Targeted disruption of the mouse phospholipase C β3 gene results in early embryonic lethality

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Abstract In order to investigate the biological function of phosphatidylinositol-specific phospholipase C (PLC) we generated mutant mice by gene targeting. Homozygous inactivation of PLCβ$_3$ is lethal at embryonic day 2.5. These mutants show poor embryonic organization as well as reduced numbers of cells. Identical phenotypes were recorded in homozygous mutants generated from two independently targeted embryonic stem cell clones. Heterozygous mutant mice, however, are viable and fertile for at least two generations. We also showed that mouse PLCβ$_3$ is expressed in unfertilized eggs, 3-cell and egg cylinder stages of embryos. In conclusion, these results indicate that PLCβ$_3$ expression is essential for early mouse embryonic development.

Key words: Phospholipase C β3; Signal transduction; Targeted disruption; Early embryonic development; Preimplantational lethality

1. Introduction

Phosphatidylinositol-specific phospholipase C (PLC) is implicated to be activated in the mammalian oocyte at fertilization [1]. Preincubation of mouse eggs with the PLC inhibitor, U73122, greatly reduces their Ca^{2+} responsiveness to acetylcholine as well as spermatozoa activation [2]. Blastocele formation in the mouse is also inhibited by U73122 [3]. The activated PLC generates inositol 1,4,5-trisphosphate (IP_3) which triggers a series of cytosolic Ca^{2+} transients [4,5]. Intracellular repetitive Ca^{2+} oscillations are critical for egg activation and may contribute to the regulation of mouse preimplantational development [6,7]. The Ca^{2+} release occurs predominantly through the IP_3 receptor from intracellular stores [8,9]. Inhibition of the IP_3 receptor by a monoclonal antibody inhibits the Ca^{2+} oscillations and blocks egg activation [4,10]. Acetylcholine, known to activate PLCβ, could induce repetitive Ca^{2+} transients of mouse oocytes [11].

The human PLCβ$_3$ gene contains 31 exons and encodes a 1235 amino acid protein [12,13]. The X and Y regions comprise 170 and 260 amino acids, respectively. PLCβ$_3$ is the most widely expressed member of the PLCβ family [14,15].

The mouse PLCβ$_3$ gene is located on chromosome 19B [16] and shows an overall 92% amino acid identity to the human homolog [17]. PLCβ$_3$ in situ hybridization of rat embryos shows expression in the neural tube at embryonic day (E) 8, and in the thymus as well as lung tissues at E20 [18]. The regulation of PLCβ$_3$ in early embryonic development has not been studied. In an attempt to investigate the biological function of PLCβ$_3$, we generated mutant mice by gene targeting.

2. Materials and methods

2.1. Construction of the targeting vector

A 1.9 kb 3′ human PLCβ$_3$ cDNA fragment was used to screen a 129SV genomic library (Stratagene 946306, USA). The isolated clones were analyzed by digestion with different restriction enzymes (*Xba*I, *Eco*RI, *Eco*RV, *Sac*I, *Bam*HI, *Hin*dIII). Partial cleavage [19], polymerase chain reaction (PCR) analysis and DNA sequencing were performed to determine the restriction map and genomic organization.

A 4 kb 5′ *Sac*I genomic fragment of the PLCβ$_3$ gene was cloned into the *Sac*I site of pBluescript II KS$^-$ (Stratagene). A 1.4 kb 3′ *Sac*I/*Hin*dIII genomic fragment of the PLCβ$_3$ gene was blunt-end ligated into a T4 DNA polymerase treated *Xho*I site. Finally, neomycin phosphotransferase expression cassette was blunt-end ligated in sense orientation into the T4 DNA polymerase treated *Not*I site, between the 5′ and 3′ fragments of PLCβ$_3$ genomic homologies. This resulted in a deletion of a 4 kb genomic DNA fragment containing PLCβ$_3$ exons 11–17, as well as the introduction of an *Eco*RI and an *Eco*RV site.

