Normal Lysosomal Morphology and Function in LAMP-1-deficient Mice*

(Received for publication, August 11, 1998, and in revised form, January 20, 1999)

Nicole Andrejewski, Eeva-Liisa Punnonen, Gundula Guhde, Yoshitaka Tanaka, Retane Lüllmann-Rauch, Dieter Hartmann, Kurt von FiguRat, and Paul Saftig**

From the Zentrum Biochemie und Molekulare Zellbiologie, Abteilung Biochemie II, Universität Göttingen, 37073 Göttingen, Germany, the Institute of Biotechnology and Electron Microscopy, University of Helsinki, Finland, and the Anatomisches Institut der Universität Kiel, Otto-Hahn-Platz 8, 24043 Kiel, Germany.

Lysosomal membranes contain two highly glycosylated proteins, designated LAMP-1 and LAMP-2, as major components. LAMP-1 and LAMP-2 are structurally related. To investigate the physiological role of LAMP-1, we have generated mice deficient for this protein. LAMP-1-deficient mice are viable and fertile. In LAMP-1-deficient brain, a mild regional astrogliosis and altered immunoreactivity against cathepsin-D was observed. Histological and ultrastructural analyses of all other tissues did not reveal abnormalities. Lysosomal properties, such as enzyme activities, lysosomal pH, osmotic stability, density, shape, and subcellular distribution were not changed in comparison with controls. Western blot analyses of LAMP-1-deficient and heterozygote tissues revealed an up-regulation of the LAMP-2 protein pointing to a compensatory effect of LAMP-1 in vivo.

Western blot analyses of LAMP-1-deficient and heterozygote tissues revealed an up-regulation of the LAMP-2 protein pointing to a compensatory effect of LAMP-1 in vivo. LAMP-1-deficient brain, a mild regional astrogliosis and altered immunoreactivity against cathepsin-D was observed. Histological and ultrastructural analyses of all other tissues did not reveal abnormalities. Lysosomal properties, such as enzyme activities, lysosomal pH, osmotic stability, density, shape, and subcellular distribution were not changed in comparison with controls. Western blot analyses of LAMP-1-deficient and heterozygote tissues revealed an up-regulation of the LAMP-2 protein pointing to a compensatory effect of LAMP-1 in vivo.
LAMP-1 and that the expression of both proteins is tightly regulated in vivo.

**EXPERIMENTAL PROCEDURES**

Isolation of a Genomic Clone and Targeting Vector Construction—An EMBL-3-129SV mouse phage library from Stratagene Inc. (La Jolla, CA) was screened with a 550-bp genomic amplification product of mouse lamp-1, corresponding to DNA positions 118–350 (8). The probe contained exon 2 and exon 3 of mouse lamp-1 interrupted by a small intron. The isolated mouse lamp-1 phage clone mouse lamp-1/4B containing the 5′-region of the gene with exons 2–5. DNA sequence analyses revealed complete sequence identity of four exons with the nucleotide sequence of the mouse lamp-1 cDNA (data not shown). For construction of a 5.3-kbp KpnI genomic DNA restriction fragment (Fig. 1A), a lamp-1 covering exons 2 and 3 (see Fig. 1A, 1I) was subcloned into the plasmid vector pBlueScript SK1+ (Stratagene). The neo expression cassette from pMC1NeoA (Ref. 29; Stratagene) was inserted as a BamHI DNA restriction fragment into a BgIII restriction site located in exon 3 of the KpnI fragment (nucleotide position 323 of the lamp-1 cDNA; amino acid 107 of 382 amino acids; Ref. 8). The insertion of the neo cassette introduces a premature translational stop codon in the open reading frame of the lamp-1 gene. Additionally, for negative selection with gancyclovir, a thymidine kinase cassette was inserted at the 3′ site of the KpnI fragment.

Selection of Targeted Embryonic Stem (ES) Cells and Generation of Mutant Mice—The targeting vector was linearized with XhoI and introduced into the cell line E14–ES cells were cultured as described by Köster et al. (30). G418- and neomycin-resistant colonies were screened by Southern blot analysis of DNA digested with HindIII and hybridized with the 3′ probe (Fig. 1A). Two ES cell lines were homologous recombination were confirmed by digesting DNA with BgIII and hybridization with the 5′ probe (Fig. 1A). The mutated E5 lines were microinjected into blastocysts of C57BL/6j mice. Chimeric males were mated to C57BL/6j females. Mice were genotyped for the lamp-1 gene mutation by Southern blot analysis of HindIII-digested genomic DNA, using the 3′ probe or by PCR analyses using a neo-specific PCR (31) and an exonic-specific PCR with primers (LAMP-1/4B 5′-cttgatgtcagttgtggaatccat-3′ and LAMP-1/3E 5′-ttttccctgccagcctctgcagaag-3′) flanking the exon used for interruption. Homozygous mutant mice were obtained by mating heterozygous or homozygous mutant mice. For initial phenotype testing, litters from the F2 and F3 generations were used. In later experiments, offspring of homozygote-deficient mice were compared with age- and sex-matched offspring of control mice. The mice were kept in a conventional animal facility at the Zentrum für Biochemie und Molekulare Zellbiologie, Universität Göttingen (Göttingen, Germany).

