Targeted disruption of the gene encoding the proteolipid subunit of mouse vacuolar H$^+$-ATPase leads to early embryonic lethality

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Abstract

Vacuolar H$^+$-ATPase (V-ATPase) is responsible for acidification of intracellular compartments in eukaryotic cells. Its 16-kDa subunit (proteolipid, PL16) plays a central role in V-ATPase function, forming the principal channel via which protons are translocated. To elucidate physiological roles of V-ATPase in mammalian cell function and embryogenesis, we attempted to generate a PL16 null mutant mouse by gene-targeting. Mice heterozygous (PL16$^{+/-}$) for the proteolipid mutation were intercrossed and their offspring were classified according to genotype. There were no homozygous (PL16$^{-/-}$) pups among 69 neonates examined, but a few PL16$^{-/-}$ embryos were found during the pre-implantation stages of embryonic development, up to day 3.5 post-coitum. These results suggested that PL16 (and hence V-ATPase) may play an essential role in cell proliferation and viability during early embryogenesis. PL16$^{+/-}$ mice were indistinguishable from their wild-type littermates and displayed no discernible abnormalities, although the PL16 mRNA level in PL16$^{+/-}$ mice decreased to about one-half of wild-type levels. © 1999 Elsevier Science B.V. All rights reserved.

1. Introduction

Vacuolar H$^+$-ATPases (V-ATPases) are ubiquitous in eukaryotic cell endomembranes, such as those enclosing vacuoles, lysosomes, coated vesicles, secretory granules and the *trans*-Golgi network [1–8]. In these organellar membranes, V-ATPases pump H$^+$ into the lumen of the organelles in a process coupled with ATP hydrolysis, leading to acidification of organellar compartments. In a number of differentiated cell types, including osteoclasts [9], kidney epithelial cells [10] and macrophages [11], V-ATPases are also enriched in the plasma membranes, where they actively secrete H$^+$ from the cells and establish an acidic extracellular environment. This process is involved in bone resorption, urinary acidification and cytoplasmic pH regulation. In situ hybridization studies have shown increasing specificity of localization of the 16-kDa V-ATPase subunit (proteolipid, PL16) mRNA to sites of mesenchymal differentiation and epithelium–mesenchyme interaction from about day 17 of rat embryogenesis, suggesting the specific involvement of V-ATPase in mammalian late embryonic development [12]. However, the functional im-

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portance of V-ATPase during mammalian early embryogenesis remains to be determined.

V-ATPases are composed of multiple subunits and assembled as a large membrane complex that has a structure similar to that of H^+-ATP synthase (F_1F_o-ATPase) [1–8]. V-ATPases have two distinct multisubunit sectors, a hydrophilic sector (V_1) carrying a catalytic center for ATP hydrolysis and a hydrophobic sector (V_o) which functions as an H^+ channel. In mammalian cells, V_1 appears to have at least eight distinct subunits (designated A–H). Although the number of essential subunits in V_o is not certain, at least four distinct subunits of 115, 39, 20 and 16 kDa are involved. Of these, the 16-kDa subunit PL16 plays a central role in H^+ translocation across the membrane [13].

In microorganisms, especially budding yeast, disruption of subunit genes has provided much information concerning the physiological roles of V-ATPase. Disruption of the budding yeast gene encoding a V-ATPase subunit was conditionally lethal, showing sensitivity to environmental factors such as alkaline pH and several metal ions [14]. Dow et al. reported that deletion of the gene encoding the B subunit of V-ATPase in *Drosophila* lead to larval lethality, whereas point mutations caused defective phenotypes ranging from subvital to embryonic lethal [15]. However, no mammalian V-ATPase gene knockouts have yet been described.

In order to elucidate the physiological roles of mammalian V-ATPase, we attempted to disrupt the proteolipid gene in mice. Although we readily obtained heterozygotes (PL16$^{+/-}$), homozygous knockout mice were not produced, indicating that V-ATPase is essential in very early embryogenesis.

2. Materials and methods

2.1. Construction of the targeting vector

The mouse PL16 gene was isolated from a 129/Sv genomic library (Stratagene) by a standard plaque hybridization method [16], using rat proteolipid cDNA [17] as a probe. The targeting vector contained approximately 10.4 kb of genomic sequence. The initiation codon AUG of the proteolipid gene in the targeting vector was replaced with an *Eco*RI rec-

ognition sequence, into which the neomycin-resistance gene (*neo*) without a promoter and a polyA sequence were inserted. The gene for diphtheria toxin A fragment with an MC1 promoter [18] was inserted into the construct at a *Hin*dIII site to provide a marker for negative selection. The targeting vector was linearized with *Xho*I before being transfected into ES cells (Fig. 1).

