Tubular proteinuria in mice and humans lacking the intrinsic lysosomal protein SCARB2/Limp-2

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Desmond MJ, Lee D, Fraser SA, Katerelos M, Gleich K, Martinello P, Li YQ, Thomas MC, Michelucci R, Cole AJ, Saftig P, Schwake M, Stapleton D, Berkovic SF, Power DA. Tubular proteinuria in mice and humans lacking the intrinsic lysosomal protein SCARB2/Limp-2. Am J Physiol Renal Physiol 300: F1437–F1447, 2011. First published March 23, 2011; doi:10.1152/ajprenal.00015.2011.—Deficiency of the intrinsic lysosomal protein human scavenger receptor class B, member 2 (SCARB2; Limp-2 in mice) causes collapsing focal and segmental glomerular sclerosis (FSGS) and myoclonic epilepsy in humans, but patients with no apparent kidney damage have recently been described. We now demonstrate that these patients can develop tubular proteinuria. To determine the mechanism, mice deficient in Limp-2, the murine homolog of SCARB2, were studied. Most low-molecular-weight proteins filtered by the glomerulus are removed in the proximal convoluted tubule (PCT) by megalin/cubilin-dependent receptor-mediated endocytosis. Expression of megalin and cubilin was unchanged in Limp-2−/− mice, however, and the initial uptake of injected Alexa Fluor 555-conjugated bovine serum albumin (Alexa-BSA) was similar to wild-type mice, indicating that megalin/cubilin-dependent, receptor-mediated endocytosis was unaffected. There was a defect in proteolysis of reabsorbed proteins in the Limp-2−/− mice, demonstrated by the persistence of Alexa-BSA in the PCT compared with controls. This was associated with the failure of the lysosomal protease cathepsin B to colocalize with Alexa-BSA and endogenous retinol-binding protein in kidneys from Limp-2−/− mice. The data suggest that tubular proteinuria in Limp-2−/− mice is due to failure of endosomes containing reabsorbed proteins to fuse with lysosomes in the proximal tubule of the kidney. Failure of proteolysis is a novel mechanism for tubular proteinuria.

albuminuria; lysosome

MUTATIONS IN THE LYOSOSMAL membrane protein human scavenger receptor class B, member 2 (SCARB2) have recently been found to cause action myoclonus renal failure syndrome, a rare syndrome of progressive neurological disease associated with nephrotic-range proteinuria, kidney failure, and the histological appearances of collapsing focal and segmental glomerular sclerosis (FSGS) (2, 3). Although Limp-2−/− (the murine homolog of SCARB2) mice develop albuminuria, FSGS has not been observed (3, 15). At 16 mo of age, mice have diffuse foot process effacement, suggesting that the albuminuria is glomerular in origin (3). At 3 mo of age, however, there is significant albuminuria in the absence of foot process effacement (Power DA, Berkovic SF, unpublished data). This suggested that some of the albuminuria noted in the Limp-2−/− mice could be due to tubular dysfunction.

Limp-2 is an intrinsic membrane protein of the lysosome, with its N- and C-terminal tails located within the cytoplasm and a heavily glycosylated loop located within the lysosome (32). Its function has been only partly defined, although overexpression of Limp-2 leads to formation of enlarged lysosomal structures, indicating a role in lysosomal biogenesis and maintenance (17). Interestingly, Limp-2−/− mice have a conductive deafness associated with a failure to express megalin on luminal surfaces of marginal cells within the stria vascularis on the inner ear (16). Megalin is important in reabsorption of filtered proteins, including albumin, from the tubular filtrate in the proximal tubule (4, 11). Some proteins such as retinol binding protein (RBP) require expression of megalin on the tubular cell surface while others such as transferrin require coexpression of megalin and its associated receptor cubulin (4, 27).