Northern Blot Analysis—Total RNA of liver and kidney from 3-month-old mice was prepared using the Qiagen RNeasy system. Ten micrograms of total RNA were separated on a formaldehyde agarose gel and processed as described by Isbrandt et al. (32). Filters were hybridized with a lamp-1 5′ probe (a lamp-1 5′ probe used for genomic library screening, a lamp-2 cDNA probe (33), and a 280-bp cDNA fragment from glyceraldehyde-3-phosphate dehydrogenase (34)). Hybridization and washing of filters were performed as described by Lehmann et al. (35).

Western Blot Analyses—Expression of LAMP-1, LAMP-2, and LIMP-2 (lysosomal membrane glycoprotein 85) was analyzed in tissue homogenates (liver, kidney, spleen, brain, heart, adult, and embryonic fibroblasts). Frozen tissues were homogenized in Tris-buffered saline (w/v) at 4°C using an Ultra-Turrax, analyzed for protein (36), and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (7.5% polyacrylamide) under reducing conditions. Proteins were transferred to a polyvinylidene difluoride membrane (Schleicher und Schuell, Dassel, Germany), which was subsequently blocked with 10 mM PBS, pH 7.4, 0.8 ml of 0.5% saponin. Cathepsin-D was immunostained using a rabbit anti-mouse lamp-1 antibody (1D4B, Developmental Studies Hybridoma Bank, Iowa City, IA) in a 1:250 dilution, an anti-mouse lamp-2 antibody (Ab1 39; Developmental Studies Hybridoma Bank) in a 1:100 dilution, and a polyclonal anti-rat LIMP-2 antibody (1:7500 for LAMP-1 and LIMP-2) or with horseradish peroxidase-coupled anti-rat antibody (1:20,000 for LIMP-2) was hybridized for 1 h at room temperature followed by washing six times for 5 min in 10 mM PBS, pH 7.4, 0.1% Tween 20. Subsequently, incubation with horseradish peroxidase-coupled anti-rat antibody (1:7500 for LAMP-1 and LIMP-2) or with horseradish peroxidase-coupled anti-rabbit antibody (1:20,000 for LIMP-2) was performed for 1 h at room temperature following washing six times for 5 min in 10 mM PBS, pH 7.4, 0.1% Tween 20. Blots were finally analyzed using the ECL Detection System (Amersham Pharmacia Biotech, Buckingham, England). Band intensity was determined by densitometry (Hewlett-Packard Scan Jet 4c/T; WinCam 2.2).

Immunofluorescence—Mouse embryonic fibroblasts, mouse adult fibroblasts, and peritoneal macrophages were grown on glass coverslips for 1 day. The cells were fixed with methanol or paraformaldehyde with 0.5% saponin. Cathepsin-D was immunostained using a rabbit anti-mouse lamp-1 antibody (1D4B, Developmental Studies Hybridoma Bank), and LIMP-2 was immunostained using a monoclonal anti-mouse rat hybridoma medium (1D4B and Ab1 93; Developmental Studies Hybridoma Bank), and LIMP-2 was immunostained using a monoclonal rabbi anti-rat-lysosomal membrane glycoprotein 85 antisem (37). The primary antibodies were detected with goat anti-rabbit Texas Red, goat anti-rat Texas Red, and goat anti-rat 5(4,6-dichloroazin-2-yl)aminofluorescein (Dianova, Hamburg, Germany). After embedding in Mowiol (Calbiochem-Novabiochem GmbH), fluorescence was examined using a confocal laser scanning microscope (LSM 2; ZEISS, Oberkochen, Germany) with the filter combination described by Schütz-Garg et al. (39).

Subcellular Fractionation on Percoll Gradient—Tissue homogenates of liver, kidney, and fibroblasts were prepared in 0.25 M sucrose buffer (pH 7.4) at 5 ml homogenate/100 mg of protein. The supernatant was applied onto 11.2 ml of a 20% Percoll solution (40) and centrifuged for 30 min at 20,000 rpm in the vertical rotor VTI 65.1 (Beckman). Density and β-hexosaminidase activity were determined in each of 12 fractions collected. Fractions corresponding to dense vesicles were pooled according to the distribution of β-hexosaminidase activity and centrifuged for 1 h at 100,000 × g. The membrane layer (lysozome-enriched pellet) floating above the pelleted silica was collected (41).

Osmotic Stability—50 μl of the lysosome-enriched pellet were suspended in 250 μl of 0.25 M sucrose, pH 7.0, and 0.25 M glucose, pH 7.0, respectively. The reactions were incubated at 37°C, and samples (30 μl) were withdrawn for assay at 0, 5, 10, 15, and 20 min. Immediately after the withdrawal, the samples were suspended in 270 μl of ice-cold 0.25 M sucrose solution and subjected to centrifugation for 10 min at 3000 rpm and 4°C in a Beckman TL-100 centrifuge. 150 μl of the resulting supernatants were collected and kept at 20°C until β-hexosaminidase enzyme activity measurements. To calculate the total β-hexosaminidase activity (100% of the possible β-hexosaminidase enzyme activity), one sample was incubated in 0.25 M sucrose in the presence of 1% Triton X-100, β-hexosaminidase activities in the supernatants were calculated as percentages of total activity.

