LIMP-2/LGP85 deficiency causes ureteric pelvic junction obstruction, deafness and peripheral neuropathy in mice

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In previous overexpression studies we revealed a role for the lysosomal membrane protein LIMP-2/LGP85 in lysosomal biogenesis. LIMP-2-deficient mice show an increased postnatal mortality which is associated with a development of a uni- or bilateral hydronephrosis caused by an obstruction of the ureteropelvic junction. An accumulation of lysosomes in epithelial cells of the ureter adjacent to the ureteral lumen and a disturbed apical expression of uroplakin was observed, suggesting an impairment of membrane transport processes. Serious hearing impairment in LIMP-2-deficient animals was indicated by deficits in acoustic startle responses, in brainstem evoked auditory potentials and a reduced endochondral potential. LIMP-2-deficient mice suffer from a massive decline of spiral ganglia in the cochlea concomitant with that of the inner and outer hair cells. These pathological changes begin at the age of 3 months and are probably secondary to a degeneration of the stria vascularis. LIMP-2-deficient mice are also characterized by a peripheral demyelinating neuropathy. Demyelination was found to be associated with a massive loss of peripheral myelin proteins and an increased activity and expression of lysosomal proteins highlighting a hitherto unknown role of the lysosomal compartment in the development of this myelination disorder.

The phenotype of LIMP-2-deficient mice stimulates the search for mutations in human disorders associated with degeneration of the stria vascularis and/or demyelination of peripheral nerves.

INTRODUCTION

The limiting membrane of the lysosomal compartment fulfils multiple functions. It is responsible for acidification of the interior, sequestration of the active lysosomal enzymes (1), and transport of degradation products from the lysosomal lumen to the cytoplasm (2–4). The lysosomal membrane contains several highly N-glycosylated proteins (5,6) whose functions remain largely unknown. The lysosome-associated membrane proteins I and II (LAMP I and II) are distinguished from the lysosomal integral membrane proteins I and II (LIMP-1 and -2). LIMP-2 is regarded as a member of the CD36 family. This gene family is evolutionary conserved and includes cell adhesion molecules and lipid receptors at the cell surface as well as lysosomal membrane proteins. Members of this family include the mammalian gene Cla-1 (CD36 and LIMP-2 analogous-1) (7), which is also known as SR-BI (scavenger receptor class B-I) (8), the invertebrate genes Drosophila croquemort (dCD36) (9)
and emp (epithelial membrane protein) (10), and Caenorhabditis elegans cm08 h8 (11).

All members of the CD36 family share a common topology: LIMP-2 is a type III membrane glycoprotein that transverses the membrane twice with an aminoterminal transmembrane anchor corresponding to an uncleaved signal peptide, a large luminal domain and a second membrane-spanning domain preceding a 20 amino acid cytoplasmic tail at the COOH-terminus. A leucine-isoleucine motif within the C-terminal cytoplasmic tail determines lysosomal localization of LIMP-2 (12–14). The dileucine-motif of LIMP-2 interacts with the heterotetrameric adaptor-complex 3 (AP3) (15).

In vitro studies suggested an association of LIMP-2 with thrombospondin (16), although the biological significance of such an interaction is still unclear. The LIMP-2 homologue in Dictyostelium discoideum (DdLIMP) has been shown to be an effective suppressor of the profilin-minute phenotype (17,18). Profilin is a G-actin binding protein. Profilin deficient Dictyostelium discoideum cells show defects in pinocytosis, macropinocytosis, exocytosis and secretion of hydrolases.

We recently showed that overexpression of LIMP-2 caused an enlargement of early endosomes and late endosomes/lysosomes. Such morphological alterations were not observed after overexpression of other lysosomal membrane proteins. Overexpression of LIMP-2 impaired the endocytic membrane traffic out of these enlarged compartments, probably causing an accumulation of cholesterol in these compartments. Co-transfection of LIMP-2 and dominant-negative form of Rab5b inhibited the formation of the enlarged vacuoles, suggesting an interaction of the GTP-bound active form of Rab5b with LIMP-2 (19).

In the present study we have generated LIMP-2 knockout mice to address the in-vivo function of this lysosomal membrane protein. LIMP-2-deficient mice are viable and fertile but show a triad of phenotypical alterations. They develop an ureteric pelvic junction obstruction, deafness and a peripheral neuropathy making the limp-2 gene an interesting candidate gene for human diseases exhibiting one or several of these phenotypic alterations.

RESULTS

Targeted disruption of the limp-2 gene and generation of deficient mice

A 14 kb genomic clone from the limp-2 gene region was isolated (Fig. 1A). The targeting vector (Fig. 1AI) with an insertion of a neo cassette in a Nhel site at exon 8 of the murine limp-2 gene was used for disruption of the limp-2 gene in embryonic stem (ES) cells. The targeting construct was introduced into ES cells and in two out of 83 independent clones a homologous recombination event was observed (Fig. 1B). All of the generated chimeric males transmitted the mutated allele through the germline. Heterozygote mice exhibit a normal phenotype and normal fertility (data not shown).

