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# Role of Phospholipase C-L2, a Novel Phospholipase C-Like Protein That Lacks Lipase Activity, in B-Cell Receptor Signaling

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**Phospholipase C (PLC) plays important roles in phosphoinositide turnover by regulating the calcium-protein kinase C signaling pathway. PLC-L2 is a novel PLC-like protein which lacks PLC activity, although it is very homologous with PLC δ. PLC-L2 is expressed in hematopoietic cells, but its physiological roles and intracellular functions in the immune system have not yet been clarified. To elucidate the physiological function of PLC-L2, we generated mice which had a genetic *PLC-L2* deficiency. PLC-L2-deficient mice grew with no apparent abnormalities. However, mature B cells from PLC-L2-deficient mice were hyperproliferative in response to B-cell receptor (BCR) cross-linking, although B2 cell development appeared to be normal. Molecular biological analysis revealed that calcium influx and NFATc accumulation in nuclei were increased in PLC-L2-deficient B cells. Extracellular signal-regulated kinase activity was also enhanced in PLC-L2-deficient B cells. These mice had a stronger T-cell-independent antigen response. These results indicate that PLC-L2 is a novel negative regulator of BCR signaling and immune responses.**

Phospholipase C (PLC) is responsible for phosphoinositide turnover through the hydrolysis of phosphatidylinositol 4,5-bisphosphate. The two products of this process, inositol 1,4,5-trisphosphate and diacylglycerol, trigger transient calcium mobilization and protein kinase C activation, respectively, resulting in events such as cell proliferation, differentiation, adhesion, and secretion. To date, 12 isoforms have been cloned, and they are classified into five isotypes (β, γ, δ, ε, and ζ), depending on the sequence homology and the mechanism of activation (12, 22, 23, 24).

PLC-L2, as well as its homologue, PLC-L1 (13) (also called p130 [10]), is a PLC-like protein that has the sequence that is most similar to PLC-δ (17). Interestingly, however, this protein has no catalytic activity due to replacement of the conserved histidine residue necessary for hydrolytic activity with threonine (17). PLC-L2 was first shown to be abundant in skeletal muscle by Northern blot analysis (17). However, further studies have revealed that this molecule is also expressed in hematopoietic cells, including B cells and T cells. In B cells and T cells, PLC-γ2 and PLC-γ1 play important roles downstream of the B-cell receptor (BCR) and T-cell receptor (TCR) (14), respectively. BCR and TCR signals are tightly regulated in normal lymphocytes, and recent gene targeting studies have shown that irregular signals often result in impaired lymphocyte development or enhanced lymphocyte activation, sometimes leading to immune deficiency or autoimmune disease (16, 25, 27). The unique features of PLC-L2, i.e., the loss of PLC activity and its expression in lymphocytes, led us to speculate that it may also act downstream of the above-mentioned receptors, probably in a negative regulatory fashion. To assess the role of PLC-L2 in the immune system, we generated and analyzed mice which had a targeted deletion of the *PLC-L2* gene.

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## MATERIALS AND METHODS

**Generation of PLC-L2-deficient mice.** Genomic clones containing exons (II) and (III) of mouse PLC-L2 were isolated from a 129 genomic library. Exon (II) (corresponding to amino acids 87 to 941) was replaced by a neomycin phosphotransferase cassette. The targeting construct was introduced into E14 embryonic stem (ES) cells by electroporation, and chimeric mice were generated from PLC-L2$^{+/-}$ ES cells. Germ line transmission was confirmed by Southern blot analysis. Genotyping of mice was also done by PCR analysis using primers as follows: for the wild-type mice, 5′-AAG AAT AGA TGC TCC CCG AAG C-3′; for the mutant mice, 5′-CCT GTG CTC TAG CTT TAG G-3′; and for both, 5′-GAA GGA ATC TGT ACT CGG CTA G-3′.

**Flow cytometric analysis.** Spleen, thymus, bone marrow, and peritoneal cells from 10- to 13-week-old mice were stained with direct fluorescence-conjugated antibodies (all from PharMingen).

**Measurement of antibody level.** Serum immunoglobulin M (IgM) concentrations were determined by enzyme-linked immunosorbent assay (ELISA). To determine T-independent (T-I) type II response, mice were injected with trinitrophenyl-conjugated Ficoll (TNP Ficoll) (Biosearch Technologies). TNP-specific antibody production was analyzed with TNP-bovine serum albumin (BSA) (a gift from S. Nakae) as the capture reagent and horseradish peroxidase-conjugated subclass-specific immunoglobulins (Southern Biotechnology Associates). For measurement of the T-I response, the serum dilution factors were as follows: 1:2,700 for IgM, IgG1, and IgG3 and 1:900 for IgG2b. The titers of anti-nuclear antigen IgM from aged mice were determined with a mouse anti-nuclear antibody ELISA kit (Alpha Diagnostic).

**Proliferation assay.** Mature B cells (CD23$^{+}$/CD21$^{int}$) were collected with FACSVantage (Becton Dickinson). These B cells ($10^5$/well) in 10% fetal bovine serum–RPMI 1640 medium containing 50 μM 2-mercaptoethanol and antibiotics