In two inherited forms of tubular disease, Dent disease (9, 28) and Lowe syndrome (19), reduced expression of megalin has been identified as the most likely explanation for proteinuria. Other causes of tubular proteinuria such as nephropathic cystinosis and the autosomal dominant form of renal Fanconi syndrome (31), however, have not been associated with changes in megalin expression.

In view of the evidence that megalin expression is reduced in the inner ear of Limp-2 null mice, we attempted to determine whether mice and humans lacking SCARB2/Limp-2 have tubular proteinuria.

METHODS

Animal and human studies. The Austin Hospital Animal Ethics Committee approved the experimental protocols in mice. The generation of Limp-2−/− mice has been previously described (15). Controls were littermate siblings.

Human studies were approved by the Austin Hospital Human Research Ethics Committee. Urine was shipped to Australia on dry ice and stored at −80°C.

Antibodies. Primary antibodies used included rabbit anti-Limp-2 (Novus Biologicals), rat anti-LAMP-1 and LAMP-2 (DSHB), goat
anti-cathepsin B, goat anti-megalin and cubilin (Santa Cruz Biotechnology), and mouse anti-
β-tubulin (Sigma, St. Louis, MO). For proximal tubular antigens, rabbit anti-transferrin, rabbit anti-
Gc globulin (vitamin D-binding protein [VDBP]), rabbit anti-RBP, rabbit anti-α1 microglobulin, and rabbit anti-β2 microglobulin antibodies (all from Dako) were used. Secondary antibodies were directed against the primary antibody species and conjugated with horseradish peroxidase (HRP; Dako), Alexa 488, or Alexa 594 (Molecular Probes) for Western blotting and immunofluorescence microscopy, respectively.

**Urine protein analysis.** Urine samples were separated on 12.5 or 4–20% SDS-PAGE gels. For Western blot analysis, proteins were electrophoretically transferred from the SDS-PAGE gel to polyvinylidene difluoride membranes (Millipore), washed, blocked, and immunoreactive proteins were detected with West Pico Chemiluminescence (Pierce) according to the manufacturer’s instructions. RBP was assayed by ELISA (GenWay Biotech, San Diego, CA). For human urine samples, volumes were corrected for creatinine concentration. For mouse urine, timed collections were used to normalize differences in urine volumes, so that a sample of urine collected over the same time was added to the gel.

**Urine excretion of albumin, amino acids, glucose, phosphate, and uric acid in mice and humans.** Wild-type (WT) and Limp-2 mouse urine samples were run on 10% SDS-PAGE and stained with Coomassie dye. For identification by mass spectrometry, proteins were excised from a dried gel and subsequently digested with 250 ng proteomics grade trypsin (Sigma). Tryptic peptides were analyzed on a LC/MSD Trap XCT Plus mass spectrometer (Agilent) coupled to an Agilent 1100 series HPLC. Peptide separation was achieved using an HPLC-Chip (Agilent) comprising a 40-μl enrichment column and a 75-mm separation column packed with 5 μm Zorbax 300SB-C18. Tandem mass spectrometry (MS/MS) ions were searched using MASCOT (software version 2.1.03, Matrix Science).

**Harvesting mouse kidneys.** Mice were anesthetized, and kidneys were harvested for immunofluorescence by open nephrectomy and immersion fixation in 4% paraformaldehyde. For fine structural morphology on electron microscopy and some immunofluorescence microscopy, the kidneys were perfusion fixed by retrograde cannulation of the abdominal aorta inferior to the renal arteries with 2.5% glutaraldehyde and 4% paraformaldehyde, respectively.

**Immunofluorescence microscopy.** Kidney sections were incubated with primary antibody in 10% BSA for 60 min at room temperature, washed three times in PBS, then incubated in secondary antibodies conjugated with Alexa 594 (red) or Alexa 488 (green) in 10% BSA for 30 min. Following three further washes in PBS, sections were mounted with fluorescent mounting medium (Dako). Images were generated and collected on a confocal laser microscope (Leica Microsystems, Heidelberg, Germany).

