The mouse gene encoding the peroxisomal membrane protein 1-like protein (PXMP1-L): cDNA cloning, genomic organization and comparative expression studies

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Abstract PXMP1-L (synonyms: PMP69, P70R) is a peroxisomal protein that belongs to the ABC-transporter superfamily. Its closest homolog is the peroxisomal membrane protein 1 (PMP70). We have cloned the mouse PXMP1-L gene. It encodes a 606 amino acid protein. In contrast to the human and the rat, mouse PXMP1-L is predominantly expressed in the liver. The mouse PXMP1-L gene consists of 19 exons and spans 21 kb of genomic sequence. No obvious peroxisome proliferator response element has been found in 1.1 kb of the putative promoter region. No coordination of constitutive or fenofibrate-induced expression of PXMP1-L with other peroxisomal ABC transporters was observed so that an obligate exclusive heterodimer formation is not likely to occur. The data presented will be particularly useful for the generation of a mouse model defective in PXMP1-L in order to elucidate the yet unknown function of this protein.

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Key words: Peroxisome; ABC transporter; Fatty acid transport; Adrenoleukodystrophy

1. Introduction

We and others have recently cloned the cDNA of the human peroxisomal membrane protein 1-like protein (synonyms: peroxisomal membrane protein 69, PMP69; PMP70-related protein, P70R), a novel human membrane protein which belongs to the superfamily of integral membrane transporter proteins containing an ATP binding cassette (ABC transporter) [1,2]. PXMP1-L is localized exclusively within the peroxisomal membrane. Members of the ABC-transporter superfamily are involved in the transport of a variety of substrates across biological membranes [3]. Three other members of the family have been located within the peroxisomal membrane: the adrenoleukodystrophy protein (ALDP) [4], the peroxisomal membrane protein 1 (PXMP1, PMP70) [5] and the adrenoleukodystrophy-related protein (ALDRP) [6,7]. Defects in the ALD gene are obligate in X-linked adrenoleukodystrophy (ALD; McKusick 300100), a demyelinating disorder of the nervous system with marked heterogeneity of clinical manifestations even between affected individuals carrying the same mutation [8,9]. ALDP is likely to be involved in the peroxisomal import of very long chain fatty acids (VLCFA; $>$C22) into the peroxisome since VLCFA accumulate in tissues of ALD patients. This hypothesis is supported by yeast studies [10]. The functions of ALDRP, PXMP1 (PMP70) and PXMP1-L are not known. All four peroxisomal ABC transporters conform to the model of an ABC half-transporter so that dimerization is necessary to constitute a functional transporter. It is currently not known in which way peroxisomal ABC transporters dimerize. Heterodimer formation as well as homodimer formation or both is conceivable. Induction of peroxisomal ABC transporters (ALDP, ALDRP, PXMP1) by fenofibrate, a potent peroxisome proliferator activating PPARα [14], has previously been studied in the rat. Upon treatment with fenofibrate expression of the ALD gene was not altered whereas that of PXMP1 and ALDR genes was strongly increased in intestine and liver, respectively [15]. The data available on the tissue expression pattern of individual peroxisomal ABC transporters [6,7,16] or their induction by fenofibrate [15] did not reveal a clue to distinct pairs among these half-transporters. Expression analysis of the fourth transporter (PXMP1-L) was not possible at this time. The knowledge of primary structure similarities to ALDP, a putative lipid transporter, and to peroxisomal lipid transporters (Pxa1p, Pxa2p) in *Saccharomyces cerevisiae* have led to the hypothesis that the other half-transporters ALDRP, PXMP1, and PXMP1-L might also be involved in lipid translocation across the peroxisomal membrane [1,2]. Several groups have independently established a mouse model for ALD by targeted disruption of the ALD gene [11–13]. These models do not show neurologic abnormalities but are clearly affected by a defect of VLCFA β-oxidation. This phenotype might be influenced by the overlap or redundancy of function between ALDP and the other related peroxisomal ABC half-transporters. One strategy to prove such hypotheses is the generation of mouse models deficient for more than one peroxisomal ABC transporter. As a prerequisite for this strategy we report here the cloning of the mouse PXMP1-L cDNA encoding the fourth known peroxisomal ABC half-transporter. We have analyzed the complete exon-intron structure, the expression of PXMP1-L mRNA in various tissues of mouse, rat and human and the putative promoter sequence. We also report the comparison of constitutive and fibrate-inducible levels of expression of peroxisomal ABC transporters in rodents and man.

