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Mutational and Protein Analysis of Patients and Heterozygous Women with X-Linked Adrenoleukodystrophy

Valérie Feigenbaum,[1,*] Gäel Lombard-Platet,[2,*] Sylvie Guidoux,[1] Claude-Olivier Sarde,[2] Jean-Louis Mandel,[2] and Patrick Aubourg[1]

[1]INSERM U342, Hôpital Saint-Vincent de Paul, Paris; and [2]Institut de Génétique et de Biologie Moléculaire et Cellulaire, INSERM, CNRS, Université Louis Pasteur, Illkirch

Summary

X-linked adrenoleukodystrophy (ALD), a neurodegenerative disorder associated with impaired β-oxidation of very-long-chain fatty acids (VLCFA), is due to mutations in a gene encoding a peroxisomal ATP-binding cassette (ABC) transporter (ALD protein [ALDP]). We analyzed the open reading frame of the ALD gene in 44 French ALD kindred by using SSCP or denaturing gradient-gel electrophoresis and studied the effect of mutations on ALDP by immunocytofluorescence and western blotting of fibroblasts and/or white blood cells. Mutations were detected in 37 of 44 kindreds and were distributed over the whole protein-coding region, with the exception of the C terminus encoded in exon 10. Except for two mutations (delAG1801 and P560L) observed four times each, nearly every ALD family has a different mutation. Twenty-four of 37 mutations were missense mutations leading to amino acid changes located in or close to putative transmembrane segments (TMS 2, 3, 4, and 5), in the EAA-like motif and in the nucleotide fold of the ATP-binding domain of ALDP. Of 38 ALD patients tested, 27 (71%) lacked ALDP immunoreactivity in their fibroblasts and/or white blood cells. More than half of missense mutations studied (11 of 21) resulted in a complete lack of ALDP immunoreactivity, and six missense mutations resulted in decreased ALDP expression. The fibroblasts and/or white blood cells of 15 of 15 heterozygous carrier from ALD kindred with no ALDP showed a mixture of positive- and negative-ALDP immunoreactivity due to X-inactivation. Since 5%–15% of heterozygous women have normal VLCFA levels, the immunodetection of ALDP in white blood cells can be applicable in a majority of ALD kindred, to identify heterozygous women, particularly when the ALD gene mutation has not yet been identified.

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Address for correspondence and reprints: Patrick Aubourg, Inserm U342, Hôpital Saint-Vincent de Paul, 82 Avenue Denfert-Rochereau, 75014 Paris, France.

*These two authors contributed equally to this work.

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Introduction

X-linked adrenoleukodystrophy (ALD) is a neurodegenerative disorder characterized by progressive demyelination within the CNS, adrenal insufficiency, accumulation of very-long-chain fatty acids due to an impairment of peroxisomal β-oxidation, and marked phenotypical variation within the same kindred (Moser et al. 1995). The ALD gene encodes a 75-kD protein (ALDP) with six putative membrane-spanning regions and one ATP-binding domain (Mosser et al. 1993). ALDP belongs to the superfamily of ATP-binding cassette (ABC) proteins (Higgins 1992) and is located in the membrane of peroxisome (Contreras et al. 1994; Mosser et al. 1994; Watkins et al. 1995). ALDP shows significant homology to a growing family of peroxisomal ABC transporters that includes the human and rat 70-kD peroxisomal protein (PMP70) (Kamijo et al. 1990; Gärtner et al. 1992); ALDPR, a closer relative of ALDP whose gene has been recently cloned in human and mouse (Lombard-Platet et al. 1996); and two yeast proteins, PXA1 (Shani et al. 1995) and YKL 741 (Bossier et al. 1994), that are orthologues of ALDP and PMP70. Yet the function of ALDP is unknown. ALDP could play a role in the transport of cofactors necessary to very-long-chain fatty acids (VLCFA)–CoA synthetase activity or import of VLCFA-CoA into peroxisomes.

Intragenic deletions of the ALD gene were initially identified in ≈6% of ALD patients (Mosser et al. 1993). Since then, heterogeneous point mutations have been detected in many ALD kindred (Cartier et al. 1993; Barceló et al. 1994; Berger et al. 1994; Fanen et al. 1994; Fuchs et al. 1994; Kemp et al. 1994; Uchiyama et al. 1994; Braun et al. 1995; Kok et al. 1995; Ligtenberg et al. 1995; Yasutake et al. 1995). Moreover, ALDP could not be detected in lymphoblasts and/or fibroblasts lines of several ALD patients by using western blotting or immunocytofluorescence (Mosser et al. 1994; Watkins et al. 1995).