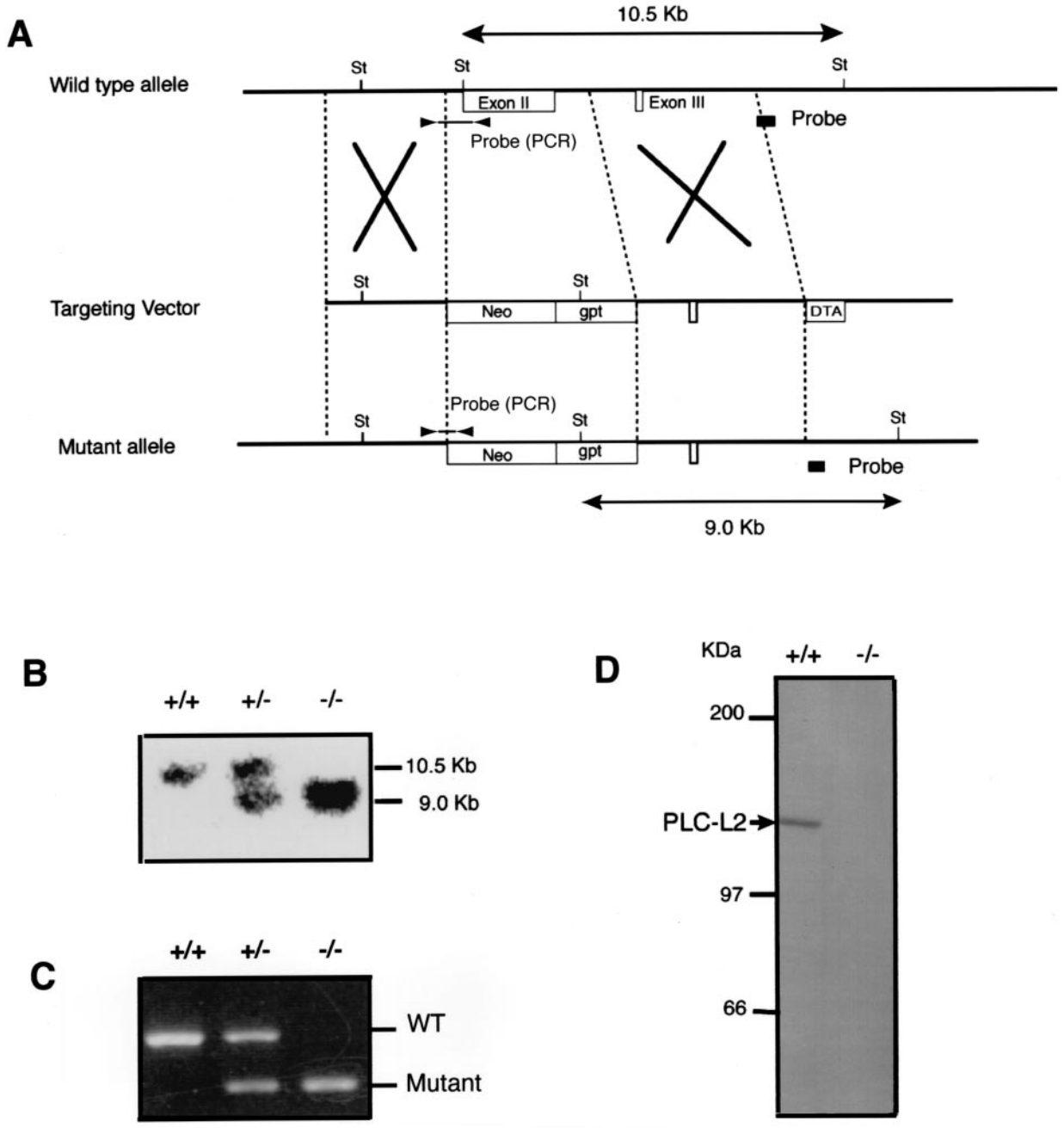
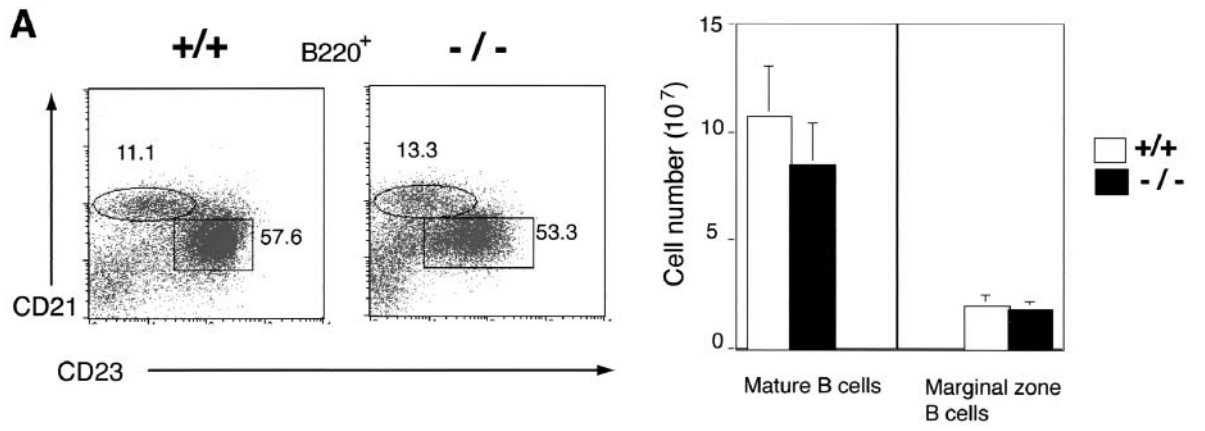
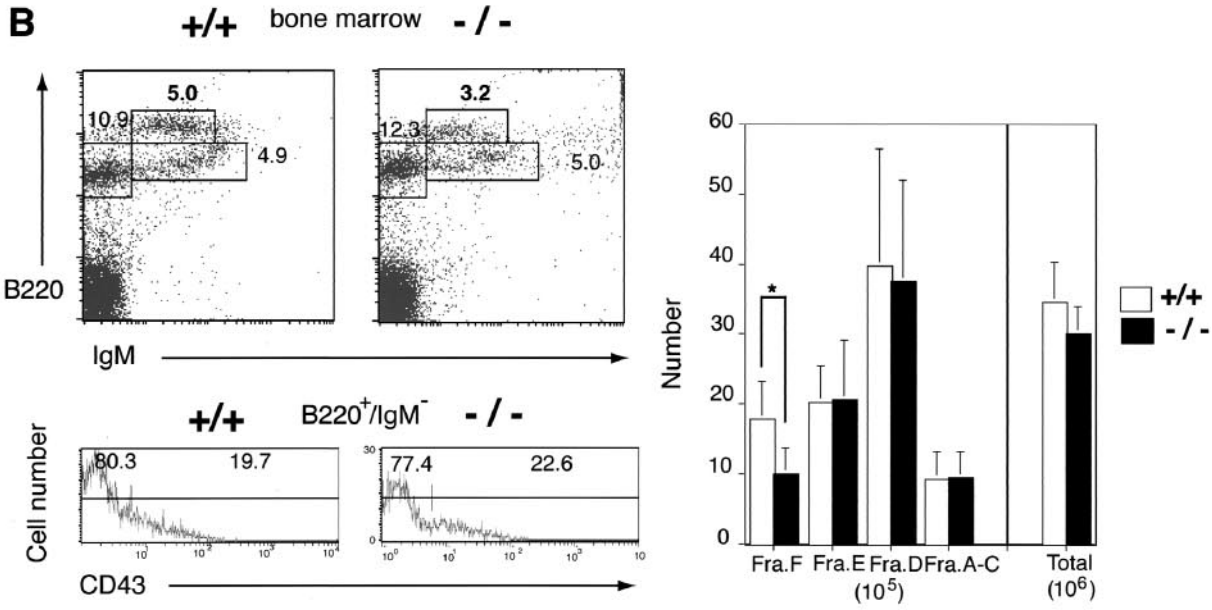



FIG. 1. Targeted disruption of the *PLC-L2* gene in mice. (A) Gene targeting strategy. Exon (II) was replaced with the *neo* gene. St, *Stu*I. (B) Southern blot analysis of mouse genomic DNA using C-terminal probes. *Stu*I digestion yielded a 10.5-kb fragment for the wild-type allele and a 9.0-kb fragment for the mutant allele. (C) PCR analysis of genomic DNA using N-terminal probes. (D) Western blotting of protein extracts from spleens of wild-type and PLC-L2-deficient mice.

