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The protein coded by the X-adrenoleukodystrophy gene is a peroxisomal integral membrane protein

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Abstract

The gene for adrenoleukodystrophy (X-ALD), a peroxisomal disease characterized by excessive accumulation of very long-chain (VLC) fatty acids ($> C_{22\ 0}$), has recently been identified by positional cloning, and it is predicted to encode a protein (ALD-P) of 745 amino acids [(1993) Nature 361, 726]. Using Western blot analysis of subcellular organelles purified by isopycnic density gradient centrifugation from X-ALD and control fibroblasts, we show that the monoclonal antibodies directed against ALD-P cross-react with a 75 kDa protein in intact peroxisomes and that ALD-P is an integral component of the peroxisomal membrane. Moreover, no signal for ALD-P was detected in peroxisomes from X-ALD patients with deletion of the ALD gene.

Key words: Peroxisome; X-adrenoleukodystrophy; Lignoceric acid oxidation; X-adrenoleukodystrophy gene product

1. Introduction

Adrenoleukodystrophy (X-ALD), a peroxisomal disease, is an X-linked progressive degenerative neurological disorder affecting the central nervous system and adrenal cortex [1–5]. It is associated with the pathognomonic accumulation of saturated very long-chain (VLC) fatty acids ($> C_{22}$) as a constituent of cholesterol esters, phospholipids and gangliosides [3–5]. Although X-ALD presents as various clinical phenotypes, including childhood X-ALD, adrenomyeloneuropathy (AMN), and Addison's disease, all forms of X-ALD accumulate VLC fatty acids at similar concentrations in fibroblasts and plasma [4,5]. The increased levels of VLC fatty acids has been used as a diagnostic tool both in prenatal and postnatal diagnosis [1,4–6].

To delineate the specific enzyme defect, initial studies focused on the metabolism of cholesterol esters since there is excessive accumulation of VLC fatty acids in the cholesterol ester fraction. However, no defect was observed [7,8]. The accumulation of VLC fatty acids in lipids other than cholesterol esters [4–6] and normal metabolism of cholesterol esters suggested that the defect in X-ALD may be in the metabolism of VLC fatty acids. A number of laboratories, including ours, showed that oxidation of VLC fatty acids is deficient in X-ALD cultured skin fibroblasts, whereas oxidation of palmitic acid is normal [7,9–13]. The identification of peroxisomes as the site of oxidation of VLC fatty acids [10] suggested that X-ALD might be a peroxisomal disease. Direct demonstration of defective oxidation of VLC fatty acids in peroxisomes isolated from cultured X-ALD skin fibroblasts established that X-ALD is a disease associated with a defect in the peroxisomal β-oxidation system [14].

The deficient oxidation of free lignoceric acid (substrate for the first step in the oxidation of fatty acids) as compared to the normal oxidation of lignoceroyl-CoA and α-,β-unsaturated lignoceroyl-CoA (substrates for the 2nd and 3rd step in the β-oxidation of fatty acids) suggested that the abnormality in oxidation of VLC fatty acids (lignoceric acid) may be due to deficient activity of lignoceroyl-CoA ligase required for the activation of lignoceric acid to lignoceroyl-CoA [12,15]. The deficient activity of lignoceroyl-CoA ligase as compared to the normal oxidation of lignoceroyl-CoA in purified peroxisomes supported this conclusion [14,17]. These observations suggested that the gene coding for lignoceroyl-CoA ligase may be a candidate for the X-ALD gene; however, the recent identification of the ALD gene by positional cloning [18–20] led to a different conclusion. The protein sequence encoded by this gene (ALD-P) has significant homology with the 70 kDa peroxisomal membrane protein [18,21], a member of the ATP-binding cassette (ABC) superfamily of the transporters [22]. This suggests that ALD-P could be responsible for incorporation of lignoceroyl-CoA ligase in the membranes of peroxisomes [18]. However, the levels of mRNA for the

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ALD gene did not correlate with the amount of peroxisomes in various organs [18]. For example, the amount of peroxisomes in liver are relatively much higher as compared to muscle, but the muscle contained higher amounts of mRNA compared to liver. This raised the question of whether the protein transcribed by the ALD gene is in fact a peroxisomal component. To answer this question we used monoclonal antibodies raised against ALD-P [23] to determine its distribution in various subcellular organelles purified by the isopycnic density gradient technique from control and cell lines from patients with deletion of the ALD gene.