2.2. Isolation of targeted ES cell clones

ES cell line CJ7 [19], kindly provided by Dr. T. Gridley (Roche Institute of Molecular Biology), was cultured on an inactivated embryonic fibroblast feeder layer. Gene transfer experiments were carried out by electroporation [20] and transfected clones were cultured on a G418-resistant feeder layer (Gibco). G418-resistant ES clones were isolated after 10–14 days of culture in medium containing 150 μg/ml of geneticin (Gibco) and expanded further in 24-well plates.

Homologous recombinants were identified by Southern blot analysis [16]. Genomic DNA was prepared from cells cultured in the absence of feeder cells and subjected to Southern blot analysis. Total DNA (10 μg) was cleaved with *Bam*HI and *Eco*RI, resolved by agarose gel electrophoresis, blotted onto Hybond N+ membrane (Amersham), and hybridized to probe A (Fig. 1). Probe A is a 1.0-kb *Hin*dIII–*Hin*dIII fragment flanking the 3′-end of the targeting vector. The probe hybridized to a 6.3-kb fragment of the wild-type allele and a 1.8-kb fragment of the mutant allele. To ensure integration of a single targeting vector in the transformed ES clones, DNAs were digested with *Sac*I, Southern blotted as above and hybridized with probe B (Fig. 1), which is the complete coding sequence of the neomycin-resistance gene (0.8 kb).

2.3. Generation of chimeric and heterozygous mice

Eight-cell embryos were collected from pregnant Crj:CD-1 (ICR) or C57BL/6 females 2.5 days post-coitum (dpc). Approximately 8–10 ES cells carrying a homologous recombination were microinjected into the embryos [21] and implanted into pseudopregnant ICR foster mothers. Chimeras identified by the presence of an agouti coat color were test-mated with

C57BL/6 females. Agouti offspring were tested for the targeted PL16 gene by Southern blot analysis using probe A as above (Fig. 1).

2.4. Genotyping of embryos

Postimplantation embryos (8.5 and 14.5 dpc) obtained from heterozygous intercrosses were genotyped by Southern blot analysis as described above. Preimplantation embryos (1.5–3.5 dpc) were analyzed using a PCR-based method [22]. Briefly, preimplantation embryos collected from oviducts and uteri were washed several times with phosphate-buffered saline (PBS; pH 7.3), suspended in 7.5 μl of double-distilled water, frozen with liquid nitrogen, thawed and digested with proteinase K (0. 25 mg/ml final concentration) for 1 h at 55°C. After incubation for 10 min at 95°C, the embryo extracts were used as templates for PCR analysis. Nested PCR was performed using primers P1 (5′-TTG CCT CCT CGC TCG CTG TCC CGT T-3′) and P2 (5′-GCG CCC ATG ACA CCG AAA AAC GAA G-3′), which are complementary to the 5′-non-coding region and exon 1 of the PL 16 gene, respectively, followed by primers P3 (5′-CCT TGA ATT CAT GGG ATC GGC CAT TGA AC-3′) and P4 (5′-CCT TGA ATT CCC CTC AGA AGA ACT CGT C-3′), which match the N-terminal and C-terminal ends of the neomycin-resistance gene, respectively (Fig. 3). The first round of nested PCR was carried out using 10 μl of embryo extract under the following conditions: first cycle, 180 s at 95°C, 120 s at 55°C and 90 s at 72°C, followed by 40 cycles, each consisting of 60 s at 95°C, 30 s at 55°C and 90 s at 72°C. The product of the first-round PCR was diluted 1:100 and 2 μl of this dilution was used as the target of the second PCR (30 cycles, each consisting of 60 s at 95°C, 30 s at 55°C and 90 s at 72°C). The resulting PCR products were resolved by 5% polyacrylamide gel electrophoresis (PAGE) and visualized with UV light after staining with ethidium bromide.

