

# Cbl Suppresses B Cell Receptor-mediated Phospholipase C (PLC)- $\gamma$ 2 Activation by Regulating B Cell Linker Protein-PLC- $\gamma$ 2 Binding

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## Abstract

Accumulating evidence indicates that the Cbl protein plays a negative role in immune receptor signaling; however, the mode of Cbl action in B cell receptor (BCR) signaling still remains unclear. DT40 B cells deficient in Cbl showed enhanced BCR-mediated phospholipase C (PLC)- $\gamma$ 2 activation, thereby leading to increased apoptosis. A possible explanation for the involvement of Cbl in PLC- $\gamma$ 2 activation was provided by findings that Cbl interacts via its Src homology 2 (SH2) domain with B cell linker protein (BLNK) after BCR ligation. BLNK is a critical adaptor molecule for PLC- $\gamma$ 2 tyrosine phosphorylation through its binding to the PLC- $\gamma$ 2 SH2 domains. As a consequence of the interaction between Cbl and BLNK, the BCR-induced recruitment of PLC- $\gamma$ 2 to BLNK and the subsequent PLC- $\gamma$ 2 tyrosine phosphorylation were inhibited. Thus, our data suggest that Cbl negatively regulates the PLC- $\gamma$ 2 pathway by inhibiting the association of PLC- $\gamma$ 2 with BLNK.

Key words: adaptor molecule • antigen receptor • lymphocyte • negative regulator • signaling

## Introduction

Cbl is the cellular homologue of the v-Cbl oncoprotein (1), which was originally identified in the murine Cas NS-1 retrovirus (2). v-Cbl induces pre-B cell and myeloid tumors in mice (2), and transforms rodent fibroblasts (3). Recently, Cbl has been identified as a prominent substrate of protein tyrosine kinases (PTKs)<sup>1</sup> that is rapidly phosphorylated after engagement of several different receptors on hematopoietic cells. Cbl has no known catalytic function, but it contains a RING finger domain, an extensive proline-rich region, and a COOH-terminal leucine zipper (for reviews, see references 4–8). Although it has been thought that Cbl contains a novel NH<sub>2</sub>-terminal phosphotyrosine-binding domain (9), recent crystal structure analyses indi-

cate that the Cbl NH<sub>2</sub>-terminal region (Cbl-N) is not similar to a phosphotyrosine-binding domain (10, 11). Instead, this region comprises three domains: an NH<sub>2</sub>-terminal four-helix bundle, a calcium-binding domain, and an unusual Src homology 2 (SH2) domain. Moreover, this study clarifies that G306E mutation in Cbl is located inside its unusual SH2 domain, and that this mutation disrupts binding of Cbl to phosphotyrosine-containing proteins (10).

The functional importance of Cbl in hematopoietic cell signaling was initially suggested by the finding that Cbl binds to several critical signaling molecules, e.g., PTKs such as Src family PTK (12–14), Syk/Zap-70 family PTK (8, 15–18), and Btk (19); adaptor molecules, including Grb2 (13, 20), Crk (21–23), and Nck (24); and effector molecules, including phosphatidylinositol 3-kinase (PI3-K [16, 25]) and Vav (26). The most revealing clue about the function of Cbl came from genetic studies in *Caenorhabditis elegans*, where SLI-1, a *C. elegans* Cbl homologue, negatively regulates signaling downstream of LET-23, a *C. elegans* epidermal growth factor receptor homologue (27, 28). Recent studies have provided evidence that Cbl is involved in regulating PTKs rather than downstream effectors. Indeed, overexpression of Cbl in mast cells suppresses Syk

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<sup>1</sup>Abbreviations used in this paper: BCR, B cell receptor; BLNK, B cell linker protein; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; Cbl-N, NH<sub>2</sub>-terminal region of Cbl; ECL, enhanced chemiluminescence; GST, glutathione S-transferase; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; PI3-K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PTK, protein tyrosine kinase; SH2, Src homology 2.

function upon FcεRI engagement (29). This is presumably because of the interaction of the Cbl SH2 domain with the phosphorylation site in human Syk Tyr-323 (equivalent to Tyr-316 in porcine Syk), since either inactivation of the Cbl SH2 domain (G306E mutation) or Syk-Y323F mutation leads to abrogation of Cbl's negative influence on Syk in a heterologous COS cell system (30). Moreover, this study demonstrates the possibility that Cbl targets the auto-phosphorylated Syk for degradation, leading to downregulation of the expression level of Syk.

Consistent with the negative role of Cbl in Syk function, Cbl-deficient mouse thymocytes exhibited hyperactivation of Zap-70 after CD3 cross-linking (31–33). However, contrary to the expectation from the hyperactivated Zap-70 in the Cbl-deficient thymocytes, neither PLC-γ1 nor PI3-K activities were enhanced in these thymocytes (32). Thus, it is still unclear whether the target molecule of Cbl in anti-gen receptor signaling is solely a PTK such as Syk/Zap-70.

To address this issue, we have used the DT40 B cell system. First, by generating DT40 B cells deficient in Cbl, we have shown that Cbl negatively regulates the B cell receptor (BCR)-induced PLC-γ2 pathway. Given the previous evidence that BCR-mediated PLC-γ2 activation requires its association with BLNK (alternatively named SH2 domain-containing leukocyte protein of 65 kD [SLP-65] or B cell adaptor containing the SH2 domain [BASH]) (34–38), as well as two types of PTKs (Syk and Btk; for reviews, see references 39–42), two models could be put forward to account for this phenotype. In the first model, Cbl acts on PTKs, particularly Syk, which in turn inhibits PLC-γ2 tyrosine phosphorylation. In the second model, Cbl binds to the phosphorylated BLNK through its SH2 domain, possibly to competitively inhibit the association of the PLC-γ2 SH2 domain with BLNK, leading to inhibition of PLC-γ2 phosphorylation. Our findings suggest that Cbl dominantly utilizes the second mechanism in the BCR signaling context.

## Materials and Methods

**Cells and Abs.** Wild-type and various mutant chicken DT40 B cells were maintained in RPMI 1640 supplemented with 10% FCS, 1% chicken serum, 50 μM β-mercaptoethanol, 2 mM l-glutamine, penicillin, and streptomycin. Anti-PLC-γ2 Ab (34), anti-Syk Ab (43), anti-BLNK Ab (34), and anti-chicken IgM mAb M4 (44) were described previously. Anti-Cbl Ab and anti-glutathione S-transferase (GST) mAb were purchased from Santa Cruz Biotechnology. Anti-T7 mAb and antiphosphotyrosine mAb 4G10 were purchased from Novagen and from Upstate Biotechnology, respectively.