FIG. 2. Flow cytometric analysis of B-cell subpopulations in PLC-L2-deficient mice. (A) Splenocytes were stained with B220, CD21, and CD23. The oval regions show the marginal-zone B cells, and the rectangular regions indicate the mature B cells. Note that the rectangular region of mature B cells of PLC-L2-deficient mice is defined differently from that of wild-type mature B cells, because the expression level of CD23 is reduced in PLC-L2-deficient mature B cells. The numbers of mature B cells and marginal-zone B cells are shown on the graph. For both wild-type and PLC-L2-deficient mice, $n = 7$. (B) Bone marrows cells were stained with B220, IgM, and CD43. The classification of bone marrow cells into the fractions A through F was done according to the methods described previously by Hardy et al. (6). For wild-type and PLC-L2-deficient mice, $n = 6$. *, $P < 0.05$. (C) The peritoneal cells were stained with CD5, Mac-1, and IgM. The number of B-1b cells was obtained by subtracting the number of B-1a cells from the total number of B-1 ($Mac^+$ and $IgM^+$) cells. For wild-type mice, $n = 5$; for PLC-L2-deficient mice, $n = 4$. **, $P < 0.01$.


A
+/+
B220+
- / -
11.1
57.6
13.3
53.3
CD21
CD23
Cell number (10^7)
15
10
5
0
+/+
- / -
Mature B cells
Marginal zone B cells


B
+/+
bone marrow
- / -
5.0
10.9
4.9
3.2
12.3
5.0
B220
IgM
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B220+/IgM-
- / -
Cell number
80.3
19.7
77.4
22.6
CD43
Number
60
50
40
30
20
10
0
*
Fra.F
Fra.E
Fra.D
Fra.A-C
Total
(10^5)
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+/+
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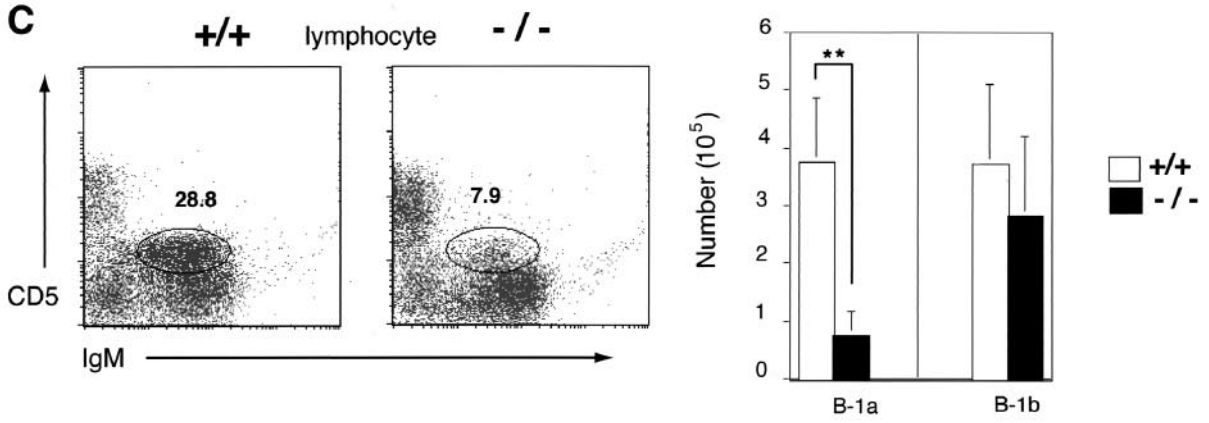
C
+/+
lymphocyte
- / -
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7.9
CD5
IgM
Number (10^5)
6
5
4
3
2
1
0
**
B-1a
B-1b
+/+
- / -

were stimulated with goat anti-mouse anti-IgM F(ab′)$_2$ (Jackson Immuno Research), anti-CD40 (PharMingen), interleukin 4 (PharMingen), lipopolysaccharide and ionomycin (Sigma), and phorbol myristyl acetate and cultured for 48 h in 96-well plates. During the last 12 h of culture, cells were pulse-labeled with [$^3$H]thymidine (0.5 μCi/well), and [$^3$H]thymidine uptake was measured with a Matrix 96 scintillation counter (Packard). For the T-cell proliferative assay, purified splenic T cells were stimulated with anti-CD3ε, anti-CD28 (PharMingen), concanavalin A, ionomycin, and phorbol myristyl acetate and cultured for 48 h and pulse-labeled with [$^3$H]thymidine (0.5 μCi/well) for 8 h. Data are the average of three independent experiments with triplicate culture.

**Measurement of intracellular calcium concentrations.** Purified CD23$^+$ B cells (MACS magnetic cell sorting system; Miltenyi Biotec) or thymocytes ($10^7$/ml) were loaded with fura-2/AM (Dojindo) in Hanks' balanced salt solution (Invitrogen) supplemented with 1 mM $CaCl_2$, 10 mM HEPES–NaOH (pH 7.2), and 0.025% BSA for 20 min at 37°C. Cells were washed twice and suspended in this buffer. For measurement of extracellular calcium influx, CD23$^+$ B cells ($10^7$/ml) were loaded with 3 μM fura-2/AM in Hanks' balanced salt solution supplemented with 10 mM HEPES–NaOH (pH 7.2) and 0.025% BSA for 20 min at 37°C. Cells were washed twice and suspended in the calcium-free medium (125 mM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES–NaOH [pH 7.2]). Just before the preincubation for stimulation, EGTA was added to the solution at a final concentration of 1 mM. The calcium concentration was monitored with a CAF-110 spectrofluorometer (Nippon Bunko). Data are representative of at least three independent experiments.

**Determination of NFATc1 level.** Purified B220$^+$ cells ($10^7$/ml) were suspended in RPMI 1640 medium and not stimulated (time zero) or stimulated for 6 h in the presence of 10 μg of anti-IgM F(ab′)$_2$ per ml with or without 20 ng of cyclosporine A (CSA; Sigma) per ml. The cytoplasmic and nuclear fractions were isolated as described previously (4). Western blotting was performed with anti-NFATc1 (7A6; Santa Cruz) or antiactin (Chemicon) antibody. A dephosphorylated or phosphorylated form of NFATc1 was evaluated as previously described (8).

**ERK phosphorylation assay.** Purified splenic B220$^+$ cells ($5 \times 10^7$/ml) were stimulated with 15 μg of anti-IgM F(ab′)$_2$ per ml, and the reactions were stopped by adding cold phosphate-buffered saline containing 1 mM $Na_3VO_4$. Cells were lysed with cell lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 50 mM HEPES [pH 7.4], 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$). Western blotting was done with anti-extracellular signal-regulated kinase (ERK) polyclonal antibody (Cell Signaling) and anti-phospho-ERK antibody (E4; Santa Cruz). Relative intensities of phosphorylated ERK2 were measured with NIH image software (version 1.62).

