

# Cbl-b Positively Regulates Btk-mediated Activation of Phospholipase C- $\gamma$ 2 in B Cells

Tomoharu Yasuda,<sup>1,4</sup> Tohru Tezuka,<sup>1</sup> Akito Maeda,<sup>2</sup> Tetsuya Inazu,<sup>3</sup>  
Yuji Yamanashi,<sup>4</sup> Hua Gu,<sup>3</sup> Tomohiro Kurosaki,<sup>2</sup>  
and Tadashi Yamamoto<sup>1</sup>

<sup>1</sup>Department of Oncology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

<sup>2</sup>Department of Molecular Genetics, Institute for Liver Research, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan

<sup>3</sup>Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852

<sup>4</sup>Department of Cell Regulation, Medical Research Institute, Tokyo Medical Dental University, Bunkyo-ku, Tokyo 113-8510, Japan

## Abstract

Genetic studies have revealed that Cbl-b plays a negative role in the antigen receptor-mediated proliferation of lymphocytes. However, we show that Cbl-b-deficient DT40 B cells display reduced phospholipase C (PLC)- $\gamma$ 2 activation and Ca<sup>2+</sup> mobilization upon B cell receptor (BCR) stimulation. In addition, the overexpression of Cbl-b in WEHI-231 mouse B cells resulted in the augmentation of BCR-induced Ca<sup>2+</sup> mobilization. Cbl-b interacted with PLC- $\gamma$ 2 and helped the association of PLC- $\gamma$ 2 with Bruton's tyrosine kinase (Btk), as well as B cell linker protein (BLNK). Cbl-b was indispensable for Btk-dependent sustained increase in intracellular Ca<sup>2+</sup>. Both NH<sub>2</sub>-terminal tyrosine kinase-binding domain and COOH-terminal half region of Cbl-b were essential for its association with PLC- $\gamma$ 2 and the regulation of Ca<sup>2+</sup> mobilization. These results demonstrate that Cbl-b positively regulates BCR-mediated Ca<sup>2+</sup> signaling, most likely by influencing the Btk/BLNK/PLC- $\gamma$ 2 complex formation.

Key words: BCR • Cbl family • calcium • GEMs • adaptor protein

## Introduction

The B cell receptor (BCR)\* complex, consisting of membrane-bound immunoglobulin and Ig- $\alpha$ / $\beta$  heterodimer, transmits signals that regulate the survival, differentiation, and proliferation of B lymphocytes (1–3). The Ig- $\alpha$ / $\beta$  heterodimer, an integral component of the surface expression and signaling function of BCR, contains immunoreceptor tyrosine-based activation motif (ITAM), a common motif

also found in the receptor complexes of other cells in the immune system including T, NK, and mast cells (4, 5). Engagement of the BCR initiates signaling cascades beginning with the activation of cytoplasmic protein tyrosine kinases (PTKs) and the phosphorylation of ITAM. Three distinct families of nonreceptor PTKs, Src family PTKs, Syk, and Bruton's tyrosine kinase (Btk), are activated upon BCR engagement and contribute to the activation of multiple downstream effectors such as Ras-mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C (PLC)- $\gamma$ 2 (3, 6–8). PLC- $\gamma$ 2 converts phosphatidylinositol 4,5-bisphosphate into the two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP<sub>3</sub>), which leads to the activation of protein kinase C and Ca<sup>2+</sup> mobilization, respectively. PLC- $\gamma$ 2 is activated through its tyrosine phosphorylation by Syk and Btk after BCR stimulation (9–11). Recent studies have demonstrated that BCR-mediated PLC- $\gamma$ 2 activation also requires association with B cell linker protein (BLNK), which is responsible for PLC- $\gamma$ 2 membrane translocation (12).

A. Maeda's present address is Department of Molecular Immunology and Allergy, Kyoto University, Sakyo-ku, Kyoto 606-8315, Japan.

Address correspondence to Tadashi Yamamoto, Department of Oncology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-5449-5301; Fax: 81-3-5449-5413; E-mail: tyamamoto@ims.u-tokyo.ac.jp

\*Abbreviations used in this paper: BCR, B cell receptor; BLNK, B cell linker protein; Btk, Bruton's tyrosine kinase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GEM, glycolipid-enriched microdomain; GST, glutathione S-transferase; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; ITAM, immunoreceptor tyrosine-based activation motif; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PTK, protein tyrosine kinase; RBD, Rap1 binding domain; TKB, tyrosine kinase-binding.

The mammalian Cbl family is made up of Cbl, Cbl-b, and Cbl-c/Cbl-3. They all have a tyrosine kinase-binding (TKB) domain, a RING finger domain, and a proline-rich region (13–15). The TKB domain, also called the Cbl-N domain, is an integrated phosphopeptide-binding platform composed of a four-helical bundle, a  $\text{Ca}^{2+}$ -binding EF hand, and an SH2 domain (16). The Cbl family proteins are tyrosine phosphorylated upon the stimulation of epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor, as well as ITAM-related receptors, such as TCR, BCR, and Fc receptors. The importance of Cbl family proteins in intracellular signal transduction is additionally emphasized by the observation that Cbl and Cbl-b interact with critical signaling molecules, such as Src family PTKs, Zap-70/Syk family PTKs, p85 subunit of PI3K, Vav, and Slp-76/BLNK family linker proteins (13, 17–19). Recent studies have also shown that Cbl family proteins recruit ubiquitin-conjugating enzymes (E2) via the RING finger domain, allowing ubiquitination and degradation of phosphorylated substrates (20).

Cbl and Cbl-b are abundantly expressed in a variety of immune cells. Although they share an evolutionary conserved  $\text{NH}_2$ -terminal region, Cbl and Cbl-b play distinct roles in the activation and development of lymphocytes. Thymocytes of Cbl-deficient mice exhibit enhanced positive selection of  $\text{CD4}^+$  cells, increased surface expression of TCR/CD3 and CD4, and hyperphosphorylation of Zap-70 after CD3 stimulation (21, 22). In contrast, Cbl-b-deficient mice show normal thymic development, but mature T and B cells of Cbl-b-deficient mice are hyperproliferative. These mice develop spontaneous autoimmunity, and Cbl-b deficiency uncouples T cell proliferation, IL-2 production, and phosphorylation of Vav from the requirement for CD28 costimulation (17, 23). In addition, recent studies show that Cbl-b not only regulates Vav, but also other signaling molecules such as PI3K downstream of TCR (24). To further dissect the roles of Cbl-b in BCR signaling, we established Cbl-b-deficient DT40 B cell lines by the gene targeting method. In contrast to Cbl-deficient DT40 cells, Cbl-b-deficient DT40 cells exhibited attenuated activation of PLC- $\gamma$ 2,  $\text{Ca}^{2+}$  mobilization, and c-Jun  $\text{NH}_2$ -terminal kinase (JNK) in response to BCR stimulation. Our data suggest that Cbl-b is important for Btk/BLNK/PLC- $\gamma$ 2 complex formation, which is required for downstream  $\text{Ca}^{2+}$  mobilization.

## Materials and Methods

**Cells and Abs.** DT40 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% chicken serum, and 50  $\mu\text{M}$   $\beta$ -mercaptoethanol. Lyn-, Syk-, Btk-, and Cbl-deficient DT40 cells have been described previously (10, 11, 18). WEHI-231 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50  $\mu\text{M}$   $\beta$ -mercaptoethanol. B cells from the spleens of 8–13-wk-old mice were enriched by negative selection with mAbs against CD3 $\epsilon$ , CD11b, and NK1.1. The resulting cells were >85% B220 $^+$ , as assessed by flow cytometry. The Abs for chicken PLC- $\gamma$ 2, chicken Syk,

chicken BLNK, and chicken Lyn were described previously (10, 25). Mouse monoclonal IgM toward chicken  $\mu$  chains (M4) was used for IgM cross-linking on DT40 cells (26). The following Abs were purchased: anti-Cbl-b (C-20), anti-Cbl-b (G-1), anti-mouse PLC- $\gamma$ 2, anti-Rap1, anti-extracellular signal-regulated kinase (ERK)2, and anti-p38 (Santa Cruz Biotechnology, Inc.); anti-JNK1 (BD PharMingen), anti-T7 epitope (Novagen), anti-phosphotyrosine (4G10; Upstate Biotechnology), anti- $\alpha$ -tubulin (Oncogene Research Products); and anti-mouse IgM ( $\mu$  chain specific; Sigma-Aldrich). The Abs against Cbl-b used in this study specifically recognized Cbl-b but not Cbl. Anti-T7 mAb did not react to chicken Cbl-b, BLNK, and PLC- $\gamma$ 2. Flow cytometric analysis was performed as described previously (18).

**Generation of Cbl-b-deficient DT40 Cells.** A partial chicken Cbl-b cDNA was cloned by reverse transcription-PCR with RNA from DT40 cells and degenerate primers in the conserved region between Cbl and Cbl-b (amino acids 67–418 in human Cbl-b). On the basis of the chicken Cbl-b cDNA sequence, chicken genomic fragments were obtained by PCR with chicken genomic DNA as a template. The targeting vectors, pCbl-b-neo and pCbl-b-hisD, were constructed by replacing the genomic fragment-containing exon corresponding to human Cbl-b amino acids 242–281 with *neo* and *hisD* cassettes, respectively. These cassettes were flanked by 3.5- and 3.0-kb chicken Cbl-b genomic sequences on the 5' and 3' side, respectively. The targeting vector pCbl-b-neo was linearized and introduced into wild-type DT40 cells by electroporation at 550 V and 25  $\mu\text{F}$ . Selection was done in the presence of 2 mg/ml G418, and G418-resistant clones were screened by Southern blot analysis for the targeted allele. pCbl-b-hisD was transfected into the neo-targeted clone and selected with both 1 mg/ml G418 and 1 mg/ml histidinol. Introduction of a single copy of each targeting vector was verified by reprobing the blots with internal *neo* or *hisD* probe.

**Expression Constructs.** Alternatively spliced form of human Cbl-b cDNA, which lacks 767–810 amino acids (provided by S. Lipkowitz, Bethesda Naval Hospital, Bethesda, MD; reference 27), was subcloned into the pApuro expression vector (10). Mutation of Gly-298 to Glu, designated GE, and Cys-373 to Ala, designated CA, were introduced into the human Cbl-b cDNA by PCR-directed mutagenesis, respectively. The human Cbl-b sequence encoding amino acids 1–444, designated N, was generated by SphI site-mediated deletion. GE, N, and CA were subcloned into the pApuro expression vector. Transfection into B cells was performed by electroporation at 550 V and 25  $\mu\text{F}$ . Selection was performed in the presence of 0.5  $\mu\text{g}/\text{ml}$  puromycin. T7-tagged Btk cDNA (28) subcloned into the pAhygro expression vector was transfected into wild-type or Cbl-b-deficient (C3-3) DT40 cells. The pAhygro vector was generated by replacing a puromycin-resistant gene of the pApuro vector by a hygromycin-resistant gene. Selection was performed in the presence of 2 mg/ml hygromycin B. Expression of Cbl-b or T7-Btk in each transfectant was confirmed by Western blot analysis.

**Northern Blot Analysis.** RNA was prepared using the guanidium thiocyanate method. Total RNA (20  $\mu\text{g}$ ) was separated on a 1.2% formaldehyde gel, transferred to Hybond N $^+$  nylon membrane (Amersham Pharmacia Biotech), and probed with  $^{32}\text{P}$ -labeled chicken *cbl-b* and chicken *actin* cDNAs.