2.5. Northern blot analysis of heterozygous mice

Total cellular RNA was extracted from various tissues of wild-type and heterozygous (PL16$^{+/-}$) mice using Isogen solubilizing solution (Wako, Osa-

ka, Japan), which is based on the guanidium isothiocyanate method [23]. For Northern blot analysis, 15 μg of total RNA from different mouse tissues was denatured with formaldehyde, separated by electrophoresis on 1% agarose gels, and transferred onto Hybond N+ membrane (Amersham). Membranes were prehybridized with buffer containing Denhardt's solution [24], and hybridized with radiolabeled rat DNA encoding PL16 as a probe. Hybridizing bands were visualized and quantified using a Bio-Image Analyzer BAS1000 (Fuji). In order to normalize total RNA loading, the membranes were stripped by boiling and then reprobed with a cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [25].

2.6. DNA manipulation and sequencing

Preparation of plasmids, digestion with restriction endonucleases, ligation of the DNA fragments with T4 DNA ligase and other techniques related to handling of DNA were performed according to standard procedures [16,26]. The nucleotide sequences of inserts cloned into the various expression plasmids in this study were determined by dideoxynucleotide chain termination reactions using α-^{35}S deoxy-CTP (37 TBq/mol, Amersham) and T7 DNA polymerase [27] and subsequent autoradiography, or by automated sequencing using fluorescent primers (Pharmacia Biotech).

2.7. Reagents and enzymes

Restriction endonucleases, T4 DNA ligase, Tth and Pfu DNA polymerases and T7 DNA polymerase were purchased from Toyobo, New England Biolabs, and Takara. Oligonucleotides were synthesized by Pharmacia Biotech. Other reagents and materials were of the highest grade commercially available.

3. Results

3.1. Generation of ES cells and mice with a mutated proteolipid gene

We found that only one copy of functional gene and two defective pseudogenes of 16 kDa proteolipid
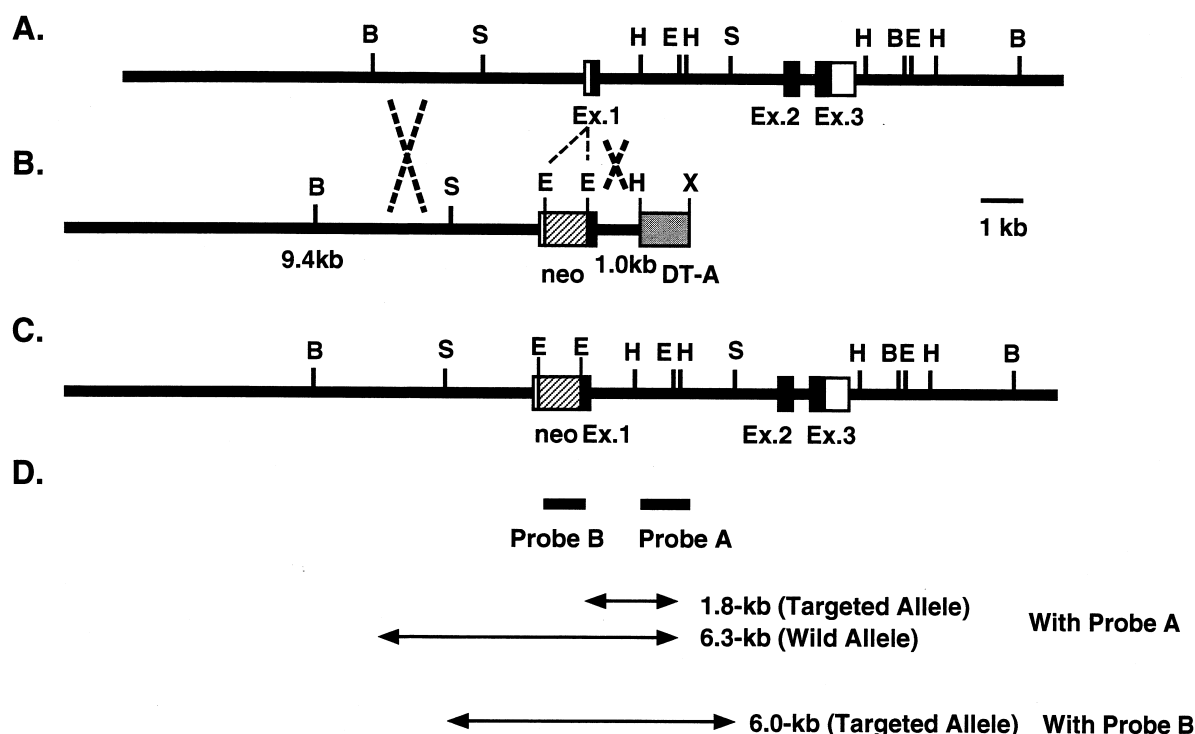
Fig. 1. Structures of proteolipid gene in mouse genome, targeting vector, homologous recombinant allele and Southern blotting probes. (A) Structure of proteolipid gene in mouse genome. Ex.1, Ex.2 and Ex.3 indicate exons 1–3. Closed and open boxes indicate coding and non-coding regions, respectively. (B) Structure of targeting vector. The coding region of the neomycin-resistance gene (*neo*) was inserted into the translation initiation site of the proteolipid gene, which had been mutated to create an *Eco*RI restriction site. DT-A indicates diphtheria toxin A fragment gene with MC1 promoter. (C) Structure of homologous recombinant (targeted) allele. (D) DNA fragment used as a probe for Southern blot analysis and the sizes of bands expected in the analyses shown in Fig. 2. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; X, *Xba*I.