**Gel shift assay.** Splenic B220$^+$ cells ($5 \times 10^6$/ml) in 2% fetal bovine serum–RPMI medium were stimulated with 25 μg of anti-IgM F(ab′)$_2$ per ml for 4 h. Nuclear fractions were extracted as described previously (20). Nuclear extracts (~2 μg) were incubated with $^{32}$P-labeled oligonucleotide DNA probe (~$1.0 \times 10^6$ cpm) in a reaction solution containing 20 mM HEPES (pH 7.9), 50 mM KCl, 5% glycerol, 1 mM EDTA (pH 8.0), 10 mM dithiothreitol, 0.5 mg of BSA per ml, and 0.1 mg of dI-dC per ml for 30 min at room temperature and resolved in 5% polyacrylamide gel. The oligonucleotide DNAs used in this assay were as follows: AP-1 sense, 5′-GCC CCT CTG ACT CAT GCT-3′; AP-1 antisense, 5′-GTC AGC ATG AGT CAG AGG-3′; mutation AP-1 sense, 5′-GCC CCT CTG CAG CAT GCT-3′; and mutation antisense, 5′-GTC AGC ATG CTG CAG AGG-3′ (for the first two, underlining indicates the AP-1 binding sites, and for the last two, underlining indicates the mutation bases).

**Statistics.** Student's *t* test was used for the statistical analysis.

## RESULTS

**Generation of PLC-L2-deficient mice.** PLC-L2 has a pleckstrin homology domain, an X-Y domain, and a C2 domain (17). Exon (II), which encodes the pleckstrin homology, X-Y, and C2 domains, was replaced with a neomycin phosphotransferase cassette (Fig. 1A). Targeted homologous recombinant and germ line transmission were verified by Southern blot analysis using C-terminal probes (Fig. 1B) and by PCR analysis using N-terminal probes (Fig. 1C). Western blot analysis of spleens with an antibody specific for PLC-L2 confirmed that none of this protein was expressed in mutant mice (Fig. 1D). PLC-L2-deficient mice were born in approximately the expected Mendelian ratio and were fertile. They also grew with no apparent abnormalities. Because PLC-L2 was expressed in the immune organs, we investigated its role in the immune system.

**Flow cytometric analysis of immune organs of PLC-L2-deficient mice.** Flow cytometric analysis was performed on cells from the thymus, spleen, bone marrow, and peritoneal cavity. In the spleen, IgD-IgM staining appeared to be normal (data not shown), suggesting that splenic B-cell development was not significantly affected in PLC-L2-deficient mice. We further analyzed the PLC-L2-deficient splenic B-cell subpopulations by CD21-CD23 staining. The absolute numbers of marginal-zone B cells (CD21$^{hi}$-CD23$^{low\sim neg}$) and mature B cells (CD21$^{int}$-CD23$^+$) of PLC-L2-deficient mice were not different from those of wild-type mice (Fig. 2A), though we observed the reduced expression levels of CD23 on PLC-L2-deficient mature B cells (Fig. 2A).

In the bone marrow, we observed a slight, but significant, reduction of mature recirculating B cells (fraction F), while early development of B cells (fractions A through C, pro-B cells; fraction D, pre-B cells; fraction E, immature B cells) was not affected (Fig. 2B). This result, taken together with the results in the spleen, indicates that PLC-L2 is not important for B2 cell development.

In contrast to B2 cells, however, we found a reduction of B-1a cells in the peritoneal cavity of PLC-L2-deficient mice (Fig. 2C). This reduction of peritoneal B-1 cells was restricted to the CD5$^+$-IgM$^+$ B-1a population, while the population of Mac1$^+$-CD5$^-$-IgM$^+$ B-1b cells (11) was approximately the same in PLC-L2-deficient mice and wild-type mice (Fig. 2C). These data suggested that, unlike B2 cells, PLC-L2 might be involved in B-1a cell development or in maintenance.

In contrast to B-cell lineage, we did not find any significant differences of T-cell surface markers between wild-type and PLC-L2-deficient mice. CD4 and CD8 FACS profiles of thymus and spleen were not different between two genotypes (Fig. 3A). The expression levels of various maturation or activation markers such as CD5, CD69, CD3ε, TCRβ, and CD24 on single- or double-positive thymocytes from PLC-L2-deficient mice and wild-type mice were not different (data not shown). In addition, thymocytes from PLC-L2-deficient mice showed normal calcium mobilization after TCR stimulation (Fig. 3B). Splenic T cells from PLC-L2-deficient mice also had comparable proliferative responses in response to several stimuli, including anti-CD3 (Fig. 3C). These results strongly suggested that loss of PLC-L2 did not affect the T-cell development and function. Western blot analysis showed that PLC-L1, a homologue of PLC-L2, was expressed in thymus and T cells, but it was only a slightly detected in B cells (Fig. 3D). In contrast, PLC-L2 was highly expressed in B cells, just as was true in thymus and T cells (Fig. 3D), suggesting the possibility that the major phenotype of PLC-L2 deficiency might be found in B-cell lineages but not in T-cell lineages. Thus, we investigated in detail the function of B cells in PLC-L2-deficient mice.

**PLC-L2-deficient B cells have increased proliferative responses after BCR cross-linking.** It is known that different B-cell subpopulations have different ligand sensitivities. To minimize subpopulation difference, we used fluorescence-activated cell-sorted CD21$^{int}$-CD23$^+$ mature B cells (Fig. 4A, left) instead of whole B220$^+$ cells for the proliferation assay. Those

