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Cbl-b Negatively Regulates B Cell Antigen Receptor Signaling in Mature B Cells through Ubiquitination of the Tyrosine Kinase Syk

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Abstract

Members of the Cbl family of molecular adaptors play key roles in regulating tyrosine kinase-dependent signaling in a variety of cellular systems. Here we provide evidence that in B cells Cbl-b functions as a negative regulator of B cell antigen receptor (BCR) signaling during the normal course of a response. In B cells from Cbl-b–deficient mice cross-linking the BCRs resulted in sustained phosphorylation of Igα, Syk, and phospholipase C (PLC)-γ2, leading to prolonged Ca^{2+} mobilization, and increases in extracellular signal–regulated kinase (ERK) and c-Jun NH_2-terminal protein kinase (JNK) phosphorylation and surface expression of the activation marker, CD69. Image analysis following BCR cross-linking showed sustained polarization of the BCRs into large signaling-active caps associated with phosphorylated Syk in Cbl-b–deficient B cells in contrast to the BCRs in Cbl-b–expressing B cells that rapidly proceeded to form small, condensed, signaling inactive caps. Significantly, prolonged phosphorylation of Syk correlated with reduced ubiquitination of Syk indicating that Cbl-b negatively regulates BCR signaling by targeting Syk for ubiquitination.

Key words: B cells • antigen receptor • ubiquitination • tyrosine kinase • capping

Introduction

B lymphocyte responses are initiated by the binding of multivalent antigens to the B cell antigen receptors (BCRs),* an event that triggers signaling cascades resulting in the transcription of a variety of genes associated with B cell activation (1). The BCR is composed of antigen binding chains, the Ig molecules, and a noncovalently associated signal-transduction complex, Igα/Igβ, containing in its cytoplasmic domain immunoreceptor tyrosine-based activation motifs (ITAMs; reference 2). Cross-linking the BCR results in the phosphorylation of the ITAMs by the Src family kinase, Lyn followed by recruitment and activation of the nonreceptor protein tyrosine kinase, Syk (3–5). Recruitment of Syk by the phosphorylated BCR is a key event in the assembly of the BCR signalosome composed of the adaptor protein BLNK and key downstream signaling components including phospholipase C (PLC)-γ2, Vav, and Bruton's tyrosine kinase (Btk) (6, 7). Syk deficiencies in mice result in aberrant B cell development (8, 9), and chicken DT40 B cells lacking Syk are unresponsive to BCR stimulation (10). Given the central role of Syk in B cell activation, regulation of Syk is likely to be essential to set the appropriate signaling thresholds for both the initiation and dampening of immune responses. However, the two known negative regulators of BCR signaling, namely SHIP and SHP-1, do not appear to target Syk. SHIP is a lipid phosphatase that influences the levels of PIP-3 (11–13) and SHP-1, a protein phosphatase, regulates BCR signaling by dephosphorylating Lyn (14).

Recent studies have provided evidence that two members of the Cbl family of molecular adaptors, c-Cbl and Cbl-b, function to regulate signaling in immune cells downstream of the immune receptors (15, 16). Members of the Cbl family share several highly conserved features including: a tyrosine kinase binding (TKB) domain composed of a four-helix bundle, a Ca^{2+}-binding EF hand motif and an unusual SH2 domain; a RING finger domain that interacts with ubiquitin conjugating enzymes allowing Cbl proteins to function as ubiquitin ligases; a proline rich region involved in SH3-domain interactions; multiple tyrosine residues that when phosphorylated allow interactions with SH2 domains and luicine zippers (16). Current evidence indicates that Cbl proteins regulate the activity of protein

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**Abbreviations used in this paper:* BCR, B cell antigen receptor; Btk, Bruton's tyrosine kinase; RT, room temperature; TKB, tyrosine kinase binding; Ub, ubiquitin.

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tyrosine kinases in part through ubiquitination and degradation of their targets (17–19). Evidence for the role of c-Cbl and Cbl-b in lymphocyte cell signaling was provided by the phenotypes of c-Cbl– and Cbl-b–deficient mice, the most dramatic of which were in T cell compartments (20–23). These phenotypes provided evidence that c-Cbl and Cbl-b have distinct functions in T cells. Loss of c-Cbl resulted in severe defects in developing thymocytes but only mild defects in mature peripheral T cells. Thymocytes from c-Cbl$^{-/-}$ mice showed enhanced activation of ZAP-70 after TCR engagement that was uncoupled from the need for CD4 costimulation and Lck activation. Consequently, positive selection of CD4^{+} thymocytes was enhanced in c-Cbl$^{-/-}$ mice (20, 21). In contrast, thymocyte development was normal in Cbl-b–deficient mice but the peripheral T cells were hyperreactive. Activation of mature peripheral T cells from Cbl-b$^{-/-}$ mice was independent of the engagement of the coreceptor CD28 and consequently Cbl-b$^{-/-}$ mice were highly susceptible to autoimmunity (22, 23). The effect of Cbl deficiencies in B cells has not been as rigorously explored. B cells in c-Cbl$^{-/-}$ mice appeared in normal numbers although in some mice an increase in the number of immature IgM^{+} IgD^{-} cells was noted (20), hinting at a developmental defect. B cells from Cbl-b$^{-/-}$ mice showed enhanced proliferative responses to BCR cross-linking and to CD40 stimulation analogous to the T cell phenotype in these mice (23). Recent evidence indicates that Cbl-b plays a positive role in signaling in immature DT40 B cells (24). Here we provide evidence that in mature splenic B cells Cbl-b is a negative regulator of BCR-mediated B cell activation in part through its ubiquitination of Syk.

Materials and Methods

Mice. Cbl-b$^{-/-}$ mice on a mixed genetic background between 129 and C57BL/6 were as described (22). Cbl-b$^{+/+}$ mice on a mixed genetic background between 129 and C57BL/6 were obtained from Taconic. For some analyses, Cbl-b$^{+/-}$ and Cbl-b$^{-/-}$ littermates on the same mixed genetic background were used.

Reagents and Abs. The mouse IgM-specific rat IgG2a mAb (R6–60.2) used for BCR cross-linking was purchased from BD Biosciences. F(ab′)$_2$ goat Abs specific for mouse Igμ or for IgM + G, biotin-conjugated F(ab′)$_2$ goat Abs specific for rat Ig, HRP-conjugated goat Abs specific for mouse Ig, Rhodamine Red X (RRX)-conjugated Fab goat Abs specific for mouse Igμ, PE-conjugated F(ab′)$_2$ donkey Abs specific for rabbit IgG, FITC-conjugated F(ab′)$_2$ goat Abs specific for mouse IgG, and Texas-Red–conjugated streptavidin were purchased from Jackson ImmunoResearch Laboratories. Latrunculin B, Piceatannol and PP2 were purchased from Cal Biochem Co. Cytochalasin D was purchased from Sigma-Aldrich. The following Abs were purchased: rabbit Abs specific for Syk, Cbl-b, Vav1, BLNK, Lyn, PLC-γ2, ubiquitin, phospho-ERK, ERK or JNK, goat Abs specific for Btk, and a mouse mAb specific for BLNK (2B11) from Santa Cruz Biotechnology, Inc.; rabbit Abs specific for phospho-JNK from Biosource International; rabbit Abs specific for phospho-Syk (tyrosine 519/520), phospho-Btk (tyrosine 223), phospho-Akt (serine 473), and Akt from Cell Signaling Technology; and the phosphotyrosine specific PY20 mAb from Transduction Lab. AlexaFluor 488-conjugated to streptavidin, phalloidin and goat Abs specific for rabbit IgG were purchased from Molecular Probe. The WASP-GBD-GFP fusion protein used for the measurement of active CDC42 (25) was a kind gift of Dr. Michael Rosen (University of Texas Southwestern Medical Center, Dallas, TX). Rabbit Abs specific for Igα was prepared as described (26).

B Cell Activation Assay. B cells were purified from red cell depleted spleen cells by complement-mediated cytotoxicity after incubation with a Thy-1–specific mAb. The resulting cells were >90% B220^{+}. The cells were incubated with a rat mAb specific for mouse IgM (1 μg/10^{6} cells) on ice for 30 min, washed and incubated with F(ab′)$_2$ goat Abs specific for rat IgG Fcγ at 37°C. Alternatively, cells were incubated with F(ab′)$_2$ goat Abs specific for mouse IgM at 37°C for the indicated times.

Immunoprecipitation, Immunoblotting, and Lyn Kinase Assays. Immunoprecipitation and immunoblotting of cell lysates were as described previously (27). For immunoprecipitation of Syk, Igα, BLNK, phospho-Btk, Vav1, and PLC-γ2, 5×10^{7} cells per time point were lysed in the Tris or MES-buffered saline containing 1% NP-40, sodium orthovanadate, and protease inhibitors. For immunoprecipitation of Cbl-b and Lyn, 2×10^{7} cells were lysed in the RIPA buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, and protease inhibitors. Lyn kinase activity was measured in Lyn immunoprecipitates by the transfer γP^{32} into a peptide substrate using the SignaTECT protein tyrosine kinase assay system according to the manufacturer's protocol (Promega). Briefly, the Lyn immunoprecipitates were incubated with kinase buffer including biotin-conjugated Src kinase–specific peptide substrates and γP^{32}-ATP for 20 min at 25°C. The reaction was stopped and the reaction mixtures dotted on to membranes to which streptavidin was coupled. The membranes were washed and counted using a β-scintillation counter. For immunoblotting of unfractionated lysates, cells were lysed in the RIPA buffer and 20 μg of protein per time point was analyzed. The immunoblot bands were quantified by densitometry and expressed as a ratio of phosphotyrosine containing protein to total protein.

Measurement of Intracellular Ca^{2+} Mobilization. For detection of cytosolic Ca^{2+}, 10^{7} splenocytes were incubated with 0.75 μM Indo-1 acetoxy-methyl ester (Indo-1; Molecular Probe) in 1% HBSS-FBS containing 0.01% F-127 (Molecular Probe) for 45 min at 30°C. Cells were subsequently stained with PE-conjugated B220 specific mAb to detect B220^{+} cells in flow cytometry. The primary IgM-specific Ab was added followed by the secondary Ab and cytosolic Ca^{2+} fluxes were recorded in real time using a FACS vantage (Becton Dickinson) and analyzed by the FlowJo software program (True Star, Inc.).