2.2. Generation of PLCβ$_3$ mutant mice

The embryonic stem (ES) cell line E14.1, derived from the mouse strain 129/Ola, was cultured as described [20]. The targeting vector was linearized with *Pvu*II and electroporated into the ES cells. The neomycin-resistant (G418) ES cell clones were isolated and analyzed by Southern blotting. Two different clones were used for generation of chimeras by injecting the targeted ES cells into C57BL blastocysts and transferred to recipient mice [21]. Both attempts were successful and the targeted ES cells contributed to the germ line of two chimeras.

2.3. Polymerase chain reaction

Routinely, neonates were genotyped by PCR using primers a, b, and c. In selected cases, the genotype was verified by Southern blot analyses, which were consistent with PCR results. All embryos were genotyped by PCR amplification. Embryos or mouse tails were digested with proteinase K. PCR amplifications were performed using three primers (a, b, and c) in a single reaction mixture for 35 cycles (60 s at 94°C, 90 s at 55°C, 120 s at 72°C). The common primer was located in the 3′ fragment for homologous recombination (a, 5′-ttgtactcaaacacgcctgcgttga-3′). The primer for the wild type allele was located in the deleted exon 17 (b, 5′-agcagctcagccgcatctaccctaa-

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Abbreviations: PLC, phosphatidylinositol-specific phospholipase C; IP_3, inositol 1,4,5-trisphosphate; ES, embryonic stem; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; Neo, neomycin

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3′). The mutant allele primer was in the neomycin resistance gene (c, 5′-cgctatcaggacatagcgttggcta-3′) (Fig. 1). In order to further amplify and specify the signals a nested PCR was performed on 2 μl aliquots of the first PCR products using either primers a′ (5′-acacgcctgcgttgagctgcattggc-3′) and b′ (5′-tgccagcttgttgccctcaacttcca-3′), or a′ together with c′ (5′-actgtgccttctagttgccagccat-3′), each located inside the primers a, b and c. PCR products were resolved on 1% agarose gels.

2.4. Culture of preimplantational embryos

Mating was achieved by caging paired heterozygous males and females representing offspring from both chimeras respectively. Embryos were collected on day 2.5 and cultured for 24 or 48 h [22]. Conditions for culturing encompassed M16 medium and a +37°C humidified incubator maintaining a 5% CO_2 atmosphere. Individual embryos were collected and suspended in lysis buffer before PCR amplification.

2.5. Histology and immunohistochemistry

2.5.1. Embryos. Embryos from wild type mouse crossings were collected and fixed overnight in 3% formalin. The fixative was embedded in a drop of melted 2% agar and then cut into 1 mm^3 blocks. The agar blocks were dehydrated in ethanol and embedded in paraffin [23]. Mounted sections were stained for the presence of PLCβ$_3$ using polyclonal antibody against the C-terminal portion of PLCβ$_3$ (sc-403, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibody, and biotinylated anti-rabbit as secondary antibody. The immunoreaction was visualized with avidin-horseradish peroxidase and DAB as the chromogen [24]. No positive staining was observed in the absence of the primary antibody.

2.5.2. Tumor tissues. Tumor specimens were fixed in 4% paraformaldehyde and embedded in paraffin. For histological analysis, 5 μm thick sections were stained with hematoxylin and eosin.

2.6. Western blot analysis

Western blotting was performed according to a modified method [25]. Briefly, 10 μg of the total protein (quantified by protein assay kit, Pierce, Rockford, IL, USA) was loaded on 4–15% SDS-polyacrylamide gradient gels, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated overnight at 4°C with anti-PLCβ$_3$ (above) diluted 1:1000. After rinsing and washing twice for 10 min with PBS containing 0.1% Tween 20, the filters were incubated for 1 h with a 1/1000 dilution of donkey anti-rabbit IgG conjugated to horseradish peroxidase at room temperature. Subsequently, the filters were washed as above and developed using the ECL Western blotting kit (Amersham, UK). After stripping (according to the ECL Western blotting kit manual) the filters were reprobed with anti-actin monoclonal antibody (Boehringer Mannheim, Germany). The filters were scanned and target bands were quantified with NIH Image software.