Lysosomal Enzyme Assays—Lysosomal enzymes were detected using fluorimetric assays as described by Köster et al. (42). Arylsulfatase A was measured using p-nitrophenyl sulfate as substrate (43).

Metabolically Labeling of Cells and Immunoprecipitation of Cathepsin-D and LAMP-2—Mouse embryonic fibroblasts and mouse adult fibroblasts were cultured in methionine-free medium for 24 h. They were then labeled with [35S]methionine/cysteine (Amersham Pharmacia Biotech) in the same medium containing 5% dialyzed fetal calf serum. During the following chase for 1, 2, 4, and 6 h, the medium was supplemented with 0.25 mg/ml l-methionine. Immunoprecipitation from cells and media was carried out as described previously (44) with antiserum specific for mouse cathepsin-D (38).

For LAMP-2 immunoprecipitation cells were labeled for 2 h with [35S]methionine and chased for 24, 48, 72, and 96 h, respectively. Immunoprecipitation of cells was done as described for cathepsin-D with overnight incubation of 2 μg of monoclonal antibody Abl 93 and subsequent incubation for 1 h with 20 μg of a “goat anti-rat” bridge antibody. Densitometric quantification of cathepsin-D and LAMP-2 was done with a phosphor imager (Fuji) and the program MacBas.

Histology—For standard light microscopic histology and immunohistochemistry, tissues were fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer and shock-frozen or fixed by perfusion with Bouin’s solution diluted 1:3 with 0.1 M phosphate buffer and embedded in paraffin. Sections were cut at 7 μm, de waxed in xylene, and embedded in a graded series of alcohol, air dried, and coated with 0.5% gold by ion sputtering with 0.75% BSA and quenching of endogenous peroxidase activity with 3% H2O2 in methanol, sections were incubated with antibodies against cathepsin-D (38); glial fibrillary acidic protein (Dako, Hamburg, Germany); F4/80 (clone obtained from the Developmental Studies Hybridoma Bank); LAMP-1 (clone obtained from the Developmental Studies Hybridoma Bank); MHC-II (Pharmingen, Hamburg, Germany); and lectins RCA-I, GS-I B4, and Solanum tuberosum (all

The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); pc, polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
FIG. 1. Targeted disruption of the lamp-1 gene. A, strategy for inactivation of the lamp-1 gene by homologous recombination in ES cells. I, partial structure of the genomic locus representing about 13 kbp of the lamp-1 gene region. Exons are indicated by open boxes, and flanking introns are indicated by solid lines. Bars labeled ‘‘5’’ and ‘‘3’’ denote DNA probes used for Southern blot analysis. II, targeting vector pCK-Lamp-1(neo) with 5.3-kbp homology to the lamp-1 gene locus. The neo cassette was inserted into a BglII restriction site in exon 3. The arrow marks the direction of transcription of the neo gene and thymidine kinase cassette. III, predicted lamp-1 gene locus after homologous recombination. B, Southern blot analysis of ES cell clones. The 3’ probe was hybridized to HindIII-digested genomic DNA from ES cell clones (EL-18, EL-19, and EL-20). An additional 5.3-kbp DNA fragment indicates a targeted allele. C, PCR analysis of tail genomic DNA with an exon-specific PCR amplifying a 0.6-kb fragment in ‘+/−’, a 0.6- and a 1.8-kbp fragment in ‘+/−’, and a 1.8-kbp fragment in ‘−/−’ mice, respectively. A neo cassette-specific PCR amplifies a 0.4-kb fragment in ‘+/−’ and ‘−/−’ mice, respectively. D, Northern blot analysis of lamp-1 expression. Total RNA (10 μg) was hybridized using a lamp-1 genomic probe containing exons 2 and 3 and a murine glyceraldehyde-3-phosphate dehydrogenase probe. E, Western blot analysis of Lamp-1 expression using a hybridoma supernatant against mouse LAMP-1 (1D4B; Developmental Studies Hybridoma Bank). In ‘+/−’ tissue extracts, the glycosylated Lamp-1 molecules were detected. In ‘−/−’ tissues, no Lamp-1 product was found.

LAMP-1-deficient Mice

Lectin reagents from Vector Laboratories, Inc. (Burlingame, CA). Detection of bound primary reagents was performed by either the avidin-biotin complex technique (reagents from Vector Laboratories), gold-labeled secondary antibodies followed by silver intensification (British Biocell), or tyramide signal amplification (all reagents from NEN Life Science Products, Bad Homburg, Germany).

For transmission electron microscopy, animals were perfused with 6% glutaraldehyde in phosphate buffer and stored in fixative until further processing. Tissue blocks were rinsed in phosphate buffer, post-fixed in OsO4 for 2 h, and embedded in Araldite or Epon 812 according to routine procedures. Ultrathin sections were collected on copper grids, contrasted with uranyl acetate and lead citrate, and observed with Zeiss EM 900 and EM 902 microscopes.

