Dominant-negative action of the jimpy mutation in mice complemented with an autosomal transgene for myelin proteolipid protein

(disease model/hypomyelination/glia/apoptosis/X chromosome-linked gene)

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ABSTRACT Mutations in genes encoding membrane proteins have been associated with cell death of unknown cause from invertebrate development to human degenerative diseases. A point mutation in the gene for myelin proteolipid protein (PLP) underlies oligodendrocyte death and dysmyelination in jimpy mice, an accurate model for Pelizaeus–Merzbacher disease. To distinguish the loss of PLP function from other effects of the misfolded protein, we took advantage of the X chromosomal linkage of the gene and have complemented jimpy with a wild-type PLP transgene. In this artificial heterozygous situation, the jimpy mutation emerged as genetically dominant. At the cellular level oligodendrocytes showed little increase in survival although endogenous PLP gene and autosomal transgene were truly coexpressed. In surviving oligodendrocytes, wild-type PLP was functional and immunodetectable in myelin. Moreover, compacted myelin sheaths regained their normal periodicity. This strongly suggests that, despite the presence of functional wild-type PLP, misfolded jimpy PLP is by itself the primary cause of abnormal oligodendrocyte death.

The architecture of compacted myelin is determined by abundant membrane proteins that are specific to oligodendrocytes and Schwann cells (1–3). The major myelin component of the central nervous system is proteolipid protein (PLP), accounting for up to 50% of total myelin protein in the adult mammalian brain (4). PLP and its smaller isof orm DM-20 (5, 6) are highly hydrophobic proteins with similar overall topology and presumably four transmembrane domains (7, 8).

The X chromosome-linked jimpy mutation (jp) of the mouse results in a severe dysmyelinating phenotype that causes tremors and seizures, followed by premature death at 3–4 weeks of age (9, 10). In jimpy mice, a point mutation in the PLP gene causes aberrant splicing of exon 5, resulting in a frameshift of translation (11–15). Consequently, the putative fourth transmembrane domain of jimpy PLP is replaced by an aberrant Cys-rich C terminus (12), rendering a misfolded protein that is degraded after biosynthesis (16). Additional mutations of the PLP gene have been identified in several mammalian species including human patients with Pelizaeus–Merzbacher disease (for review, see ref. 17).

At the cellular level, dysmyelination of jimpy mice is associated with increased oligodendrocyte death (18, 19) and elevated numbers of proliferating glial precursor cells (20, 21). The paucity of fully mature oligodendrocytes is thus a major cause of myelin deficiency. It is still unproven, however, that the complex jimpy phenotype reflects a simple loss of PLP function. It is also possible that the expression of misfolded jimpy PLP is, by itself, deleterious to oligodendrocytes. The mutant membrane protein could accumulate intracellularly and constitute a rather unspecific trigger of cell death. Alternatively, misfolded PLP could interfere with normal oligodendrocyte function through PLP-specific protein interactions and by virtue of its structural similarity to the wild-type protein.

The jimpy phenotype can be observed only in male hemizygous mice (jp/Y) expressing a single PLP gene. Because of X chromosome inactivation in females, heterozygous jimpy mice (jp/+) develop as mosaics in which jimpy cell death occurs cell-autonomously (22, 23). To compare the survival and differentiation of oligodendrocytes expressing both the wild-type and jimpy allele, we generated transgenic jimpy mice with functional autosomal copies of the intact PLP gene. These transgenic animals provide experimental evidence that the jimpy phenotype has, at the cellular level, all features of a genetically dominant disorder. However, expression of wild-type PLP results also in a small degree of rescue. To explain this complex phenotype, we propose that wild-type and misfolded PLP assemble into a protein complex.

EXPERIMENTAL PROCEDURES

Transgenic Complementation. The generation and characterization of PLP-transgenic/wild-type mice used in this study have been reported in ref. 24. Pronuclear DNA microinjection of ferti lized BDF2 hybrid mouse oocytes was performed as described (25). Heterozygous transgenic mice in lines 66 and 72 express the PLP transgene with similar efficiency relative to the endogenous gene and remain healthy (24), as the reported arrest of myelination is only observed in mice homozygous for these transgenes. In the present study, heterozygous PLP transgenic males were mated to jimpy carrier females (The Jackson Laboratory). Offspring were genotyped after tail biopsies and PCR analysis.

