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# cDNA Cloning and mRNA Expression of the Human Adrenoleukodystrophy Related Protein (ALDRP), a Peroxisomal ABC Transporter[1]

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**We have cloned the cDNA containing the complete coding region of the human adrenoleukodystrophy related (ALDR) gene. The 2220-bp open reading frame encodes a 740-amino-acid polypeptide with a predicted molecular weight of 83.3 kDa. The human ALDR protein displays high similarity (62.8% identical amino acid residues) to the human adrenoleukodystrophy (ALD) gene. Analysis of ALDR expression revealed the presence of ALDR mRNA in a variety of human tissues, predominantly in brain and heart. This expression pattern is different from all other known peroxisomal ABC-transporters. Defects in the ALD gene are the primary cause of adrenoleukodystrophy, a demyelinating disorder of the central nervous system. The ALD protein (ALDP) and the ALDR gene product are peroxisomal membrane proteins belonging to the superfamily of transporters containing an ATP-binding cassette (ABC-transporters). All known peroxisomal ABC-transporters represent only one-half of a functional transporter. They are expected to form dimers either as a homodimer or as a heterodimer. ALDRP is a potential dimerization partner of ALDP or other peroxisomal ABC-transporters. The ALDR gene is a candidate for a modifier gene, accounting for the strikingly varying clinical courses of ALD observed even within a family.** 

X-linked adrenoleukodystrophy (ALD), a demyelinating disorder of the nervous system, is characterized by the elevation of very long chain fatty acids in tissues and plasma (1). ALD is the most frequent human peroxisomal disease affecting one in 20 000 males. In addition to the pathology of the central nervous system ALD is a major cause of adrenal insufficiency. The primary cause of ALD is a defect in the ALD gene, however, striking variations of the clinical course have been observed in affected members from the same family. Additional genetic factors may play a role in the pathogenesis of the disease and account for this observation (2). The cloning of the ALD-gene (3) has revealed that its product (ALDP) belongs to a superfamily of membrane transporter proteins carrying an ATP-binding cassette (ABC-transporters) (4). The high homology to PMP70, a human peroxisomal ABC-transporter of unknown function (5), was immediately recognized. We have recently cloned PMP69, another human ABC-transporter which, by homology, was also placed into this subgroup (6). These molecules (also referred to as "half-transporters") represent only one half of a functional transporter such as the p-glycoprotein or the cystic fibrosis transmembrane conductance regulator (CFTR) and are therefore thought to associate with another half-transporter molecule to form a homodimer or a heterodimer. For the yeast peroxisomal ABC-transporters, which are involved in fatty acid $\beta$-oxidation, heterodimerization has been demonstrated (7-9). Lombard-Platet and collegues (10) have isolated the cDNA of a mouse gene (mALDR) by a PCR-based approach searching for ABC-transporters. Mouse ALDRP displays very high homology (63.7 % amino acid identity) to ALDP from the same species. ALDRP is a potential dimerization partner of ALDP. In this article we report the cloning of the full coding region of the human ortholog of the mouse ALDR gene and data on tissue distribution of ALDR mRNA-expression.

## METHODS

*cDNA cloning.* Based on a human ALDR cDNA fragment reported by Lombard-Platet *et al.* in their original description of the

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mouse ALDR gene (9), we designed primers for 5′ RACE-PCR in order to obtain the 5′ end of the cDNA. Human liver adaptor-ligated cDNA (Marathon PCR-Ready cDNA, Clontech) was used as a template in nested PCR-reactions. The PCR product resulting from oligonucleotide P1r (5′-AGTATTTCTGTGTTCTCTGCAG-3′) and adaptor primer AP1 (Clontech) was boosted in a 1:100 dilution with primers P2r (5′-CTTGCCAATGATGGGATAGAGG-3′) and AP2 (Clontech). The resulting products were cloned into the vector pGemT-easy (Promega) and sequenced using vector primers. A 1300-bp cDNA fragment 3′ from the previously reported cDNA fragment was amplified using a human-specific forward primer P3f (5′-AGGACTTGTGGTGTATGCCACTGC-3′) and a reverse primer (mP4r) derived from mouse cDNA (5′-CAGTTGCATTTTGGGAATTCCAGC-3′). This fragment was also cloned and sequenced. Forward primers from the 3′ end of the resulting consensus sequence P5f (5′-GTGAGGGAGGTTGGCGCTTTG-3′) and P6f (5′-GCTTTGAACAATTGGATACTG-3′) were used in a nested 3′ RACE-PCR procedure that yielded 3′ sequence beyond the termination signal. All PCR reactions were performed with Amplitaq Gold (Perkin Elmer). DNA-sequencing was performed on an ABI 377 sequencer using the dideoxy dye terminator technology.