Mouse fibroblasts were grown on plastic tissue culture wells until semiconfluency. The cells were fed with 5-nm gold particles coated with bovine serum albumin and diluted in serum-free Dulbecco’s modified Eagle’s medium to an optical density of 5.0, for 2 h. The cells were then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and prepared for conventional Epon embedding. Thin sections were photographed with a Jeol 1200EX transmission electron microscope.

Immunocytochemistry of Kidney—Mouse kidneys were fixed by perfusion with 4% paraformaldehyde in 0.2 w Heps, pH 7.4. Fixation was continued in immersion for 2 h at room temperature, and the tissue cubes were then stored in 2% paraformaldehyde. For cryosectioning, tissue cubes from the cortex were infiltrated in 18% polyvinylpyrrolidone, 1.66 M sucrose in PBS overnight at 4 °C, mounted on specimen holders, and frozen in liquid nitrogen. Semithin sections (500 nm) and thin sections (80 nm) were cut at −100 °C for immunofluorescence and immunoelectron microscopy, respectively. The sections were labeled with rat LAMP-1 (1D4B), rat LAMP-2 (Abi 93), or rabbit cathepsin-D antibodies, which were detected using goat anti-rat IgG-fluorescein, goat anti-rabbit-lissamine rhodamine (J. Jackson Immunoresearch Laboratories, West Grove, PA), or rabbit anti-rat IgG (J. Jackson Immunoresearch Laboratories) followed by protein A coupled to 10-nm colloidal gold (University of Utrecht, The Netherlands) or directly with protein A-gold. Semithin sections were mounted in Mowiol containing Diazobicycloundecan, and thin sections were mounted in 1.5% methyl cellulose containing 0.4% uranyl acetate. Two control and two LAMP-1-deficient mice were used for the immunocytochemical analysis.

RESULTS

Targeted Disruption of the lamp-1 Gene and Generation of Deficient Mice—A 12-kbp genomic clone from the lamp-1 structural gene region was isolated by screening a genomic 129/SvJ mouse phage library with a genomic PCR fragment as probe containing exons 2 and 3 of lamp-1, corresponding to amino acid residues 37–114 of the LAMP-1 protein (Fig. 1A, I; for details see “Experimental Procedures”). The targeting vector (Fig. 1A, II) was used for disruption of the lamp-1 structural gene in ES cells. The open reading frame of the gene is disrupted by insertion of a neo cassette in a BglII site at lamp-1 exon 3 corresponding to cDNA nucleotide position 323 and amino acid 107 of 382 amino acids, respectively (8).

The targeting construct was introduced into E14-1 ES cells (45), and G418- and gancyclovir-resistant colonies were ana-
lyzed by Southern blotting. Using the 3’ external probe (Fig. 1A), in two out of 96 independent clones tested an additional HindIII DNA restriction fragment was detected, indicating a homologous recombination in one of the lamp-1 gene alleles (Fig. 1B). These results were confirmed by hybridizing BglII-digested DNA with the 5’ external probe (data not shown). The targeted ES cell clones were microinjected into C57BL/6J blastocysts, and five chimeric males were generated. Only the ES cells from ES cell clone EL20 transmitted the mutated allele to offspring and normal fertility (data not shown). Heterozygotes exhibit a normal phenotype and normal fertility (data not shown).

Genotyping of 98 offspring from heterozygote crosses (Fig. 1C) revealed a frequency of 27.2% for homozygous mutant mice (LAMP-1−/−), resembling the expected Mendelian frequency (25%). Hence, disruption of the lamp-1 gene does not result in embryonic lethality.

Inactivation of the lamp-1 Gene—To test for expression of the lamp-1 gene in LAMP-1−/− mice, Northern blot analyses were performed. A single lamp-1-specific transcript was detectable in liver and kidney total RNA from wild-type animals, whereas no lamp-1-specific transcripts were detectable in homozygous mutant animals (Fig. 1D).

LAMP-1 protein was not detectable in brain, heart, liver, spleen, and kidney homogenates from LAMP-1−/− animals, whereas it was readily detectable in the respective homogenates from wild-type mice (Fig. 1E).

The Northern blot and Western blot analyses demonstrate that the lamp-1 gene has been inactivated and that homozygous mutant mice are devoid of LAMP-1.

Phenotype of LAMP-1-deficient Mice—Homozygous mutant, heterozygous, and wild-type mice resulting from heterozygote crosses did not exhibit differences in growth and weight development (data not shown). LAMP-1-deficient mice were fertile and did not show an elevated mortality up to an age of 19 months. X-ray analyses and determination of clinical blood and serum parameters did not reveal abnormalities (data not shown). Macroscopically, no differences were observed in shape and size of individual organs prepared from knockout mice as compared with controls. Light microscopic investigation of liver, kidney, lung, spleen, and brain did not reveal any apparent change in tissue structure. Likewise, density and distribution of macrophages and microvascular architecture taken as indicators for tissue damage were unchanged. In the brain (and there most notably within the dorsal neocortical region), the normally high neuronal expression of cathepsin-D (Fig. 2A) was replaced by a more irregular distribution pattern with a still high immunoreactivity in superficial laminae II and deep lamina VI, but now being irregularly reduced in the more central strata (Fig. 2B). It should be pointed out that this does not correlate with neuronal cell degeneration, but it could be associated with an early disturbance of lysosomal metabolism in a subpopulation of cells. In addition, astroglrosis was observed in circumscribed dorsal cortical areas spanning about 500–800 μm (Fig. 2C,D; control shown in Fig. 2E), which overlapped only partially with the regions featuring alterations in their cathepsin-D expression.

