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DEVELOPMENT AND DISEASE

Deficiency of phospholipase C-γ1 impairs renal development and hematopoiesis

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SUMMARY

Phospholipase C-γ1 (PLC-γ1) is involved in a variety of intracellular signaling via many growth factor receptors and T-cell receptor. To explore the role of PLC-γ1 in vivo, we generated the PLC-γ1-deficient (*plc-γ1*$^{-/-}$) mice, which died of growth retardation at embryonic day 8.5-9.5 in utero. Therefore, we examined *plc-γ1*$^{-/-}$ chimeric mice generated with *plc-γ1*$^{-/-}$ embryonic stem (ES) cells for further study. Pathologically, *plc-γ1*$^{-/-}$ chimeras showed multicystic kidney due to severe renal dysplasia and renal tube dilation. Flow cytometric analysis and glucose phosphate isomerase assay revealed very few hematopoietic cells derived from the *plc-γ1*$^{-/-}$ ES cells in the mutant chimeras. However, differentiation of *plc-γ1*$^{-/-}$ ES cells into erythrocytes and monocytes/macrophages in vitro was observed to a lesser extent compared with control wild-type ES cells. These data suggest that PLC-γ1 plays an essential role in the renal development and hematopoiesis in vivo.

Key words: PLC-γ1, Chimeric mice, Renal dysplasia, Hematopoiesis, In vitro differentiation

INTRODUCTION

Phospholipase C (PLC) hydrolyzes phosphoinositides to generate the second messengers, inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol (DAG). Then, InsP_3 increases intracellular Ca^{2+} levels and DAG activates protein kinase C (PKC). These initial events lead to cell activation, differentiation, proliferation and apoptosis. Ten isoforms of mammalian PLC have been described to date: β1-4, γ1,2 and δ1-4 (Majeras, 1992; Rhee and Choi, 1992). Recently, PLCε has been reported to represent a distinct family of these enzymes (Lopez et al., 2000; Song et al., 2000). Of these isoforms, the γ-type isozymes are very unique in that they contain SH2 and SH3 domains and are activated by a number of receptor tyrosine kinases (RTKs) of growth factors (Ullrich and Schlessinger, 1990) and T-cell receptors (TCR) (Cantrell, 1996). PLC-γ1, one of the γ-type isozymes, is widely expressed in a variety of cell types (Homma et al., 1989) and there are several reports that have investigated the function of PLC-γ1 in vitro. Nevertheless, the significance of PLC-γ1 in the growth factor-initiated responses such as mitogenesis, cell migration and transformation in cell culture system is controversial. For example, it was shown that PLC-γ1 was essential in canine kidney epithelial TRMP cells (Valius and Kazlauskas, 1993) and in NIH3T3 cells (Roche et al., 1996), but dispensable in rat L6 myoblasts (Mohammadi et al., 1992; Peters et al., 1992) and in porcine aortic endothelial cells (Rönnstrand et al., 1992) for the growth factor-induced mitogenic response. In recent work, an antisense approach and gene targeting studies have also produced controversial results about the importance of PLC-γ1 in vitro (Ji et al., 1998; Nebigil, 1997; Xie and Bikle, 1999). Hence, we thought that PLC-γ1-deficient mice would be a very useful tool to investigate the function of PLC-γ1 in vivo. However, these mice died at approximately embryonic day 9 (E9.0) as previously reported (Ji et al., 1997). It is obvious that PLC-γ1 is essential for normal development of mice, although the precise reason(s) of the death of PLC-γ1-deficient mice remain unclear.

Mouse embryonic stem (ES) cells have pluripotential to differentiate into all cell types of primary germ layers

(Doetschman et al., 1985). Chimeric mice constructed with homozygous mutant ES cells enables the examination of the function of targeted genes that would cause embryonic lethality in adult homozygous mutant mice. For example, both platelet-derived growth factor B (PDGFB)- and PDGF receptor β (PDGFRβ)-deficient mice exhibited hematological disorders, failure of cardiac and smooth muscle development and defects in kidney glomeruli because of the absence of mesangial cells (Levéen et al., 1994; Soriano, 1994). Some phenotypes of homozygous mutant mice are preserved and represented in the adult *pdgf*$^{-/-}$ and *pdgfrβ*$^{-/-}$ chimeric animals (Crosby et al., 1998; Lindahl et al., 1998).

Recently, the pluripotentiality of ES cells has received a great deal of attention not only from those working in biological sciences but also from those in regenerative medicine. Nakano et al. demonstrated that ES cells could differentiate to erythroid and myeloid lineages by co-culturing with the stromal cell line, OP9 (Nakano et al., 1994). In the present study, we investigated the function of PLC-γ1 in vivo using *plc-γ1*$^{-/-}$ chimeric mice and the potentiality of differentiation of *plc-γ1*$^{-/-}$ ES cells in vitro. These approaches allowed us to assess the developmental effects of the loss of PLC-γ1 protein by exploring the contribution of *plc-γ1*$^{-/-}$ cells in vivo and in vitro.

MATERIALS AND METHODS

Gene targeting of the PLC-γ1 targeting vector

Four independent phage clones corresponding to the *plc-γ1* locus were obtained from a genomic DNA library of 129/Sv mouse (Stratagene). The *Xba*I-*Hin*dIII fragment (~17 kb) coding the functional domains (X, SH2, SH3 and Y) of *plc-γ1* was deleted and replaced with a *PGK-neo*-polyadenylate [poly(A)] cassette. The targeting vector contains 1.0 kb of homology 5′, and 8.0 kb 3′ of the drug resistant gene and a *PGK-TK*-poly(A) cassette. The linearized targeting vector was transfected into E14 ES cells (100 μF, 300V, 2 pulse). Cells were selected in G418 (300 μg/ml) plus gancyclovir (1 μM) for 7~9 days. Homologous recombination events were screened by PCR and reconfirmed by Southern blot analysis. Two independent *plc-γ1*$^{+/-}$ clones were obtained. Furthermore, three independent *plc-γ1*$^{-/-}$ ES clones were obtained by culturing the *plc-γ1*$^{+/-}$ ES clones at the concentration of 1.8 mg/ml of G418 for 7 days.

