

Identification of Mutations in the Putative ATP-binding Domain of the Adrenoleukodystrophy Gene

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Abstract

The recently identified adrenoleukodystrophy (ALD) gene is predicted to encode a peroxisomal protein of 745 amino acids that includes one domain for ATP-binding, termed nucleotide-binding fold (NBF). To determine whether mutations occur in the putative NBF of ALD protein, we analyzed by denaturing gradient gel electrophoresis (DGGE) exon 6 and 8 that encode most part of this domain in 50 ALD patients. Four amino acid substitutions, three frameshift mutations leading to premature termination signal, and a splicing mutation were identified. These amino acid substitutions occurred at residues highly conserved in other ATP-binding cassette (ABC) proteins. In addition, a nonsense mutation was detected in exon 4. (*J. Clin. Invest.* 1994; 94:516–520.) **Key words:** peroxisomal disorder • point mutations • ABC transporter • denaturing gradient gel electrophoresis • X-linked genetic disease

Introduction

X-linked adrenoleukodystrophy (ALD)¹ is a neurodegenerative disorder characterized by impaired β -oxidation and abnormal accumulation of very-long-chain fatty acids (VLCFA) in neural white matter, adrenal glands, fibroblasts, and plasma (1). Using positional cloning strategies, the *ALD* gene has recently been identified and is predicted to encode a protein of 745 amino acids consisting of six membrane-spanning segments and a putative hydrophilic ATP-binding domain termed nucleotide-binding fold (NBF) (2). The NBF consensus sequences of ALD protein (ALDP) is almost identical to that in the mammalian 70-kD peroxisomal membrane protein (PMP) (3) and highly homologous to the NBFs of other ATP-binding cassette (ABC)

proteins (4). The majority of these ABC proteins are involved in transport events. In human, the ABC transporter family includes the cystic fibrosis transmembrane conductance regulator (CFTR) (5), the multidrug-resistant (MDR) gene product (6), TAP1 and TAP2 peptide transporters encoded in the MHC cluster (7, 8) and the 70-kD PMP (9). The function of ALDP is unknown but could serve the import of VLCFA-CoA synthetase whose activity is deficient in ALD (1). As observed for CFTR and MDR (10–12), mutations in the putative ATP-binding domain of ALDP could alter the coupling of ATP hydrolysis to the transport process. To search for such mutations, we amplified the corresponding exons by polymerase chain reaction (PCR), then assayed the amplified products for sequence variation using denaturing gradient gel electrophoresis (DGGE). This approach allowed us to identify four amino acid substitutions, three frameshift mutations leading to premature termination signal, and a splicing mutation. In addition, we report the first observation of a nonsense mutation in *ALD* gene that occurred in exon 4.

Methods

Patients. DNA samples were obtained from members of 50 ALD families selected among the 85 previously reported (2). All affected boys or men had increased level of VLCFA in plasma and/or fibroblasts. Clinical classification of their ALD phenotype was performed according to current criteria (1). Heterozygous status of women was determined by the demonstration of intermediate levels of VLCFA in their plasma and/or fibroblasts. Linkage analysis with the DXS52 probe (13) was performed in 7 families where the VLCFA results were equivocal.

Denaturing gradient gel electrophoresis design. DGGE parameters were determined using the MELT87 and SQHTX programs kindly provided by L. Lerman and colleagues (14). These computer algorithms predict the melting behavior of a DNA fragment on the basis of its base composition and nucleotide sequence. This information was used to select the positions of PCR primers suitable to generate fragments adapted to DGGE analysis, the range of denaturant concentrations and the electrophoresis run time that provides maximum resolution. One of the two amplification primers bore an additional 5' GC-rich oligomer required to create a high-temperature melting domain (15). The sequence of interest is then contained within the first melting domain. The gel apparatus used has been described elsewhere (16). 15 μ l of each amplified DNA sample was subjected to electrophoresis at 160 V (10 V/cm) in a 6.5% polyacrylamide gel containing a linearly increasing denaturant gradient (45–90%) for 4 h to analyze exon 6 and 6 h for exon 8 at 60°C. The gel was thereafter stained with ethidium bromide.