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Sequence data from this article have been deposited at the EMBL/NCBI-GenBank Databases under accession number AJ001166.

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Fig. 1. Alignment of the amino acid sequences of mouse PXMP1-L with human PXMP1-L and mouse PXMP1 (PMP70).

2. Materials and methods

2.1. cDNA cloning

The dbEST expressed sequence database was probed with the human PXMP1-L amino acid sequence using the tblastn program at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). A C57BL/6 mouse clone from the Wash-U-HHMI Mouse EST Project (GenBank accession number AA105879), the ORF of which demonstrated significant similarity to the human sequence, was obtained from the Resource Center of the German Human Genome Project (Berlin) [17]. Since the 3′ untranslated end of this clone contained a B1 repetitive element that might have been a cloning artifact, a 473 bp cDNA probe was amplified from the above EST clone avoiding this repetitive sequence using primers 1f (5′-GCCATTCTTCACTGATGGGACA-3′) and 1r (5′-GAGCTCCGCTGCTCAGATG-3′). A 9 day mouse embryonic cDNA library was screened with this probe labeled by [α-^{32}P]dATP random priming (Screening Service of the Resource Center of the German Human Genome Project). A positive clone containing a PXMP1-L cDNA fragment (MPMGc559E0541Q3) was isolated and sequenced. The 5′ end of the cDNA was completed by RACE-PCR using mouse liver adapter-ligated cDNA (Marathon-Ready mouse liver cDNA, Clontech).

For fenofibrate induction studies in rats, a rat PXMP1-L cDNA fragment was amplified from random-primed rat cardiomyocyte cDNA with primers 1fh (5′-CTTCAGACCCAGAGGGAGCTG-3′) and 2rh (5′-CCCGTGTTGCCTGTGATGAGC-3′) derived from the human cDNA sequence. The resulting 474 bp product was cloned and sequenced to confirm orthologous sequence. Fenofibrate treatment of rats was performed as described previously [15].

2.2. Northern blots

A BALB/c mouse multiple tissue Northern blot containing 2 μg poly(A)$^+$ mRNA per lane was purchased from Clontech. The 473 bp cDNA mentioned above was [α-^{32}P]dATP labeled by random priming and purified from unincorporated nucleotides by size exclusion chromatography using Sephadex G50 or ProbeQuant G-50 Micro Columns (Pharmacia). Signals obtained in Northern analysis were compared to those detected by human actin or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control probes. Ethidium bromide staining of gels prior to blotting was also used as loading control. RNA extraction and Northern blot analysis of rat tissues were performed as described previously [15].

2.3. Genomic structure analysis

A 129/Ola mouse genomic DNA cosmid library was screened with the 473 bp cDNA probe described above. Three positive clones were isolated. Cosmid DNA was prepared using ion exchange column purification (Midiprep Kit, Qiagen). Multiple exon-exon PCR reactions were performed using cosmid DNA as template to amplify complete introns and to obtain intron sizes. In order to get the precise intron-exon boundaries including exon-flanking intronic sequences the resulting PCR products were sequenced from either end using the amplification primers.

2.4. Sequencing of the putative promoter region

The putative promoter region was directly sequenced by fluorescent dideoxy dye terminator technology using cosmid DNA preparations as templates. Sequencing of long templates involved the use of BigDye terminator sequencing technology (ABI). All DNA sequencing was performed on an ABI 377 automated sequencer.