The wild-type and *plc-γ1*$^{-/-}$ ES clones were microinjected into the blastocysts from C57/BL6 (B6) mice to generate the control and the *plc-γ1*$^{-/-}$ chimeric mice. The *plc-γ1*$^{+/-}$ ES clones were used to generate the PLC-γ1 homozygous null mutant mice.

Western blot analysis of PLC-γ1 and -γ2

The *plc-γ1*$^{+/+}$, *plc-γ1*$^{+/-}$ and *plc-γ1*$^{-/-}$ ES cells were lysed in lysis buffer [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 50 mM NaF, 2 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF]. Each cell lysate (50 μg/lane) was fractionated on 7.5% SDS-PAGE, transferred to an immobilone™ membrane (Millipore) and blotted with a rabbit polyclonal antibody generated against the SH2-SH2-SH3 region of the rat PLC-γ1 protein (Santa Cruz) or against a synthetic peptide corresponding to residues 1213-1232 of the human PLC-γ2 protein (Santa Cruz). Specific proteins were visualized with an ICL western blotting kit (Amersham) according to the instructions of the manufacturer.

Histopathological analysis

For histopathological analysis, after anesthesia by perioneal infusion of Nembutal, mice were perfused with physiological saline followed by 4% paraformaldehyde (PFA) in PBS. And all tissues were dissected rapidly, placed in the same fixative overnight at 4°C, dehydrated and embedded in paraffin wax. The tissues were then sectioned (6 μm thickness) and used in histological examination and in situ hybridization. The detection of the Y-chromosome DNA was carried out according to the method of Grounds et al. (Grounds et al., 1991). Briefly, the specific probe for Y-chromosome of BALB/c mice was labeled with digoxigenin-deoxy UTP using PCR labeling (forward primer; 5′-GGACAACTGGAGTGGGAT-GGTGCT-3′, reverse primer; 5′-GACACCAGAGTATAGACATAGATA-3′). Tissue paraffin sections were deparaffinized, fixed with 4% PFA/PBS, and treated with proteinase K (400 μg/ml). After prehybridization, tissue sections were hybridized with hybridization buffer (45% formamide, 5× SSC, 10% dextran sulfate, 100 μg/ml salmon sperm DNA) containing 1 ng/l of digoxigenin-labeled specific probe at 42°C overnight, and washed with 2×, 1× or 0.1× SSC at 37°C for 15 minutes. The signal was detected using the digoxigenin signal detection kit (Roche Diagnostics) in accordance with the industrial instruction manual.

Tissue contribution of the *plc-γ1*$^{-/-}$ ES cells in *plc-γ1*$^{-/-}$ chimeric mice

Detection of glucose phosphate isomerase (GPI) isozymes was performed as described previously (Bradley, 1987). Red blood cells were separated by density centrifugation on fresh blood (diluted 1:4 with 0.38% (w/v) trisodium citrate in PBS) on a lymphocyte separation medium (WAKO) according to standard procedures. The isolated tissue samples were quickly frozen, thawed into equal volumes of distilled water and homogenized with a micropestle. These homogenates were frozen in liquid nitrogen and then lysed by three rounds of freezing and thawing. After centrifugation, the supernatants were diluted 10- to 50-fold with distilled water and applied on Titan III Zip Zone cellulose acetate membranes (Helena Laboratories). Samples were electrophoresed in a Zip Zone chamber (Helena Laboratories) for 1.5 hours at 180 V at 4°C. To 1.0% agarose containing 15 mg fructose 6-phosphate (Sigma), 2 mg nicotinamide adenine dinucleotide phosphate (NADP; Sigma), 0.36 mg phenazine methasulfate (PMS; SIGMA), 2 mg methylthiazolium tetrazolium (MTT; SIGMA) at 55°C, 10 Units of glucose 6-phosphate dehydrogenase (SIGMA) were added. The overlay was mixed and poured over the cellulose acetate membranes. The GPI isozyme bands appeared in darkness in a few minutes. Relative levels of the GPI isozymes were quantified by densitometry software (PDI).

Flow cytometric analysis of thymocytes, lymph node cells, splenocytes and bone marrow cells

Thymocytes, lymph node (LN) cells, splenocytes and bone marrow (BM) cells (1×10^6 cells/each) were prepared from the control and *plc-γ1*$^{-/-}$ chimeric mice and were stained with anti-Ly9.1-fluorescein isothiocyanate (FITC) monoclonal antibody (PharMingen). The cells isolated from B6 mice were used as a negative control. Dead cells were excluded by staining with propidium iodide (Roche Diagnostics). Flow cytomeric analysis was carried out using a FACScan computer and software (Becton Dickinson).

In vitro differentiation of the *plc-γ1*$^{+/+}$, *plc-γ1*$^{+/-}$ and *plc-γ1*$^{-/-}$ ES cells

In vitro differentiation of the *plc-γ1*$^{+/+}$, *plc-γ1*$^{+/-}$ and *plc-γ1*$^{-/-}$ ES cells was performed as described (Nakano et al., 1994). 1×10^4 cells of each clone were cultured on OP9 cells with or without erythropoietin (EPO). After 4 days, cells were trypsinized and 1×10^5 cells were replated. Then the number of primitive erythrocytes (Ery^P) was counted on the seventh day in culture. After 10 days, adherent cells were replated and the number of definitive erythrocytes (Ery^D) was counted on the 13th day in culture.

1×10^5 cells of each clones were cultured on OP9 cells. After 5 days, the cells were trypsinized; 1×10^5 cells were replated and cultured with

or without macrophage colony-stimulating factor (M-CSF). The number of adherent cells was counted on the 10th day in culture.

