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A Point Mutation at the X-Chromosomal Proteolipid Protein Locus in Pelizaeus-Merzbacher Disease Leads to Disruption of Myelinogenesis

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Summary: A group of inherited neurological disorders are the X-chromosome linked dysmyelinoses, in which myelin membranes of the CNS are missing or perturbed due to a strongly reduced number of differentiated oligodendrocytes. In animal dysmyelinoses (jimpy mouse, msd-mouse, md rat, shaking pup) mutations of the main integral myelin membrane protein, proteolipid protein, have been identified. Pelizaeus-Merzbacher disease (PMD) or sudanophilic leucodystrophy is an X-linked dysmyelinosis in humans.

We report here on the molecular basis of the defect of affected males of a PMD kindred. Rearrangements of the PLP gene were excluded by Southern blot hybridisation analysis and PCR amplification of overlapping domains of the PLP gene. Sequence analysis revealed one single C→T transition in exon IV, which leads to a threonine→isoleucine substitution within a hydrophobic intramembrane domain. The impact of this amino-acid exchange on the structure of PLP in the affected *cis* membrane domain is discussed. A space filling model of this domain suggests a tight packing of the α-helices of the loop which is perturbed by the amino-acid substitution in this PMD exon IV mutant.

The C→T transition in exon IV abolishes a *Hph* I restriction site. This mutation at the recognition site for *Hph* I (RFLP) and allele-specific primers have been used for mutation screening the PMD kindred.

Eine Punktmutation im X-chromosomalen Proteolipidprotein-Gen bei der Pelizaeus-Merzbacher-Krankheit führt zur Unterbrechung der Myelinogenese

Zusammenfassung: Eine Gruppe erblicher neurologischer Krankheiten bilden die X-Chromosom-gebundenen Dysmyelinosen, bei denen Myelinmembranen des CNS aufgrund einer stark reduzierten Zahl von Oligodendrozyten fehlen oder in ihrem Aufbau gestört sind. Bei Tier-Dysmyelinosen (jimpy-Maus, msd-Maus, md-Ratte, shaking pup) stellten sich Mutationen des wichtigsten integralen Membranproteins, des Proteolipid-Proteins, als Ursache heraus. Die Pelizaeus-Merzbacher-Krankheit (PMD) oder sudanophile Leucodystrophie ist eine X-chromosomale Dysmyelinose des Menschen.

Wir beschreiben die molekulare Basis des Defekts bei betroffenen männlichen Mitgliedern innerhalb einer Familie mit Auftreten von PMD-Fällen. Reorganisationen des PLP-Gens konnten durch Southern-Blot-Hybridisierungsanalyse und durch PCR-Amplifikation von überlappenden Domänen des PLP-Gens ausgeschlossen werden. Die Sequenzanalyse ergab einen einzigen C→T-Übergang im Exon IV, der zu einer Threonin→Isoleucin-Substitution innerhalb eines hydrophoben intramembranalen Abschnitts führt. Die Auswirkung dieses Aminosäuren-Austausches auf die PLP-Struktur in der betroffenen *cis*-

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Abbreviations:
CNS, central nervous system; MBP, myelin basic protein; PCR, polymerase chain reaction; PLP, proteolipid protein; PMD, Pelizaeus-Merzbacher disease; lmp, low melting point.

Membrandomäne wird besprochen. Auf einem Kalottenmodell dieser Domäne basiert die Annahme, daß die α-Helices innerhalb dieses Abschnitts dicht aneinandergelagert sind und dieser Aufbau durch den Aminosäuren-Austausch in der PMD-Exon IV-Mutante gestört wird.

Der C→T-Übergang im Exon IV führt zum Verlust einer *Hph* I-Restriktionsstelle. Diese Mutation an der Erkennungsstelle für *Hph* I (RFLP) und Allel-spezifische Primer wurden dazu eingesetzt, die Mitglieder der Familie auf das Vorliegen der PMD-Krankheit zu untersuchen.

Key words: Myelinogenesis, Pelizaeus-Merzbacher disease, point mutation, protein conformation, proteolipid protein.

The main function of oligodendrocytes in vertebrate central nervous system (CNS) is the synthesis of the components of the myelin sheath which protrudes from the plasma membrane and enwraps the axon spirally as a multilayer membrane system. This compacted lamellar structure is organized and stabilized by the two main protein components of CNS myelin, myelin basic protein (MBP), a peripheral membrane protein, and proteolipid protein (PLP), a strongly hydrophobic integral membrane protein. MBP contributes to the compaction of the cytosolic space, and PLP for the tight apposition of the extracytosolic membrane surfaces[1]. Any disturbance of this ensheathment of the axons disrupts the normal nerve conductance. The organization of the human PLP gene has been elucidated[2]. Seven exons and six introns span about 17 kb. The nucleotide sequence of the exons and adjacent sequences of the introns have been determined.