**Quantification of protein distribution and colocalization.** Fluorescence of LAMP-1 and LAMP-2 in the proximal convoluted tubule (PCT) was quantified using the software NIH ImageJ 1.34 (http://rsweb.nih.gov/ij/), as described previously (30). A region of interest (ROI) was drawn for the apical (excluding the lumen) and basal areas, respectively, divided by a circumferential line midway between the brush border and basolateral surface of the cross section of each PCT. The fluorescence intensity for Alexa 488 was measured for the apical and basal halves of each tubule, and the mean intensity was calculated. The mean intensity of the background fluorescence was determined by measuring the ROIs in the basal region of each tubule clear of LAMP vesicles. The corrected mean fluorescence intensity was calculated by subtracting the background intensity from that of the apical and basal halves of each tubule, respectively. A ratio of the corrected basal:apical fluorescence intensity was then determined for each tubule from Limp-2+/− or −/− animals.
To quantify the proportion of RBP colocalizing with LAMP-1 or cathepsin B in the PCT, the software NIH ImageJ 1.34 was used to measure the area of pixels of RBP (in the red channel) and that of LAMP-1 or cathepsin B (in the green channel) after adjustment of the threshold to minimize background noise while retaining the signal of

Fig. 2. Characteristics of urinary protein excretion in Limp-2/−/− mice compared with wild-type (WT) mice and mice with anti-glomerular basement membrane (GBM) disease. A: Coomassie blue stain of nonreduced urine samples run on a 10% SDS-PAGE reveals markedly increased low-molecular-weight proteinuria in Limp-2/−/− mice compared with the other mice. There was also an increase in albuminuria compared with WT, but this is not as marked as the anti-GBM disease model. Multiple bands in the urine from mice with anti-GBM disease were found to be albumin, marked with arrows in the lanes run with urine from mice with anti-GBM disease (GBM). In the Limp-2/−/− lanes, arrows depicting albumin (small, top) and α2u-globulin (large, bottom) are shown. The higher α2u-globulin band is probably a dimer.

B: albumin-to-creatinine ratio showed a 13-fold increase in Limp-2/−/− mouse urine compared with WT; n = 12/group. *P < 0.05 compared with WT.

C: Western blot of 10% SDS-PAGE of mouse urine. Limp2/−/− mouse urine contains low-molecular weight proteins including recognized ligands of the megalin [vitamin D-binding protein (VDBP), α1-microglobulin, and β2-microglobulin] and cubilin (transferrin) receptor complex.

Fig. 3. Expression of megalin and cubilin in Limp-2/+/+ and −/− mouse kidneys. A: immunofluorescence microscopy with anti-megalin antibody (red) shows similar staining at the brush border of proximal tubules of WT (+/+) and Limp-2/−/− (−/−) mice. Labeling with anti-cubilin antibody (green) was also similar in WT and Limp-2/−/− mice. Scale bars = 20 μm. B: Western blot of whole kidney lysates showed no difference in the amount of megalin and cubilin between the 2 groups. C: qRT-PCR revealed no difference in the amount of mRNA for megalin and cubilin, although there was a trend toward more mRNA in the Limp2/−/− mice (open bars; n = 12) compared with WT (filled bars; n = 11).
the vesicles, and this process was performed in a similar fashion for all tubules by the same operator. A ratio of the “area of pixels of RBP colocalizing with LAMP-1 or cathepsin B” to that of the total RBP in each tubule from Limp-2/+/+ or −/− animals was then calculated. Using JACoP (http://tsweb.nih.gov/ij/plugins/jacop.html), a plugin for the software ImageJ, the Manders’ coefficient for each tubule was generated, an alternative method for measuring the ratio of the “summed intensities of pixels from the RBP immunostaining where the intensities from the LAMP-1 or cathepsin B signals were above zero” to the “total intensity from RBP,” as described previously (6). All images were acquired under the same settings, in particular the same pinhole for an identical section thickness of 0.8 μm, by the Zeiss LSM 510 Meta confocal microscope (Goettingen, Germany). Images were analyzed by z-series to ensure that the areas of colocalization existed in all three dimensions and not due to pixels from two different channels at various levels of the sections.