We report here the systematic analysis of ALD gene mutations over the whole protein-coding region in 44 unrelated French ALD families. Where possible, the effect of various mutations was correlated with ALDP expression in peripheral white blood cells and/or cultured skin fibroblasts by using indirect immunofluores-

Table 1

Primers Used for Amplification and SSCP or DGGE Analysis

Exon	Forward (5′) Primer	Reverse (3′) Primer	Length (bp)	Annealing (°C)	Analysis	Primer Designation
1	5′-ACCAGCCCCAGTCCCTAC-3′	5′-GGCGGGCGCCTGAAGACC-3′	188	58	SSCP	SA1F/SA1R
1	5′-ATGGAGCCCACAAAGTCTAC-3′	5′-GCCACATACACCGACAGGAA-3′	255	58	SSCP	SA2F/SA2R
1	5′-TTCCCCCGGGTCCTGTGC-3′	5′-AGAAGTAGAGGCGGTAGGC-3′	279	58	SSCP	SB1F/SB1R
1	5′-CCCTCCCTGCTACCTTCG-3′	5′-GGCTTGGTCAGGTTGGAG-3′	231	58	SSCP	SB2F/SB2R
1	5′-CATGGACGGGCGGCTTCG-3′	5′-CCACCAGCTCCCCGAACT-3′	253	58	SSCP	SC1F/SC1R
1	5′-CGCCGGCCTCTCGTGGTGTT-3′	5′-GGCCTGCCCACACCTTTG-3′	288	58	SSCP	SC2F/SC2R
2	5′-AGCCTCTCTGTGTCTGTCACC-3′	5′-TCTTGGAGCCCTGGGTCTCAC-3′	231	64	SSCP	EX2F/EX2R
3	5′-ATTTGCAGAAGAGCCTCGCC-3′	5′-[55GC]CAGGGAGAGAAGAAGCATGGC-3′	314	60	DGGE	KS3F/SG20
4	5′-TTGCCATGCTTCTCTCCCTG-3′	5′-[55GC]CTGAGTGAGGGAGCGGGAAT-3′	405	55	DGGE	SG5/SG19
5	5′-AGAATGCAGAGGGGTCGCCA-3′	5′-[55GC]CCACCTGCCACGTACATCTA-3′	324	60	DGGE	SG24/SG21
6	5′-[55GC]AAGGGGCCTGCGCTCTCT-3′	5′-CAGGAAGAGGTGGAGGAG-3′	283	55	DGGE	SG10/SG9
7	5′-[50GC]TGGATGCGTGTGAGCGTGG-3′	5′-GCACCTGGCACTTTAGACTC-3′	409	60	DGGE	SG18/SG14
8–10	5′-AGCTCTGTGCCAAGGAACTG-3′	5′-CTGGCACGGTAGTCACATTG-3′	2001	60		SG1/SG2
8	5′-[50GC]CGGGTCTGGGTGCTGGTGG-3′	5′-GCCTCCCCACAGCTGCTACT-3′	250	64	DGGE	GCALD8/ALD8
9	5′-TGGAGGGTGCACAGACTCTC-3′	5′-[55GC]CTGCTGATGACACCCGCCT-3′	323	62	DGGE	SG25/SG21
10	5′-GTGCCGTGCCCCTGACC-3′	5′-TGGGTGCTGCTGTCTCCTT-3′	348	58	SSCP	5RP/3RP

cence and western blotting. We also tested the usefulness of studying ALDP expression in white blood cells and/or skin fibroblasts from 15 heterozygous women, as an adjunct to determine carrier status in ALD families.

Material and Methods

Genomic DNA from ALD Patients

DNA was extracted from fresh blood, skin fibroblasts, or lymphocytes immortalized with Epstein-Barr virus as described elsewhere (Aubourg et al. 1990: Mosser et al. 1993). DNA samples were obtained from at least one ALD proband and/or one obligatory carrier of 44 ALD kindreds. These 44 ALD kindreds include 23 of 50 kindreds from an earlier study in which mutation search was restricted to exons 4, 6, and 8 (the nine patients in whom mutations were identified were naturally excluded from this study [Fanen et al. 1994]) and 21 new ALD kindreds. The choice of studied kindred was based on urgency of genetic counseling and/or DNA availability. All heterozygous women and affected males had increased levels of VLCFAs in plasma and/or fibroblasts (Aubourg et al. 1985). Clinical classification of ALD phenotype was performed according to current criteria (Moser et al. 1995).

PCR Amplification

Primers were designed according to the published ALD genomic sequence (Sarde et al. 1994) and are listed in table 1. These primers cover the coding sequence and flanking intronic sequences that extends from 39 bp upstream of the ATG initiation codon to 65 bp downstream of the stop codon in exon 10.

Exon 1.—PCR reactions were run in a Perkin Elmer 9600 thermocycler with 0.2 U *Taq* DNA polymerase (Cetus), 10 pmol of two primers, 100–200 ng of genomic DNA, in a final volume of 25 µl of 1 × *Taq* polymerase buffer (Cetus), 1.5 mM $MgCl_2$, 10% glycerol, and 40 nM α^{32}P-dATP for SSCP analysis (30 cycles of 94°C for 10 s, 58°C for 10 s, and 72°C for 10 s).

Exon 2.—PCR amplifications were performed with 0.5 U *Taq* DNA polymerase (Cetus), 5 pmol of two primers, 100–200 ng of genomic DNA, in a final volume of 25 µl of 1 × *Taq* polymerase buffer (Appligene) complemented with 10% dimethyl sulfoxide (DMSO) and 40 nM α^{32}P-dATP for SSCP analysis. (30 cycles of 94°C for 10 s, 64°C for 10 s, and 72°C for 10 s).