**Immunoprecipitation and Western Blot Analysis.** Cells were solubilized in 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.5, and 1 mM EDTA) containing protease and phosphatase inhibitors as described previously (10), or in 0.2% NP-40 lysis buffer (0.2% NP-40, 10 mM Hepes, pH 7.0, 143 mM KCl, 5 mM  $\text{MgCl}_2$ , 1 mM phenylmethylsulfonyl fluoride,

10  $\mu\text{g/ml}$  leupeptin, 10  $\mu\text{g/ml}$  aprotinin, and 1 mM sodium orthovanadate). Precleared lysates were sequentially incubated with appropriate Abs and protein A/G-Sepharose beads. The immunoprecipitates were washed four times with lysis buffer. Whole cell lysates or immunoprecipitates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and detected by appropriate Abs and chemiluminescence (NEN Life Science Products).

**Preparation of Glycolipid-enriched Microdomain (GEM) Fractions.**  $5 \times 10^8$  cells were stimulated with 8  $\mu\text{g/ml}$  M4 for 3 min in 5 ml RPMI 1640 at 37°C. Cells were washed with PBS containing 4 mM EDTA, and were lysed on ice in 1 ml 1% Triton X-100 in MNE buffer (25 mM MES, pH 6.5, 150 mM NaCl, 5 mM EDTA, and 1 mM sodium orthovanadate) supplemented with protease and phosphatase inhibitors as described previously (10), and were homogenized with 20 strokes in a tight-fitting dounce homogenizer (Wheaton). The lysates were gently mixed with 1 ml of 80% (wt/vol) sucrose in MNE buffer and placed in the bottom of a centrifuge tube. The samples were then overlaid on 6.5 ml of 30% sucrose and 2.5 ml of 5% sucrose in MNE buffer and supercentrifuged for 20 h at 200,000  $g$  in a RPS40T rotor (Hitachi Instruments) at 4°C. Among 11 fractions collected from the top of the gradient, fractions 3 and 4 corresponded to GEMs as revealed by Western blot analysis using ganglioside GM1 and anti-Lyn Ab. For immunoprecipitation, 10 mg/ml digitonin was added to the fractions.

**In Vitro Kinase Assay.** The in vitro kinase assay conditions for ERK, p38, and JNK were described previously (29). In brief, lysates from  $2\text{--}5 \times 10^6$  cells were immunoprecipitated with 1  $\mu\text{g}$  anti-ERK2, anti-p38, or anti-JNK1 Abs with 40  $\mu\text{l}$  protein G-Sepharose. Half of the immune complexes were used for in vitro kinase assay and the rest were used for Western blot analysis. Immunoprecipitates were suspended in 30  $\mu\text{l}$  kinase assay buffer containing 10  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP and 5  $\mu\text{M}$  cold ATP. Glutathione S-transferase (GST)-Elk, GST-ATF2, or GST-c-Jun fusion protein (5  $\mu\text{g}$  each) was added as a substrate for ERK2, p38, or JNK1, respectively. The reactions were performed at 30°C for 20 min. For in vitro kinase assay of Syk, Syk immunoprecipitates were suspended in kinase assay buffer (20 mM Tris, pH 7.5, 10 mM  $\text{MgCl}_2$ , and 10 mM  $\text{MnCl}_2$ ) in the presence of 10  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP and 5  $\mu\text{M}$  cold ATP. Recombinant GST fusion protein containing a cytoplasmic region of mouse  $\text{Ig}\alpha$  (5  $\mu\text{g}$ ) was used as an exogenous substrate (30). The reactions were performed at 30°C for 10 min. For in vitro kinase assay of Btk, Btk immunoprecipitates were suspended in kinase assay buffer (20 mM Pipes, pH 7.5, and 20 mM  $\text{MnCl}_2$ ) containing 10  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP, 5  $\mu\text{M}$  cold ATP, and 5  $\mu\text{g}$  enolase (Sigma-Aldrich), and incubated at 25°C for 3 min. The kinase reactions were terminated by the addition of SDS sample buffer followed by boiling for 5 min. All samples were separated by SDS-PAGE. The gels were dried and subjected to autoradiography.

**Ca<sup>2+</sup> Measurements.** DT40 and  $2.5 \times 10^6$  cells were suspended in HBSS supplemented with 10 mM Hepes, pH 7.2, 0.025% BSA, and 1 mM  $\text{CaCl}_2$ , and loaded with 3  $\mu\text{M}$  acetoxy-methyl ester of Fura-2 (Molecular Probes) at 30°C for 30 min. Cytosolic Ca<sup>2+</sup> concentration of  $5 \times 10^5$  cells in 0.5 ml HBSS supplemented with 10 mM Hepes, pH 7.2, 0.025% BSA, and 1 mM  $\text{CaCl}_2$ , was recorded at 510 nm emission wavelength excited by 340 and 380 nm with a CAF110 spectrofluorometer (JASCO). Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> store was measured in 0.5 ml HBSS supplemented with 10 mM Hepes, pH 7.2, 0.025% BSA, and 1 mM EGTA. Mouse splenocytes were loaded with 5  $\mu\text{g/ml}$  Indo-1 (Molecular Probes) at 30°C for 40 min,

and then labeled with FITC-conjugated anti-B220 Ab (BD PharMingen). Ca<sup>2+</sup> mobilization of B220<sup>+</sup> cells was determined by flow cytometry.

**IP<sub>3</sub> Generation Assay.**  $2 \times 10^6$  cells were stimulated with 2  $\mu\text{g}$  M4 at 37°C for the indicated time. Analyses of IP<sub>3</sub> production were performed using the BIOTRAK IP<sub>3</sub> assay system (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

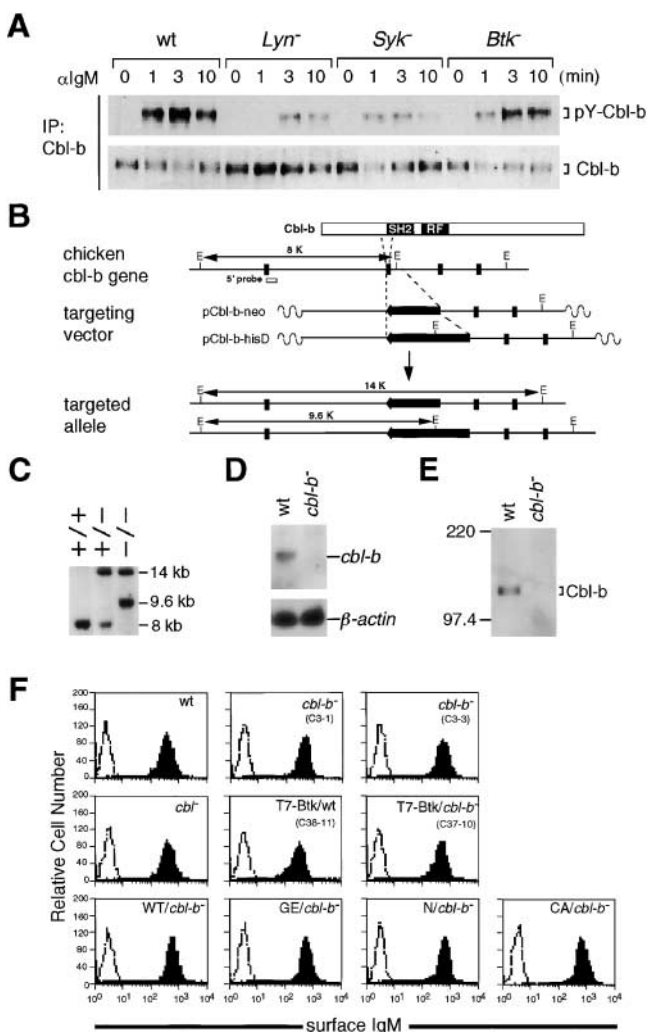
**Binding Assay with GST Fusion Proteins.** Human Btk cDNA fragments encoding amino acids 276–401 (Btk SH2) and 219–272 (Btk SH3), and rat PLC- $\gamma$ 2 cDNA fragments encoding amino acids 532–720 (PLC- $\gamma$ 2 [SH2]<sub>2</sub>) and 769–831 (PLC- $\gamma$ 2 SH3) were amplified by PCR. The PCR products were cloned in frame into pGEX4T-1 vector (Amersham Pharmacia Biotech). The constructs were verified by DNA sequencing. The pGEX-RalGDS(Rap1 binding domain [RBD]) plasmid, which encodes GST fusion protein containing the RBD of RalGDS, is as described previously (31). GST fusion proteins were produced in DH5 $\alpha$ , and affinity purified by glutathione Sepharose beads (Amersham Pharmacia Biotech). For in vitro binding assays, lysates from Cbl-b-overexpressing WEHI-231 cells were incubated with 3  $\mu\text{g}$  GST fusion proteins immobilized on glutathione Sepharose for 4 h at 4°C. Bound proteins were washed and blotted with anti-Cbl-b Ab. For Rap1 activation assay, cell lysates were incubated with 50  $\mu\text{g}$  GST-RalGDS(RBD) immobilized on glutathione Sepharose for 1 h at 4°C. Bound proteins were washed and blotted with anti-Rap1 Ab.

## Results

**Requirement of Lyn and Syk in BCR-induced Tyrosine Phosphorylation of Cbl-b.** Cbl-b becomes tyrosine phosphorylated upon stimulation of hematopoietic receptors such as the TCR, FLT3 receptor, and IL-7R (32, 33). Because Cbl-b is expressed in B cells, we tested whether Cbl-b, like Cbl (34), is tyrosine phosphorylated upon stimulation of BCR. As shown in Fig. 1 A, Cbl-b was rapidly tyrosine phosphorylated, being maximally phosphorylated 3 min after BCR stimulation in DT40 B cells. Tyrosine phosphorylation of Cbl-b was severely impaired in Lyn- and Syk-deficient cells. In Btk-deficient DT40 cells, tyrosine phosphorylation of Cbl-b was apparently suppressed at an earlier time point (i.e., at 1 min), but not at a later time point (i.e., at 10 min). This result is consistent with the previous observation that Src family PTKs and Syk lead to tyrosine phosphorylation of Cbl-b in COS-1 cells (32). After BCR stimulation, the total amount of immunoprecipitated Cbl-b was decreased in wild-type, Syk-, and Btk-deficient cells, but not in Lyn-deficient cells. Lyn-mediated tyrosine phosphorylation of Cbl-b may inhibit the binding of the anti-Cbl-b Ab. Another possibility is that Cbl-b translocates to the detergent insoluble fractions after BCR stimulation in a Lyn-dependent manner.