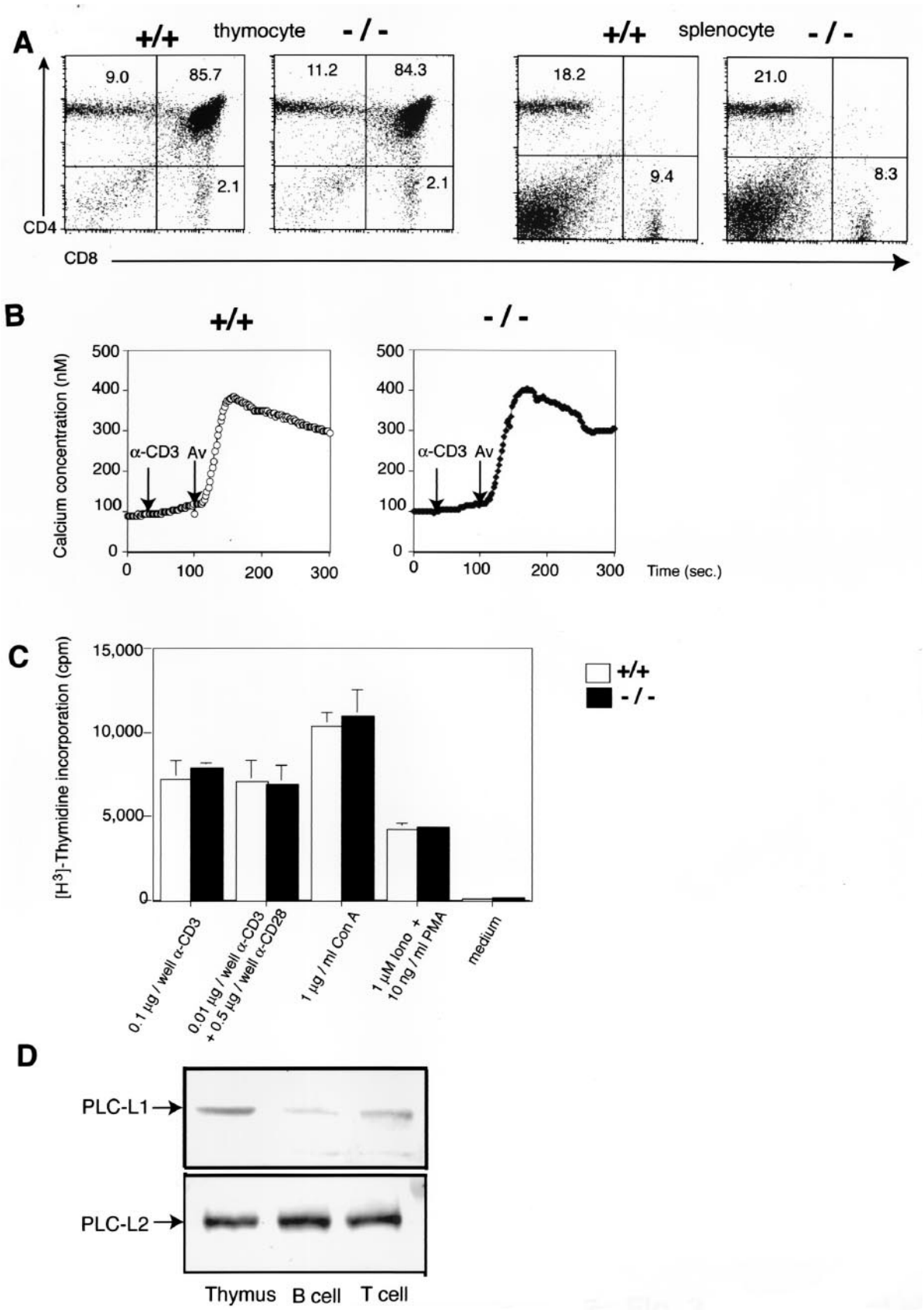
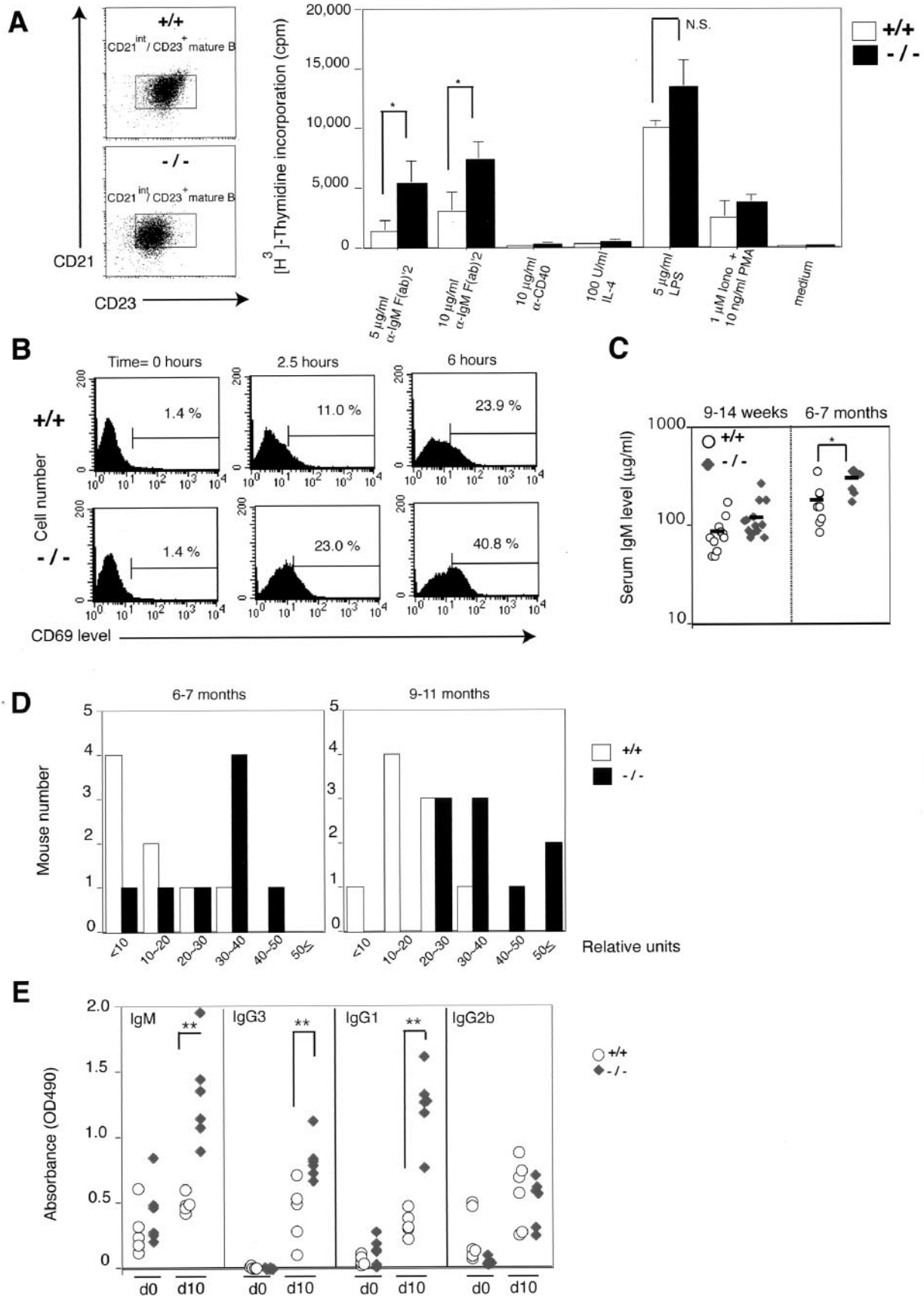



FIG. 3. Normal development and in vitro responsiveness in PLC-L2-deficient T cells. (A) Thymocytes or splenocytes were stained with CD4 and CD8. (B) Calcium mobilization in response to TCR stimulation. Thymocytes were stimulated with 5 μg of biotinylated CD3 per ml by cross-linking with 5 μg of avidin (Av) per ml. (C) Splenic T cells were cultured in the presence of indicated stimuli, and the proliferative ability was evaluated by thymidine uptake. (D) The expression levels of PLC-L1 and PLC-L2 in thymus, B cells, and T cells were examined by Western blot analysis (18 μg of protein/lane).