Cloned PLP cDNA and genomic sequences allowed the assignment of the PLP locus to the Xq12-q22 chromosome[2–4]. This made the study of the X-chromosome-linked recessive forms of dysmyelination in animal models such as the jimpy mouse, myelin-deficient (md) rat and the human sudanophilic leucodystrophy known as Pelizaeus-Merzbacher (PMD) disease feasible. The brain of the jimpy mouse, md rat and PMD-affected males is characterized morphologically [5] and biochemically[6] by the strongly reduced formation of myelin in the CNS with intact PNS myelin. Clinically symptoms like ataxia, tremor, seizures and early death are prevailing.

The dysmyelinations in the mouse and rat have been delineated to point mutations in the PLP gene. In the jimpy PLP gene an A→G transversion in the splice acceptor signal of intron IV has occurred[7]. This leads to the loss of exon V and a frame shift in the residual exons VI and VII following the splicing event with the formation of a missense PLP. The md defect of the rat is caused by an A→C transversion within exon III of the rat PLP gene leading to a structural mutation: threonine^{75} in the centre of the second transmembranal α-helix of our proposed model is substituted by the α-helix breaker proline[8]. The phenotype of these mutations is clearly pleiotropic with a reduced synthesis of the other main myelin protein MBP but also a reduced myelin membrane lipid synthesis.

The X-linkage, neurological symptoms, biochemical and morphological analyses of PMD are very similar to those of the molecularly well established jimpy mouse and md rat dysmyelinoses. This suggested studies on mutational events within the regulating and coding region of the human PLP gene, the only X-linked gene of the main proteins expressed in the oligodendrocyte during myelination.

This report describes studies on a large Swiss PMD pedigree. The study was initiated by establishing a genomic library of an affected male of this kindred in λ-phage EMBL3. Dideoxy sequencing of the coding exons revealed a C→T(G→A) transition of base pair 14 of exon IV. This substitution of the second base of the threonine^{155} codon to ATC leads to a substitution by isoleucine. The mutation has been established by four independent methods:

a) cloning of the PMD patient's proteolipid protein gene and analysis of the nucleotide sequences of exons IV to VII and large domains of adjacent introns,
b) sequence analysis of overlapping genomic PCR fragments covering exons I to VII,
c) analysis of the mutation at the recognition site introduced by the base exchange (*Hph* I variant) and
d) by allele-specific PCR amplification using specific synthetic oligonucleotide primers.

The analysis of the PLP gene of the kindred unravelled the tight linkage of the mutated PLP gene and the PMD.

Possible implications of the threonine^{155} exchange against isoleucine in the invariant transmembrane domain of PLP for the integration of PLP are proposed. During our investigations mutations in the PLP genes of three unrelated PMD cases have been reported supporting the suggestion described above[9–11]. In each case a point mutation within the coding region led to an amino-acid substitution in the proteolipid protein.

Results

PMD is a rare inherited disease. The diagnosis is often suspicious just resting on the neurological symptoms associated with the typical inheritance in affected male within the kindred.

We analysed the genetic defect of an affected male of a large PMD kindred which has previously been described[12,13] according to the following strategy:

1) Search for gene rearrangements by Southern blot hybridization analysis and by in vitro amplifying the whole PLP gene (with the exception of the first large intron) in several overlapping sections.

2) Because no gross alterations were found the nucleotide sequences of the coding regions, the intron-exon junctions and the 5'- and 3'-non-coding regions have been determined using a genomic λ-clone with direct genomic sequencing of PCR products.

Southern blot hybridization of genomic DNA of the PMD patient

Genomic DNA of one affected male, his mother and two control persons was digested with *Eco* RI and *Pst* I, respectively, fragments separated electrophoretically, transferred to Nylon membranes and hybridized with the randomly labelled 624 bp and 743 bp *Pst* I fragments of the PLP cDNA spanning from the start codon to one third of exon VII. As shown in Fig. 1 no difference in band size between the pedigree members and the controls is visible.

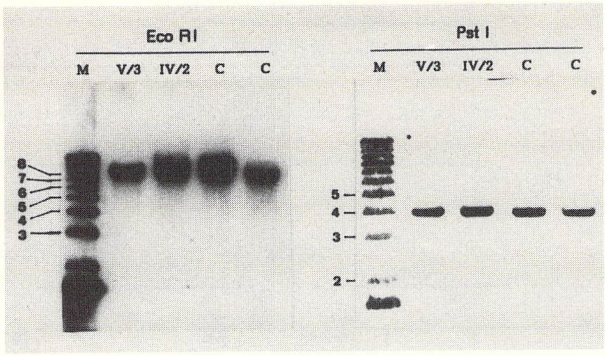


Fig. 1. Southern blot hybridization of genomic DNA of PMD patient (V/3, see pedigree in Fig. 6), his unaffected mother (IV/2) and two controls (C) restricted with *Eco* RI and *Pst* I.

Table. Oligonucleotides used as primers for PCR amplification and single strand sequencing.

