The Syk-binding Ubiquitin Ligase c-Cbl Mediates Signaling-dependent B Cell Receptor Ubiquitination and B Cell Receptor-mediated Antigen Processing and Presentation*†

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Background: c-Cbl associates with various signaling molecules to regulate diverse signaling networks via ubiquitination of receptors and/or protein tyrosine kinases.

Results: c-Cbl drives ubiquitin-dependent Ag:BCR trafficking to MIIC and BCR-mediated antigen processing and presentation.

Conclusion: c-Cbl directs BCR-mediated antigen processing and presentation.

Significance: c-Cbl coordinates antigen-induced BCR signaling and rapid BCR-mediated antigen processing and presentation to drive a strong humoral immune response.

B cell receptor (BCR)-mediated antigen (Ag) processing and presentation lead to B cell-T cell interactions, which support affinity maturation and immunoglobulin class switching. These interactions are supported by generation of peptide-MHC class II complexes in multivesicular body-like MIIC compartments of B cells. Previous studies have shown that trafficking of Ag:BCR complexes to MVB-like MIIC occurs via an ubiquitin-dependent pathway and that ubiquitination of Ag:BCR complexes occurs by an Src family kinase signaling-dependent mechanism that is restricted to lipid raft-resident Ag:BCR complexes. This study establishes that downstream Syk-dependent BCR signaling is also required for BCR ubiquitination and BCR-mediated antigen processing and presentation. Knockdown studies reveal that of the two known Syk-binding E3 ubiquitin ligases c-Cbl and Cbl-b, only c-Cbl appears to have a central role in BCR ubiquitination, trafficking to MIIC, and ubiquitin-dependent BCR-mediated antigen processing and presentation. These results establish the novel role for Syk signaling and the Syk-binding ubiquitin ligase c-Cbl in the BCR-mediated processing and presentation of cognate antigen and define one mechanism by which antigen-induced BCR ubiquitination is modulated to impact the initiation and maturation of the humoral immune response.

B lymphocytes are unique antigen-presenting cells in that they are the only antigen-presenting cells with a clonotypically restricted antigen binding receptor, the B cell receptor (BCR)²

(1). The BCR complex is composed of a membrane-bound immunoglobulin heavy and light chain antigen-binding subunit non-covalently coupled to a CD79 signaling subunit which are converted to antigenic peptide class II complexes (18). However, the precise molecular mechanism of ligand-induced BCR ubiquitination remains unclear.

Binding of antigen to the BCR results in Ag:BCR endocytosis and trafficking to multivesicular body (MVB)-like MIIC where Ag:BCR complexes are processed to peptides that are then loaded onto MHC class II molecules for presentation to CD4 T cells (9–14). The resulting B cell-T cell interactions are essential for immunoglobulin class switching and affinity maturation, critical aspects of the humoral immune response.

Ubiquitination of cell surface receptors such as the epidermal growth factor receptor targets internalized receptors for interaction with the ESCRT protein sorting complex on the limiting membrane of MVB, resulting in receptor delivery to MVB intralumenal vesicles and, ultimately, to degradative endocytic compartments (15). Binding of antigen to the BCR results in ubiquitination of the BCR cytoplasmic tail, which targets delivery of internalized Ag:BCR complexes to MVB-like MIIC where they are converted to antigenic peptide class II complexes (16, 17). Previous reports have established that BCR ubiquitination is signaling-dependent and restricted to lipid raft-resident Ag:BCR complexes (18). However, the precise molecular mechanism of ligand-induced BCR ubiquitination remains unclear.

Binding of antigen to the BCR initiates a signaling cascade that starts with Src-family kinase-mediated phosphorylation of cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) on CD79a and CD79b (2–8). The resulting dually phosphorylated ITAMs function as a docking site for spleen tyrosine kinase (Syk) (2, 3, 7, 8, 19). Signaling cascades downstream of Syk involve several protein tyrosine kinases and adaptor proteins such as Btk, PLCγ2, BLNK, PI3 kinase, and Vav (13, 19). Moreover, Syk can regulate actin dynamics to impact the intracellular trafficking and processing of Ag:BCR complexes (20).