Three independent and blinded experiments were performed, and at least 20 proximal tubules in total from 5 pairs of mice and 18 tubules from 3 pairs were analyzed for the studies of LAMP distribution and colocalization, respectively.

HRP- and Alexa Fluor 555-conjugated BSA injections. Mice were anesthetized and injected intravenously with 120 μg/g body wt of HRP (Sigma) into the tail vein, as described previously (9, 28). At 7 min, kidneys were removed and fixed in 4% paraformaldehyde before embedding.

For studies using labeled albumin, mice were anesthetized and injected intravenously with 10 μg/g body wt of Alexa Fluor 555-labeled BSA (Alexa-BSA), as described previously (25). Kidneys were removed at 7, 30, or 60 min and immersion-fixed in neutral buffered formalin for these studies. Some kidneys were also perfusion-fixed with 4% paraformaldehyde for these studies.

Real-time PCR. Total RNA was purified from whole mouse kidney samples by using TRIZol reagent (Invitrogen) in accordance with the manufacturer’s instructions. RNA was then quantified using spectrophotometry and reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an Applied Biosystems real-time PCR system ABI-7500 (Applied Biosystems) with Brilliant II SYBR green Master mix (Stratagene) according to the manufacturers’ instructions. The sequences for the primers used were megalin 5′-AGGCCACCAGTTCACTTGCT-3′ (sense) and 5′-AGGACACGCCATTCTCTTG-3′ (antisense), cubilin 5′-GGGATCCTCTTCAGGGACACA-3′ (sense), 5′-TGCTGGCCGATTCTAAATCAAA-3′ (antisense), and the housekeeping gene used was mouse 18S 5′-AGTCCCTGCCCTTTGTACACA-3′ (sense) and 5′-GATCCAGGGCCCTCACTAAAC-3′ (antisense). Primer efficiency was measured using standard dilution, and the Pfaffl method (21) was used to calculate relative expression. Data are expressed as fold-expression relative to littermate control mice.

Statistical analysis. GraphPad InStat v.3.06 (GraphPad Software, La Jolla, CA) was used for data analysis. Statistical differences between individual experimental groups were compared by unpaired Student t-tests. Results were expressed as means ± SD. A P value of <0.05 was considered significant.

RESULTS

Tubular proteinuria in humans with SCARB2 mutations. While most of the patients described with action myoclonus renal failure syndrome had severe renal disease, a small group has subsequently been reported with disease restricted to the neurological system (13). Urine was obtained from two such individuals, and the albumin/creatinine ratio was determined. Case A had microalbuminuria defined as an albumin/creatinine ratio ≥2.5 mg/mmol, whereas case B had no detectable albuminuria (Table 1). Analysis of urine using an ELISA for RBP demonstrated that case B had tubular proteinuria, using criteria developed by Norden et al. (20), whereas for case A it was mixed glomerular and tubular. Albumin was detectable by Coomassie staining of urine from both cases compared with negative controls (Fig. 1A). Western blots revealed that both of these individuals had detectable transferrin, VDBP, and RBP.
whereas these were undetectable or only faintly present in negative controls (Fig. 1B). Interestingly, \( \alpha_{1}\)-microglobulin was easily detectable in negative controls. To determine whether there was any evidence of Fanconi syndrome, urine was assayed for the presence of aminoaciduria, phosphaturia, and glycosuria. All were within normal limits (data not shown).