Determination of F-actin Polymerization and CDC42 Activation. F-actin was detected by phalloidin staining quantified by flow cytometry. GTP-bound CDC42 was detected using WASP-GBD-GFP fusion protein as described previously (25). Cells were fixed, permeabilized with 0.05% saponin and stained with rabbit Abs specific for GFP followed by FITC-labeled goat Abs specific for rabbit Ig and analyzed by flow cytometry (FACScan™; Becton Dickinson).

Detection of Phospho-Btk. The level of intracellular phospho-Btk was determined by flow cytometry using phospho-Btk–specific antibodies. Splenic B cells (10^{7} cells/ml) were incubated in HBSS for 1 h at 37°C and stimulated by adding 20 μg/ml F(ab′)$_2$ goat Abs specific for mouse Igμ for the indicated time periods. After washing twice with PBS containing 0.2% sodium azide,

cells were fixed, permeabilized, blocked with 10% normal mouse and donkey sera on ice, stained with rabbit Abs specific for phospho-Btk (Y223)-or as a control nonspecific rabbit Ig detected using PE-conjugated $F(ab')_2$ donkey Abs specific for rabbit IgG. After acquisition by FACScan™ (Becton Dickinson), the data were analyzed by the FlowJo software program. The percentage of the phospho-Btk–positive cells relative to control Ab stained cells was calculated after gating on live cells by the forward- and side-scatter.

Immunofluorescence Microscopy. To quantify the number of cells showing patch, Cap I or Cap II BCR structures, purified splenic B cells were treated with a rat mAb specific for mouse IgM at 4°C for 30 min then transferred to poly-L-lysine treated coverslips at 4°C. Biotin-conjugated goat antibodies specific for rat IgG were added to further cross-link the BCR and the cells warmed to 37°C for the times indicated. At the end of each time point the cells were fixed with 3.7% paraformaldehyde, quenched with 50 μM NH_4Cl, blocked with PBS containing 1% BSA (PBS-BSA) for 30 min at room temperature (RT) and stained with AlexaFluor 488-conjugated streptavidin in the dark for 30 min at RT. The coverslips were mounted in Prolong Antifade (Molecular Probe) and examined by fluorescence microscopy (Olympus IX70 microscope, 100 W mercury lamp, a cooled CCD camera, 60 × 1.4 oil objectives). The cells having a BCR patch, Cap I, or Cap II morphology were counted at each time point.

To image Igμ and phosphotyrosine in patch-, Cap I–, Cap II–positive cells, splenic B cells were incubated with a rat mAb specific for IgM at 4°C, washed, allowed to settle on coverslips on ice, and further cross-linked with biotin-conjugated goat Abs specific for rat IgG for 5 min for patch and Cap I and for 30 min for Cap II at 37°C. Fixed cells were incubated with Texas Red–conjugated Streptavidin washed, permeabilized with 0.05% saponin in PBS-BSA, blocked with 50 μg/ml of purified normal goat IgG for 30 min at RT, and incubated with PY20 mAb specific for phosphotyrosine for 1 h at RT. The secondary FITC-conjugated goat $F(ab')_2$ anti–mouse, Fc_γ-specific Ab, was added for 30 min at RT. For imaging of IgM and phospho-Syk, and IgM and Syk, splenic B cells were incubated with Fab goat Abs specific for mouse Igμ conjugated with Rhodamine Red-X (RRX) for 15 min at RT, washed, and allowed to settle onto an poly-L-lysine coated 8-well glass-bottom chamber slide (Labtek/Nunc) on ice for 30 min. Cells were stimulated with 50 μg/ml of $F(ab')_2$ goat Abs specific for mouse IgM + G at 37°C for the indicated times and fixed. After quenching, cells were permeabilized with 0.05% saponin in PBS-Gelatin (PBS containing 1% fish skin gelatin), blocked with 10% normal mouse sera and 50 μg/ml of purified normal goat IgG for 30 min at RT. Cells were stained with rabbit Abs specific for Syk or phospho-Syk (Y519/520) detected using goat Abs specific for rabbit IgG conjugated with AlexaFluor 488.

For two-color confocal and differential interference contrast (DIC) images, a confocal laser scanning microscope (Zeiss Axiovert 200M LSM 510 META; Carl Zeiss Microimaging, Inc.) fitted with a 1.4 oil planapochromat ×63 objective was used. Images were acquired with configuration of Ex488/Em 505–530BP for AlexaFluor 488 or FITC and Ex543/Em 560LP for Texas Red Dye or RRX and under the scan control of fixed pixel density at 512 × 512 pixels, 8 bit (phosphotyrosine) or 12 bit (phospho-Syk and Syk) pixel depths, linear contrast of grayscale, 7.8 ms scan time, and pinhole size of 45 μm (phosphotyrosine and IgM) or 66 μm (phospho-Syk/Syk and IgM). No significant signal saturation was noted in any of the images used for analysis. To quantify the colocalization between IgM and phosphotyrosine, phospho-Syk or Syk, the LSM5 imaging examiner software program was used. To compare the intensity of phospho-Syk colocalized with IgM between Cbl-$b^{+/-}$ and Cbl-$b^{-/-}$ splenic B cells, the images from five randomly chosen fields each containing at least 400 cells were acquired as grayscale with linear contrast. The mean fluorescence intensity (MFI) of phospho-Syk colocalized with IgM was calculated based on the colocalization scatter diagrams by multiplying both IgM and phospho-Syk positive, matched pixel numbers by the MFI of the matched pixels and then dividing by the number of cells in a chosen field.

Results

Prolonged Phosphorylation of Igα and Syk after BCR Cross-linking in Cbl-$b^{-/-}$ B Cells. B cells from Cbl-b–deficient mice were shown previously to hyper-proliferate in response to BCR cross-linking suggesting a role for Cbl-b in the negative regulation of B cell signaling. One of the earliest events in BCR signaling is the tyrosine phosphorylation of the Igα/Igβ complex by the Src-family kinase Lyn (3). The phosphorylation of Igα results in the recruitment of Syk to Igα via its SH2 domains and the subsequent phosphorylation of Syk (4). The subsequent Syk-mediated phosphorylation of the B cell adaptor protein BLNK links the BCR to both PLC-γ2 and Btk. To investigate the molecular mechanisms underlying the hyper-responsiveness of B cells from Cbl-$b^{-/-}$ mice, we began by monitoring the tyrosine phosphorylation of Cbl-b, Lyn, Igα, and Syk after BCR cross-linking. B cells from Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ mice were incubated with $F(ab')_2$ IgM-specific Abs to cross-link the BCR for increasing lengths of time at 37°C and at the end of each time point the phosphorylation state of Cbl-b, Lyn, Igα, and Syk was determined by immunoprecipitation of each protein followed by immunoblots probing with a phosphotyrosine-specific mAb.

In B cells from Cbl-$b^{+/+}$ mice, Cbl-b was not detectably phosphorylated in resting cells, but became phosphorylated immediately upon cross-linking (Fig. 1 A). Phosphorylation of Cbl-b increased after 2 min at 37°C then decreased approaching unactivated levels by 30 min. In control experiments, Cbl-b was not detected in Cbl-$b^{-/-}$ B cells (Fig. 1 A). The phosphorylation of Cbl-b following BCR cross-linking suggests that it plays a direct role in BCR signaling.

In both Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ B cells Lyn showed a high level of constitutive phosphorylation that increased slightly upon BCR cross-linking and remained phosphorylated for the 30-min time course (Fig. 1 B). The level of Lyn phosphorylation, assessed by the ratio of the densities of the Lyn bands in immunoblots to the densities of the phospho-Lyn bands, was slightly higher (~10%) in B cells from Cbl-$b^{-/-}$ as compared with Cbl-$b^{+/+}$ mice. Otherwise, the absence of Cbl-b did not appear to have a significant effect on Lyn phosphorylation. Direct measurements of the kinase activity of Lyn in vitro showed no significant differences in Lyn activity in Lyn immunoprecipitates from cell lysates prepared from B cells from Cbl-$b^{+/+}$ as compared with Cbl-$b^{-/-}$ mice after BCR cross-linking (Fig. 1 C). Resting Cbl-$b^{-/-}$ B cells may have a slightly higher

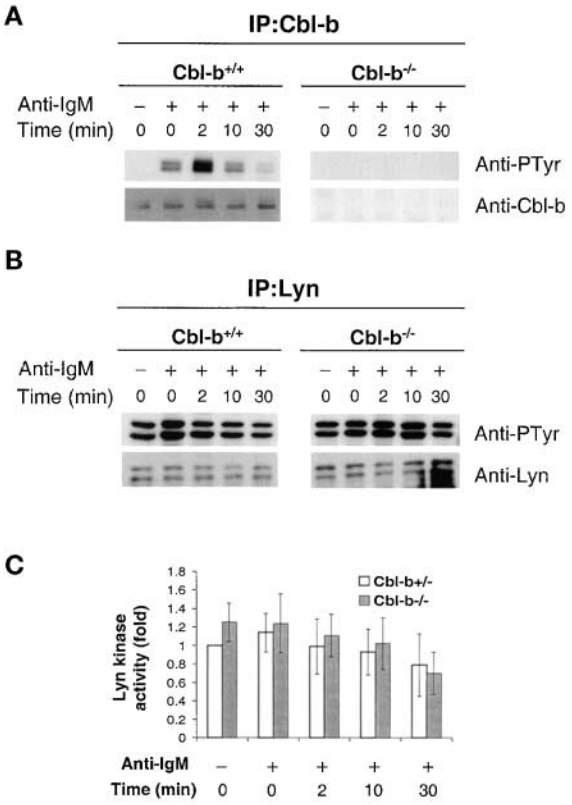

Figure 1. Cbl-b is phosphorylated following BCR cross-linking but does not affect Lyn activity. Purified splenic B cells from Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ mice were incubated with $F(ab')_2$ goat Abs specific for mouse Igμ at 37°C for the indicated times. The cells were washed, lysed, and the lysate subjected to immunoprecipitation using Abs specific for Cbl-b (A) or Lyn (B) and the immunoprecipitate analyzed by SDS-PAGE and immunoblotting probing for phosphotyrosines using the phosphotyrosine-specific mAb PY20, stripped, and reprobed for either Cbl-b (A) or Lyn (B). The results shown are representative of three independent experiments. The Lyn kinase activity was measured in Lyn immunoprecipitates of lysates of B cells from Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ mice at various times after cross-linking the BCR (C). The results from three experiments were averaged and the results expressed relative to the Lyn activity in resting Cbl-$b^{+/+}$ B cells.

level of Lyn activity as compared with Cbl-$b^{+/+}$ B cells reflecting the slightly higher level of Lyn phosphorylation in Cbl-$b^{-/-}$ B cells.