**Generation of Cbl-deficient DT40 Cells.** Chicken Cbl cDNA that corresponds to human c-Cbl amino acid residues 77–426 was isolated by reverse transcriptase PCR using RNA from chicken DT40 B cells. Cbl genomic DNA fragments were obtained by screening a chicken genomic library (Clontech Laboratories) using the isolated chicken Cbl cDNA. The targeting vectors pCbl-Bsr, pCbl-HisD, pCbl-Puro, and pCbl-Neo were constructed by replacing the genomic fragment-containing exons that correspond to human c-Cbl amino acids 250–336 with *bsr*, *hisD*, *puro*,

or *neo* cassettes. These cassettes were flanked by 3.4 and 6.6 kb of chicken Cbl genomic sequence on the 5' and 3' sides, respectively. The targeting vector pCbl-Bsr was linearized and introduced into wild-type DT40 cells by electroporation at 550 V, 25 μF. Transfectants were selected in the presence of 50 μg/ml blasticidin S, and resistant clones were screened by Southern blot analysis. pCbl-HisD was transfected into the *bsr*-targeted clone, and was selected with both blasticidin S (50 μg/ml) and histidinol (1 mg/ml). pCbl-Puro was transfected into the *bsr/his*-targeted clone, and was selected with blasticidin S (50 μg/ml), histidinol (0.5 mg/ml), and puromycin (0.5 μg/ml). pCbl-Neo was transfected into the *bsr/his/puro*-targeted clone, and was selected with blasticidin S (50 μg/ml), histidinol (0.5 mg/ml), puromycin (0.5 μg/ml), and G418 (2 mg/ml).

DT40 B cells display a stable karyotype with a modal chromosome number of 80, which comprises 11 autosomal macrochromosomes, the ZW sex chromosomes, and 67 microchromosomes. The karyotype does not show obvious abnormalities, except for a trisomy of chromosome 2 and one additional microchromosome (45). The requirement for four rounds of targeting in generating a null mutant suggested that the DT40 B cell has two alleles with a duplicated *cbl* gene, although the possibility that four copies of a *cbl* gene are integrated in a single chromosome, such as sex chromosomes, could not be excluded.

Although some critical experiments (see Fig. 3) were carried out using another independent Cbl-deficient clone (C8-12), a single Cbl-deficient clone (C8-10) was analyzed extensively and transfected with c-Cbl cDNAs.

**Expression Constructs.** The hemagglutinin-tagged human c-Cbl cDNA (provided by Dr. W.Y. Langdon, University of Western Australia, Nedlands, Australia [1]) was subcloned into the pAzeo expression vector, which was generated by replacing the CMV promoter on pcDNA3.1/Zeo (Invitrogen) with the chicken actin promoter on pApuro (43). Mutation of Gly-306 to Glu, designated G306E, was introduced into the hemagglutinin-tagged human c-Cbl cDNA by PCR-directed mutagenesis. Transfection into Cbl-deficient DT40 cells was done by electroporation at 550 V, 25 μF. Selection was performed in the presence of zeocin (1 mg/ml). T7-tagged Btk cDNA subcloned into both pApuro and pAhygro was transfected into wild-type or Cbl-deficient DT40 cells, respectively. Mutation of porcine Syk Tyr-316 to Phe (Y316F), which corresponds to human Syk Tyr-323, was produced by PCR-directed mutagenesis. Wild-type and Syk-Y316F cDNAs were subcloned into pApuro, and were transfected into Syk-deficient DT40 cells. Expression of each transfectant was confirmed by Western blot analysis.

**Northern Blot Analysis.** RNA was prepared from wild-type and Cbl-deficient DT40 cells using the guanidium thiocyanate method. Total RNA (20 μg) was separated in 1.2% formaldehyde gel, transferred to Hybond™-N<sup>+</sup> membrane (Amersham Pharmacia Biotech), and probed with <sup>32</sup>P-labeled chicken Cbl cDNA.

**Immunoprecipitation, Western Blot Analysis, and In Vitro Kinase Assay.** For immunoprecipitation, cells were solubilized in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors (43), and precleared lysates were sequentially incubated with proper Abs and protein A-agarose. The immunoprecipitates were washed four times with lysis buffer. Whole cell lysates were prepared from unstimulated or M4-stimulated cells using SDS sample buffer. Whole cell lysates or immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by appropriate Abs and the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Bio-

tech). To examine in vitro kinase activity of Syk, immunoprecipitated Syk was suspended in kinase buffer (20 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>) in the presence of 10 μCi of [ $\gamma$ -<sup>32</sup>P]ATP (>3,000 Ci/mmol). Recombinant GST fusion protein containing a cytoplasmic domain of mouse Ig $\alpha$  (5 μg) was used as an exogenous substrate (46). The reactions were allowed to incubate at 30°C for 10 min. For in vitro kinase assay of Btk, immunoprecipitated Btk was suspended in kinase buffer (20 mM Pipes, pH 7.5, 20 mM MnCl<sub>2</sub>) containing 10 μCi of [ $\gamma$ -<sup>32</sup>P]ATP (>3,000 Ci/mmol) and 5 μg of enolase (Sigma Chemical Co.), and was incubated at 25°C for 3 min. The kinase reactions were terminated by the addition of SDS sample buffer. The mixtures were separated by SDS-PAGE gel, followed by autoradiography.

**Inositol 1,4,5-Trisphosphate Generation Assay.** Cells (2 × 10<sup>6</sup>) were stimulated with mAb M4 (2 μg) at 37°C for the indicated time. Determination of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) production was performed using the Biotrak IP<sub>3</sub> assay system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Results are shown as the mean from three independent experiments. Error bars represent the SD from the mean.