**Urinary protein excretion in Limp-2\(^{-/-}\) mice.** The major differences seen in Coomassie-stained SDS-PAGE gels among urine from normal mice, Limp-2\(^{-/-}\) mice, and mice with anti-glomerular basement membrane (anti-GBM) disease were the increased low-molecular-mass species seen in Limp-2\(^{-/-}\) mice and the increased high-molecular-mass species in the anti-GBM mice (Fig. 2A). Analysis of selected bands by mass spectrometry showed that the major bands at 32 and 18 kDa in the low-molecular-mass region were \( \alpha_{2u}\)-globulin proteins. The higher band disappeared when the gels were run reduced, indicating that it was probably a dimer of two of the smaller subunits. The \( \alpha_{2u}\)-globulin proteins are synthesized by the liver in male rodents and excreted by the kidney (8). Their function is to bind pheromones (8). Previous studies have shown that they are partially reabsorbed and degraded by the proximal tubule in the kidney (8, 29). Peptides from odorant protein 1A, previously shown to be increased in urine from megalin-deficient mice (18), were not present. Albumin excretion rates were also increased in Limp-2\(^{-/-}\) mice relative to WT at 12 wk (Fig. 2B).

Urine proteins separated on SDS-PAGE were then subjected to Western blotting to determine whether there was any loss of proteins known to require tubular reabsorption. While transferrin was detected in urine from Limp-2\(^{-/-}\) and anti-GBM

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**Fig. 5.** Immunofluorescence microscopy for intrinsic lysosomal proteins LAMP-1 and LAMP-2 and their distributions in the proximal tubules from Limp-2\(^{+/+}\) and \(^{-/-}\) mice. A: there was similar intensity of staining for LAMP-1 and LAMP-2 (green) in the 2 groups. WT mice (\(^{+/+}\)) are on the left, with Limp-2\(^{-/-}\) mice on the right. Top: labeled with anti-LAMP-1 antibody; bottom, labeled with anti-LAMP-2. Scale bars = 15 \( \mu \)m. B: mean fluorescence intensity of LAMP-1-and LAMP-2-labeled vesicles showed the more diffuse distribution toward the basal areas throughout the proximal tubules in Limp-2\(^{-/-}\) compared with \(^{+/+}\) mice; \( n = 20 \) tubule measured for both groups, 5 animals/group. **\( P < 0.0001 \), *\( P = 0.0002 \).
mice, the qualitative ratio of transferrin (Fig. 2C) to albumin (Fig. 2A) was much higher in the anti-GBM mice, indicating a far higher loss of this protein compared with albumin in the Limp-2−/− mice. VDBP, α1-microglobulin, and β2-microglobulin were present in blots from the Limp-2−/− mice, but not in the anti-GBM mice. As they were not seen in the anti-GBM mice, we considered it likely that this was due to the failure of tubular uptake rather than a glomerular loss.

There was also no evidence of Fanconi syndrome in the mice, as evidenced by the lack of aminoaciduria, phosphaturia, and glycosuria (data not shown).

Expression of cubilin and megalin in Limp-2−/− mice. The megalin/cubilin reuptake system is very important for protein reabsorption by the PCT (10). Studies of the inner ear have shown that megalin is not expressed normally on cells of the stria vascularis in Limp-2−/− mice (15). In view of the importance of megalin and its associated receptor cubilin in proximal tubular reabsorption of a variety of filtered proteins, expression of megalin at the proximal tubule was examined by immunofluorescence microscopy. In normal mice, megalin was strongly expressed on the apical surface of proximal tubules. No change in expression of megalin was observed by immu-