Cross-linking the BCR resulted in the tyrosine phosphorylation of Igα in B cells from both Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ mice detected immediately upon cross-linking (Fig. 2 A, left panel). Quantitation of the bands corresponding to Igα and phosphorylated Igα showed slightly greater phosphorylation of Igα in Cbl-$b^{-/-}$ B cells (a ratio of tyrosine-phosphorylated Igα to total Igα of 1) as compared with Cbl-$b^{+/+}$ B cells (a ratio of 0.75). Significantly, the phosphorylation of Igα was prolonged in B cells lacking Cbl-b (Fig. 2 A). In Cbl-$b^{+/+}$ B cells Igα phosphorylation returned to nearly unstimulated levels 10 min following BCR cross-linking (a ratio of 0.2). In contrast, a large portion of Igα in Cbl-$b^{-/-}$ B cells remained phosphorylated at 10 min (a ratio of 0.7).

The phosphorylation of Syk was similarly prolonged in Cbl-$b^{-/-}$ B cells (Fig. 2 B). BCR cross-linking resulted in slightly higher phosphorylation of Syk immediately following cross-linking in Cbl-$b^{-/-}$ B cells (a ratio of 1.1) as compared with Cbl-$b^{+/+}$ B cells (a ratio of 0.9). Syk remained phosphorylated for a longer period of time in Cbl-$b^{-/-}$ B cells compared with Cbl-$b^{+/+}$ B cells (a ratio of 0.8 versus 0.4 measured at 10 min). The phosphorylation of Syk on Y519/520 within its activation loop is essential for Syk's activity (17, 28). Immunoblots of Syk immunoprecipitates probed with polyclonal Abs specific for Syk phosphorylated on Y519/520 showed approximately twofold more Y519/520 phospho-Syk in Cbl-$b^{-/-}$ as compared with Cbl-$b^{+/+}$ B cells 2 min after BCR cross-linking (Fig. 2 C). Significantly, phosphorylated Syk remained associated with Igα for longer periods of time in Cbl-$b^{-/-}$ B cells as compared with Cbl-$b^{+/+}$ B cells (Fig. 2 A, right panel). Syk was immunoprecipitated with Igα in resting cells, however, the Igα-associated Syk was not phosphorylated. Immediately upon BCR cross-linking the Syk associated with Igα was phosphorylated. The amount of phospho-Syk associated with Igα showed a significant decrease in Cbl-$b^{+/+}$ B cells 10 min after BCR cross-linking relative to that in Cbl-$b^{-/-}$ B cells (a ratio of 0.2 versus 0.9).

The association of phospho-Syk with the BCR was imaged in B cells following BCR cross-linking. B cells were incubated with RRX-conjugated Fab goat Abs specific for Igμ, washed and allowed to settle on coverslips before cross-linking the BCR by addition of goat Abs specific for mouse IgM. Cells were incubated at 37°C for 0 to 30 min, fixed, permeabilized, and stained for phospho-Syk using phospho-Syk (Y519/520)-specific rabbit Abs detected using AlexaFluor 488-conjugated goat Abs specific for rabbit IgG. The cells were imaged by laser scanning confocal microscopy and representative merged images acquired 2 min after BCR cross-linking are shown (Fig. 2 D, top). In untreated resting cells no phospho-Syk was detected in either Cbl-$b^{+/-}$ or Cbl-$b^{-/-}$ B cells. 2 min after BCR cross-linking the images appeared to show a larger number of cells with cap structures in which the BCR and phospho-Syk were colocalized in Cbl-$b^{-/-}$ as compared with Cbl-$b^{+/-}$ B cells. To quantify the amount of BCR and phospho-Syk that were colocalized, the images were analyzed pixel by pixel and the FI of the IgM RRX and phospho-Syk (Y519/520) AlexaFluor 488 within each pixel plotted in a histogram (Fig. 2 D, bottom). Pixels that lie along the diagonal contain equal amounts of colocalized IgM and phospho-Syk (Y519/520). For those above the diagonal the intensity of the phospho-Syk (Y519/520) is less than that of the BCR and for pixels below the diagonal the phospho-Syk (Y519/520) intensity is greater than that of the BCR. The histograms indicate less colocalization of phospho-Syk with the BCR at the 2 min time point in Cbl-$b^{+/-}$ B cells as compared with Cbl-$b^{-/-}$ B cells (Fig. 2 D, bottom). For each time point, the MFI of the phospho-Syk (Y519/520) colocalized with the BCR was quantified (Fig. 2 E). The degree of colocalization of phospho-Syk and the BCR was similar immediately after the addition of the BCR cross-