**Calcium Measurements.** Cells (5 × 10<sup>6</sup>) were suspended in PBS containing 20 mM Hepes (pH 7.2), 5 mM glucose, 0.025% BSA, and 1 mM CaCl<sub>2</sub>, and were loaded with 3 μM acetoxy-methyl ester of fura-2 (fura-2 AM) at 37°C for 45 min. Cells were washed twice, and were adjusted to 10<sup>6</sup> cells/ml. Continuous monitoring of fluorescence from the cell suspension was performed using a fluorescence spectrophotometer (model F-2000; Hitachi Instruments) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Calibration and calculation of calcium levels were done as described (47).

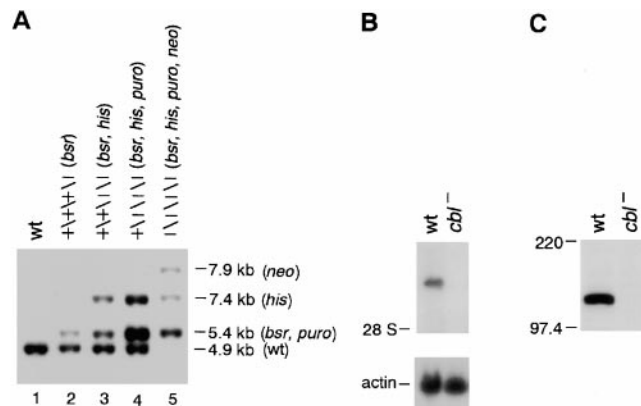
**Flow Cytometric Analysis.** For DNA content analysis, stimulated or unstimulated cells were pelleted and resuspended in hypotonic DNA staining solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100). Samples were kept at 4°C overnight, and were subjected to analysis by FACScan™ (Becton Dickinson). Debris and doublets were excluded by appropriate gating (48). For cell surface expression of BCR, DT40

cells were washed, incubated with FITC-conjugated anti-chicken IgM (Bethyl Laboratories), and analyzed using FAC-Scan™. The x and y axes for the histograms indicate fluorescence intensity (four-decade log scales) and relative cell number, respectively.

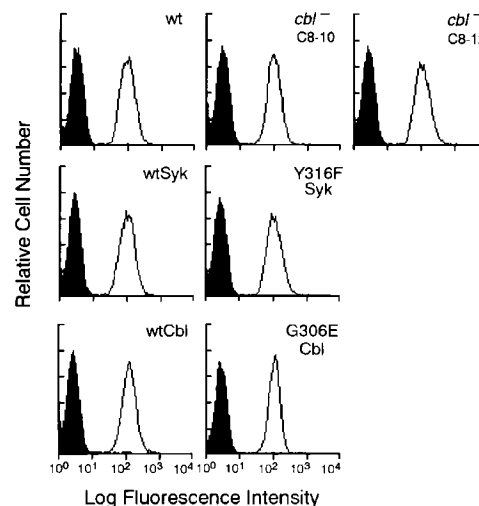
**Binding Assay with GST Fusion Proteins.** Human Cbl sequences encoding amino acids 25–351 were amplified by PCR from wild-type or G306E Cbl cDNAs, and were cloned as BamHI-XhoI fragments into the pGEX-4T-1 vector (Amersham Pharmacia Biotech). GST fusion proteins were affinity purified by glutathione-Sepharose beads (Amersham Pharmacia Biotech). For in vitro binding assays, stimulated or unstimulated DT40 cell lysates (1 × 10<sup>7</sup>) were incubated with 20 μg of purified GST fusion proteins for 1 h at 4°C. The binding mixtures were washed extensively in NP-40 lysis buffer. Bound proteins or whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected by anti-BLNK Ab and ECL. For far-Western blotting, anti-BLNK immunoprecipitates were separated on a 7% SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes. The blotted membranes were incubated with 2.5 μg/ml eluted GST fusion proteins, and were detected using anti-GST mAb and ECL.

## Results

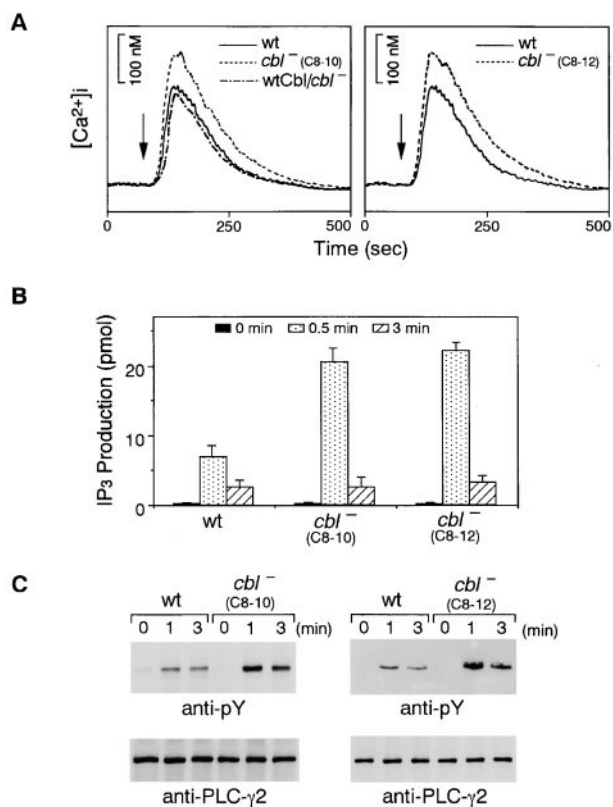
**Cbl Negatively Regulates the PLC- $\gamma$ 2 Pathway.** To disrupt the *cbl* locus in DT40 B cells, we transfected a targeting construct pCbl-Bsr into parental DT40 B cells. Southern blot analysis indicated that 10 out of 24 blasticidin-resistant clones had sustained a targeting event (data not shown). The hybridization signal of a rearranged 5.4-kb band was two- or threefold weaker than the wild-type 4.9-kb band, suggesting that DT40 cells contain more than two alleles of the *cbl* locus (Fig. 1 A). Disruption of the remaining alleles of the *cbl* locus was accompanied by the sequential transfect-



**Figure 1.** Disruption of the *cbl* gene in chicken DT40 B cells. (A) Southern blot analysis of wild-type and various targeted DT40 cells. Hind-III-digested genomic DNA separated on agarose gel was blotted and hybridized with a chicken Cbl cDNA probe. Wild-type (wt) and *bsr*-, *hisD*-, *puro*-, and *neo*-targeted alleles showed 4.9-, 5.4-, 7.4-, 5.4-, or 7.9-kb fragments, respectively. (B) Northern blot analysis using a chicken cDNA probe for Cbl (top) or  $\beta$ -actin (bottom). The position of 28 S rRNA is indicated. (C) Protein expression analysis of Cbl in wild-type (wt) and Cbl-deficient (*cbl*<sup>-/-</sup>) DT40 cells. Total cell lysates (2 × 10<sup>6</sup> cells) were prepared and analyzed by Western blotting using anti-Cbl Ab.