DNA amplification. Genomic DNA was extracted from peripheral blood cells and 1 μ g was used as template in a 100- μ l reaction mixture containing 50 mM KCl, 10 mM Tris, pH 8.4, 1 mM MgCl₂, 0.01% (wt/vol) gelatin, 200 μ M of each dNTP, 0.2 μ M of each primer, and 0.5 U Taq polymerase (ATGC Biotechnologie, Noisy le Grand, France); DMSO (2.5%) was added to amplify exon 6 and the 2,001-bp fragment. After denaturation at 94°C for 5 min, 35 cycles were then performed (1 min at 94°C, 1 min at the relevant annealing temperature, and 1.5

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1. **Abbreviations used in this paper:** ABC, ATP-binding cassette; ALD, adrenoleukodystrophy; AMN, adrenomyeloneuropathy; CFTR, cystic fibrosis transmembrane conductance regulator; DGGE, denaturing gradient gel electrophoresis; MDR, multidrug-resistant; NBF, nucleotide-binding fold; PMP, peroxisomal membrane protein; VLCFA, very-long-chain fatty acids.

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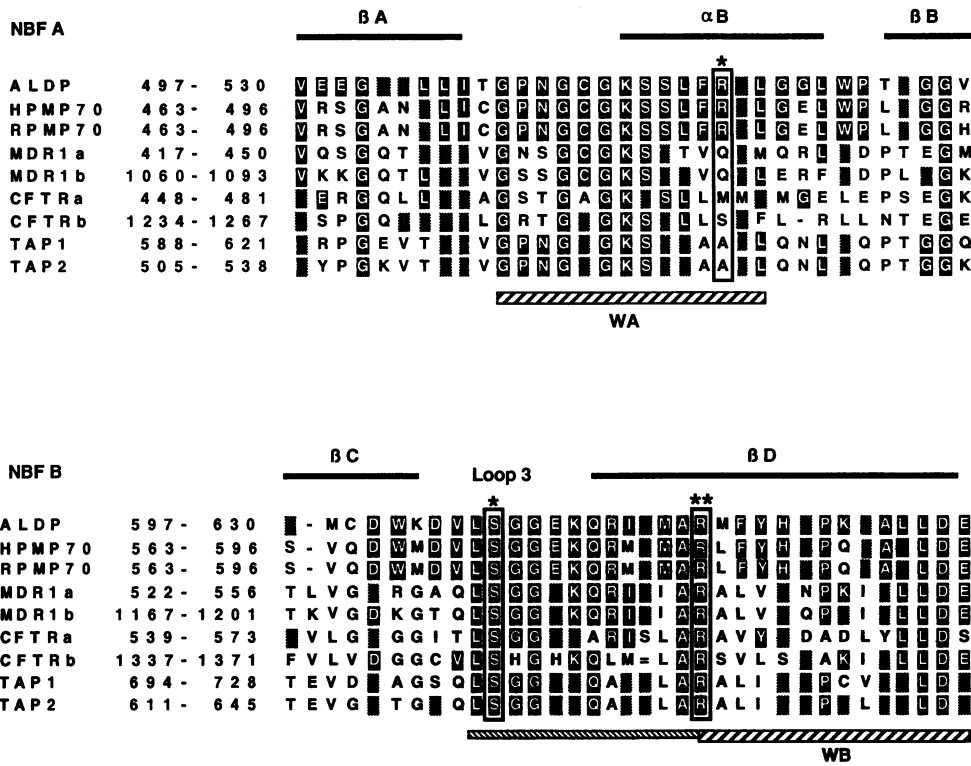