Normal Lysosomal Morphology and Function—Ultrastructural analysis of cell types known to be rich in lysosomes such as Kupffer cells (Fig. 3, A and B), cultured embryonic fibroblasts (Fig. 5, compare A and B), and kidney-proximal tubule epithelial cells (Fig. 5, C and D) did not reveal abnormalities in size, distribution, and shape of the lysosomal compartment in LAMP-1-deficient cells. These observations were also confirmed in immunofluorescence analyses of peritoneal macro-
FIG. 3. Normal lysosomal morphology and function in LAMP-1-deficient mice. An electron microscopic image of Kupffer cells derived from control (A) and LAMP-1-deficient (B) livers is shown. Electron-dense lysosomal structures are labeled with arrows. S, sinusoidal space; Ku,
phages, embryonic fibroblasts (not shown), and adult fibroblasts. Neither staining with antibodies against the lysosomal protease cathepsin-D nor with antibodies against the lysosomal membrane proteins LAMP-2 and LIMP-2 revealed differences between LAMP-1-deficient and control mice (shown for LAMP-2 in Fig. 3, C and D). To investigate if changes of lysosomal milieu have occurred affecting lysosomal hydrodases, the specific activities of five lysosomal enzymes were determined in brain, heart, liver, spleen, kidney, and serum of age- and sex-matched LAMP-1-deficient and control mice. No significant differences (four animals of each genotype were analyzed) were observed in specific enzyme activities of arylsulfatase A, β-galactosidase, β-glucuronidase, β-hexosaminidase, and β-mannosidase (Fig. 3E). In addition, Western blot analysis of major organs also revealed that the concentration of the lysosomal protease cathepsin-D was not different from controls (data not shown). Homogenates of fibroblasts, brain, liver, and kidney were subjected to subcellular fractionation using Percoll gradients. The gradient fractions were analyzed for density and β-hexosaminidase activity. No differences in the β-hexosaminidase profiles were observed between LAMP-1-deficient and control tissues, indicating that LAMP-1-deficient and control lysosomes do not differ in shape and density (data not shown).

Lysosome-enriched fractions of LAMP-1-deficient and control kidneys were used to examine (two independent experiments) the osmotic stability of lysosomes during an incubation for up to 20 min in 0.25 M glucose. Whereas lysosomes are stable at 0.25 M sucrose, they rapidly lyse during incubation in glucose due to import of glucose and osmotic swelling (46). No changes in fragility and integrity of lysosomes were observed in both genotypes (Fig. 3F). Even small differences in lysosomal pH are known to alter the processing of cathepsin-D (47). The processing of cathepsin-D was normal in LAMP-1-deficient fibroblasts from adult (Fig. 3G) and embryos of day 12 (not shown). Also the sorting of newly synthesized cathepsin-D to lysosomes was not affected by LAMP-1 deficiency (Fig. 3G).

Lysosomes were labeled by feeding BSA-gold to cultured LAMP-1-deficient and control embryonic fibroblasts for 2 h. BSA-gold-containing lysosomal structures could easily be identified in both genotypes. The morphology of the lysosomal compartment and the amount of BSA-gold found inside these organelles were similar in both kinds of fibroblasts (Fig. 5, A and B). Immunoelectron microscopy of LAMP-2 in control and LAMP-1-deficient kidney-proximal tubule cells further showed the normal size and morphology of lysosomes in the deficient cells (Fig. 5, B and C).

LAMP-2 Up-regulation in LAMP-1-deficient and Heterozygote Tissues—Since no obvious alterations of several lysosomal parameters were found, we examined whether the loss of LAMP-1 leads to a compensatory increase of other lysosomal membrane proteins. Western blot analysis of several tissues of LAMP-1-deficient mice revealed an increased immunoreactivity of LAMP-2 (Fig. 4A). The increase was most pronounced in kidney, but also clearly detectable in spleen and heart (Fig. 4A). The amount of LAMP-2 in LAMP-1-deficient liver appears to be the same as in control liver. Probing of the same membranes with an anti-LIMP-2 (37) antibody showed only moderate changes in the level of this lysosomal membrane protein (Fig. 4A). Whereas the amount of LIMP-2 appears to be reduced in LAMP-1-deficient brain and spleen, the amounts are equivalent in heart and liver but slightly increased in LAMP-1-deficient kidney. It is notable that there appears to be a slight reduction of the LIMP-2 molecular weight in LAMP-1-deficient tissues (Fig. 4A). Analyses of kidney extracts from three age- and sex-matched control and LAMP-1-deficient mice confirmed the increased immunoreactivity of LAMP-2 in LAMP-1-deficient kidneys (Fig. 4B). LIMP-2 expression was variable between the genotypes, i.e., slightly elevated in two LAMP-1-deficient kidneys but decreased in the third LAMP-1-deficient kidney extract (Fig. 4B). This analysis was extended to kidneys of heterozygotes (Fig. 4C). Loss of one lamp-1 allele decreases expression of LAMP-1, while expression of LAMP-2 is increased. Again, expression of LIMP-2 is not affected or is only moderately affected (Fig. 4C). Densitometric evaluation of Western blot experiments revealed that LAMP-2 was increased 2.7 ± 0.8-fold in LAMP-1-deficient (n = 6) and 1.7 ± 0.4-fold in LAMP-1 heterozygote (n = 3) kidneys (Fig. 4D).

