Profilin I is essential for cell survival and cell division in early mouse development

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Profilins are thought to play a central role in the regulation of *de novo* actin assembly by preventing spontaneous actin polymerization through the binding of actin monomers, and the adding of monomeric actin to the barbed actin-filament ends. Other cellular functions of profilin in membrane trafficking and lipid based signaling are also likely. Binding of profilins to signaling molecules such as Arp2/3 complex, Mena, VASP, N-WASP, dynamin I, and others, further implicates profilin and actin as regulators of diverse motile activities. In mouse, two profilins are expressed from two distinct genes. Profilin I is expressed at high levels in all tissues and throughout development, whereas profilin II is expressed in neuronal cells. To examine the function of profilin I *in vivo*, we generated a null profilin I (*pfn1*^{ko}) allele in mice. Homozygous *pfn1*$^{ko/ko}$ mice are not viable. *Pfn1*$^{ko/ko}$ embryos died as early as the two-cell stage, and no *pfn1*$^{ko/ko}$ blastocysts were detectable. Adult *pfn1*$^{ko/wt}$ mice show a 50% reduction in profilin I expression with no apparent impairment of cell function. However, *pfn1*$^{ko/wt}$ embryos have reduced survival during embryogenesis compared with wild type. Although weakly expressed in early embryos, profilin II cannot compensate for lack of profilin I. Our results indicate that mouse profilin I is an essential protein that has dosage-dependent effects on cell division and survival during embryogenesis.

Profilins are ubiquitous proteins found in mammals (1), animal cells (2), plants (3), and viruses (4). Profilins sequester actin monomers in a 1:1 complex and inhibit actin polymerization (1). When bound to actin, profilin functions as an ATP nucleotide exchange factor recharging ADP-actin with ATP (5). Phosphoinositides were shown to dissociate the profilin–actin complex, suggesting that release of free ATP-actin at the plasma membrane could promote polymerization locally (6). Because of its affinity for phosphatidylinositol 4,5-bisphosphate, profilin also functions as a regulator of the signal transduction pathway through phospholipase C-γ1 (7).

Recent biochemical studies have suggested that profilin plays a key role in promoting actin assembly at the plasma membrane that drives cell motility and other actin-linked processes (8–10). Profilin also has been implicated in the motility of the intracellular pathogen *Listeria monocytogenes* (11), although an essential role for *Listeria* motility has been brought into question by recent results (12).

Despite a wealth of biochemical data on profilin function, the *in vivo* role is still under debate. Part of the complication is that profilin interacts not only with actin but also with a large number of ligands such as Mena (13), VASP (14), dynamin I (15), gephyrin (16), and SMN (17), and that this ligand binding might be an important aspect of profilin function. The promiscuity of profilin for a plethora of signaling molecules provides links between a variety of cellular processes and actin remodeling, but it also complicates the interpretation of profilin's function *in vivo*.

Whereas profilin I has been suggested as a tumor suppressor (18), no human diseases linked to profilin have been described, and no mouse models are available yet. In mouse, two profilin genes (profilin I and profilin II) that are 62% identical have been described. Biochemically, both isoforms are related closely in respect to actin binding, phosphatidylinositol bisphosphate binding, and their affinity for poly(L-proline) (19, 20). The major differences between the two profilin isoforms are their distinct expression patterns and their isoform-specific interactions with certain ligands, suggesting unique functions of the isoforms *in vivo* (15). Recently, we identified a splice form of profilin II (profilin IIB) in mouse that is generated in very low amounts in certain tissues (21).

Loss-of-function analysis in different model systems has provided further information on the role of profilin. In *Schizosaccharomyces pombe* (22) and *Drosophila melanogaster* (23), deletion of the one profilin gene resulted in lethal phenotypes, whereas in *Saccharomyces cerevisiae* severe growth reduction was observed (24). The amoeba *Dictyostelium discoideum* carries two profilin genes and only ablation of both isoforms reveals defects in cytokinesis. Nevertheless, double mutants can grow under certain conditions without any detectable profilin (25). In *Dictyostelium* cells lacking profilin, F-actin contents are increased. This increase formally contradicts the predictions for a protein required to promote actin polymerization, but is in agreement with profilin as a regulator of the monomeric actin pool. These analyses show that evaluation of profilins' *in vivo* function very much depends on the studied cell type and the organism.