BCR antigen engagement also results in recruitment of several regulatory molecules such as the Cbl (Casitas B-lineage lymphoma) family of E3 ubiquitin ligases. Two members of this...
family, c-Cbl and Cbl-b, which have distinct functions in B cells (21, 22), interact with several BCR signaling molecules such as PLCγ2, BLNK, PI3 kinase, Lyn, Vav, and, most relevant to this report, Syk (21–23). Subsequent to ITAM binding, Syk is phosphorylated on tyrosine 323, which generates a binding site for c-Cbl (24). Binding of c-Cbl results in Syk ubiquitination and down-regulation of BCR signaling (24). Because BCR ubiquitination is Src-dependent (18) and both Syk and Cbl (c-Cbl and Cbl-b) are downstream of Src signaling, it was of interest to determine the role of Syk and Cbl in BCR ubiquitination and BCR-mediated antigen processing. Here we establish that the E3 ubiquitin ligase c-Cbl mediates Ag-driven BCR ubiquitination and drives BCR-mediated Ag processing. These results define the mechanism by which c-Cbl functions to coordinate antigen-induced BCR signaling and ubiquitination as well as the rapid processing and presentation of Ag-BCR complexes to support the development of the humoral immune response.

EXPERIMENTAL PROCEDURES

Animals—MD4.B10.Br mice were bred at Taconic Farms, Inc. B10.BR/SgSnJ (B10.Br) mice were purchased from The Jackson Laboratory. Mice were housed in the Albany Medical College Animal Resource Facility under specific-pathogen-free conditions. The Albany Medical College Institutional Animal Care and Use Committee approved all reported protocols.

Cells—A20uWT B cells (murine B cell line expressing a wild-type human IgM BCR specific for phosphorylcholine (PC) (25)) were maintained in α modified Eagle’s medium, 5% FBS, and 50 μM 2-mercaptoethanol with 500 μg/ml of G418 to maintain human IgM expression. Splenocytes from B10.Br (expressing a non-HEL-specific IgM BCR and I-Ak class II) and MD4 B10.Br (expressing a HEL-specific IgM BCR and I-Ak class II) mice were isolated and maintained in tissue culture as reported previously (16). DO11.10 T cells were cultured in DMEM with 10% FBS, sodium pyruvate, 1-glutamine, 50 μg/ml penicillin-streptomycin and 50 μM 2-mercaptoethanol. Cells were maintained at a density of < 1 × 10⁶ viable cells/ml at 37 °C, 5% CO₂.

Reagents—The following reagents were used for this study: hen egg lysozyme (HEL, catalog no. L-6876, Sigma), HEL₄₆–₆₁-I-Ak-specific monoclonal antibody C4H3 (26), goat anti-mouse IgG F(ab')₄ (catalog no. 115-006-006, Jackson ImmunoResearch, West Grove, PA), FITC rat anti-mouse IgG₁ (A85-1, catalog no. 553443, BD Biosciences), FITC anti-mouse IgM (DS-1, IgG₁, catalog no. 553516, BD Biosciences), PE anti-CD45R/B220 (RA3-6B2, IgG₂b, catalog no. 553090, BD Biosciences), goat anti-human IgM F(ab')₂ (catalog no. 109-006-129, Jackson ImmunoResearch), rabbit anti-human IgM Btn (catalog no. 309-065-095, Jackson ImmunoResearch), biotin anti-mouse IgM (catalog no. 553515, BD Biosciences), c-Cbl antibody (catalog no. 2747, Cell Signaling Technology), purified mouse anti-c-Cbl (catalog no. 610442, BD Biosciences), Cbl-b (clone G1, catalog no. sc-8006), Cbl-b (clone C20, catalogue no. sc-1435), purified rat anti-mouse CD107b (LAMP-2) monoclonal antibody (BD Biosciences, catalog no. 558756), SA-Alexa Fluor 594 (Molecular Probes, catalog no. S21374); Alexa Fluor 647 anti-rat IgG (H+L) (Molecular Probes, catalog no. A-21247), fluoro-gel (Electron Microscopy Sciences, catalog no. 17985-10), RNeasy mini kit (Qiagen, catalog no. 74104), Transcriptor First Strand cDNA synthesis kit (Roche, catalog no. 04 379 012 001), and AccuPrime TaqDNA polymerase (Invitrogen, catalog no. 12339016).