Genotyping of offspring from heterozygote crosses (Fig. 1C) revealed a frequency of 27% for homozygous mutant mice (limp-2<sup>−/−</sup>), resembling the expected Mendelian frequency (25%). Hence, disruption of the limp-2 gene does not result in embryonic lethality.

To test for expression of the limp-2 gene in LIMP-2<sup>−/−</sup> mice northern blot analyses (not shown) and RT–PCR analyses (Fig. 1D) were performed. No limp-2 specific amplification products were detectable in homozygous mutant animals (Fig. 1D). The ubiquitously expressed LIMP-2 protein was not detectable in tissue homogenates from LIMP-2<sup>−/−</sup> animals (Fig. 1E).

Phenotype of LIMP-2-deficient mice

Homozygous mutant and heterozygous mice did not exhibit differences in growth, weight development and fertility (data not shown). Twenty-eight percent of LIMP-2-deficient mice died within the first 200 days of life (Fig. 2A). Determination of blood and serum parameters did not reveal obvious abnormalities (data not shown). LIMP-2-deficient mice consumed about twice as much drinking water compared with control mice (Fig. 2BII). This difference is also reflected by an increased urine volume of about 6 ml/day in LIMP-2-deficient males compared with 0.9 ml/day in control male mice (Fig. 2BII), leading to a severely reduced osmolality of the urine collected from LIMP-2-deficient mice (Fig. 2BIII). Normalized to osmolality, the level of electrolytes, urea and creatinin were comparable between the genotypes, whereas a decreased level of glucose was observed (not shown). In addition, protein electrophoresis and silver staining of control and LIMP-2-deficient urine, samples revealed the presence of a protein of about 70 kDa representing albumin, indicating a defect in glomerular filtration (Fig. 2BIV). Using standard urine sticks, increased number of leucocytes were observed in LIMP-2-deficient urine, suggesting infection of the urogenital tract system (not shown). With increasing age, LIMP-2-deficient mice were characterized by an altered body shape, i.e. a grossly enlarged, ball-like trunk.

Urinary tract pathology in LIMP-2-deficient mice

At autopsy, most of the LIMP-2-deficient mice at ages of 3–16 months displayed massive hydronephrosis, which was either unilateral (with 65% preference of the left side) or bilateral (Fig. 2C). The upper portion of the ureter was thickened, while the distal portion was inconvincing macroscopically (Fig. 2D). The thin-walled, giant pelvic cyst was filled with clear urine, and the kidney was reduced to a flat structure within the wall of the cyst. The pelvic mucosa in the vicinity of the uretero-pelvic junction displayed a papillary surface (Fig. 2E), which appeared to continue into the upper ureter.

The thickening of the upper ureter of severely hydronephrotic kidneys was due to proliferation of the mucosa, reducing the ureteric lumen to a narrow cleft (Fig. 2F and G). The mucosa displayed features reminiscent of lesions known in human pathology as Brunn’s nests or as ureteritis glandularis (20). The epithelium was stratified, with prismatic cells at the surface rather than umbrella cells as seen in the urothelium of wild-type mice. The lamina propria and the surrounding connective tissue were infiltrated with polymorphonuclear leucocytes and plasma cells. Even in non-hydronephrotic kidneys, the mucosa at the
ureteropelvic junction displayed papillary proliferations, whereas controls showed an entirely smooth surface (Fig. 2H and G).

On the cellular level, the urothelium was altered all the way down to the urinary bladder. At the ureteropelvic junction, the surface cells clearly had undergone metaplasia, since at the apical surface they showed short microvilli and a symmetric plasma membrane with a glycocalyx. This was in contrast to wild-type mice, where the apical membrane of the umbrella cells showed a serrate outline and an asymmetric trilaminar substructure known to be due to the presence of uroplakins (21). Additionally, the surface cells of LIMP-2-deficient mice displayed vacuoles filled with some floccular material, membrane-limited elongated inclusions filled with multilamellated material (Figs 2G and 3C–E), altered mitochondria (not shown), and sometimes greatly dilated cisternae of the endoplasmic reticulum (not shown).

In the distal ureter, there was a mixture of (i) cells altered as above and (ii) cells reminiscent of umbrella cells, with an asymmetric adluminal membrane. In contrast to wild-type mice, however, the so-called discoid vesicles with an asymmetric membrane (Fig. 3A and B), which are known to represent reserve membrane material inserted into the adluminal membrane, were no longer flat and discoid. Instead they were roundish and often contained small amounts of polymorphic material (Fig. 3C and D).

In the urinary bladder the superficial cells resembled those described under (ii) for the ureter. Additionally, huge solitary vacuoles occurred in some cells (not shown).