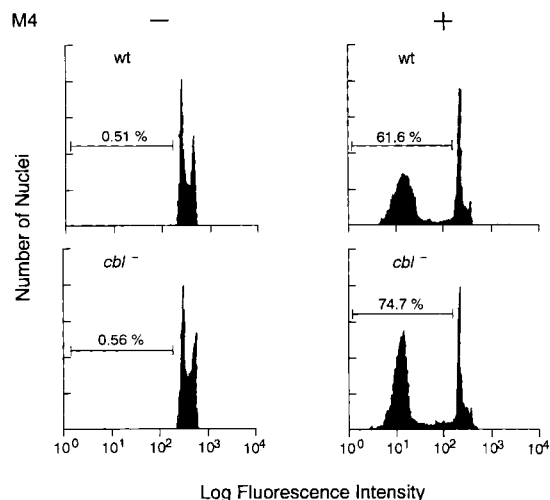
**Figure 2.** Cell surface expression of BCR. BCR expression on the surface of wild-type (wt), Cbl-deficient (*cbl*<sup>-/-</sup>), and various mutant DT40 cells was determined by flow cytometry. Unstained cells were used as negative controls (filled histogram). Syk-deficient cells expressing wild-type and Y316F mutant of Syk are indicated as wtSyk and Y316FSyk, respectively. Cbl-deficient cells (C8-10) expressing wild-type Cbl and its G306E mutant are shown as wtCbl and G306ECbl, respectively.



**Figure 3.** Characterization of Cbl-deficient DT40 cells. (A) Calcium mobilization in wild-type (wt) DT40 cells, Cbl-deficient clones (C8-10 and C8-12), and C8-10 clone expressing wild-type Cbl (see Fig. 8).  $[Ca^{2+}]_i$  levels were monitored by a spectrophotometer after stimulation with M4 (2  $\mu\text{g}/\text{ml}$ ). Arrows indicate the time point for adding M4. (B)  $IP_3$  generation. Cells were stimulated with M4 for the indicated amount of time, and  $IP_3$  production was measured. (C) Tyrosine phosphorylation of PLC- $\gamma$ 2. At the indicated time points after the M4 stimulation (4  $\mu\text{g}/\text{ml}$ ), immunoprecipitates with anti-PLC- $\gamma$ 2 Ab were separated on a 7% SDS-PAGE gel, and were analyzed by Western blotting with antiphosphotyrosine mAb (top, anti-pY) or anti-PLC- $\gamma$ 2 Ab (bottom).

tion of three other targeting constructs, pCbl-HisD, pCbl-Puro, and pCbl-Neo (Fig. 1 A). Hybridization with a *bsr*, *hisD*, *puro*, or *neo* probe indicated that the targeted clone had incorporated a single copy of each construct (data not shown). Lack of Cbl was verified by Northern and Western analyses (Fig. 1, B and C). The level of cell surface expression of BCR on the targeted DT40 cells was the same as that of parental cells (Fig. 2).

One of the hallmarks of BCR-induced signaling is calcium mobilization. Fura-2-loaded wild-type and Cbl-deficient (C8-10) DT40 cells were stimulated with anti-BCR mAb M4, and the rise of intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) was measured. As shown in Fig. 3 A, the peak height of the  $[Ca^{2+}]_i$  increase upon receptor stimulation was enhanced by  $\sim 1.3$ -fold by loss of Cbl. Because  $IP_3$  generation mediated by PLC- $\gamma$ 2 is primarily important for BCR-induced calcium mobilization through its binding to  $IP_3$  receptors, BCR-induced  $IP_3$  generation in wild-type and Cbl-deficient B cells was examined. Cbl-deficient cells exhibited increased  $IP_3$  generation compared with wild-type cells;



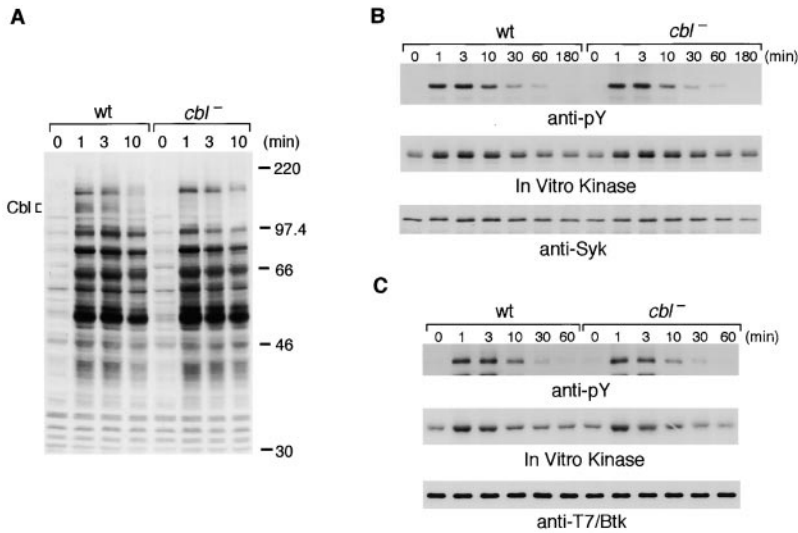
**Figure 4.** Induction of apoptosis in wild-type and Cbl-deficient DT40 cells. Wild-type (wt) and Cbl-deficient (C8-10, *cbl*<sup>-</sup>) DT40 cells were cultured with (+) or without (-) M4 (10  $\mu\text{g}/\text{ml}$ , 24 h), treated in hypotonic DNA staining solution containing 50  $\mu\text{g}/\text{ml}$  propidium iodide, and subjected to analysis by FACScan™ (Becton Dickinson). The percentage of fragmented nuclei is indicated.

the peak stimulation after BCR ligation was threefold more than wild-type DT40 cells (Fig. 3 B). We also examined another independent Cbl-deficient clone (C8-12), which indicated essentially the same abnormalities as clone C8-10. Furthermore, transfection of wild-type Cbl cDNA into clone C8-10 restored normal BCR-mediated calcium mobilization (Fig. 3 A), as well as  $IP_3$  generation (see Fig. 8 B). Based on these data, we conclude that Cbl negatively regulates PLC- $\gamma$ 2 activation after BCR stimulation.