BCR Signaling—Intracellular calcium measurements were performed as described previously (18, 27).

BCR Internalization—The kinetics of antigen–BCR endocytosis were determined as described previously (18, 27).

BCR Ubiquitination—Analysis of BCR ubiquitination by ubiquitin pull-down and SDS-PAGE Western blot analysis was done as described previously (18, 27).

BCR Processing—Analysis of BCR processing by Western blot analysis was done as described previously (18, 27). For BCR stimulation, biotinylated rabbit anti-human IgM was used at 10 μg/ml, and the cells were pulsed with ligand for the indicated times.

Flow Cytometric Antigen Processing and Presentation Assay—Flow cytometric analysis of HEL₄₆–₆₁-I-Ak expression was done as reported previously (16, 18, 27–29), except for the following modifications. Splenocytes from either MD4.B10.Br or B10.Br mice were pretreated with inhibitors (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine; or piceatannol) diluted from a 10 mM DMSO stock or vehicle control for 1 h at 37 °C before a 2-hour antigen pulse with antigen (HEL, 100 nM HEL for MD4.B10.Br B cells and 100 μM HEL + anti-mulgM₁₁₁ for B10.Br B cells). The cells were then washed and returned to culture in complete media for an additional 24 h. The level of HEL₄₆–₆₁-I-Ak expression at 24 h was determined by staining with HEL₄₆–₆₁-I-Ak-specific mAb C4H3 followed by anti-rat IgG₂b–FITC (26). The MFI of C4H3 staining for live B cells (propidium iodide-negative, B220⁺) was normalized to the vehicle-treated control. 10–3.6-FITC was used to monitor the total I-Ak expression in parallel samples.

c-Cbl and Cbl-b Knockdown in A20uWT Cells—High-titer (1.8–4.72 × 10⁸ TU/ml) viral particles containing Expression Arrest™ GIPZ lentiviral shRNA mir particles to c-Cbl (three clones, catalog nos. V3LMM_478102, V2LMM_3834, and V3LMM_478107; GenBank™ accession nos. NM_007619, AK029826, and AK153915), Cbl-b (three clones, catalog nos. V2LMM_132235, V2LMM_140774, and V2LMM_160020; GenBank™ accession nos. NM_001033238, AK050005, BC035536, and AK084162), GAPDH (positive control, pool of six, catalog no. RHS4372, GenBank™ accession no. NM_008048.2) and non-silencing or scrambled (negative control, pool of 10, catalog no. RHS4348) were obtained from Thermo Scientific Open Biosystems. The binding sites of the shRNA sense strands on c-Cbl and Cbl-b are shown in Fig. 3A. Cells were infected with lentiviral particles at 37 °C with constant inversion for 30 min and returned to culture in complete media to allow viral integration for 48 h. After 48 h, flow cytometric analysis confirmed a transfection efficiency of 8–15% on the basis of GFP expression. The cells were cultured in selection media containing 2 μg/ml puromycin for 5–8 days until > 95% transfection efficiency (on the basis of GFP expression) was achieved. SDS-PAGE and Western blot analysis were used to
confirm the efficiency and selectivity of protein knockdown (30).