Exons 3–9.—PCR amplifications were performed in a Perkin Elmer 480 thermocycler with 0.5 U *Taq* DNA polymerase (Appligene), 20 pmol of each primer, 200 pmol of each dNTP, 500 ng of genomic DNA in a final volume of 100 µl of 1 × *Taq* polymerase buffer (Appligene). (35 cycles of 94°C for 60 s, 60 s at melting temperature [T_m] of corresponding exon, 72°C for 90 s). T_m's were 60°C, 55°C, 60°C, and 55°C, respectively, with 2.5% DMSO for exons 3–6, and 60°C, 64°C, and 62°C, respectively, for exons 7–9; T_m was 60°C with 2.5% DMSO for the amplification of the 2,001-bp fragment comprising exons 8–10 (Fanen et al. 1994).

Exon 10.—PCR amplifications were performed in a Perkin Elmer 480 thermocycler with 0.125 U *Taq* DNA polymerase (Appligene), 5 pmol of each primer, 250 ng of genomic DNA in a final volume of 25 µl of 1 × *Taq* polymerase buffer (Appligene) complemented with 10% DMSO, and 40 nM α^{32}P-dATP for SSCP analysis (30 cycles of 94°C for 60 s, 58°C for 60 s, and 72°C for 90 s).

Mutation Detection

SSCP (exons 1, 2, and 10).—PCR fragments were heat denatured in formamide and loaded on 0.2-mm-thick 6% acrylamide:bis-acrylamide (49:1), 10% glycerol, 90

mM Tris, 90 mM borate, 2 mM EDTA. Electrophoresis was performed below 40 W for 180 Wh, at room temperature for exons 1 and 2 and at 4°C for exon 10. Gels were dried on Wattman 3MM and exposed to x-ray films. DNA samples from the seven patients with no mutation were reanalyzed for the first exon by SSCP, using Pharmacia PhastSystem®, but no additional mutation could be detected.

Denaturing Gradient-Gel Electrophoresis (DGGE) (Exons 3–9).—This step was performed as described elsewhere (Fanen et al. 1994). To generate heteroduplexes, DNA from ALD and normal males was mixed, was denatured at 94°C for 10 min, and was heated for 30 min at the annealing temperature of the exon. Running time was 4 h for exon 3; 5 h for exons 4 and 6; 6 h for exons 5, 7, and 9; and 7 h for exon 8.

DNA Sequence Analysis

DNA fragments showing variant migration with SSCP or DGGE were amplified by PCR, were phenol-chloroform extracted, were purified on Centricon® 100 (Amicon), and were sequenced by the dideoxynucleotide chain termination method by using their own primers either manually with Sequenase (Amersham) or on an ABI 373A automated DNA sequencer. Mutant alleles that did not show unequivocal determination of nucleotide sequence alterations were subcloned using the TA Cloning system (Stratagene), and positive clones were sequenced manually. Suspected mutations were confirmed at least on two independent PCR amplifications and on sequence determination, and eventually by restriction fragment analysis when mutations affected or created restriction sites.

Western Blotting and Immunocytofluorescence

Whole fibroblast and/or white blood cell protein extract, SDS-PAGE, and western blotting were performed as described elsewhere (Cartier et al. 1995). Peripheral white blood cells (lymphocytes and monocytes) were obtained by centrifugation of total blood sample (5 ml) on Ficoll gradient. After washing and resuspension in PBS (1×10^6 cells/ml), cells were cytospinned at 100 *g* for 10 min. Immunocytofluorescence of fibroblasts and white blood cells with anti-ALDP and anti-catalase antibodies as well double localization studies were performed as described elsewhere (Cartier et al. 1995). The two anti-ALDP monocolonal antibodies (mAbs) used in this study (1D6 and 2B4) have been characterized elsewhere (Mosser et al. 1994).

Results

Analysis of Mutations on Genomic DNA

At least one affected male and/or one heterozygous women from each of 44 kindred were systematically analyzed for the 10 exons by using SSCP or DGGE. In four families where DNA from a male ALD proband was not available, the study was performed on DNA from at least one obligate heterozygote and one normal woman of the family

A mutation of the ALD gene was detected in 37 of 44 families (table 2). Twenty-nine different mutations were found, and five of them were present in more than one family (S98L, R518W, and R660W in two families, and 1801 delAG and P560L in four families each). Apart from delAG 1801-1802 mutations, all recurring mutations were found at CpG sites. There was no indication that families in which recurring mutations occurred were related. A de novo mutation was identified in 2 of 37 cases (patients 29 and 76). The mutation found in patient 29 was likely due to a germinal or somatic mosaicism: no ALD gene mutation was found in the fibroblasts or lymphocytes of the mother, who has normal plasma VLCFA levels, while patient 29 has a heterozygous sister.

Twenty-five families showed a single nucleotide substitution: 24 lead to a single amino acid substitution and one to a stop codon. Ten mutations resulted in a frameshift leading to a premature stop codon that included a recurring dinucleotide mutation in exon 5 (del AG position 1801-1802) found in four families. One trinucleotide insertion leading to the addition of valine at position 491 was observed in patient 48 and his brother. This mutation was present in the heterozygous mother and absent in normal brothers and sisters.