**BCR-mediated Apoptosis Is Enhanced in Cbl-deficient DT40 Cells.** As PLC- $\gamma$ 2 activation is required for BCR-induced apoptosis in DT40 B cells (49), we reasoned that the receptor-induced apoptosis might be augmented by the enhanced PLC- $\gamma$ 2 activation in Cbl-deficient DT40 cells. As shown in Fig. 4, the BCR-induced apoptosis was clearly enhanced by loss of Cbl, demonstrating that Cbl negatively regulates BCR-mediated apoptosis through the PLC- $\gamma$ 2 pathway.

**Enhancement of BCR-induced Tyrosine Phosphorylation of PLC- $\gamma$ 2 by Loss of Cbl.** To explore the mechanism by which Cbl mediates its inhibitory effect on PLC- $\gamma$ 2 activation, the BCR-mediated tyrosine phosphorylation status of PLC- $\gamma$ 2 was determined in wild-type and Cbl-deficient cells. Compared with wild-type cells, PLC- $\gamma$ 2 tyrosine phosphorylation in Cbl-deficient DT40 B cells was increased by about three- to fivefold, as assessed by antiphosphotyrosine mAb blotting analysis (Fig. 3 C). These observations indicate that PLC- $\gamma$ 2 hyperactivation in Cbl-deficient DT40 cells is most likely due to the enhanced tyrosine phosphorylation of PLC- $\gamma$ 2 in the BCR signaling context.

As Syk and Btk are the PTKs responsible for BCR-induced PLC- $\gamma$ 2 tyrosine phosphorylation (43, 50), one possible explanation for the enhanced PLC- $\gamma$ 2 phosphorylation in Cbl-deficient cells is that Syk and/or Btk are



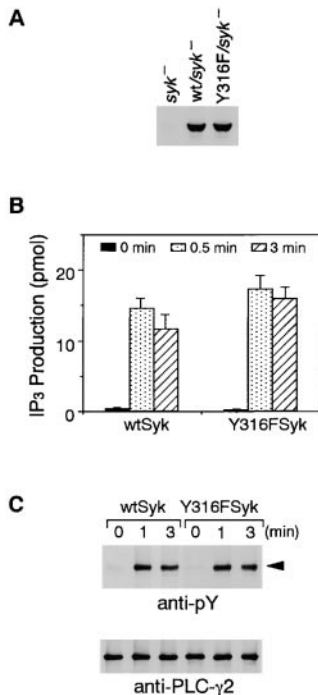
**Figure 5.** BCR-induced tyrosine phosphorylation in wild-type and Cbl-deficient DT40 cells. C8-10 was used for a Cbl-deficient clone. (A) Tyrosine phosphorylation in whole cell lysates from wild-type (wt) and Cbl-deficient (*cbl*<sup>-</sup>) DT40 cells. At the indicated time points after addition of M4 (4  $\mu$ g/ml), whole cell lysates prepared from  $2 \times 10^6$  cells were loaded onto an 8% SDS-PAGE gel. The blotted membrane was incubated with antiphosphotyrosine Ab. (B) Tyrosine phosphorylation and in vitro kinase activity of Syk. Wild-type and Cbl-deficient DT40 cells were stimulated with M4 for the indicated amount of time, and were immunoprecipitated with anti-Syk Ab. Immunoprecipitates were divided, and one third was used for Western blotting with antiphosphotyrosine mAb (top, anti-pY) or anti-Syk Ab (bottom). The remaining third was used for the in vitro kinase assay (middle). (C) Tyrosine phosphorylation and in vitro kinase activity of Btk. Wild-type and Cbl-deficient DT40 cells expressing T7-tagged Btk were stimulated with M4 for the indicated amount of time, and were immunoprecipitated with anti-T7 mAb. Immunoprecipitates were divided, and one third was used for Western blotting with antiphosphotyrosine mAb (top) or anti-T7 mAb (bottom). The remaining third was used for the in vitro kinase assay (middle).

hyperactivated in Cbl-deficient DT40 cells, leading to hyperphosphorylation of PLC- $\gamma$ 2. To evaluate this possibility, the BCR-induced whole tyrosine phosphorylation pattern was compared in wild-type and mutant cells. As shown in Fig. 5 A, there were no significant changes between wild-type and Cbl-deficient cells, except that the band corresponding to Cbl itself was absent in the mutant cells. Consistent with the whole tyrosine phosphorylation data, both Syk and Btk were inducibly tyrosine phosphorylated to an almost similar extent in wild-type and Cbl-deficient DT40 cells. In addition, the BCR-induced in vitro

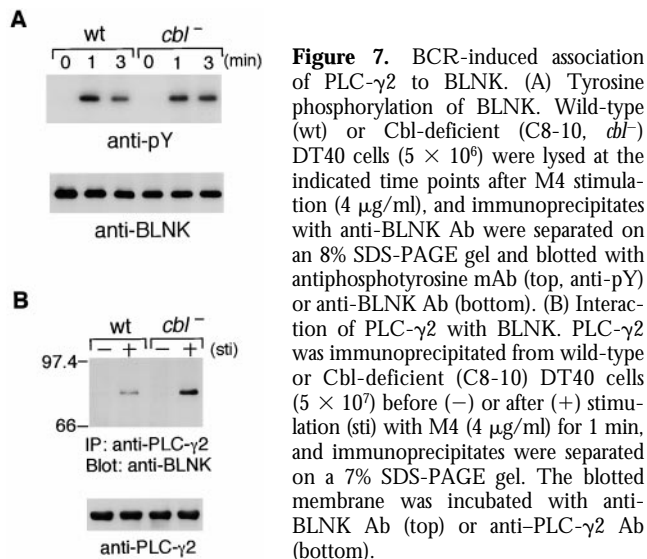
kinase activities of Syk and Btk were almost the same between wild-type and mutant DT40 cells (Fig. 5, B and C).

