, pmp4 and pmp5), three in *Trypanosoma brucei* (GAT1, GAT2, and GAT3), and two in *Arabidopsis thaliana* (CTS/AtABCD1 and AtPMP1/AtABCD2). The following section describes the current state of knowledge on the ABCD proteins in plants, protozoa, nematoda and fungi.

6.1. A. thaliana

In *A. thaliana*, a full-size peroxisomal ABCD transporter with the characteristics of a fused heterodimer is reported [94–96]. *AtABCD1* (also known as COMATOSE (CTS), *Peroxisome defective 3* (*PED3*), *Peroxisomal ABC transporter 1* (*PXA1*), *Acetate non-utilising 2* (*ACN2*) and *A. thaliana Peroxisomal Membrane Protein 2* (*AtPMP2*)) is involved in the transport of variety of substrates such as fatty acids and hormone precursors into peroxisomes. Both halves of AtABCD1 have significant sequence identity to human ABCD1. The NBD phylogenetic tree showed that the N- and C-terminal halves of AtABCD1 cluster with Pxa2p and Pxa1p, respectively (Fig. 4). In the *AtABCD1* mutant, free fatty acids and acyl-CoA, which are predominantly derived from triacylglycerol, accumulate in seeds and seedlings [95]. Furthermore, the *AtABCD1* mutant under prolonged dark conditions exhibited an accumulation of free fatty acids in mature leaves, including palmitic acid, 7,10,13-hexadecatrienoic acid and α-linolenic acid in mature leaves [97]. These results indicate that AtABCD1 has a role in the transport of acyl-CoA, at least in mature leaves and germinating seeds. This is supported by the result that AtABCD1 exhibits ATPase activity and recovers from the β-oxidation of saturated or unsaturated fatty acids with the carbon chain length of C16–C24 when expressed in a yeast *pxa1*/*pxa2*-deficient mutant [98]. AtABCD1 is also known to function in the transport into peroxisomes of 2,4-dichlorophenoxybutyric acid (2,4-DB), indole butyric acid (IBA) and 12-oxo-phytodienoic acid (OPDA), precursors of jasmonic

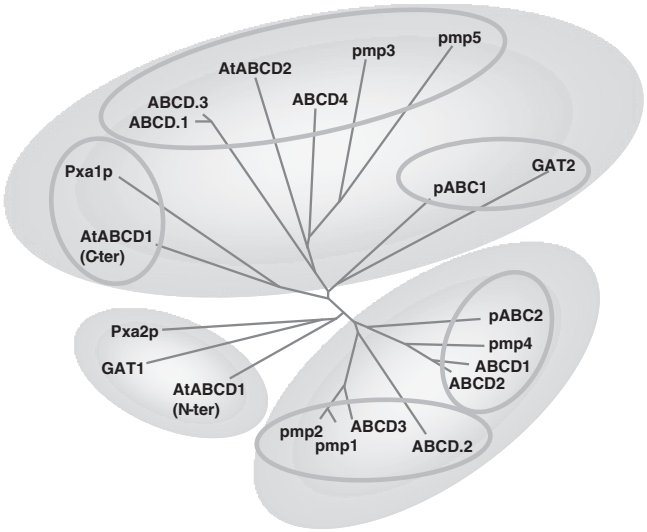

Fig. 4. Phylogenetic relation of the various ABCD transporters, including the human (ABCD1, ABCD2, ABCD3 and ABCD4), plant (AtABC1 and AtABCD2 in *Arabidopsis thaliana*), protozoa (GAT1, GAT2, and GAT3 in *Trypanosoma brucei*), nematoda (pmp1, pmp2, pmp3, pmp4 and pmp5 in *Caenorhabditis elegans*), and fungi (Pxa1p and Pxa2p in *Saccharomyces cerevisiae*, pABC1 and pABC2 in *Podospora anserina*, ABCD.1, ABCD.2 and ABCD.3 in *Dictyostelium discoideum*). The protein sequences of the nucleotide-binding domains (NBDs) from various ABCD proteins were assembled and aligned using ClustalW. Aligned sequences were used to generate matrices of the mean distances between proteins, and these matrices were used to generate phylogenetic trees according to the neighbor-joining algorithm.

acid [94,99]. These results demonstrate that AtABCD1 supports the β-oxidation of a wide range of fatty acids having a broad range of substrate specificity [98], whereas the mammalian ABCD transporters have much more restricted substrates.