**Inhibition of BCR-induced PLC- $\gamma$ 2 Activation in Cbl-b-deficient DT40 B Cells.** To evaluate the function of Cbl-b in B cells, we generated Cbl-b-deficient DT40 B cells by the gene targeting method. Disruption of the chicken *cbl-b* gene was accomplished by targeted mutation of an exon corresponding to amino acids residues 242–281 of human Cbl-b (Fig. 1, B and C). Lack of the *cbl-b* mRNA and Cbl-b protein was verified by Northern and Western blot anal-





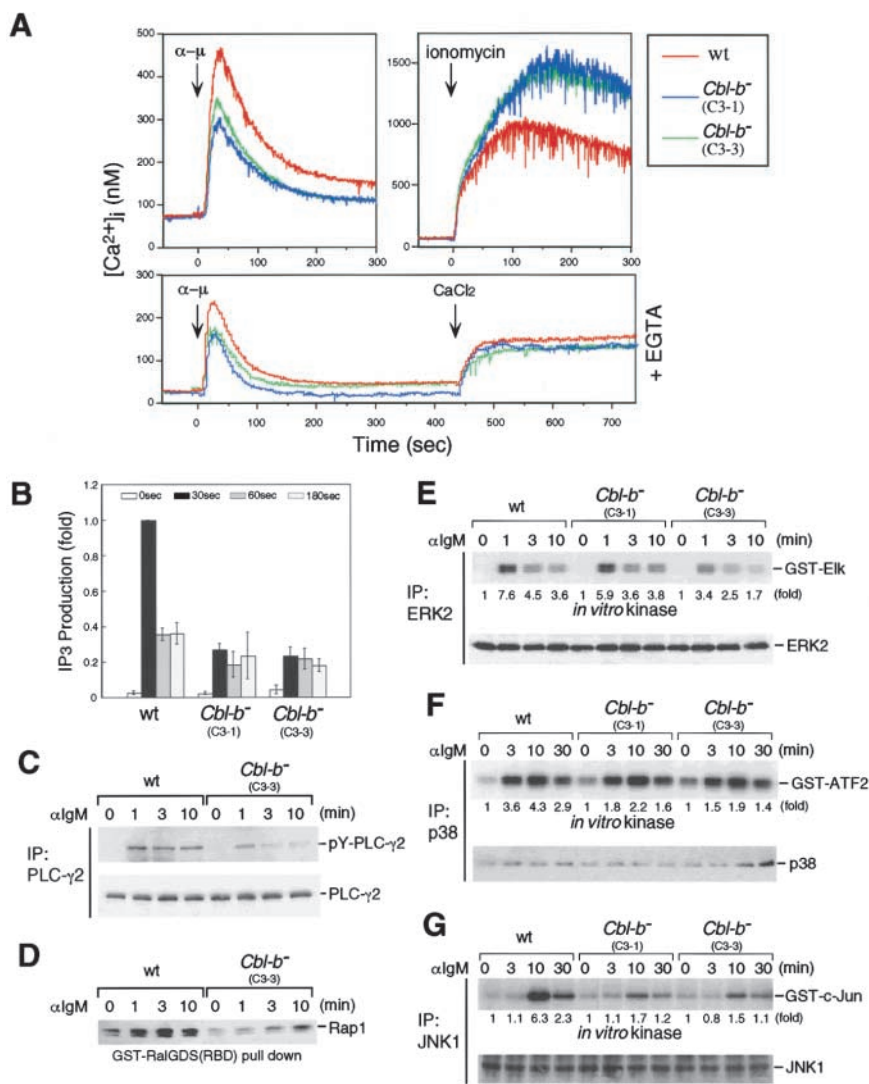
**Figure 1.** Tyrosine phosphorylation of Cbl-b and generation of Cbl-b-deficient DT40 B cells. (A) At the indicated time points after stimulation of 8  $\mu\text{g/ml}$  M4, wild-type, *Lyn*<sup>-</sup>, *Syk*<sup>-</sup>, or *Btk*<sup>-</sup> deficient DT40 cells ( $2.5 \times 10^7$ ) were lysed in 1% NP-40 lysis buffer containing 10 mg/ml digitonin. Anti-Cbl-b (C-20) immunoprecipitates from the lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb (top) and anti-Cbl-b (C-20) Ab (bottom). (B) Structure of the chicken *cbl-b* allele, the targeting vector, and the mutated allele. Restriction sites for *EcoRI* (E) are indicated. (C) Southern blot analysis of wild-type and targeted DT40 cells. *EcoRI*-digested genomic DNAs were separated on an agarose gel, blotted, and hybridized with the chicken *cbl-b* cDNA probe (B, 5' probe). (D) Northern blot analysis of wild-type and Cbl-b-deficient DT40 cells using chicken cDNA probe for *cbl-b* (top) or  $\beta$ -actin (bottom). (E) Protein expression of Cbl-b in wild-type and Cbl-b-deficient DT40 cells. Immunoprecipitates with anti-Cbl-b (C-20) Ab were prepared from wild-type and Cbl-b-deficient DT40 cells and subjected to Western blot analysis using anti-Cbl-b (C-20) Ab. (F) BCR expression on the surface of wild-type (wt), Cbl-b-deficient (*cbl-b*<sup>-</sup>, C3-1, and C3-3), and various DT40 derivatives were monitored by flow cytometry. Unstained cells were used as the negative controls (dashed histogram). Wild-type and Cbl-b-deficient cells expressing T7-tagged Btk are indicated as T7-Btk/wt and T7-Btk/*cbl-b*<sup>-</sup>, respectively. Cbl-b-deficient cells (C3-3) expressing wild-type Cbl-b, G298E mutant Cbl-b, COOH-terminal deletion mutant Cbl-b (1-444 amino acids), and C373A mutant Cbl-b are shown as WT/*cbl-b*<sup>-</sup>, GE/*cbl-b*<sup>-</sup>, N/*cbl-b*<sup>-</sup>, and CA/*cbl-b*<sup>-</sup>, respectively. The x and y axes for the histograms indicate fluorescence intensity (four-decade log scales) and relative cell number, respectively.

yses, respectively (Fig. 1, D and E). The level of cell surface expression of BCR on the targeted DT40 cells was comparable to that of parental cells (Fig. 1 F).

One of the crucial biochemical cascades in BCR signaling is the PLC- $\gamma$ 2/ $\text{Ca}^{2+}$  pathway. As shown in Fig. 2 A, BCR stimulation of DT40 cells deficient for Cbl-b showed impaired  $\text{Ca}^{2+}$  mobilization compared with wild-type cells. To evaluate the effect of Cbl-b on calcium release from internal stores as well as extracellular calcium influx, cells in medium containing EGTA were stimulated by anti-IgM Ab, followed by the addition of molar excess of calcium. The results showed that  $\text{Ca}^{2+}$  release from internal stores was impaired in Cbl-b-deficient cells. This was not due to the lesser amount of  $\text{Ca}^{2+}$  storage in the ER of Cbl-b-deficient cells than in wild-type cells, because ionomycin-induced calcium mobilization was not suppressed in the absence of Cbl-b. Consistent with this observation, BCR-induced IP<sub>3</sub> production was remarkably impaired in Cbl-b-deficient cells (Fig. 2 B). Because the activity of PLC- $\gamma$ 2 is critically regulated by its tyrosine phosphorylation, we evaluated the BCR-induced tyrosine phosphorylation status of PLC- $\gamma$ 2 in wild-type and Cbl-b-deficient cells. The results showed that Cbl-b-deficient cells exhibited markedly decreased levels of PLC- $\gamma$ 2 tyrosine phosphorylation (Fig. 2 C).

**Suppression of Signaling Downstream of PLC- $\gamma$ 2 in the Absence of Cbl-b.** As PLC- $\gamma$ 2-mediated generation of diacylglycerol results in the activation of Rap1 (31), we examined whether Rap1 activation is impaired in Cbl-b-deficient cells. BCR stimulation rapidly increased the amount of activated Rap1 in wild-type cells. However, Rap1 activation was suppressed or delayed in Cbl-b-deficient cells (Fig. 2 D), which suggests that PLC- $\gamma$ 2 activation was impaired in the absence of Cbl-b. PLC- $\gamma$ 2 is also implicated in the BCR-induced activation of MAPKs, ERK, JNK, and p38 (29). As shown in Fig. 2, E and F, the levels of BCR-induced activation of ERK2 and p38 were only slightly less in Cbl-b-deficient cells than in wild-type cells. In contrast, BCR-induced JNK activation was dramatically reduced by the loss of Cbl-b (Fig. 2 G). Because PLC- $\gamma$ 2 and JNK are relevant to apoptosis (35–37), we examined the possible involvement of Cbl-b in the BCR-induced apoptosis of DT40 cells. The population of apoptotic cells in the absence of Cbl-b was  $\sim 20\%$  less than that in the presence of Cbl-b, as measured by the level of endonucleolytically cleaved DNA (unpublished data). Thus, BCR-induced apoptosis was also suppressed, in part, in Cbl-b-deficient cells. Taken together, we conclude that BCR-induced tyrosine phosphorylation and the activation of PLC- $\gamma$ 2 are suppressed in the absence of Cbl-b.

**Effect of Cbl-b on the BCR-induced Activation of Syk and Btk.** It is possible that the BCR-induced activation of Syk and Btk is down-regulated in Cbl-b-deficient cells, which results in the attenuation of PLC- $\gamma$ 2 phosphorylation. To examine this possibility, the overall BCR-induced tyrosine phosphorylation pattern was first compared between wild-type and Cbl-b-deficient cells. As shown in