**Anti-c-Cbl and Cbl-b Western Blotting**—B cells (WT, Δc-Cbl, ΔCbl-b, ΔGAPDH, and ΔScrambled) were lysed at 1 × 10^7 viable cells/ml in radioimmunoprecipitation assay buffer for 10 min on ice. Postnuclear supernatants were generated by centrifugation for 15 min at 16,000 × g at 4 °C. Samples were analyzed by SDS-PAGE and Western blot analysis (8% gel) using wet transfer conditions. Antibodies anti-c-Cbl (catalog 27477, Cell Signaling Technology) and anti-Cbl-b (clone C20, catalog sc-1435, Santa Cruz Biotechnology) were used to probe for these ~120-kDa proteins. β-Actin was used as a loading control.

**Immunofluorescence Microscopy**—A20μWT B cells and knockdown cells were pulsed with 10 μg/ml biotinylated Rb-anti-hulgM-btn on ice and washed twice to remove excess ligand. Cells were then stained with SA-Alexa Fluor 594. Ag-BCR complexes were then allowed to internalize at 37 °C for 0–60 min. Cells were attached to Alcian blue-treated cover-slips, fixed, and permeabilized (or not) as described previously (29). For staining of the LAMP-2 positive compartment, cells were permeabilized in 0.01% saponin and stained with anti-LAMP-2 (1:100) and anti-rat IgG (H+L) Alexa Fluor 647. Nuclei were stained with 1 μg/ml DAPI before final washing and mounting with Fluoro-gel (Electron Microscopy Sciences) mounting media. Samples were visualized with an Olympus Fluoview FV1000 microscope (×60 numerical aperture 1.25 water immersion lens).

**Measuring Colocalization**—Quantiation of BCR/LAMP-2 colocalization was done by determining the Pearson’s coefficient, which measures the strength of the linear relationship between two variables, for each sample. The analysis was applied to all Z-stack slices. For each condition, 100–150 cells were analyzed.

**In Vitro Antigen Processing and Presentation Assay**—B cells (WT or knockdowns) and Ova-specific T cells (DO11.10) were cocultured at a 1:1 ratio with an indicated concentration of either Ova or PC-Ova for 24 h at 37 °C in a 96-well plate. After 24 h, supernatants were collected, and IL-2 levels were determined using a mouse IL-2 ELISA (Ready-SET-Go! eBioscience, catalog no. 88-7024).

**Statistical Analysis**—Data were analyzed using Student’s t test for differences in densitometry (Figs. 3 and 5), BCR endocytosis (Fig. 4), and in vitro antigen presentation (Figs. 2 and 6) using Microsoft Excel 2008 for Mac Version 12.3.0. Statistical significance is indicated as p ≤ 0.05.

**RESULTS**

**Syk Is Required for Antigen-induced BCR Ubiquitination and BCR-mediated Antigen Processing**—BCR interaction with cognate antigen results in lipid raft-dependent BCR signaling involving raft-resident Src-family kinases and the downstream kinase Syk. BCR ubiquitination, which is critical for the trafficking of Ag-BCR complexes to MVB-like antigen processing compartments, occurs via an Src-family kinase-dependent mechanism and is restricted to lipid raft-resident Ag-BCR complexes (18, 31). Although Syk activity has been implicated in BCR-mediated antigen processing and presentation (5), the role of Syk in BCR ubiquitination remains unclear.

To establish the role of Syk in BCR ubiquitination, B cells were treated with the Syk inhibitor piceatannol, and the impact on Ag-BCR ubiquitination was determined. As expected, treatment of either the A20μWT B cell line (Fig. 1A) or splenic B cells (supplemental Fig. S1A) with piceatannol completely blocks BCR signaling at concentrations greater than 10 μM. Moreover, as reported previously (27), inhibition of Syk activity fails to alter BCR expression or change the kinetics of BCR-mediated ligand internalization (data not shown). To determine the effect of Syk inhibition on Ag-BCR ubiquitination, cell surface BCR molecules were “tagged” with biotinylated anti-BCR antibodies. We then employed an ubiquitin pull-down approach to follow the ubiquitination of tagged BCR molecules (Fig. 1B) (16). Consistent with the signaling result, treatment of A20μWT B cells with ≥ 10 μM piceatannol blocks BCR ubiquitination at all time points tested (Figs. 1, C and D) and blocks degradation of Ag-BCR complexes (Fig. 1D). Similar results were obtained in splenic B cells (supplemental Fig. S1C). These findings, together with data published previously (18), establish that ubiquitination of lipid raft-resident Ag-BCR complexes occurs via a mechanism that involves both Src- and Syk-dependent BCR signaling.