exist in mouse based on sequence analyses of cloned genes and hybridization analyses of the whole mouse genome (Noumi et al., manuscript in preparation). A targeting vector for disrupting the proteolipid gene was constructed using the first exon of the only functional gene derived from 129Sv genomic DNA (Noumi et al., manuscript in preparation). The genes encoding neomycin resistance (*neo*) and diphtheria toxin A fragment were included in the construct as positive and negative selection markers [18], respectively. The coding region of *neo* was inserted into an *Eco*RI site which was created by substitution of the initiation codon of the proteolipid gene (Fig. 1). Consequently, the first AUG of the proteolipid gene was replaced by that of the *neo* gene. The targeting vector was introduced into ES cells by electroporation and ES cells showing increased resistance to G418 were selected. Homologous recombination in the selected clones was identified by Southern blot

analysis with probe A (Fig. 1). The presence of the targeted alleles was confirmed by detection of a 1.8-kb *Eco*RI–*Eco*RI fragment created by recombination, compared with a 6.3-kb *Eco*RI–*Bam*HI fragment from the wild-type allele (Fig. 1, Fig. 2A). Six clones carrying the targeted allele were found in 297 colonies. In all of the selected clones, a probe con-

Table 1
Genotypes of liveborn offspring and embryos derived from heterozygote intercrosses

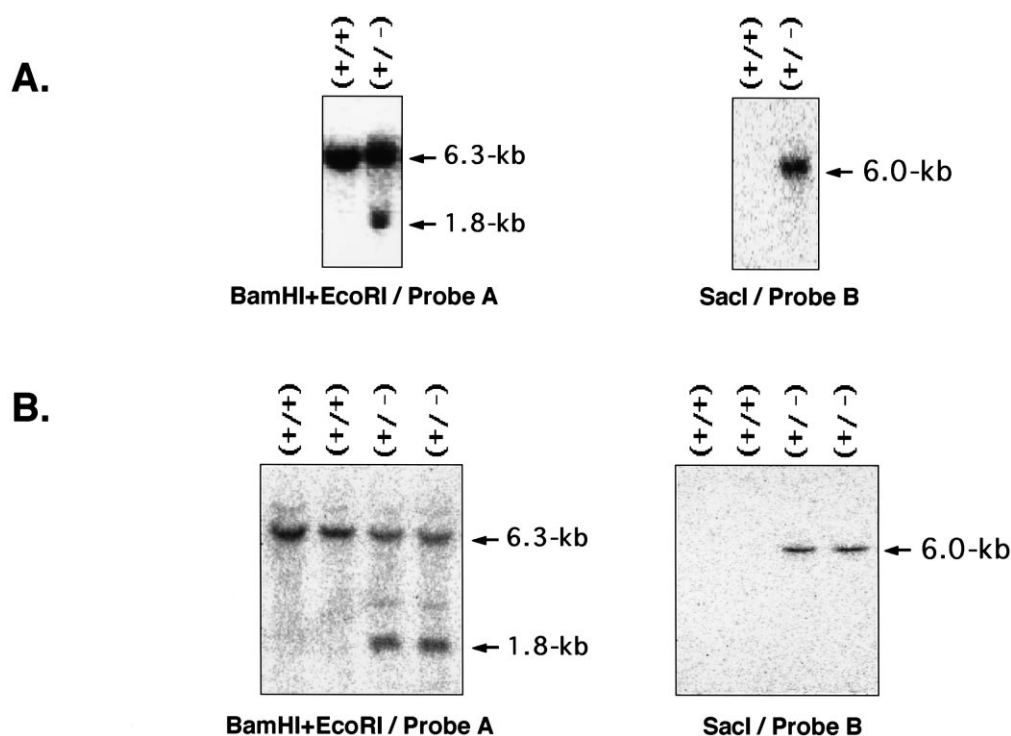|	(+/+)	(+/−)	(−/−)	Total
Liveborn offspring	22	47	0	69
Embryos				
E14.5	10	20	0	30
E8.5	13	23	0	36
E3.5	8	23	1	32
E2.5	9	24	4	37
E1.5	2	10	3	15