Here we compare the expression pattern of mouse profilin I and profilin II, and report the generation of a conventional gene knockout for the profilin I gene. Our results show an essential role for profilin I in early mouse development during the first cleavages. Profilin II is not able to compensate and most likely has a more specific function in neuronal cells.

Materials and Methods

Generation of Profilin I Knockout Mice. Embryonic stem (ES) cell culture of J1 cells (26) and blastocyst injections were performed as described (27). Three independent, targeted ES clones were used to produce transgenic mice. Two clones were injected into BALB/c blastocysts and maintained in the BALB/c background, and the third clone was injected into C57 blastocysts. In all three lines, the profilin I mutation proved to be an early embryonic lethal mutation.

Genotyping of Blastocysts. Blastocysts were collected and individually lysed by adding Chelex-100 beads and boiling for 5 min. The supernatant was used directly for PCR. Two primer pairs were used in combination to amplify a 140-bp fragment from the wild-type profilin I allele (pfn1-A, 5′-CCA TGG CCG GGT GGA ACG CC-3′; pfn1-B, 5′-GTA CCG TAA TGC TAA CGA AGG-3′) and a 400-bp fragment from the neomycin-resistance gene in the disrupted allele (neo-A, 5′-ATT GAA CAA GAT GGA TTG CAC-3′; neo-B, 5′-CGT CCA GAT CAT CCT GAT

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Abbreviations: ES, embryonic stem; E*n*, embryonic day *n*.

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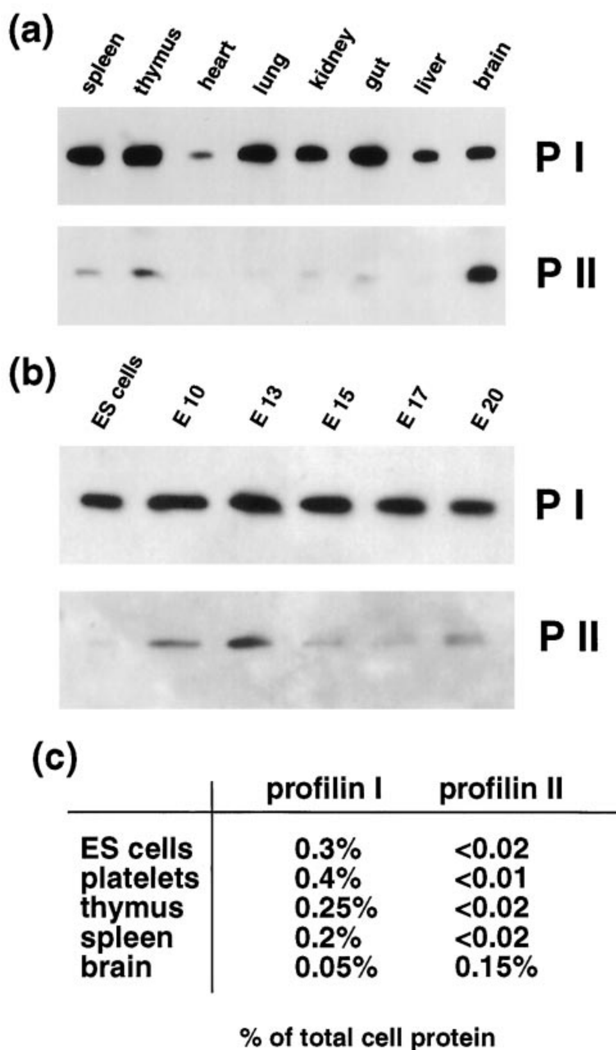

	profilin I	profilin II
ES cells	0.3%	<0.02
platelets	0.4%	<0.01
thymus	0.25%	<0.02
spleen	0.2%	<0.02
brain	0.05%	0.15%

% of total cell protein

Fig. 1. Expression of profilin I and profilin IIA protein in mouse tissues and during development. (*a*) Equal amounts of tissue extracts from adult mice were separated by SDS/PAGE, transferred, and probed with affinity-purified polyclonal antibodies raised against profilin I (P I) and profilin II (P II). (*b*) Lysates from ES cells and embryos at different stages [embryonic days 10–20 (E10–E20)] were tested for levels of profilins I and IIA. (*c*) Absolute amounts of profilins I and IIA expressed in various mouse tissues in percentage of total cellular protein.