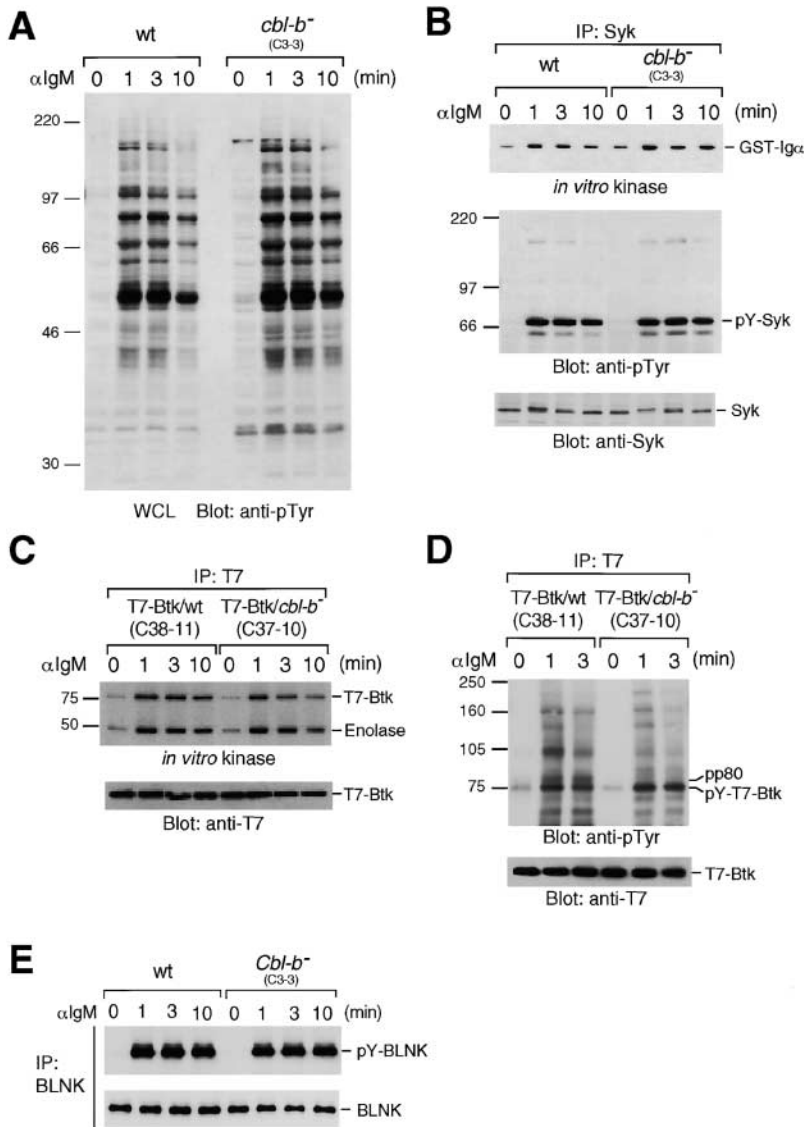
**Figure 2.** Characterization of the PLC- $\gamma$ 2/Ca<sup>2+</sup> signaling, Rap1 activation, and MAPKs activation in Cbl-b-deficient DT40 cells. (A) Ca<sup>2+</sup> mobilization in wild-type DT40 cells (red) and Cbl-b-deficient cells, and clones C3-1 (blue) and C3-3 (green). [Ca<sup>2+</sup>]<sub>i</sub> were monitored by spectrophotometer after stimulation with 2  $\mu$ g/ml M4 or 0.5  $\mu$ g/ml ionomycin. Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> store was monitored after stimulation with 2  $\mu$ g/ml M4 in the presence of 1 mM EGTA (+EGTA). Ca<sup>2+</sup> was restored to the media to evaluate extracellular Ca<sup>2+</sup> influx. Arrows indicate the time point of the addition of M4 ( $\alpha$  -  $\mu$ ), ionomycin, and CaCl<sub>2</sub>. Similar results were obtained using two other Cbl-b-deficient clones. (B) IP<sub>3</sub> production in wild-type and Cbl-b-deficient DT40 cells. 2  $\times$  10<sup>6</sup> cells were stimulated with 2  $\mu$ g M4 for the indicated time period and IP<sub>3</sub> production was measured. Results are the mean  $\pm$  SEM of three independent experiments. (C) Tyrosine phosphorylation of PLC- $\gamma$ 2 in wild-type and Cbl-b-deficient DT40 cells. 10<sup>7</sup> cells were lysed in 1% NP-40 lysis buffer at the indicated time points after stimulation of 4  $\mu$ g/ml M4. Anti-PLC- $\gamma$ 2 immunoprecipitates from the lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb (top) and anti-PLC- $\gamma$ 2 Ab (bottom). (D) Rap1 activation in wild-type and Cbl-b-deficient DT40 cells. 1.25  $\times$  10<sup>7</sup> cells were lysed in 1% NP-40 lysis buffer at the indicated time points after stimulation of 5  $\mu$ g/ml M4. Cell lysates were precipitated with GST-RalGDS(RBD) immobilized on glutathione Sepharose, and bound proteins were analyzed by anti-Rap1 immunoblotting. (E-G) BCR-induced ERK2, p38, and JNK activation in wild-type and Cbl-b-deficient DT40 cells. 2.5  $\times$  10<sup>7</sup> cells were lysed at the indicated time points after stimulation of 4  $\mu$ g/ml M4. (E) Anti-ERK2, (F) anti-p38, or (G) anti-JNK1 immunoprecipitates from the lysates

were subjected to kinase reaction using GST-Elk, GST-ATF2, or GST-c-Jun as an exogenous substrate, respectively. The protein levels in the immunoprecipitates are shown in E-G. All experiments were performed more than three times.

Fig. 3 A, the kinetics and extent of BCR-induced tyrosine phosphorylation of cellular proteins were nearly comparable, or the extent was marginally enhanced, in Cbl-b-deficient cells. BCR-induced activation of Syk and Btk was then examined *in vitro*. Because the Ab raised against chicken Btk does not immunoprecipitate Btk efficiently, we stably expressed a T7 epitope-tagged Btk (designated T7-Btk) in wild-type and Cbl-b-deficient cells. Clones expressing similar levels of T7-Btk and surface IgM were selected (Fig. 1 F). The activities and kinetics of Syk and Btk were nearly the same between the wild-type and Cbl-b-deficient cells (Fig. 3, B and C). Moreover, Syk and Btk were inducibly tyrosine phosphorylated to an almost similar extent in wild-type and Cbl-b-deficient cells (Fig. 3, B and D). Consistently, BCR-induced phosphorylation of BLNK, a prominent substrate of Syk (38), was almost comparable between wild-type and mutant cells (Fig. 3 E). Therefore, we concluded that the loss of Cbl-b had no no-

table effects on either Syk or Btk tyrosine kinase activities in stimulated B cells.

**Role of Cbl-b on the Association of Btk, BLNK, and PLC- $\gamma$ 2 in BCR Signaling.** BCR-induced activation of the Ca<sup>2+</sup> pathway requires Syk-dependent assembly of signaling components such as Btk, BLNK, and PLC- $\gamma$ 2 (39). Because Cbl-b contains various protein-protein interaction domains, it may serve as a scaffolding protein in BCR signaling. To evaluate this possibility, tyrosine-phosphorylated proteins coimmunoprecipitated with Syk or Btk were compared between wild-type and Cbl-b-deficient cells. We found no significant differences in the pattern of tyrosine-phosphorylated proteins coimmunoprecipitated with Syk between wild-type and Cbl-b-deficient cells (Fig. 3 B). In contrast, a tyrosine-phosphorylated protein, pp80, was coimmunoprecipitated with Btk in a Cbl-b-dependent manner after BCR stimulation (Fig. 3 D). Because the Btk SH2 domain interacts with phosphorylated BLNK in a



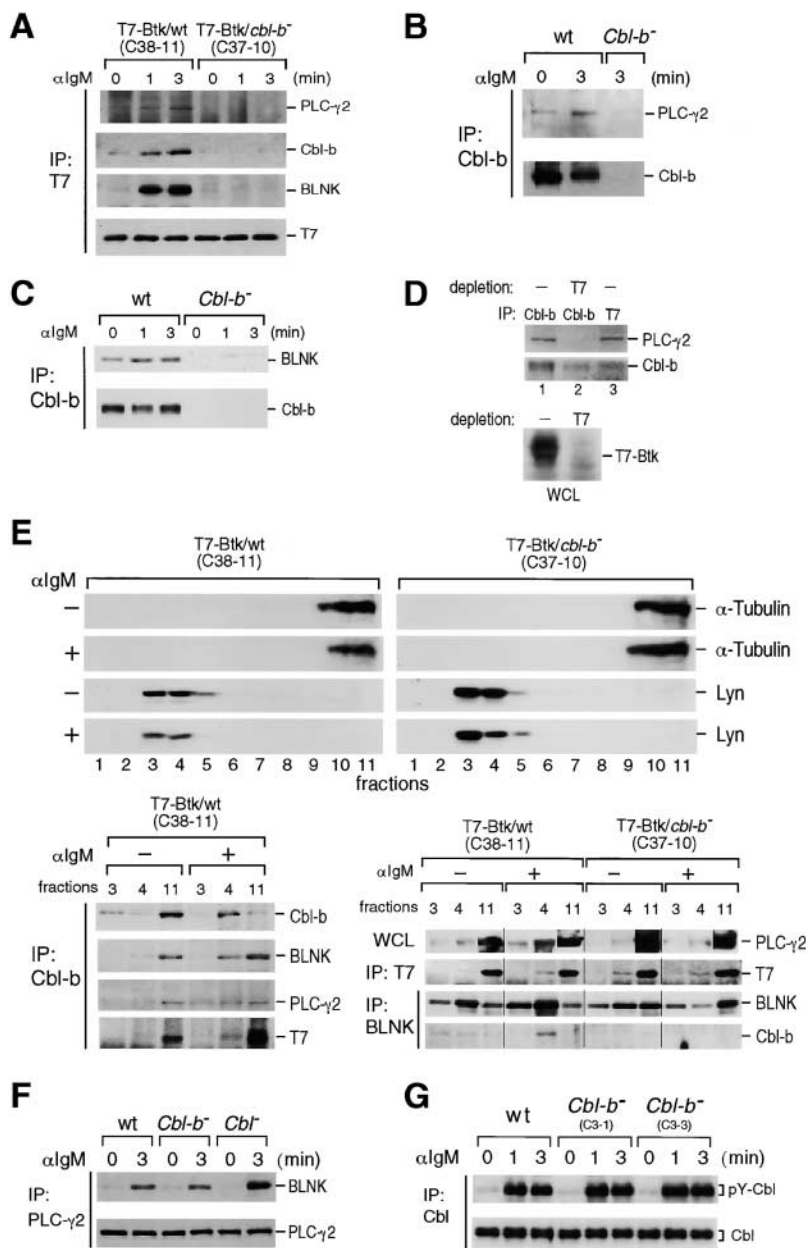
**Figure 3.** BCR-induced activation of Syk and Btk. (A) Tyrosine phosphorylation profiles of wild-type and Cbl-b-deficient (C3-3) DT40 cells. Whole cell lysates were prepared at the indicated time points after stimulation of 4  $\mu\text{g/ml}$  M4. The lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb. (B) Tyrosine phosphorylation and kinase activity of Syk. Wild-type or Cbl-b-deficient (C3-3) DT40 cells were lysed at the indicated time points after stimulation of 4  $\mu\text{g/ml}$  M4. Anti-Syk immunoprecipitates from the lysates were equally divided, and aliquots (one third of the lysates) were subjected to in vitro kinase assay with GST-Ig $\alpha$  as a substrate (top), and the remaining immunoprecipitates were analyzed by Western blot analysis with anti-phosphotyrosine mAb (middle) and anti-Syk Ab (bottom). (C) Kinase activity of Btk. Wild-type or Cbl-b-deficient DT40 cells expressing T7-tagged Btk were lysed at the indicated time points after stimulation of 4  $\mu\text{g/ml}$  M4. Anti-T7 immunoprecipitates from the lysates were equally divided, and half of them were subjected to in vitro kinase assay with enolase as a substrate (top), and the remaining half were analyzed by Western blot analysis with anti-T7 mAb (bottom). (D) Tyrosine phosphorylation of Btk. Wild-type or Cbl-b-deficient DT40 cells expressing T7-tagged Btk were lysed in 0.2% NP-40 lysis buffer at the indicated time points after stimulation of 4  $\mu\text{g/ml}$  M4. Anti-T7 immunoprecipitates from the lysates were used for Western blot analysis with anti-phosphotyrosine mAb (top) and anti-T7 mAb (bottom). (E) Tyrosine phosphorylation of BLNK. Wild-type or Cbl-b-deficient (C3-3) DT40 cells ( $5 \times 10^6$ ) were lysed in 1% NP-40 lysis buffer at the indicated time points after stimulation of 4  $\mu\text{g/ml}$  M4. Anti-BLNK immunoprecipitates from the lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb (top) and anti-BLNK Ab (bottom). All experiments were performed more than three times.

BCR-dependent manner, and chicken BLNK is estimated to be 80 kD (25, 40), we assumed that the 80-kD protein might be BLNK. Indeed, BCR-induced association of Btk with BLNK was inhibited by the loss of Cbl-b (Fig. 4 A), despite comparable levels of BLNK phosphorylation in the wild-type and Cbl-b-deficient cells (Fig. 3 E). Phosphorylated BLNK binds to PLC- $\gamma$ 2 and then the resultant BLNK-PLC- $\gamma$ 2 complex translocates to the membrane, which leads to PLC- $\gamma$ 2 activation (25, 41). The recruitment of PLC- $\gamma$ 2 to Btk was also impaired in Cbl-b-deficient cells (Fig. 4 A). Thus, Cbl-b positively regulates the recruitment of both BLNK and PLC- $\gamma$ 2 to close proximity of Btk in activated B cells.