AtABCD2 (also known as *A. thaliana Peroxisomal Membrane Protein 1* (*AtPMP1*)) encodes a half-size, putative plastid ABC transporter that has not yet been characterized [100,101]. The NBD phylogenetic tree indicates that AtABCD2 clusters with human ABCD4 (Fig. 4).

6.2. T. brucei

In *T. brucei*, three ABC transporters (termed GAT1, 2 and3) have been identified in peroxisome-like organelles called glycosomes [102]. The identity between the *Trypanosoma* ABCD transporters and human ABCD transporters is approximately 25% [102]. The NBD phylogenetic tree shows that GAT1 clusters with Pxa2p (Fig. 4). GAT1 and GAT3 are expressed both in the bloodstream and procyclic trypanosomes, while in contrast GAT2 is mainly or exclusively expressed in bloodstream-form cells [103]. In mammals, *T. brucei* live in the bloodstream and glucose in the blood is converted into pyruvate by the glycolytic pathway in their glycosomes for energy intake. Igoillo-Esteve et al. have demonstrated that the silencing of GAT1 in procyclic cells cultured in a glucose-free medium was lethal [103]. Furthermore, glycosomes purified from procyclic wild-type cells incorporate oleoyl-CoA in an ATP-dependent manner, indicating the functional role of GAT1 in the transport of oleoyl-CoA into the glycosomal lumen. The functions of GAT2 and GAT3 have not yet been reported.

6.3. C. elegans

In *C. elegans*, five ABCD transporters (pmp1–5) have been identified. The similarity of pmp1, pmp2 and pmp4 to human ABCD2 and ABCD3 is 55–78% [104]. The NBD phylogenetic tree shows that pmp1 and pmp2 cluster with human ABCD3, and pmp4 clusters with human ABCD1 and ABCD2 (Fig. 4). Interestingly, pmp3 and pmp5 cluster with human ABCD4, which localizes to the ER.

The silencing of the *pmp1*, *pmp2* and *pmp4* genes caused a substantial delay in the development of the nematode, indicating that these ABCD transporters were important for development. The β-oxidation of polyunsaturated fatty acid and the biosynthesis of plasmalogen are essential for normal development in *C. elegans*, suggesting that these ABCD transporters might be involved in the transport of PUFA and plasmalogen precursor molecules. In *C. elegans*, peroxisomal VLCFA β-oxidation is essential for detoxification and biosynthesis of the dauer pheromone, ascaroside, (−)-6-(3,5-dihydroxy-6-methyltetrahydropyran-2-yloxy) heptanoic acid [105]. Dysfunction of either of the two peroxisome enzymes, DHS-28 and DFA-22, results in severe developmental defects, probably because of the massive accumulation of saturated fatty acids (C20–24) and their acyl-CoAs (C23–26). It is thus possible that an ABCD transporter such as pmp1, pmp2 or pmp4 is involved in the transport of VLCFA-CoA [104]. Further studies will be needed to elucidate the individual function of these ABCD transporters in *C. elegans*.