Figure 1. Alignment of the NBF of ALD protein (2) with sequences of other members of the ABC protein superfamily, including the human and rat 70-kD peroxisomal membrane protein (PMP70) (3, 9), the cystic fibrosis transmembrane conductance regulator (CFTR) (5), the human multidrug-resistant gene product (MDR) (6) and the peptide transporters TAP1 and TAP2 (7, 8). The conserved sequences include the two Walker motifs (indicated WA and WB), and the 12 amino acid segment highly conserved in other ABC proteins. Walker motif A corresponds to the glycine-rich P-loop known to be involved in phosphoryl transfer. Structural motifs (α helix indicated by αB and β sheets indicated by βA , βB , βC , and βD) are as in the structural model of Hyde et al. (4). Loop 3 extends from the core NBF. Asterisks indicate the position of the four missense mutations in ALD protein (ALDP): R518W substitution occurs at the same amino acid position as in the

CFTR mutant S1255P, and S606L at the same position as in the S549I or S549R CFTR mutants; R617C and R617H have no equivalents in CFTR mutants. Identity of amino acid between ALDP and other proteins are boxed in black and conservative amino acid changes are boxed in grey.

min at 72°C) and finally a 7-min extension at 72°C. The amplified products were analyzed on a 6% nondenaturing polyacrylamide gel electrophoresis and stained with ethidium bromide.

DNA sequencing of PCR products. PCR amplified fragments of exon 4 with a new BglII restriction site were directly sequenced after asymmetric amplification (17). For exons 6 and 8, direct sequencing of PCR-amplified genomic DNA did not allow unequivocal determination of nucleotide sequence alterations, and cloning of the mutant allele was necessary. The 251 or 283 bp PCR amplified fragments were subcloned using the TA Cloning system (Invitrogen, San Diego, CA) and positive clones were sequenced by the dideoxynucleotide chain termination method (18).

Results

Denaturing gradient gel electrophoresis analysis of exons 6 and 8. The structure of the *ALD* gene was recently determined (18a). This gene is 21 kb long and contains ten exons ranging in size from 86 to 1286 bp. The determination of flanking intron

sequences allowed PCR amplification of individual exons from genomic DNA for analysis of mutations. We have initially directed our effort towards the two exons that encode most of the well conserved NBF. Exon 6 (residues 497–546) includes the Walker A motif (Fig. 1). Exon 8 (residues 595–621) encodes most of the second part of the NBF including the 12 amino acid segment which is highly conserved in other ABC proteins and 6/14 amino acids of the Walker B motif.

Exons 6 and 8 were amplified making use of pairs of oligonucleotide primers (Table I) and single base pair differences were screened by denaturing gradient gel electrophoresis (DGGE) analysis (19). We first analyzed the obligate heterozygous mothers of 47 unrelated ALD patients who had no detectable deletion of the *ALD* gene. Each sample studied contains the mutant and the wild-type *ALD* alleles, a situation favoring the formation of heteroduplexes during the amplification process and enhancing the resolution of DGGE (Fig. 2). In three cases of de novo mutation, DNA from the ALD patient and

Table I. Primers Used for Amplification and DGGE Analysis of *ALD* Gene Exons

Exon	Upstream (5') primer	Downstream (3') primer	Annealing temperature °C	Amplified product bp
4	5'-TTGCCATGCTTCTCTCCCTG-3'	5'-CTGAGTGAGGGAGCGGGAAT-3'	60	325
6	5'-[55GC]AAGGGGCTGCGCTCTCT-3'	5'-CAGGAAGAGGTGGAGGAG-3'	55	283
8	5'-[50GC]CGGGTCTGGGTGCTGGTGG-3'	5'-GCCTCCCCACAGCTGCTACT-3'	64	251
8-10	5'-AGTCTGTGCCAAGGAAGCTG-3'	5'-CTGGCACGGTAGTCACATTG-3'	60	2001

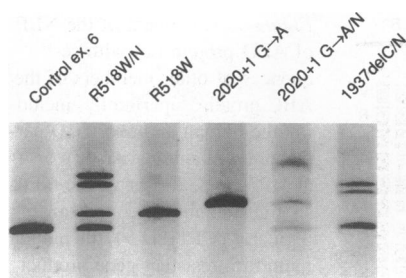


Figure 2. DGGE analysis of exon 6. ALD patients hemizygous for mutations R518W and 2020 + 1 G → A and their heterozygous mothers are shown. The pattern of 1937delC mutation exemplifies the efficiency of heteroduplexes to detect a nucleotide change that does not modify migration of homoduplexed molecules.