The Ery^P, Ery^D and monocyte/macrophage colonies were identified by using specific expression markers; Ter-119 for Ery^P and Ery^D, and Mac-1 for monocyte/ macrophage, respecively.

Statistical analysis

All results were analyzed by paired or unpaired Student's *t*-test. *P* values lower than 0.05 were considered statistically significant.

RESULTS

Gene targeting of the PLC-γ1 gene in mouse ES cells

The PLC-γ1 gene was disrupted by deleting the gene segment encoding the functional domain as described in Materials and Methods (Fig. 1A,B). The *plc-γ1*$^{+/-}$ ES clones were used to generate germline knockout mice and *plc-γ1*$^{-/-}$ ES clones. Consistent with a previous report (Ji et al., 1997), the germline PLC-γ1 homozygous mutant mice were embryonic lethal and died at E8.5-E9.5 (data not shown). Although PLC-γ1 is clearly indispensable for normal mouse development, the precise cause(s) of embryonic death remains unclear. To further investigate the role of PLC-γ1 in vivo, we generated *plc-γ1*$^{-/-}$ ES cells from the *plc-γ1*$^{+/-}$ ES cells by culturing with high doses of G418 as described in Materials and Methods. The homozygous mutant ES clones were confirmed by Southern blotting (Fig. 1B). The PLC-γ1 protein was not detected by western blotting (Fig. 1C). In addition, lack of PLC-γ1 protein did not affect the expression level of PLC-γ2 protein. The *plc-γ1*$^{-/-}$ ES cells looked morphologically normal and grew as well as wild-type ES cells. Two independent *plc-γ1*$^{-/-}$ ES clones and the wild-type ES clones were then injected into B6 blastocysts to create *plc-γ1*$^{-/-}$ and control chimeric mice, respectively.

Histopathological analysis of the *plc-γ1*$^{-/-}$ chimeric mice

The contribution of the ES cells to chimeric mice (chimerism) can be roughly estimated from the extent of agouti coat color. We analyzed the age-matched (6-8 weeks of age) control and *plc-γ1*$^{-/-}$ chimeric mice with agouti coat color ranging from 30 to 70%. We could not obtain *plc-γ1*$^{-/-}$ chimeric mice showing over 70% agouti coat color, suggesting that extensive chimerism is lethal, as observed in the case of *gata1*$^{-/-}$ chimeric mice (Pevney et al., 1991).

There was no obvious growth retardation in *plc-γ1*$^{-/-}$ chimeric mice compared with control chimeric ones (*plc-γ1*$^{+/+}$). Surprisingly, most of the *plc-γ1*$^{-/-}$ chimeras showed clinical signs of abdominal distention and some of these became moribund by three months postpartum. As shown in Fig. 2A, macroscopic examination of the *plc-γ1*$^{-/-}$ chimeras revealed marked enlargement of both kidneys with multicystic