Using immunohistological localization of LAMP-1 (Fig. 4A and B) and cathepsin-D (Fig. 4C and D), we could show an increased expression in these cell layers of LIMP-2-deficient ureters, suggesting that the vesicles accumulating in LIMP-2-deficient cells represent lysosomes. This is also supported by immunoblot analyses showing that the major lysosomal membrane proteins LAMP-1 and LAMP-2 are upregulated in LIMP-2-deficient kidney, ureter and bladder (Fig. 4G). Enzyme activities of different lysosomal enzymes (not shown) were also elevated. An altered lysosomal compartment became also apparent after subcellular fractionation of control and LIMP-2-deficient ureter. In control ureters the lysosomal fraction is found in the dense part of the gradient, whereas in LIMP-2-deficient ureters the lysosomal marker is shifted towards the light fractions of the gradient (Fig. 4H). Interestingly the usually apical distribution of uroplakin along the ureter (Fig. 4E) is disturbed or absent in LIMP-2-deficient mice (Fig. 4F), suggesting defective membrane traffic between the uroplakin containing vesicles and the apical plasmamembrane of the urothelium.

**Deafness in LIMP-2-deficient mice**

During routine weekly cage cleaning, it became apparent that older LIMP-2-deficient mice did not react to noise or other acoustic stimuli. For detailed assessment of their response abilities, we have determined acoustic startle responses (ASRs) in 7- (Fig. 5A) and 2-month-old (not shown) LIMP-2-deficient and control mice. Seven-month-old LIMP-2-deficient mice showed no or only very weak reaction to the stimuli (Fig. 5A). The failure to respond to auditory stimuli appeared to be based on some neuronal deficit as the 7-month-old LIMP-2-deficient mice also showed severely impaired brainstem auditory evoked potential (BAEP) responses (Fig. 5B). On the other hand 2-month-old LIMP-2-deficient mice were still able to show ASR as well as BAEP responses (not shown), suggesting that LIMP-2-deficient mice become severely hearing impaired by developing a complete deafness between 3 and 7 months of age.

To further analyse the reason for the hearing impairment in LIMP-2-deficient mice we examined the cochlea of wild-type and LIMP-2−/− mice histologically (Fig. 5C–H). The most conspicuous alterations in the cochlea of limp2-deficient mice were the following: (a) atrophy of the stria vascularis beginning at the age of 2 months; (b) gradual reduction and finally loss of the outer hair cells, beginning around the age of 6 months, and finally also loss of the inner hair cells; (c) severe reduction of the neurons of the spiral ganglion in animals older than 9 months. Figure 5D–F shows the end stage seen in a 16-month-old animal.

The fibres of the acoustic nerve and the neuronal perikarya of the spiral ganglion representing the first neuron of the auditory pathway, became reduced in number with increasing age of the LIMP-2-deficient animals. By the age of 9 months, the neurons amounted to 85% of the age-matched controls. In two older animals of 12 and 16 months, the number was reduced to about 20% of age-matched controls.

The stria vascularis of the LIMP-2-deficient mice became reduced in width. At the ultrastructural level, the three cell layers were still present, but the interdigitating processes of the marginal cells, one of the most characteristic features of the normal stria, were totally lost (Fig. 6). The pericapillary extracellular matrix had increased in amount and electron density and extended into the intercellular clefts between the epithelial cells.

The assumption that a primary stria vascularis defect underlies the progressive deafness of LIMP-2-deficient mice is supported by the preliminary finding of a drastically reduced endocochlear potential (EP) at the time when threshold of auditory evoked brain-stem responses are strongly elevated. Thus, two 6-month-old LIMP-2-deficient mice showed EPs between 10 and 20 mV. In a 9-month-old LIMP-2-deficient mouse we could not detect any EP. Control mice of the same age recorded in the same set-up displayed robust EPs of 100–110 mV, which are expectedly higher than those previously reported in the second turn (22).

Also in the vestibular organ of LIMP-2-deficient mice characteristic abnormalities were noted. The stastolith membrane was severely reduced in height, otoliths were absent in LIMP-2-deficient mice and a layer of cells of unknown nature was found at the area were normally the otoliths are functioning (not shown).