*Northern blot analysis of ALDR expression.* A human multiple tissue Northern blot containing 20 $\mu$g of poly $A^+$ RNA per lane was purchased from Clontech. The 1300-bp DNA fragment derived from PCR amplification with primers P3f and mP4r and cloned into the pGemT-easy vector was excised with *Not*I, purified by gel electrophoresis and [$\alpha$-$^{32}$P]dATP labeled by random priming. This probe was used for Norhern blot hybridization following the method described previously (11).

*RT-PCR analysis of ALDR expression.* RNA from a variety of human tissues and cultured cells was extracted using the RNAzol reagent (Biozol). Approximately 1$\mu$g of RNA was reverse-transcribed in a 15 $\mu$L reaction using the First Strand cDNA Synthesis Kit from Pharmacia. 1 $\mu$L of the reaction was used in a 50 $\mu$L PCR with ALDR-specific primers P6f (5′-GAAGCCTTTACCACTGCT-3) and P7r (5′-CCTGCTGGTGTAATTATGGG-3′). This primer combination was selected such that an ALDR-specific 311-bp product was amplified from cDNA but two introns of 2800 and 800 bp respectively are positioned between them on genomic DNA level (Holzinger *et al.* unpublished data). This strategy prevented amplification of genomic DNA potentially contaminating the RNA extracts. RT-PCR was not performed under conditions allowing quantitative analysis. Amplification of a DNA fragment corresponding to the mRNA of PMP69 was performed from the same random-primed cDNAs as a control for cDNA integrity.

*Computational analysis.* The *ClustalW* online program from the Baylor College of Medicine was used for the generation of protein alignments. Protein similarity scores were determined using the program *Align* from EERIE, Montpellier, France.

## RESULTS

### *cDNA Cloning and Primary Protein Structure*

We have cloned a 2395-bp cDNA containing the full coding region of the human ALDR gene. The open reading frame of 2220 bp encodes a 740-amino acid protein with a predicted molecular weight of 83.3 kDa. This protein displays 93.5 % amino acid identity in an alignment with mouse ALDRP and can therefore be regarded the human ortholog. Homology with human ALD is 62.8 % (Fig.1) which is almost identical to the close relationship of 63.7 % amino acid identity in an alignment of mouse ALDRP with mouse ALDP. Human ALDRP conforms to the model of an ABC-half-transporter containing the highly conserved Walker A an B motifs (12) as well as the C-sequence which are regarded the inclusion criteria for the superfamily of ABC-transporters. The EEA-like motif (13), shared by all peroxisomal ABC-transporters, was also identified (Fig. 1).

### *Tissue Distribution of ALDR mRNA Expression*

The distribution of ALDR mRNA expression was examined by virtue of Northern blot analysis as well as RT-PCR. Northern blot analysis demonstrates the presence of an ALDR-specific signal of an approximate size of 5.5 kb predominantly in brain but also in heart tissue. Additionally a transcript of roughly 4.4 kb was detected (Fig. 2). No signal was detected in any other tissue analyzed.

In RT-PCR, a 311-bp fragment corresponding to ALDR mRNA could be amplified from random-primed cDNA derived from human uterus, lung, liver, placenta, thy-