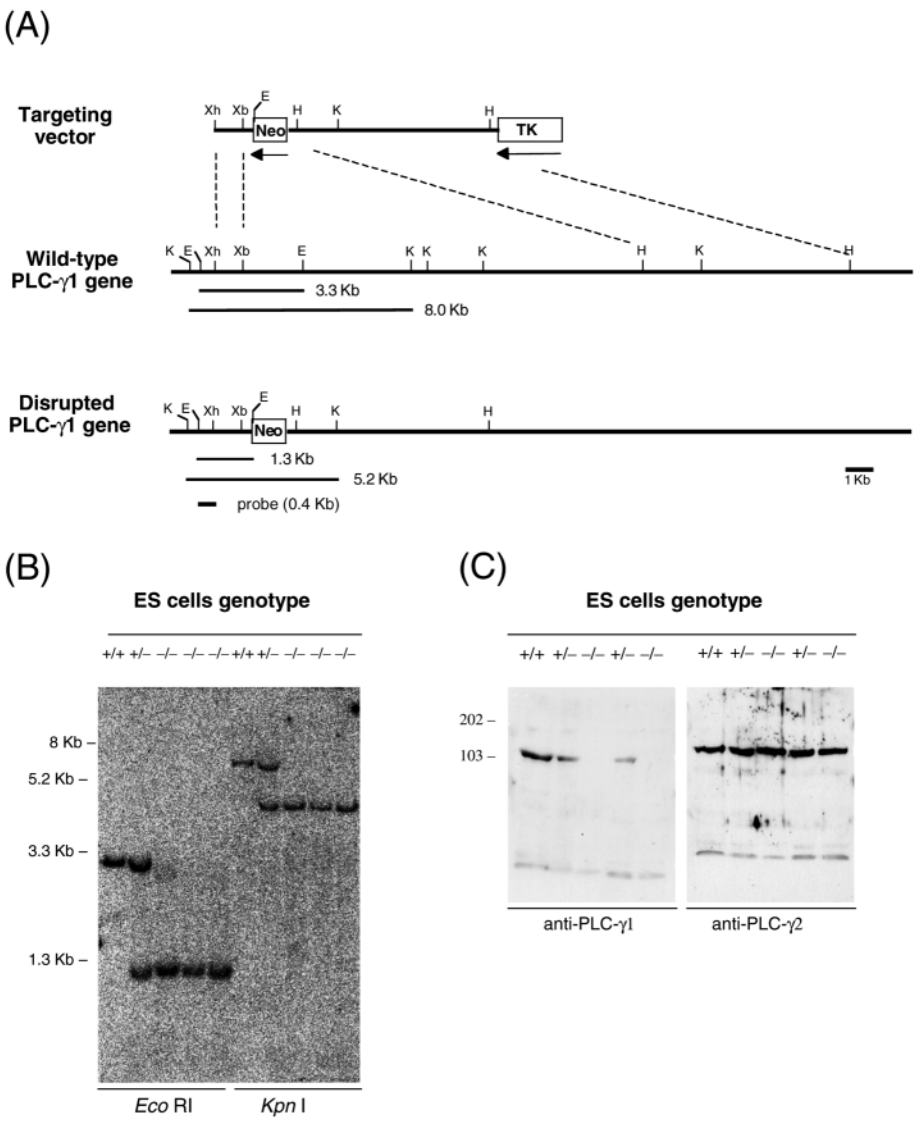

Fig. 1. Disruption of the PLC-γ1 gene in mouse ES cells. (A) Genomic structure coding the functional domains of the mouse PLC-γ1 protein, the structure of the targeting vector and predicted structure of the targeted *plc-γ1* locus. The location of the hybridization probe, 0.4 kb *Xho*I-*Eco*RI fragment, and the expected sizes of the *Eco*RI and the *Kpn*I fragments that hybridize with the probe are indicated. E, *Eco*RI; Xh, *Xho*I; Xb, *Xba*I; H, *Hin*dIII; K, *Kpn*I. (B) Southern blot analysis of the ES cell clones. The *plc-γ1*$^{-/-}$ ES cells were selected by culturing the *plc-γ1*$^{+/-}$ ES cells in the increased dose of G418 as described in Materials and Methods. Genomic DNA isolated from wild-type (+/+), *plc-γ1*$^{+/-}$ (+/–) and *plc-γ1*$^{-/-}$ (–/–) ES cells were digested with *Eco*RI (left panel) or *Kpn* I (right panel) and hybridized with the probe shown in (A). The expected sizes of the wild-type and the mutated fragments are 3.3 kb and 1.3 kb, respectively, when digested with *Eco*RI, 8.0 kb and 5.2 kb, respectively, with *Kpn*I. (C) Expression of the PLC-γ1 (left) and PLC-γ2 (right) proteins in the wild-type (+/+), *plc-γ1*$^{+/-}$ (+/–) and *plc-γ1*$^{-/-}$ (–/–) ES clones.

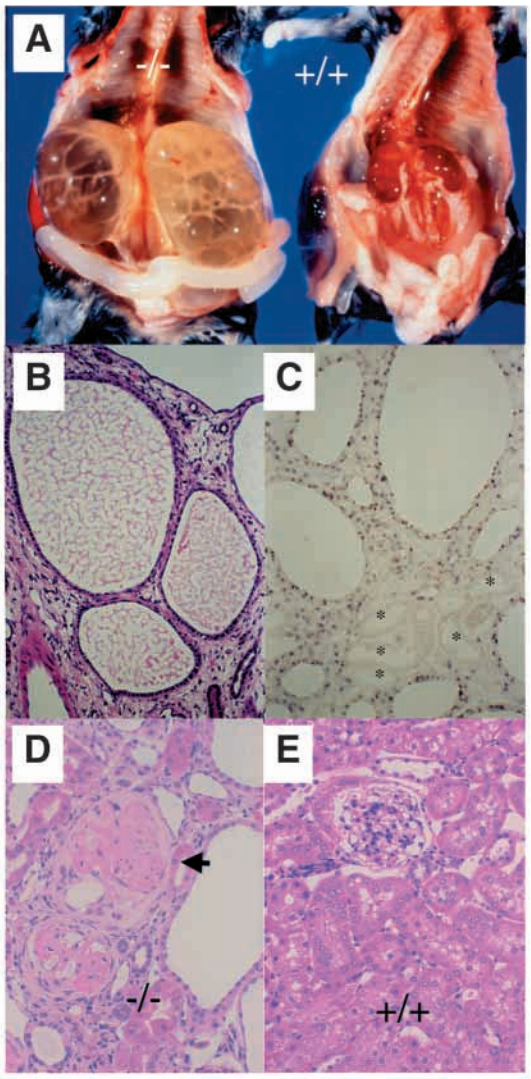

Fig. 2. Abnormal appearance of the kidneys of *plc-γ1*$^{-/-}$ chimeric mouse. (A) Abdominal cavities of the control (right) and *plc-γ1*$^{-/-}$ (left) chimeras. (B) Histopathology of the kidney from *plc-γ1*$^{-/-}$ chimeric mice. The specimens of the kidney from *plc-γ1*$^{-/-}$ chimeric mice were stained with Haematoxylin and Eosin. (C) In situ hybridization of the kidney with a Y-chromosome-specific probe. Dark spots show digoxigenin-positive *plc-γ1*$^{-/-}$ cells. *Normal looking tubules were negative for digoxigenin signals. (D,E) Representative kidney glomeruli in the *plc-γ1*$^{-/-}$ and control chimeras. The arrow indicates a sclerotic glomeruli.

appearance. There was no gross abnormality in other organs or tissues including the ureter. Histopathological examinations demonstrated renal cell dysplasia and dilation of tubules in the kidneys of *plc-γ1*$^{-/-}$ (Fig. 2B). In order to investigate the relationship between the histopathological changes of the kidney and the deletion of PLC-γ1 gene in the chimeric mice, in situ hybridization assay with a Y-chromosome-specific probe was performed in the *plc-γ1*$^{-/-}$ chimeric females. Since a parental ES cell carries a Y chromosome whereas a B6 blastocyst has XX or XY, the probe can specifically detect the somatic cells derived from ES cells in female chimeric mice (Ground et al., 1991). The intense signal of the Y-chromosome-specific probe was recognized in the epithelial cells of the

Table 1. Relationship between the percentage chimerism and renal dysplasia

% Chimerism		
Coat color	GPI assay*	Renal dysplasia†
70	63	Severe
70	37	Moderate
60	56	Severe
50	41	Severe
50	30	Moderate
40	46	Severe
40	38	Moderate
40	41	Moderate
40	26	None
30	44	Moderate
30	30	None

*Each value was extracted from the data of Fig. 3.
†Renal dysplasia was judged by the extent of cyst formation: severe, many cyst in the whole kidney; moderate, some cyst partially in the kidney; none, no obvious abnormality.