1 S	AACGGATCCT TTTTCTTGG GGCTGATACA
1 A	ACTGAATTCC TGTGTCCTCT TGAATCTTCA ATG
2 S	CTGCTTTCAG AGCCTGTGAC TTCTTGTGTG
II S	GTTTGTTAGA GTGCTGTGCT AGATGTCTGC T
2 A	TGCACAGAGG GAAGACTCGG GAATCCGGTC
3 S	CTAGAAAATC CCTAGCCTTG TTAAGGTGCT
wt III A	GGCCCCATAA AGGAAGAAGA AAGAGGCAGT
3 A	CCAGAACATT GTAAATTTAC CTTGGAGTTA
4 S	AGTGGATCCT CCTCATTCTT CCCCTACCCA TTC
4 A	AACTAAGACC CAAGAATTCA TCATCTTATA
5 S	AAGAGGATCC TAATTGTTGG TAGCATCCAA
5 A	CGGCGAATTC AAAATCCAGC AAAGGGAAAC
VI S	TTCCAAATGA CCTTCCACCT GTTTATTGCT GCATTT
6 A	TCCCTCCAGC ATTTCAAGGA TGGAAGCAGT
7 (301 S)	ATACAGAGTC AAGTAATTTC TCACCTTGTA
VII A	GAACTTGGTG CCTCGGCCCA TGAGTTTAAG GACGGC
7i S	ATTCAGTCAT CGTAGGTGAT TTGAAGGTCT
7i A	GAATTATTCT CCAGACATTT CTGATGCAAC
7 A	TCTTGAGAAG GTACGATTAT TAAGTTTCTA

Overlapping DNA amplification of the PLP gene in PMD

Oligonucleotide primers were designed for the amplification of the complete PLP gene from genomic DNA of the PMD patient and a control person in overlapping domains (appr. 9 kb) except of the large 8 kb intron I, the sequence of which has not yet been determined completely.

Fig. 2 visualizes the position of the hybridizing oligonucleotides and the table shows the oligonucleotide sequences. The size of the PCR fragments of the affected male (P) and the normal individual (C) was identical over the whole range of the amplified PLP gene. Therefore sizable deletions of or insertions into the PLP gene described above can be excluded. The overlapping amplification method used here has some advantages over the commonly used Southern method provided the sequence of the gene is known for the construction of suitable oligonucleotide primers:

1) avoidance of radioactive material,
2) less genomic DNA (< 100 ng per PCR) is needed as compared to Southern hybridization,
3) this method is less laborious, reproducible results can be obtained in one or two days.

Analysis of the coding regions (exons I to VII) and the 5'-noncoding region of the PLP gene

a) Genomic library of PMD patient

Genomic DNA of the PMD patient was partially restricted with *Sau* 3A, size-fractionated over a linear

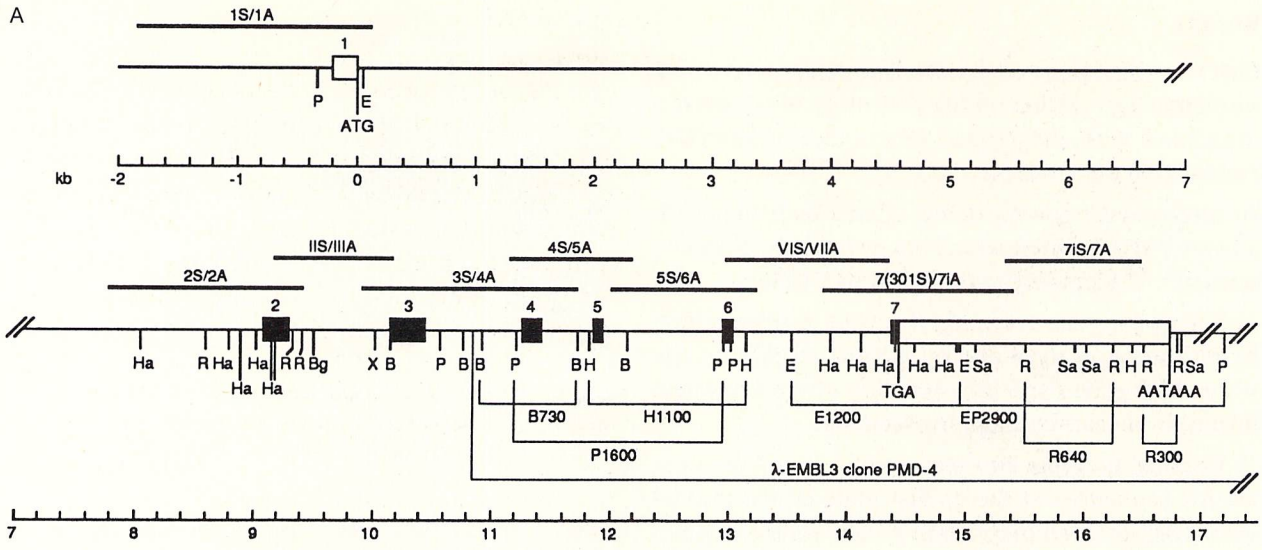
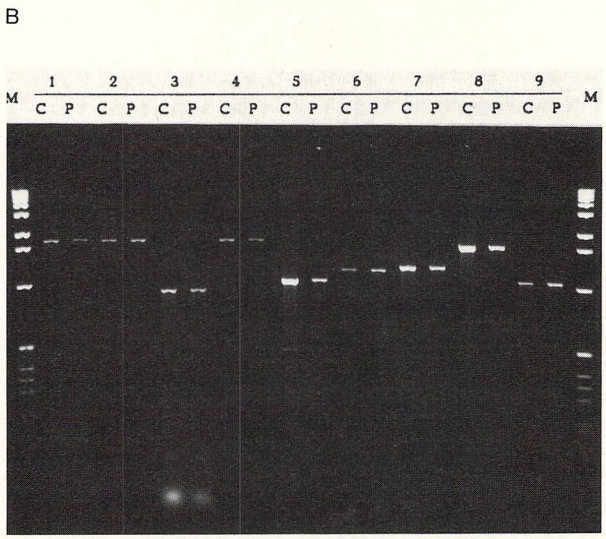