C-3′). Because of the 1.8-kb insertion, only the internal neo-fragment would be amplified in homozygous mutants. In wild-type embryos, only the 140-bp product was detected. PCR products were separated, transferred onto membrane, and probed with the neomycin-resistance gene and profilin-specific probes.

Quantitation of Profilins I and II in Tissues and Embryos. Defined amounts of total lysates were subjected to SDS/PAGE and were transferred to membranes, and the filters were probed with the respective antibodies. Profilin antibodies were raised in rabbits against recombinant human profilins I and II. Both antibodies were isoform specific and crossreacted with mouse profilin I and the major mouse profilin II isoform, profilin IIA. Expression of the profilin IIB isoform is very low (21) and can be neglected for the absolute quantitation of profilin II in tissues. Serial dilutions of recombinant native profilins I and IIA were run as standards in parallel on the same gel. Signals were detected with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia) and the intensities were determined by using IMAGE 1.49.

Results

We chose to study the *in vivo* functions of mammalian profilins by using the mouse as a model system. To begin, we examined the relative expression of profilins I and II in different tissues and at different developmental stages by using isoform-specific antibodies (Fig. 1). Profilin I was expressed in all tissues, and was particularly high in the spleen, thymus, lung, and gut, but relatively low in the heart (Fig. 1*a*). In contrast, profilin II was expressed at high levels only in the brain (Fig. 1*a*). During embryonic development, profilin I was expressed highly at all stages, including ES cells, whereas profilin II was expressed at low levels in ES cells and early embryos, with peak expression around E13 (Fig. 1*b*). This increase in profilin IIA expression correlates with rapid development of the brain, supporting the notion that profilin II has acquired a specific function in neural cells and development.

Quantitative analysis indicated that profilin I occurred at highest levels in mouse platelets (0.4% of total cell protein, Fig. 1*c*) with comparable levels in other tissues, apart from brain. Profilin I expression in skeletal muscle is very low (data not shown). In contrast, profilin II expression was complementary to that of profilin I, being very high in brain (0.15% of total cell protein) and very low elsewhere. In summary, all tested mouse tissues—except skeletal muscle—contained 0.2–0.4% profilin I (30–60 μM), which is about half the concen-

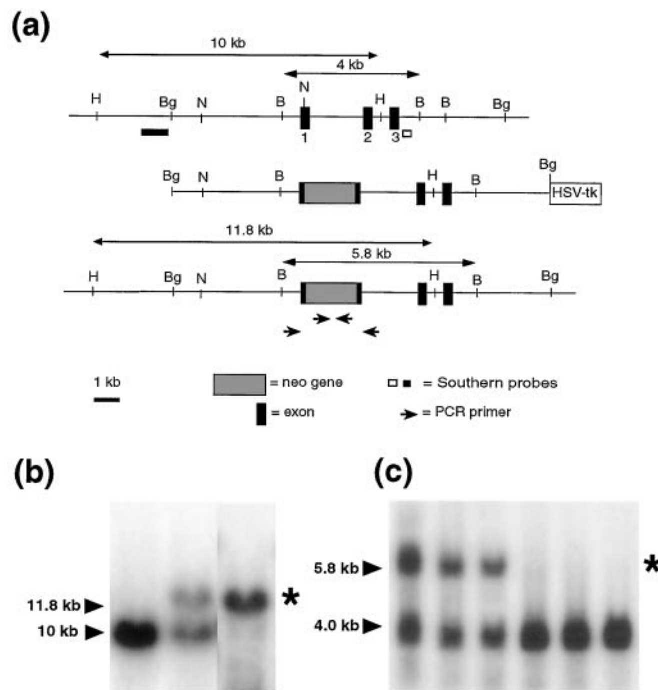

Fig. 2. Generation of profilin I knockout mice. (*a*) Map of the mouse profilin I gene (*Top*), the targeting vector (*Middle*) consisting of an 8.5-kb *Bgl*II (Bg) fragment, and the targeted locus (*Bottom*) are shown. Filled rectangles indicate coding exons 1, 2, and 3. The arrows (*Bottom*) indicate the primers used to identify the targeted alleles for the PCR analysis of blastocyst DNA. (*b*) Homologous recombination in ES cells is indicated by generation of an 11.8-kbp fragment (marked with an asterisk) caused by insertion of the *neo* gene into the wild-type allele of 10 kbp. DNA was tested with the indicated probes (lanes 1 and 2, profilin probe; lane 3, *neo* probe) after restriction digestion with *Hin*dIII (H). (*c*) Analysis of DNA from litters of a heterozygous breeding. DNA was digested with *Bam*HI and probed with the indicated probe. The targeted allele (5.8 kbp) is marked with an asterisk.

