Cbl-b Positively Regulates Btk-mediated Activation of Phospholipase C-γ2 in B Cells

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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)-γ2 activation and Ca2+ 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 Ca2+ mobilization. Cbl-b interacted with PLC-γ2 and helped the association of PLC-γ2 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 Ca2+. Both NH2-terminal tyrosine kinase-binding domain and COOH-terminal half region of Cbl-b were essential for its association with PLC-γ2 and the regulation of Ca2+ mobilization. These results demonstrate that Cbl-b positively regulates BCR-mediated Ca2+ signaling, most likely by influencing the Btk/BLNK/PLC-γ2 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-α/β heterodimer, transmits signals that regulate the survival, differentiation, and proliferation of B lymphocytes (1–3). The Ig-α/β 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)-γ2 (3, 6–8). PLC-γ2 converts phosphatidylinositol 4,5-bisphosphate into the two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP3), which leads to the activation of protein kinase C and Ca2+ mobilization, respectively. PLC-γ2 is activated through its tyrosine phosphorylation by Syk and Btk after BCR stimulation (9–11). Recent studies have demonstrated that BCR-mediated PLC-γ2 activation also requires association with B cell linker protein (BLNK), which is responsible for PLC-γ2 membrane translocation (12).

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*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; IP3, inositol 1,4,5-trisphosphate; ITAM, immunoreceptor tyrosine-based activation motif; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; PDK, phosphatidylinositol 3-kinase; PLC, phospholipase C; PTK, protein tyrosine kinase; RBD, Rα1 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 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 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 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-γ2, Ca^{2+} mobilization, and c-Jun NH_{2}-terminal kinase (JNK) in response to BCR stimulation. Our data suggest that Cbl-b is important for Btk/BLNK/PLC-γ2 complex formation, which is required for downstream 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 μM β-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 μM β-mercaptoethanol. B cells from the spleens of 8–13 wk-old mice were enriched by negative selection with mAbs against CD3ε, CD11b, and NK1.1. The resulting cells were >85% B220{sup}, as assessed by flow cytometry. The Abs for chicken PLC-γ2, chicken Syk, chicken BLNK, and chicken Lyn were described previously (10, 25). Mouse monoclonal IgM toward chicken κ 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-γ2, 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–α-tubulin (Oncogene Research Products); and anti–mouse IgM (μ 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-γ2. 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 chicken 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 μF. 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. Lipkowtiz, 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–337 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 μF. Selection was performed in the presence of 0.5 μg/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 guanidinium thiocyanate method. Total RNA (20 μg) was separated on a 1.2% formaldehyde gel, transferred to Hybond N{sup}+, nylon membrane (Amersham Pharmacia Biotech), and probed with 32P-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 MgCl_2, 1 mM phenylmethylsulfonyl fluoride,
10 μg/ml leupeptin, 10 μg/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 × 10^6 cells were stimulated with 8 μg/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 2–5 × 10^6 cells were immunoprecipitated with 1 μg anti-ERK2, anti-p38, or anti-JNK1 Abs with 40 μg 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 μl kinase assay buffer containing 10 μCi [γ-^32^P]ATP and 5 μM cold ATP. Glutathione S-transferase (GST)-Elk, GST-ATF2, or GST-c-Jun fusion protein (5 μg 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 MgCl2, and 10 mM MnCl2) in the presence of 10 μCi [γ-^32^P]ATP and 5 μM cold ATP. Recombinant GST fusion protein containing a cytoplasmic region of mouse Igα (5 μg) 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 MnCl2) containing 10 μCi [γ-^32^P]ATP, 5 μM cold ATP, and 5 μg 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^{2+} Measurements. DT40 and 2.5 × 10^6 cells were suspended in HBSS supplemented with 10 mM Hepes, pH 7.2, 0.025% BSA, and 1 mM CaCl2, and loaded with 3 μM acetoxy-methyl ester of Fura-2 (Molecular Probes) at 30°C for 30 min. Cytosolic Ca^{2+} concentration of 5 × 10^6 cells in 0.5 ml HBSS supplemented with 10 mM Hepes, pH 7.2, 0.025% BSA, and 1 mM CaCl2, was recorded at 510 nm emission wavelength excited by 340 and 380 nm with a CAF110 spectrofluorometer (JASCO). Ca^{2+} release from intracellular Ca^{2+} 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 μg/ml Indo-1 (Molecular Probes) at 30°C for 40 min, and then labeled with FITC-conjugated anti-B220 Ab (BD PharMingen). Ca^{2+} mobilization of B220^+ cells was determined by flow cytometry.