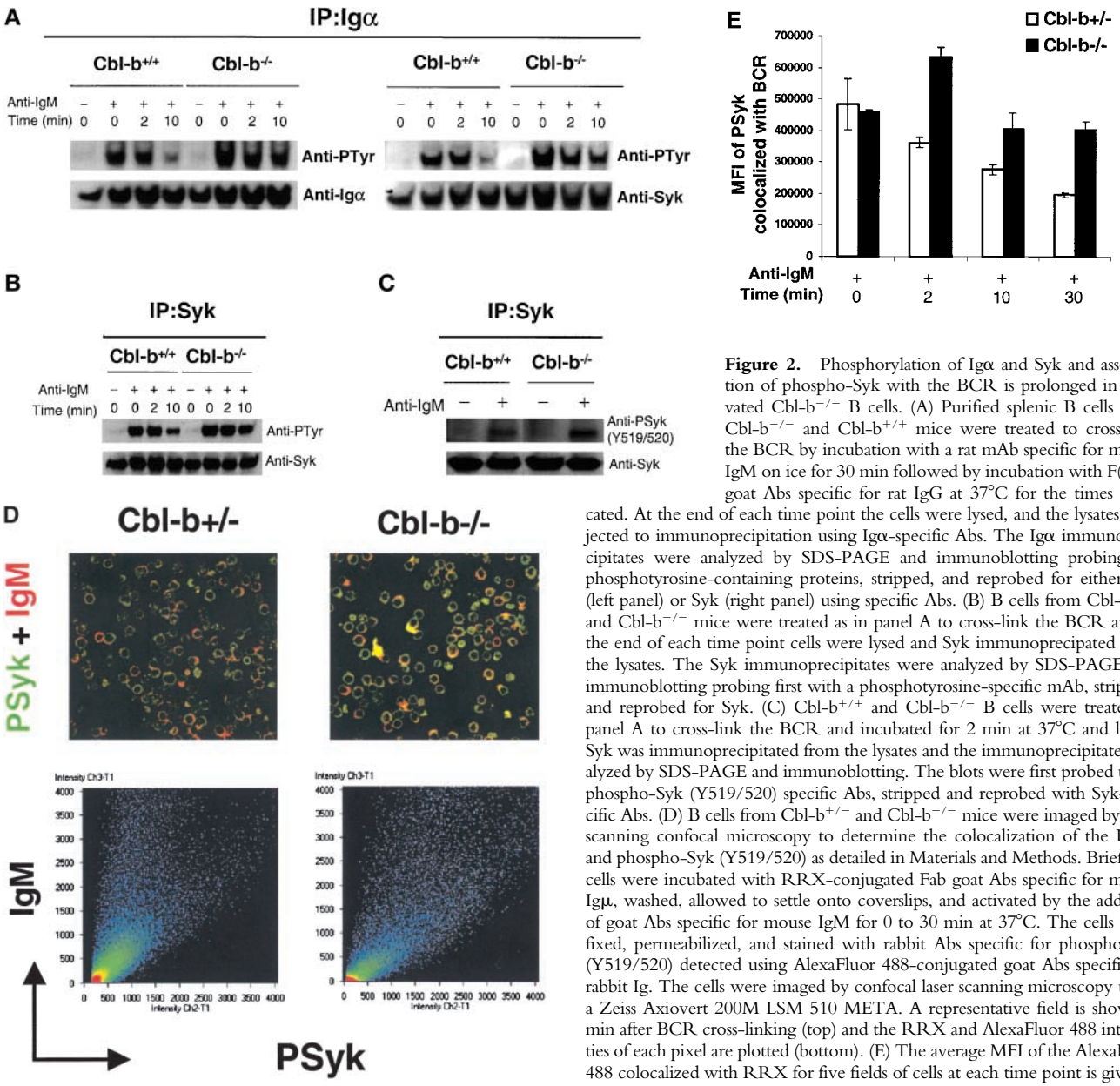

Figure 2. Phosphorylation of Igα and Syk and association of phospho-Syk with the BCR is prolonged in activated Cbl-b$^{-/-}$ B cells. (A) Purified splenic B cells from Cbl-b$^{-/-}$ and Cbl-b$^{+/+}$ mice were treated to cross-link the BCR by incubation with a rat mAb specific for mouse IgM on ice for 30 min followed by incubation with F(ab′)$_2$ goat Abs specific for rat IgG at 37°C for the times indicated. At the end of each time point the cells were lysed, and the lysates subjected to immunoprecipitation using Igα-specific Abs. The Igα immunoprecipitates were analyzed by SDS-PAGE and immunoblotting probing for phosphotyrosine-containing proteins, stripped, and reprobed for either Igα (left panel) or Syk (right panel) using specific Abs. (B) B cells from Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ mice were treated as in panel A to cross-link the BCR and at the end of each time point cells were lysed and Syk immunoprecipitated from the lysates. The Syk immunoprecipitates were analyzed by SDS-PAGE and immunoblotting probing first with a phosphotyrosine-specific mAb, stripped, and reprobed for Syk. (C) Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ B cells were treated in panel A to cross-link the BCR and incubated for 2 min at 37°C and lysed. Syk was immunoprecipitated from the lysates and the immunoprecipitates analyzed by SDS-PAGE and immunoblotting. The blots were first probed using phospho-Syk (Y519/520) specific Abs, stripped and reprobed with Syk-specific Abs. (D) B cells from Cbl-b$^{+/-}$ and Cbl-b$^{-/-}$ mice were imaged by laser scanning confocal microscopy to determine the colocalization of the BCR and phospho-Syk (Y519/520) as detailed in Materials and Methods. Briefly, B cells were incubated with RRX-conjugated Fab goat Abs specific for mouse Igμ, washed, allowed to settle onto coverslips, and activated by the addition of goat Abs specific for mouse IgM for 0 to 30 min at 37°C. The cells were fixed, permeabilized, and stained with rabbit Abs specific for phospho-Syk (Y519/520) detected using AlexaFluor 488-conjugated goat Abs specific for rabbit Ig. The cells were imaged by confocal laser scanning microscopy using a Zeiss Axiovert 200M LSM 510 META. A representative field is shown 2 min after BCR cross-linking (top) and the RRX and AlexaFluor 488 intensities of each pixel are plotted (bottom). (E) The average MFI of the AlexaFluor 488 colocalized with RRX for five fields of cells at each time point is given.

linking Abs (the 0 min time point) in Cbl-b$^{+/-}$ and Cbl-b$^{-/-}$ B cells. 2 min after BCR cross-linking the amount of phospho-Syk (Y519/520) associated with the BCR increased significantly in Cbl-b$^{-/-}$ B cells and then decreased with time. In contrast, in Cbl-b$^{+/-}$ B cells the amount of phospho-Syk (Y519/520) colocalized with the BCR had already decreased 2 min after BCR cross-linking. These results taken together with those above indicate that Syk is a target of Cbl-b activity after BCR cross-linking resulting in the rapid turnover of active phospho-Syk (Y519/520) associated with Igα.

Increased Syk Ubiquitination in Cbl-b$^{+/+}$ B Cells. The members of the Cbl family have been shown to function as E3 ubiquitin ligases and to target protein tyrosine kinase substrates for degradation (17). To investigate the possibility that Cbl-b influences Syk through ubiquitination, Syk was immunoprecipitated from lysates of Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ B cells treated to cross-link the BCR for varying lengths of time and the immunoprecipitates analyzed by immunoblot probing for ubiquitin, Syk and phosphotyrosine. The results from the 2-min time point are shown (Fig. 3). In resting B from both Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ a portion of Syk is tyrosine phosphorylated. A high molecular weight band reacting with both Syk- and phosphotyrosine-specific antibodies appears to be ubiquitinated and may represent a product of constitutive turnover. Cross-linking the BCR resulted in increased tyrosine phosphorylation of Syk and heterogeneous and poly-ubiquitination of Syk in Cbl-b$^{+/+}$ B cells. In contrast, although Syk is phosphorylated the ubiquitination of Syk was significantly less

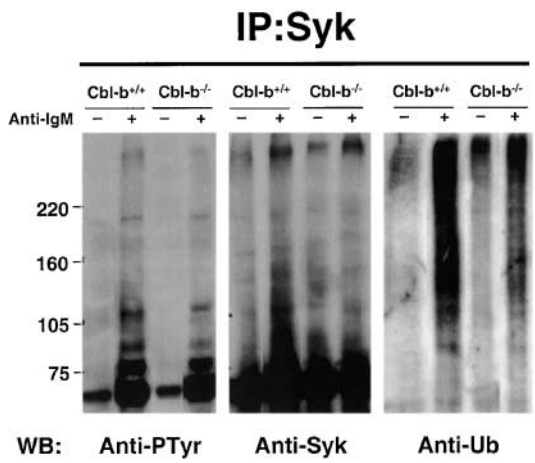

Figure 3. The activation-induced ubiquitination of Syk is reduced in Cbl-b$^{-/-}$ B cells. Splenic B cells from Cbl-b$^{-/-}$ and Cbl-b$^{+/+}$ mice were treated as in Fig 2, A–C, to cross-link the BCR and warmed to 37°C for increasing lengths of time. The cells were lysed, and Syk was immunoprecipitated and analyzed by SDS-PAGE and immunoblotting. Duplicate immunoblots were probed for either ubiquitin (anti-Ub) or phosphotyrosine (Anti-PTyr). The phosphotyrosine blot was stripped and reprobed for Syk (anti-Syk). The results for the 2-min time point are shown at which point ubiquitination of Syk was maximal. The result shown is representative of three independent experiments.

in Cbl-b$^{-/-}$ B cells and appeared to involve primarily the most highly ubiquitinated forms of Syk detected in resting cells. In controls, there was no detectable ubiquitination of Igα in Cbl-b$^{+/+}$ or Cbl-b$^{-/-}$ B cells at any time following BCR cross-linking (unpublished data). However, it is possible that in addition to Syk proteins coimmunoprecipitated with Syk are ubiquitinated. Ubiquitination of Syk was maximal at two min following BCR cross-linking (unpublished data) at a time at which the levels of phospho-Syk (Y519/520) began decreasing in Cbl-b$^{+/-}$ B cells (Fig. 2, D and E) suggesting that Cbl-b targets phospho-Syk for ubiquitination resulting in its subsequent degradation.

Prolonged Phosphorylation of BLNK, Btk and PLC-γ2 and Ca^{2+} Fluxes in Cbl-b$^{-/-}$ B Cells. A key downstream effector of BCR signaling is PLC-γ2 that cleaves PIP2 (4, 5) releasing IP3 and DAG resulting in release of intracellular Ca^{2+} stores. PLCγ-2 is phosphorylated by Syk and Btk which are activated by phosphorylation by Lyn (6). BLNK plays a key role in Ca^{2+} signaling by functioning as a scaffold for the assembly of complexes of PLC-γ2 and Btk after Syk-mediated phosphorylation. Recent evidence indicates that normal Ca^{2+} signaling requires BLNK-dependent PLC-γ2–BLNK interactions and BLNK-independent Btk activation as well as the assembly of BLNK-PLC-γ2–Btk complexes (29). To determine if a Cbl-b deficiency influenced these events the levels of phosphorylation of BLNK, Btk, and PLC-γ2 and Ca^{2+} fluxes were measured in Cbl-b$^{+/+}$ or Cbl-b$^{+/-}$ and Cbl-b$^{-/-}$ B cells after BCR cross-linking.

In Cbl-b$^{-/-}$ B cells the phosphorylation of BLNK was prolonged as compared with Cbl-b$^{+/-}$ B cells (Fig. 4 A). In Cbl-b$^{+/-}$ B cells maximal BLNK phosphorylation occurred immediately following BCR cross-linking and decreased rapidly thereafter. In contrast, BLNK remained significantly phosphorylated 10 min after BCR cross-linking in Cbl-b$^{-/-}$ B cells. Thus, Cbl-b influenced the duration of the phosphorylation of BLNK.

Cbl-b also affects the phosphorylation state of Btk (Fig. 4 B). B cells from Cbl-b$^{+/-}$ and Cbl-b$^{-/-}$ mice were treated with IgM-specific antibodies to cross-link the BCR and at various times afterward the cells were permeabilized, stained with Abs specific for phospho-Btk (Y223) and analyzed by flow cytometry. The levels of phosphorylated Btk increased immediately after BCR cross-linking in B cells from both Cbl-b$^{-/-}$ and Cbl-b$^{+/-}$ mice. However, Btk phosphorylation reached higher levels in Cbl-b$^{-/-}$ B cells as compared with Cbl-b$^{+/-}$ B cells 2–10 min following BCR cross-linking.

To further characterize the phospho-Btk (Y223) in terms of its association with PLC-γ2 and Syk, cells were lysed at various times after BCR cross-linking and the lysates subjected to immunoprecipitation using phospho-Btk (Y223)-specific Abs. The immunoprecipitates were immunoblotted probing for phosphotyrosine, stripped and reprobed for Btk, PLC-γ2, and Syk using specific antibodies (Fig. 4 C). The phospho-Btk (Y223) immunoprecipitates contained Btk, only weakly detected in immunoblot, and phospho-PLC-γ2 and phospho-Syk. Significantly, the phospho-Btk/phospho-PLC-γ2/phospho-Syk complex remains assembled for longer periods of time in Cbl-b$^{-/-}$ as compared with Cbl-b$^{+/-}$ B cells (Fig. 4 C).

The direct effect of Cbl-b on PLC-γ2 phosphorylation was measured. In resting B cells from both Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ mice there was no significant phosphorylation of PLC-γ2 (Fig. 5, top). Upon BCR cross-linking PLC-γ2 was phosphorylated in Cbl-b$^{+/+}$ mice and by 15 min following BCR cross-linking the amount of phosphorylated PLC-γ2 had decreased significantly. In Cbl-b$^{-/-}$ B cells, PLC-γ2 was phosphorylated upon BCR cross-linking but in contrast to the PLC-γ2 in Cbl-b$^{+/+}$ B cells, PLC-γ2 was strongly phosphorylated at 15 min and a decrease in phosphorylation was not detected until 30 min after BCR cross-linking. Consistent with this observation Ca^{2+} fluxes after BCR cross-linking in Cbl-b$^{-/-}$ B cells were more persistent as compared with those in Cbl-b$^{+/+}$ B cells (Fig. 5, bottom). Taken together these results indicate that in the absence of Cbl-b, complexes containing phosphorylated Syk, BLNK, Btk and PLC-γ2 persist leading to prolonged Ca^{2+} fluxes.