A
+/+
CD21int / CD23+ mature B
- / -
CD21int / CD23+ mature B
CD21
CD23
[H3]-Thymidine incorporation (cpm)
20,000
15,000
10,000
5,000
0
N.S.
+/+
- / -
5 μg/ml α-IgM F(ab')2
10 μg/ml α-IgM F(ab')2
10 μg/ml α-CD40
100 U/ml IL-4
5 μg/ml LPS
1 μM Iono + 10 ng/ml PMA
medium
B
Time= 0 hours
2.5 hours
6 hours
+/+
1.4 %
11.0 %
23.9 %
- / -
1.4 %
23.0 %
40.8 %
Cell number
CD69 level
C
9-14 weeks
6-7 months
+/+
- / -
Serum IgM level (μg/ml)
1000
100
10
D
6-7 months
9-11 months
Mouse number
+/+
- / -
<10
10~20
20~30
30~40
40~50
50≤
Relative units
E
IgM
IgG3
IgG1
IgG2b
Absorbance (OD490)
2.0
1.5
1.0
0.5
0
d0
d10
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- / -

purified PLC-L2-deficient mature B cells showed a significantly increased proliferative response to anti-IgM $F(ab')_2$ stimulation (Fig. 4A right) compared with wild type cells. We further evaluated the sensitivity of activation of PLC-L2-deficient B cells in response to BCR cross-linking. CD69 is an early lymphocyte activation marker, the expression of which is induced by stimulation (28). The expression levels of CD69 after BCR stimulation were more rapidly increased on PLC-L2-deficient B cells than on wild-type cells, indicating that up-regulation of CD69 was more rapidly induced in PLC-L2-deficient B cells (Fig. 4B). These results also showed that PLC-L2-deficient B cells exhibit a hyperreactive phenotype downstream of BCR.

**Loss of PLC-L2 results in hyper-B-cell response in vivo.** Despite the reduced proportion of B-1a cells (Fig. 2C), PLC-L2-deficient mice had a normal range of serum IgM levels (Fig. 4C). Furthermore, when aged mice (6 to 7 months old) were assessed, PLC-L2-deficient mice had a slight, but significant, increase of serum IgM (Fig. 4C). Those aged PLC-L2-deficient mice had also higher titers of anti-nuclear antigen IgM than did wild-type mice (Fig. 4D). This tendency was also observed when we further assessed mice that were 9 to 11 months old (Fig. 4D). When mice were immunized with T-I II antigens (TNP Ficoll), we observed a significant increase of anti-TNP IgM, IgG1, and IgG3 in PLC-L2-deficient mice compared with wild-type mice (Fig. 4E). These enhanced T-I immune responses were consistent with the hyperreactivity of B cells from PLC-L2-deficient mice in vitro. In contrast to T-I responses, when immunized with T-cell-dependent antigen, PLC-L2-deficient mice showed nearly normal immune responses (not shown). These data show that PLC-L2-deficient mice have an enhanced T-I immune response, indicating that loss of PLC-L2 results in a hyperreactive phenotype of B cells in vivo as well as in vitro.

**Enhanced calcium signaling in PLC-L2-deficient B cells.** To explore the cell biological events leading to hyperreactive phenotypes in PLC-L2-deficient B cells, we investigated the effects of PLC-L2 deficiency on the B-cell signal transduction pathway. First, we examined calcium signaling, which is an important signal downstream of BCR (2).

In the presence of extracellular calcium, a remarkable increase of intracellular calcium levels was observed in PLC-L2-deficient mature B cells (Fig. 5A). Calcium mobilization is initially induced by release from the endoplasmic reticulum (ER), followed by the influx of extracellular calcium, which is mediated by store-operated calcium entry. In the absence of extracellular calcium, calcium mobilization induced by BCR cross-linking was small in both wild-type and PLC-L2-deficient mature B cells, suggesting that PLC-L2 does not affect intracellular calcium mobilization from the ER (Fig. 5B). However, when calcium was added to the medium, greater calcium influx was observed in PLC-L2-deficient mature B cells than in wild-type cells (Fig. 5B). Enhanced calcium influx was also observed in PLC-L2-deficient cells when cells were treated with thapsigargin, an inhibitor of sarcoplasmic and ER calcium-ATPases that depletes intracellular calcium stores and activates store-operated calcium channels (SOCs) (Fig. 5C). These results indicated that enhancement of calcium mobilization in PLC-L2-deficient mature B cells was due to the increased influx of extracellular calcium.