2. Experimental

The subcellular organelles were prepared by isopycnic density gradient centrifugation in Nycodenz (Accurate Chemical and Scientific Corp., Westbury, NY) from cultured skin fibroblasts from controls and X-ALD patients as described previously [14]. Thirty or more confluent flasks (75 cm^2) of cultured skin fibroblasts from control and X-ALD patients were harvested, cell pellets were washed with homogenizing medium (0.25 M sucrose, 1 mM EDTA, 0.5 μg/ml of antipain, 0.5 μg/ml of leupeptin, 1 μg/ml of aprotinin, 0.7 μg/ml pepstatin, 0.2 mM phenylmethylsulfonylfluoride, 0.1% ethanol in 3 mM imidazole buffer (pH 7.4)) at 4°C and the subcellular organelles were purified by differential and isopycnic density gradient centrifugation [14]. The gradient was collected from the bottom and analyzed by the marker enzyme activities of catalase for peroxisomes, NADPH cytochrome *c* reductase for endoplasmic reticulum and cytochrome *c* oxidase for mitochondria. The purity of peroxisomes was between 90–92% with small concentration of mitochondria (5.4 ± 2.4%), endoplasmic reticulum (2.2 ± 1.2%) and lysosomes (0.9 ± 0.4%) [25]. The peroxisomal membrane and matrix components were prepared by carbonate treatment of the peroxisomal peak from the Nycodenz gradient as described previously [24]. Monoclonal antibodies (1D6) raised against protein sequence corresponding to codons Leu^{279}–Val^{482} [18,20] were used to detect the ALD-P in various subcellular organelles by Western blot analysis.

3. Results and discussion

Fig. 1 shows the subcellular fractionation of cultured skin fibroblasts from control (Fig. 1A) and X-ALD (Fig. 1B). The distribution of activities of catalase for peroxisomes, cytochrome *c* oxidase for mitochondria and NADPH cytochrome *c* reductase for endoplasmic reticulum were similar from control and X-ALD cells. The peroxisomes were very well resolved from other subcellular organelles. This procedure allows us to purify peroxisomes of over 90% purity [25]. The antibodies against ALD-P recognized only one protein with an apparent molecular mass of 75 kDa in gradient fractions containing peroxisomes, as identified with catalase, in control cultured skin fibroblasts (Fig. 2). The antibodies against ALD-P react with a protein which migrates in SDS-PAGE at 75 kDa instead of 83 kDa as expected from the coding sequence (Figs. 2 and 3). This difference in electrophoretic mobility may be due to hydrophobicity, as previously observed with the 70 kDa peroxisomal membrane protein [21]. These results demonstrate that ALD-P is a component of peroxisomes. Peroxisomes are made of limiting membrane and matrix proteins.

Next we examined the distribution of ALD-P in peroxisomal membrane and peroxisomal matrix components isolated from purified peroxisomes using the sodium carbonate procedure [24]. This procedure releases all the matrix and membrane-associated proteins from the peroxisomes and leaves behind membranes containing phospholipids and integral proteins [24]. The peroxisomal membrane prepared by this procedure represents only 12% of the peroxisomal protein with phospholipid-to-protein ratio of 200 nmol/mg [24]. The antibodies against ALD-P cross-reacted with a 75 kDa protein in peroxisomal membranes, but no signal was detected in matrix proteins (Fig. 3). To further establish that the ALD-P is in fact a component of the peroxisomal membrane, we searched for the presence of the ALD-P in different subcellular organelles and peroxisomal membranes and matrix isolated from purified peroxisomes (Fig. 3) from X-ALD which were previously shown to have a deletion of the X-ALD gene [18] and absence of mRNA of the ALD gene [19]. The antibodies against ALD-P did not recognize any protein in peroxisomal membranes or matrix (Fig. 3) or in any of the other subcellular organelles in the gradient (data not shown here) from X-ALD fibroblasts with a deletion of the X-ALD gene.