**Peripheral neuropathy in LIMP-2-deficient mice**

The third prominent feature of LIMP-2-deficient mice is the development of a neuropathy which is restricted to peripheral nerves but absent in neurons from the central nervous system. At gross dissection, the nerves of LIMP-2-deficient mice appeared thicker than those of age-matched controls, which was confirmed in histological sections. For the phrenic nerve, the diameters were determined and found to be increased to about 150% of controls (Fig. 7). This was accompanied by...
Figure 1. Targeted disruption of the limp-2 gene. (A) Strategy for inactivation of the limp-2 gene by homologous recombination in ES cells. (I) Partial structure of the genomic locus representing about 14 kb of the limp-2 gene region. Exons are indicated by solid boxes and flanking introns are indicated by solid lines. The bar labeled '5' probe' denotes a DNA probe used for Southern blot analysis. (II) Targeting vector II pBSK-LIMP-2(neo) with 5.3 kb homology to the limp-2 gene locus. The neo cassette was inserted as an XbaI fragment into an NheI restriction site at Tyr 343 in exon 8. (III) Predicted limp-2 gene locus after homologous recombination. (B) Southern blot analysis of ES cell clones. The 5' probe was hybridized to SacI–BglII-digested genomic DNA from ES cell clones (E-14, E-24 and E-37). An additional 5.0 kbp DNA fragment indicates a targeted allele. (C) PCR analysis of tail-genomic DNA with an exon-specific PCR amplifying a 0.4 kb fragment in +/+, and a 1.6 kb fragment in −/− mice. (D) RT-PCR analysis of limp-2 expression. Total RNA was used for reverse transcription followed by PCR amplification of the LIMP-2-cDNA ORF. A 1.4 kbp fragment is amplified in +/+ and absent in LIMP-2-deficient mice, respectively. (E) Western blot analysis of LIMP-2 expression using a C-terminal specific antibody against mouse LIMP-2. (I) Glycosylated LIMP-2 molecules were ubiquitously detected in +/+ tissues and absent in tissues of LIMP-2−/− mice. (II) Coomassie-staining of a gel run in parallel demonstrates equal protein load.
reduction of the total number of nerve fibres per phrenic nerve by about 20%. In younger LIMP-2-deficient animals, the increase of nerve diameter was due to a widening of the empty space between the nerve fibres. In animals at the age of 6 months or older, the amounts of endoneurial collagen fibrils were increased. Furthermore, every nerve fibre was surrounded by concentric layers of Schwann cells as identified by the basal lamina (Fig. 7B). The nerve fibres were markedly thinner. Occasional medium-sized axon profiles were surrounded by a Schwann cell without myelin sheath indicating incipient remyelination (asterisks in Fig. 7B). The histologic picture observed in the nerves of limp2-deficient mice corresponded to that of the hypertrophic neuropathy, which is generally interpreted as a consequence of repeated de- and remyelination. Within a given animal, the examined nerves were affected to differing degrees, with the following decreasing order: saphenous nerve > sciatic nerve > phrenic nerve > facial nerve embedded in petrous bone; the acoustic nerve did not show indications of hypertrophic neuropathy.

The Schwann cells often contained abnormal dense inclusions and vacuoles with the outer cytoplasmic zone (Fig. 7C and D). The dense inclusions resembled those shown in the urothelium (see Fig. 4E).

To analyse lysosomal protein involvement and content of peripheral nerve proteins the expression of LAMP-1 and cathepsin-D (Fig. 8A) as well as MBP, PO and PMP22 (Fig. 8B) was examined in sciatic nerve extracts. Whereas the lysosomal proteins LAMP-1 and cathepsin D are increased in LIMP-2+/− mice the peripheral myelin proteins MBP and PMP22 are severely diminished, the decrease being less pronounced for P0 (Fig. 8B). The activity of various lysosomal hydrolases from LIMP-2+/− mice were also increased in LIMP-2+/− sciatic nerve homogenates (Fig. 8C). To correlate the apparent increase of the lysosomal system in LIMP-2-deficient peripheral nerves with the severely decreased expression of myelin proteins we performed immunohistochemistry of P0 and Cathepsin D on paraffin-embedded cross-sections of sciatic nerves (Fig. 9A and B). In controls myelinated nerve fibres are tightly packed. P0 is localized to the thick myelin sheaths and cathepsin D to cytoplasmic compartments at the periphery of Schwann cells (Fig. 9A). LIMP-2+/− animals showed thinner P0-positive myelin sheaths and more cathepsin D-containing compartments. This was confirmed by teased-fibre preparation of sciatic nerves followed by immunofluorescence analysis (Fig. 9C and D). P0 antigens are localized to the thick myelin sheath in control sciatic nerves, interrupted by myelin-free nodes of Ranvier. Cathepsin D localizes preferentially to the nodes of Ranvier (Fig. 9C). In LIMP-2-deficient sciatic nerves no nodes of Ranvier could be observed (Fig. 9D). The myelinization appeared much thinner since P0 fluorescence was markedly diminished.

**DISCUSSION**

It has been estimated that LIMP-2 contributes to about 4% of all lysosomal membrane proteins (23). Whereas the deficiency of the more abundant lysosomal membrane protein LAMP-1 (24) causes no overt phenotype in mice, the lack of LAMP-2 (25) causes a characteristic phenotype with cardiomyopathy and myopathy resembling human patients with Danon Disease (26,27). This was also one of the first reports showing that alterations in a lysosomal membrane protein were associated with human disease.

LIMP-2-deficient mice develop a dilatation of the renal pelvis and calyces from the third month of life. This uni- or bilateral hydronephrosis is caused by an obstruction of the urinary tract between renal pelvis and ureter. Secondary to this obstruction the kidney functions may be affected (28). The severely decreased osmolality and altered urine parameters in LIMP-2-deficient mice point towards a renal dysfunction. Additionally, the high quantity of albumin in the urine of LIMP-2−/−, but not control mice, may be explained by a glomerular filtration damage secondary to the hydronephrosis.