Previous studies have established that BCR ubiquitination is necessary for delivery of Ag-BCR complexes to intracellular antigen processing compartments and subsequent class II-mediated antigen presentation to CD4 T cells, whereas the fluid-phase (F-P) processing of non-cognate antigen is independent of BCR ubiquitination, even in anti-BCR stimulated cells (16–18). Therefore, it was of interest to determine the role of Src/ Syk signaling in BCR-mediated versus F-P antigen processing and presentation. In the case of F-P antigen processing, BCR-mediated signaling (normally elicited by the binding of cognate antigen to the BCR) is provided in parallel by ligation of the BCR with anti-BCR antibody.

As shown by the results presented in Fig. 2A, blockade of either Src- or Syk-mediated BCR signaling profoundly inhibits BCR-mediated antigen processing and formation of derivative peptide-class II complexes, whereas there is no significant inhibitory effect of signaling inhibition on F-P antigen processing in the anti-BCR stimulated B cells. Moreover, analysis of surface I-A^k expression (Fig. 2B) establishes both that the block in BCR-mediated antigen processing is not due to a global ablation of MHC class II expression and demonstrates that the lack of inhibition of F-P antigen processing is not due to a lack of inhibitor effect, as all inhibitor treatments blocked the BCR signaling-induced up-regulation of class II surface expression in both systems. Together with the results presented in Fig. 1, these results establish the critical role of Syk-dependent BCR signaling in BCR ubiquitination and BCR-mediated antigen processing and presentation.

**A Unique Role for c-Cbl in BCR-mediated Antigen Processing and Presentation**—Cbl-b and c-Cbl are two related ubiquitin ligases known to interact directly with the BCR signaling molecule Syk (23, 32, 33). These two RING finger-type E3 ubiquitin ligases are ~50% similar at the amino acid level and share a general overall domain structure (Fig. 3A). Nevertheless, they
have been shown to have distinct functions within B cells (23, 34). Intriguingly, Syk is a target of c-Cbl-mediated ubiquitylation upon B-cell receptor stimulation (35). Given the role of Syk-dependent signaling in BCR ubiquitination established above, it was of interest to investigate the role of these two Syk-binding ubiquitin ligases in BCR ubiquitination.

A20\(^{\text{WT}}\) B cells express both c-Cbl and Cbl-b (Fig. 3A). To selectively knock down the levels of either c-Cbl or Cbl-b, A20\(^{\text{WT}}\) cells were infected with a lentivirus encoding both GFP and one of six Cbl shRNA (three targeting c-Cbl and three targeting Cbl-b, Fig. 3A). After 7 days of post-infection growth, parallel samples were stained with a pan-reactive anti-I-A\(^{\text{a}}\) monoclonal antibody (10-3.6-FITC) and analyzed by flow cytometry. Shown is the normalized MFI of 10-3.6 staining of B220\(^{\text{+}}\) cells, mean ± 1 S.E. for three independent experiments. Each experimental sample was compared with the vehicle (DMSO) control by a Student’s t test. *, p < 0.05. Pice, piceatannol.