Table 1. Genotype of adult mice and embryos from profilin I mutant crossing

Crossing	No. of progeny +/+	+/−	−/−	Expected
Mixed background $pfn1^{ko/wt} \times pfn1^{wt/wt}$				
3–6 weeks	102	133	0	1:2:1
E9.5–11.5	22	42	0	1:2:1
E3.5	14	34	0	1:2:1
Mixed background $pfn1^{ko/wt}$				
+/− (m) × +/+ (f)	49	49	0	1:1
+/− (m) × +/− (f)	54	38	0	1:2:1
Congenic N10 C57BL/6 $pfn1^{ko/wt}$				
+/− × +/−	61	33	0	1:2:1
+/+ (m) × +/− (f)	37	13	–	1:1
+/− × +/− (E8–E18)	24	26	0	1:2:1

Lines 1–3: Results for heterozygous breeding pairs. No $pfn1^{ko/ko}$ mutants were found after weaning. Retrograde analysis on midgestation embryos (E9.5–E11.5) confirmed that $pfn1^{ko/ko}$ mutants die early in development. PCR analysis on E3.5 embryos suggests that no homozygous mutant embryos survive to the blastocyst stage. Lines 4 and 5: Embryos derived from a profilin I heterozygous oocyte have a disadvantage in development. Wild-type males (m) and females (f) were crossed with $pfn1^{ko/wt}$ females and males. Note that in case of a wild-type mother, the expected ratio of 1:1 was found, whereas in case of a heterozygous mother, a significant number of $pfn1^{ko/wt}$ embryos were lost. Lines 6–8: Congenic N10 C57 BL/6 $pfn1^{ko/wt}$ intercrosses. $pfn1^{ko/wt}$ mice were crossed 10 times against a C57BL/6 background, and then again $pfn1^{ko/wt}$ offspring were intercrossed. Note that the ratio of $pfn1^{ko/wt}$ to $pfn1^{wt/wt}$ is skewed further compared with the mixed background.

tration of actin in cells. This high-level expression suggests it has a general function in all tissues and throughout development. Profilin I expression is particularly high in tissues undergoing active proliferation, consistent with a role in cell growth or division.

To test the *in vivo* role of mouse profilin I, we generated a $pfn1^{ko}$ allele by gene targeting in ES cells. In the gene-targeting construct, the neomycin-resistance cassette was inserted at the ATG start codon of the *pfn1* locus, avoiding the production of any possible truncated protein (Fig. 2*a*). ES clones bearing a