The exact reasons leading to the obstruction are currently unknown, but our microscopic studies point towards a hyperproliferation of the mucosa at the ureteropelvic junction. Also in human patients with inherited ureter obstruction a proliferation of these cells was observed (28,29). On light- and electron-microscopic examination, surface cells of the urothelium of LIMP-2−/− mice show a massive accumulation of vesicular structures, most likely of lysosomal origin. It has been suggested that these cells have an active endocytosis to ensure reuptake of low- and high-molecular-weight substances from the urine which are then transported to the lysosomes for degradation and recycling to the organism (30). The observed increased lysosomal enzyme activities and expression levels in LIMP-2 deficient ureter extracts would favour an impaired membrane/vesicular transport in LIMP-2 deficient umbrella cells. In a previous study we described that overexpression of LIMP-2 in
Figure 3. Abnormal morphology of urothelial cells in LIMP-2-deficient mice. Umbrella cells of the urothelium (ultrastructure in the ureters of a wild-type (A, B) and a LIMP-2-deficient mouse (C, D), both at the age of 16 months. In (A) numerous discoid vesicles are seen in the cytoplasm, which at higher magnification show an asymmetric membrane. In the limp-2-deficient umbrella cell, the vacuoles are round, contain low amounts of polymorphic material, and the membrane is not clearly asymmetric. (E) Abnormal lysosome-like inclusion with multilamellated material (arrow) in an umbrella cell of the urothelium (ureter of a 3-month-old limp-2-deficient mouse). Arrowheads point to the limiting membrane. Bars: (A) 3.8 μm; (B) 0.2 μm; (C) 4.1 μm; (D) 0.16 μm; (E) 0.11 μm.
Figure 4. Disordered expression of lysosomal proteins and uroplakin III in LIMP-2-deficient mice. (A) Immunohistological analysis of LAMP-1 expression in control and LIMP-2-deficient (B) ureter. (C) Immunohistological analysis of cathepsin-D expression in control and LIMP-2-deficient (D) ureter. (E) Immunohistological analysis of uroplakin 3 expression in control and LIMP-2-deficient (F) ureter: inserts in C–F represent higher magnification images. (G) Expression of lysosomal membrane proteins LAMP I and LAMP II in urogenital tract tissues of LIMP-2+/+ and −/− mice revealed by immunoblot. (H) Subcellular fractionation of postnuclear supernatants of ureter tissue from +/+ and −/− mice on a 30% Percoll gradient. Twenty fractions were assayed for β-hexosaminidase activity. The profile of +/+ ureter shows peak activity in fractions 16–19, corresponding to the lysosome-containing fractions. Endosome-containing fractions show less activity in fractions 2–3. Fractionation of LIMP-2−/− ureter reveals a shift of peak activity towards light fractions 1–3. Bars in (A)–(F) are 0.6 μm.
Figure 5. Deafness in LIMP-2-deficient mice. (A) ASR recorded in three +/+ and LIMP-2−/− mice aged 7.5 months. Control mice show normal response to acoustic stimulation, LIMP-2−/− mice display decreased or absent reaction to noise. (B) BAEP of the same mice show absent or decreased signals in LIMP-2-deficient mice. Cochlea of a wild-type (C–E) and a limp-2-deficient mouse (F–H), both at the age of 16 months. CD, cochlear duct. In the deficient animal, the stria vascularis (SV) is atrophic as shown in higher magnification in (H). The neurons of the spiral ganglion (SG) and the nerve fibres (NF) are greatly reduced in number. In (D) the inner and outer hair cells (IHC, OHC) are seen. In (G) the arrows point to the sites where the sensory cells are missing; the nuclei, which are seen, belong to supporting cells. Bars: (C, F): 100 μm; (D, G) 20 μm; (E, H) 40 μm.
different cell types causes an enlargement of early and late endosomes/lysosomes and that LIMP-2 may play a role in the biogenesis and maintenance of endosomal/lysosomal compartments (19). The assumption of an impaired vesicular trafficking is also supported by a severely disturbed expression of uroplakin III in LIMP-2-deficient urothelium. Uroplakins are involved in stabilization of the apical surface of the mammalian urothelium thus preventing urothelial rupture during bladder distention (31), by regulating the apical surface area through reversible retrieval from and insertion into the apical surface (32) and in regulation of the remarkable permeability barrier function of the urothelium (33). The ablation of uroplakin III in mice (21) was shown to cause primary vesicoureteral reflux (VUR) associated with the development of hydronephrosis, a hereditary disease affecting approximately 1% of newborns and representing a leading cause of renal failure in infants. It needs to be evaluated why the distribution and expression of uroplakin III is affected in the urothelium of LIMP-2-deficient mice.

Interestingly a similar ureter phenotype has been described in mice deficient for the metalloproteinase ADAMTS-1 (34). This protease has been described to be involved in the proteolytic processing of cell surface molecules and extracellular matrix molecules. ADAMTS-1 contains thrombospondin type I motifs (35) making an interaction between LIMP-2 and ADAMTS-1 possible. In vitro experiments already suggested such interactions (16) and it is tempting to speculate that LIMP-2 may be involved in the modulation of the activity of metalloproteinases of the ADAMTS family. The occasional presence of LIMP-2 and other lysosomal membrane proteins at the plasmamembrane has been demonstrated (36–39).