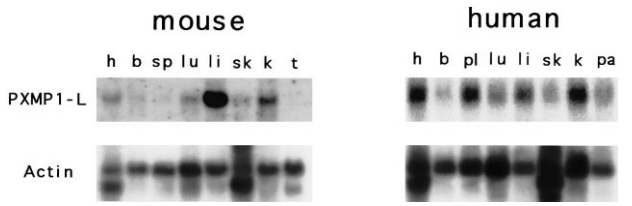

Fig. 2. Northern analysis in various mouse tissues and comparison to the human. h: heart, b: brain, sp: spleen, lu: lung, li: liver, sk: skeletal muscle, k: kidney, t: testis, pl: placenta, pa: pancreas. In the mouse PXMP1-L mRNA is predominantly expressed in liver. Hybridization with a human actin probe was performed as loading control.

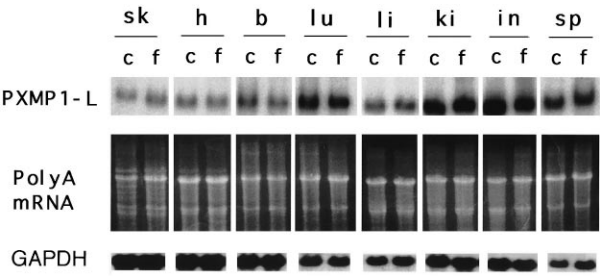

Fig. 3. Northern analysis for the presence of PXMP1-L mRNA in various tissues from fenofibrate-treated and untreated rats. Pretransfer ethidium bromide staining of poly(A) RNA and hybridization with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe were used as loading controls. c: untreated, f: fenofibrate-treated rats; sk: skeletal muscle, h: heart, b: brain, lu: lung, li: liver, ki: kidney, in: small intestine, sp: spleen.

3. Results

3.1. Mouse PXMP1-L cDNA and primary protein structure

We have cloned a 2308 bp cDNA of the mouse ortholog of the human PXMP1-L gene. The 1818 bp open reading frame encodes a protein of 606 amino acids with a predicted molecular weight of 69 kDa. The deduced amino acid sequence shows 89.4% identity to human PXMP1-L (Fig. 1). The 3′ untranslated region of the cDNA contains a B1 repetitive element ranging from bp 2173 to 2309.

3.2. PXMP1-L transcription in various tissues of mouse and rat

Northern blot analysis demonstrates the presence of PXMP1-L specific mRNA in all tissues examined in mouse (Fig. 2) and rat (Fig. 3). A single transcript of approximately 2.4 kb was observed in both species. In mouse, PXMP1-L is predominantly expressed in liver whereas the rat expression pattern shows a more even distribution similar to the pattern previously reported for the human [1].

3.3. Effects of fenofibrate diet on the transcription of PXMP1-L in rats

In contrast to other peroxisomal ABC transporters such as PXMP1 (PMP70) and ALDR, PXMP1-L transcription was not induced by fenofibrate in rats (Fig. 3). A similar result (i.e. no change in transcription by fenofibrate) has previously been described for the adrenoleukodystrophy gene [15].

3.4. Characterization of the region upstream of the transcriptional start

1.1 kb of genomic sequence immediately upstream of the transcriptional start have been obtained. A TATA box was not observed which is consistent with a housekeeping function of PXMP1-L. Potential Sp1 binding sites have been found at positions −32 to −20, −121 to −117 and −347 to −342. The putative promoter region did not reveal apparent peroxisome proliferator responsive elements (PPRE) [18–20]. Such an element, however, may theoretically be positioned further upstream [20].