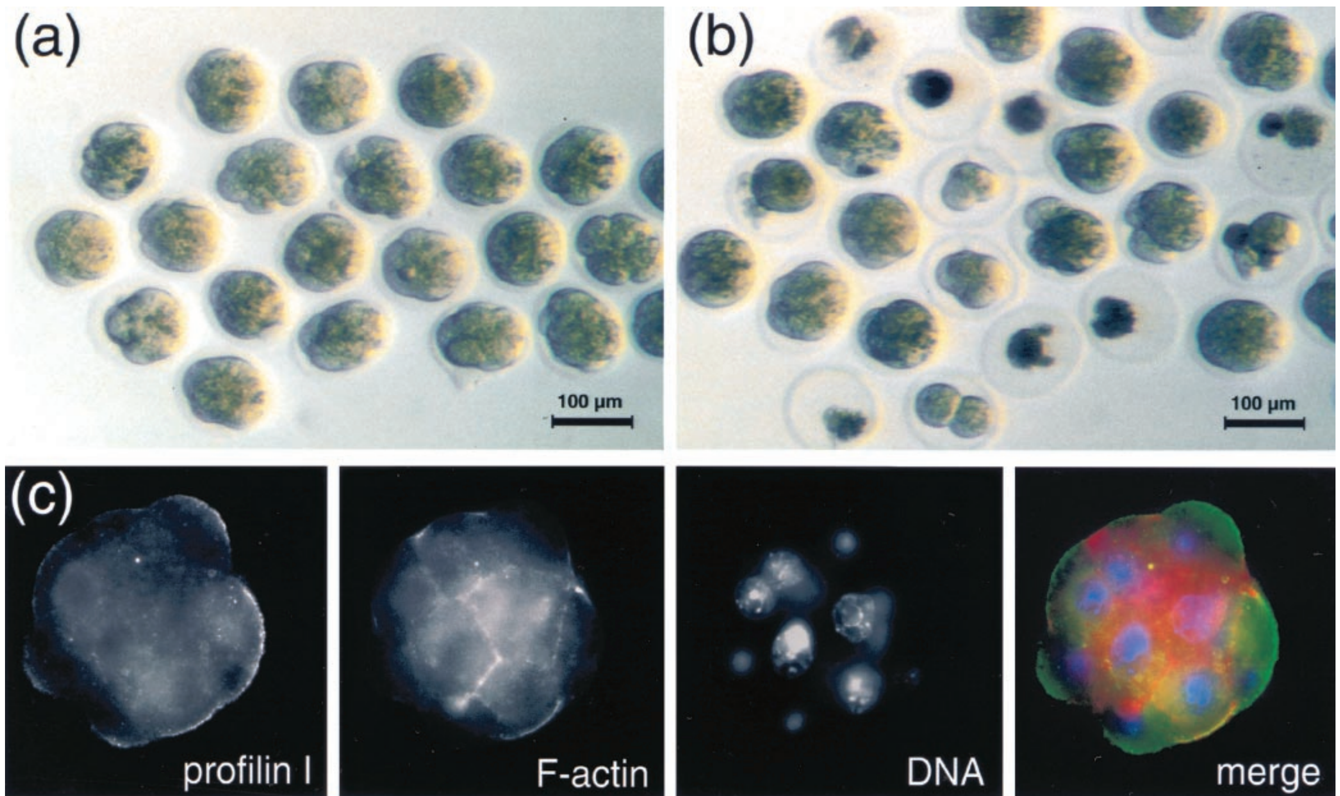

Fig. 3. *In vitro* development of two-cell embryos from wild-type ($pfn1^{wt/wt}$) and heterozygous ($pfn1^{ko/wt}$) crossings. Embryos were flushed at E1.5 and cultured for 2 days according to standard procedures (34) to allow development into morulas/blastocysts. (*a*) Embryos from a $pfn1^{wt/wt}$ mating after 3.5 days. (*b*) Embryos from a $pfn1^{ko/wt}$ mating after 3.5 days. Note that a significant number of embryos from the mutant cross are growth-arrested and deteriorated. (*c*) Staining of a wild-type morula for profilin I (green), F-actin (red), and DNA (blue). Embryos were stained with affinity-purified anti-profilin I antibody and an FITC-labeled secondary antibody, tetramethylrhodamine B isothiocyanate-phalloidin for F-actin, and 4′,6-diamidino-2-phenylindole for DNA. Details of the immunostaining protocol are available upon request.

targeted $pfn1^{ko}$ allele were identified at a frequency of 1/18 neomycin-resistant clones by Southern blot analysis (Fig. 2*b*). Three independent ES clones were injected into blastocysts, and all resulting chimeras passed on the mutation to offspring. Subsequent crosses of heterozygous $pfn1^{ko/wt}$ mice did not yield any homozygous $pfn1^{ko/ko}$ offspring, indicating that the mutation leads to an embryonic lethal phenotype (Table 1). This result was seen in mice derived from all three independently targeted ES clones, excluding a nonspecific effect from secondary mutation (data not shown).

$pfn1^{ko/wt}$ intercross embryo analysis showed that no $pfn1^{ko/ko}$ embryos were present at midgestation (E9.5–11.5), or even at E3.5, the preimplantation blastocyst stage (Table 1). These results show that profilin I is indispensable for the earliest steps in mouse development. To explore this phenomenon in greater detail, we cultured embryos at the 2-cell stage from wild type and $pfn1^{ko/wt}$ intercross matings, allowing them to differentiate toward blastocysts. Almost all (92.5%; $n = 81$) of the wild-type embryos developed into morula/blastocysts (Fig. 3*a*). In contrast, only 52.5% ($n = 97$) of $pfn1^{ko/wt}$ intercross-derived embryos developed properly (Fig. 3*b*), consistent with the premature death of about 1/4 of the $pfn1^{ko/ko}$ embryos, and perhaps some of the heterozygotes (see below). These failing embryos displayed a consistent pattern of condensation and fragmentation, with some variation in the time of death ranging from the two-cell to the four-cell stage.