Genotype Analysis. Presence of the autosomal transgene was revealed by a PCR amplification product obtained with a primer at the 3′ end of the PLP transgene (5′-CAGGTGGTT-GAGTCGCTAGTCTACACAAG-3′) and primer T7cos (5′-GC-ATAAATACGACTCACTATAGGGATC-3′) directed against the T7 promoter in the PLP cosm id cos901 (24). The jimpy allele was distinguished from the wild-type gene by the loss of a Dde1 site. After the amplification of exon 5 with primers I-4 (5′-GGCCTCTAGGGTTATGAAG-3′) and I-5 (5′-CCTGGTCATCTAGCGATTCA-3′), Dde1 digestion results in three fragments (74 bp, 65 bp, and 53 bp) for the wild-type and two fragments (127 bp and 65 bp) for the jimpy allele. For autoradiography, PCR products were trace-labeled with [32P]dCTP (Amersham) and size-fractionated on 5% poly-

Abbreviations: PLP, proteolipid protein; RT-PCR, reverse transcription-PCR; MBP, myelin basic protein.

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acrylamide gels. To determine the sex of newborn mice, we performed a Y chromosome-specific PCR with primers sry1s (5'-GGGACTGTTGACAATTTTGC-3') and sry1a (5'-CAC-TGACGAAAGTGTTCATC-3'). All reactions were carried out simultaneously with denaturation at 94°C (45 s), annealing at 55°C (45 s), and extension at 72°C (45 s), in a reaction mixture containing 100 ng of genomic DNA, each primer at 0.4 μM, all four dNTPs (each at 200 μM), 2 mM MgCl₂, and 2 units of Taq polymerase (Promega).

**Quantitation of Transgene Expression.** Brains were removed and frozen in liquid nitrogen, and RNA was prepared by standard procedures (27). For reverse transcription (RT)–PCR analysis, brain cDNA was synthesized from total brain RNA with random hexamer primers and avian myeloblastosis virus reverse transcriptase (Stratagene) and quantified by incorporating trace label amounts of [32P]dCTP. For quantification of mRNA, exons 2–7 of PLP and DM-20 cDNA were amplified with primers located in exon 2 (5'-GCTCTCAGTGGTACAGA-3') and the 3' untranslated region (5'-ATGGATCCACAAAGGGCAGTCTTATGGAGACTC-3').

To better distinguish jimpy and wild-type fragments on gels, PCR products were digested with Nco I (GIBCO) because there is a Nco site only in exon 5 that is absent in the jimpy sequence. PCR parameters were as before except for 20 ng of cDNA, 2 μCi of [32P]dCTP (Amersham; 1 Ci = 37 GBq), and 4 units of Taq polymerase. All RT–PCR quantitations were normalized to an internal standard by coamplifying ubiquitin cyclic transcript with primers cycl5 (5'-ACCC-CACCT CCTCTAGGGA-3') and acyc300 (5'-CATTGGCATGGGACAAGTGA-3') as described (24). Aliquots of the reaction mixture were removed after 15, 19, 23, 27, and 31 cycles and size-separated on a 5% polyacrylamide gel, and products were quantified by using a phosphoimage analyzer (Fuji BAS 1000). Readings were normalized for C+G nucleotide content and relative expression ratios were calculated from the linear range of a half-logarithmic plot.

**Electron Microscopy and Immunocytochemistry.** Mice were anesthetized with avertin and perfused through the left ventricle with saline followed by either 5% (vol/vol) glutaraldehyde/4% (wt/vol) paraformaldehyde in cacodylate buffer (pH 7.2) or 10% (vol/vol) buffered neutral formalin. Brains, optic nerves, and spinal cords were removed and trimmed. Blocks for araldite embedding were postfixed in OsO₄ and processed routinely for electron microscopy. After dehydration and Epon embedding, 1-μm sections were processed for light microscopy and thin sections were processed for electron microscopy. Formalin-fixed tissue was embedded in paraffin wax. Resin sections of the thoracic spinal cord were stained with methylene blue/azure II. Immunocytochemistry was done by the peroxidase-antiperoxidase technique on 5-μm paraffin sections and 1-μm resin sections of spinal cord and brain. Antisera were provided by N. P. Groome (Oxford) (anti-PLP) and J. M. Mathieu (Lausanne) (anti-myelin basic protein (MBP)) or were commercially available (glial fibrillary acidic protein, Dako).