No mutations were detected in seven families. In six of these seven kindreds, there was evidence of X-linked inheritance of the disease; the plasma VLCFA levels of obligate heterozygotes were typical of values that are observed in X-linked ALD carriers, and the plasma VLCFA levels of patients' fathers studied in five of seven cases were normal. Patient 49 was the only affected member in his family and likely has a de novo ALD gene mutation, since his mother, father, aunts, and sister had normal VLCFA levels.

ALDP Expression in Fibroblasts and/or Peripheral White Blood Cells from ALD Patients

The fibroblasts and/or white blood cells of 38 of 44 ALD patients were screened for the presence or absence of ALDP by immunocytofluorescence (38 of 38) and/or western blotting (30 of 38). We used two mAbs (1D6 and 2B4) that detect a 75-kD protein in normal fibroblasts and white blood cells and show a punctate pattern that is identical to that observed with other peroxisomal proteins (catalase, acyl-CoA oxidase, 3-oxo-thiolase, and PMP70) (Mosser et al. 1994; Watkins et al. 1995). ALDP is expressed at a higher level in monocytes than in lymphocytes (see fig. 1*E*). mAb 1D6 recognizes one epitope of ALDP between codon Leu 279 and Val 482, and 2B4 recognizes one epitope of ALDP between Ile 495 and Lys 648. Fibroblasts and/or peripheral white

Table 2

Mutations in the ALD Gene in Studied Patients

Kindred Number	Clinical Phenotype[a]	Mutations[b]			Localization in Protein[c]	Amino Acid Alteration	Amino Acid Homologue in[d]		ALDP by IF/WB[e]
		DNA	CpG	Exon			h/m ALDRP	hPMP70	
64	CALD, AMN	G416A		1		W10 X			Absent
84	CALD	Ins T524		1		Frameshift at L46			Not done
53	CALD	C679T	+	1	TMS2	S98L	S	A	Present
33	CALD, AS	C679T	+	1	TMS2	S98L	S	A	Present
39	AD	C700T		1		T105I	T	T	Absent
18	CALD, AMN	C709G		1		S108W	S	D	Decreased
4	CALC	G732A	+	1		G116R	G	T	Absent
55	AD	A829G		1	TMS3	N148S	N	N	Present
27	AD	C840T	+	1	TMS3	R152C	R	K	Present
43	AD	Del TA927-28		1		Frameshift at Y180			Absent
75	ALMD	A928G		1		Y181C	Y	Y	Not done
66	CALD	A985T		1	TMS4	D200V	D	D	Not done
19	CALD, AMN	A1048G		1	TMS4	D221G	D	D	Absent
63	CALD	DelGC1080-81		1		Frameshift at R231			Absent
32	CALD, AMN, AD	C1174T		1		P263L	P	R	Decreased
47	AMN	G1266A	+	1	EAA-like	A294T	A	A	Not done
67	ALMD	ins C1521		3		Frameshift at V378			Absent
26	CALD	1636delC		4		Frameshift at T416			Absent
1	ALMD	DelAG 1801-02		5		Frameshift at E471			Absent
61	CALD	DelAG 1801-02		5		Frameshift at E471			Absent
11	AMN	DelAG 1801-02		5		Frameshift at E471			Absent
58	ALD	DelAG 1801-02		5		Frameshift at E471			Absent
48	AD, AMN, AS	ins TGG 1848		5		ins val 491			Absent
59	CALD, AS	G 1920 A		6	Walker A	G512S	G	G	Absent
73	CALD	C1938T	+	6	Walker A	R518W	R	R	Absent
76	CALD	C1938T	+	6	Walker A	R518W	R	R	Decreased
83	AD	G1950A		6		G 522 W	G	E	Absent
69	CALD	C2065T	+	7		P560L	P	P	Decreased
5	AMN, ALMD	C2065T	+	7		P560L	P	P	Decreased
37	CALD	C2065T	+	7		P560L	P	P	Decreased
13	CALD	C2065T	+	7		P560L	P	P	Absent
65	AMN, ALMD	C2065G	+	7		P560R	P	P	Absent
56	CALD	G 2166+1 A		7		Splice at G593			Not done
78	CALD, AMN, ALMD	T2202C		8	Walker B	S606P	S	S	Absent
29	CALD	DelGC 2335	+	9		Frameshift at D649			Absent
22/51	CALD, ALMD, AS	C2364T	+	9		R660W	R	R	Absent
10	ALMD	C2364T	+	9		R660W	R	R	Absent
79	CALD	No mutation found							Not done
68	AMN	No mutation found							Absent
3	CALD, AMN	No mutation found							Absent
16	AD	No mutation found							Absent
49	AD	No mutation found							Present
52	AMN	No mutation found							Absent
57	CALD	No mutation found							Absent

[a] CALD = cerebral ALD (5–15 years); AMN = adrenomyeloneuropathy; ALMD = adrenomyeloneuropathy with cerebral involvement; AD = Addison disease; AS = Asymptomatic.

[b] Nucleotide numbers refer to the published sequence of ALD cDNA (Mosser et al. 1993; European Molecular Biology Library database z21876). In a case of frameshift or splice defect mutation, the last amino acid residue that is not altered in the expected protein is indicated.

[c] Walker A and B motives refer to a consensus stretch of amino acids that are present in all ABC proteins, including CFTR and MDR (Walker et al. 1982).