6.4. S. cerevisiae, D. discoideum and P. anserina

A search for the yeast homologs of human ABCD1 and ABCD3 resulted in two ABCD1 homologs in *S. cerevisiae*. They are *PXA1* (*peroxisomal ABC-transporter 1*), also known as *PTA1* or *PAL1*, and *PXA2*, also known as *PAT1* or *YKL741*. The NBD phylogenetic tree shows that Pxa1p and Pxa2p cluster with AtABCD1 (C-terminal) and AtABCD1 (N-terminal), respectively (Fig. 4). Disruption of *PXA1* and/or *PXA2* resulted in impaired growth of these mutants on oleic acid as a sole carbon source, and also a reduced ability to oxidize oleate [106]. Furthermore, Verleur et al. showed that Pxa2p is directly responsible for the ATP-dependent transport of long-chain acyl-CoA across peroxisomal membranes by using a semi-intact yeast cell system [107]. These results clearly indicate that Pxa1p/Pxa2p functions as a transporter of acyl-CoA. Since fatty acid β-oxidation is restricted to peroxisomes in *S. cerevisiae*, a *PXA1*/*PXA2*-knockout yeast mutant is particularly useful in studying the substrate specificity of other ABCD transporters.

In *D. discoideum*, the dysfunction of the peroxisomal multifunctional enzyme MFE1 led to cAMP-relay and developmental defects, with an accumulation of excess cyclopropane fatty acids derived from ingested bacteria [108,109]. There are three ABCD transporters in *Dictyostelium*, ABCD.1, ABCD.2 and ABCD.3 [110]. Among them, the *Dictyostelium* ABCD.2 protein clusters with the human ABCD3 protein, and *Dictyostelium* ABCD.1 and ABCD.3 cluster with human ABCD4 (Fig. 4). Although further studies are needed to elucidate the function of these transporters, *Dictyostelium* ABCD.2 might be involved, at least in part, in the transport of cyclopropane fatty acids into peroxisomes for degradation.

In *P. anserina*, there are only two genes encoding members of the ABCD transporter family, pABC1 and pABC2. The sequence similarity is 45% with human ABCD1, and pABC2 clusters with both human ABCD1 and ABCD2 (Fig. 4). β-Oxidation of fatty acids occurs in both mitochondria and peroxisomes, which is different from plants and other fungi. In addition to the putative function as a fatty acyl-CoA transporter, pABC1 and pABC2 might have other roles during developmental processes [111]. A defect in pABC1 or pABC2 suppresses the germination defect in peroxisomal fatty acid β-oxidation deficient ascospores, suggesting that the absence of pABC1 and/or pABC2 stimulates an unknown metabolic pathway that results in a compensation for the germination defects.

Studies of ABCD proteins in other organisms provide clues for understanding function of mammalian ABCD transporters. *C. elegans* possesses a nervous system and has a well known life cycle, which makes it highly suitable for studying peroxisomal disorders. Therefore, *C. elegans* is a valuable model system for investigating defects in the ABCD transporters. On the other hand, ABCD transporter function in cellular activities such as germination and sporulation can be

easily examined in fungi. Studies of their physiological roles using such systems could lead to the discovery of new functions.

7. Concluding remarks

Further investigation is needed to understand how peroxisomal ABC proteins work and regulate cellular function. However, in the research reported over the last few years, considerable progress has been made on the targeting, assembly and function of the peroxisomal ABC transporters. The expression of human peroxisomal ABC transporters in yeast lacking *PXA1* and *PXA2* has clearly demonstrated the substrate specificity of human peroxisomal ABC transporters. However, to understand the detailed transport of substrate by the peroxisomal ABC transporters, X-ray crystallographic studies will be required. In addition, to fully understand the regulation of peroxisomal ABC transporters, proteomic approaches which identify interaction partners are also going to be needed. Characterization of peroxisomal ABC transporters in other organisms would be helpful in elucidating the role of mammalian ABCD transporters in cellular function and regulation. Dysfunction of ABCD1 results in X-ALD and the accumulation of VLCFA seems to be closely associated with the disease. However, the mechanism by which the accumulation of VLCFA in the brain causes neurodegeneration remains obscure. Novel approaches may be required to identify the regulatory networks of the various proteins as well as the micro RNAs that change in association with the dysfunction of ABCD1 and the onset of X-ALD in targets cells.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan, and for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20590054, 22590060, 23590072). Pacific Edit reviewed the manuscript prior to submission.

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