Fig. 2. Genotype analysis of ES cell lines and liveborn offspring derived from heterozygote intercrosses by Southern blot analysis. (A) Genomic DNA prepared from wild-type (+/+) and targeted (+/−) ES clones were digested with both *Bam*HI and *Eco*RI, or with *Sac*I alone, and hybridized to probes A and B as indicated. (B) Genomic DNA prepared from liveborn offspring of heterozygote intercrosses were digested and probed as above. (+/+) and (+/−) represent wild-type and heterozygous mice, respectively.

taining the *neo* gene (probe B; Fig. 1) hybridized with fragments of the expected sizes on Southern blots (Fig. 2A).

The mutant ES clones were microinjected into eight-cell embryos of ICR or C57BL/6 mice. A germ-line chimera (male) was obtained from embryos injected with one of the mutant ES clones. The chimeric mouse was then bred to produce mice heterozygous for the mutation. The heterozygous mice (PL16$^{+/-}$) thus created were viable and fertile. The PL16$^{+/-}$ mice were then intercrossed and the genotypes of the resulting offspring determined. A typical result is shown in Fig. 2B. Among 69 offspring produced by mating of PL16$^{+/-}$ mice, none was found to be homozygous for the mutation (PL16$^{-/-}$). The ratio of wild-type (PL16$^{+/+}$) to PL16$^{+/-}$ mice was approximately 1:2, based on the result shown in Table 1. Crosses of PL16$^{+/-}$ and wild-type mice (either male or female) produced PL16$^{+/-}$ and wild-type offspring in a ratio of about 1:1 (data not shown). These results indicate that PL16^{-} germ cells, both spermatozoa and ova, were of normal

fertility and that PL16$^{-/-}$ mice died during development.