3. Results and discussion

In order to design the targeting construct, genomic DNA clones encompassing the mouse PLCβ$_3$ gene were isolated from a λ phage library by probing with a human PLCβ$_3$ cDNA fragment. Eight positive clones were isolated and phage DNA purified. Two overlapping clones spanning the PLCβ$_3$ coding sequence were restriction mapped by partial cleavage. The genomic structure of mouse PLCβ$_3$ was determined by DNA sequencing, restriction analysis and genomic PCR. The organization of the mouse gene is similar to that of the human, encompassing 31 exons spanning about 16 kb (Fig. 1A). All analyzed exon-intron boundaries followed the GT-AG intron donor-acceptor splice rule. The Profile Scan Program (http://isrec-insect.unil.ch/cgi-bin/Callmollusc.pl) revealed two bipartite nuclear localization signals (aa 469–486 and aa 1100–1117). The amino acid sequence also contains the X (aa 318–469) and Y (aa 590–707) domains [17]. The X region comprises exons 10–13, and the Y region consists of exons 15–21. The X and Y regions have been reported to be essential for enzymatic activity [26].

The PLCβ$_3$ gene was disrupted in ES cells by a targeting vector, which resulted in deleting exons 11–17, amino acid sequence 338–681. Two thirds of the X and one third of the Y region as well as one nuclear targeting sequence were removed. The vector contained a neomycin resistance gene flanked by 4.0 kb of genomic DNA upstream of exon 11 as well as 1.4 kb downstream of exon 17 (Fig. 1A). Southern blot analysis showed that five out of 200 neomycin resistant ES cell clones carried a mutant PLCβ$_3$ allele. Two of these clones (Fig. 1B) were used to generate chimeric mice, and both successfully contributed to the germ line. The genotypes of the mice were determined by PCR or Southern blotting.

No viable homozygous mutant (−/−) pups were identified among 133 offspring from heterozygous (+/−) intercrosses (Table 1). This suggests that inactivation of PLCβ$_3$ is lethal to the embryo. We were able to produce heterozygous mutant pups by breeding both male and female heterozygotes with wild type mice, indicating that both paternal and maternal sources of PLCβ$_3$ could sustain embryonic development. To assess the consequences of the PLCβ$_3$ mutation on embryonic development we analyzed embryos from heterozygote intercrosses at E17.5, E8.5, E3.5, and E2.5 (Table 1). Genotyping was performed by nested PCR amplification (Fig. 1A,C). No −/− embryos were identified at E17.5 nor at E8.5 among 28 embryos. On embryonic day 3.5, 57 embryos were genotyped and only one, which showed normal morphology for date, was homozygous for the mutant allele. On E2.5 however, 14 of 99 embryos were identified as homozygous mutants. Ten of these homozygotes showed abnormalities characterized by poor embryonic organization and a substantial reduction of cell numbers compared to wild type and heterozygous littermates: mean number of cells 4.0 ± 0.6 (S.E.M.) vs. 7.5 ± 0.1 (S.E.M.) ($P < 0.0001$) (Table 1). Sixty-one of the 99 E2.5 morulas showed a heterozygous genotype and the remaining 24 represented wild types. Only two of the wild types and one heterozygote showed abnormal morphology at E2.5. Additionally, we collected day 2.5 embryos from heterozygous matings for culturing. Forty-two embryos were cultured for 24 h and both wild type and heterozygotes developed blastocele cavities. Forty-five embryos were cultured for 48 h, the wild type and heterozygotes formed late expanded blastocysts. The 11 mutants, however, failed to form blastocele cavities and four appeared distorted at 48 h (Fig. 2). Homozygous mutants generated from the two independently targeted ES cell clones showed identical phenotypes. These results demon-