IP3 Generation Assay. 2 × 10^6 cells were stimulated with 2 μg M4 at 37°C for the indicated time. Analyses of IP3 production were performed using the BIOTRAK IP3 assay system (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Binding Assay with GST Fusion Proteins. Human Btk cDNA fragments encoding amino acids 532–720 (PLC-γ2 [SH22]) and 769–831 (PLC-γ2 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 DH5α, 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 μg 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 μg 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-γ2 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 μg/ml M4, wild-type, Lyn−, Syk−, or Btk−deficient DT40 cells (2.5 × 10⁶) 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 gene, 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 (top) or β-actin (bottom). (D) 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 (bottom). (E) Protein expression of Cbl-b in wild-type and Cbl-b–deficient DT40 cells. Immuno-precipitates 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 (bottom). (F) BCR expression on the surface of wild-type (wt), Cbl-b–deficient (blb–), 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/blb–, 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/blb–, GE/blb–, N298E/blb–, and CAa/blb–, respectively. The x and y axes for the histograms indicate fluorescence intensity (4-decade log scales) and relative cell number, respectively.

One of the crucial biochemical cascades in BCR signaling is the PLC-γ2/Ca²⁺ pathway. As shown in Fig. 2 A, BCR stimulation of DT40 cells deficient for Cbl-b showed impaired Ca²⁺ 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 Ca²⁺ release from internal stores was impaired in Cbl-b–deficient cells. This was not due to the lesser amount of Ca²⁺ 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 IP₃ production was remarkably impaired in Cbl-b–deficient cells (Fig. 2 B). Because the activity of PLC-γ2 is critically regulated by its tyrosine phosphorylation, we evaluated the BCR-induced tyrosine phosphorylation status of PLC-γ2 in wild-type and Cbl-b–deficient cells. The results showed that Cbl-b–deficient cells exhibited markedly decreased levels of PLC-γ2 tyrosine phosphorylation (Fig. 2 C).

Suppression of Signaling Downstream of PLC-γ2 in the Absence of Cbl-b. As PLC-γ2–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-γ2 activation was impaired in the absence of Cbl-b. PLC-γ2 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-γ2 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 ~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-γ2 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-γ2 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
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 notable 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-γ2 in BCR Signaling. BCR-induced activation of the Ca^{2+} pathway requires Syk-dependent assembly of signaling components such as Btk, BLNK, and PLC-γ2 (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. However, an anti–PLC-γ2 Ab-dependent immunoprecipitation from the lysates was subjected to Western blot analysis with anti–phosphotyrosine mAb (top) and anti–PLC-γ2 Ab (bottom). (D) Rap1 activation in wild-type and Cbl-b–deficient DT40 cells. 1.25 × 10^6 cells were lysed in 1% NP-40 lysis buffer at the indicated time points after stimulation of 5 μg/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 × 10^6 cells were lysed at the indicated time points after stimulation of 4 μg/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.
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-γ2 and then the resultant BLNK–PLC-γ2 complex translocates to the membrane, which leads to PLC-γ2 activation (25, 41). The recruitment of PLC-γ2 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-γ2 to close proximity of Btk in activated B cells.