Previous overexpression experiments using the COS cell system have indicated that a potential target of the Cbl SH2 domain is phosphorylated tyrosine 316 of porcine Syk (30). To formally demonstrate that PLC- $\gamma$ 2 hyperactivation by loss of Cbl cannot be accounted for by the interaction between Cbl and Syk, we determined the effect of the Syk-Y316F mutation on BCR-mediated PLC- $\gamma$ 2 activation. Wild-type Syk and its mutant (Y316F) were transfected into Syk-deficient DT40 cells, and the resulting DT40 clones expressing similar levels of Syk were characterized (Fig. 6 A). As shown in Fig. 6 (B and C), BCR-mediated tyrosine phosphorylation of PLC- $\gamma$ 2 and subsequent IP<sub>3</sub> production were only marginally enhanced by this mutation. Together, these results demonstrate that activation of Syk and Btk is not significantly perturbed by loss of Cbl.

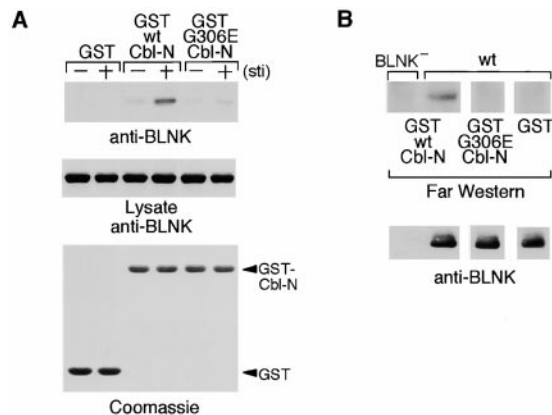
**Recruitment of PLC- $\gamma$ 2 to BLNK Is Significantly Enhanced in Cbl-deficient B Cells.** The adaptor molecule BLNK is shown to be essential for BCR-mediated tyrosine phosphorylation of PLC- $\gamma$ 2, in that phosphorylated BLNK provides docking sites for PLC- $\gamma$ 2 SH2 domains, leading to recruitment of PLC- $\gamma$ 2 into the close proximity of Btk and Syk (36). Thus, the effect of disruption of Cbl on association of PLC- $\gamma$ 2 to BLNK after BCR ligation was examined. As this association is SH2-phosphotyrosine dependent, we first checked the tyrosine phosphorylation status of BLNK (Fig. 7 A), which indicated the same level between wild-type and Cbl-deficient DT40 cells. Despite the same level of BLNK phosphorylation, BCR-induced association of PLC- $\gamma$ 2 with BLNK was significantly augmented by loss of Cbl (Fig. 7 B). Given that the extent of PLC- $\gamma$ 2 association to BLNK correlates well with the phosphorylation extent of PLC- $\gamma$ 2 (36), the enhanced association between PLC- $\gamma$ 2 and BLNK in Cbl-deficient cells is likely to cause hyperphosphorylation of PLC- $\gamma$ 2.



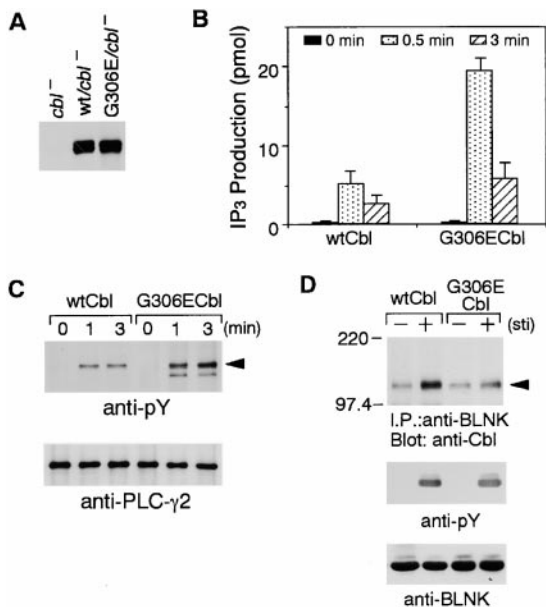
**Figure 6.** Effect of Syk-Y316F mutation on PLC- $\gamma$ 2 activation. Syk-deficient DT40 cells that stably expressed wild-type or Syk-Y316F were analyzed. (A) Expression of wild-type or Syk-Y316F in Syk-deficient cells. Total cell lysates ( $2 \times 10^6$  cells) were prepared and analyzed by Western blotting using anti-Syk Ab. (B) BCR-induced IP<sub>3</sub> generation and (C) tyrosine phosphorylation of PLC- $\gamma$ 2 were examined as described in the legend to Fig. 3.



**Figure 7.** BCR-induced association of PLC- $\gamma$ 2 to BLNK. (A) Tyrosine phosphorylation of BLNK. Wild-type (wt) or Cbl-deficient (C8-10, *cbl*<sup>-</sup>) DT40 cells ( $5 \times 10^6$ ) were lysed at the indicated time points after M4 stimulation ( $4 \mu\text{g/ml}$ ), and immunoprecipitates with anti-BLNK Ab were separated on an 8% SDS-PAGE gel and blotted with antiphosphotyrosine mAb (top, anti-pY) or anti-BLNK Ab (bottom). (B) Interaction of PLC- $\gamma$ 2 with BLNK. PLC- $\gamma$ 2 was immunoprecipitated from wild-type or Cbl-deficient (C8-10) DT40 cells ( $5 \times 10^7$ ) before (-) or after (+) stimulation (sti) with M4 ( $4 \mu\text{g/ml}$ ) for 1 min, and immunoprecipitates were separated on a 7% SDS-PAGE gel. The blotted membrane was incubated with anti-BLNK Ab (top) or anti-PLC- $\gamma$ 2 Ab (bottom).



**Figure 9.** Direct binding of Cbl-N to BLNK. (A) In vitro binding of the Cbl-N with BLNK. DT40 cells unstimulated (-) or stimulated (+) with M4 ( $4 \mu\text{g/ml}$ ) for 1 min were lysed, and were incubated with GST or the indicated GST-Cbl-N fusion proteins. The precipitates (top) or whole cell lysates (middle) were separated on a 7% SDS-PAGE gel and immunoblotted with anti-BLNK Ab. GST fusion proteins used in precipitation were visualized by Coomassie staining (bottom). (B) For far-Western blotting, wild-type (wt) or BLNK-deficient (BLNK<sup>-</sup>) DT40 cells ( $5 \times 10^7$ ) were stimulated for 1 min with M4 ( $4 \mu\text{g/ml}$ ), lysed, and immunoprecipitated with anti-BLNK Ab. Immunoprecipitates were divided, and half were separated on a 7% SDS-PAGE gel, transferred to polyvinylidene difluoride membranes, incubated with the indicated soluble GST fusion proteins, and detected using anti-GST mAb (top). The remaining half were used for Western blotting with anti-BLNK Ab (bottom).