Immunogold labeling for LAMP-2 in kidney lysosomes of LAMP-1-deficient and control animals encountered in the proximal tubuli revealed clear membrane staining and indicated that there is a somewhat higher amount of gold particles in LAMP-1-deficient lysosomal membranes (Fig. 5, C and D). Immunofluorescence analyses of kidney sections confirmed the increased LAMP-2 immunoreactivity in LAMP-1-deficient kidneys (Fig. 5, E and D). As expected, LAMP-1 immunoreactivity was absent in LAMP-1-deficient kidneys (inset in Fig. 5F), whereas it could be easily identified in control kidneys (inset in Fig. 5E). Immunoreactivity for the lysosomal protease cathepsin-D was not different in control (Fig. 5G) and LAMP-1-deficient (Fig. 5H) kidneys. This suggests that the total number of lysosomal vesicles is not affected by the LAMP-1 deficiency.

Normal LAMP-2 Expression and LAMP-2 Stability in LAMP-1-deficient Tissues—To see whether the up-regulation of LAMP-2 correlates with an increased expression of lamp-2 Northern blot analysis of kidney, brain, heart, and liver (shown for kidney in Fig. 6A) were performed. In none of these tissues were lamp-2 transcripts significantly increased in LAMP-1-deficient mice.

To determine whether an increased half-life of LAMP-2 may cause the increase of LAMP-2, fibroblasts from adult mice were metabolically labeled with [35S]methionine and then chased for up to 96 h. The LAMP-2 immunoprecipitates (Fig. 6B) were quantified by densitometry (Fig. 6C). It became apparent that the stability of LAMP-2 is not affected by LAMP-1 deficiency. It should be noted, however, that in fibroblasts from adult LAMP-1-deficient mice, the level of LAMP-2 is only 1.5 times elevated compared with controls (not shown).

DISCUSSION

LAMP-1 and LAMP-2 are the major components of the lysosomal membrane. Estimates based on immunopurification pro-
procedures calculated that both proteins account for about 50% of lysosomal membrane proteins (48) and 0.1% of total cell protein (23). Although much is known about the structure and lysosomal trafficking of LAMP-1 and LAMP-2 (9, 12, 13), the proposals for their physiological function are only of a hypothetical nature. The lysosomal membrane plays a vital role in the proper function of lysosomes by sequestering numerous acid hydrolases from the rest of the cytoplasmic components. Moreover, it is likely to be involved in maintaining an acidic intralysosomal environment, transport of amino acids, and mono- and oligosaccharides, resistance to degradation by lysosomal hydrolases and its ability to interact and fuse with other membrane organelles, including endosomes, phagosomes, and plasma membranes (3, 4). In order to better understand the possible contribution of one of the major components of the lysosomal membrane, we have generated mice that are deficient for LAMP-1.

Despite its abundance in the lysosomal membrane, the deficiency of LAMP-1 is apparently well tolerated in mice. If LAMP-1 is critical for lysosomal integrity, lysosomal pH, and lysosomal catabolism, we reasoned that a deficiency of LAMP-1 would cause alterations of the morphology, number, distribution, and stability of lysosomes as well as reduced lysosomal hydrolase activities and altered processing of lysosomal enzymes. For this reason we have first investigated possible alterations of the lysosomal compartment by morphological and functional analyses. Immunofluorescence using lysosomal markers, subcellular fractionation, activity determination of lysosomal enzymes, and Western blot as well as processing and secretion analysis of cathepsin-D, glucose loading experiments, and two-dimensional gel electrophoresis of fractions enriched in lysosomal membrane proteins (data not shown) failed to demonstrate specific differences between LAMP-1 deficient and control cells. The negative outcome of these experimental approaches suggested that LAMP-1 is either dispensable or that the loss of LAMP-1 is compensated by another protein or other proteins. Since the latter appeared to be the more likely possibility, two other lysosomal membrane proteins were quantified using immunoblot analyses.

These experiments clearly demonstrated that the structur-
ally related LAMP-2 protein is up-regulated in the majority of LAMP-1-deficient tissues tested. The specificity of this up-regulation was examined by probing the same blots for LIMP-2, another component of the lysosomal membrane. In contrast to LAMP-2, the immunoreactivity for LIMP-2 was only moderately changed, and its expression pattern in LAMP-1-deficient tissues appeared to vary from mouse to mouse. However, we cannot completely exclude the possibility that also LIMP-2 and/or other lysosomal membrane proteins (e.g., LIMP-1/CD63, macrosialin/CD68) can contribute to the compensation of the LAMP-1 deficiency. In kidney homogenates, LAMP-2 was about 2.7-fold higher than in controls. In homogenates from heterozygotes, LAMP-2 was 1.7-fold higher, while LAMP-1 was reduced to about half. This indicates that loss of a lamp-1 allele induces an elevation of LAMP-2, while expression of the remaining lamp-1 allele is apparently not affected. The increased LAMP-2 expression was also confirmed in LAMP-2 immunofluorescence analyses of kidney sections.