BCR-dependent Interaction of Cbl-b with Btk, BLNK, and PLC-γ2, and Their Translocation to GEMs. We then addressed whether Cbl-b forms a signaling complex with Btk, BLNK, and PLC-γ2. As shown in Fig. 4, A–C, Cbl-b associated with Btk, BLNK, and PLC-γ2 in the BCR-stimulated cells expressing T7-tagged Btk. The immunodepletion 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-γ2 was completely abrogated by Btk immunoprecipitation (Fig. 4 D). These results indicate that BCR–induced Btk–Cbl-b complex also includes PLC-γ2 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 α-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-

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 μg/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 μg/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-Tag 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 μg/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 μg/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 × 10⁶) were lysed in 1% NP-40 lysis buffer at the indicated time points after stimulation of 4 μg/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.
portion of Cbl-b in GEMs greatly increased after BCR stimulation (Fig. 4 E, bottom left). Small but significant amounts of PLC-γ2, 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-γ2, resulting in the activation of PLC-γ2.

We have previously shown that Cbl directly binds to phosphorylated BLNK via its TKB domain. This binding inhibits interaction between BLNK and PLC-γ2, and subsequent PLC-γ2 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-γ2 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-γ2.
Regulation of Btk-dependent Sustained Ca\(^{2+}\) Flux by Cbl-b. To further confirm that Cbl-b positively regulates Btk-mediated PLC-γ2 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\(_3\) and the tyrosine phosphorylation of PLC-γ2 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-γ2/Ca\(^{2+}\) 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-γ2/Ca\(^{2+}\) 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\(^{-}\)) showed enhanced and sustained Ca\(^{2+}\) mobilization in comparison to that of parental Cbl-b–deficient cells, confirming the positive role of Cbl-b in PLC-γ2/Ca\(^{2+}\) signaling (Fig. 6 C). The expression of a TKB mutant (Gly\(^{296}\) 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\(^{2+}\) mobilization (Fig. 6 C). The Ca\(^{2+}\) mobilization was also not restored in Cbl-b–deficient cells expressing a RING finger mutant (Cys\(^{373}\) to Ala, CA). Intriguingly, however, a low level of sustained Ca\(^{2+}\) 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 increase of extracellular Ca\(^{2+}\) influx (Fig. 6 C, bottom), which might be related to the sustained Ca\(^{2+}\) 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-γ2 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-γ2 activation. In vitro GST pull-down experiments revealed that the SH3 but not the SH2 domains of Btk and PLC-γ2 interacted with Cbl-b from the B cells with or without BCR stimulation (Fig. 6 E). These data suggest that Btk and PLC-γ2 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-γ/Ca\(^{2+}\) signaling, we generated WEHI-231 mouse B cell lines overexpressing Cbl-b,
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^{2+} mobilization. Therefore, Cbl-b regulates PLC-γ2/Ca^{2+} 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^{2+} 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^{2+} signaling, and provide evidence suggesting that Cbl-b functions as a scaffold protein to promote Btk-mediated PLC-γ2 activation.
argues that Cbl-b contributes to complex formation among PLC-γ2. In accordance with this idea, our present data strongly.

Figure 7. Role of Cbl-b on the PLC-γ2/Ca²⁺ pathway in mouse B cells. (A) Whole cell lysate (5 × 10⁶ 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 (5 × 10⁶) were stimulated with anti-IgM (10 µg/10⁶ cells) for 3 min. Anti-PLC-γ2 immunoprecipitates from the lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb (top) and anti-PLC-γ2 mAb (bottom). (C) BCR-induced Ca²⁺ mobilization in parental and Cbl-b–overexpressing (C85-11 and C85-12) WEHI-231 cells. [Ca²⁺]i were monitored by spectrophotometer after stimulation with 2 µg/ml anti-IgM. Arrows indicate the time point of the addition of anti-IgM (α–μ). (D) BCR-induced Ca²⁺ 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 µg/10⁶ cells). Data are presented as the median ratio of Ca²⁺ bound to Ca²⁺–free Indo-1 fluorescence of B220⁺ cells as measured by flow cytometry. (E) 3 × 10⁵ splenic B cells of wild-type (Btk⁺/+ or Btk–/–) or Cbl-b–deficient (Btk⁺/+ or Cbl-b⁻/⁻) mice were stimulated with anti-IgM for 1 min. Anti-PLC-γ2 immunoprecipitates from the lysates were subjected to Western blot analysis with anti-phosphotyrosine mAb (top) and anti-PLC-γ2 mAb (bottom). All experiments were performed more than three times.