The severe hearing loss caused by strial dysfunction due to an atrophic stria vascularis with a poor interdigitation of the

Figure 6. Atrophy of the stria vascularis (ultrastructure) in LIMP-2-deficient mice. Cochlea of a wild-type (A, B) and a limp-2-deficient mouse (C, D), both at the age of 6 months. In the wild-type stria, numerous interdigitating processes of marginal cells are seen around a capillary (c). BL, basal lamina; el, endolymph. In the limp-2-deficient stria, the processes of the marginal cells (mc) are absent. ic, intermediate cell; bc, basal cell; fb, fibroblast. In the cytoplasm of the marginal cell abnormal vacuoles (V) are seen. The pericapillary extracellular matrix (ECM) extends between the epithelial cells, is of increased electron density and contains deposits of globular structures, whose nature is unknown. Bars: (A) 1.2 μm; (B) 1.5 μm; (C) 2.9 μm; (D) 0.7 μm.
Figure 7. Hypertrophic neuropathy in LIMP-2-deficient mice. Phrenic nerves of a wild-type (A) and a limp-2-deficient mouse (B–D), both at the age of 16 months. The insets show cross sections through the entire nerves at identical magnification. In (B) the diameter of the total nerve, the thickness of the perineurial sheath (P) and the amount of the endoneurial collagen fibrils are increased as compared with the wild type. The nerve fibres in (B) are thinner and surrounded by several layers of Schwann cell processes ('onion bulbs'). One axon (*) is ensheathed by a Schwann cell lacking a myelin sheath. (C) Nerve fibre marked by frame in (B). In the cytoplasm of the Schwann cell several abnormal vacuoles are seen. Ax, axon. N, nucleus. d, Portion from (C) at higher resolution. Some of the ‘stiff’ vacuoles are shown. BL, basal lamina. Bars: (A) 4 μm; insert, 70 μm; (B) 4 μm; insert: 70 μm; (C) 1.4 μm; (D) 0.3 μm.
marginal cells has also been described in the naturally occurring mouse mutants shaker-2 and mix (40), as well as gene knockout strains, such as the KCNJ10 potassium channel knockout (22). Our histopathological examinations revealed that strial degeneration is very likely the earliest event in the inner ear pathology of LIMP-2-deficient mice followed by a subsequent loss of outer and inner hair cells and spiral ganglia cells. The stria vascularis is a highly vascularized multilayered epithelium consisting of two epithelial barriers: the marginal cells secrete K⁺ into the endolymph, whereas the basal cells inside stria vascularis are associated with intermediate cells and outside the spiral ligament with fibrocytes. The high K⁺ concentration of endolymph of about 150 mM drives the sensory transduction in the hair cells (41). The severe atrophy of marginal cells in LIMP-2-deficient mice may lead to a disturbed endocochlear potential and secondary damage of hair cells and neurons of the spiral ganglia. In the basolateral membrane of the marginal cells the Na⁺, K⁺-ATPase and the secretory isoform of Na⁺-K⁺-2Cl⁻ cotransporters mediate uptake of the K⁺ from the interstitial compartment (42,43) while the secretion of K⁺ in the endolymph is mediated by KCNQ1 and KCNE1, voltage-dependent channels localized in the apical membrane (44).

A third interesting feature of LIMP-2 deficient mice is the development of a progressive neuropathy which is restricted to the peripheral nervous system. The major cell type responsible for this demyelinating pathology is the Schwann cells. Different genetic defects have been associated with a dysfunction of this cell type (45), leading to peripheral nerve dysmyelination and rendering Schwann cells unable to sort bundles of axons.

Figure 8. Increased expression and activation of lysosomal proteins concomitant with decreased expression levels of peripheral myelin proteins in LIMP-2-deficient mice. (A, B) Western blot of lysosomal (A) and peripheral myelin proteins (B) using independent preparations of sciatic nerve extracts from control (+/+) and LIMP-2-deficient mice (−/−) aged 1 year. Lysosomal proteins LAMP I and Cathepsin D are increased in LIMP-2−/− mice, which do not express LIMP-2 protein (A). Peripheral myelin proteins MBP and PMP22 are strongly down-regulated in −/− mice, the effect is less pronounced for P0 (B). The size of the corresponding proteins is indicated by arrows. (C) Activity of lysosomal hydrolases in sciatic nerve homogenates from control and LIMP-2−/− mice. All measured enzymes show increased activity in −/− mice. β-Hex, β-hexosaminidase; β-Gal, β-galactosidase; β-Mann, β-mannosidase; β-Gluc, β-glucuronidase; ASA, arylsulfatase A.
Interestingly, LIMP-2-deficient Schwann cells exhibit an upregulation of lysosomal enzymes concomitantly with a downregulation of peripheral myelin proteins. Although it remains to be determined if the decrease of peripheral myelin proteins is due to mis-sorting and degradation in the lysosomal compartment, it highlights a somewhat unexpected role of LIMP-2 in the regulation of these myelin proteins. A possible role of lysosomes in demyelinating processes is also highlighted in defective myelination in other animal models. In Trembler^1^-neuropathy, with a pmp-22 mutation (46), the activation of the endosomal/lysosomal degradation pathway of myelin has been discussed, leading to an instability of myelin and subsequent downregulation of other myelin proteins such as P0 and MBP. How LIMP-2 deficiency causes the apparent up-regulation of