The Association of Vav-1 with Syk Is Prolonged in Cbl-b$^{-/-}$ B Cells. Vav, a guanine-nucleotide exchange factor or GEF for the Rho/Rac family of GTPases, plays a central role in integrating signaling from the BCR. Vav activity is regulated, in part, by phosphorylation by Syk (30). Evidence for a role for Cbl-b in regulating the function of Vav in lymphocytes was provided by the observation that the phosphorylation of Vav-1 was increased in Cbl-b$^{-/-}$ T cells as compared with Cbl-b$^{+/+}$ T cells (22, 23). To investigate the effect of a Cbl-b deficiency on Vav-1 phosphorylation, Vav was immunoprecipitated from Cbl-b$^{+/+}$ and

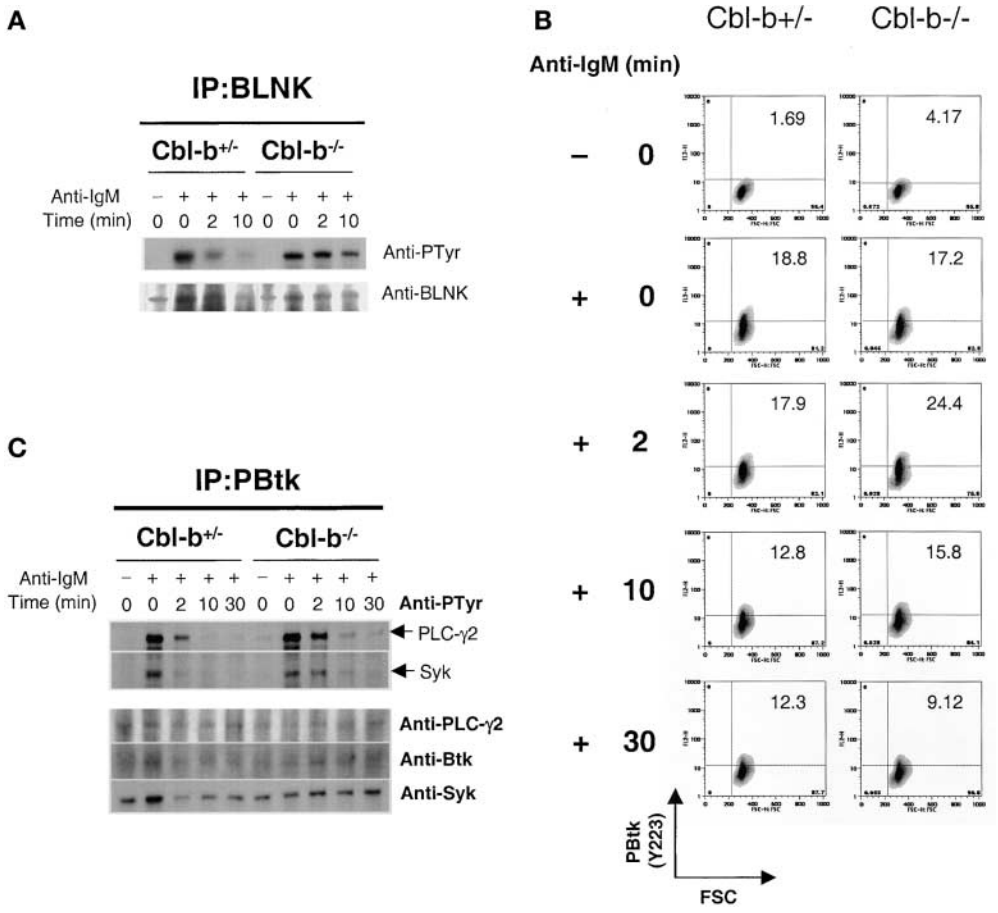

Figure 4. The phosphorylation of BLNK and Btk is influenced by Cbl-b. (A) B cells from Cbl-$b^{+/-}$ and Cbl-$b^{-/-}$ mice were treated as in Fig. 1 to cross-link the BCR at 37°C for the times indicated. The cells were lysed and BLNK was immunoprecipitated from the lysates. The BLNK immunoprecipitates were analyzed by SDS-PAGE and immunoblotting probing with a phosphotyrosine-specific mAb or with a BLNK-specific mAb. (B) B cells from Cbl-$b^{+/-}$ and Cbl-$b^{-/-}$ mice were treated as in Fig. 1 to cross-link to BCR at 37°C for 0 to 30 min, fixed, permeabilized, and stained with rabbit phospho-Btk (Y223)-specific Abs detected using PE-conjugated Abs specific for rabbit Ig. The cells were analyzed by flow cytometry and the percent phospho-Btk positive cells relative to the negative control is given for each time point. (C) B cells from Cbl-$b^{+/-}$ and Cbl-$b^{-/-}$ mice were treated as in B to cross-link the BCR, lysed and the lysates immunoprecipitated using the phospho-Btk (Y223)-specific Abs and the immunoprecipitates analyzed by immunoblot probing first using a phosphotyrosine-specific mAb, stripped, and re-probed using antibodies specific for PLC-γ2, Btk, or Syk.

Cbl-$b^{-/-}$ B cells before and at various times after BCR cross-linking and the immunoprecipitates analyzed by immunoblot probing for phosphotyrosine. In resting B cells from both Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ mice low levels of phosphorylated Vav-1 were detected (Fig. 6 A). BCR cross-linking resulted in the rapid phosphorylation of Vav-1 in both Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ B cells which persisted for the 30-min course of the experiment. Densitometry analyses of the intensities of the bands in the Vav and phosphotyrosine immunoblots in Fig. 6 A indicated a small increase in the level of phosphorylation of Vav at the 2 and 5 min time point in Cbl-$b^{-/-}$ versus Cbl-$b^{+/+}$ B cells, however, this increase was not completely reproducible. Significantly, the association of Vav with Syk was prolonged in Cbl-$b^{-/-}$ B cells as compared with Cbl-$b^{+/+}$ B cells. Probing immunoblots of Vav-1 immunoprecipitates for Syk showed larger amounts of Syk associated with Vav at early time points after BCR cross-linking that persisted for longer in Cbl-$b^{-/-}$ as compared with Cbl-$b^{+/+}$ B cells (Fig. 6 A). Probing the immunoblot for phosphotyrosine-containing proteins indicated that the Syk associated with Vav was phosphorylated (Fig. 6 A). In addition to phosphorylated Syk, a phosphoprotein migrating at 77 kD was also more strongly associated with Vav-1 in Cbl-$b^{-/-}$ as compared with Cbl-$b^{+/+}$ B cells. The identity of this protein is not known, however, given that Btk is phosphorylated upon BCR cross-linking (Fig. 4) and associates with phospho-Syk (Fig. 4) and based on its MW, Btk is a good candidate for this 77 kD phosphoprotein.

Syk has also been shown to play an important role in activating PI3K through phosphorylation (31). Little differences were detected in Cbl-$b^{+/+}$ versus Cbl-$B^{-/-}$ B cells in the phosphorylation of PI3K (unpublished data) or Akt (Fig. 6 B), a downstream effector of PI3K. Thus, Cbl-b deficiencies do not appear to significantly affect the phosphorylation of proteins in the PI3-K pathway downstream of Syk.

Prolonged Phosphorylation of ERK and JNK in Cbl-$b^{-/-}$ B Cells. The MAP kinases, ERK and JNK, that regulate nuclear transcription events are also major downstream targets of BCR signaling cascades leading to B cell activation (4). To determine if these pathways were influenced by Cbl-b, the level of tyrosine phosphorylation of ERK 1 and 2 and JNK 1 and 2 were monitored at various times after BCR cross-linking. As compared with Cbl-$b^{+/+}$ B cells, ERK was more strongly phosphorylated throughout a 20 min time course in Cbl-$b^{-/-}$ B cells (Fig. 7 A). Similarly, both forms of JNK were more strongly phosphorylated in Cbl-$b^{-/-}$ as compared to Cbl-$b^{+/+}$ B cells (Fig. 7 A). Thus, the signaling pathways involving ERK and JNK appear to be regulated by Cbl-b. Consistent with the increased activation of ERK in Cbl-$b^{-/-}$ B cells, Cbl-$b^{-/-}$ B cells showed greater expression of the early activation

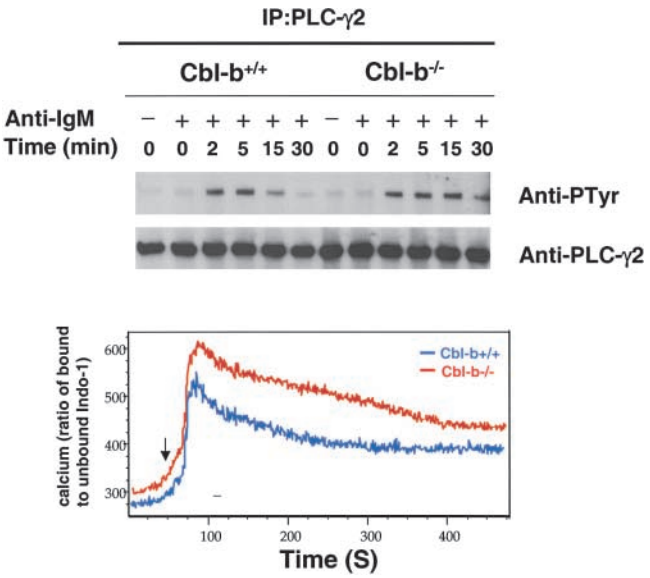

Figure 5. PLC-γ2 phosphorylation and downstream Ca^{2+} fluxes are prolonged in activated Cbl-$b^{-/-}$ B cells. (Top) B cells from Cbl-$b^{-/-}$ and Cbl-$b^{+/+}$ mice were treated to cross-link the BCR as in Fig. 1, lysed, and the lysates subjected to immunoprecipitation using PLC-γ2–specific antibodies. The immunoprecipitates were analyzed by immunoblot for phosphotyrosine-containing proteins, stripped, and reprobed for PLC-γ2. A representative result of three independent experiments is shown. (Bottom) B cells were incubated with Indo-1 for 45 min at 30°C and stained with a PE-conjugated B220-specific mAb to allow gating on the B cells. The cells were treated at 37°C with a rat mAb specific for IgM for 30 s followed by $F(ab')_2$ goat antibodies specific for rat IgG to cross-link the BCR. The cells were monitored for 512s by flow cytometry for bound (violet) and unbound (blue) Indo-1. The arrow indicates the time of addition of the secondary antibody.