[d] h = human; and m = mouse.

[e] IF/WB = immunofluorescence/western blotting.

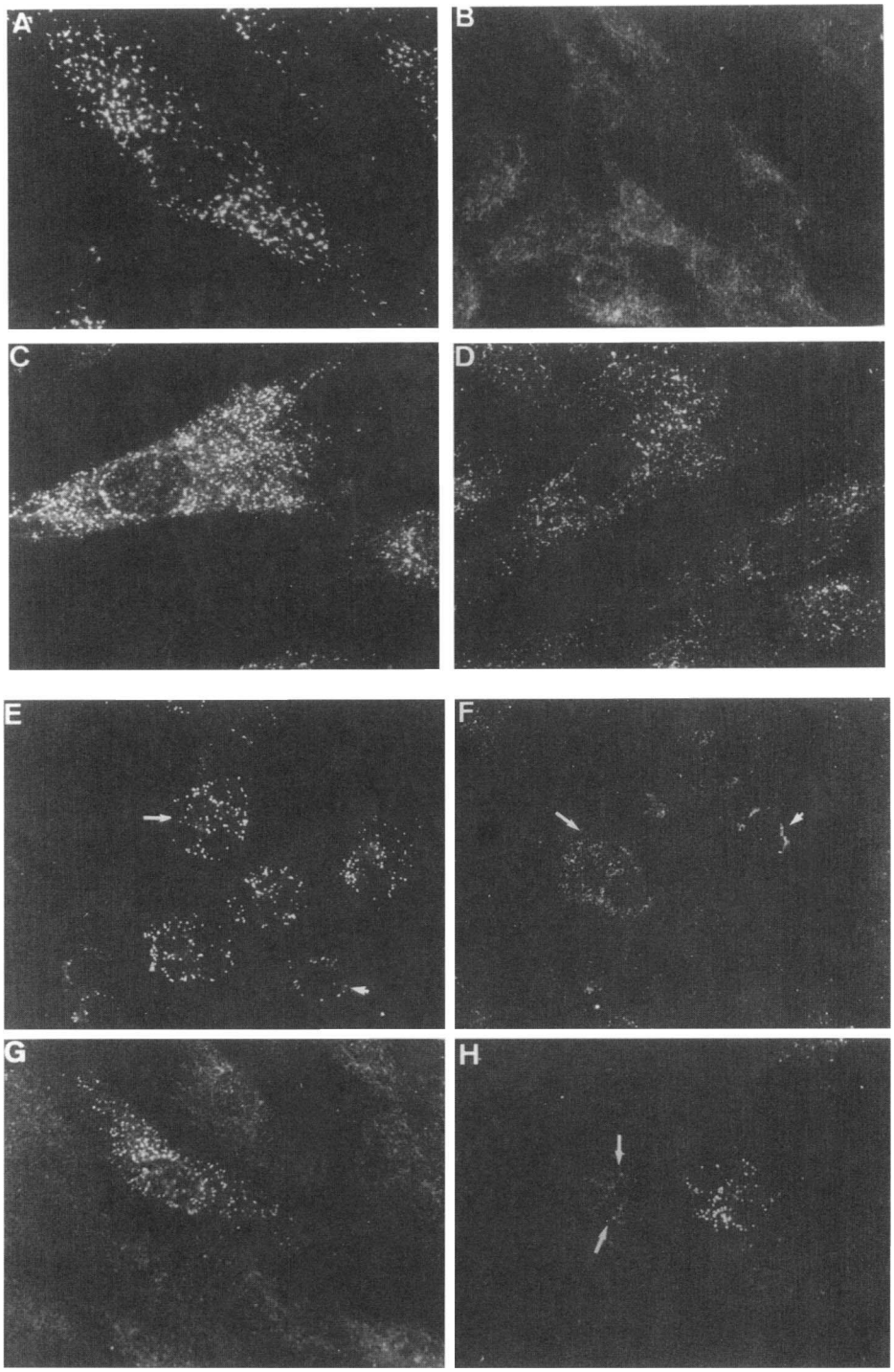

Figure 1 ALDP immunofluorescence of fibroblasts and white blood cells from ALD patients and ALD heterozygous women. Skin fibroblasts and/or white blood cells from 38 ALD patients and 15 heterozygous women were examined by indirect immunocytofluorescence, as described in Material and Methods. The results are summarized in table 2. *A,* normal fibroblasts immunostained with mAb 1D6, showing normal punctate pattern. *B,* ALD fibroblasts (delAG 1801-1802) immunostained with mAb 1D6, showing a lack of ALDP immunoreactivity. *C,* ALD fibroblasts (R152C mutation) immunostained with mAb2B4, showing normal ALDP immunoreactivity. *D,* ALD fibroblasts (P560L mutation), showing weakly positive immunoreactivity with mAb 2B4 (and 1D6, not shown). *E,* normal white blood cells immunostained with mAb 1D6. Note that ALDP is expressed at higher level in monocytes (*arrow*) than in lymphocytes (*short arrow*). *F,* ALD white blood cells (R660W mutation) immunostained with mAb 2B4, showing the absence of ALDP immunoreactivity. *G* and *H,* fibroblasts and white blood cells of heterozygous women (D221G mutation) immunostained with mAb 1D6 in panel G and 2B4 in panel H. Arrows in panel H show a monocyte with a complete absence of ALDP immunoreactivity close to a monocyte with normal ALDP immunoreactivity. Similarly, in panel G, a two-cell population of fibroblasts is seen: one with normal ALDP immunoreactivity and one with a complete lack of ALDP expression.