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**Fig. 6.** Costaining of proximal tubules with antibodies against LAMP-1, cathepsin B, and RBP. **A:** immunofluorescence microscopy of kidneys from Limp-2+/+ and Limp-2−/− mice with anti-RBP (red) and anti-LAMP-1 antibodies (green). **B:** immunofluorescence microscopy of kidneys from Limp-2+/+ and Limp-2−/− mice with anti-RBP (red) and anti-cathepsin B antibodies (green). **A** and **B:** boxes in the merged figures indicate the areas magnified to create the panels on the far right. Scale bars = 15 μm (5 μm for magnified figures). **C:** quantification studies of colocalization showed that there was a smaller proportion of RBP-labeled vesicles colocalizing with cathepsin B in the proximal tubules from Limp-2−/− compared with +/+ mice whereas there was no difference in the degree of colocalization of RBP with LAMP-1 between the 2 groups. Numbers of tubules measured: cathepsin B, n = 29 for +/+ mice, n = 25 for −/− mice; LAMP-1, n = 18 for both groups, 3 animals/group. *P < 0.0001. **D:** Western blot of kidney lysates from WT and Limp-2−/− mice. Blots were probed with antibodies against cathepsin B and β-tubulin, the latter as a loading control.
nofluorescence microscopy in kidneys from Limp-2−/− mice (Fig. 3A). Western blots demonstrated similar quantities of megalin in whole kidney lysates (Fig. 3B). Quantification of megalin mRNA transcript numbers by qRT-PCR, moreover, revealed a nonsignificant increase in transcripts in the Limp-2−/− mice (Fig. 3C). Similar findings were obtained for cubilin, with unchanged expression and similar levels of protein by immunofluorescence (Fig. 3A), Western blotting (Fig. 3B), and a nonsignificant increase in mRNA transcript numbers by qRT-PCR (Fig. 3C).

Expression of RBP in mouse proximal tubules. RBP is reabsorbed in the PCT via a megalin-dependent pathway. Immunofluorescence microscopy of kidneys from Limp-2−/− mice showed that the pattern of staining was similar in both groups in the early part of the PCT (Fig. 4, top). In the distal PCT, however, the vesicles containing endogenous RBP were larger in the Limp-2−/− mice and not concentrated on the apical surface of the cells, as in the WT mice (Fig. 4, bottom).

Lysosomal structure in PCT from Limp-2−/− mice. To determine whether the absence of Limp-2 was associated with any change in lysosomal structure, kidneys were stained with antibodies against the two major intrinsic lysosomal proteins, LAMP-1 and LAMP-2, and examined by immunofluorescence microscopy. There was a similar intensity of staining in both WT and Limp-2−/− kidneys (Fig. 5A), supporting previously published data from Gamp et al. (15) using Western blotting. There was a significantly greater distribution of LAMP-1 and LAMP-2 throughout the tubular cells in the Limp-2−/− mice,
whereas in the WT they were more localized to the apical surface (Fig. 5B).

**Costaining of RBP with LAMP-1 and cathepsin B.** Since lysosomal structure appeared similar in WT and Limp-2<sup>−/−</sup> mice, we next determined whether fusion of the lysosomes with endosomes containing reabsorbed proteins was likely to be different in the two strains. Both LAMP-1 and LAMP-2 may be found in late endosomes, before their fusion with lysosomes. To identify lysosomal proteins, we used an anti-cathepsin B antibody (Fig. 6). In WT and Limp-2<sup>−/−</sup> mice, colocalization of LAMP-1 with RBP was similar (Fig. 6A). In the sections labeled with anti-cathepsin B antibody, however, there was an excess of RBP that did not colocalize with cathepsin B in the Limp-2<sup>−/−</sup> mice (Fig. 6B). Quantification studies confirmed these observations that there was no difference in the degree of RBP colocalizing with LAMP-1 between WT and Limp-2<sup>−/−</sup> mice but a lower proportion of RBP colocalizing with cathepsin B in the Limp-2<sup>−/−</sup> mice (Fig. 6C). The mean Manders’ coefficient was also significantly lower for RBP and cathepsin B in Limp-2<sup>−/−</sup> compared with +/+ mice whereas that for RBP and LAMP-1 was not different between the two groups (data not shown). This suggested that lysosomes containing cathepsin B did not fuse with endosomes containing reabsorbed protein in the Limp-2<sup>−/−</sup> mice to the same extent as in the WT mice. Expression of cathepsin B appeared similar in the two strains as determined by immunofluorescence microscopy (Fig. 6B) and Western blotting of kidney lysates (Fig. 6D).