*BCR-dependent Interaction of Cbl-b with Btk, BLNK, and PLC- $\gamma$ 2, and Their Translocation to GEMs.* We then addressed whether Cbl-b forms a signaling complex with Btk, BLNK, and PLC- $\gamma$ 2. As shown in Fig. 4, A-C, Cbl-b associated with Btk, BLNK, and PLC- $\gamma$ 2 in the BCR-stimulated cells expressing T7-tagged Btk. The immuno-

depletion of Btk resulted in a 30–50% decrease in the amount of Cbl-b in the anti-Cbl-b immunoprecipitates. Furthermore, the interaction between Cbl-b and PLC- $\gamma$ 2 was completely abrogated by Btk immunoprecipitation (Fig. 4 D). These results indicate that BCR-induced Btk-Cbl-b complex also includes PLC- $\gamma$ 2 as a component. Recent evidence shows that the activation of immune receptors, such as BCR, TCR, and FcR, is followed by the accumulation of tyrosine-phosphorylated receptors and the associated signaling molecules in the GEMs (42–45). The disruption of GEMs results in the attenuation of antigen receptor-mediated calcium mobilization. Therefore, we examined whether Cbl-b and its associated proteins are recruited to GEMs after BCR stimulation. Fractionation of the cell lysates in sucrose density gradients showed that Lyn was enriched in Triton-insoluble GEMs (fractions 3 and 4) and  $\alpha$ -tubulin in Triton-soluble fractions (fractions 9–11; Fig. 4 E, top). A small fraction of Cbl-b was present in the GEMs of unstimulated cells. Interestingly, the pro-





precipitates from the lysates were used for Western blot analysis with anti-BLNK Ab (top) and anti-PLC- $\gamma$ 2 Ab (bottom). (G) Tyrosine phosphorylation of Cbl in wild-type and Cbl-b-deficient DT40 cells.  $10^7$  cells were lysed in 1% NP-40 lysis buffer at the indicated time points after stimulation of 4  $\mu$ g/ml M4. Anti-Cbl immunoprecipitates from the lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb (top) and anti-Cbl Ab (bottom). All experiments were performed more than three times.

portion of Cbl-b in GEMs greatly increased after BCR stimulation (Fig. 4 E, bottom left). Small but significant amounts of PLC- $\gamma$ 2, BLNK, and Btk in GEMs were also increased after BCR stimulation in the presence but not the absence of Cbl-b (Fig. 4 E, bottom right). Importantly, these proteins in GEMs were coimmunoprecipitated with Cbl-b. These data suggest that Cbl-b translocates to GEMs in a manner dependent on BCR stimulation. Cbl-b in GEMs likely serves as a scaffold protein for Btk, BLNK, and PLC- $\gamma$ 2, resulting in the activation of PLC- $\gamma$ 2.

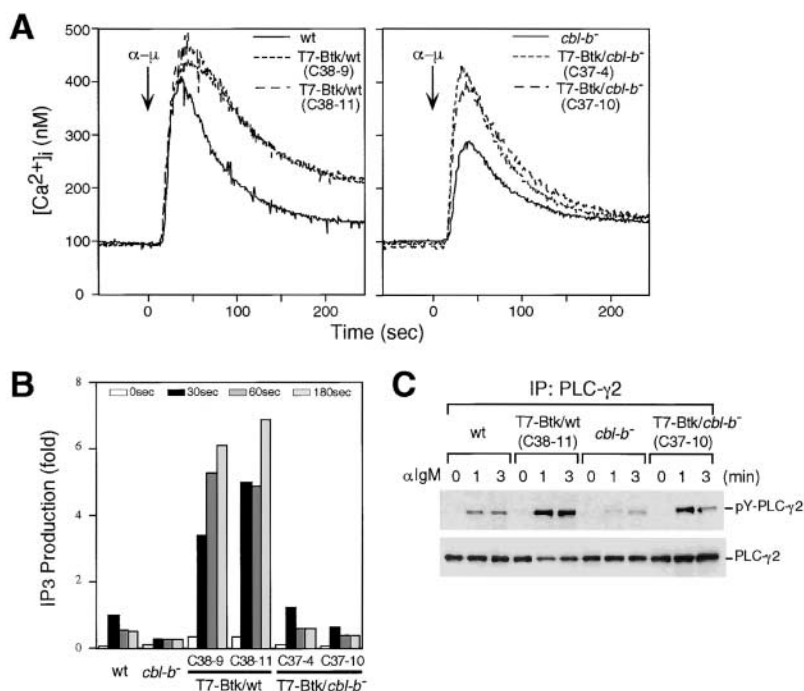
We have previously shown that Cbl directly binds to phosphorylated BLNK via its TKB domain. This binding inhibits interaction between BLNK and PLC- $\gamma$ 2, and subsequent PLC- $\gamma$ 2 activation (18). Despite the high homology between Cbl and Cbl-b (82% identity in the amino acid sequences at the TKB domains), the BLNK-PLC- $\gamma$ 2 interaction was not affected by the loss of Cbl-b (Fig. 4 F). Both the protein level and tyrosine phosphorylation status of Cbl were not affected by the absence of Cbl-b (Fig. 4 G). These data suggest that Cbl and Cbl-b have distinct roles in the regulation of PLC- $\gamma$ 2.

**Regulation of Btk-dependent Sustained  $Ca^{2+}$  Flux by Cbl-b.** To further confirm that Cbl-b positively regulates Btk-mediated PLC- $\gamma$ 2 activation, we examined the role of Cbl-b in Btk-dependent intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) increase upon BCR stimulation. Consistent with the findings of Takata and Kurosaki (11) and Fluckiger et al. (46), the overexpression of Btk in wild-type DT40 cells resulted in an enhanced and sustained increase in  $[Ca^{2+}]_i$  after BCR stimulation. In contrast, the overexpression of Btk in Cbl-b-deficient cells induced an initial but not sustained increase in  $[Ca^{2+}]_i$ . Within 4 min after BCR stimulation,  $[Ca^{2+}]_i$  of these cells decreased to a level comparable with that in parental Cbl-b-deficient cells (Fig. 5 A). The production of IP<sub>3</sub> and the tyrosine phosphorylation of PLC- $\gamma$ 2 were enhanced and sustained in wild-type DT40 cells overexpressing Btk, but those in Cbl-b-deficient cells overexpressing Btk were transient (Fig. 5, B and C). Thus, we concluded that Cbl-b is a positive regulator of Btk-dependent activation of PLC- $\gamma$ 2/Ca<sup>2+</sup> signaling.

**Requirement of the TKB Domain and COOH-terminal Region of Cbl-b for Cbl-b-dependent  $[Ca^{2+}]_i$  Elevation.** To determine the region(s) within Cbl-b that is responsible for the regulation of PLC- $\gamma$ 2/Ca<sup>2+</sup> signaling, we generated stable clones expressing the wild-type or mutant Cbl-b protein in Cbl-b-deficient cells (Fig. 6 A). Clones expressing similar amounts of Cbl-b and surface IgM were selected (Fig. 1 F and Fig. 6 B). The level of BCR-induced tyrosine phosphorylation of Cbl-b was not affected much by the mutations in the TKB or RING finger domain, but completely abolished by COOH-terminal deletion (Fig. 6 B). Upon BCR stimulation, Cbl-b-deficient cells expressing wild-type Cbl-b (WT/*cbl-b*<sup>-</sup>) showed enhanced and sustained Ca<sup>2+</sup> mobilization in comparison to that of parental

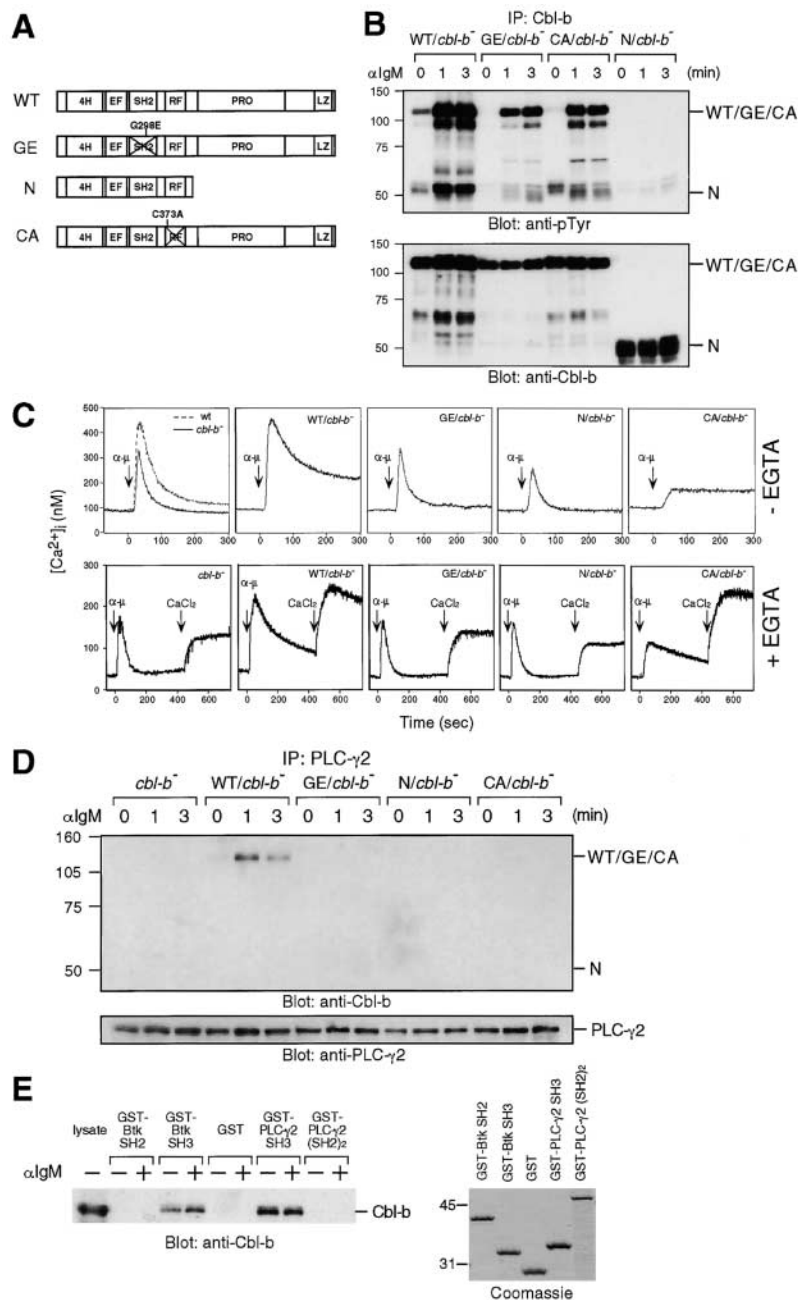
Cbl-b-deficient cells, confirming the positive role of Cbl-b in PLC- $\gamma$ 2/Ca<sup>2+</sup> signaling (Fig. 6 C). The expression of a TKB mutant (Gly<sup>298</sup> to Glu in the SH2 domain, GE), or a COOH-terminal deletion mutant (1–444 amino acids, N), did not restore the defect of Cbl-b-deficient cells in BCR-induced Ca<sup>2+</sup> mobilization (Fig. 6 C). The Ca<sup>2+</sup> mobilization was also not restored in Cbl-b-deficient cells expressing a RING finger mutant (Cys<sup>373</sup> to Ala, CA). Intriguingly, however, a low level of sustained Ca<sup>2+</sup> mobilization was observed in the CA mutant-expressing cells. It should be noted that the introduction of wild-type or CA mutant Cbl-b into Cbl-b-deficient cells resulted in an excessive increase of extracellular Ca<sup>2+</sup> influx (Fig. 6 C, bottom), which might be related to the sustained Ca<sup>2+</sup> mobilization seen in these cells. The mechanism underlying the increased influx remains to be addressed. In contrast to wild-type Cbl-b, none of these Cbl-b mutants interacted with PLC- $\gamma$ 2 upon BCR stimulation (Fig. 6 D). These results show that the TKB domain, the COOH-terminal region, and the RING finger domain of Cbl-b are important for Btk-mediated PLC- $\gamma$ 2 activation. In vitro GST pull-down experiments revealed that the SH3 but not the SH2 domains of Btk and PLC- $\gamma$ 2 interacted with Cbl-b from the B cells with or without BCR stimulation (Fig. 6 E). These data suggest that Btk and PLC- $\gamma$ 2 could interact with Cbl-b via its SH3 domains in direct or indirect mechanisms. The mode of interaction through the TKB domain likely requires the modification or structural alteration of Cbl-b (see Discussion).