blood cell from ALD patients and controls showed a normal distribution and presence of peroxisomes after immunocytofluorescence with anticatalase antibody (not shown). A similar punctate pattern as observed for catalase was observed in normal fibroblasts or white blood cells by using 1D6 or 2B4 (fig. 1*A* and *E*). No specific signal was observed with both ALDP antibodies in 10 of 10 studied ALD fibroblasts or white blood cells in which a frameshift or nonsense mutation has been identified, indicating that the full-length ALDP is not stably expressed in these cells (fig. 1*B*). ALDP was not detectable by immunostaining in 11 (52.3%) of 21 ALD fibroblasts or white blood cells with an independent missense mutation in the ALD gene (fig. 1*F*). While 4 (19%) of 21 fibroblasts or white blood cells with missense mutations had a normal immunofluorescence pattern (fig. 1C), 6 (28.6%) of 21 showed a weak and decreased immunoreactivity with both ALDP antibodies (fig. 1*D*). Double staining with anti-ALDP and anticatalase was performed in two ALD fibroblast lines with normal ALDP immunocytofluorescence (S98L and R152C mutants). In both cases, the mutant ALDP colocalized with catalase in peroxisomes (not shown).

Variation in the immunoreactivity of the ALD mutant protein was observed for two mutations, P560L and R518W: immunoreactivity of mutant protein P560L was markedly decreased in fibroblasts and peripheral white blood cells from patients 69, 5, and 37 and was completely absent in patient 13. Although the 10 exons were studied in the last family, we cannot formally exclude that an additional and yet undetected mutation is responsible for the complete absence of ALDP. Similarly, ALDP immunoreactivity of mutant protein R518W was markedly decreased in fibroblasts from patient 76 and completely absent in fibroblasts from another patient (73) with the same mutation.

The results of western blotting were concordant in all cases when ALDP immunoreactivity was completely absent or normally present in ALD fibroblasts and/or white blood cells (fig. 2). Five of the six ALD fibroblasts or white blood cells (patients 5, 18, 32, 69, and 76) with decreased ALDP immunoreactivity showed a weak 75-kD ALDP band on western blotting. The 75-kD band was not visible in the last patient (37). No mobility shift was observed for patients with a frameshift or nonsense mutation.

Overall, 33 (86.8%) of 38 of ALD fibroblasts or white blood cells showed a complete lack or marked decrease of ALDP immunoreactivity by immunofluorescence or western blotting.

ALDP Expression in Fibroblasts and/or Peripheral White Blood Cells of ALD Heterozygous Women

Fibroblasts and/or peripheral white blood cells of 15 unrelated heterozygous women were studied using immunocytofluorescence. These women belong to families in which fibroblasts and/or peripheral white blood cells showed a complete absence of ALDP by immunocytofluorescence. Fibroblasts and white blood cells from these 15 women showed normal peroxisomes with catalase antibody (not shown). In 15 of 15 fibroblasts and/or peripheral white blood cells, two populations of cells were observed after immunostaining with ALDP antibodies (fig. 1*G* and *H*): one had normal-appearing punctate pattern and one completely lacked ALDP immunoreactivity. While 100% of normal fibroblasts are ALDP positive, 23%–86% of heterozygous fibroblasts or white blood cells/monocytes lacked ALDP immunoreactivity. VLCFA levels were not reevaluated at the time of the immunocytofluorescence study in these fibroblasts or white blood cells. Therefore, a correlation could not be established between the intracellular levels of VLCFA and the proportion of cells that did not express ALDP. In five cases, ALDP expression was studied in both fibroblasts and white blood cells of the same woman. Fibroblasts and white blood cells from these women showed the two cell populations, but the fraction of cells that did not express ALDP differs significantly in fibroblasts and peripheral white blood cells from the same woman in four of five cases. There was no obvious shift favoring the mutant allele. Thus, we found no support for the suggestion that there may be a selection process favoring fibroblasts with an ALD mutation on the active X chromosome (Migeon et al. 1981). Five of these 15 women had clinical symptoms ranging from mild to severe adrenomyeloneuropathy. There was no apparent correlation between the clinical expression of the disease and the fraction of fibroblasts that did not express ALDP.