Syk and Btk are required for tyrosine phosphorylation and activation of PLC-γ2. 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-γ2/Ca²⁺ pathway requires the assembly of signaling components Btk, BLNK, and PLC-γ2. A crucial adaptor protein, BLNK, couples Syk and Btk to PLC-γ2 activation in B cells (39). A possible explanation for the attenuated phosphorylation of PLC-γ2 in Cbl-b–deficient cells is that Cbl-b acts as a scaffolding protein to promote a complex formation including PLC-γ2. In accordance with this idea, our present data strongly argues that Cbl-b contributes to complex formation among Btk, BLNK, and PLC-γ2, which leads to the activation of PLC-γ2. 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-γ2 to GEMs where Cbl-b interacts with these proteins. Because the translocation of PLC-γ 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-γ2 into GEMs suggests the importance of Cbl-b in the regulation of PLC-γ2/Ca²⁺ signaling (Fig. 4). Overexpression of Btk in B cells significantly enhanced the sustained increase in [Ca²⁺]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 [Ca²⁺]i in the early phase after BCR stimulation (Fig. 5). Obviously, Cbl-b is important for the sustained activation of PLC-γ2 through Btk that is likely to occur in GEMs.

Here we show that Cbl-b–dependent positive regulation of Ca²⁺ mobilization requires its TKB domain and COOH-terminal half, and that these domains are essential for its interaction with PLC-γ2. Although Cbl suppresses BCR-induced PLC-γ2 activation by inhibiting BLNK–PLC-γ2 interaction (18), Cbl-b does not inhibit this interaction. These differential effects on the binding of PLC-γ2 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-γ2 interacts with Cbl-b in the absence of BCR stimulation. However, the interaction of Cbl-b with Btk,
BLNK, and PLC-γ2 occurred in a manner dependent on BCR stimulation, which suggests that tyrosine phosphorylation events are required for the interaction in vivo. Because the NH₂-terminal 418-amino acid sequence of Cbl-b, including the TKB domain, did not bind to either Btk, BLNK, or PLC-γ2 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-γ2 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-γ2/Ca²⁺ signaling between mouse and chicken, because the overexpression of Cbl-b enhanced tyrosine phosphorylation of PLC-γ2 as well as Ca²⁺ mobilization in WEHI-231 mouse B cells. Intriguingly, TCR-induced tyrosine phosphorylation of PLC-γ1 is unaffected in the absence of Cbl-b (17, 23). In contrast, TCR-induced tyrosine phosphorylation of PLC-γ1 is reduced in Cbl-deficient T cells (22). These data suggest that both Cbl and Cbl-b could positively regulate PLC-γ/Ca²⁺ signaling and Cbl compensates antigen receptor–mediated activation of PLC-γ in lymphocytes of Cbl-b–deficient mice. Indeed, TCR-stimulated tyrosine phosphorylation of PLC-γ1 and Ca²⁺ 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-γ/Ca²⁺ 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-γ1/Ca²⁺ signaling (unpublished data). Taken together, we assume that PLC-γ/Ca²⁺ 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 ubiquitination of EGFR and the p85 regulatory subunit of PI3K (20, 24). However, the protein levels of Syk, Btk, BLNK, and PLC-γ2 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-γ1, 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 (Cys²⁷³ to Ala) did not bind to PLC-γ2 upon BCR stimulation, the RING finger domain of Cbl family proteins may be used to regulate protein–protein interaction that are relevant to Ca²⁺ 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), β₃ 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-γ2/Ca²⁺ signaling. Similarly, TCR-induced PLC-γ/Ca²⁺ 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 FcγRI stimulation of myeloid cells (19). Likewise, Cbl-b may be involved in the ITAM signaling downstream of FcγR, FcεR, 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.

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