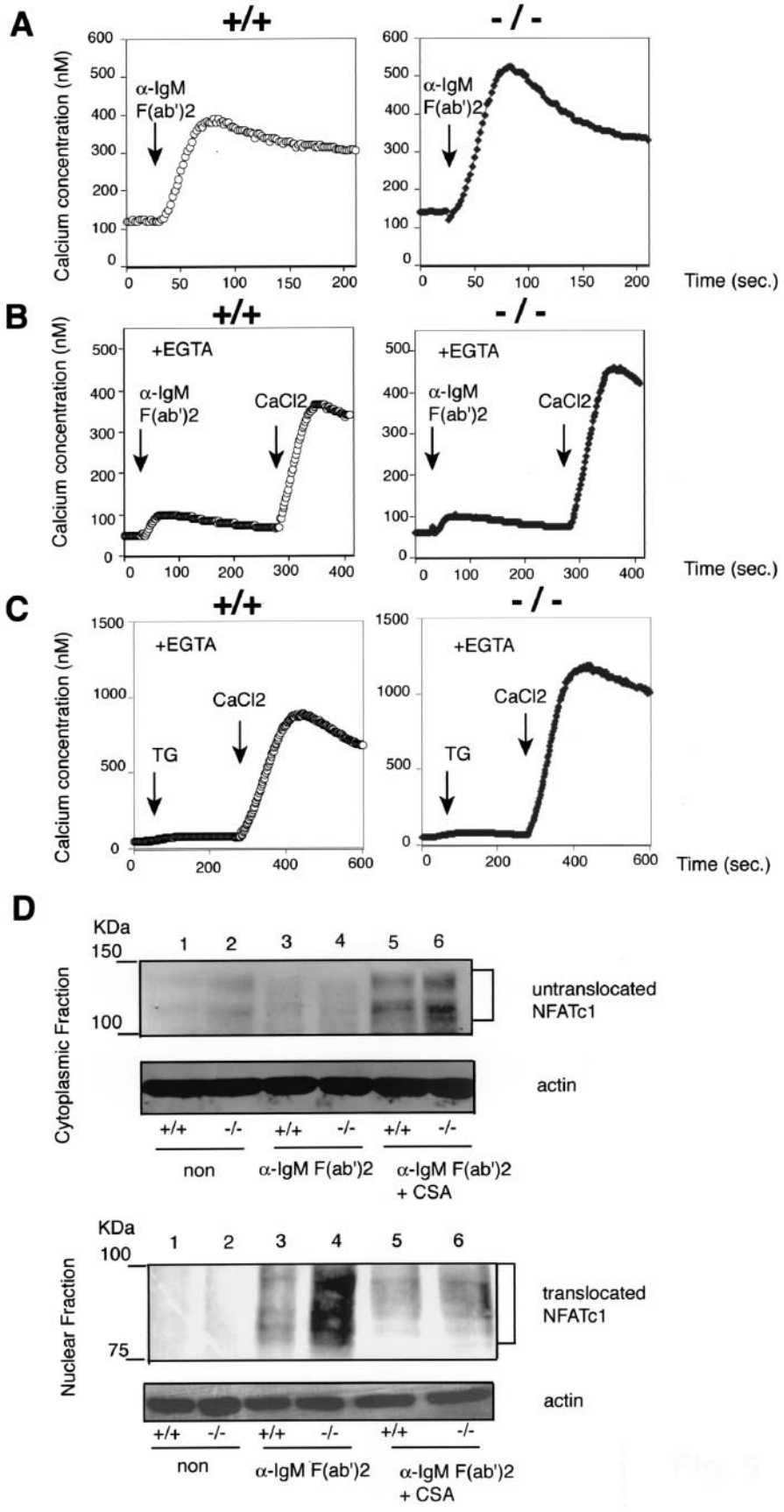
Translocation of NFAT into nuclei is regulated by a calcium-dependent protein phosphatase, calcineurin. Recent data have suggested that NFATc1 regulates normal homeostasis and differentiation of B cells (19). Western blot analysis showed that the dephosphorylated forms (molecular mass, 80 to 100 kDa) of nuclear NFATc1 were more prominent in the PLC-L2-deficient B cells than in wild-type cells (Fig. 5D, lower panel, lanes 3 and 4) after BCR stimulation, indicating that the active form of NFATc1 was accumulated in the nuclei of PLC-L2-deficient B cells. In the presence of CSA, an inhibitor of calcineurin, NFATc1 was present mainly in the cytoplasmic fractions of both cells (Fig. 5D, upper panel, lanes 5 and 6), indicating that enhanced nuclear NFATc accumulation in PLC-L2-deficient B cells was CSA sensitive (i.e., calcium dependent). These results show that calcium signaling is increased in PLC-L2-deficient B cells.

**Enhanced MAPK pathway in PLC-L2-deficient B cells.** The increased proliferative ability following BCR cross-linking of mature PLC-L2-deficient B cells (Fig. 4A) suggested that loss of PLC-L2 also affected the mitogen-activated protein kinase (MAPK) pathway, which is involved in cell growth (9). The results depicted in Fig. 6A show that anti-IgM $F(ab')_2$ stimulation triggered the ERK2 phosphorylation, and this phosphorylation was enhanced in PLC-L2-deficient B cells. ERK activation causes the induction of the transcription factor, activating protein 1 (AP-1) (26). To examine the downstream pathway of ERK signaling, we investigated the AP-1 activity of BCR-stimulated PLC-L2-deficient B cells. The results of the gel shift assay depicted in Fig. 6B showed that AP-1 activity after BCR cross-linking was increased in PLC-L2-deficient B cells, indicating that ERK signaling is also enhanced in PLC-L2-deficient B cells. These results, taken together with the data of calcium signaling, strongly suggested that PLC-L2 inhibits the B-cell signal transduction following the BCR cross-linking.

## DISCUSSION

**Physiological role of PLC-L2 in the immune system.** The loss of PLC-L2 in B cells results in apparently normal development of B2 cells, but they exhibit a hyperreactive phenotype. These results strongly suggest that the physiological role of

FIG. 4. Hyperresponsiveness in PLC-L2-deficient B cells in vitro and in vivo. (A) Purified splenic mature B cells (panel A, left) were cultured with the indicated reagents, and their proliferative ability was evaluated by thymidine uptake. *, $P < 0.05$. N.S., not significant. (B) $B220^+$ cells were stimulated with 5 μg of anti-IgM $F(ab')_2$ per ml for the indicated times, and the expression levels of CD69 were examined. (C) Normal serum IgM levels were determined by ELISA. Bars show the average values. *, $P < 0.05$. (D) The titers of anti-nuclear antigen IgM were determined by ELISA. The average titer of anti-nuclear antigen IgM from two 4-month-old control MRL/lpr male mice was defined as 100. (E) Wild-type and PLC-L2-deficient mice that were 9 to 14 weeks old were intraperitoneally immunized with 100 μg of TNP Ficoll, and the anti-TNP titers of each isotype on day 10 were determined by ELISA. **, $P < 0.01$.


A
+/+
- / -
Calcium concentration (nM)
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Time (sec.)
B
+EGTA
CaCl2
C
TG
D
KDa
Cytoplasmic Fraction
untranslocated NFATc1
actin
+/+ -/- +/+ -/- +/+ -/-
non
α-IgM F(ab')2
α-IgM F(ab')2 + CSA
Nuclear Fraction
translocated NFATc1

PLC-L2 is raising the thresholds of B-cell activation to prevent B cells from being excessively activated. In fact, aged PLC-L2-deficient mice had higher anti-nuclear antigen IgM titers. In view of these facts, PLC-L2 may be important for the maintenance of B-cell homeostasis in vivo.

Unlike B2 cells, numbers of peritoneal B-1a cells were significantly reduced in PLC-L2-deficient mice. This result strongly suggested that PLC-L2 is also involved in B-1a cell development or maintenance.

**Hyperreactive phenotype of PLC-L2-deficient B cells in vitro and in vivo.** The increased proliferative responses of PLC-L2-deficient mature B cells after BCR cross-linking strongly suggest that PLC-L2 functions mainly downstream of BCR in a negative regulatory fashion. Consistent with these in vitro results, PLC-L2-deficient splenic B cells showed enhanced immune responses in vivo. When immunized with T-I II antigen, PLC-L2 showed a stronger immune response than did wild-type mice. Recently, splenic marginal-zone B cells have been reported to play an important role in the T-I II immune responses (5). This enhanced T-I II response in PLC-L2-deficient mice is unlikely due to a different proportion of marginal-zone B cells, because the population of marginal-zone B cells in PLC-L2-deficient mice was not altered (Fig. 2A). Thus, the enhanced T-I responses may show that splenic B cells from PLC-L2-deficient mice are more subject to activation in vivo. Thus, PLC-L2-deficient B cells have enhanced responses in vitro and in vivo, indicating that PLC-L2 acts as a negative regulator of B-cell activation mainly downstream of BCR signaling.