from a normal male were mixed. The sequence of DNA samples showing a shift in mobility was determined by direct sequencing after asymmetric amplification or subcloning (see Methods) (Fig. 3). While a 6% nondenaturing polyacrylamide gel revealed a single band at 251 bp as expected for exon 8, DGGE analysis showed the presence of additional bands (data not shown) that arised likely from related or pseudogenes (18a) and prevented interpretation of the results. To circumvent this problem, we first amplified a larger fragment (2,001 bp) containing exons 8 to 10 and then performed a nested-PCR with primers suited to generate a fragment adapted to DGGE analysis. This strategy allowed us to preferentially amplify the *ALD* gene.

Mutations in the first part of the nucleotide-binding fold. Three mutations were identified in exon 6 (Table II). A frameshift mutation (a 1-bp deletion at nucleotide 1937, 1937delC) (Fig. 3) was detected in the heterozygous mother of a boy who died at 13 years from cerebral ALD. This mutation is predicted to create a premature termination signal at codon 557. A missense mutation, C1938 → T was identified in an adult who developed adrenomyeloneuropathy (AMN) at 27 years. This mutation (Arg 518 → Trp or R518W in the single letter amino acid code) (Fig. 3) causes a nonconservative change between a charged and strongly polar (basic) amino acid and a hydrophobic nonpolar amino acid (Fig. 1). A splice mutation (2020 + 1 G → A) was detected in an adult who developed AMN at 28 years and who died at 43 years from cerebral involvement. This family (AB) illustrates the marked clinical variation of ALD: two infantile cerebral forms, one pure AMN and one Addison's disease were diagnosed among the brothers or nephews of this patient (Fig. 4). In this exon, we also detected a polymorphism (G1934 → A, CTG → CTA) which does

not change the coding for a leucine at codon 516 and which occurred in 11% of 150 X chromosomes tested.

Mutations in the second part of the nucleotide-binding fold. Three different missense mutations were identified in exon 8 (Table II). Each amino acid substitution involved a highly conserved residue of ABC transporters (Fig. 1). The C2203 → T (Ser606 → Leu or S606L) mutation was identified in a patient who had Addison's disease without neurologic involvement at 20 years. This mutation causes a nonconservative substitution between a hydrophilic uncharged polar amino acid and a hydrophobic nonpolar amino acid. The C2235 → T (Arg617 → Cys or R617C) mutation was discovered in a family where the index case died of cerebral ALD at 9 years. DNA from this patient being no more available, the mutation was shown to be present in his heterozygous mother and sister and absent in his normal brother, sister, and aunt. This mutation causes a nonconservative change between a charged and strongly polar basic amino acid and a weakly polar uncharged amino acid. The third missense mutation (G2236 → A) involved the same codon (Arg617 → His or R617H) and was discovered in a patient who developed AMN with cerebral involvement at 33 years. He is the only affected male among four brothers and his sister is not a carrier. The G2236 → A mutation, that leads to a conservative change at the first amino acid of the Walker B motif, was absent in DNA from the patient's mother who had normal plasma VLCFA levels (predicting a non carrier status). Therefore, the R617H mutation presented by this patient is likely to be a de novo mutation. These three missense mutations and the R518W were not observed in 100 normal X chromosomes, strongly suggesting that they are deleterious.

Frameshift mutations in exon 8 were identified in two other ALD families. A 1-bp deletion at nucleotide 2204 (2204delG) was discovered in a family where the affected proband has isolated Addison's disease at 21 years. A 2-bp deletion at nucleotide 2177 (2177delTA) was discovered in a family where two brothers had cerebral ALD at 7 and 9 years. Both mutations are predicted to create a premature termination signal at codons 635 and 599 respectively.