Fig. 2. A) Schematic presentation of the PLP gene.

The exons are indicated by boxes and the coding sequences are black. The bars above the gene define the overlapping segments which were amplified between the primers indicated in B). A partial restriction map of λEMBL3 PMD clone ranging from intron III to exon VII is included. Below the gene the subclones are drawn. The subclone names are composed of the enzyme abbreviation and the approximate length in base pairs. Exon I to III were analysed by PCR of genomic DNA. Restriction enzymes: B *Bam* HI; Bg *Bgl* I; E *Eco* RI; H *Hin*d III; Ha *Hpa* II; P *Pst* I; Ra *Rsa* I; Sa *Sal* I; Sc *Sac* I.

B) Ethidiumbromide stained 1.5% agarose gel of 10% aliquots of the overlapping PCR's according to the numbering of A). C = control person; P = PMD patient. In the following are the primer-combinations (see table) and the size of the PCR product (in brackets).

1: 1S/1A (1.79 kb); 2: 2S/2A (1.76 kb); 3: IIS/wtIIIA (1.0 kb); 4: 3S/4A (1.7 kb); 5: 4S/5A (1.07 kb); 6: 5S/6A (1.3 kb); 7: VIS/VIIA (1.3 kb); 8: 7(301S)/7iA (1.65 kb); 9: 7iS/7A (1.08 kb); M: kb-ladder DNA size marker.



sucrose gradient (10 to 40%), dephosphorylated and ligated into the *Bam* HI-restricted λ-EMBL-3 phage DNA. For packaging, plating and plaque screening of the PMD library established procedures were used. One clone, EMBL-3-PMD4, about 15 kb in length, encoded the 3′ part of intron III and exon IV to exon VII.

b) Sequence analysis

Restriction fragments of the insert containing exons III to VII were subcloned into the multicloning site of pGEM3Z for double strand sequencing using the T7 and SP6 sequencing primers. The other sequences of exons I to III were obtained by single strand sequencing of PCR fragments after asymmetric amplification according to Gyllensten and Ehrlich[14] with some modifications: the double strand template is generated by genomic PCR amplification, the fragment separated by electrophoresis in low melting agarose, the band excised and immediately used for asymmetric amplification in which a molar ratio of the primers 50:1 was chosen. The faster running single strand DNA fragment was separated by agarose gel electrophoresis and the agarose slice with the fragment used for dideoxy sequencing. All coding sequences of exons, intron-exon junctions, approximately 1500 bp of the 3′- and 350 bp of the 5′-nontranslated region except exon IV proved to be identical with the DNA sequences described before. Sequence analysis of exon IV revealed only one substitution, a C→T (G→A) transversion in position 14 of exon IV. The C→T base substitution leads to a threonine to isoleucine amino-acid exchange within the hydrophobic sequence of exon IV.

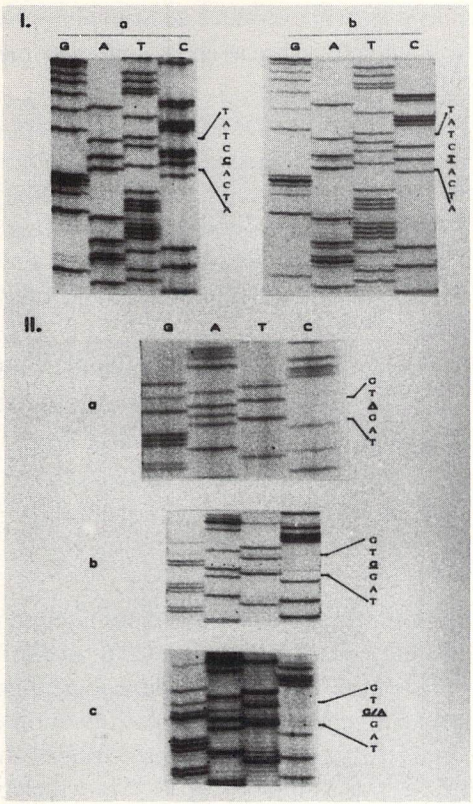


Fig. 3. I Autoradiogram of dideoxy sequencing of PCR single stranded DNA of exon IV in sense direction: a) control individual, b) PMD patient.