**Cell Counts and Myelin Morphometry.** Quantitation of glial cells and morphometric data was obtained by using groups of three to six mice for each genotype. Glial cell densities and the derived total glial cell counts were calculated in the ventral column of the cervical spinal cord. Oligodendrocytes and astrocytes were identified by using light microscopic morphological criteria. The total number of pyknotic nuclei in white matter was counted in toluidine blue-stained sections of cervical spinal cord (at least two sections per animal). The amount of myelin (myelin volume) in the lateral and ventral columns was quantified on electron micrographs (final magnification, ×6000). Myelin periodicity was measured directly on electron micrographs (final magnification, ×120,000).

**RESULTS**

The jimpy mutation is X chromosome-linked recessive (9, 10). In an attempt to rescue mutant mice by transgenic complementation, lines of PLP-transgenic mice were generated that transmit and functionally express autosomal copies of the entire mouse PLP gene. In independent experiments, heterozygous jimpy females (jp/+) were mated to phenotypically normal wild-type males, heterozygous for the PLP transgene in two different lines (24). In line 66, a total of 14 male offspring were analyzed that carried both the jimpy allele and PLP transgene (jp/Y; 66/--; Fig. 1A). All of them were phenotypically mutant and indistinguishable from nontransgenic jimpy mice with respect to onset and intensity of tremors. In spite of the presence of the PLP transgene, these mice also developed seizures and died prematurely at 3–4 weeks of age. All mice genotyped as (jp/+; 66/--) or (+/+; 66/--) were normal as expected. The same result was obtained in line 72, with 15 severely affected males subsequently genotyped as (jp/Y; 72/--), suggesting that a PLP transgene is unable to rescue the jimpy defect at the behavioral level.

By RNase protection and RT–PCR analysis of brain cDNA and by in situ hybridization experiments, the autosomal PLP transgenes were abundantly and cell-type-specifically expressed (24). At 18 days of age, which is approximately the PLP- and PLP-transgenic 66 mice with 115% efficiency relative to the endogenous PLP gene (being 100%) and in line 72 mice with 55% efficiency. Importantly, transgene expression paralleled the transcription of the endogenous mutant allele at very early (postnatal day 2) and late stages (postnatal day 17) of central nervous system myelination (Fig. 1B). We also recloned transgene-derived PLP and DM-20 cDNAs from brains of jimpy mice in both lines and ruled out any new mutation by sequence analysis (data not shown).

By macroscopic and light-microscopic examination, brains and spinal cords of all PLP transgenic mutants were severely dysmyelinated with no obvious difference from nontransgenic jimpy littermates. On closer inspection, however, we noted clear effects of the PLP transgene from both lines. By using an anti-peptide antibody against the C terminus of the wild-type protein, nearly all myelin sheaths in jimpy mice could be strongly immunostained for PLP/DM-20 in contrast to nontransgenic jimpy mice (Fig. 2 B, D, F, and H), as demonstrated also by labeling adjacent sections of the spinal cord with an antibody against MBP (Fig. 2 A, C, E, and G). Thus, transgene-derived PLP/DM-20 polypeptides are normally synthesized, transported, and assembled into the myelin membrane. A quantitative comparison of cervical cord white matter revealed that transgenic jimpy mice have an ~3-fold increased number of myelinated axons at 20 days of age, compared to nontransgenic jimpy controls (Fig. 2), but this number was variable. Thus, the additional expression of wild-type PLP increases a very low level of myelination in the mutant without restoring the normal phenotype. The thickness of myelin sheaths in transgenic mutants was also not significantly increased.

Ultrastructurally, myelin membranes show an abnormally compacted intraperiod line and a reduced periodicity in jimpy (27). We assessed the effect of transgenic complementation by electron microscopy (Fig. 3). The expression of the transgene restored the periodicity of transgenic jimpy myelin to that of the wild type [wild type, 12.43 ± 0.22 nm; (jp/Y, 11.96 ± 0.12 nm; (jp/Y; 66/++), 12.56 ± 0.24 nm; mean ± SEM), demonstrating that the altered spacing of myelin lamellae in jimpy is in fact due to the lack of normal PLP.

A major cause of dysmyelination in jimpy mice, and presumably in most cases of Pelizaeus–Mercier syndrome, is an abnormally high rate of oligodendrocyte death concurrent with an increased proliferation of immature cells (18–20). By comparing groups of PLP-transgenic and nontransgenic jimpy mice, we determined the total number of glial cells, oligoden-
drocytes, and pyknotic cells (largely dying oligodendrocytes) in spinal cord sections. In this study, the data from mice heterozygous for either transgene served as controls and were identical to those from wild-type mice. As shown in Fig. 4A, in (nontransgenic) jimpy mice the total number of glial cells was higher than in wild-type mice, confirming an earlier report (20). Expression of either PLP transgene did not change this