The peroxisomal β-oxidation system consists of four enzymes: acyl-CoA ligases, acyl-CoA oxidase, trifunctional enzyme and thiolase. Fatty acids are converted to their CoA derivatives in the peroxisomal membrane and then transported to the matrix for their oxidation [26]. The deficient oxidation of lignoceric acid as compared to the normal oxidation of lignoceroyl-CoA in X-ALD peroxisomes [12,15], the deficient activity of lignoceroyl-CoA ligase in peroxisomes isolated from X-ALD [14,16], and the normalization of oxidation of lignoceric acid when peroxisomes from X-ALD were supplemented with exogenous lignoceroyl-CoA ligase [17], demonstrate that the abnormality in the oxidation of VLC fatty acids in X-ALD is due to a functional defect in the activity of lignoceroyl-CoA ligase present in the peroxisomal membranes.

Peroxisomal membranes contain at least three distinct acyl-CoA ligases with enzyme activities for palmitic acid [27–29], lignoceric acid [28,29] and phytanic acid [30]. The active site of palmitoyl-CoA ligase [27–29] and phytanoyl-CoA ligase [31] is on the cytoplasmic surface, and that of lignoceroyl-CoA ligase is on the luminal surface of the peroxisomal membrane [28]. Consistent with the topology of the active site of acyl-CoA ligases, phytanic acid [31] and palmitic acid are transported into peroxisomes as acyl-CoA derivatives [31,32], whereas lignoceric acid is transported into peroxisomes as free fatty acid and then activated to lignoceroyl-CoA on the luminal surface prior to its β-oxidation in the matrix [32].

This implies that CoASH, ATP and lignoceric acid, required for synthesis of lignoceroyl-CoA, need to be transported into peroxisomes for the activation of lignoceric acid by lignoceroyl-CoA ligase on the luminal surface of the peroxisomal membrane. The data reported in this manuscript clearly demonstrate that ALD-P is a component of the peroxisomal integral membrane. Therefore, the abnormality in the degradation of VLC fatty acids observed in X-ALD peroxisomes may be the result of the absence of or an abnormality in ALD-P in peroxisomal membranes.

Based on the possible function of ALD-P as a transporter protein, ALD-P may influence the activity of lignoceroyl-CoA ligase by either restricting the entry of cofactors required for its activity or as a result of an abnormality in the enzyme's activity. The deficient activity of lignoceroyl-CoA ligase in peroxisomes from X-ALD may not be due to restricted import of fatty acid or nucleotides into peroxisomes because molecules smaller than 800 Da (e.g. CoASH and ATP) are freely permeable through the non-specific pores present in this membrane [33], and no abnormality was observed in the transport of lignoceric acid into peroxisomes from X-ALD [32]. ALD-P is more likely to be involved in the transport of lignoceroyl-CoA ligase or may serve a structural function necessary for the activity of lignoceroyl-CoA ligase in the peroxisomal membrane. The fact that ALD-P could transport lignoceroyl-CoA ligase is questionable at least for two reasons; first, if we assume that ALD-P is indeed a transporter, it would seem unlikely