Figure 9. Altered expression and distribution of Cathepsin D, associated with decreased levels of myelin. (A, B) Immunohistochemistry of P0 and Cathepsin D on paraffin-embedded cross-sections of sciatic nerves from 1-year-old +/+ (A) and −/− (B) mice. Control nerves show P0 fluorescence (red) localized to thick myelin sheaths formed around axons by Schwann cells. Myelinated fibres are tightly packed. Cathepsin D fluorescence (green) localizes to cytoplasmic compartments at the periphery of Schwann cells. The same nerve in LIMP-2^−/− mice shows thinner P0-positive myelin sheaths and more Cathepsin D-containing compartments (marked by arrow). Bar = 10 μm. (C, D) Teased fibres of sciatic nerves from LIMP-2^+/+ (C) and LIMP-2^−/− (D) mice. P0 fluorescence (green) localizes to the thick myelin sheath of control sciatic nerves, interrupted by myelin-free nodes of Ranvier (marked by arrow). Cathepsin D (red) localizes preferentially to the nodes of Ranvier. LIMP-2-deficient sciatic nerves are thinner, and P0 fluorescence is markedly diminished. Cathepsin D fluorescence is distributed in vesicle-like structures along the nerve. No nodes of Ranvier could be found in −/− sciatic nerves. Bars: (A, B) 30 μm (C, D) 20 μm.
lysosomal enzymes in Schwann cells remains obscure, but again a defective lysosomal biogenesis as suggested by LIMP-2 overexpression studies (19) could account for the observed alterations.

Charcot–Marie–Tooth disease (CMT), a peripheral neuropathy, is one of the most frequent inherited neurological disorders, with a prevalence of approximately one in 2500 people. We have initiated a study to screen for possible mutations in the human LIMP-2 gene in patients suffering from a combination of symptoms similar to the phenotypic alterations described in LIMP-2-deficient mice.

MATERIALS AND METHODS

Generation of mutant mice

A EMBL3-129SV mouse phage library from Stratagene Inc., La Jolla, USA, was screened with a partial cDNA of rat limp-2 (lgp85). The isolated mouse limp-2 phage clones contained the murine limp-2 gene containing exons 7–11. The neo expression cassette (Stratagene) was inserted into a Nhel restriction site located in exon 8 of the KpnI-SacI fragment (Tyrosine 343 of the limp-2 cDNA). The targeting vector was introduced into the ES cell line E14-1 by electroporation. G418-resistant colonies were screened by Southern blot analysis of DNA digested with SacI-BglII and hybridized with the 5′ probe (Fig. 1A). The mutated ES line E1I24 was microinjected into blastocysts of C57BL/6J mice. Chimeric males were mated to C57BL/6J females. Mice were genotyped for the limp-2 gene mutation by Southern blot analysis of DNA digested with SacI–BglII and hybridized with the 5′ probe (Fig. 1A).

RT–PCR analysis

One microgram of total liver and kidney RNA was used for cDNA synthesis using the Qiagen Omniscript Reverse Transcriptase system. For amplification of the limp-2 cDNA-specific primers [LIMP-2 3′(1), 5′-CCA AGC TCA GGC AAC AGG TAA GAC CCC-3′, LIMP-2 3′(2), 5′-TTA GGT TCG TAT GAG GGG TGC TCT-3′, LIMP-2 5′(1), 5′-ATG GGC AGA TGC TCG TTC TAC ACG-3′] were used in a standard PCR reaction (30 min at 94°C, 30 min at 55°C, 120 min at 72°C, 30 cycles).

Urine analysis

The amount of water uptake and urine excretion were measured every 24 h for 2 days. Osmolality of the urine was measured using a freezing-point osmometer (Knauer Osmometer automatic) and excretion of electrolytes was measured on a VT250 Chemical Analyzer.

Measurements of auditory startle responses and brainstem auditory-evoked potentials

ASR were recorded using computerized startle response apparatus and software (Med Associates Inc., Vermont, USA). The stimulation protocol consisted of a series of 12 non-startling 60 dB tones randomly alternated with 12 startle 120 dB tones. Tone frequency and duration were 10 kHz and 60 ms, respectively. Startle ballistograms were recorded for 300 ms following the onset of the tone. For each animal, 9–12 checked ASR ballistograms were averaged offline, and mean peak time and amplitude of the first ASR peak were determined on the tracings.