The normal nucleotide (C) at position 14 of exon IV and the substituted T are underlined. II Sequencing in the antisense direction. a) PMD patient V/3, showing the mutation at the underlined position. b) Father of PMD patient (IV/1), with the normal PLP sequence. c) Mother of the PMD patient (VI/2), with both nucleotides (G and A) at the same position indicating that she is heterocygote for the mutation. Roman and arabic numbers identify members of the pedigree given in Fig. 6.

In order to exclude a cloning artifact exon IV was additionally sequenced with the PCR method. Fig. 3 I compares the sequencing pattern of a control individual, (a), and of the PMD patient (b). Sequencing has also been carried out in the antisense direction which is shown in Fig. 3 II. It can be seen that the father of the patient has the normal PLP gene whereas the mother is heterozygous for PMD showing two bands at the same position on the sequencing gel.

Further support for the point mutation in exon IV came from

a) the deletion of the *Hph* I restriction site (TCACC) in the wild-type exon IV due to the substitution of C by T (TCATC) and
b) PCR amplification of the genomic DNA with allele-specific oligonucleotide primers.

Hph I RFLP

The C→T substitution causes the destruction of a *Hph* I restriction site (TCACC→TCATC). This variant was used to further support the point mutation and to screen the kindred for it. Exon IV was amplified between the primers 4S and 4A, the product was isolated and digested with *Hph* I. Fig. 4 presents the band pattern obtained after gel electrophoresis. The normal PCR product has three restriction sites for *Hph* I leading to four fragments after complete digestion (243, 178, 85, and 34 bp) whereas the mutated product yields only three fragments of 421, 85, and 34 bp. Female PMD carriers (IV/2, IV/3, V/4, and V/5, see the pedigree of Fig. 6 below) can be identified because both patterns are superimposed.

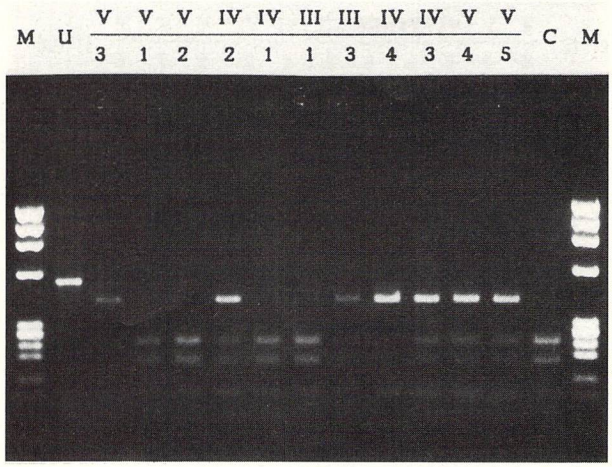


Fig. 4. *Hph* I RFLP- screening of the PMD kindred.

Exon IV was amplified between primers 4S and 4A, the DNA was ethanol-precipitated and digested with *Hph* I. The roman and arabic numbers refer to the position in the pedigree (Fig. 6). U: undigested PCR product; C: digested PCR product of a control; M: *Hae* III digested ΦX-174 DNA.

PCR of PMD genomic DNA with allele-specific primers

Two synthetic allele-specific oligonucleotide primers designated "Mut4 N" and "Mut4 PMD" hybridizing at the intron III-exon IV junction were used for the PCR with genomic DNA of the PMD kindred:

5' ATGTCAATCATTTTAGTTTGTGGGCATCAC 3' (normal primer)

5' ATGTCAATCATTTTAGTTTGTGGGCATCAT 3' (PMD-specific primer)

The primers Mut4 N und Mut4 PMD differ only in their 3' base producing C/A- and T/G mismatches when Mut4 N is hybridized to a mutated gene or when Mut4 PMD is used with the normal gene. In this case under appropriate conditions no amplification product is obtained in a PCR with the same antisense primer (5A). In the reverse combination the allele-

specific primers match perfectly and lead to the formation of a PCR product. Fig. 5 shows the result of these experiments which are in agreement with the *Hph* I RFLP results. This method is a modification of that previously described by Newton et al.[15]. Although these authors reported that C/A and T/G mismatches are not refractory to extension under their conditions the desired specificity of the allele-specific primers was obtained here simply by increasing the annealing temperature to 69 °C. It was therefore neither necessary to introduce additional mismatches near the 3′ end nor to use a second pair of primers for coamplification.