**Uptake of HRP- and fluorescent-conjugated albumin.** To determine whether fluid-phase endocytosis was abnormal in Limp-2<sup>−/−</sup> mice, they were injected intravenously with HRP and euthanized 7 min later. Compared with WT mice, there was no difference in the presence of HRP in the PCT (Fig. 7). Results were similar for Alexa-BSA when mice were euthanized after 7 min, indicating that receptor-mediated endocytosis was similar in WT and Limp-2<sup>−/−</sup> mice (Fig. 7). At 30 min after injection, however, there was persistence of the marker in the Limp-2<sup>−/−</sup> mice compared with controls (Fig. 7), indicating an inability to degrade albumin reabsorbed from the tubular lumen. At 60 min, there was still a small amount of label in the KO mice but not in WT mice (data not shown).

Perfusion-fixed sections from Limp-2<sup>−/−</sup> mice containing persisting Alexa-BSA at 45 min postinjection were colabeled with antibodies against LAMP-1 or cathepsin B (Fig. 8). At this time point, there was too little signal in the WT kidneys to enable similar studies. Whereas most of the Alexa-BSA appeared to colocalize with LAMP-1 (Fig. 8A and B), there were many areas of Alexa-BSA that did not colocalize with cathepsin B (Fig. 8C and D), indicating a failure of fusion of lysosomes with late endosomes containing reabsorbed Alexa-BSA.

**Structural studies of kidneys from Limp-2<sup>−/−</sup> mice.** To determine whether there was any abnormality in lysosomal

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Fig. 8. Costaining of Alexa-BSA 45-min postinjection with LAMP-1 and cathepsin B in Limp-2<sup>−/−</sup> mice. Alexa-BSA is shown in red and anti-LAMP-1 antibody or anti-cathepsin B in green. A and B: LAMP-1 colocalizes (arrows) with Alexa-BSA. C and D: cathepsin B does not colocalize (arrows) with Alexa-BSA. Boxes in A and C indicate the areas magnified to create B and D. Scale bars = 15 μm (A and C) and 5 μm (B and D).
structure, kidneys from WT and Limp-2\(^{-/-}\) mice were examined by electron microscopy (Fig. 9).

**DISCUSSION**

These studies have demonstrated that Limp-2 is required for normal protein reabsorption by the PCT. The abnormality found was considerably less severe than that seen with megalin or CIC-5 knockout mice (9, 18, 22), or that in humans with Dent’s disease (20). There was also a qualitative difference as the most prominent low-molecular-mass proteins seen in the Limp-2\(^{-/-}\) mice were the male-specific, \(\alpha\)-2u-globulins that are subject to uptake and proteolysis in the proximal convoluted tubule of the kidney (8, 29). These proteins do not appear to be removed by the megalin-dependent pathway, since they did not appear to be increased in the urine of megalin-deficient mice (18).

Albumin excretion was increased in the urine of Limp-2\(^{-/-}\) mice, as has been previously described (15), but this study suggests that some of it might originate from the failure of the PCT to reabsorb proteins normally. Since proteins such as VDBP and RBP undergo megalin- or cubilin-dependent uptake, failure to express these proteins on the luminal surface of the PCT was initially considered the mostly likely reason for the presence of tubular proteinuria. Surprisingly, megalin and cubilin were expressed at similar levels in the PCT in Limp-2\(^{-/-}\) mice compared with WT mice. The presence of normal levels of functional megalin was further suggested by an unchanged early uptake of labeled albumin in Limp-2\(^{-/-}\) mice, as well as the presence of RBP in vesicular structures in the PCT.