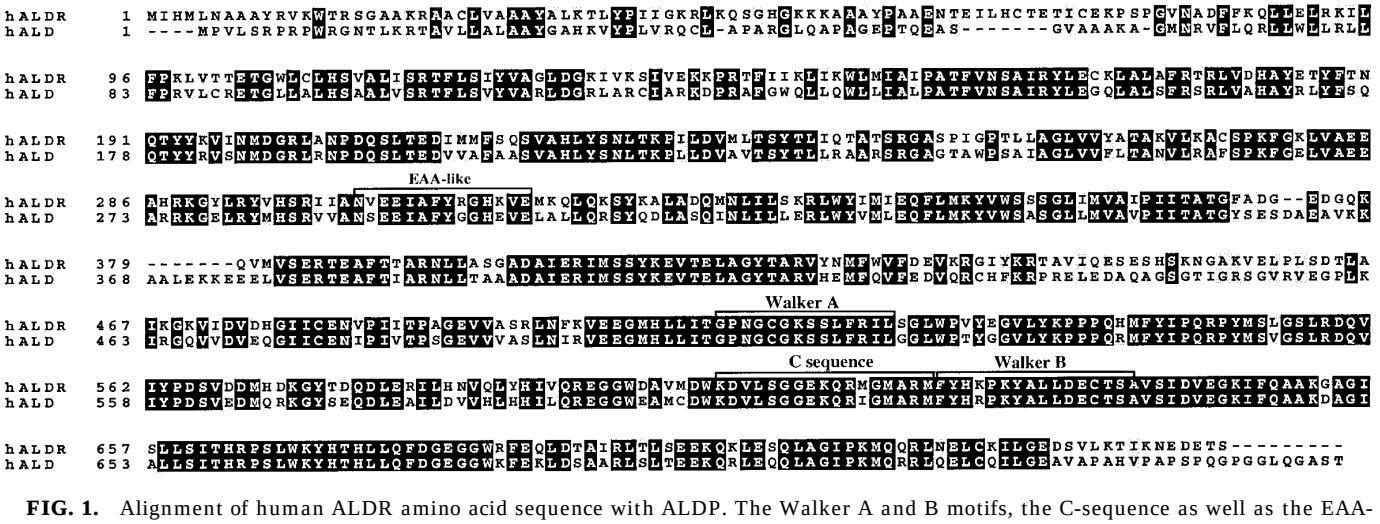


**FIG. 1.** Alignment of human ALDR amino acid sequence with ALDP. The Walker A and B motifs, the C-sequence as well as the EAA-like motif shared by peroxisomal ABC-transporters are present in ALDRP. The $NH_2$-terminus represents the region of weakest similarity.

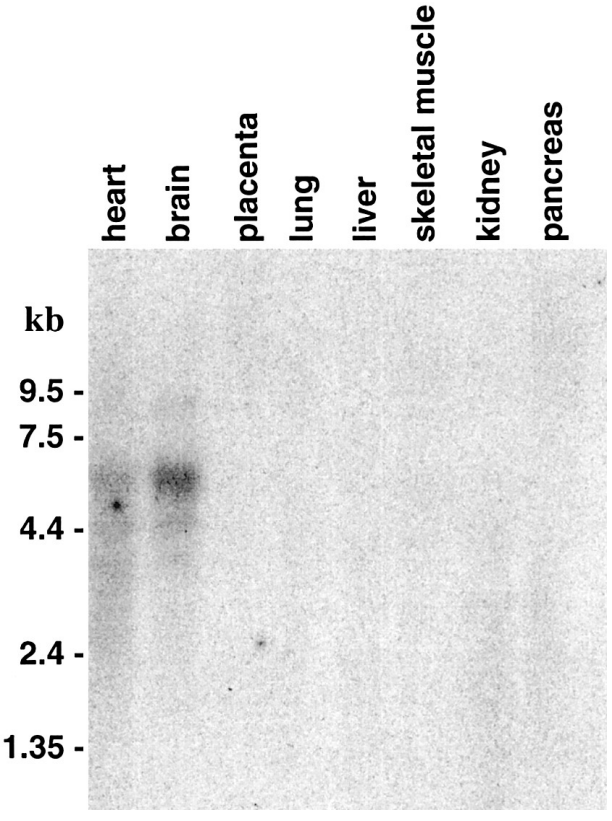


**FIG. 2.** Northern blot analysis of ALDR mRNA expression in various tissues. The predominant transcript of approximately 5.5 kb and an additional transcript of 4.4 kb were detected in brain and heart tissue.

mus, colon, and leukocytes but not from cultured skin fibroblasts (Fig. 3). Control PCR amplifications of a PMP69 fragment yielded a product of correct size in amplifications from all cDNAs examined (data not shown).

## DISCUSSION

We have cloned the cDNA of the human adrenoleukodystrophy-related protein. Protein alignments demonstrate 93.5 % amino acid identity to mouse ALDR which was previously cloned by Lombard-Platet *et al.* (10) who also showed the exclusively peroxisomal localization. A predominant band of 5.5 kb was detected in Northern blot analysis in addition to a transcript of 4.4 kb. This is in contrast to the previously reported single human ALDR transcript size of 4.2 kb (14). The ALDR-specific signal was predominantly found in brain but also in heart. The relatively high expression levels in brain are consistent with data from mouse ALDR (10). In Northern blot, no specific signal was detected in other tissues including lung, liver and placenta. ALDR mRNA could be readily detected in these organs using RT-PCR. The higher sensitivity of RT-PCR compared to Northern blot analysis enabled detection of ALDR mRNA in all tissues examined with the exception of cultured skin fibroblasts. This study, however, did not include morphological analysis to determine ALDR expression in certain cell types within organs. It is conceivable that low level ALDR mRNA detected in liver, placenta and lung might be derived from specific low abundant cell types only, thus explaining the low expression level.