Discussion

In this study, we identified mutations in 37 of 44 ALD patients examined so far, and we have demonstrated the heterozygous state of the mutations in 31 of 37 patients' mothers. A de novo mutation was observed in 3 of 44 ALD patients. This frequency (6.8%) is in accordance with the previous estimation (7.8%), which was based on VLCFA analysis in ALD kindred (7.8%; Fanen et al. 1994). This frequency appears lower than the 20% expected for an X-linked disease with a reproductive fitness of ~.4 (estimated proportion of childhood cerebral form of .6), and may indicate that late-onset forms are in fact more prevalent, as suggested by a recent Dutch study (van Geel et al. 1994), and/or that more ALD mutations arise more frequently in the paternal than maternal germ line. As reported elsewhere (Fanen et al. 1994; Braun et al. 1995; Kok et al. 1995; Ligtenberg et al. 1995), 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. Phenotypic variation of

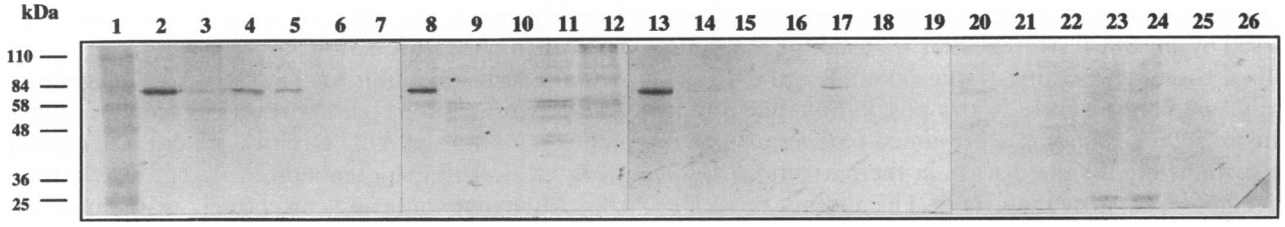

Figure 2 Immunoblot analysis of ALDP in ALD fibroblasts or white blood cells. Fibroblasts (lanes 2–12) and/or white blood cells (lanes 13–26) from 5 controls and 30 ALD patients were examined with mAb 1D6 (lanes 2–12) and 2B4 (lanes 13–26) as described in Material and Methods. Lane 1, protein markers; lane 2, control; lane 3, patient 18 (S108W); lane 4, patient 32 (P263L); lane 5, patient 5 (P560L); lane 6, patient 4 (G116R); lane 7, patient 19 (D221G); lane 8, patient 33 (S98L); lane 9, patient 78 (S606P); lane 10, patient 3 (no mutation found); lane 11, patient 37 (P560L); lane 12, patient 22 (R660W); lane 13, control; lane 14, patient 39 (T105I); lane 15, patient 4 (G116R); lane 16, patient 43 (frameshift at Y180); lane 17, patient 5 (P560L); lane 18, patient 59 (G512S); lane 19, patient 29 (frameshift at D649); lane 20, patient 69 (P560L); lane 21, patient 19 (D221G); lane 22, patient 64 (W10X); lane 23, patient 63 (frameshift at R231); lane 24, patient 52 (no mutation found); lane 25, patient 61 (frameshift at E471); and lane 26, patient 83 (G522W).

ALD is likely due to the influence of modifier genes (Maestri and Beaty 1992; Moser et al. 1992) but also to stochastic factors (Sobue et al. 1994). The majority of mutations were found only once in the kindred investigated. Exceptions are a dinucleotide deletion (del 1801-1802) and P560L missense mutation, which were both observed in four kindreds, and S98L, R518W, and R660W missense mutations, which were each observed in two kindreds. As shown in ALD Dutch families (Kemp et al. 1994), there is no indication that the French ALD families in whom the dinucleotide deletion (del 1801-1802) or the P560L mutation was observed were related. The frequency of delAG 1801-1802 mutation was quite similar in French (11%), U.S. (12%), and Dutch (14%) ALD families (this study; Ligtenberg et al. 1995; Kok et al. 1995).

In 24 (64.9%) of 37 kindreds, the mutation affected a single amino acid residue (table 2 and fig. 3). Since no direct functional assay of ALDP is available, definitive proof is lacking that these mutations cause ALD. However, it is unlikely that these alterations are merely polymorphisms. In all cases, only one alteration was found, and except for the S98L, R518W, P560L, and R660W mutations, these alterations differed from each other. None of these missense mutations were detected in families with a frameshift, nonsense, or splice mutation. The R518W mutation occurred as a de novo mutation in one ALD patient, and the R518W and P560L mutations were not observed in 100 normal X chromosomes. Moreover, the missense mutations observed affect 17 amino acid positions, which are all identical between ALDP and ALD-related protein (ALDRP, 66% sequence identity) (Lombard-Platet et al. 1996), while 11 of these are also identical in PMP70 (38% overall identity with ALDP), suggesting that they indeed affect functionally or structurally important residues (see table 2).

Four missense mutations (G512S, R518W, G522W, and S606P) were found in the ATP-binding domain. Three of these mutations (G512S, G522W, and S606P) affect amino acid residues that are identical among other ABC transporters (see fig. 4 in Mosser et al. 1993 and fig. 1 in Fanen et al. 1994). One missense mutation (A294T) was found in an EAA-like motif that is highly conserved in ALDP, ALDPR, and PMP70 proteins (Shani et al. 1995) and whose mutations can cause loss of transporter function of an ABC protein (Köster and Braun 1992). One mutation (Y181C) was found in the loop between the third and fourth transmembrane domain, and two mutations (D200V and D221G) were found in the third transmembrane domain. Recent studies have suggested a role in the corresponding region for targeting and inserting PMP70 in the peroxisomal membrane (Leiper et al. 1995).