Table 1
Genotypic and phenotypic analysis of neonates and embryos from PLCβ$_3$ heterozygous intercrosses

Stage	Total number	Genotype[a]		
		+/+	+/−	−/−
Neonate	133	40 (0)	93 (1)	0
E17.5	5	2 (0)	3 (0)	0
E8.5	23	8 (0)	15 (1)	0
E3.5	57	16 (1)	40 (2)	1
E2.5	99	24 (1)	61 (2)	14 (10)

The embryos were collected on embryonic days 17.5, 8.5, 3.5, and 2.5. Abnormal phenotypes encompassed poor embryonic organization and reduced cell numbers in comparison with control littermates.
[a]Number of abnormal individuals in parentheses.

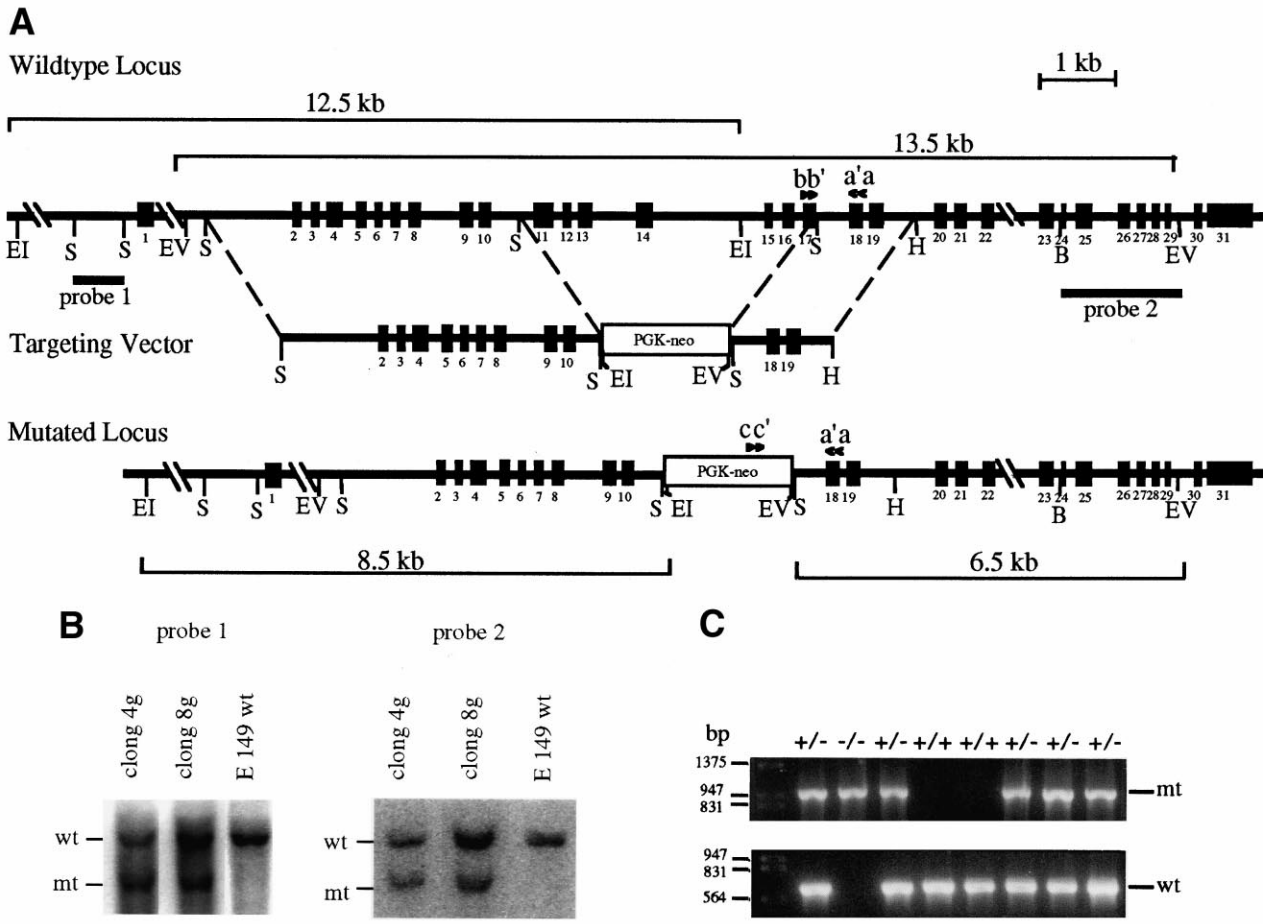

Fig. 1. Targeted disruption of the mouse PLCβ$_3$ gene. A: Genomic organization and targeting construct. Exons are depicted by filled boxes. Probe location for Southern blotting (probes 1, 2) and the PCR primers a, b and c, as well as the nested primers a′, b′ and c′ are shown. The neomycin transferase gene linked to the phosphoglycerate kinase promoter (PGK-neo); EI, *Eco*RI; EV, *Eco*RV; S, *Sac*I; H, *Hin*dIII; B, *Bam*HI. B: Southern blots showing homologous recombination at the PLCβ$_3$ locus for two of the targeted ES clones. The structure of the targeted locus was verified on both sides. Genomic DNA was digested with *Eco*RI and hybridized with probe 1. The probe hybridized to a 12.5 kb fragment derived from the wild type allele, or a 8.5 kb fragment from the allele with a correctly targeted replacement. Similarly, genomic DNA digested with *Eco*RV and hybridization with probe 2 resulted in either a 13.5 kb fragment from the wild-type allele, or a 6.5 kb fragment from the properly disrupted gene. C: Genotype analysis of embryos by PCR. Sizes of marker fragments (lane 1) are indicated. Primers a′, b′ generate a 900 bp (wild type) PCR product, and primers a′, c′ generate a 1200 bp (mutant) PCR product.