Nonsense mutation in exon 4. Spontaneous deamination of 5-methylcytosine to thymine on the coding strand results in C to T transitions and is responsible for recurrent mutations at CpG sites in various genetic diseases (20, 21). In particular, in the case of CGA (arginine) codons, a CGA to TGA transition creates a premature termination codon. Only one such codon was found in the *ALD* gene, at nucleotide 1776 in exon 4. This C to T transition creates a new BglII site. DNA from 50 ALD patients was amplified using specific primers (Table I) and digested with BglII restriction enzyme. The R464X mutation was found in one patient who had Addison's disease at 10

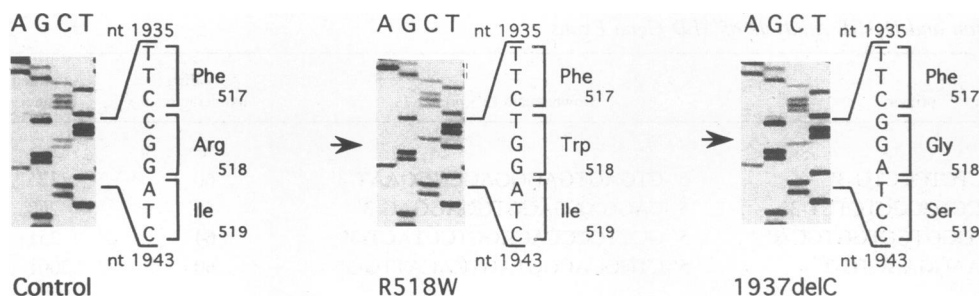


Figure 3. Partial nucleotide sequence of exon 6 in control, in the AMN patient with the R518W mutation and in the abnormal allele of the heterozygous mother with the 1937delC mutation.

years and developed adrenomyeloneuropathy at 15 years. This mutation is the first nonsense mutation identified in the *ALD* gene.

Discussion

The recently identified *ALD* gene was found partially deleted in 6 of 85 independent patients (2). The abnormal expression of messenger RNA in ALD fibroblasts, as well as the identification of a missense mutation between the fourth and the fifth transmembrane segment provided additional evidence that this gene is indeed responsible for ALD (22). Sequence homology between the ALD and the 70-kD PMP proteins suggests that they both belong to the family of ABC proteins (2). Typical ABC transporters are composed of four domains, two of which consist of six (α -helical) membrane-spanning segments (23). The other two domains are peripherally located at the cytoplasmic face of the membrane, bind ATP, and couple ATP hydrolysis to the transport process. Individual domains of ABC transporters are frequently expressed as separate polypeptides but almost every conceivable type of fusion has now been reported. For example, MDR and CFTR represent "full" transporters whereas TAP1 and TAP2, and likely ALDP and the 70-kD PMP represent "half-transporters." The presence, in ALD protein, of one nucleotide-binding fold which includes the five hydrophobic β sheets and the glycine-rich P-loop (Walker A) suggests that it is indeed an ATP-dependent transporter.

Previous studies (2) have shown that 7% of ALD patients (6/85) have a detectable deletion of *ALD* gene. We have now identified nine point mutations in the *ALD* gene. Of the NBF mutations, three are frameshift mutations leading to premature termination signal, one is a splicing mutation and four are amino acid substitutions. The three frameshift mutations and the 5' splice site defect are expected to result in a loss of function of the ALD protein. Analysis of a CpG site contained in an Arginine codon led to the characterization of a nonsense mutation in exon 4.

The four amino acid substitutions are located in a region which is highly conserved in the ABC family of transporters. Three of them (S606L, R617C, and R617H) involve invariant residues in all ABC proteins studied so far (Fig. 1). The molecular mechanisms by which all these missense mutations cause a loss of function are only speculative at this time. Nevertheless, in the light of *in vitro* studies performed on other ABC transporters, several hypotheses can be made.