The weak phenotype of LAMP-1-deficient mice suggests that the increased LAMP-2 can efficiently compensate for the loss of LAMP-1. The LAMP-2 up-regulation is not due to an increased expression of the lamp-2 gene or stability of lamp-2 transcripts. Either mechanism would have caused an increased amount of lamp-2 mRNA detectable in Northern blot (49). An increased half-life of LAMP-1 has been correlated with an increased activity of the enzyme β13 3-N-acetylglucosaminyltransferase,
a key enzyme in the synthesis of N-acetyllactosamine side chains (14). Such an increase in LAMP-2 stability was not observed in LAMP-1-deficient fibroblasts, where LAMP-2 was only moderately increased. LAMP-2 expression may therefore be translationally regulated.

Of interest is the astrogliosis and altered cathepsin-D distribution in LAMP-1-deficient brains. Since almost no LAMP-2 is detectable in mouse brain (Fig. 3A), one might speculate that brain is a tissue where LAMP-1 deficiency cannot be compensated for by an increase of LAMP-2 and hence develop the observed alterations. In mice made deficient for the lysosomal acid phosphatase, similar alterations were observed and were thought to be early markers for lysosomal alterations (50). However, future work to determine the nature and extent of these lesions is in progress.

Taken together, this study demonstrates that the morphology and function of lysosomes appears to be normal in LAMP-1-deficient mice and that the deficiency of LAMP-1 neither produces an overt phenotype nor affects viability and fertility of mice. The up-regulation of the structurally related LAMP-2 suggests a functional overlap between both LAMP molecules. These overlapping functions are supported by the phenotype of LAMP-1/LAMP-2 double deficient mice. Whereas the single deficient mice are fertile and viable, the loss of both LAMP-molecules leads to embryonic lethality.\footnote{P. Saftig, unpublished data.}

Acknowledgments—We thank N. Hartelt, M. Grell, and D. Niemeyer for excellent technical assistance; K. Rajewski, (Universität Köln, Köln, Germany) for providing the E-14–1 cell line; and O. Schunck and K. Nebendahl for veterinary advice.

REFERENCES
1. Melmann, I., Fuchs, R., and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700
2. Peters, C., and von Figura, K. (1994) FEBS Lett. 346, 108–114
3. Fukuda, M. (1991) J. Biol. Chem. 266, 21327–21330

FIG. 6. Unchanged lamp-2 expression and LAMP-2 stability in LAMP-1-deficient tissues. A, Northern blot analyses of lamp-2 expression. Kidney total RNA was hybridized with LAMP-2 murine cDNA (33) and a murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (34) probe. Two separate experiments are shown. B, immunoprecipitation of adult control and LAMP-1-deficient fibroblasts with a hybridoma supernatant specific for LAMP-2 (Ab 93). Cells were metabolically labeled and chased for up to 96 h. C, densitometric evaluation of the immunoprecipitation experiment described for B. Shown is the percentage of LAMP-2 protein resistant to degradation. Closed symbols represent values derived from control fibroblasts, and open symbols represent values from LAMP-2-deficient fibroblasts. The immunoprecipitation experiment shown in B was confirmed by an independent experiment (not shown).
LAMP-1-deficient Mice