strate that homozygosity for the PLCβ$_3$ mutant allele results in embryonic lethality at E2.5.

Immunohistochemical staining using a polyclonal PLCβ$_3$ antibody showed high expression PLCβ$_3$ in unfertilized wild type mouse eggs as well as 3-cell stage embryos and egg cylinders (Fig. 3). No PLCβ$_3$ immunoreactivity was observed in primitive streak embryos.

The expression of PLCβ$_3$ protein in wild type and heterozygous mice was investigated. Since the vast majority of homozygotes could not be identify beyond E2.5 the material was too limited for Western blot analysis. We screened different mouse tissues including brain, lung, heart, liver and kidney by Western blot. The results demonstrate that PLCβ$_3$ protein is widely expressed in various adult mouse tissues, and lung tissue show the highest level (data not shown). Six wild type and six heterozygous mice were killed. The proteins were extracted from each individual lung and used for Western blot. PLCβ$_3$ protein of the expected size, 152 000, could be detected on Western blot from both wild type and heterozygous mice. The signal intensities were quantified with NIH Image software and normalized with corresponding signals of actin. Strikingly, the expression of PLCβ$_3$ protein in heterozygous mice was significantly reduced ($P < 0.05$), about 50% compared to that of wild type mice (Fig. 4). This result may indicate that both PLCβ$_3$ alleles normally are responsible for production of similar amounts of the protein. Under normal laboratory circumstances one allele can generate enough PLCβ$_3$ protein to sustain the life of heterozygous mice. Whether this reduced amount of PLCβ$_3$ would be sufficient in more challenging conditions is unclear.