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Genetic complementation of jimpy mice with a wild-type PLP/DM-20 transgene. (A) Genotype analysis of transgenic and mutant mice obtained by mating a PLP transgenic male (from line 72) with a jimpy carrier female. Lanes: 1, wild-type male; 2, PLP-transgenic/wild-type male; 3, female jimpy carrier; 4, PLP transgenic jimpy carrier female; 5, jimpy male; 6, PLP-transgenic/jimpy male; 7, wild-type female; 8, jimpy male; 9, jimpy female; 10, wild-type male; 11, wild-type female; 12, nontransgenic jimpy male. (B) Dde digestion of jimpy transgene DNA and wild-type DNA. **Fig. 2.** Expression of functional PLP in transgenic jimpy mutant mice (line 72). Identical results were obtained in both transgenic lines. (A–D) Immunostaining of myelin proteins (A and C, MBP; B and D, PLP) in the lateral cervical spinal cord of a 22-day-old PLP-transgenic/jimpy mouse (A and B) and an age-matched (nontransgenic) jimpy control (C and D). Note the very sparse myelination of fiber tracts in jimpy (C), which is only moderately increased upon PLP transgene expression (A). By using a C-terminal-specific antibody, PLP is undetectable in the absence of a wild-type PLP transgene (D). (×190.) (E–H) Same as in A–D. Note that at higher magnification, immunoreactive MBP (E and G) and wild-type PLP (F and H) is strictly colocalized to individual myelinated axons of transgenic jimpy mice (E and F), indicating that transgene-derived PLP is assembled into compacted myelin. (×1132.)

The number significantly. When only morphologically identifiable oligodendrocytes were counted, there was no significant difference among PLP-transgenic/jimpy mice, nontransgenic jimpy mice, and controls, presumably reflecting two opposite trends: the increase of immature oligodendrocytes and loss of differentiated cells (data not shown).

To analyze the role of mutant PLP expression in abnormal oligodendrocyte death, we determined the number of pyknotic cells in the white matter of transgenic and nontransgenic mutants relative to the basal level of cell death in wild-type controls (in these experiments we found no difference between controls heterozygous for either transgene and nontransgenic mice) (Fig. 4B). We confirmed the previous finding that 3- to 4-week-old jimpy mice have a dramatically increased number of pyknotic cells (18, 21), suggesting that most newly generated jimpy oligodendrocytes die prior to terminal differentiation. In comparison, jimpy mice expressing the PLP transgene of either line 66 or line 72 showed a small but significant reduction in oligodendrocyte death, measuring ~50%. This decrease (reflecting a moderate increase of oligodendrocyte survival) is obviously insufficient to generate the normal number of myelin-forming cells.

Thus, we conclude that functional expression of the wild-type PLP gene can be achieved in jimpy mice by using
transgenic complementation; however, normal myelination cannot be restored in the presence of the mutant PLP allele. Preliminary experiments suggest that this genetic "dominance" of a PLP gene mutation is not restricted to the specific jimpy defect.

**DISCUSSION**

Point mutations in the mammalian PLP gene are associated with a lethal myelin deficit, best studied in the jimpy mouse. To distinguish between the loss of PLP function and gain-of-function effects of the misfolded protein, we took advantage of the X chromosomal linkage of the gene. By autosomal transgenic complementation, we generated "homozygous" mutants in which genotypic male oligodendrocytes expressed both the jimpy and the wild-type PLP genes. This allowed us to compare the effect of misfolded PLP in the hemizygous (mutant gene only) and the heterozygous (mutant plus wild-type gene) situation. Transgenic jimpy mice were phenotypically indistinguishable from nontransgenic jimpy mice despite subtle improvements of myelination at the cellular level. In this respect, the jimpy defect has revealed typical features of an autosomal-dominant disease.

Recessive mutations in mice can, in principle, be rescued by transgenic complementation, as demonstrated by shiverer mice transgenic for the MBP gene (25). We strongly believe that our opposite results obtained with jimpy reflect a previously unrecognized dominant action of mutations in the PLP gene rather than a functional deficit of the autosomal transgenes. In two lines of mice, expression of the reintroduced intact PLP gene was monitored at the RNA and protein levels and found to parallel qualitatively and quantitatively the expression of the X chromosomal-linked endogenous gene (24). We ruled out any new mutation in either transgene by recloning and sequencing PLP cDNAs from transgenic mutants (data not shown). It is important to note that expression

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**FIG. 3.** Restoration of normal myelin periodicity in PLP-transgenic/jimpy mice (line 72). Identical results were obtained in both transgenic lines. At high magnification, PLP-deficient jimpy mice (B) reveal an abnormal ultrastructure of myelin, including a fused intraperiod line (28) and reduced periodicity when compared to wild-type controls (A). Upon PLP-transgene expression (C), the normal periodicity is restored, but the intraperiod line appears more irregular and less electron dense than in wild-type myelin.