The cloned cDNA of 2395 basepairs contains the full coding region (2220 bp) but apparently lacks most of the 3′ untranslated region since a polyA stretch immediately following the termination codon has been recognized by polyT oligonucleotides during the generation of cDNA. A consensus polyadenylation signal (ATTAAA) can be found 30 bp upstream from this polyA such that it is probably a result of posttranscriptional polyadenylation. The larger transcript detected in Northern blot analysis, however, must result from utilization of a polyadenylation signal localized further downstream and/or from a transcriptional start further upstream. The short transcript detected during PCR cloning apparently represents only a small fraction of all existing ALDR transcripts.

The similarity to ALDP is weak at the $NH_2$-terminus given the high overall conservation (Fig. 2), which is true for comparisons among all peroxisomal ABC-

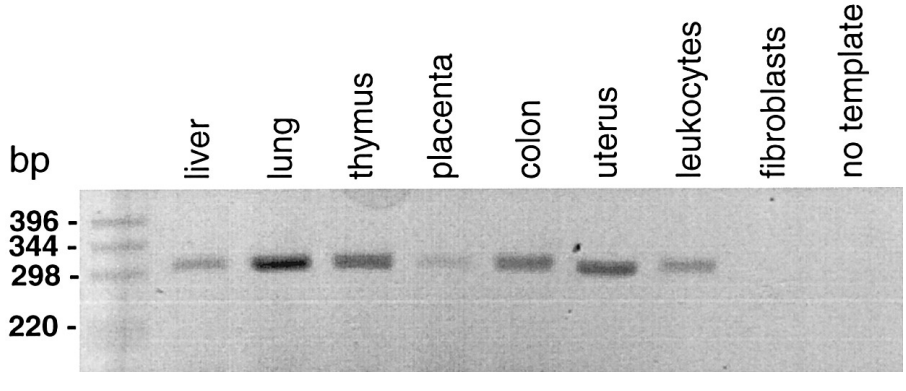


**FIG. 3.** RT-PCR analysis of mRNA-Expression in various tissues. An ALDR-specific 311-bp DNA-fragment could be amplified from all tissues and cells examined with the exception of cultured skin fibroblasts.

transporters. This sequence is of particular interest with regard to specific function. Highly conserved regions are probably needed for purposes shared by all peroxisomal ABC-transporters such as general structure formation, targeting, insertion into the membrane and nucleotide binding. The 55 $NH_2$-terminal aminoacids are not necessary for targeting and membrane insertion of a COOH-terminally epitope-tagged mouse ALDR fusion protein (10). General transmembrane topology models of peroxisomal ABC-transporters predict the $NH_2$-terminus to be localized on the cytoplasmic side of the peroxisomal membrane (12). It might therefore be justified to speculate that a specific receptor and/or substrate-binding function is associated with the $NH_2$-terminus of peroxisomal ABC-transporters.

The striking similarity to the adrenoleukodystrophy protein (ALDP), the peroxisomal localization and the general concept that ABC-half-transporters require a partner protein from the same family have led to the hypothesis that ALDR might be a heterodimer partner of ALD. Evidence such as an expression pattern of ALDR different from ALDP (10) or differential inducibility by fenofibrate (14) indicates, that ALDR is at least not the exclusive partner. For example, in this study we could not detect ALDR mRNA in cultured human skin fibroblasts. This is in strong contrast to ALDP which can be readily detected on RNA and protein level and the absence of which from fibroblast cell lines derived from ALD-patients is associated with a $\beta$-oxidation defect and the accumulation of VLCFA. Apparently ALDR is not needed for VLCFA $\beta$-oxidation in cultured human fibroblasts. The expression pattern of ALDR is different from all other known peroxisomal ABC-transporters such that obligate exclusive heterodimerization with any of the other known peroxisomal ABC-transporters is unlikely. Functionally related ABC-transporters such as the TAP proteins can sometimes be found to cluster in specific chromosomal localizations (15). The chromosomal localization of the human ALDR gene has been determined to be chromosome 12q11-12q12 (16). All known peroxisomal ABC-transporters, however, are localized on different chromosomes (3, 6, 16, 17) such that the location of these genes in the genome does not yield any information on functional relations. ALDR is predominantly expressed in the central nervous system, the major location of disturbances in ALD. Therefore it might play a role as a brain-specific "isoform" of a peroxisomal ABC-transporter involved in functions related to VLCFA $\beta$-oxidation. In addition to a specific spatial function, a temporal one during ontogenesis is also conceivable. Future experiments will include complementation of $\beta$-oxidation by expression of ALDRP in ALDP-deficient cell lines which naturally do not express ALDRP to determine a possible role in $\beta$-oxidation. If ALDRP is in fact involved in $\beta$-oxidation it might be an important factor accounting for the variations of the clinical course observed in ALD. The ALDR gene might thus be a modifier gene of adrenoleukodystrophy. Protein-protein interaction studies with other peroxisomal ABC-transporters will also be of importance to clarify the physiological role of ALDRP.

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