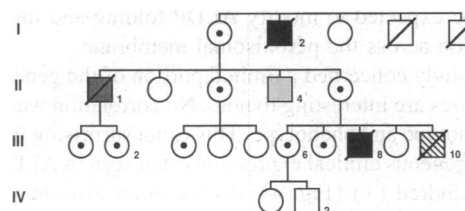


Figure 4. Pedigree of family AB. The 2020 + 1 G \rightarrow A mutation was observed in subject II₁ and III₂. A prenatal diagnosis was performed for subject IV₂ because his mother (subject III₆) was heterozygous. The Roman numerals represent the generations; the numbers beneath the symbols identify the individuals. Squares, male; circles, female. Clinical phenotypes of affected subjects are represented by the following symbols: (■) cerebral ALD (subjects I₂ and III₈); (▣) adrenomyeloneuropathy with cerebral involvement (subject II₁); (▧) adrenomyeloneuropathy (subject II₄); (⊞) Addison's disease (subject III₁₀ who died from adrenal insufficiency); (○) heterozygous women.

Mutations in the NBF can modify activity and/or regulation of ABC transporters through abnormal interaction with cytosolic nucleotides. The chloride channel activity of the CFTR mutant S1255P, which involves the same amino acid position in the NBF as in the ALD mutant R518W, is less sensitive to ATP stimulation (24) and the MDR mutants K433M or K1076M within the same Walker motif are unable to hydrolyze ATP (25). Alignment of sequences of the ATP-binding domain of the ALDP with those of other members of the ATP-binding protein superfamily (Fig. 1) predicts that arginines 518 and 617 would form part of the NBF core and serine 606 would be part of loop 3 (4, 26). This loop extends from the core and interacts probably with other components of the transporter to couple the energy of ATP hydrolysis to the transport process (23). Therefore mutations at these amino acids are likely to prevent binding and/or hydrolysis of ATP.

Another molecular mechanism leading to protein dysfunction could be a defective processing of the protein. The CFTR mutants S549I and S549R, that involve the same amino acid position as in the ALD mutant S606L, fail to produce mature CFTR and therefore prevent trafficking to the correct cellular localization (12). Since the three amino acid substitutions reported in this study involved residues that are presumed to be exposed at the surface of the ALD protein, the drastic changes

Table II. Mutations Detected in the *ALD* Gene

Name	Nucleotide change	Effect on coding sequence	Exon	Clinical phenotype
R464X	C \rightarrow T at 1776	Arg \rightarrow Stop at 464	4	AMN*
1937delC	Deletion of C at 1937	Frameshift	6	cerebral ALD
R518W	C \rightarrow T at 1938	Arg \rightarrow Trp at 518	6	AMN
2020 + 1 G \rightarrow A	G \rightarrow A at 2020 + 1	5' splice signal	Intron 6	ACMN [†]
2177delTA	Deletion of TA at 2177	Frameshift	8	cerebral ALD
S606L	C \rightarrow T at 2203	Ser \rightarrow Leu at 606	8	Addison
2204delG	Deletion of G at 2204	Frameshift	8	Addison
R617C	C \rightarrow T at 2235	Arg \rightarrow Cys at 617	8	cerebral ALD
R617H	G \rightarrow A at 2236	Arg \rightarrow His at 617	8	ACMN

* Adrenomyeloneuropathy; [†]adrenomyeloneuropathy with cerebral involvement.

we detected can be expected to modify ALDP folding and impair its translocation across the peroxisomal membrane.

Although our study concerned a limited portion of the gene, some genetic features are interesting to note. No correlation was found between genotype and phenotype. This is not surprising in view of the heterogeneous clinical expression often seen in ALD within the same kindred (1) (Fig. 4). As for other disorders, phenotypic variability of ALD is likely to be influenced by environmental factors and/or modifying genes.

The findings of different mutations in different families (this study, and references 2 and 22) is a strong indication of the highly heterogeneous nature of ALD mutations. This was indeed expected, as a recent study of 90 French ALD families over three generations, by assay of plasma VLCFA, established that a de novo mutation occurred in 7.8% of ALD cases and that the first recognized mutation occurred in the mother of 42% of index cases (F. Rocchiccioli and P. Aubourg, unpublished results). It is thus unlikely that a major mutation will be found, as this is the case for the $\Delta F508$ defect in cystic fibrosis. Mutation screening will thus be important for genetic counselling and prenatal diagnosis, especially when biochemical analysis and/or indirect linkage analysis are not informative.

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