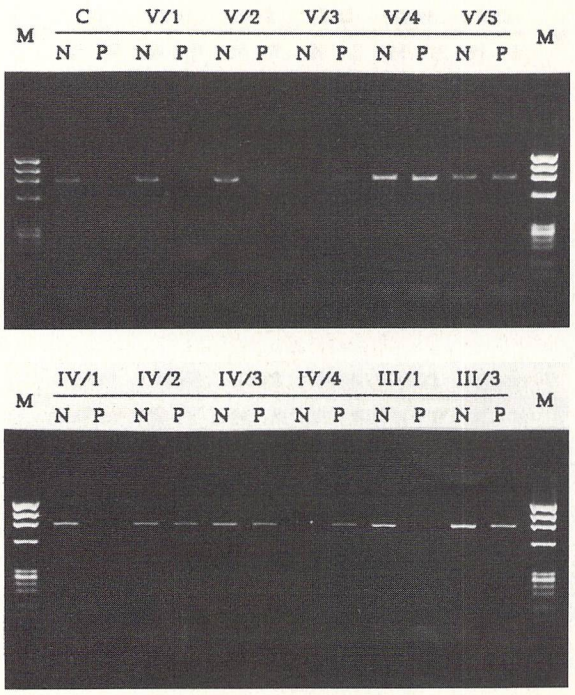


Fig. 5. Screening of the PMD kindred by PCR with allele-specific primers.

The roman and arabic numbers refer to the position in the pedigree. N: indicates a PCR with primers Mut4 N and 5A; P: PCR with primers Mut4 PMD and 5A; C: control individual; M· *Hae* III digested ΦX-174 DNA.

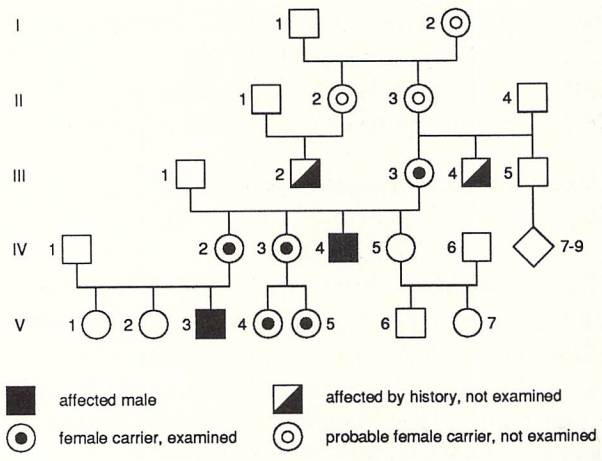


Fig. 6. Linkage pedigree of the examined swiss PMD kindred.

With these primers the DNA of the members of the large Swiss PMD pedigree were screened. Fig. 6 presents the linkage pedigree, PCR analyses of the genomic DNA with the allele-specific primers of six members of the PMD kindred with siblings of the fifth, parents of the fourth and a grandmother of the third generation.

The PMD diagnosis with allele-specific PCR is faster and less laborious than the investigation of the restriction variant and has the same reliability.

Discussion

Proteolipid protein (PLP) is the main integral membrane protein constituent in the CNS myelin membrane. Structural analyses on the polypeptide and DNA level revealed the extreme conservation of the amino-acid structure of this protein during evolution. We interpret this homology of PLP in widely divergent species such that even minor mutations are incompatible with the function of this membrane protein. Several animal models indeed demonstrate the effect of point mutations within the PLP gene. The jimpy mouse carries an A→G transversion at the splice acceptor site of intron IV of the PLP gene which leads to alternative splicing (loss of exon V) associated with a frame shift and a nonsense mutated protein[7]. Point mutations in the coding region of the PLP gene have been found in the msd mouse leading to the amino acid exchange Ala^{242}→Val in the third transmembrane domain[16], in the myelin-deficient rat (threonine^{75}→proline mutation within the second transmembrane helix of PLP), again with fatal consequences due to oligodendrocyte death[8]. In the shaking pup histidine^{36} at the end of the first transmembrane helix is exchanged against proline[17].

PMD is an X-chromosome-linked dysmyelinosis in humans with a lack of myelin in CNS as proven morphologically and biochemically. The disease occurs in a severe and a mild form in affected males. The PMD patient described in this communication carries an exchange of threonine^{155} against isoleucine due to a C→T base transition within the threonine triplett in exon IV. The point mutational events described above must be correlated to structural distortions of PLP caused even by one amino-acid exchange. The Thr→Ile exchange of this PMD exon IV variant occurs in the large *cis*-membranal loop of our proposed model. The intramembrane arrangement of this domain is defined by the position of the proline^{172} resi-