dilated tubules and the dysplastic renal cells of the *plc-γ1*$^{-/-}$ kidney (Fig. 2C). As summarized in Table 1, the higher the percentage of chimerism the more severe was the phenotype in the kidneys of mice, suggesting that lack of PLC-γ1 directly influenced renal dysplasia. In contrast to the PDGFB- and PDGFRβ-deficient mice (Levéen et al., 1994; Soriano, 1994), we could not recognize any defects in glomeruli in the *plc-γ1*$^{-/-}$ kidney, although some chimeras with severe renal dysplasia showed glomerular sclerosis (Fig. 2D,E), probably a secondary effect of renal dysfunction. Renal cell dysplasia occurred around 2 weeks of age and dilation of tubules was recognized later, suggesting that the primary abnormality of the *plc-γ1*$^{-/-}$ kidney is renal cell dysplasia.

Tissue contribution of *plc-γ1*$^{-/-}$ ES cells in *plc-γ1*$^{-/-}$ chimeric mice

PLC-γ1 is widely expressed in a variety of tissues (Homma et al., 1989). We investigated the contribution of *plc-γ1*$^{-/-}$ ES cells to various tissues, including lymphoid organs, by analyzing the GPI isozymes. The 129/Sv mouse is homozygous for the allele of *gpi-1s*^{a} (encoding GPI-AA), while B6 is homozygous for *gpi-1s*^{b} (encoding GPI-BB) (Lyon and Searle, 1990). These isozymes can be separated on a membrane as described in Materials and Methods (Bradley, 1987). Fig. 3A shows the representative pattern of electrophoresis of GPI isozymes. The GPI-AA isozyme could not be detected in the thymi, spleens, BM or red blood cells (RBC) of the *plc-γ1*$^{-/-}$ chimeric mice. We summarized the relative contribution of the ES-derived cells in the control and *plc-γ1*$^{-/-}$ chimeric mice in Fig. 3B. The *plc-γ1*$^{-/-}$ ES-derived cells contributed to most of the adult tissues examined except for the lymphoid and hematopoietic organs in the *plc-γ1*$^{-/-}$ chimeric mice to a similar extent with wild-type ES cells as in the control chimeric mice. These data suggest that PLC-γ1 is essential for development of hematopoietic stem cells. Interestingly, we observed here similar amounts of *plc-γ1*$^{-/-}$ cells as wild-type cells in the kidney of the mutant chimeras, in spite of severe dysplasia.

To clarify further the effect of loss of function of PLC-γ1 in lymphocytes, we performed flow cytomeric analyses of thymocytes, LN cells, splenocytes and BM cells of control and *plc-γ1*$^{-/-}$ chimeric mice (Fig. 4). The lymphocytes derived from

129/Sv ES cells express Ly-9.1 on their membrane surface, while the lymphocytes from B6 blastocysts do not (Nakayama et al., 1993). Thus, the lymphocytes originating from ES cells can be identified by staining with anti-Ly-9.1 monoclonal antibody. The mean proportions of the Ly-9.1$^+$ cells in the thymi, the LN, the spleens and the BM of the control chimeric mice were 8.1, 36.4, 41.2 and 23.9%, respectively. In contrast, consistent with the results of the GPI isozyme assay, very few Ly-9.1$^+$ cells in these lymphoid organs of the *plc-γ1*$^{-/-}$ chimeric mice were detected (Fig. 4; Table 2). The total number of cells was, however, comparable between control and mutant chimeric mice.

In addition, we observed that hematopoiesis in PLC-γ1 embryos at E9.5 were extremely reduced in the macroscopic analysis (Fig. 5). Impaired hematopoiesis may, at least in part, be the cause of death in the PLC-γ1 homozygous mutant mice.

Table 2. Percentage of Ly9.1-positive cells and total number of thymocytes, lymph node (LN) cells, splenocytes and bone marrow (BM) cells in control (*n*=8) and *plc-γ1*$^{-/-}$ (*n*=11) chimeric mice

	Ly9.1-positive cells (%)		Total cell numbers ($\times 10^7$)	
	Control	*plc-γ1*$^{-/-}$	Control	*plc-γ1*$^{-/-}$
Thymocytes	7.7±2.5	0.2±0.1*	11.8±1.3	16.3±2.3
LN cells	36.4±6.9	1.6±0.7**	2.1±0.5	1.7±0.3
Splenocytes	41.2±5.9	1.3±0.2**	13.3±2.7	13.3±1.9
BM cells	23.9±5.8	1.6±0.2**	2.1±0.5	2.3±0.5

Mean chimerism judged from coat color was 57.5±5.3% for control and 45.5±4.6% for *plc-γ1*$^{-/-}$ chimeric mice, respectively. Data represents mean ± s.e.m. *P<0.05, **P<0.01 versus control mice (Student's *t*-test).

In vitro differentiation of the *plc-γ1*$^{+/+}$, *plc-γ1*$^{+/-}$ and *plc-γ1*$^{-/-}$ ES cells into hematopoietic cells

The differentiation of *plc-γ1*$^{-/-}$ ES cells into hematopoietic cells might be suppressed by competition with wild-type host cells in the chimeric mice. Therefore, we investigated whether the *plc-γ1*$^{-/-}$ ES cells had the potential to differentiate into hematopoietic cells in vitro. We cultured the *plc-γ1*$^{+/+}$, *plc-γ1*$^{+/-}$ and *plc-γ1*$^{-/-}$ ES cells on OP9 cells with or without EPO or M-CSF. The production of both primitive and definitive erythrocytes from the *plc-γ1*$^{-/-}$ ES cells significantly decreased as compared to those of the *plc-γ1*$^{+/+}$ and *plc-γ1*$^{+/-}$ ES cells (P<0.01) (Fig. 6A). The number of the adherent cells recovered from the *plc-γ1*$^{-/-}$ ES cells culture was also significantly less than those from the *plc-γ1*$^{+/+}$ and *plc-γ1*$^{+/-}$ ES cells culture (P<0.01) (Fig. 6B). However, differentiation of the *plc-γ1*$^{-/-}$ ES cells into erythrocytes or the monocyte/macrophage lineage was not completely abrogated. We initially measured EPO-stimulated InsP_3 production in the *plc-γ1*$^{+/+}$ (112.6 pmol/mg protein) and *plc-γ1*$^{-/-}$ cells (30.4 pmol/mg protein), and observed that the response in the mutant cells was lower, but not completely attenuated, than that in wild-type cells. Therefore, the generation of phosphoinositides seems to be retained in *plc-γ1*$^{-/-}$ cells.