4. Granger, B. L., Green, S. A., Gabel, C. A., Howe, C. L., Meltman, I., and Helin, A. (1990) J. Biol. Chem. 265, 12036–12043
5. Fambrough, D. M., Takeyasu, K., Lippincott-Schwartz, J., and Siegel, N. R. (1988) J. Cell Biol. 106, 61–67
6. Howe, C. L., Granger, B. L., Hull, M., Green, S. A., Gabel, C. A., Helin, A., and Meltman, I. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7577–7581
7. Himeno, M., Nogushi, Y., Sasaki, H., Tanaka, Y., Furuno, K., Kono, A., Sasaki, Y., and Kato, K. (1989) FEBS Lett. 244, 351–356
8. Chen, J. W., Cha, Y., Yuki, K. U., Gracy, R. W., and August, J. T. (1988) J. Biol. Chem. 263, 8754–8758
9. Viitala, J., Carlsson, S. R., Siebert, P. D., and Fukuda, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3743–3747
10. Fukuda, M., Viitala, J., Matteson, J., and Carlsson, S. R. (1988) J. Biol. Chem. 263, 18920–18928
11. Kornfeld, S., and Mellman, I. (1989). Ann. Rev. Cell Biol.
12. Guarnieri, F. G., Arterburn, L. M., Penno, M. B., Cha, Y., and August, J. T. (1993). J. Biol. Chem. 268, 1941–1946
13. Höning, S., and Hunziker, W. (1995) J. Biol. Chem. 270, 464–473
14. Lee, N., Wang, W. C., and Fukuda, M. (1990) J. Biol. Chem. 265, 20476–20487
15. Carlsson, S. R., and Fukuda, M. (1989) J. Biol. Chem. 264, 20526–20531
16. Youakim, A., Romero, P. A., Yee, K., Carlsson, S. R., Fukuda, M., and Herson, A. (1989) Cancer Res. 49, 6889–6895
17. Yamashita, K., Ohkura, T., Tachibana, Y., Takasaki, S., and Kobata, A. (1984) J. Biol. Chem. 259, 10834–10840
18. Pierce, M., and Arango, J. (1986) J. Biol. Chem. 261, 10772–10777
19. Yousef, S., Higgins, E., Dadling, Z., Pollex-Krüger, A., Hindsaul, O., and Dennis, J. W. (1990) J. Biol. Chem. 265, 1772–1782
20. Zot, A. S., and Fambrough, D. M. (1990) J. Biol. Chem. 265, 20988–20995
21. Laferte, S., and Dennis, J. W. (1989) Biochem. J. 259, 569–576
22. Helft, M., Yousef, S., and Dennis, J. W. (1989) Cancer Res. 49, 6077–6084
23. Chen, J. W., Pan, W., D’Souza, M. P., and August, J. T. (1985). Arch. Biochem. Biophys. 239, 374–386
24. Helin, A., and Silverstein, R. L. (1990) J. Biol. Chem. 265, 18531–18537
25. Dahlgren, C., Carlsson, S. R., Karlsson, A., Lundquist, H., and Sjölin, C. (1995) Biochem. J. 311, 667–674
26. Peters, J. P., Broc, J., Oort, E., Fukuda, M., Krähenbühl, O., Tschopp, J., Slet, J. W., and Geuze, H. J. (1991) J. Exp. Med. 173, 1099–1109
27. Sawada, R., Jardine, K. A., and Fukuda, M. (1993) J. Biol. Chem. 268, 9014–9022
28. Acevedo-Schermerhorn, C., Gray-Bablin, J., Gama, R., and McCormick, P. J. (1997) Exp. Cell Res. 236, 510–518
29. Thomas, K. R., and Cappechi, M. R. (1987) Cell 51, 503–512
30. Köster, A., Safitg, P., Matzen, U., von Figura, K., Peters, C., and Pohlmann, R. (1993) EMBO J. 12, 5219–5223
31. Saftig, P., Peters, C., von Figura, K., Craessenaerts, K., van Leuven, F., and De Strooper, B. (1996) J. Biol. Chem. 271, 27241–27244
32. Isbrands, H., Arit, O., Brooks, D. A., Hopwood, J. J., von Figura, K., and Peters, C. (1994) Am. J. Hum. Genet. 54, 454–463
33. Cha, Y., Holland, S. M., and August, J. T. (1990) J. Biol. Chem. 265, 5008–5013
34. Lyons, K., Graycar, J. L., Lee, A., Hashmi, S., Lindquist, P. B., Chen, E. Y., Hogen, B. L. M. et al. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4554–4558
35. Lehmann, L. E., Eberle, W., Krull, S., Prill, V., Schmidt, B., Sander, C., von Figura, K., and Peters, C. (1992) EMBO J. 11, 4391–4399
36. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
37. Okazaki, I., Himeno, M., Ezaki, J., Ishikawa, T., and Kato, K. (1992) Biochem. (Tokyo) 111, 763–769
38. Pohlmann, R., Boecker, M. W. C., and von Figura, K. (1995) J. Biol. Chem. 270, 27311–27318
39. Schulze-Garg, C., Boker, C., Nadimpalli, S. K., von Figura, K., and Hille-Rehfeld, A. (1993). Cell Biol. 122, 541–551
40. Lemanski, P., Gieselmann, V., Hasilik, A., and von Figura, K. (1984) J. Biol. Chem. 259, 10129–10135
41. Bresciani, R., and von Figura, K. (1996) Eur. J. Biochem. 238, 669–674
42. Köster, A., von Figura, K., and Pohlmann, R. (1994) Eur. J. Biochem. 224, 685–689
43. Porter, M. T., Fluhrty, A. L., and Kihara, H. (1969) Proc. Natl. Acad. Sci. U. S. A. 62, 887–891
44. Waheed, A., Gottschalk, S., Hille, A., Krentler, C., Pohlmann, R., Braulke, T., Hauser, H., Geuze, H., and von Figura, K. (1988) EMBO J. 7, 2351–2358
45. Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987) Nature 326, 292–295
46. Docherty, K., and Brenchley, G. V. (1979) Biochem. J. 178, 361–366
47. Gieselmann, V., Hasilik, A., and Figura, K. (1985) J. Biol. Chem. 260, 3215–3220
48. Marsh, M., Schindl, S., Kern, H., Harms, E., Male, P., Meltman, I., and Helin, A. (1987) J. Cell Biol. 104, 875–886
49. Jacobson, A., and Petz, S. W. (1996) Ann. Rev. Biochem. 65, 693–739
50. Saftig, P., Hartmann, D., Lüllmann-Rauch, R., Wolff, J., Evers, M., Köster, A., Hetman, M., von Figura, K., Peters, C. (1997) J. Biol. Chem. 272, 18628–18635