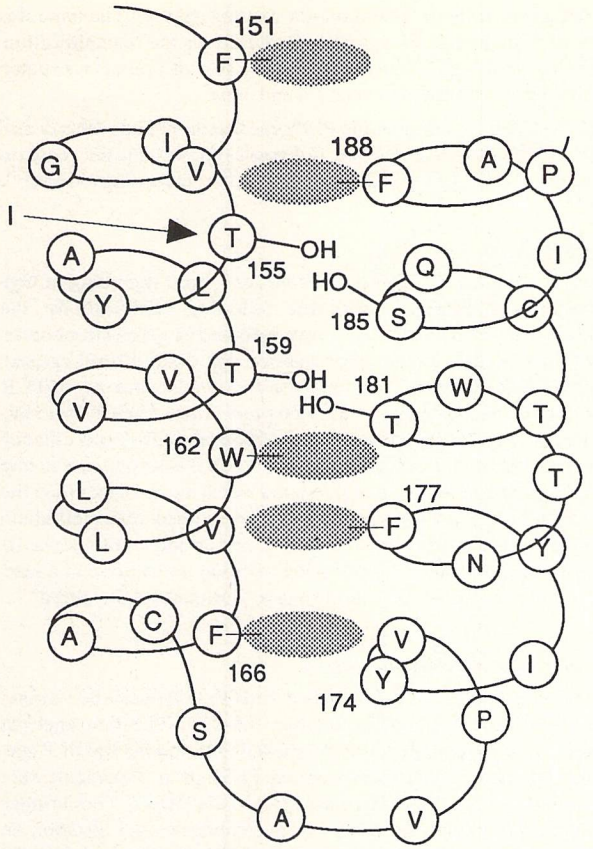


Fig. 7. Schematic presentation derived from a space-filling model of the intramembranal cis-loop of PLP encoded by exon IV! The shaded areas represent the stacked aromatic side chains of Phe and Trp. The possible H-bonding between Thr and Ser residues is indicated. Thr^{155} is exchanged against Ile in this PMD case (arrow).

due which causes the turn of the α-helix. In order to test the structural effect of the Thr-Ile exchange we constructed a space-filling model of the sequence of amino-acid residues 151-189 with an α-helical structure between Phe^{151}-Cys^{168} and Val^{171}-Pro^{189}. Twisting the model led to a very stable conformation. A further stabilization is suggested by hydrogen bonding of two pairs of neighbouring hydroxyl-containing side-chains (Thr^{155}:Ser^{185} and Thr^{159}:Thr^{181}) and aromatic stacking of three aromatic side-chains (Trp^{162}, Phe^{166} and Phe^{177}) below these and two above (Phe^{151} and Phe^{188}). Fig. 7 shows a schematic picture of the space-filling model. The β-turn structure is sterically facilitated by the exclusive presence of small amino acids (Ser-Ala-Val-Pro-Val) at this site. We therefore suggest a compact hairpin structure for this PLP domain requiring a correct arrangement of all amino acids. Even minor disturbances e.g. the elimination of one H-bond in our PMD patients PLP causes gross structural alterations of the protein with impaired structure. Additionally isoleucine has been recognized as an α-helix destabilizing residue and could therefore contribute to the structural changes. Recently a PMD patient with an exchange of Trp^{162} by arginine has been described[9]. This Trp residue contributes to the aromatic stacking described in our model. It is likely that the introduction of a charged amino acid into this *cis*-loop might strongly hinder its insertion into the lipid bilayer. The postulation of a *cis*-membrane domain in PLP is based on topobiochemical experiments[1], investigations using antibodies against synthetic peptides of the PLP[18] and has been supported by the work of Kahan and Moscarello[19] who also identified the three transmembranal domains of our model with photolabelling studies using a hydrophobic probe.

The fact that the whole PLP amino-acid sequence is extremely conserved between different species (100% between rat and man) during evolution and that several single amino-acid substitutions in man and animals PLP cause severe dysmyelinosis suggest a compact three-dimensional structure of PLP with several critical intra- and/or intermolecular interactions between the various membrane domains. It is indeed very striking that mutational events in the animal models (md-rat, msd-mouse and shaking pup and described in man all have occurred so far in the hydrophobic domains of PLP.

Besides the two exon IV variants a PMD exon II variant in which $proline^{14}$ is substituted by leucine[10] and in an exon V variant with $proline^{215}$ substituted by serine[11] have been described. It would be very informative to correlate the seriousness of the clinical symptoms and morphology of the myelin membrane with the position of the mutation within the PLP polypeptide chain, its location in the proposed model and impact on the PLP integration in the myelin lipid bilayer. It is not clear whether each PLP mutation leads to oligodendrocyte death. The degree of the dysmyelination may be due to a reduced number of oligodendrocytes or a reduced expression of mutated PLP or the integration or membrane interaction of mutated PLP leading to the assembly of a functionally altered myelin membrane. The information obtained by the analysis of different animal models with a mutated PLP locus and four variants of PMD clearly indicate that unique mutations occur at the PLP locus in unrelated families leading to a unique genetic defect at the PLP locus each of which causes a different structural alteration of PLP. The answer of the oligodendrocyte to those mutations leads to a similar pathological phenotype differing however in the degree of the expression of the symptoms.