No mutations of the ALD gene were detected in seven families. We cannot exclude the possibility that some mutations occurred in the promoter region or

Figure 3 Distribution of mutations in the ALD gene. The boxes represent the coding region distributed over the 10 exons (Sarde et al. 1994); the lines between these boxes represent introns (not in scale). Shaded boxes in exons 1 and 2 represent regions encoding the six putative transmembrane segments; horizontal-hatched box in exon 1 indicate an EAA-like motif (Koster and Bohm 1992); hatched-boxes in exons 6, 8, and 9 represent the 2-nt-binding fold of the ATP-binding domain. Each vertical bar represents the location of a mutation detected. For the frameshift mutations, the last amino acid that is not altered is indicated. The arrow represent the alteration of a splice donor site.

in introns of the ALD gene. Others may have been missed by the SSCP strategy that we used for scanning half of the open reading frame (exon 1 and 2). Formally, we cannot exclude the possibility that mutations in a gene encoding a presumed partner of ALDP are responsible for the disease in the patients with no detectable ALD gene mutations. The absence of ALDP immunoreactivity that was demonstrated in five of six studied fibroblasts and/or white blood cells from these patients could reflect instability of ALDP in the absence of a putative partner within the peroxisomal membrane. This possibility has recently been demonstrated for PXA1 and YKL 741, two peroxisomal ABC transporter homologues of ALDP in yeast. PXA1 becomes nearly or completely undetectable when its partner YKL is absent in the peroxisomal membrane (Shani et al. 1995). A close relative of ALDP, recently cloned (Lombard-Platet et al. 1996), could therefore be a candidate disease gene in these patients. This possibility is, however, unlikely, because the ALDR gene is autosomal, while there was strong evidence of X-linked inheritance of the disease in five of six patients. Last, retroviral-mediated transfer of ALD cDNA in fibroblasts from patient 3 corrected completely their VLCFA metabolism (Cartier et al. 1995).

Fibroblasts and/or white blood cells from 27 (71%) of 38 ALD patients lacked punctate immunoreactive material or the 75-kD ALDP band when examined with two anti-ALDP antibodies, by immunocytofluorescence and western blotting. Correlation of immunocytofluorescence and western blotting with specific mutations was possible in 32 patients. All patients with nonsense or frameshift mutations lacked ALDP in their fibroblasts and/or white blood cells. Fibroblasts and or white blood cells from 50% (11 of 21) of patients with missense mutations also showed negative immunocytofluorescence and no detectable ALDP in western blotting. These missense mutations probably result in the synthesis of an unstable protein that is rapidly degraded in the cytosol. Another possibility is that some of these mutations (in particular, Y181C, D220V, and D221G) may affect the targeting of ALDP into peroxisomal membrane. Missense mutations leading to a lack of ALDP included three mutations located in the ATP-binding domain (G512S, G522W, and S606P). Four missense mutations (S108W, P263L, R518W, and P560L) resulted in decreased ALDP immunoreactivity reflecting likely instability and/or partial deficiency in the peroxisomal targeting of ALDP. Three missense mutations (S98L, N148S, and R152C) resulted in the synthesis of a stable but presumably nonfunctioning protein. These mutations are in hydrophobic areas of putative transmembrane domain (TMS2 and TMS3) and alter the charge and/or hydrophobicity of the affected residue. Since these mutations did not prevent synthesis of full-length protein, the affected residues are likely to be critical for ALDP function.

Our results on mutational analysis of ALD gene and ALDP expression have important consequence for genetic counseling of ALD families. As did other groups (Kok et al. 1995; Ligtenberg et al. 1995), we found that ALD gene mutations are largely distributed over the whole coding region and that a majority (78%) of ALD kindreds have different ALD gene mutations. As discussed above, our failure to detect mutations in all ALD families may be at least in part explained by the strategy we used to identify mutations in the first and second exons. It may be preferable to use direct sequencing of PCR products derived from fibroblasts or lymphocyte cDNA to search for mutations in the ALD gene (Ligtenberg et al. 1995). Since the plasma VLCFA assay currently used to identify ALD carriers is not totally satisfactory (5%–15% of heterozygous women have normal VLCFA levels), screening for the mutation would be the most reliable assay to establish the carrier status in ALD kindreds. The distribution of the mutations over the whole coding region, however, complicates such detection. Study of ALDP expression in white blood cells (or fibroblasts) may help to circumvent this problem. Fibroblasts and/or white blood cells of all obligate heterozygous ALD kindreds with absent ALDP showed a mixture of positive and negative ALDP immunoreactivity due to X-inactivation. Seventy-six (84%) of 90 fibroblasts or white blood cells of ALD hemizygous patients we have examined so far lack ALDP immunoreactivity. This diagnostic procedure, which is rapid (3 h) and requires a fresh blood sample of only 5 ml, would therefore be applicable to a large majority of ALD families. Although we did not find evidence for skewed-X inactivation in fibroblasts or lymphocytes of ALD carriers and Watkiss et al. (1993) reached the same conclusions when using the M27-beta probe (DXS225) to differentiate between the active and inactive X chromosome in 12 ALD carriers, one should keep in mind the rare possibility of a false-negative diagnosis in case of a biased inactivation resulting in the normal X being active in a large majority of cells. Recently, we also have successfully used this immunofluorescence test as an adjunct to VLCFA assay in the prenatal diagnosis of seven ALD pregnancies.

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