3.2. Loss of proteolipid leads to early embryonic lethality

Embryo genotypes were analyzed by Southern blot or PCR (Fig. 3) at different times during gestation in order to determine when embryos of the PL16$^{-/-}$ mice died. No PL16$^{-/-}$ embryos were found at postimplantation stages 8.5 and 14.5 dpc (Table 1). However, when embryos were recovered at preimplantation stages (1.5–3.5 dpc) from the oviducts and uteri of naturally mated PL16$^{+/-}$ mice, a few PL16$^{-/-}$ embryos were detected: one at 3.5 dpc (blastocyst stage), four at 2.5 dpc (eight-cell stage) and three at 1.5 dpc (two-cell stage) (Table 1). The ratio of PL16$^{-/-}$ to total embryos was clearly lower than the Mendelian ratio even at 2.5 dpc (eight-cell stage). These data indicate that loss of function of the endogenous proteolipid gene resulted in early embryonic lethality during pre-implantational develop-
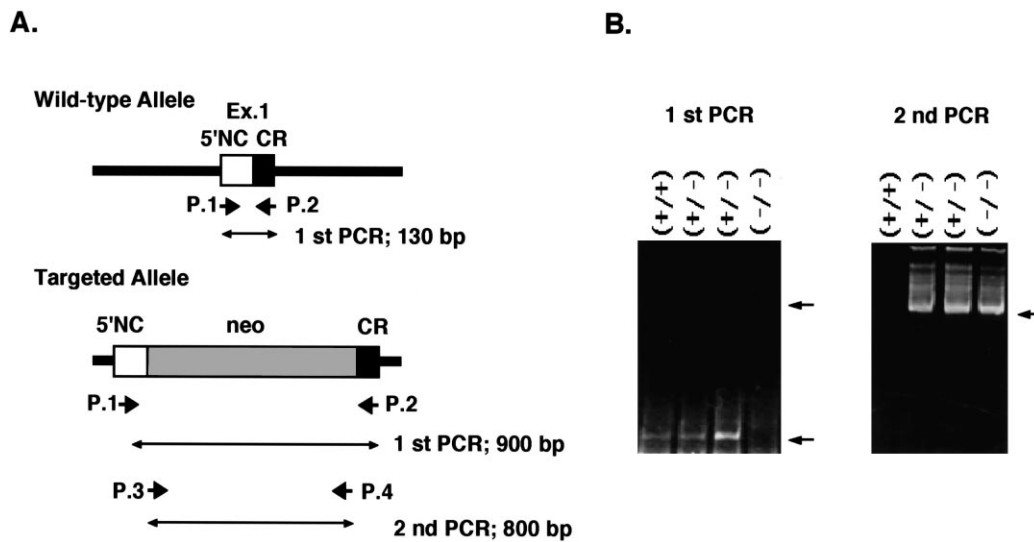
Fig. 3. Genotype analysis of embryos derived from heterozygote intercrosses by PCR. (A) Schematic representation of the wild-type and targeted alleles, and the primers used for genotyping of embryos. PCR primers P1–P4 are indicated by arrows. A 130-bp DNA fragment is amplified from the wild-type allele by the first PCR using P1 and P2 as primers, whereas a 900-bp fragment is obtained from the targeted allele. Since the 900-bp fragment was very faint, the targeted allele was detected as an 800-bp fragment by nested PCR using primers P3 and P4 for the second round. Ex.1, exon 1 of the proteolipid gene; 5′NC, 5′-non-coding region; CR, coding region of exon 1. (B) Genotype analysis of E2.5 embryos. PCR was performed as above. The lower and upper arrows represent 130- and 900-bp products, respectively, in the first PCR. An arrow indicates the 800-bp DNA product of the second PCR. (+/+), wild-type; (+/−), heterozygote; (−/−), homozygote.

ment, and that proteolipid (and hence V-ATPase activity) may be essential for basal cell growth in mammalian cells. No abnormalities were observed in the blastocyst or eight-cell PL16$^{-/-}$ embryos by phase contrast microscopy.

3.3. mRNA level of proteolipid gene in heterozygous mice

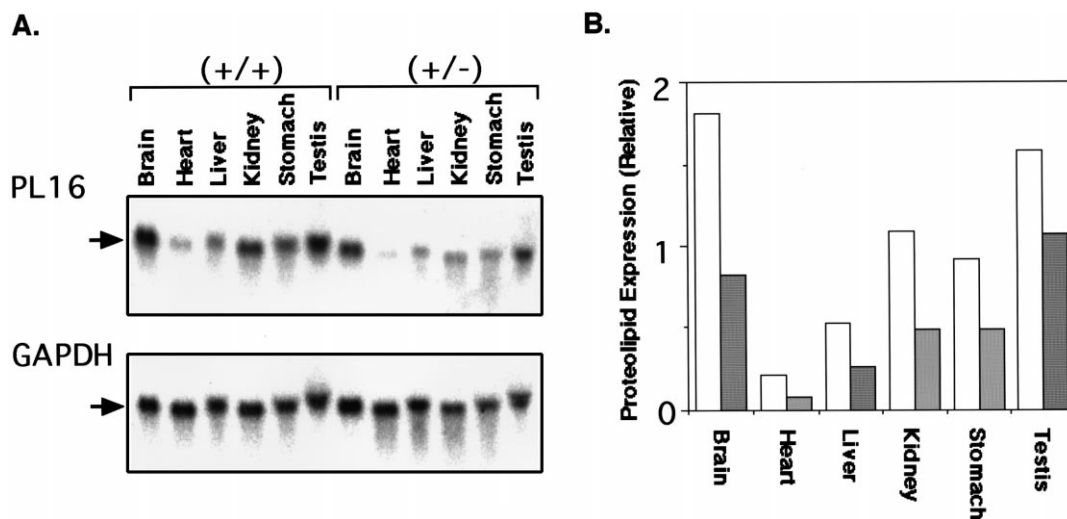Mice heterozygous for the PL16 mutation were indistinguishable from wild-type mice with respect