Immunostaining of wild-type compacted morulas with purified profilin I antibodies revealed a diffuse cytoplasmic localization in the blastomeres with some enrichment in the cell cortex (Fig. 3*c*), consistent with its proposed role in early cleavages. Experiments to stain earlier embryos to determine their genotype were problematic because of the maternal contribution of profilin I and the rapid necrosis in homozygous mutants. Similarly, the severe deterioration and small size of these embryos made it impossible to analyze morphological alterations in the cytoskeleton in any detail.

These results suggested clearly that the profilin I null state was a cell-lethal event, which occurred rapidly after zygote formation, presumably as cellular pools of maternally derived profilin mRNA and protein declined. Additional evidence for the early embryonic lethality because of lack of profilin I also was seen in that we were unable to isolate $pfn1^{ko/ko}$ ES cell lines by high-dose G-418 selection, in contrast to several other genes for which null alleles have been generated by this method (27, 28).

$pfn1^{ko/wt}$ mice were viable, had normal longevity, and normal phenotype under standard mouse colony conditions. Histological analysis of tissues from $pfn1^{ko/wt}$ mice showed no abnormalities (data not shown). Western blot analysis confirmed that in $pfn1^{ko/wt}$ mice, profilin I expression was reduced by about 50% (Fig. 4), and was independent of the parent of origin of the $pfn1^{ko}$ allele (data not shown). Several $pfn1^{ko/wt}$ cell types were studied and all appeared normal. For example, $pfn1^{ko/wt}$ platelets had resting and stimulated F-actin levels similar to those of wild type, and $pfn1^{ko/wt}$ embryonic fibroblasts had normal growth characteristics and cytoskeletal architecture (data not shown).

Although $pfn1^{ko/wt}$ mice were viable, there was a selective loss of such embryos during embryonic development (Table 1). The ratio of wild-type to $pfn1^{ko/wt}$ mice in the viable offspring from $pfn1^{ko/wt}$ intercrosses was 1:1.3 instead of the expected 1:2 ($P < 0.001$ by χ^2 test), and this effect was seen in mixed BALB/c-129/SvJae and mixed C57BL/6–129/SvJae backgrounds. To examine whether the survival of $pfn1^{ko/wt}$ embryos was affected by parental genotype, we crossed wild-type males with $pfn1^{ko/wt}$ females and *vice versa*. The loss of $pfn1^{ko/wt}$ embryos appeared to occur exclusively when the mother was $pfn1^{ko/wt}$ and therefore contributed the $pfn1^{ko}$ allele to the embryo (Table 1), suggesting

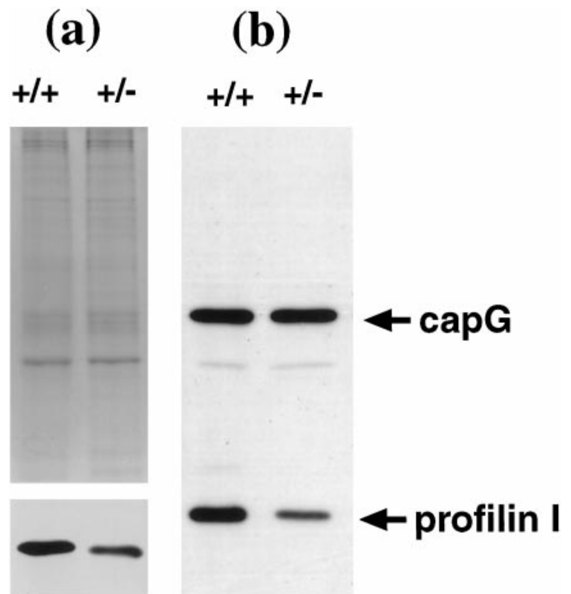

Fig. 4. Levels of profilin I in platelets and thymocytes from heterozygous mutant mice. (*a*) Equal amounts of platelet lysate from $pfn1^{wt/wt}$ and $pfn1^{ko/wt}$ mice were separated by SDS/PAGE, transferred, and probed with a profilin I-specific antibody (*Bottom*), or the gel was stained with Coomassie blue (*Top*). The amount of profilin I is reduced by about 50% in the heterozygotes. (*b*) Equal amounts of thymocyte lysate from $pfn1^{wt/wt}$ and $pfn1^{ko/wt}$ mice were subjected to SDS/PAGE and probed with a profilin I-specific antibody. The filter was reprobed with a (capG)-specific antibody to normalize for loaded protein amounts. Note the reduction of profilin I in the mutant thymocytes.