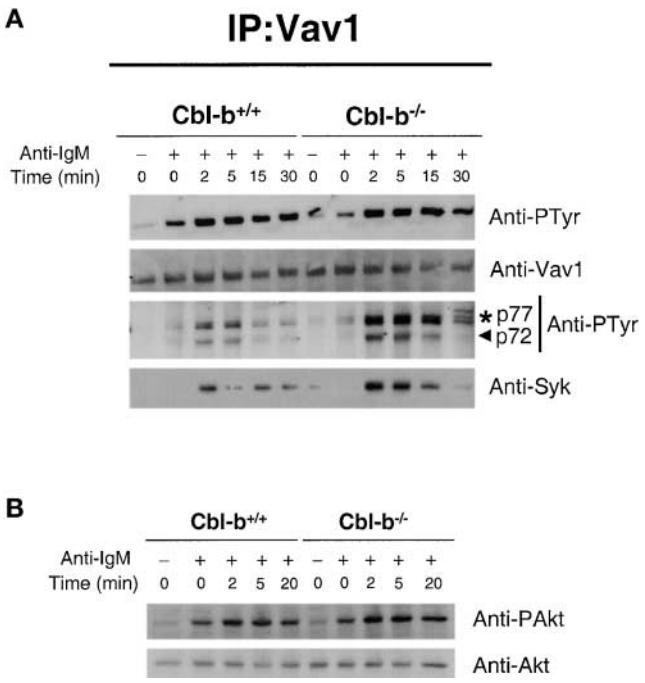

Figure 6. The association of phosphorylated Syk with Vav1 is prolonged in Cbl-$b^{-/-}$ B cells. Purified splenic B cells from Cbl-$b^{-/-}$ and Cbl-$b^{+/+}$ mice were treated to cross-link the BCR as in Fig. 1. (A) The cells were lysed and the lysates subjected to immunoprecipitation using Vav1-specific Abs. The immunoprecipitates were analyzed by immunoblotting probing for phosphotyrosine using the mAb PY20. The blots were stripped and probed for Vav1 or Syk. (B) Alternatively, the unfractionated lysates were analyzed by immunoblot probing for phospho-Akt (S473) stripped and reprobed for Akt.

marker CD69 that is partially dependent on ERK activation (32). Maximal expression of CD69 was induced by treating the cells with 10 μg/ml IgM-specific antibody to cross-link the BCR in both Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ B cells but maximal CD69 expression was over twofold greater in Cbl-$b^{-/-}$ B cells (Fig. 7 B).

The Formation of Signaling Active BCR Cap Structures Is Prolonged in Cbl-$b^{-/-}$ B Cells. After cross-linking, the BCR clusters to form patches that subsequently move to one pole of the B cell to form a cap that is ultimately condensed and internalized (33). Recent evidence suggests that the cap is a region of active signaling and that Syk promotes and stabilizes cap formation in B cells (34). As described above (Fig. 2, D and E) after BCR cross-linking the BCR remained colocalized with phospho-Syk for a longer period of time in Cbl-$b^{-/-}$ as compared with Cbl-$b^{+/-}$ B cells. The cross-linking-induced capping of the BCR and colocalization of the BCR with tyrosine phosphorylated proteins, specifically with Syk and phospho-Syk, was further characterized. B cells were incubated at 4°C with IgM-specific rat mAbs followed by biotin-conjugated goat Abs specific for rat Ig, washed, and warmed to 37°C for increasing lengths of time. The cells were fixed, and incubated with Texas Red–conjugated streptavidin to detect the BCR. To detect phosphotyrosine-containing proteins, the cells were permeabilized and incubated with the phosphotyrosine-specific mAb, PY20, detected using FITC-conjugated $F(ab')_2$ goat Abs specific for mouse IgG. To characterize the patched and capped structures with regard to Syk and phospho-Syk colocalization, B cells were incubated with RRX-conjugated Fab goat Abs specific for Igμ, washed, and the BCR cross-linked by the addition of goat Abs specific for mouse IgM. The cells were warmed to 37°C for varying lengths of time, fixed, permeabilized, and stained for Syk and phospho-Syk using rabbit Abs specific for Syk or phospho-Syk (Y519/520) detected using AlexaFluor 488-conjugated Abs specific rabbit Ig. The cells were imaged by confocal laser scanning microscopy and the merged images of the BCR and either phosphotyrosine, phospho-Syk or Syk are shown (Fig. 8, a–c). The images were analyzed pixel by pixel as described above (Fig. 2 D) and the data displayed in histograms. Similar structures were observed in B cells from Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ mice and shown are the images of Cbl-$b^{-/-}$ B cells. Cross-linking the BCR resulted immediately in BCR patches (Fig. 8, a–c, top) that with time at 37°C formed large caps at one pole of the cell (Cap I structures; Fig. 8, a–c, middle) that proceeded to form tight, condensed Cap II structures (Fig. 8, a–c, bottom). The BCR in the patches and large Cap I structures were nearly completely coincident with phosphotyrosine-containing proteins as indicated in the merged

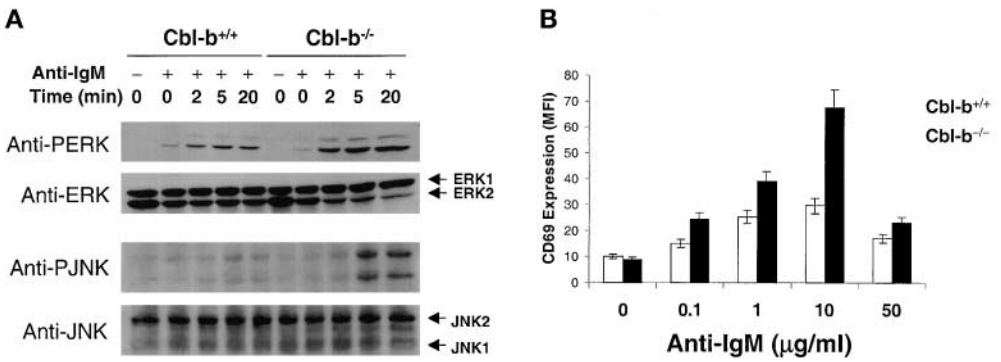

Figure 7. ERK and JNK phosphorylation are prolonged and downstream CD69 expression increased in Cbl-b$^{-/-}$ B cells. (A) Purified splenic B cells were treated to cross-link the BCR as in Fig. 1. The cells were lysed and equal amounts of whole-cell lysates subjected to immunoblotting using Abs specific for phospho-ERK (anti-PERK) and phospho-JNK (anti-PJNK). The blots were stripped and reprobed for ERK1 and 2 and JNK1 and 2. (B) After BCR cross-linking the cells were incubated for 20 h at which time the surface expression of CD69 was quantified by flow cytometry. The results are the mean of duplicate cultures of one of three representative experiments.

image showing colocalization of the red and green fluorescent dyes yielding yellow (Fig. 8 a, top, middle). In addition, the analyses of the distribution of the fluorescence intensities of the green and red dyes showed that most pixels lie along the diagonal of the histogram indicating colocalization of IgM and phosphotyrosine-containing proteins (Fig. 8 a, top, middle). Thus, both the patched and Cap I structures appear signaling active. In contrast, the Cap II BCR structures showed no staining for phosphotyrosine indicating that these BCR structures are signaling inactive (Fig. 8 a, bottom). The analyses of the red and green fluorescence intensities showed little colocalization of red and green dye with most pixels lying significantly above the diagonal (Fig. 8 a, bottom). The analysis of cells stained with Syk-specific Abs showed near complete colocalization of Syk with the BCR in patched and Cap I structures yielding yellow in the image (Fig. 8 c, top, middle) and quantification of the image showed a near complete colocalization of the BCR and Syk. However, not all of the Syk associated with the BCR in the patched and Cap I structures was active phospho-Syk (Y519/520). The images of cells stained to detect phospho-Syk (Y519/520) and the BCR show distinct red areas indicating that the BCR was not completely colocalized with phospho-Syk (Y519/520). The quantification of the image showed significant proportion of the pixels lie above the diagonal indicating regions that contain the BCR but not phospho-Syk (Y519/520). The Cap II structures showed virtually no staining with either the Syk-specific Abs (Fig. 8 c, bottom panel) or with the phospho-Syk (Y519/520)-specific Abs (Fig. 8 b, bottom). The condensed Cap II structures are often associated with intracellular vesicles containing the BCR as shown in Fig. 8 c, bottom panel. Although the Cap II structures showed no staining for Syk the vesicles containing internalized BCR stained for Syk. The quantitation of the MFI of the image showed two clear BCR areas one of which contains Syk and one of which did not. This observation suggests that the internalized BCR associated with Syk enters the cell from active Syk-containing Cap I structures and not from the signaling inactive Cap II structures. The BCR in the Cap II structure may also be internalized but degraded too rapidly to be imaged.

The number of patch, Cap I and Cap II structures formed by B cells from Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ B cells after BCR cross-linking were counted and compared (Fig. 8 d). Similar numbers of B cells from Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ mice formed patches with nearly all B cells showing a patched morphology immediately after BCR cross-linking which decreased with time at 37°C (Fig. 8 d, top). Significantly, Cbl-b$^{-/-}$ B cells formed BCR Cap I structures more rapidly as compared with Cbl-b$^{+/+}$ B cells such that after 2 min at 37°C the BCR was in Cap I structures in 62% of Cbl-b$^{-/-}$ B cells as compared with 32% of Cbl-b$^{+/+}$ B cells (Fig. 8 d, middle). In several experiments the time course of polarization varied by 2–3 min but the accelerated rate of polarization in Cbl-b$^{-/-}$ versus Cbl-b$^{+/+}$ was observed in each experiment. The presence of BCR in Cap I structures was sustained in B cells over the course of the experiment in Cbl-b$^{-/-}$ as compared with Cbl-b$^{+/+}$ B cells (50% versus 32%) in agreement with the observation above (Fig. 2, D and E) that the BCR remained colocalized with phospho-Syk for longer in Cbl-b$^{-/-}$ as compared with Cbl-b$^{+/-}$ B cells. In addition, more Cbl-b$^{+/+}$ B cells progressed to form signaling inactive Cap II structures as compared with Cbl-b$^{-/-}$ B cells (Fig. 8 d, bottom) suggesting that Cbl-b by negatively regulating the BCR signaling promoted the condensing of the BCR into signaling inactive Cap II structures.