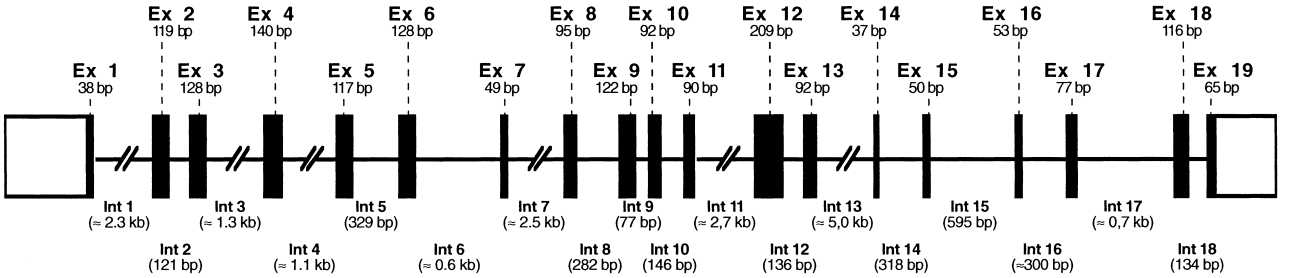

Fig. 4. Genomic organization of the mouse PXMP1-L gene. Exon sizes (in bp) are indicated above, intron sizes below the horizontal line.

3.5. Determination of the complete exon-intron structure

The genomic organization was determined by PCR amplification of genomic fragments with cDNA-derived primers using a cosmid clone containing the entire murine PXMP1-L gene. Overlapping fragments spanning the complete coding region were generated and sequenced. Comparison of the obtained genomic sequence with the cDNA sequence revealed that the PXMP1-L gene consists of 19 exons and 18 introns (Fig. 4). The last exon is the largest one containing 506 bp. The other exons range in length between 37 bp and 209 bp. All exon-intron boundaries conform to the GT-AG rule. We have fully sequenced nine introns (introns 2, 5, 8–10, 12, 14, 15 and 18) and determined the exon-flanking intronic sequences of all other introns. The approximate sizes of introns not fully sequenced were determined by agarose gel electrophoresis of corresponding PCR products. The total size of the murine PXMP1-L gene is approximately 21 kb (Fig. 4).

4. Discussion

We and others have previously reported the cloning of PXMP1-L a novel human membrane protein belonging to the group of peroxisomal ABC half-transporters [1,2]. Its closest homolog is the peroxisomal membrane protein 1 (PXMP-1) also known as PMP70. In addition to ALDP, ALDRP and PXMP1, it constitutes the fourth peroxisomal ABC transporter known to date. The function of PXMP1-L is still unknown, but the similarity to other peroxisomal ABC transporters suggests a role in lipid translocation across the peroxisomal membrane. The complete loss of peroxisomal functions in peroxisome biogenesis defects (PBD) in humans is not lethal prenatally. This has also been shown in mouse models of PBD [21,22]. Therefore it is to be expected that disruption of any peroxisomal gene will yield a phenotype viable until birth and accessible to further characterization. The example of ALD mouse models [11–13] has demonstrated that the function of a peroxisomal ABC transporter can be studied by gene disruption. As a prerequisite to studying PXMP1-L in an animal model we have cloned the orthologous gene of the mouse. The length of the cloned cDNA corresponds to the transcript size of 2.4 kb observed in Northern blot analysis so that it is probably full length. This transcript size is identical in all tissues and also identical to rat and human so that the formation of alternative transcripts (as have been observed for ALDRP) appears not to play a role in PXMP1-L function. The homology of 89.4% identical amino acids as compared with human PXMP1-L (Fig. 1) proves that it is in fact the murine ortholog. A B1 repetitive element [23] is part of the transcript in the 3′ untranslated region. The role of such a mobile element becoming part of the transcript is not known. The insertion of this element into the transcript might have led to an alteration in stability and thus expression level of this gene during evolution. The composition of the coding region of the PXMP1-L gene from 19 exons is very similar to that of the human ortholog in that all exon-intron boundaries are conserved whereas intron sizes are variable [24]. No apparent similarities to the genomic structure of the human ALD gene [25], the human ALDR gene (Holzinger et al., unpublished data) or the human PXMP1 gene [26] were found, so that the genomic organization data do not provide a clue to an interaction of PXMP1-L with other peroxisomal ABC transporters.