Since Limp-2 is an intrinsic lysosomal protein, an alternative explanation for the presence of tubular proteinuria is that lysosomal degradation of proteins in endosomes does not occur normally and that these proteins are then recycled back to the tubular lumen, as can occur with membrane proteins (24). Evidence for this possibility includes the prolonged survival of labeled albumin in the PCT of Limp-2\(^{-/-}\) mice. The absence of Limp-2 in Limp-2\(^{-/-}\) mice has previously been associated

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Fig. 9. Representative electron microscopy of proximal tubules from Limp-2\(^{+/+}\) and Limp-2\(^{-/-}\) mice. Cross section of proximal tubules from Limp-2\(^{+/+}\) (A) and Limp-2\(^{-/-}\) (B and C) mice is shown. Scale bars = 15 \(\mu\)m (A and B).
with impaired lysosomal biogenesis (14, 15, 23). The lysosomal markers LAMP-1 and LAMP-2, however, were expressed normally in the PCT, indicating normal lysosome abundance. Although Limp-2 also has a role in trafficking proteins such as the Gaucher’s disease protein β-glucocerebrosidase to the lysosome (23), trafficking of most proteases does not require Limp-2 (24). Gamp et al. (15) have demonstrated increased enzymatic activity in the kidneys of Limp-2−/− mice, and in the present study expression of cathepsin B was not reduced. These data suggest that the lysosomes in Limp-2−/− mice are likely to contain normal levels of most proteases. Evidence that cathepsin B colocalizes with albumin or RBP in the PCT to a lesser extent, however, suggests that endosomes are unable to fuse with lysosomes containing proteases such as cathepsin B. This would then lead to the persistence of proteins reabsorbed from the lumen and the appearance of endosomes containing reabsorbed proteins that are not degraded.

Until recently, the specific functions of Limp-2 have not generally been considered to include a role in lysosome fusion (14). Recent data, however, have demonstrated that Limp-2 controls fusion of the late endosomal/lysosomal compartment with phagosomes containing the parasite L. monocytogenes (7). In that system, Limp-2 is present on phagosomes, requires Rab5a-GTP, and promotes interaction with late endosomes/lysosomes. Limp-2 has been considered to have an intracellular specificity for late-trafficking events following endocytosis, a specificity probably due to the presence of a coiled-coil domain (8). LAMP-1 and LAMP-2, however, were expressed normally in the PCT, indicating normal lysosome abundance. Although Limp-2 also has a role in trafficking proteins such as the Gaucher’s disease protein β-glucocerebrosidase to the lysosome (23), trafficking of most proteases does not require Limp-2 (24). Gamp et al. (15) have demonstrated increased enzymatic activity in the kidneys of Limp-2−/− mice, and in the present study expression of cathepsin B was not reduced. These data suggest that the lysosomes in Limp-2−/− mice are likely to contain normal levels of most proteases. Evidence that cathepsin B colocalizes with albumin or RBP in the PCT to a lesser extent, however, suggests that endosomes are unable to fuse with lysosomes containing proteases such as cathepsin B. This would then lead to the persistence of proteins reabsorbed from the lumen and the appearance of endosomes containing reabsorbed proteins that are not degraded.

A potential confounding reason for the differences in the distribution of RBP and labeled albumin seen in the current study is increased filtration of these molecules in Limp-2−/− mice, leading to greater luminal concentrations and increased uptake relative to WT mice. This possibility is suggested by the severe FSOS seen in humans with mutation of SCARB2 (1). In mice, however, Limp-2 mutation does not produce a severe glomerular phenotype, even in very elderly mice (e.g., 16 mo of age), which showed foot process effacement by electron microscopy but no evidence of FSOS (3). Although mice used in the current studies had no detectable foot process effacement (data not shown), it is impossible to exclude a contribution from increased glomerular filtration to the differences seen in handling of protein uptake in the proximal tubule.

In summary, these data suggest that Limp-2−/− mice have tubular proteinuria that is not due to a failure of protein uptake but an inability to fuse lysosomes with endosomes and degrade reabsorbed proteins. It emphasizes the importance of protein degradation in tubular handling of proteins reabsorbed by the PCT.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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