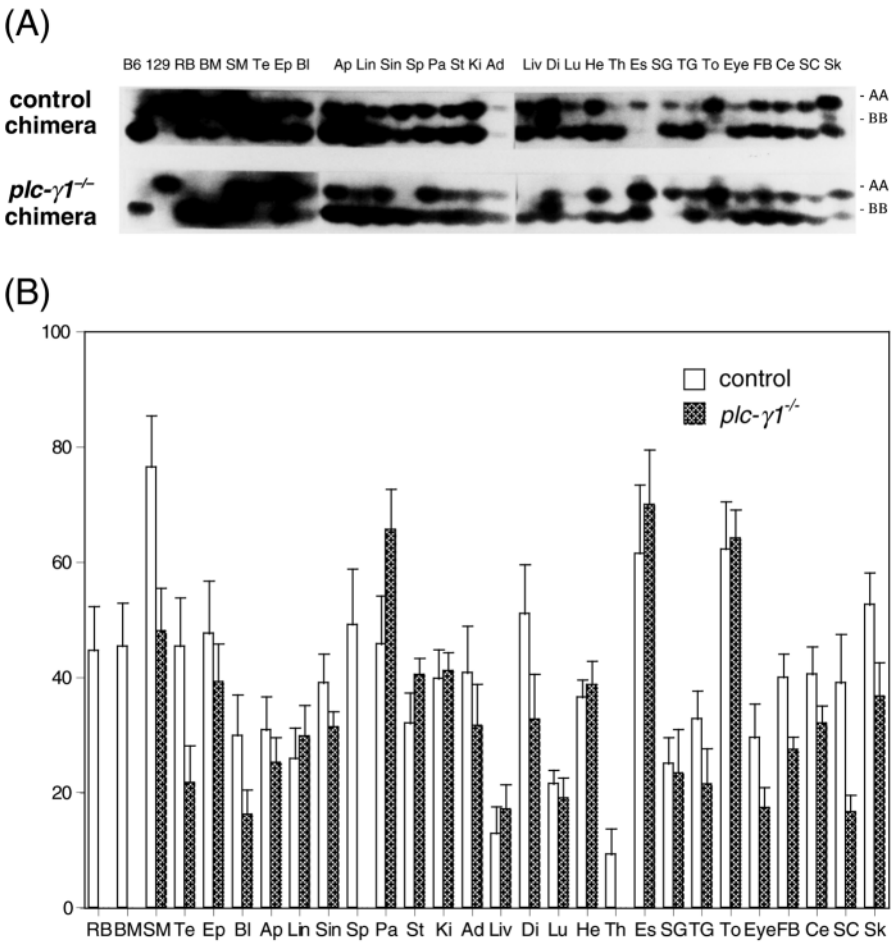

Fig. 3. GPI isozyme analysis. (A) Representative pattern of GPI assay in the control and *plc-γ1*$^{-/-}$ chimeric mice. Lysates from several tissues isolated from the chimeric mice were separated by cellulose acetate electrophoresis and stained for GPI activity as described in Materials and Methods. RB, red blood cells; BM, bone marrow, SM, skeletal muscle; Te, testis; Ep, epididymis; Bl, bladder; Ap, appendix; Lin, large intestine; Sin, small intestine; Sp, spleen; Pa, pancreas; St, stomach; Ki, kidney; Ad, adrenal; Li, liver; Di, diaphragm; Lu, lung; He, heart; Th, thymus; Es, esophagus; SG, submaxillay gland; TG, thyroid gland; To, tongue; Ey, Eye; FB, forebrain; Ce, cerebellum; SC, spinal cord and Sk, skin. (B) Quantification of the tissue distribution of the wild-type (open bars; *n*=8) and *plc-γ1*$^{-/-}$ (filled bars; *n*=12) ES cells. Relative levels of the GPI isozymes were quantified by densitometry software. Each column shows the percentage contribution of wild-type or *plc-γ1*$^{-/-}$ ES cells to the representative tissues isolated from the control and *plc-γ1*$^{-/-}$ chimeric mice and is expressed as the mean±s.e.m.

DISCUSSION

In concurrence with a previous report (Ji et al., 1997), germline *plc-γ1*$^{-/-}$ mice died between E8.5 and E9.5 of gestation and a delay of development was observed (data not shown), and other isoforms of PLCs,