**Effect of Cbl-b on BCR-mediated Calcium Signaling in Mouse B Cells.** To examine whether or not Cbl-b positively regulates PLC- $\gamma$ /Ca<sup>2+</sup> signaling, we generated WEHI-231 mouse B cell lines overexpressing Cbl-b,



**Figure 5.** Role of Cbl-b on Btk-dependent Ca<sup>2+</sup> mobilization, IP<sub>3</sub> production, and tyrosine phosphorylation of PLC- $\gamma$ 2. Wild-type cells, Cbl-b-deficient cells, wild-type cells expressing T7-tagged Btk (C38-9 and C38-11), and Cbl-b-deficient cells expressing T7-tagged Btk (C37-4 and C37-10) were analyzed for (A) BCR-induced Ca<sup>2+</sup> mobilization, (B) IP<sub>3</sub> production, and (C) tyrosine phosphorylation of PLC- $\gamma$ 2, as described in Fig. 2. Similar results were obtained using two other Cbl-b-deficient clones. All experiments were performed more than three times.



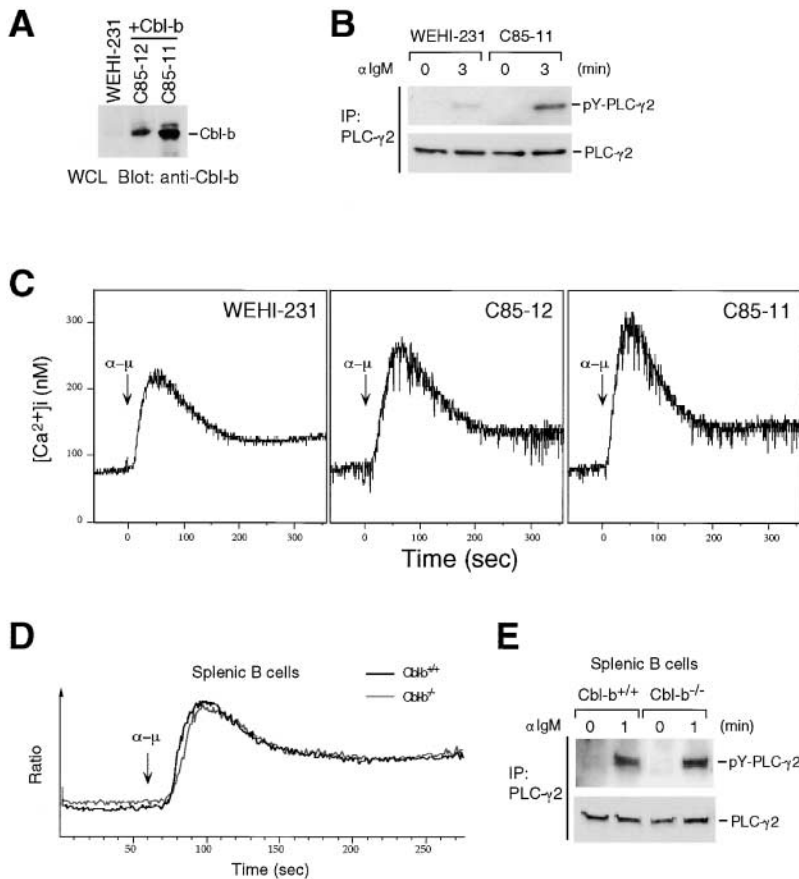


**Figure 6.** Effects of mutations in Cbl-b on BCR-induced Ca<sup>2+</sup> mobilization. (A) Schematic representation of the wild-type and mutant Cbl-b proteins. 4H, four-helix bundle; EF, EF hand; SH2, Src homology 2; RF, RING finger; PRO, proline-rich region; LZ, leucine zipper. (B) Expression and tyrosine phosphorylation of wild-type or mutant Cbl-b in Cbl-b-deficient cells. The indicated cells were lysed in 1% NP-40 lysis buffer at 0, 1, and 3 min after stimulation of 4 μg/ml M4. Anti-Cbl-b (G-1) immunoprecipitates from the lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb (top) and anti-Cbl-b (G-1) mAb (bottom). The positions of WT, GE, N, and CA Cbl-b are indicated. (C) BCR-induced Ca<sup>2+</sup> mobilization in wild-type cells, Cbl-b-deficient cells (C3-3), and Cbl-b-deficient cells expressing wild-type Cbl-b (WT/*cbl-b*<sup>-</sup>), G298E Cbl-b (GE/*cbl-b*<sup>-</sup>), a COOH-terminal deletion mutant (N/*cbl-b*<sup>-</sup>), or C373A Cbl-b (CA/*cbl-b*<sup>-</sup>). [Ca<sup>2+</sup>]<sub>i</sub> were monitored by spectrophotometer after stimulation with 2 μg/ml M4 (−EGTA). Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> store was monitored after stimulation of 2 μg/ml M4 in the presence of 1 mM EGTA (+EGTA), and Ca<sup>2+</sup> was restored to the media to evaluate extracellular Ca<sup>2+</sup> influx. Arrows indicate the time point of the addition of M4 (α − μ) and CaCl<sub>2</sub>. The representative results from four independent clones of each cell type are shown. (D) Association of Cbl-b with PLC-γ2. The indicated cells were lysed in 1% NP-40 lysis buffer at 0, 1, and 3 min after stimulation of 4 μg/ml M4. Anti-PLC-γ2 immunoprecipitates from the lysates were subjected to Western blot analysis with anti-Cbl-b (G-1) mAb (top) and anti-PLC-γ2 Ab (bottom). The positions of WT, GE, N, and CA Cbl-b are indicated. (E) In vitro binding of Cbl-b with the SH3 domains of Btk and PLC-γ2. WEHI-231 cells overexpressing Cbl-b (C85-11) and unstimulated (−) or stimulated (+) with anti-IgM (10 μg/10<sup>7</sup> cells) for 3 min, were lysed in 1% NP-40 lysis buffer, and the lysates were incubated with the indicated GST fusion proteins immobilized on glutathione Sepharose. The bound proteins or lysates (1/10 input) were separated by SDS-PAGE and immunoblotted with anti-Cbl-b (G-1) mAb (left). Expression of the GST fusion proteins was confirmed by Coomassie staining (right). (C–E) Experiments were performed more than three times.

termed C85-11 and C85-12 (Fig. 7 A). As shown in Fig. 7, B and C, BCR stimulation of these cells resulted in enhanced PLC-γ2 phosphorylation and Ca<sup>2+</sup> mobilization. Therefore, Cbl-b regulates PLC-γ2/Ca<sup>2+</sup> signaling positively in mouse B cells as well. Unexpectedly, however, the extent of calcium flux (Fig. 7 D) and tyrosine phosphorylation of PLC-γ2 (Fig. 7 E) in BCR-stimulated splenic B cells from Cbl-b-deficient mice was similar to that in wild-type mice. These data suggest the presence of compensatory mechanisms for the PLC-γ/Ca<sup>2+</sup> signaling pathway in mouse splenic B cells (see Discussion).

## Discussion

Cbl family proteins Cbl and Cbl-b have been implicated in signal transduction of lymphocyte antigen receptors (13). In this study, we show that both Lyn and Syk are required for tyrosine phosphorylation of Cbl-b after BCR stimulation. Because Syk, but not Lyn, is dispensable for BCR-induced tyrosine phosphorylation of Cbl (34), tyrosine phosphorylation of Cbl and Cbl-b induced by BCR stimulation is likely to be regulated by distinct mechanisms. We show that Cbl-b positively regulates BCR-induced PLC-γ2/Ca<sup>2+</sup> signaling, and provide evidence suggesting that Cbl-b functions as a scaffold protein to promote Btk-mediated PLC-γ2 activation.



**Figure 7.** Role of Cbl-b on the PLC- $\gamma$ 2/ $\text{Ca}^{2+}$  pathway in mouse B cells. (A) Whole cell lysate ( $5 \times 10^5$  cells) from parental and Cbl-b-overexpressing (C85-11 and C85-12) WEHI-231 mouse B cells were analyzed by Western blot analysis using anti-Cbl-b (G-1) mAb. (B) Parental and Cbl-b-overexpressing (C85-11) WEHI-231 cells ( $3 \times 10^6$ ) were stimulated with anti-IgM ( $10 \mu\text{g}/10^7$  cells) for 3 min. Anti-PLC- $\gamma$ 2 immunoprecipitates from the lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb (top) and anti-PLC- $\gamma$ 2 mAb (bottom). (C) BCR-induced  $\text{Ca}^{2+}$  mobilization in parental and Cbl-b-overexpressing (C85-11 and C85-12) WEHI-231 cells.  $[\text{Ca}^{2+}]_i$  were monitored by spectrophotometer after stimulation with  $2 \mu\text{g}/\text{ml}$  anti-IgM. Arrows indicate the time point of the addition of anti-IgM ( $\alpha - \mu$ ). (D) BCR-induced  $\text{Ca}^{2+}$  mobilization in B cells from wild-type (Cbl-b $^{+/+}$ ) and Cbl-b-deficient (Cbl-b $^{-/-}$ ) mice. Splenocytes were loaded with Indo-1, labeled with B220-FITC, and stimulated with anti-IgM ( $10 \mu\text{g}/10^7$  cells). Data are presented as the median ratio of  $\text{Ca}^{2+}$ -free Indo-1 fluorescence of B220 $^{+}$  cells as measured by flow cytometry. (E)  $3 \times 10^6$  splenic B cells of wild-type (Cbl-b $^{+/+}$ ) or Cbl-b-deficient (Cbl-b $^{-/-}$ ) mice were stimulated with anti-IgM for 1 min. Anti-PLC- $\gamma$ 2 immunoprecipitates from the lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb (top) and anti-PLC- $\gamma$ 2 mAb (bottom). All experiments were performed more than three times.

Syk and Btk are required for tyrosine phosphorylation and activation of PLC- $\gamma$ 2. A previous report shows that the Syk-related protein Zap-70 is positively regulated by Cbl-b in Jurkat T cells (47). Therefore, it is possible that Syk activity is suppressed in the absence of Cbl-b. However, this is unlikely because in vitro kinase activities and tyrosine phosphorylation of Syk were nearly the same between wild-type and Cbl-b-deficient cells. Consistently, recent findings show no significant differences in the TCR-induced tyrosine phosphorylation of Zap-70 and Lck in T cells from Cbl-b-deficient mice (17, 23). We also show that Btk activity is not affected in the absence of Cbl-b.