The capping of the BCR has been shown to be dependent on the association of the BCR with the actin cytoskeleton (35). The difference observed in the rate of BCR caps was reflected in the rate of activation of CDC42 and of F-actin polymerization (Fig. 9). CDC42 is a member of the Rac family of GTPases that when activated binds to WASP which promotes actin cytoskeleton association. The level of activated CDC42 in B cells from Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ mice at various times after BCR cross-linking was determined by permeabilizing the cells and incubating with the recombinant fusion protein WASP-GBD-GFP. The GFP signal of the recombinant protein was amplified by incubation with GFP-specific Abs detected using FITC-conjugated secondary Abs. Cbl-b$^{-/-}$ B cells showed an immediate increase in active CDC42 which decreased by 2 min and remained at an elevated level for the course of the

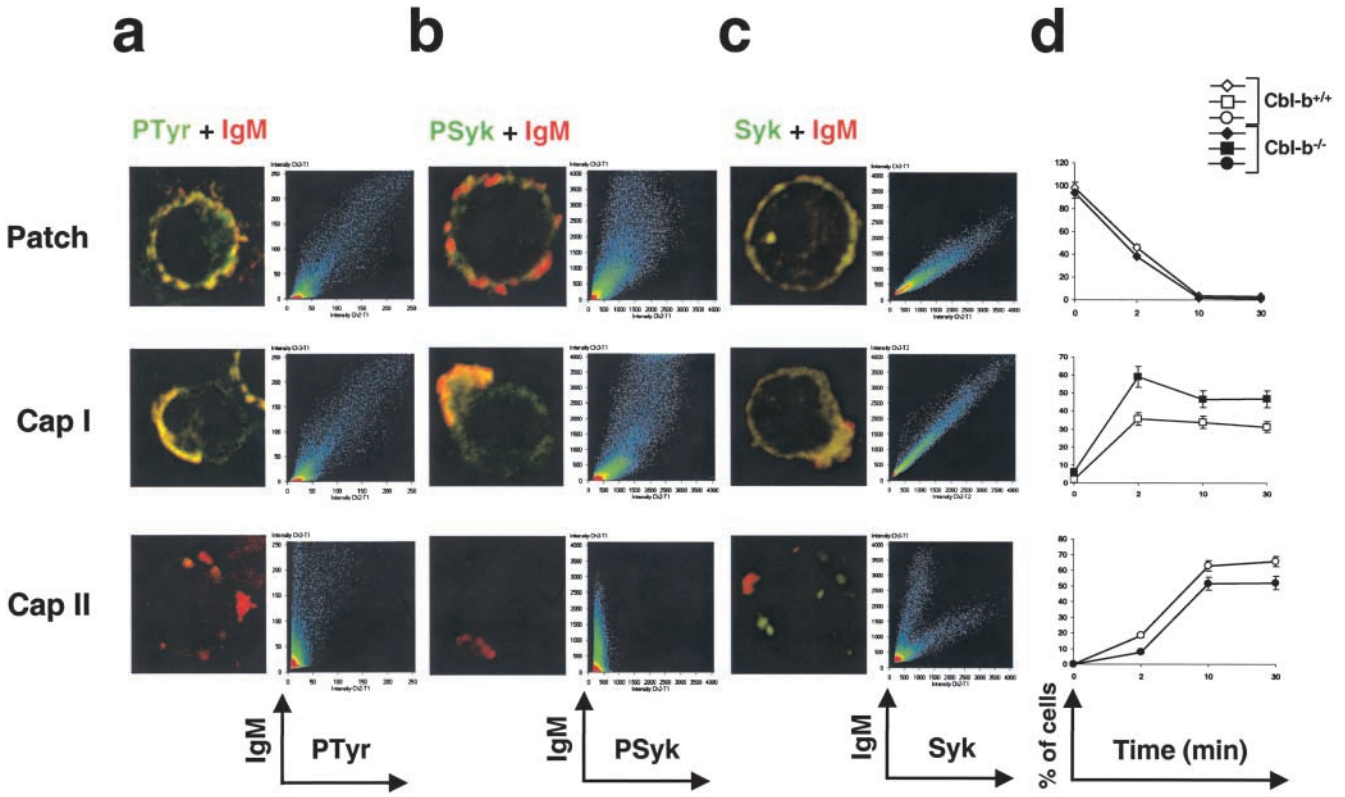

Figure 8. Signaling active BCR caps are prolonged in Cbl-b$^{-/-}$ B cells. (a) Splenic B cells from Cbl-b$^{-/-}$ mice were incubated with a rat mAb specific for IgM at 4°C, washed and incubated with biotin-labeled goat antibodies specific for rat IgG and warmed to 37°C for increasing lengths of time. At the end of each time point the cells were fixed and incubated with Texas Red–conjugated streptavidin to detect the BCR. The cells were permeabilized and incubated with the phosphotyrosine-specific mAb PY20 detected using FITC-labeled F(ab′)$_2$ goat Abs specific for mouse IgG (b and c). Splenic B cells from Cbl-b$^{-/-}$ mice were incubated with RRX-conjugated Fab goat Abs specific for Igμ for 15 min at 25°C and the BCR was cross-linked by the addition of goat Abs specific for mouse IgM. The cells were incubated at 37°C for increasing lengths of time, permeabilized, and incubated with either rabbit phospho-Syk (Y519/520)-specific Abs (b) or with rabbit Syk-specific Abs (c) each detected using AlexaFluor 488-labeled goat Abs specific for rabbit IgG. In each case, the cells were examined by confocal laser scanning microscopy and shown are the merged images of the optimal single planes at a magnification of ×63. Shown are typical images of patch, Cap I and Cap II structures taken for phosphotyrosine images at 5 min for patch and Cap I structures and 30 min for Cap II structures and for Syk and phospho-Syk images at 0 min for patch, 2 min for Cap I, and 30 min for Cap II structures. The images were analyzed by the LSM 5 software program to quantify the colocalization of red and green fluorescence and the data plotted in histograms. (d) Purified splenic B cells from Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ mice were treated as in panel a to cross-link the BCR and cells were incubated at 37°C for increasing lengths of time. At the end of each time point the cells were fixed with 3.7% paraformaldehyde, stained with Alexa 488-conjugated streptavidin, and examined by fluorescence microscopy. The mean percentage (±SD) of B cells exhibiting the patch, Cap I and Cap II structures with time after warming to 37°C is given. At least 500 cells at random were counted per time point.

experiment (Fig. 9 A). In contrast Cbl-b$^{+/+}$ B cells showed no immediate rapid increase in active CDC42 but rather active CDC42 increased slowly in Cbl-b$^{+/+}$ B cells reaching levels comparable to those in Cbl-b$^{-/-}$ B cells by 2 min. Actin polymerization was measured after BCR cross-linking in permeabilized and fixed cells using Alexa 488-conjugated phalloidin. Cbl-b$^{-/-}$ B cells showed slightly accelerated actin polymerization as compared with Cbl-b$^{+/+}$ B cells (Fig. 9 B). Consistent with these observations a small but significantly larger proportion of the BCR was found associated with the actin cytoskeleton in a high-density pellet from lysates of Cbl-b$^{-/-}$ as compared with Cbl-b$^{+/+}$ B cells (unpublished data).

To directly assess the requirements for signaling and the actin cytoskeleton in the formation of patch, Cap I and Cap II structures, Cbl-b$^{-/-}$ B cells were treated with the following inhibitors: latrunculin (LN; reference 36) or Cytochalasin D (CytD; reference 36), inhibitors of the actin cytoskeleton; PP2, an inhibitor of Src family kinases (37); and piceatannol (Pic), an inhibitor of Syk (38) that has also been reported to inhibit additional protein tyrosine kinases (39). As shown in Fig. 9 C, 2 min after BCR cross-linking the BCR were patched on the majority of untreated cells. Patching at 2 min after BCR cross-linking was not affected by any inhibitor indicating that patching was not dependent on Src or Syk signaling nor on an intact cytoskeleton. This is despite the observation that phospho-Syk (Y519/520) colocalized with the BCR in patches (Fig. 8 b). 10 min following BCR cross-linking the BCR were observed in Cap I (60%) and Cap II structures (25%). Each inhibitor significantly blocked the formation of Cap I structures and the BCR in treated cells remained patched. Thus, the formation of Cap I structures required both Src and Syk signaling and an intact actin cytoskeleton. Significantly, in B