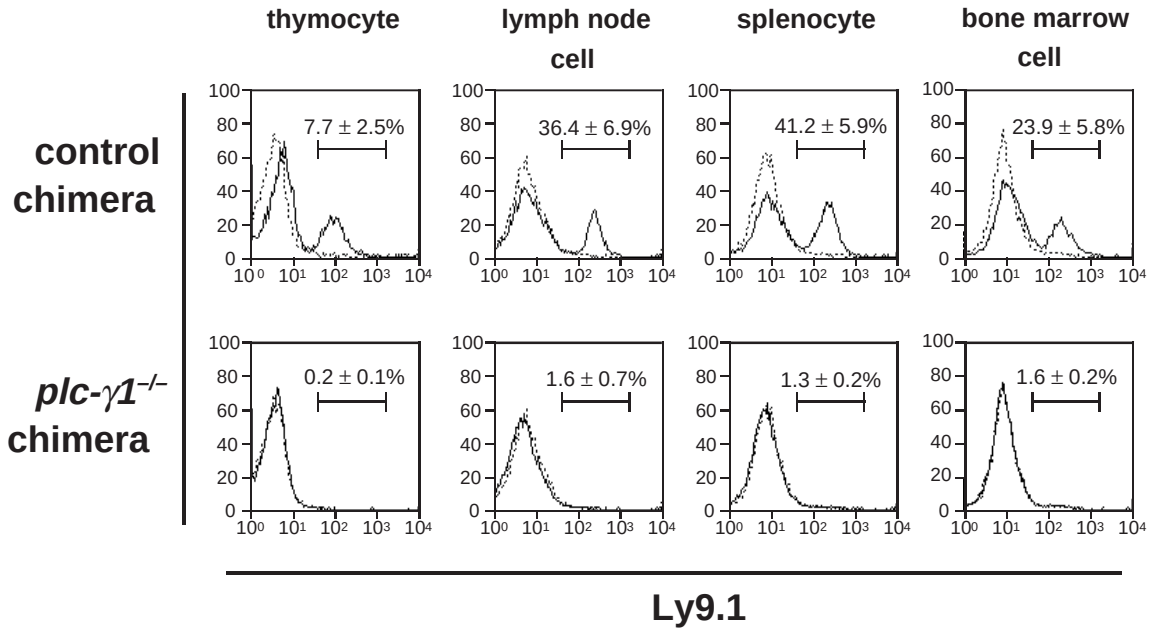

Fig. 4. Flow cytometric analysis of lymphocytes. Thymocytes, lymph node (LN) cells, splenocytes and bone marrow (BM) cells isolated from control (upper panel; *n*=7) and *plc-γ1*$^{-/-}$ chimeric mice (lower panel; *n*=12) were stained for Ly9.1. The dotted lines indicate the staining pattern of the cells isolated from C57/BL6 mice. Each value was expressed as the mean percentages±s.e.m. of the Ly9.1^{+} cells.

especially PLC-γ2, could not compensate for a deficiency of PLC-γ1. For further investigation, we generated and analyzed *plc-γ1*$^{-/-}$ chimeras. The mutant chimeric mice showed severe renal dysplasia and *plc-γ1*$^{-/-}$ cells were detected along the enlarged renal tubules. In addition, from the results of GPI isozyme assay and flow cytometoric analysis (Fig. 3 and Fig. 4), we could observe very few hematopoietic cells derived from *plc-γ1*$^{-/-}$ ES cells in the chimeras. Fewer *plc-γ1*$^{-/-}$ ES cells, however, differentiated into hematopoietic cells than did wild-type cells in vitro. These data suggest that PLC-γ1 plays an important role in renal development and hematopoiesis in mice and that the failure of embryonic blood cell development might cause lethality of PLC-γ1-deficient mice.

Vainio and Müller summarized molecular-based renal organogenesis (Vainio and Müller, 1997). Results from gene targeting in mice showed that some growth factors are required for renal development; glial cell line-derived neurotropic factor/Ret and fibroblast growth factor for initial organogenesis (Moore et al., 1996; Perantoni et al., 1995; Pichel et al., 1996; Sánchez et al., 1996; Schuchardt et al., 1996), PDGFB/PDGFRβ for mesenchymal-to-epithlial differentiation (Levéen et al., 1994; Soriano, 1994) and EGF for maintenance of the tubular epithelial cells (Threadgill et al., 1995). In addition, several lines of studies indicated that renal expression of EGF was reduced in polycystic kidney disease (PKD) in both human (Weinstein et al., 1999) and animal models (Cowley and Rupp, 1995; Gattone et al., 1990; Horikoshi et al., 1991; Nakamura et al., 1993), and that this disease was ameliorated by the exogenous administration of EGF to the PKD mice (Gattone et al., 1995). The expression level of the EGF receptor was 30-fold higher in fetal kidney membranes than in the adult and PLC-γ1 was tyrosine phosphorylated in fetal kidneys but not in the adult (Cybulsky et al., 1994). We observed that renal cell dysplasia occurred around 2 weeks of age and dilation of tubules was apparent later. The dilation was limited to the tubules lined with *plc-γ1*$^{-/-}$ cells (Fig. 2C). Hence, we assumed that the lack of PLC-γ1 aborted EGF signaling, resulting in dilation of tubules in the kidney of *plc-γ1*$^{-/-}$ chimeric mice. It is likely that PLC-γ1 is preferentially involved in the mid to late stage of renal development, probably mediated by EGF. Recently, El-Dahr et al. (El-Dahr et al., 2000) reported that renal structure was destroyed in salt-stressed bradykinin B_2 receptor-deficient mice, which resembled our observation in *plc-γ1*$^{-/-}$ chimeric mice. Since the bradykinin B_2 receptor stimulates PLC-γ1 (Venema et al., 1998), the abnormal signaling via this receptor may be also partly involved in the renal dysplasia in the *plc-γ1*$^{-/-}$ chimeric mice.

Our observations on the kidneys of *plc-γ1*$^{-/-}$ chimeric mice are reminiscent of human multicystic renal disorder and hydronephrosis, including enlarged organs, dilation of tubules and thin walled spherical cysts (Wen et al., 1999; Winyard et al., 1997). The precise etiology of these malformations is not yet known but there is an evidence for a genetic cause. Genetic

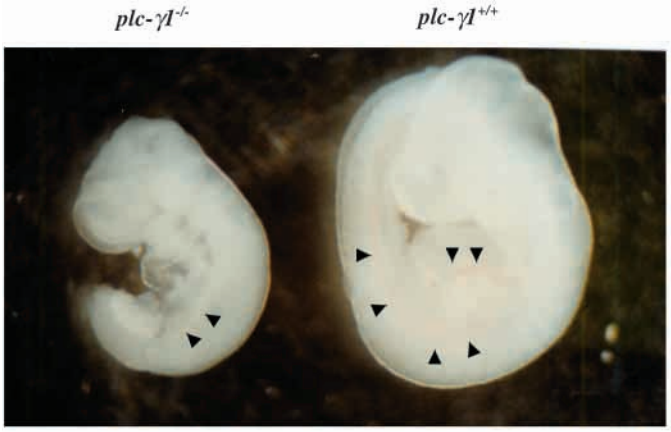

Fig. 5. Hematopoiesis in the PLC-γ1-deficient mice at E9.5.