a maternal effect because of the decreased level of supplied profilin I in the oocytes. Interestingly, the haploid $pfn1^{ko}$ sperms are apparently viable and fully functional as indicated by the numbers (Table 1).

The reduction in $pfn1^{ko/wt}$ viability was even more dramatic in an N10 (10 backcrosses) C57BL/6 congenic strain derived from C57BL/6 and 129/SvJae (Table 1). In these mice, the proportion of $pfn1^{ko/wt}$ offspring fell to 35% from the expected 67% in $pfn1^{ko/wt}$ intercrosses, and the fetal loss was again much more severe when the mother carried the $pfn1^{ko}$ allele. Timed embryo analysis in these congenic $pfn1^{ko/wt}$ intercrosses indicated that at all stages from E8 to E18, the proportion of $pfn1^{ko/wt}$ embryos was reduced from the expected 67% but not as low as that seen in newborns. These observations suggested there were two stages at which $pfn1^{ko/wt}$ embryos failed—an early time point before E8 and a second time point in the perinatal interval. The early phase of $pfn1^{ko/wt}$ embryo loss could be caused by reduced levels of profilin in the early embryo, because the eggs from $pfn1^{ko/wt}$ mothers began with a one-half complement of profilin mRNA and protein. The perinatal mortality could be caused by defects in the mother or the fetus or a combination of the two.

Discussion

In summary, the profilin I gene exerts a dosage-dependent effect on viability in the mouse. Profilin I null zygotes die shortly after fertilization, whereas $pfn1^{ko/wt}$ mice have reduced survival, which is accentuated by maternal passage of the $pfn1^{ko}$ allele. The reduced survival of $pfn1^{ko/wt}$ mice in mixed genetic backgrounds, which is further decreased on formation of a congenic, suggests that there are several loci that interact with the profilin I locus to influence the viability of these mice. One candidate gene, alleles of which might modify the viability of $pfn1^{ko/wt}$ mice, is *Mena*. *Mena*-null mice display axonal pathfinding defects resulting in subtle brain structural abnormalities but are viable with normal fertility, whereas the

addition of one *pfn1*^{ko} allele to the *Mena*-null background leads to embryonic death in late gestation (29). Mena is a profilin I ligand and functions through the profilin pathway to regulate actin dynamics (13).

Although profilin II is expressed weakly in ES cells and early embryos, it is not sufficient to rescue the deficiency in profilin I, suggesting a unique function in brain. This possibility is also supported by preliminary results on profilin II-deficient mice (A. Di Nardo and W.W., unpublished data). Analysis of combined profilin I and II alleles may resolve whether any functional redundancy exists between the two proteins, and conditional loss-of-function alleles for profilin I will facilitate our further analysis of roles in development and in nondividing somatic cells.

Profilin is known to participate in the actin dynamics that characterize many forms of cellular motility, has multiple binding partners, and also may affect phospholipid metabolism and signaling. It is currently uncertain which of these properties contributes to the marked effects of profilin loss in mouse. Biochemical studies in *Acanthamoeba* suggest that actin binding is the primary function of profilin (30). However, the binding of other ligands via the poly(L-proline) binding site in profilin is also necessary as revealed by rescue experiments in yeast (31) and *Dictyostelium* (32). Much as profilin can sequester actin monomers, the interaction with ligands such as Arp2/3 complex (33), VASP (14), Mena (13), drebrin, gephyrin (16), SMN (17), and others may serve to sequester these ligands until they are needed to trigger a specific cellular process. Because the viability of profilin I null embryos is so limited, we speculate that leaving all of the profilin I ligands unlocked could be one cause for the cell lethal phenotype. The conditional replacement of wild-type profilin I by mutant alleles in mouse may define the relationship between the actin and poly(L-proline) ligand-binding properties of profilin I.

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