**FIGURE 2.** BCR-mediated antigen presentation requires Src-family kinase and Syk signaling. A, splenocytes from MD4.B10.Br (black bars, BCR-mediated) or B10.Br (white bars, fluid-phase mediated) mice were preincubated for 1 h with indicated inhibitors or diluted vehicle (DMSO). Cells were then pulsed with antigen (MD4.B10.Br, 100 nM HEL for BCR-mediated processing; B10.Br, 100 μM HEL + 10 μg/ml anti-murine IgM for F-P processing and BCR signaling) for 24 h, harvested, and HEL\(^{\text{4E-6E}}\)/I-A\(^{\text{a}}\) complex expression was determined by staining with the HEL\(^{\text{4E-6E}}\)/I-A\(^{\text{a}}\) complex-specific mAb C4H3 and analysis flow cytometry. Shown is the normalized MFI of C4H3 staining of B220\(^{\text{+}}\) cells, mean ± 1 S.E., for three independent experiments. PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo [3,4-d]-pyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine. B, parallel samples were stained with a pan-reactive anti-I-A\(^{\text{a}}\) monoclonal antibody (10-3.6-FITC) and analyzed by flow cytometry. Shown is the normalized MFI of 10-3.6 staining of B220\(^{\text{+}}\) cells, mean ± 1 S.E. for three independent experiments. Each experimental sample was compared with the vehicle (DMSO) control by a Student’s t test. *, p < 0.05. Pice, piceatannol.
in selective media, surviving B cells were > 95% GFP-positive. To establish the degree and specificity of Cbl knockdown, whole cell lysates from all six Cbl knockdown lines as well as control cells were probed for both c-Cbl and Cbl-b by Western blot analysis (Fig. 3). The results reveal that expression of all six shRNA results in 90% knockdown of the targeted Cbl protein with essentially no change in the level of the non-targeted Cbl molecule. On the basis of Cbl expression profiles, cells expressing c-Cbl #3 and Cbl-b 3# shRNA (called c-Cbl and Cbl-b from here on) were selected to be the focus of further analysis. A20 WT cells expressing either GAPDH and scrambled (non-silencing) shRNA (as well as non-infected A20 WT cells) were used as controls for subsequent experiments.

To characterize the Cbl knockdown cell lines, the impact of Cbl deletion on BCR expression, signaling, and endocytosis was determined. Knockdown of both c-Cbl and Cbl-b has no impact on BCR expression, as all six of the knockdown cells expressed the same level of human IgM as A20 WT cells. Knockdown of c-Cbl, an established negative regulator of Syk activity (23, 34), results in an increase in BCR-driven calcium signaling (Fig. 4A and supplemental Fig. S2A). In contrast, knockdown of Cbl-b has no detectable impact on this early aspect of BCR signaling (Fig. 4A and supplemental Fig. S2A). Consistent with the published literature establishing that BCR internalization is a signaling-independent event (25, 27, 36, 37), knockdown of either c-Cbl or Cbl-b has no significant impact on the kinetics of BCR internalization (Fig. 4C and supplemental Fig. S2B). These results are consistent with previous reports on an inhibitory role for c-Cbl in early BCR signaling (21, 23, 38), and demonstrate that BCR internalization is a Cbl-independent event. Although these results appear to contradict a previous report on the role of c-Cbl in BCR internalization (38), that study used a different cell line (the chicken DT-40 B cell line), and the slight delay in BCR internalization was only seen at early time points.

To address the roles of c-Cbl and Cbl-b in Syk-dependent BCR ubiquitination, we returned to tagging cell surface BCR....
molecules with biotinylated anti-BCR antibodies and our established ubiquitin pull-down assay (Fig. 1B). As shown by the results presented in Fig. 5 (upper panels), a robust level of BCR ubiquitination is observed in wild-type, ΔCbl-b-, ΔGAPDH-, and scrambled shRNA-expressing B cells. In stark contrast, there is no detectable BCR ubiquitination in the Δc-Cbl B cells, even though these cells bind as much BCR ligand as the wild-type cells. These results establish a unique role for the Syk-binding ubiquitin ligase c-Cbl in BCR ubiquitination.