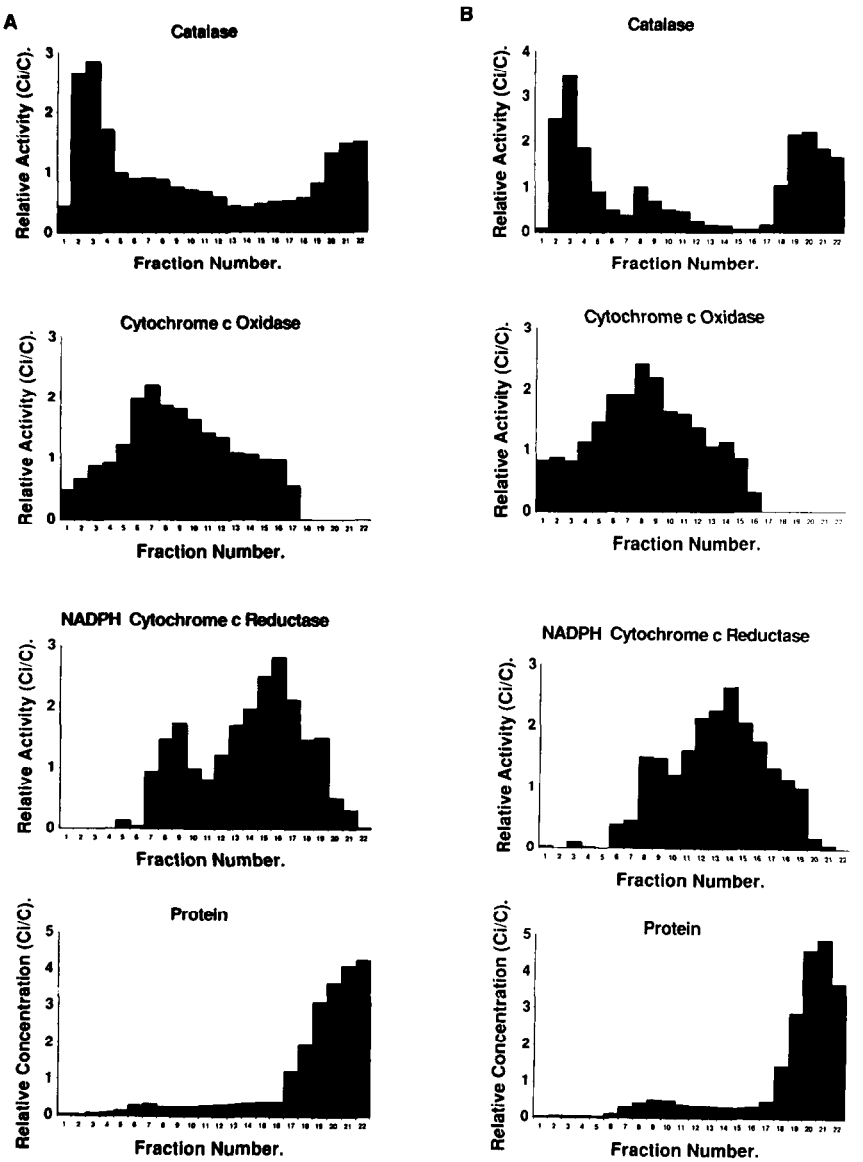

Fig. 1. Fractionation of the post-nuclear fraction from control (A) and X-ALD (B) cultured skin fibroblasts by isopycnic density gradient centrifugation. The distribution of subcellular organelles in the gradient was identified by their marker enzymes: catalase for peroxisomes, cytochrome *c* oxidase for mitochondria and NADPH cytochrome *c* reductase for endoplasmic reticulum.

that the function of ALD-P could be devoted to the import of only one protein (e.g. lignoceroyl-CoA ligase) because all other known functions of peroxisomes are normal in X-ALD. Secondly, based on what is known about the transport functions of ABC proteins [22], it also seems unlikely that ALD-P can import lignoceroyl-CoA ligase if lignoceroyl-CoA ligase is an integral membrane protein [28]. The ALD-P may rather serve a structural function necessary for the activity of lignoceroyl- CoA ligase, possibly as an anchor for the enzyme. Absence or a change in conformation of ALD-P in peroxisomal membranes may influence the activity of lignoceroyl-CoA ligase. Both models raise an intriguing possibility, namely, the function of ALD-P may not only be limited to the transport or the activity of lignoceroyl-CoA ligase. The definite function of ALD-P in peroxisomal membranes will await the purification of lignoceroyl-CoA ligase.