Heterozygous mice were apparently normal at young age. However, at a mean age of 15.8 months (range 9.5–21.5), eight of 50 PLCβ$_3$ +/− mice, all derived from the first chimera, were killed because of signs of distress. Necropsy showed enlarged lymph nodes and spleen in all eight mice. Histopathology revealed lymphomas in six of the animals. All six lymphomas showed the same histological features resembling human lymphoplasmacytoid lymphoma. One heterozygous mouse revealed a pheochromocytoma and another harbored a pancreatic microadenoma. Fourteen wild type controls were killed at a mean age of 15.7 months (range 14–19.5) and four of them had the same type of lymphoid

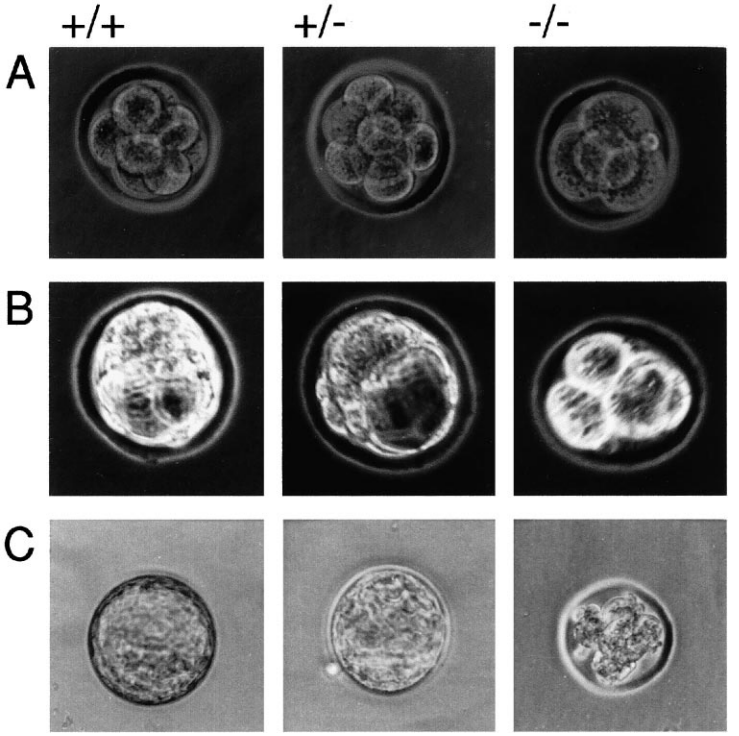

Fig. 2. Morphology of embryos. A: E2.5 embryos. The wild type and heterozygous mutant embryos showed the 8-cell stage. The mutant remained at the 4-cell stage. B: E2.5 embryos cultured for 24 h. Both wild type and heterozygote developed blastocele cavities, but the mutant failed to do so. C: E2.5 embryos cultured for 48 h. Wild type and heterozygotes had developed up to the expanded blastocysts. The mutant appeared distorted and no blastocele cavity was formed.

tumor although in less severe stages of the disease. A larger number of mice need to be investigated in order to fully understand the nature of these tumors. Heterozygotes (mean age 16 months) descending from our second chimera have not developed lymphomas, suggesting that the observed tumors are not specifically related to the mutant PLCβ_3 allele.

The data presented here show that PLCβ_3 deficient embryos suffer from a developmental arrest at E2.5 indicating that PLCβ_3 is essential for cellular proliferation at early embryonic stages. The mutant phenotype was most likely caused by disruption of the PLCβ_3 gene rather than alterations elsewhere in the ES cell genome since the offspring of the second chimera, derived from independently targeted ES cells, showed the same early embryonic lethality and presented no signs of lymphomas. Only 14% of E2.5 embryos presented a homozygous mutant genotype, implying that some of the −/− mutants disappeared before the first morphological event of differentiation. Alternatively, this unexpectedly low frequency could constitute an underestimation due to PCR contamination. Unfortunately, we had difficulties obtaining consistent results from genotyping E1.5 embryos, again, mainly due to the limited DNA templates. High expression of PLCβ_3 was found in wild type unfertilized eggs, 3-cell stage embryos and egg cylinders. Substantial expression in eggs and morulae possibly reflects that PLCβ_3 function is essential for cleavage of zygotes and early embryos. It seems likely that maternal sources of PLCβ_3 are inadequate in sustaining embryos beyond the 4-cell stage since 10 out of 14 homozygous mutants encompassed four cells or fewer. Several laboratories have found that inositol trisphosphate-induced Ca^{2+} oscillations are associated with developmental events, including egg activation and fertilization [8,27,28]. Han et al. [29] observed that the blas-