Because previous studies have established a central role for BCR ubiquitination in the delivery of Ag-BCR complexes to MIIC antigen processing compartments and the resultant formation of antigenic peptide-MHC class II complexes, the impact of Cbl knockdown on BCR-mediated antigen processing was investigated. As shown by the results presented in Fig. 5, pulsing either WT or ΔCbl-b B cells with biotinylated anti-BCR antibody leads to degradation of up to 80% of the BCR-bound antibody (numbers in upper panel) and formation of anti-BCR antibody heavy chain-derived degradation products (lower panel, Deg Bands). However, degradation of the anti-BCR-btn heavy chain and formation of derivative degradation bands is blocked in the Δc-Cbl cells, revealing an altered processing of BCR-bound ligands in these B cells.

To determine whether the altered degradation of BCR-bound ligand in Δc-Cbl cells is the result of altered BCR trafficking, delivery of internalized ligand-BCR complexes to late endocytic compartments was analyzed by confocal immunofluorescence microscopy. In wild-type and ΔCbl-b B cells, internalized ligand-BCR complexes are delivered to LAMP-2 at any time point during the course of the experiment. Taken together with the results presented in Fig. 5, these results firmly establish a unique role for the Syk-binding ubiquitin ligase c-Cbl in the intracellular trafficking and degradation of internalized ligand-BCR complexes.

To take this analysis one step further, we established the impact of Cbl knockdown on BCR-mediated antigen processing and presentation to CD4 T cells (Fig. 7 and supplemental Fig. S4). In A20 WT cells, BCR-mediated processing of PC-Ova (bound to the PC-specific IgM BCR) is about 100-fold more efficient than the F-P processing on non-PC modified Ova. Knockdown of Cbl-b, GAPDH or scrambled has no effect on either form of antigen processing. In stark contrast, the BCR-mediated processing of PC-Ova is dramatically altered in Δc-Cbl cells so that there is no difference in the dose-response curve of BCR-mediated versus F-P antigen processing in these cells. These results are consistent with the selective block in ligand-BCR ubiquitination (Fig. 5), processing (Fig. 5) and Ag-BCR trafficking to MIIC compartments (Fig. 6) in the Δc-Cbl cells. Taken in total, the results in this report firmly establish a critical role for the Syk-binding ubiquitin ligase c-Cbl in BCR ubiquitination and the BCR-mediated processing and presentation of cognate antigen.

**DISCUSSION**

Trafficking of internalized Ag-BCR complexes to MVB-like MIIC where they are converted into antigenic peptide-MHC class II complexes is dependent on BCR ubiquitination (16–18). Previous studies from this laboratory have established a role for Src-family kinase-dependent BCR signaling in receptor ubiquitination and revealed that ubiquitination is restricted to lipid raft-resident Ag-BCR complexes (18). Here, those studies are extended to establish a role for Syk and the Syk-binding
ubiquitin ligase c-Cbl in BCR ubiquitination and BCR-mediated antigen processing and presentation.

The roles of two Syk-binding ubiquitin ligases c-Cbl and Cbl-b in BCR biology are controversial, and there is no overwhelming consensus on the precise role of either of these proteins in BCR function. Although there are conflicting viewpoints, a considerable number of reports do suggest a role for c-Cbl as a negative regulator of BCR signaling (21, 23, 38). The findings in this report are consistent with this scenario and, for the first time, establish c-Cbl as a central player in the molecular mechanism of BCR ubiquitination and the rapid processing and presentation of BCR-bound cognate antigen.