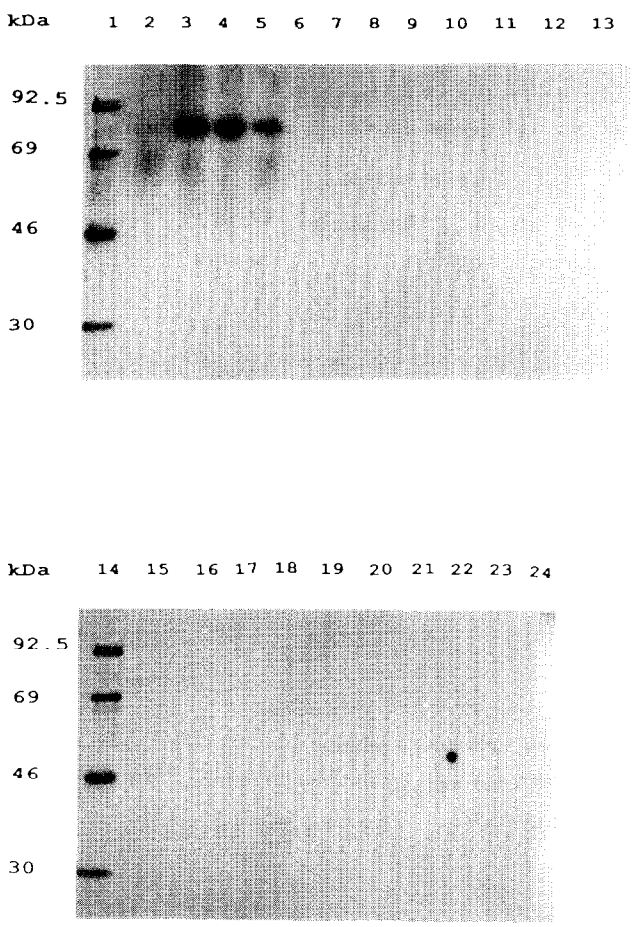

Fig. 2. Immunoblot analysis of subcellular organelles in fractions from control cultured skin fibroblast from a Nycodenz gradient with antibodies against ALD-P. 50 μg of proteins from each gradient fraction was resolved by electrophoresis and immunoblotted with antibodies against ALD-P. Lanes 1 and 14 are [^{14}C]methylated molecular weight markers (ICN Biomedicals Inc.) Lanes 2–13 and 15–24 are gradient fractions protein as shown in Fig. 1.

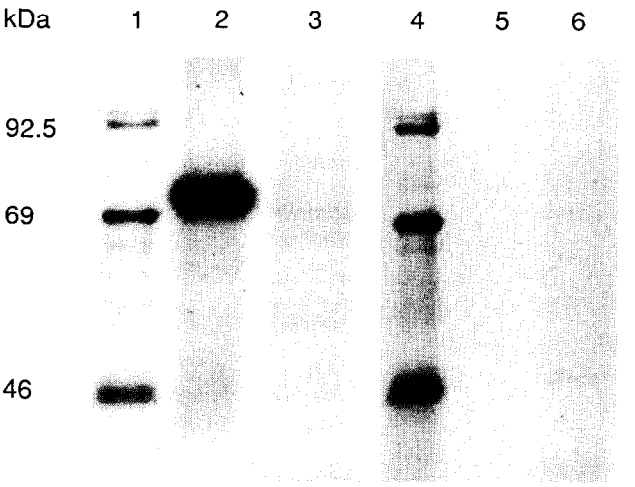

Fig. 3. Immunoblot analysis of membrane and matrix components of purified peroxisomes with antibodies against ALD-P. Lanes 1 and 4 represent molecular weight markers. Lanes 2 and 5 represent membrane and matrix proteins from control peroxisomes, and lanes 3 and 6 represent membrane and matrix proteins from X-ALD peroxisomes. 50 μg of protein was charged to each lane.

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