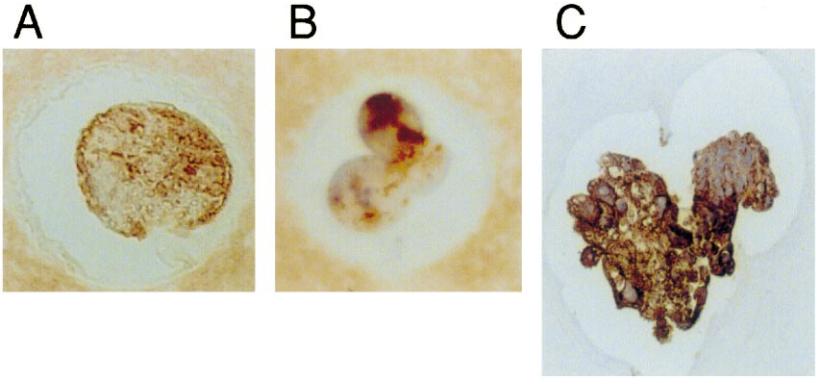

Fig. 3. Immunohistochemical detection of the PLCβ_3 protein in mouse embryos. A: Unfertilized egg. B: 3-cell embryo. C: Egg cylinder.

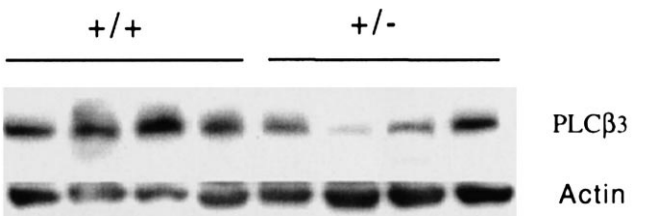

Fig. 4. PLCβ_3 expression in lung tissue of wild type and heterozygous mice analyzed by Western blot. The top panel shows an immunoblot with PLCβ_3 antibody and the lower with monoclonal actin. Lanes 1–4 contain extracts from wild type tissue and lanes 5–8 from heterozygous.

tomere stopped dividing after inhibition of PLC suggesting that intracellular calcium release is mediated through a PLC-dependent pathway. Becchetti and Whitaker reported that lithium, an inhibitor of the phosphoinositide pathway, blocked the cell cycle in sea urchin embryos at the 4-cell stage. The block was reversed by IP_3 indicating that phosphoinositide signaling is involved in the cell cycle of the early embryo [30]. Both the tyrosine kinase and G-protein coupled pathways are believed to be involved in egg activation and preimplantational development [31]. Deficiency studies of PLCβ_1, β_4 and PLCγ_1 in mice have recently been performed [32–34]. Lethality before birth was not seen in PLCβ_1 or PLCβ_4 null mice. Instead, impaired visual processing abilities and ataxia were noted in PLCβ_4 null mice [32,33]. The PLCβ_1 mutant pups developed epilepsy and showed failure to thrive [33]. Homozygous disruption of PLCγ_1 resulted in embryonic lethality at E9 [34].

In conclusion, targeted disruption of PLCβ_3 was performed in mice. Homozygous deletion of the PLCβ_3 gene leads to preimplantational embryonic lethality, hence, PLCβ_3 cannot be compensated by other isozymes or signaling pathways.

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