BCR-induced activation of the PLC- $\gamma$ 2/ $\text{Ca}^{2+}$  pathway requires the assembly of signaling components Btk, BLNK, and PLC- $\gamma$ 2. A crucial adaptor protein, BLNK, couples Syk and Btk to PLC- $\gamma$ 2 activation in B cells (39). A possible explanation for the attenuated phosphorylation of PLC- $\gamma$ 2 in Cbl-b-deficient cells is that Cbl-b acts as a scaffolding protein to promote a complex formation including PLC- $\gamma$ 2. In accordance with this idea, our present data strongly argues that Cbl-b contributes to complex formation among Btk, BLNK, and PLC- $\gamma$ 2, which leads to the activation of PLC- $\gamma$ 2. GEMs have been proposed to function as platforms for the formation of multicomponent signaling complexes (48). Our data in Fig. 4 suggest that BCR stimulation induces the translocation of Cbl-b, Btk, BLNK, and PLC- $\gamma$ 2 to GEMs where Cbl-b interacts with these pro-

teins. Because the translocation of PLC- $\gamma$  to GEMs is essential for its tyrosine phosphorylation and subsequent activation in antigen receptor signaling (43, 44), Cbl-b-dependent translocation of Btk, BLNK, and PLC- $\gamma$ 2 into GEMs suggests the importance of Cbl-b in the regulation of PLC- $\gamma$ 2/ $\text{Ca}^{2+}$  signaling (Fig. 4). Overexpression of Btk in B cells significantly enhanced the sustained increase in  $[\text{Ca}^{2+}]_i$  after BCR activation (Fig. 5; references 11 and 46). In Cbl-b-deficient B cells, however, Btk overexpression only induced a transient increase of  $[\text{Ca}^{2+}]_i$  in the early phase after BCR stimulation (Fig. 5). Obviously, Cbl-b is important for the sustained activation of PLC- $\gamma$ 2 through Btk that is likely to occur in GEMs.

Here we show that Cbl-b-dependent positive regulation of  $\text{Ca}^{2+}$  mobilization requires its TKB domain and COOH-terminal half, and that these domains are essential for its interaction with PLC- $\gamma$ 2. Although Cbl suppresses BCR-induced PLC- $\gamma$ 2 activation by inhibiting BLNK-PLC- $\gamma$ 2 interaction (18), Cbl-b does not inhibit this interaction. These differential effects on the binding of PLC- $\gamma$ 2 to BLNK could be due to the sequence divergence at the COOH-terminal region between Cbl and Cbl-b, and would account for the functional difference between the two proteins. In vitro binding analysis revealed that the SH3 domain, but not the SH2 domain, of both Btk and PLC- $\gamma$ 2 interacts with Cbl-b in the absence of BCR stimulation. However, the interaction of Cbl-b with Btk,

BLNK, and PLC- $\gamma$ 2 occurred in a manner dependent on BCR stimulation, which suggests that tyrosine phosphorylation events are required for the interaction *in vivo*. Because the NH<sub>2</sub>-terminal 418-amino acid sequence of Cbl-b, including the TKB domain, did not bind to either Btk, BLNK, or PLC- $\gamma$ 2 *in vitro* (unpublished data), the binding of Cbl-b TKB domain to target proteins may require its structural modification, such as tyrosine phosphorylation and the help of the COOH-terminal sequence of Cbl-b.

Unlike in DT40 B cells, the extent of tyrosine phosphorylation of PLC- $\gamma$ 2 and calcium flux in BCR-stimulated splenic B cells from Cbl-b-deficient mice was similar to that from wild-type mice (Fig. 7). This is unlikely to be due to the difference in the mechanisms underlying PLC- $\gamma$ 2/Ca<sup>2+</sup> signaling between mouse and chicken, because the overexpression of Cbl-b enhanced tyrosine phosphorylation of PLC- $\gamma$ 2 as well as Ca<sup>2+</sup> mobilization in WEHI-231 mouse B cells. Intriguingly, TCR-induced tyrosine phosphorylation of PLC- $\gamma$ 1 is unaffected in the absence of Cbl-b (17, 23). In contrast, TCR-induced tyrosine phosphorylation of PLC- $\gamma$ 1 is reduced in Cbl-deficient T cells (22). These data suggest that both Cbl and Cbl-b could positively regulate PLC- $\gamma$ /Ca<sup>2+</sup> signaling and Cbl compensates antigen receptor-mediated activation of PLC- $\gamma$  in lymphocytes of Cbl-b-deficient mice. Indeed, TCR-stimulated tyrosine phosphorylation of PLC- $\gamma$ 1 and Ca<sup>2+</sup> mobilization were severely impaired in mouse T cells lacking both Cbl and Cbl-b (unpublished data). It should also be noted that Cbl-b-deficient lymphocytes are hyperproliferative in the mouse system despite that PLC- $\gamma$ /Ca<sup>2+</sup> signaling is unaffected in these lymphocytes (Fig. 7; references 17 and 23). Moreover, mouse T cells lacking both Cbl and Cbl-b were hyperproliferative upon TCR stimulation, even in the impairment of PLC- $\gamma$ 1/Ca<sup>2+</sup> signaling (unpublished data). Taken together, we assume that PLC- $\gamma$ /Ca<sup>2+</sup> signaling is regulated by Cbl family proteins and that it may not necessarily be linked to the proliferative activity of lymphocytes *in vivo*. It appears that mouse B cells deficient for Cbl-b are hyperproliferative upon BCR stimulation due to yet unknown mechanisms downstream of Cbl-b deficiency. The PI3K pathway may be negatively regulated by Cbl-b in B cells, like in T cells (24), which would, at least in part, explain the hyperproliferation of Cbl-b-deficient splenic B cells. In addition, consistent with the enhanced phosphorylation of Vav1 and Vav2 in TCR-stimulated Cbl-b-deficient thymocytes (23, 49), we observed enhanced tyrosine phosphorylation of Vav2 in BCR-stimulated Cbl-b-deficient DT40 B cells (unpublished data). Because Vav1 and Vav2 are indispensable for BCR-stimulated B cell proliferation (50), the hyperproliferative nature of Cbl-b-deficient B cells may due to the hyperactivation of Vav. It is also possible that the internalization of activated surface BCR is suppressed in the absence of Cbl-b. This would extend the duration of BCR signaling, which results in enhanced B cell proliferation.

Cbl has the RING-type ubiquitin protein ligase activity and promotes the ubiquitination of activated receptor PTKs such as EGFR (20). Cbl-b promotes the ubiquitina-

tion of EGFR and the p85 regulatory subunit of PI3K (20, 24). However, the protein levels of Syk, Btk, BLNK, and PLC- $\gamma$ 2 are not affected in Cbl- (18) or Cbl-b-deficient (Figs. 2 and 3) DT40 B cells. In addition, in both Cbl- and Cbl-b-deficient T cells, the protein levels of Zap-70, Lck, Slp-76, LAT, PLC- $\gamma$ 1, and Vav1 are also not affected before or after TCR stimulation (17, 21–23). These data suggest that Cbl family-dependent ubiquitination and subsequent proteolysis of these signaling proteins are less likely to be involved in antigen receptor-mediated signaling. Because a RING finger mutant of Cbl-b (Cys<sup>373</sup> to Ala) did not bind to PLC- $\gamma$ 2 upon BCR stimulation, the RING finger domain of Cbl family proteins may be used to regulate protein-protein interaction that are relevant to Ca<sup>2+</sup> flux (Fig. 6).

Cbl negatively regulates various tyrosine kinase signaling pathways such as EGFR (20), Zap-70 (51), and Syk (52). Cbl, on the other hand, positively regulates IL-4 receptor signaling (53),  $\beta_1$  integrin signaling in macrophage (54), c-Src-mediated signaling in bone resorption (55), and TNF receptor superfamily signaling (56). Our present findings show that Cbl-b can positively regulate BCR-induced PLC- $\gamma$ 2/Ca<sup>2+</sup> signaling. Similarly, TCR-induced PLC- $\gamma$ /Ca<sup>2+</sup> signaling appears to be positively regulated by Cbl-b as well as by Cbl, as is evidenced by the analysis of T cell signaling in mice lacking Cbl and/or Cbl-b (17, 22, 23, and unpublished data). It should be noted that Cbl is involved in ITAM-based signaling and forms a signaling complex containing SLP-76 upon Fc $\gamma$ RI stimulation of myeloid cells (19). Likewise, Cbl-b may be involved in the ITAM signaling downstream of Fc $\gamma$ R, Fc $\epsilon$ R, as well as BCR. Thus, Cbl family proteins play diverse roles depending upon the signaling pathways in which they are involved. The balance between the levels of Cbl and Cbl-b, as well as the dynamics of their compartmentalization in signaling complexes, would be important for the surface receptor-mediated activation of hematopoietic cells.

We would like to thank M. Kurosaki, K. Gotoh, and K. Nakamura for technical assistance; Drs. K. Holmes and D. Stephany for FACS<sup>®</sup> analyses; Drs. M. Ishiai, I. Jang, M. Kim, K. Hironaka for critical discussions; and K. Yokoyama and members of the Department of Oncology for suggestions and support.

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

Submitted: 15 January 2002

Revised: 29 April 2002

Accepted: 14 May 2002

## References

1. Healy, J.I., and C.C. Goodnow. 1998. Positive versus negative signaling by lymphocyte antigen receptors. *Annu. Rev. Immunol.* 16:645–670.
2. Rajewsky, K. 1996. Clonal selection and learning in the antibody system. *Nature.* 381:751–758.
3. Reth, M., and J. Wienands. 1997. Initiation and processing of