Interestingly the tissue distribution of constitutive mRNA expression in BALB/c mice appears to be different from the rat and the human in that the liver is the predominant site of expression in the mouse. This finding might well be related to species differences in peroxisomal lipid metabolism not proven to date. Some of the most important functions of peroxisomes include the synthesis of plasmalogens, bile acids, cholesterol and dolichol, and the oxidation of fatty acids $>$C22, branched chain fatty acids, dicarboxylic acids, unsaturated fatty acids and prostaglandins [27]. Although standard laboratory diet was given to these mice changes in response to the diet might also be of importance. Dietary polyunsaturated fatty acids, for example, have been shown to modulate the transcription of several hepatic lipogenic genes [28]. The investigation of the effect of lipid enriched or depleted dietary regimens might yield a clue to the function of PXMP1-L. In contrast to BALB/c mice analyzed in this study, Albet et al. (unpublished data) have observed expression of PXMP1-L in the Ico:OF1 mouse kidney at higher levels than in the liver so that there are apparent inter-strain differences. Interspecies differences in tissue expression have been observed for other ABC transporters: in human, ALDP is expressed stronger in skeletal muscle than in lung and heart [4] but the reverse observation was made in mouse (Albet et al., unpublished data) and rat [15]; PMP70 is strongly expressed in the liver of rat, mouse and human. In the mouse expression levels of kidney and heart are almost as high as those of liver (Albet et al., unpublished data). The comparison of peroxisomal lipid metabolism between strains and species in relation to expression levels of peroxisomal ABC transporters will be an interesting field of further investigations and may contribute to assessing the precise function of these transporters. It appears to be of importance that differences between mouse and human expression patterns are kept in mind when mouse models are generated by targeted gene disruption.

Three of the four peroxisomal ABC transporters have pre-

viously been examined for induction of mRNA transcription in rats treated with fenofibrate, a potent peroxisome proliferator [14,15]. PXMP1 (PMP70) transcripts were found to be induced in liver and intestine, ALDR was induced in liver only and ALD was not found to be induced in any tissues [15]. The cloning of the PXMP1-L cDNA enabled us to compare all four peroxisomal ABC transporters with respect to fenofibrate induction in the rat. As was the case with ALD [15], we did not see any change in the level of PXMP1-L mRNA after fenofibrate treatment of rats. This would be in accordance with the absence of PPREs in the putative promoter region of the mouse gene although species differences in the action of peroxisome proliferators have been observed [29–31]. From these data and the analysis of tissue expression it can be concluded that there is no apparent pair of peroxisomal ABC half-transporters showing coordinate expression so that an obligate exclusive dimerization between any two of the four peroxisomal ABC transporters appears to be unlikely. In accordance, Troffer-Charlier and colleagues [32] have found strikingly different expression of ALDP and ALDRP in mouse tissues and various human cell lines.

In *S. cerevisiae* two ABC half-transporters (Pxa1p and Pxa2p) were found to form heterodimers [33]. Pxa2p was demonstrated to be responsible for C18:1-CoA, but not C8:0-CoA import into the peroxisome [10]. This could be interpreted as an indication of the co-existence of homo- and heterodimers of peroxisomal ABC transporters in this organism.

In this study, the comparison of expression data of PXMP1-L in rodents with the other peroxisomal ABC transporters did not reveal any evidence of coordinate expression or induction of any pair of these proteins. These findings are consistent with the hypothesis that peroxisomal ABC transporters form homodimers. Due to the high conservation and identical subcellular localization it has previously been proposed that ALDRP, PXMP1 and PXMP1-L might play a role as modifiers of the clinical course of adrenoleukodystrophy. This hypothesis would imply a similar, possibly overlapping function of these membrane proteins. In fact it has recently been demonstrated that the β-oxidation defect present in cell lines derived from ALD patients can be corrected not only by transfection of ALD cDNA but also by overexpression of PXMP1 [34].

The genomic organization of the murine PXMP1-L gene presented here is a prerequisite for the generation of mouse models lacking either PXMP1-L alone or in combination with the other peroxisomal ABC transporters.

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