**Figure 8.** Effect of mutation in the Cbl SH2 domain on PLC- $\gamma$ 2 activation. Cbl-deficient DT40 cells that stably expressed wild-type or G306E Cbl were analyzed. (A) Expression of wild-type or G306E Cbl in Cbl-deficient cells (C8-10). Total cell lysates ( $2 \times 10^6$  cells) were prepared and analyzed by Western blotting using anti-Cbl Ab. (B) BCR-induced IP<sub>3</sub> generation and (C) tyrosine phosphorylation of PLC- $\gamma$ 2 were examined as described in the legend to Fig. 3. (D) Recruitment of Cbl to BLNK. BLNK was immunoprecipitated from  $5 \times 10^7$  cells before (-) or after (+) stimulation (sti) with M4 ( $4 \mu\text{g/ml}$ ) for 1 min, and immunoprecipitates were separated on a 7% SDS-PAGE gel. The blotted membrane was incubated with anti-Cbl Ab (top), antiphosphotyrosine mAb (middle, anti-pY), or anti-BLNK Ab (bottom). Phosphorylated BLNK is shown in the middle panel.

**Association of Cbl with BLNK Is Dependent on the Cbl SH2 Domain.** The above observations suggest that Cbl acts through its SH2 domain to competitively inhibit the association between PLC- $\gamma$ 2 and BLNK. To verify this possibility, the effect of the Cbl SH2 mutant (G306E) on BCR-mediated PLC- $\gamma$ 2 activation was determined. Wild-type Cbl and its SH2 mutant (G306E) were transfected into the Cbl-deficient DT40 clone (C8-10), and DT40 clones expressing similar expression levels of Cbl, as assessed by immunoblotting analysis (Fig. 8 A), were selected and further characterized. Wild-type Cbl reverted both PLC- $\gamma$ 2 tyrosine phosphorylation and IP<sub>3</sub> generation upon BCR cross-linking, whereas the SH2 mutant could not, demonstrating the importance of the SH2 domain in Cbl's negative influence on PLC- $\gamma$ 2 activation (Fig. 8, B and C). Moreover, recruitment of Cbl to phosphorylated BLNK was dependent on the Cbl SH2 domain (Fig. 8 D).

To further examine the ability of the Cbl SH2 domain to bind to phosphorylated BLNK in vitro, we used GST fusion protein containing the Cbl-N. Binding of GST-Cbl-N to BLNK in lysates of unstimulated DT40 cells was low, but it increased substantially upon BCR stimulation. Importantly, G306E mutation in Cbl-N completely abrogated binding to phosphorylated BLNK (Fig. 9 A). These results establish that the Cbl SH2 domain interacts with phosphorylated BLNK in a lysate binding assay. However, it is possible that this interaction is indirectly mediated by an adaptor molecule. To determine if the Cbl SH2 domain directly binds to phosphorylated BLNK, a far-Western blotting was performed. As seen in Fig. 9 B, GST-Cbl-N

directly bound to phosphorylated BLNK; in contrast, no binding was observed with GST or GST-Cbl-N (G306E). These data demonstrate that the Cbl SH2 domain can directly bind to phosphorylated BLNK.

## Discussion

In this study, we have addressed three questions regarding the function of Cbl: (a) does Cbl function as a negative regulator in BCR signaling, as well as in the case of TCR or Fc $\epsilon$ RI (29, 31, 33); (b) if so, which signaling pathway(s) is a physiological target of Cbl in the BCR signaling context; and (c) what is the molecular mechanism by which Cbl influences the BCR signaling pathway?

DT40 B cells deficient in Cbl exhibited hyperactivation of PLC- $\gamma$ 2 (Fig. 3) and hyperapoptosis (Fig. 4) in response to BCR ligation, supporting the previous contention that Cbl functions as a negative regulator in immune receptor signaling. Since the previous evidence has shown that BCR-mediated apoptosis in DT40 B cells requires the PLC- $\gamma$ 2 pathway (49), BCR-mediated hyperapoptosis in Cbl-deficient DT40 cells is most likely accounted for by hyperactivation of the PLC- $\gamma$ 2 pathway. In contrast to threefold enhancement of IP<sub>3</sub> production in Cbl-deficient cells compared with wild-type DT40, the BCR-mediated calcium mobilization was increased by  $\sim$ 1.3-fold by loss of Cbl, suggesting that the molecule(s) lying between PLC- $\gamma$ 2 activation and calcium mobilization, such as IP<sub>3</sub> receptors, may have a limiting factor for transmission from IP<sub>3</sub> generation to calcium mobilization in the DT40 B cell system. Indeed, we observed previously that the expression level of PLC- $\gamma$ 2 in DT40 cells correlated well with BCR-mediated IP<sub>3</sub> production, whereas calcium mobilization was not linearly correlated (49).

It has been shown recently that Cbl regulates ubiquitination of receptor-type PTKs, such as platelet-derived growth factor receptor, in a manner dependent on its SH2 and RING finger domains (51–55). Indeed, Cbl has the capacity to act as a component of ubiquitin–protein ligases (E3s [54]). The expression levels of TCR components in Cbl-deficient thymocytes are higher than in wild-type mice (31–33); in contrast, the expression level of BCR in Cbl-deficient DT40 cells is not significantly upregulated (Fig. 2). Thus, one explanation for the functional difference of Cbl in TCR versus BCR signaling may be that targeting signals for degradation by Cbl are comprised in CD3  $\zeta$  chain components, but not BCR Ig $\alpha$ /Ig $\beta$  subunits. Cbl-b, another Cbl-related molecule, is expressed in hematopoietic cells, and the NH<sub>2</sub>-terminal half of Cbl-b, including SH2 and RING finger domains, is highly homologous to Cbl (56). These structural characteristics of Cbl-b suggest that Cbl-b plays a redundant role in ubiquitin-mediated degradation. Thus, it is also possible that relatively high expression of Cbl-b in B cells compared with T cells (57) may compensate for the degradation function of Cbl in B cells.