- signals from the B cell antigen receptor. *Annu. Rev. Immunol.* 15:453–479.
4. Reth, M. 1989. Antigen receptor tail clue. *Nature.* 338:383–384.
  5. Cambier, J.C. 1995. New nomenclature for the Reth motif (or ARH1/TAM/ARAM/YXXL). *Immunol. Today.* 16:110.
  6. DeFranco, A.L. 1997. The complexity of signaling pathways activated by the BCR. *Curr. Opin. Immunol.* 9:296–308.
  7. Tamir, I., and J.C. Cambier. 1998. Antigen receptor signaling: integration of protein tyrosine kinase functions. *Oncogene.* 17:1353–1364.
  8. Kurosaki, T. 1999. Genetic analysis of B cell antigen receptor signaling. *Annu. Rev. Immunol.* 17:555–592.
  9. Coggeshall, K.M., J.C. McHugh, and A. Altman. 1992. Predominant expression and activation-induced tyrosine phosphorylation of phospholipase C- $\gamma$ 2 in B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 89:5660–5664.
  10. Takata, M., H. Sabe, A. Hata, T. Inazu, Y. Homma, T. Nukada, H. Yamamura, and T. Kurosaki. 1994. Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca<sup>2+</sup> mobilization through distinct pathways. *EMBO J.* 13:1341–1349.
  11. Takata, M., and T. Kurosaki. 1996. A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C- $\gamma$ 2. *J. Exp. Med.* 184:31–40.
  12. Kurosaki, T., A. Maeda, M. Ishiai, A. Hashimoto, K. Inabe, and M. Takata. 2000. Regulation of the phospholipase C- $\gamma$ 2 pathway in B cells. *Immunol. Rev.* 176:19–29.
  13. Thien, C.B., and W.Y. Langdon. 2001. Cbl: many adaptations to regulate protein tyrosine kinases. *Nat. Rev. Mol. Cell Biol.* 2:294–307.
  14. Lupper, M.L., N. Rao, M.J. Eck, and H. Band. 1999. The Cbl proto-oncoprotein: a negative regulator of immune receptor signal transduction. *Immunol. Today.* 20:375–382.
  15. Kim, M., T. Tezuka, Y. Suziki, S. Sugano, M. Hirai, and T. Yamamoto. 1999. Molecular cloning and characterization of a novel cbl-family gene, *cbl-c*. *Gene.* 239:145–154.
  16. Meng, W., S. Sawadkisol, S.J. Burakoff, and M.J. Eck. 1999. Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase. *Nature.* 398:84–90.
  17. Bachmaier, K., C. Krawczyk, I. Kozieradzki, Y.Y. Kong, T. Sasaki, A. Oliveira-dos-Santos, S. Mariathasan, D. Bouchard, A. Wakeham, A. Itie, et al. 2000. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature.* 403:211–216.
  18. Yasuda, T., A. Maeda, M. Kurosaki, T. Tezuka, K. Hironaka, T. Yamamoto, and T. Kurosaki. 2000. Cbl suppresses B cell receptor-mediated phospholipase C (PLC)- $\gamma$ 2 activation by regulating B cell linker protein-PLC- $\gamma$ 2 binding. *J. Exp. Med.* 191:641–650.
  19. Chu, J., Y. Liu, G.A. Koretzky, and D.L. Durden. 1998. SLP-76-Cbl-Grb2-Shc interactions in Fc $\gamma$ RI signaling. *Blood.* 92:1697–1706.
  20. Levkowitz, G., H. Waterman, S.A. Ettenberg, M. Katz, A.Y. Tsygankov, I. Alroy, S. Lavi, K. Iwai, Y. Reiss, A. Ciechanover, et al. 1999. Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell.* 4:1029–1040.
  21. Murphy, M.A., R.G. Schnall, D.J. Venter, L. Barnett, I. Bertonecello, C.B.F. Thien, W.Y. Langdon, and D.D.L. Bowtell. 1998. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. *Mol. Cell. Biol.* 18:4872–4882.
  22. Naramura, M., H.K. Kole, R.J. Hu, and H. Gu. 1998. Altered thymic positive selection and intracellular signals in Cbl-deficient mice. *Proc. Natl. Acad. Sci. USA.* 95:15547–15552.
  23. Chiang, Y.J., H.K. Kole, K. Brown, M. Naramura, S. Fukuhara, R.J. Hu, I.K. Jang, J.S. Gutkind, E. Shevach, and H. Gu. 2000. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature.* 403:216–220.
  24. Fang, D., and Y.C. Liu. 2001. Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. *Nat. Immunol.* 2:870–875.
  25. Ishiai, M., M. Kurosaki, R. Pappu, K. Okawa, I. Ronko, C. Fu, M. Shibata, A. Iwamatsu, A.C. Chan, and T. Kurosaki. 1999. BLNK required for coupling Syk to PLC $\gamma$ 2 and Rac1-JNK in B cells. *Immunity.* 10:117–125.
  26. Chen, C.L.H., J.E. Lehmeyer, and M.D. Cooper. 1982. Evidence for an IgD homologue on chicken lymphocytes. *J. Immunol.* 129:2580–2585.
  27. Keane, M.M., O.M. Rivero-Lezcano, J.A. Mitchell, K.C. Robbins, and S. Lipkowitz. 1995. Cloning and characterization of cbl-b: A SH3 binding protein with homology to the c-cbl proto-oncogene. *Oncogene.* 10:2367–2377.
  28. Kurosaki, T., and M. Kurosaki. 1997. Transphosphorylation of Btk on tyrosine 551 is critical for B cell antigen receptor function. *J. Biol. Chem.* 272:15595–15598.
  29. Hashimoto, A., H. Okada, A. Jiang, M. Kurosaki, S. Greenberg, E.A. Clark, and T. Kurosaki. 1998. Involvement of guanosine triphosphatases and phospholipase C- $\gamma$ 2 in extracellular signal-regulated kinase, c-Jun NH<sub>2</sub>-terminal kinase, and p38 mitogen-activated protein kinase activation by the B cell antigen receptor. *J. Exp. Med.* 188:1287–1295.
  30. Saouaf, S.J., S. Mahajan, R.B. Rowley, S.A. Kut, J. Fargnoli, A.L. Burkhardt, S. Tsukada, O.N. Witte, and J.B. Bolen. 1994. Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. *Proc. Natl. Acad. Sci. USA.* 91:9524–9528.
  31. McLeod, S.J., R.J. Ingham, J.L. Bos, T. Kurosaki, and M.R. Gold. 1998. Activation of the Rap1 GTPase by the B cell antigen receptor. *J. Biol. Chem.* 273:29218–29223.
  32. Elly, C., S. Witte, Z. Zhang, O. Rosnet, S. Lipkowitz, A. Altman, and Y.C. Liu. 1999. Tyrosine phosphorylation and complex formation of Cbl-b upon T cell receptor stimulation. *Oncogene.* 18:1147–1156.
  33. Lavagna-Sevenier, C., S. Marchetto, D. Birnbaum, and O. Rosnet. 1998. The CBL-related protein CBLB participates in FLT3 and interleukin-7 receptor signal transduction in pro-B cells. *J. Biol. Chem.* 273:14962–14967.
  34. Tezuka, T., H. Umemori, N. Fusaki, T. Yagi, M. Takata, T. Kurosaki, and T. Yamamoto. 1996. Physical and functional association of the cbl proto-oncogene product with an Src-family protein tyrosine kinase, p53/56<sup>lck</sup>, in the B cell antigen receptor-mediated signaling. *J. Exp. Med.* 183:675–680.
  35. Takata, M., Y. Homma, and T. Kurosaki. 1995. Requirement of phospholipase C- $\gamma$ 2 activation in surface immunoglobulin M-induced B cell apoptosis. *J. Exp. Med.* 182:907–914.
  36. Dong, C., D.D. Yang, M. Wysk, A.J. Whitmarsh, R.J. Davis, and R.A. Flavell. 1998. Defective T cell differentiation in the absence of Jnk1. *Science.* 282:2092–2095.
  37. Sabapathy, K., Y. Hu, T. Kallunki, M. Schreiber, J.P. David, W. Jochum, E.F. Wagner, and M. Karin. 1999. JNK2 is re-

- quired for efficient T-cell activation and apoptosis but not for normal lymphocyte development. *Curr. Biol.* 9:116–125.
38. Fu, C., C.W. Turck, T. Kurosaki, and A.C. Chan. 1998. BLNK: a central linker protein in B cell activation. *Immunity.* 9:93–103.
  39. Kurosaki, T., and S. Tsukada. 2000. BLNK: connecting Syk and Btk to calcium signals. *Immunity.* 12:1–5.
  40. Hashimoto, S., A. Iwamatsu, M. Ishiai, K. Okawa, T. Yamadori, M. Matsushita, Y. Baba, T. Kishimoto, T. Kurosaki, and S. Tsukada. 1999. Identification of the SH2 domain binding protein of Bruton's tyrosine kinase as BLNK—functional significance of Btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. *Blood.* 94:2357–2364.
  41. Ishiai, M., H. Sugawara, M. Kurosaki, and T. Kurosaki. 1999. Association of phospholipase C- $\gamma$ 2 SH2 domains with BLNK is critical for B cell antigen receptor signaling. *J. Immunol.* 163:1746–1749.
  42. Cheng, P.C., M.L. Dykstra, R.N. Mitchell, and S.K. Pierce. 1999. A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J. Exp. Med.* 190:1549–1560.
  43. Aman, M.J., and K.S. Ravichandran. 2000. A requirement for lipid rafts in B cell receptor induced  $Ca^{2+}$  flux. *Curr. Biol.* 10:393–396.
  44. Xavier, R., T. Brennan, Q. Li, C. McCormack, and B. Seed. 1998. Membrane compartmentation is required for efficient T cell activation. *Immunity.* 8:723–732.
  45. Sheets, E.D., D. Holowka, and B. Baird. 1999. Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of Fc $\epsilon$ RI and their association with detergent-resistant membranes. *J. Cell Biol.* 145:877–887.
  46. Fluckiger, A.C., Z. Li, R.M. Kato, M.I. Wahl, H.D. Ochs, R. Longnecker, J.P. Kinet, O.N. Witte, A.M. Scharenberg, and D.J. Rawlings. 1998. Btk/Tec kinases regulate sustained increases in intracellular  $Ca^{2+}$  following B-cell receptor activation. *EMBO J.* 17:1973–1985.
  47. Zhang, Z., C. Elly, L. Qiu, A. Altman, and Y.C. Liu. 1999. A direct interaction between the adaptor protein Cbl-b and the kinase Zap-70 induces a positive signal in T cells. *Curr. Biol.* 9:203–206.
  48. Langlet, C., A.M. Bernard, P. Drevot, and H.T. He. 2000. Membrane rafts and signaling by the multichain immune recognition receptors. *Curr. Opin. Immunol.* 12:250–255.
  49. Krawczyk, C., K. Bachmaier, T. Sasaki, R.G. Jones, S.B. Snapper, D. Bouchard, I. Kozieradzki, P.S. Ohashi, F.W. Alt, and J.M. Penninger. 2000. Cbl-b is a negative regulator of receptor clustering and raft aggregation in T cells. *Immunity.* 13:463–473.
  50. Tedford, K., L. Nitschke, I. Girkontaite, A. Charlesworth, G. Chan, V. Sakk, M. Barbacid, and K.D. Fischer. 2001. Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling. *Nat. Immunol.* 2:548–555.
  51. Thien, C.B.F., D.D.L. Bowtell, and W.Y. Langdon. 1999. Perturbed regulation of ZAP-70 and sustained tyrosine phosphorylation of LAT and SLP-76 in c-Cbl-deficient thymocytes. *J. Immunol.* 162:7133–7139.
  52. Ota, Y., and L.E. Samelson. 1997. The product of the proto-oncogene *c-cbl*: a negative regulator of the Syk tyrosine kinase. *Science.* 276:418–420.
  53. Ueno, H., K. Sasaki, H. Honda, T. Nakamoto, T. Yamagata, K. Miyagawa, K. Mitani, Y. Yazaki, and H. Hirai. 1998. c-Cbl is tyrosine-phosphorylated by interleukin-4 and enhances mitogenic and survival signals of interleukin-4 receptor by linking with the phosphatidylinositol 3'-kinase pathway. *Blood.* 91:46–53.
  54. Meng, F., and C.A. Lowell. 1998. A  $\beta$ 1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. *EMBO J.* 17:4391–4403.
  55. Tanaka, S., M. Amling, L. Neff, A. Peyman, E. Uhlmann, J.B. Levy, and R. Baron. 1996. c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption. *Nature.* 383:528–531.
  56. Arron, J.R., M. Vologodskaja, B.R. Wong, M. Naramura, N. Kim, H. Gu, and Y. Choi. 2001. A positive regulatory role for Cbl family proteins in tumor necrosis factor-related activation-induced cytokine (TRANICE) and CD40L-mediated Akt activation. *J. Biol. Chem.* 276:30011–30017.