Brainstem auditory-evoked potentials (BAEPs) were recorded as described previously (48). Clicks of 0.1 ms and 90 dB were delivered at a rate of 10 Hz from a single speaker placed 5 cm in front of the animal. The BAEPs wave tracings displayed in the figures were the average of 2000 sweeps.

Measurement of endocochlear potential

Mice where anaesthetized, the osseous cochlear capsule over the first cochlear turn was locally thinned out, a glass micropipette filled with artificial endolymph (125 mmol KCl, 25 mmol KHCO3, 5 mmol HEPES, 2 mmol CaCl2, 1 mmol NaCl) (49) was gently inserted into the scala media and the endolympathic potential was recorded.

Western blot analysis

Expression of lysosomal proteins LAMP I, LAMP II and LIMP-2 was analysed in tissue homogenates as described before (24). Blots were incubated with a monoclonal anti-mouse LAMP-1 (1D4B) and an anti-mouse LAMP-2 (Abl 93) antibody (Developmental Studies Hybridoma Bank, Iowa, USA) in a 1:200 dilution, a polyclonal anti-rat LIMP-2 (lgp85) (50) antibody in a 1:1000 dilution, a monoclonal anti-rat LAMP-1 antibody in a 1:200 dilution, a polyclonal anti-rat LAMP-2 (lgp85) (50) antibody in a 1:1000 dilution, a polyclonal anti-rat LIMP-2 (lgp85) (50) antibody in a 1:500 dilution, a monoclonal anti-mouse Cathepsin D (S II-9) (51) antibody in a 1:400 dilution, a monoclonal anti-mouse LAMP-1 antibody in a 1:500 dilution, a monoclonal anti-mouse LAMP-2 (Abl 93) antibody in a 1:2500 dilution, a monoclonal anti-mouse LAMP-2 (Abl 93) antibody in a 1:5000 dilution, and Cx5, 5′- GGA TAC AGC AGG TGA CAG-3′ and Cx3, 5′-ATA GGC TAC GGA CCA CAA-3′ flanking exon 8 used for interruption.

Subcellular fractionation on Percoll gradient

Tissue homogenates of ureters were prepared using a Dounce-homogenizer. The postnuclear supernatant was mixed with a Percoll (Amersham)/0.25 M sucrose/3 mM imidazole pH 7.4 solution to give a final concentration of 30% (v/v) Percoll. The gradient was built by centrifugation for 1.5 h at 4°C and 30000g; 20 fractions were collected using a fraction collector. Individual fractions were assayed for β-hexosaminidase activity.

Lysosomal enzyme assays

Lysosomal enzymes were detected using fluorimetric assays as described (52). Arylsulfatase A was measured using p-nitrocatechol-sulfate as substrate (53).

Histology

For conventional light and electron microscopic examination, 12 limp2-deficient mice (ages 2–16 months) and eight
age-matched wild-type mice were used. Tissues were perfused with Bouin solution (diluted 20–25% with PBS) or glutaraldehyde (6%, in 0.1 M phosphate buffer, pH 7.4). Tissue blocks were processed for light and electron microscopic examination (embedding in paraffin or araldite) according to routine procedures. Sections were incubated with antibodies against cathepsin-D (SII-9), GFAP (Dako, Hamburg, Germany), F4/80 (clone obtained from DSHB, Iowa, USA), LAMP-1 (1D4B), MHC-II (Pharmingen, Hamburg, Germany), uroplakin (Progen, Heidelberg, Germany) and lectins RCA-1, GS-I B4 and Solanum tuberosum (all lectin reagents from Vector, Burlingame, USA).

**Immunofluorescence on paraffin embedded sections and “teased fibres” of peripheral nerves**

For paraffin-embedded sections of peripheral nerves, 1-year-old mice were perfused with 30 ml HBSS (Dulbecco) followed by 50 ml 1/4 Bouin solution. Sciatic nerves were embedded into paraffin blocks and processed according to routine procedures. Primary antibodies used were anti-mouse cathepsin D (SII-9) in a 1:50 dilution and anti-rat P0 in a 1:700 dilution. Photographs were taken using a confocal laser scanning microscope (LSM 2; ZEISS, Oberkochen, Germany).

**Electron microscopy**

For transmission electron microscopy, animals were perfused with 6% glutaraldehyde in phosphate buffer. Tissue blocks were fixed in 4% paraformaldehyde, followed by 3 min ice-cold methanol (95:5), 2 min acetone and 30 min methanol-0.2% acetic acid (25% with PBS) or glutaraldehyde (6%, in 0.1 M phosphate buffer, pH 7.4). Tissue blocks were processed for light and electron microscopic examination (embedding in parafﬁn) or glutaraldehyde (6% in 0.1 M phosphate buffer, pH 7.4). Tissue blocks were fixed in OsO4 for 2 h, and contrasted with uranyl acetate and lead citrate, and observed with a transmission electron microscope (Zeiss EM 900 and EM 902 microscopes).

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