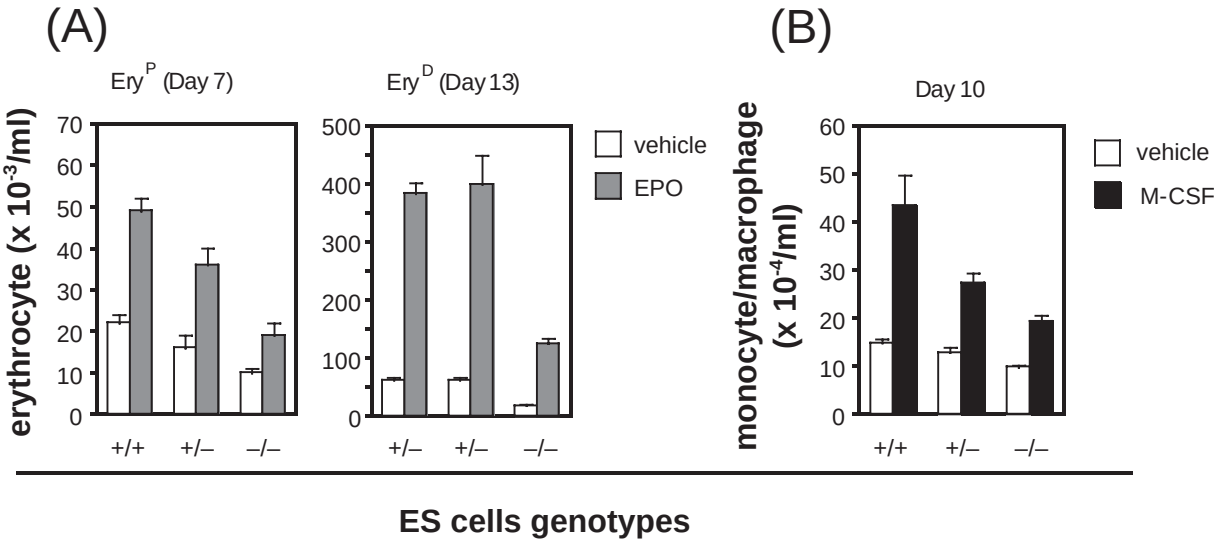

Fig. 6. In vitro differentiation of the wild-type (+/+), *plc-γ1*$^{+/-}$ (+/–) and *plc-γ1*$^{-/-}$ (–/–) ES cells. (A) ES cells differentiated into primitive erythrocytes (Ery^{P}) and definitive erythrocytes (Ery^{D}) with or without erythropoietin (EPO). Ery^{P} production at the 7th day (left) and Ery^{D} production at the 13th day (right) were investigated. (B) ES cells were differentiated to monocytes/macrophage with or without macrophage colony-stimulating factor (M-CSF) by co-culture with the stromal cell line, OP9. Total numbers of adherent cell on the tenthth day were counted. Data represent mean±s.e.m. from six independent experiments.

linkage to a single locus on chromosome 6p has been found for congenital hydronephrosis (Wen et al., 1999) and chromosome 19p for multicystic renal dysplasia (Groenen et al., 1996). However, it does not seem that the lack of PLC-γ1 explains these disorders in human since the human *plc-γ1* gene is located on chromosome 20p (Rothschild et al., 1992).

Various factors simultaneously or systemically participate in hematopoiesis (Morrison-Graham and Takahashi, 1993; Levéen et al., 1994; Shalaby et al., 1995; Soriano, 1994). GATA2-deficient mice die of severe anemia between E9.5 and E11.5 of gestation and the differentiation of *gata2*$^{-/-}$ ES cells to RBC in vitro was considerably reduced as compared to that of wild-type ES cells (Tsai et al., 1994). Likewise, hematopoietic cells derived from *gata2*$^{-/-}$ ES cells were not observed in the *gata2*$^{-/-}$ chimera. Thus, this discrepancy between chimeras and cell culture may be due to competition in vivo between wild-type and mutant cells. In our study, *plc-γ1*$^{-/-}$ cells responded to EPO and M-CSF. Since the stimulation of EPO and M-CSF receptors recruited and activated both PLC-γ1 (Ren et al., 1994) and PLC-γ2 (Boudot et al., 1999; Bourette et al., 1997), the latter might compensate for the former; PLC-γ1-deficient erythrocytes and monocytes/macrophages were recovered in vitro. However, PLC-γ2-deficient mice, unlike PLC-γ1-deficient mice, were viable and the responses of BM hematopoietic progenitor cells to EPO and CSFs were not affected even in the absence of PLC-γ2 (Wang et al., 2000). Thus, it is likely that PLC-γ2 was not sufficient to compensate for the lack of PLC-γ1 in the hematopoiesis in vivo.

It remains unclear why developmental abnormalities were observed only in the kidney and hematopoietic cells of *plc-γ1*$^{-/-}$ chimeric mice. It may be possible that many more growth factors are simultaneously involved in renal development and hematopoiesis than in other organs, or that PLC-γ1 is essential for the growth factor-dependent mitogenesis in the hematopoietic and renal cells but dispensable for others. A deficiency of PLC-γ1 impaired hematopoiesis in the chimeras and this may, at least in part, be the cause of death in the PLC-γ1 homozygous mutant mice.

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