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**FIG. 4.** Glial cell death in PLP-transgenic/jimpy mice. (A) In sections of cervical cord whiter matter, the total number of glial cells (mean ± SEM), which includes immature oligodendrocytes, is higher in 18- to 22-day-old jimpy mice (solid bar) than in transgenic nonjimpy controls (open bars). The number of glia in PLP-transgenic/jimpy mice (shaded bars) is not significantly different from nontransgenic jimpy mutants. (B) The number of pyknotic cells (as percent of total glial cells) is severalfold increased in jimpy (solid bar) over controls (open bars) as observed (18). Expression of either PLP transgene reduces jimpy cell death to ~50%. The difference between lines 66 and 72 is not statistically significant. The data for the heterozygous transgenic mice (open bars) are not significantly different from wild-type mice (data not shown), indicating that expression of the transgene does not perturb glial cell kinetics.
of either PLP transgene was still below the threshold level of overexpression above which transgenic wild-type mice show a premature arrest of myelination (24, 29). Our data also agree with a recent report that transgenic expression of a human PLP cDNA and DM-20 cDNA construct is insufficient to cure the jimpy defect (26).

By immunocytochemical analysis, transgene-derived PLP polypeptides were correctly assembled into myelin, whereas in (nontransgenic) jimpy mice misfolded PLP is degraded shortly after synthesis (16). Our data thus provide direct evidence that myelin deficiency of jimpy mice results from the paucity of mature oligodendrocytes (18) rather than the absence of PLP from myelin membranes. Additionally, the myelin sheaths remained disproportionately thin despite the incorporation of PLP. At the ultrastructural level, however, PLP proved to be a major determinant of the intraperiod line of compacted myelin.

The cause of glial cell death in PLP mutant mice is unknown. Relative to nontransgenic jimpy mice, we calculated an ~2-fold increase of oligodendrocyte survival in PLP-transgenic mutants. Since the number of glial cells was unchanged, we believe that cell death in the jimpy central nervous system is not secondary to the overproduction of immature oligodendrocytes competing for limited survival factors. We suggest instead that cell death results from the expression of jimpy PLP itself, a presumably misfolded and unstable protein. Its aberrant C terminus is highly Cys-rich (12) and could exert cytotoxic effects in oligodendrocytes—for example, by crosslinking to proteins in the endoplasmic reticulum. The number of dying oligodendrocytes, however, was not dictated in a strictly dominant fashion by the mutant allele, as predicted if jimpy PLP was an unspecific trigger of cell death. Instead cell death was moderately reduced after coexpression of a wild-type PLP transgene. This shows that normal PLP inhibits the toxic effect of jimpy PLP to some extent.

PLP belongs to a diverse group of membrane proteins in which transmembrane domains form 4-helix-bundle structures (7, 8), many of which assemble into oligomers. Highly conserved in evolution, PLP may also form multimeric complexes involving homotypic or heterotypic protein interactions. Our data are compatible with a model in which functional PLP oligomerizes in the endoplasmic reticulum, a final step of quality control of other multimeric membrane proteins. According to this model, misfolded jimpy PLP is nonfunctional and cytotoxic (possibly only to oligodendrocytes and at a critical stage of development). Interaction of normal and misfolded polypeptides in the transgenic system generates mixed protein complexes that would be nonfunctional, except for a minor fraction of PLP oligomers. The toxicity of jimpy PLP may be caused by stable interactions of the misfolded protein with other proteins in the endoplasmic reticulum. The partial rescue of oligodendrocyte survival may be attributable to a specific shielding of PLP toxicity in a mixed protein complex. Our model allows testable predictions and is in agreement with an abnormal distribution of misfolded PLP overexpressed in fibroblasts (30). Moreover, cases of Pelizaeus–Merzbacher disease stemming from a complete deletion of the gene have a strikingly milder course than cases stemming from missense mutations (ref. 31 and O. Boespflug–Tanguy, personal communication). Cellular toxicity of misfolded membrane proteins, rather than a 50% reduction of functional protein (haplo-insufficiency), may be a general mechanism in other genetic diseases, such as in autosomal dominant retinitis pigmentosa (32).

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