Fig. 4. PL16 mRNA levels in heterozygous knockout and wild-type mice. Northern blot analysis (A) of wild-type and heterozygous mice was performed using rat proteolipid cDNA as a probe. Expression levels of the proteolipid gene were normalized using GAPDH as an internal control. (B). Open and closed bars represent relative expression levels in wild-type and heterozygous mice, respectively.

to body weight and posture, and did not exhibit anatomical abnormalities (data not shown). Although we had expected that the heterozygotes might show some abnormality because of the early embryonic lethality of the homozygotes and the importance of V-ATPase in cellular functions [28–30], this was not the case. We therefore compared PL16 mRNA levels in the PL16$^{+/-}$ and wild-type mice. Northern blotting and hybridization of total RNA prepared from various organs revealed that PL16 mRNA levels were 50% lower in the PL16$^{+/-}$ mice than in the PL16$^{+/+}$ mice, suggesting that the proteolipid content of PL16$^{+/-}$ embryos may be lower than that of wild-type embryos (Fig. 4).

4. Discussion

We attempted to generate mice with a null mutation of the gene for the proteolipid subunit of V-ATPase. In result, newborns and embryos homozygous for the mutant proteolipid gene were not detected after the blastocyst stage. V-ATPase may therefore be essential for early embryogenesis. It should be noted that the proteolipid gene is one of relatively few genes whose knockout caused early embryonic lethality. Disruption of genes encoding Rad51, a factor involved in DNA repair and homologous recombination, and E-cadherin, a cell adhesion molecule, is known to cause pre-implantational lethality [21,31–33]. Tsuzuki et al. proposed that mutants leading to peri-implantational death might be divided into two categories: those that cause abnormal formation of tissues, and those that cause loss of basic molecular functions required for general cell viability [21]. E-cadherin might belong in the former category, which also includes abnormal cell–cell communication, and Rad51 might belong to the latter group. It has been reported that lysosome-like acidic vacuoles in blastocyst trophoblast cells play an important role in implantation of embryos [34]. The defect caused by disruption of the PL16 gene may therefore involve abnormal formation of tissue. However, we found that even at the eight-cell stage the ratio of PL16$^{-/-}$ to total embryos does not fit Mendelian predictions. In eight-cell embryos, the cells do not inter-communicate extensively and are relatively independent of each other [35,36]. It there-

fore appears that early embryonic lethality caused by dysfunction of V-ATPase may in fact be due to a defect in cell growth and viability.

Specific inhibitors of V-ATPase function (bafilomycin and concanamycin and its derivatives) cause inhibition of vesicle transport and protein sorting in cultured cells [28,29,37,38]. Recently, antibiotics were shown to cause programmed cell death in several cultured cell lines [39–41]. The mechanism underlying these observations has remained obscure, but there are a few clues available. Acidification of cytoplasmic pH caused by inhibition of V-ATPase induces apoptosis-like cell death in the murine B lymphoma cell line WEHI-231 [39]. Moreover, expression of p53, a tumor suppresser gene, is enhanced when apoptosis is induced by inhibition of V-ATPase in cardiomyocytes [41]. These observations suggest that an apoptotic mechanism may underlie the early embryonic lethality observed in proteolipid knockout mice.

Mice heterozygous for p53 [42–44], neurofibromatosis type I or II [45–47] or vascular endothelial growth factor (VEGF) [48,49] genes show serious defects, such as tumorigenesis or abnormal blood vessel development, respectively, although most of the heterozygote knockout mice reported to date have been indistinguishable from wild-type littermates. Mice heterozygous for the proteolipid null mutation displayed no discernible abnormalities, although the PL16 mRNA level in the heterozygotes was approximately one-half of the level observed in their wild-type littermates. It would be of interest to compare the internal pH of various cellular compartments in wild-type and PL16$^{+/-}$ mice, and to survey histochemical abnormalities in the heterozygous mice.

Here we report that a null mutation of the proteolipid gene caused early embryonic lethality, suggesting that V-ATPase is essential for early embryogenesis. In order to further analyse the function(s) of V-ATPase in the later stages of embryogenesis and in specific tissues in postnatal mice, stage- and tissue-specific conditional disruptions will be required. Heterozygous knockout mice constructed in this study will be a useful tool for such work. Null mutations of other subunits of V-ATPase will also be important for further evaluation of the gene knockout reported in this article, and of V-ATPase function.

Acknowledgements

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