**The intracellular function of PLC-L2 in B cells.** What is the intracellular function of PLC-L2 in B cells? PLC-L2 is a catalytically inactive form of PLC-like protein. This feature proposed the possibility that it was involved in PLC-mediated calcium mobilization in a negative regulatory manner. Our results, obtained by using PLC-L2-deficient B cells, showed that calcium influx but not calcium release from the ER was increased in PLC-L2-deficient B cells. This suggests that PLC-L2 can negatively regulate SOC-mediated calcium influx but not inositol 1,4,5-trisphosphate-mediated calcium release from the ER. Though the mechanism by which depletion of calcium stores leads to calcium influx and the precise molecular components of SOCs in B cells have not been well understood yet, some positive and/or negative regulatory mechanisms for SOCs have been reported (1, 7). PLC-L2 might be a novel molecule which down-regulates the activity of SOCs. Recently, it was reported that a lipase-inactive form of PLC-γ could induce calcium influx, and the src homology 3 domain of PLC-γ is required for this process (18, 21). PLC-L2 does not have a src homology 3 domain, but this finding suggests the possibility that PLC-L2 may directly or indirectly associate with channels on the plasma membrane through an unidentified motif and thus regulate the calcium influx.

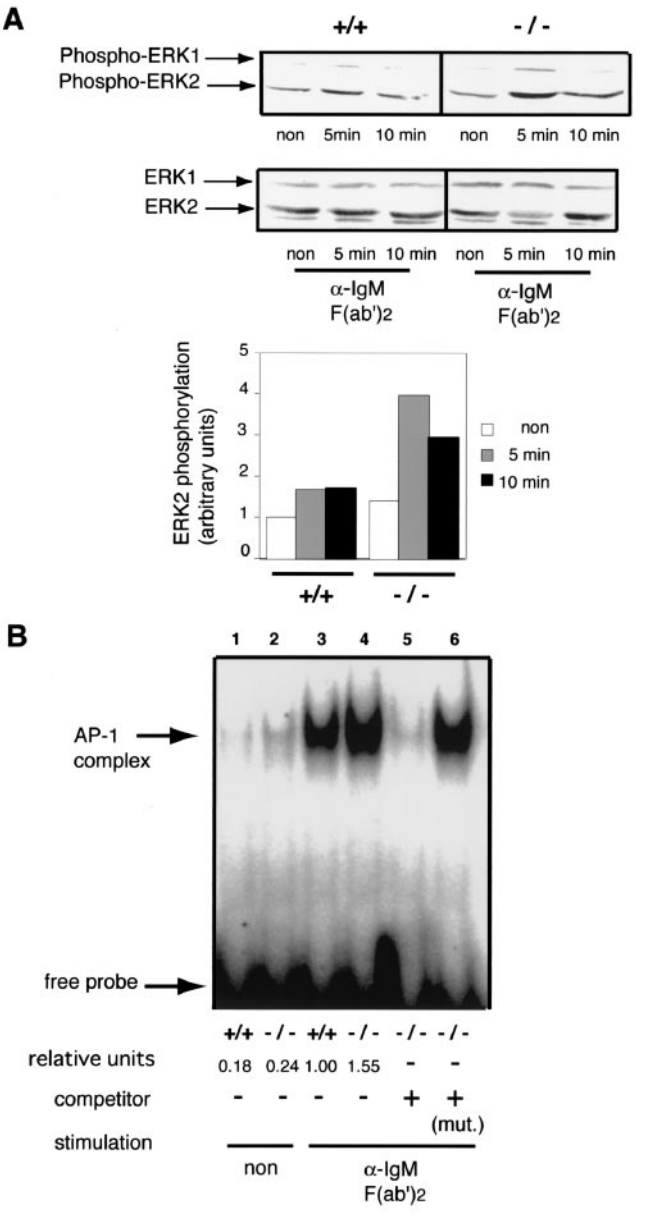


FIG. 6. Enhanced MAPK signaling in PLC-L2-deficient B cells. (A) Enhanced ERK2 phosphorylation of PLC-L2-deficient B cells. Splenic B cells were stimulated with 15 μg of anti-IgM $F(ab')_2$ per ml, and instances of ERK phosphorylation were detected by Western blot analysis. Results of one of the three independents experiments with similar results are shown. The average relative units of ERK2 phosphorylation in these three experiments are shown on the graph. (B) AP-1 activity is increased in PLC-L2-deficient B cells after BCR cross-linking. Splenic B cells were stimulated with 25 μg of anti-IgM $F(ab')_2$ per ml for 4 h, and AP-1 activity was determined by a gel shift assay. Representative data from one of three independent experiments are shown.

FIG. 5. Enhanced calcium signaling in PLC-L2-deficient B cells. (A) Changes in calcium concentrations in mature B cells ($CD23^+$ B cells) upon BCR cross-linking. The arrows indicate the time of stimulation with 10 μg of anti-IgM $F(ab')_2$ per ml. (B) BCR-mediated intracellular calcium mobilization from the ER and calcium influx in mature B cells were measured by treatment with 10 μg of anti-IgM $F(ab')_2$ per ml in the presence of 1 mM EGTA, followed by the addition of 4 mM calcium to the medium. (C) Mature B cells were stimulated with 2 μM thapsigargin (TG) in the presence of 1 mM EGTA, followed by the addition of 4 mM calcium to the medium. (D) Enhanced NFATc1 levels in nuclei of PLC-L2-deficient B cells. NFATc1 levels in cytoplasmic and nuclear cell lysates from splenic B cells were analyzed by Western blot analysis. $B220^+$ cells were either not stimulated or were stimulated in the presence of anti-IgM $F(ab')_2$ without or with cyclosporine A (CSA). Actin levels were examined as a loading control. The data are representative of two independent experiments.

We also observed the enhanced ERK activity in PLC-L2-deficient B cells. Because a calcium-dependent protein tyrosine kinase Pyk2 was reported to activate the ERK pathway downstream of calcium mobilization (3, 15), enhanced ERK activity in PLC-L2-deficient B cells may be due to enhanced calcium mobilization of PLC-L2-deficient B cells.

**T-cell phenotypes in PLC-L2-deficient mice.** Though PLC-L2 is expressed in T cells (Fig. 3D), we did not find significant changes in PLC-L2-deficient T cells. In addition, the in vitro responsiveness of PLC-L2-deficient T cells appeared to be normal (Fig. 3B and C). These results indicated that PLC-L2 is not required for T-cell development or for the regulation of T-cell function, suggesting that another molecule might compensate for PLC-L2 deficiency in T-cell lineages or that the actions of PLC-L2 are restricted to the B-cell lineage. Since a PLC-L2 homologue, PLC-L1, is expressed in the thymus and T cells (Fig. 3D), PLC-L1 might compensate in T-cell lineages of PLC-L2-deficient mice.

Our present study showed the physiological function of a novel catalytically inactive PLC-like protein, PLC-L2, as a negative regulator of BCR signaling and immune responses. Further study is required to clarify the molecular mechanisms through which PLC-L2 functions in B-cell signal transduction pathways.

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