These results indicate that the DNA sequence analysis of each newly discussed PMD kindred is required in order to define the disease and genetic state of each member of the kindred.

Materials and Methods

All cloning procedures not further described here were carried out according to Maniatis et al.[20], Davies et al.[21] or Ausubel et al.[22].

Isolation of genomic DNA

DNA from 10 m*l* blood or skin fibroblasts grown in tissue culture of male and female members of the PMD-affected kindred was prepared following the guanidine-HCl method of Bowtell[23], modified by Jeanpierre[24]. Residual protein contaminations after ethanol precipitation were removed by an additional proteinase K digestion.

Southern blot hybridization analysis

Southern blot hybridizations were carried out according to Southern[25]. 5–10 μg of genomic DNA was digested with *Eco* RI and *Pst* I respectively, electrophoresed and transferred onto a Nylon membrane (Gene Screen Plus, NEN). The blot was hybridized with 2×10^5 dpm of ^{32}P-labelled *Pst* I fragments of a PLP cDNA clone.

Construction of the genomic library

Genomic DNA of PMD was partially restricted with *Sau* 3 AI, dephosphorylated, size-fractionated by sucrose gradient centrifugation (10 to 40%) and fractions of 15–20 kb cloned into *Bam* HI-digested EMBL3 phage which had previously been dephosphorylated with alkaline phosphatase. The library contained 8×10^6 independent clones.
Screening of the library was carried out with randomly primed and labelled *Pst* I restriction fragments of our PLP-specific cDNA clone[26].

Subcloning and plasmid sequencing

DNA sequences of genomic DNA cloned into the *Eco* RI-restricted λEMBL3 phage were excised and cloned into pGEM3Z, *Eco* RI-digested and dephosphorylated. Minipreps of DNA[27] were prepared for supercoil double strand sequencing[28] using T7 and SP6 primers. Competent E. coli DH5α cells were prepared for transformation according to Hanahan[29].

Isolation of DNA fragments

DNA fragments were separated by agarose gel electrophoresis and isolated either by transfer to DEAE cellulose membrane NA45 (Schleicher & Schüll, W-5160 Düren, Germany) or recovered from low melting agarose gels (BRL).

Synthetic oligonucleotides

Oligonucleotide primers and allele-specific oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer model 381A. Primers contained about 30 nucleotides. Up to 2 kb domains were spanned by PCR to cover the whole gene between exon II to VII and exon I.

Primers were chosen which included naturally occurring restriction sites. A list of primer sequences used and their respective position within the PLP gene is given in the table.

PCR amplification

PCR amplifications were carried out on an automated DNA thermal cycler, Perkin Elmer. The reaction conditions were essentially those of Saiki et al.[30]: denaturing temperature of 94 °C for 90 s, annealing temperature between 40 and 69 °C depending on the T_m of the primer, 2–4 min extension at 72 °C depending on the length of the DNA sequence, cyclic repetition of the last three steps over 30 to 35 cycles, 10 min extension after the PCR and cooling to 4 °C. Reamplification was done with 8 μ*l* of the 100 μ*l* primary PCR after separation of the fragment on low melting agarose. The band was excised, melted at 60 °C and 1–2 μ*l* used for the reamplification. The optimal $MgCl_2$ concentration for a pair of primers was determined in a test series between 1.5 and 3mM.

The GC-rich 5′ region of the PLP gene was amplified with a deaza-dNTP mix which contained 7-deaza-2′-deoxiguanosine triphosphate (C7-dGTP) and dGTP in a ratio of 3:1 according to Innis[31].

Single-strand sequencing

Single-stranded PCR products were sequenced according to Gyllensten and Ehrlich[14] with the following modifications: the double-stranded PCR product was separated by gel electrophoresis with low melting point (lmp) agarose, the desired band excised, melted at 60 °C and a 2-μ*l* aliquot used as template in a second PCR with an asymmetric molar ratio (50 pmol : 1 pmol in a 100-μ*l* reaction) of the same primers. After 35 cycles the DNA was ethanol-precipitated and subsequently separated by electrophoresis in lmp agarose. The single-strand DNA band which moved faster than the corresponding double-strand DNA was excised under ethidium bromide visualization, melted at 60 °C and an aliquot of 8 μ*l* was directly used for dideoxy sequencing with *Taq* polymerase at a reaction temperature of 55 °C according to Gorman and Steinberg[32].

Labelling of hybridization probes

Oligonucleotides were 5′-labelled with T_4 polynucleotide kinase and [γ-^{32}P]ATP. Genomic Southern blot hybridization analyses were carried out with randomly labelled (420 and 743 bp) PLP-specific *Pst* I fragments isolated from the PLP cDNA. Fragments were labelled with the "random primed DNA labelling kit" (Boehringer, Mannheim), separated from free nucleotides by gel filtration on Biogel P30 (BioRad) and used for the hybridization following the standard procedures.

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