Previously, a report from the group of Marcus Clark implicated the ubiquitin ligase itch in ubiquitination of the CD79b subunit of the BCR complex and BCR-mediated antigen processing (17). In that report, itch-deficient B cells were shown to exhibit alterations in the intracellular trafficking of Ag:BCR complexes and impaired BCR-mediated antigen presentation, which was correlated with a change in ubiquitination of CD79b (17). Although the finding in this work may initially appear to be at odds with that previous work, that is not necessarily the case. Previous studies by others have demonstrated that c-Cbl is a substrate of itch (39, 40), establishing a functional interaction between the two proteins. Thus, it is possible that Syk recruits both ubiquitin ligases to the BCR through a multistep process. First, Syk is recruited to the BCR via binding to dually phosphorylated CD79 ITAMs. As a consequence, Syk becomes phosphorylated on tyrosine 323, forming a docking site for c-Cbl (24). Itch may then bind BCR-associated c-Cbl, bringing both ubiquitin ligases into close proximity to the BCR cytoplasmic tail. This raises the question of which ubiquitin ligase is directly responsible for BCR ubiquitination to drive its delivery to antigen-processing compartments. Because B cells deficient in both c-Cbl (this report) and itch (17) activity show altered BCR-mediated antigen processing and presentation, it is possible that both ligases contribute. However, additional studies will be necessary to better establish the precise roles of each of these proteins in BCR ubiquitination and BCR-mediated antigen processing and presentation.

It is now apparent that BCR ubiquitination is a signaling-dependent event restricted to lipid raft-resident BCR molecules. In a previous report it was established that physiological range temperature change between 33 °C and 39 °C has a profound effect on lipid raft-dependent BCR functions such as signaling and BCR ubiquitination (18). In that report, it was established that temperature change has inverse effects on BCR signaling and ubiquitination. Specifically, lowering the temperature from 39 °C to 33 °C results in an increase in BCR calcium signaling but a decrease in BCR ubiquitination and a delay in BCR-mediated antigen processing. In this report, it was found that there is a similar inverse effect in Δc-Cbl cells, where BCR calcium signaling is increased, whereas BCR ubiquitination is decreased, and BCR-mediated antigen processing is impaired. This inverse correlation between BCR calcium signaling and signaling-dependent BCR ubiquitination and processing suggests that although BCR signaling is required for receptor ubiquitination, it is not a simple straightforward relationship, and that subtle changes in the structure and spatial organization of the lipid raft-resident BCR signaling complex can have profound effects both on BCR signaling and B cell activation as well as signaling-dependent BCR ubiquitination and BCR-mediated antigen processing and presentation.

When combined with our previous report (18), the findings in this report establish that BCR ubiquitination, which drives delivery of Ag:BCR complexes to MVB-like MIIC for conversion of peptide-class II complexes, is a signaling-dependent event involving two key BCR signaling molecules, Src family kinases and Syk (supplemental Fig. S5). Within lipid rafts, Src family kinases phosphorylate the ITAMs within the cytoplasmic tail of CD79, forming a binding site for Syk. Syk then likely acts as a docking molecule to recruit the ubiquitin ligase c-Cbl (and possibly also itch), bringing one or more ubiquitin

FIGURE 6. c-Cbl mediates Ag BCR trafficking to LAMP-2+ endocytic compartments. A, B cells were pulsed on ice with 10 μg/ml anti-hulgM-btm followed by SA-Alexa Fluor 594, washed, and then incubated at 37 °C for 30 min. Cells were then fixed, permeabilized, and stained with anti-CD107b (LAMP-2). BCR staining is shown in red, LAMP-2 in pseudo-color green, and DAPI in blue. Shown are representative results from one of three independent experiments. Scale bar = 10 μm. B, for each cell type, the Pearson’s coefficient of BCR-LAMP-2 colocalization at 30 min was measured for 100–150 cells. Shown is the average Pearson’s coefficient of BCR-LAMP-2 colocalization for three independent experiments (mean ± 1 S.E.). Student’s t test was used to compare the Pearson’s coefficient of knockdown versus WT cells. *, p < 0.05.
ligases in close enough proximity to drive BCR ubiquitination and rapid delivery of these Ag/BCR complexes to MIIC. Future studies will provide a more detailed understanding of the mechanism of BCR ubiquitination and its precise role in the BCR-mediated processing and presentation of cognate antigen.

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