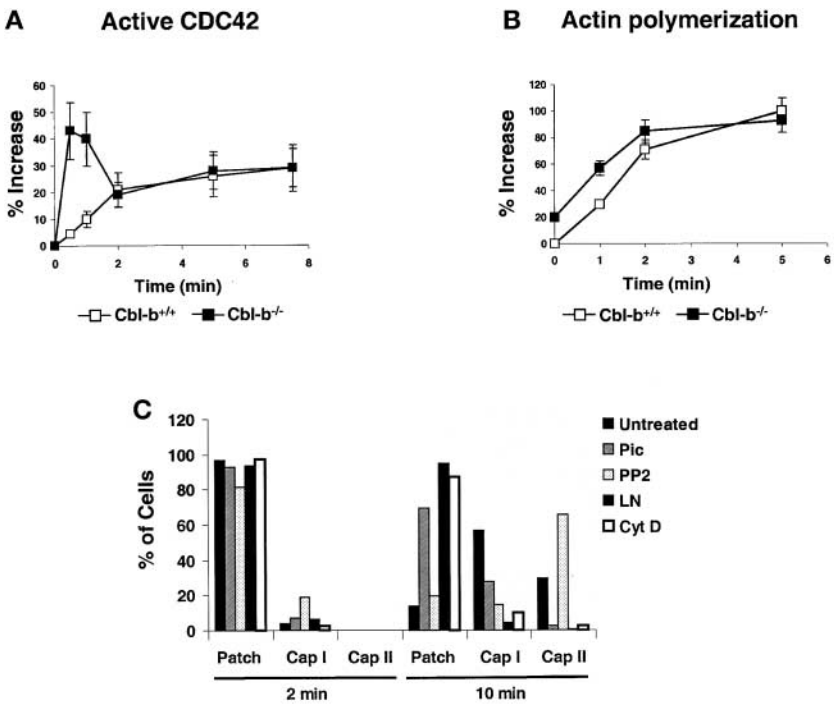

Figure 9. CDC42 activation and actin polymerization are enhanced in Cbl-b–deficient B cells. (A) Purified splenic B cells from Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ mice were treated to cross-link the BCR as in Fig. 2, A–C, incubated at 37°C for the times indicated, fixed, permeabilized, and incubated with the WASP-GBD-GFP fusion protein followed by incubation with FITC-conjugated-rabbit antibodies specific for GFP and then FITC-conjugated goat antibodies specific for rabbit Ig. The cells were analyzed by flow cytometry and the results presented as the percent increase in mean fluorescence of activated B cells relative to the mean fluorescence of resting B cells. (B) B cells were treated as in panel A and permeabilized cells were stained with Alexa 488–conjugated phalloidin and analyzed by flow cytometry. Shown is the percent increase of the mean fluorescence of stimulated B cells relative to the mean fluorescence intensity of resting cells at each time point. (C) Splenic B cells from Cbl-b$^{-/-}$ mice were pretreated with the following inhibitors: piceatannol (100 μM for 1 h at 37°C) a Syk inhibitor(38); PP2 (100 μM for 1 h at 37°C) a Src-family kinase inhibitor (reference 37); and Cytochalasin D (10 μM for 1 h at 0°C; reference 36) or Latrunculin (10 μM for 30 min at 37°C; reference 36) inhibitors of the actin cytoskeleton. The pretreated cells were incubated with a rat mAb specific for IgM, washed and incubated for 2 or 10 min at 37°C with biotin-labeled F(ab′)$_2$ goat antibodies specific for rat IgG, fixed and stained with Alexa 488–labeled streptavidin to visualize the BCR. The cells were examined by fluorescence microscopy and the number of cells showing a patch, Cap I or Cap II morphology were scored.

cells treated with PP2 the number of cells with Cap I structures decreased and the number of cells with a Cap II morphology increased indicating that when Src-dependent signaling is blocked the BCR progresses to signaling inactive Cap II structures.

Discussion

Here we provide evidence that in B cells Cbl-b functions to negatively regulate BCR signaling by targeting Syk for ubiquitination. In Cbl-b–deficient B cells Syk fails to be ubiquitinated following BCR cross-linking in contrast to B cells from wild-type mice in which case BCR cross-linking leads to rapid ubiquitination of Syk. The results presented suggest that Cbl-b ubiquitinates active phosphorylated Syk and thus functions to dampen BCR signaling after signaling is initiated and thus plays a role in the normal down modulation of BCR signaling. Two additional negative regulators of BCR signaling have been described recently, namely SHIP and SHP-1. However, the targets of their regulation appear distinct from that described here for Cbl-b. SHIP, a 5′ inositol phosphatase, was first described to block BCR signaling when recruited to the low affinity Fc receptor, FcγRIIb, after the coligation of the BCR and FcγRIIb by the binding of immune complexes (40). SHIP dephosphorylates PIP(3,4,5)P3, the phospholipid product of PI-3K activity, to PI(3,4)P2. Recently, SHIP-deficient B cells were shown to have elevated levels of PI(3,4,5)P3 (11) increased recruitment of Btk to the plasma membrane (12) and enhanced Ca^{2+} signaling (13). However, ERK activation appeared unaffected in SHIP-deficient cells (13). In contrast, Cbl-b does not appear to effect PI3K activity or PI3K-downstream effectors but does influence ERK activity. SHP-1, a tyrosine phosphatase, was recently shown to down-regulate the activation of Lyn and Lyn-induced tyrosine phosphorylation of the CD19 receptor in B cells resulting in reduced B cell activation (14). As shown here, Cbl-b does not appear to affect Lyn activity. Thus, these three negative regulators of BCR signaling appear to target different elements of the BCR signaling pathways leading to down modulation of B cell responses. It will be of interest to understand the mechanisms which trigger the activity of these regulators and how their activities are coordinated.

Lymphocytes express both c-Cbl and Cbl-b. In T cells Cbl-b and c-Cbl appear to function at different stages of development and to target different substrates for ubiquitination. c-Cbl has been reported to target Syk for ubiquitination in human Ramos B cells upon BCR cross-linking and in this way to function as a negative regulator of B cell activation (17). Thus, Cbl-b and c-Cbl may have somewhat redundant functions in regulating mature B cell activation through the ubiquitination of Syk. However, recent studies suggest that Cbl-b and c-Cbl may function differently and regulate different targets in developing versus mature B cells. In immature DT40 chicken B cells evidence was provided that c-Cbl negatively regulates BCR signaling through its affect on the essential adaptor protein BLNK resulting in a block in the recruitment of PLC-γ2 to BLNK and PLC-γ2 phosphorylation (41). In contrast, Cbl-b was shown to positively regulate Btk-mediated activation of PLC-γ2 in immature DT40 B cells (24). Evidence was also provided that Cbl-b functioned similarly in

the immature mouse WEHI B cells. In control experiments, PLC-γ2 was shown to be phosphorylated to similar levels in mature Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ B cells 1 min after BCR cross-linking consistent with the results presented here. Taken together these findings suggest that Cbl-b and c-Cbl may provide overlapping functions in the regulation of Syk in mature B cells but have distinct targets in immature B cells resulting in both positive and negative regulation.

The molecular mechanism by which Cbl-b facilitates the ubiquitination of Syk is not known. Ubiquitination is initiated by the linkage of ubiquitin (Ub) to an Ub-activating enzyme, E1. Ub is then transferred by E1 to an Ub conjugating enzyme, E2, that subsequently conjugates the COOH terminus of Ub to a lysine on the target protein through a isopeptide bond. The specificity of ubiquitination is largely determined by Ub protein ligases, E3s, which interact with both E2s, through RING finger domains, and the E2 substrates and thus facilitate the conjugation of Ub to the target protein (42). Members of the Cbl family are E3 ligases that bind to the kinases targeted for ubiquitination through their TKB domains (16). The ubiquitination of Syk by c-Cbl minimally requires the TKB and RING finger domain of c-Cbl. The TKB domain of c-Cbl binds to tyrosine 317(Y317) in the linker region between the SH2 and the catalytic domains of mouse Syk when it is phosphorylated following BCR cross-linking. A mutant form of Syk that lacks the Y317 showed an enhanced ability to interact with Igα suggesting that the association of c-Cbl with Syk blocks its ability to interact with Igα and to couple the BCR to downstream signaling pathways (43). Based on these observations a model can be proposed for the regulation of BCR signaling by Cbl-b in which BCR cross-linking results in the phosphorylation of Igα, the recruitment of Syk to Igα through the SH2 domains of Syk and phosphorylation of Syk at multiple tyrosines including Y317. Cbl-b would bind to Syk Y317 through its TKB domain resulting in both the modulation of Syk/Ig association and the ubiquitination of Syk by an E2 conjugating enzyme bound to the RING finger domain of Cbl-b. The ubiquitinated Syk would be subsequently degraded. Consistent with this model we observe that in Cbl-b–deficient B cells Syk is not ubiquitinated and the phosphorylation of Igα and association of Igα with phosphorylated Syk is prolonged. These events correlated with the prolonged association of phosphorylated Syk with Vav, the prolonged phosphorylation of BLNK, PLC-γ2, ERK, and JNK, increased and prolonged Ca^{2+} fluxes, and increases in the expression of the activation marker CD69. Also consistent with a model in which Cbl-b targets Syk for degradation was the observation that the activation of Lyn was not significantly affected in Cbl-b-deficient B cells. Thus, by targeting Syk for degradation and modulating its association with Igα, Cbl-b regulates several downstream signaling pathways of Syk. It is of interest that the PI3K pathway was apparently not influenced in Cbl-b–deficient B cells. It remains to be determined if this is due to a functional redundancy of Cbl-b and c-Cbl.

Cbl-b also appeared to influence the formation of stable, signaling active BCR caps after BCR cross-linking. In T cells the polarization of the TCR after ligation and the formation of the supramolecular activation complex and the immunological synapse appear to be crucial prerequisites for T cell activation (44). Evidence has been provided that Cbl-b influences this process by negatively regulating the coupling of the TCR to Vav-1 and downstream CDC42 and WASP, leading to actin cytoskeleton-dependent TCR clustering (45). In B cells, the BCR has been shown to polarize after cross-linking into structures that concentrate Syk, Vav, Btk, and Rac and thus appear analogous to the T cell immunological synapses (46). Here we show that in B cells from Cbl-b–deficient mice as compared with wild-type B cells the BCR remains in a signaling active Cap I structure for longer periods of time and less BCR is found condensed in signaling inactive Cap II structures. Although the functional significance of the Cap II structures is not known, it is possible that they represent preendocytotic structures involved in the removal of signaling inactive BCR from the surface. However, although Cap II structures often appear near intracellular vesicles that contain internalized BCR we show here that the intracellular BCR is associated with Syk while the Cap II BCR is not. Earlier studies showed that internalization of the BCR is dependent on BCR signaling although the exact nature of the signals required is not known. The studies presented here suggest that BCRs may be internalized from active Cap I structures as a part of normal down modulation of the response. As receptors are internalized and signaling dampened the BCRs remaining on the surface may condense into Cap II structures which are then internalized and degraded.

In summary, the studies presented here provide evidence that Cbl-b negatively regulates Syk through ubiquitination. Thus, Cbl-b has the potential to block BCR signaling at an early step effectively uncoupling the BCR from many downstream signaling pathways. Consequently, it will be of significant interest to determine the factors that induce and regulate Cbl-b activity.

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