Given that phosphatidylinositol 3,4,5-trisphosphate, a product of PI3-K, participates in positive modulation of PLC- $\gamma$  activation (58–60), one possible explanation for hy-

peractivation of PLC- $\gamma$ 2 in Cbl-deficient DT40 cells is that the PI3-K pathway is hyperactivated, leading to enhancement of PLC- $\gamma$ 2 activation. However, the observations that BCR-mediated Akt activation, a readout of the PI3-K pathway, was not affected in Cbl-deficient DT40 cells (data not shown) suggest that this hyperactivation of PLC- $\gamma$ 2 is mediated through a PI3-K-independent mechanism.

Recent experiments have demonstrated that the adaptor molecule BLNK, in addition to Syk and Btk, is required for tyrosine phosphorylation of PLC- $\gamma$ 2 and its subsequent activation (34). Indeed, the phosphorylated BLNK by Syk provides docking sites for PLC- $\gamma$ 2 SH2 domains and its subsequent phosphorylation (36, 61). Thus, one possible explanation for the hyperphosphorylation of PLC- $\gamma$ 2 by loss of Cbl is that Cbl negatively regulates Syk function, as proposed previously in mast cell and COS cell systems (29, 30). However, this possibility is unlikely in the DT40 B cell system based on the following data: (a) overall tyrosine phosphorylation was almost the same between wild-type and Cbl-deficient DT40 cells; (b) a Syk substrate, BLNK, was normally phosphorylated in Cbl-deficient DT40 cells; and (c) although the Cbl SH2 domain was essential for exerting Cbl's negative effect on PLC- $\gamma$ 2 phosphorylation, Syk-Y316F (disruption mutant for binding to the Cbl SH2 domain) did not exhibit significant enhancement of PLC- $\gamma$ 2 phosphorylation, as observed in Cbl-deficient cells. BCR-mediated Btk activation, as in the case of Syk, occurred normally in Cbl-deficient DT40 B cells. The present results differ from previous studies using a transfected rat basophilic leukemia mast cell system, which suggested that Cbl directly inhibits the enzymatic activity of Syk (29). It is possible that when Syk and Cbl proteins are overexpressed using the vaccinia virus system in rat basophilic leukemia cells, SH2-independent binding of Cbl to Syk could be promoted, thereby leading to a direct negative impact on Syk enzymatic activity. Another potential mechanism, enhanced degradation of Syk by Cbl, was proposed based on overexpression experiments in COS cells (30). The degradation effect of Cbl on Syk may be dependent on the expression level of the activated Syk; overexpressed Syk could be more susceptible to degradation. Thus, it is possible that the effect of Cbl on the endogenous expression level of Syk in DT40 cells may be too subtle to be detected by our assay system.

Despite the same levels of BLNK tyrosine phosphorylation in wild-type and Cbl-deficient DT40 cells, association of PLC- $\gamma$ 2 with BLNK was increased by about threefold in Cbl-deficient cells, indicating that Cbl negatively modulates the PLC- $\gamma$ 2–BLNK association. Since the Cbl SH2 domain was also recruited to phosphorylated BLNK after BCR ligation (Fig. 8 D and Fig. 9), it would be reasonable to anticipate that the Cbl SH2 domain and the PLC- $\gamma$ 2 SH2 domains are competitively recruited to the phosphorylated BLNK. This idea is further supported by the reported preferred sequences of these SH2 domains. A phosphopeptide library screen with the Cbl NH<sub>2</sub>-terminal region demonstrated that the binding motif for this region could be D(N/D)XpY (9). Indeed, chicken BLNK com-

prises this binding motif (DDSY<sup>115</sup>), and this motif is well conserved in both human and mouse BLNK (34). Interestingly, the sequences that match well the motif selected by the COOH-terminal SH2 domain of PLC- $\gamma$ 2 (Y<sup>103</sup>VVP and Y<sup>194</sup>IVP) are found near this Cbl binding motif (62). Thus, our biochemical data, together with previous evidence that the extent of BLNK-PLC- $\gamma$ 2 association correlates with the level of BCR-mediated PLC- $\gamma$ 2 tyrosine phosphorylation (36), suggest that enhanced BLNK-PLC- $\gamma$ 2 association by loss of Cbl competition causes increased PLC- $\gamma$ 2 phosphorylation, leading to its hyperactivation. Additional studies are underway to define exactly the in vivo phosphorylation sites on BLNK for binding to the PLC- $\gamma$ 2 SH2 domains and the Cbl SH2 domain, and to examine the functional consequences of phosphorylation on these sites. The importance of the Cbl SH2 domain in TCR signaling has been suggested by recent results that TCR-mediated nuclear factor of activated T cells modulation by oncogenic Cbl mutant 70Z was abolished by its SH2 mutation (63, 64). As nuclear factor of activated T cells activation is dependent on both PKC activation and calcium mobilization, this notion is further supported by our findings that the Cbl SH2 domain is essential for its influence on BCR-mediated PLC- $\gamma$ 2 activation. Although a D(N/D) XpY motif in Zap-70 (Y292) and Syk (Y316) binds to the Cbl SH2 domain (9, 10, 30, 65), recent studies indicate that this interaction cannot fully account for the effect of the Cbl SH2 domain in immune cells (64, 66, 67). Thus, our identification here of BLNK as a target of the Cbl SH2 domain provides another potential mode of Cbl action.

In contrast to the thymic T cell changes in Cbl-deficient mice, the B cell development in bone marrow appears to be normal, suggesting differential requirements for Cbl in T and B cell development (31). Developmental changes in T cells might reflect the fact that Cbl downregulates the expression levels of TCR components. In support of this idea, the BCR expression level in Cbl-deficient mice seems to be normal (31). Despite no apparent B cell developmental changes, the peripheral lymph nodes of Cbl-deficient mice demonstrated increases in both B and T cells (31). This observation suggests that Cbl functions as a negative regulator in peripheral B and T cells. Thus, our finding that Cbl is a negative regulator in the BCR-mediated PLC- $\gamma$ 2 pathway may reflect the status of murine peripheral B cells rather than developing B cells. Comparative studies using Cbl-deficient mice will be needed to test this hypothesis.

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